

Apoptosis induced by a human milk protein

(epithelial cells/thymocytes/cancer)

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ABSTRACT To the breast-fed infant, human milk is more than a source of nutrients; it furnishes a wide array of molecules that restrict microbes, such as antibodies, bactericidins, and inhibitors of bacterial adherence. However, it has rarely been considered that human milk may also contain substances bioactive toward host cells. While investigating the effect of human milk on bacterial adherence to a human lung cancer cell line, we were surprised to discover that the milk killed the cells. Analysis of this effect revealed that a component of milk in a particular physical state—multimeric α -lactalbumin—is a potent Ca^{2+} -elevating and apoptosis-inducing agent with broad, yet selective, cytotoxic activity. Multimeric α -lactalbumin killed all transformed, embryonic, and lymphoid cells tested but spared mature epithelial elements. These findings raise the possibility that milk contributes to mucosal immunity not only by furnishing antimicrobial molecules but also by policing the function of lymphocytes and epithelium. Finally, analysis of the mechanism by which multimeric α -lactalbumin induces apoptosis in transformed epithelial cells could lead to the design of antitumor agents.

Through programmed cell death the human body eliminates unwanted cells without evoking an inflammatory response. Cells undergoing programmed cell death show a distinct morphological appearance characterized by cell shrinkage, membrane blebbing, nuclear condensation, and fragmentation with the formation of “apoptotic bodies”; this type of cell death is generally referred to as apoptosis (1, 2).

Human milk provides the breast-fed child not only with nutrients but also with a mucosal immune system. Milk contains a wide array of molecules with antimicrobial activity: antibodies to bacterial, viral, and protozoal antigens (3–5); potentially bactericidal molecules like lysozyme and lactoferrin; fatty acids that lyse bacteria and viral particles; and glycoconjugates that inhibit bacterial adherence to epithelial cells (6–8). These components reach mucosal surfaces in the respiratory and gastrointestinal tracts of the breast-fed child and are thought to interfere with various steps in the pathogenesis of infections at these sites (8, 9). As a result, breast-feeding protects the infant from respiratory and gastrointestinal infections.

During our recent studies of human milk and its antibacterial properties, we observed that transformed cell lines exposed to human milk underwent morphological changes compatible with apoptosis. The active component was identified as multimeric α -lactalbumin (MAL), which was found to induce apoptosis in a variety of transformed and immature mammalian cells but not in other cells. This report describes these effects and provides preliminary characterization of the cytotoxic mechanism.

MATERIALS AND METHODS

Materials. Human and bovine α -lactalbumin, bovine serum albumin (BSA), human and chicken lysozyme, human lactoferrin, and cycloheximide were from Sigma. Thapsigargin was from Gibco.

Cells. The A549, NCI H292, A-498, J 82, CaCO-2, HT-29, 5637, GMK, Vero, MDCK, B9, and WEHI 164 cell lines were from the American Type Culture Collection (ATCC). The cell lines were cultured as described (10). Human embryonic lung cells (Hel) and human foreskin fibroblasts (HFF) were kindly provided by the Division of Virology, Department of Medical Microbiology, Lund University, Lund, Sweden. Human peripheral blood granulocytes and lymphocytes were isolated from heparin-treated blood of healthy human volunteers by using a Polymorphprep density gradient (Nycomed, Oslo). Rat thymocytes were harvested as described (11).

Measurement of Intracellular Ca^{2+} Concentration ($[\text{Ca}^{2+}]_i$). A549 cells (4×10^7 cells) or rat thymocytes (5×10^7 cells) were incubated in Krebs–Henseleit buffer (pH 7.2) with 10 mM Hepes, 15 mM glucose, and 1% BSA. Cells were loaded with 5 μM fura-2 AM for 25 min at 37°C, washed for 30 s at 1500 $\times g$, and resuspended in Krebs medium (11, 12). After a steady baseline of $[\text{Ca}^{2+}]_i$ was obtained, inducing agents were added. Control cells had only carrier solvent.

Milk Fractionation. Purification of the active component was according to H.S. (unpublished observation). Briefly, casein was precipitated from ≈ 5 liters of milk (13) and fractionated by using an ion-exchange column packed with DEAE-Trisacryl M (BioSeptra, Villeneuve la Garenne, France) attached to an FPLC instrument (Pharmacia-LKB). The lyophilized casein was dissolved in 0.01 M Tris-HCl (pH 8.5) and applied to the column. The run was in 0.01 M Tris-HCl (pH 8.5) with increasing concentrations of NaCl (0–1.0 M). The peaks were monitored at 280 nm. Fractions (3 ml) were pooled (pools I–VI), desalted by dialysis (membrane cutoff, 3.5 kDa) against distilled water for at least 48 h, lyophilized, and diluted in medium to a concentration of 10 mg/ml.

Analysis of the Milk Fractions. The active fraction (fraction VI) was analyzed by polyacrylamide gradient gel electrophoresis (PAGE) using 4–20% precast gels (Bio-Rad) on a Bio-Rad Mini Protean II cell. After SDS/PAGE, protein bands were transferred by Western blotting onto poly(vinylidene difluoride) membranes (Pro Blott membranes; Applied Biosystems) and subjected to protein sequencing by Edman degradation in an automated pulse liquid sequencer (model 477A; Applied Biosystems) (14). Purified proteins were analyzed by electrospray ionization mass spectrometry (ESI-MS) (15) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (16). For ESI-MS a VG Bio-Q

Abbreviations: MAL, multimeric α -lactalbumin; $[\text{Ca}^{2+}]_i$, intracellular $[\text{Ca}^{2+}]$; PAGE, polyacrylamide gradient gel electrophoresis; ESI-MS, electrospray ionization mass spectrometry; MALDI, matrix-assisted laser desorption ionization; FIGE, field-inversion gel electrophoresis.

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ESI mass spectrometer (Fisons/VG, Manchester, U.K.) was used. The electrospray carrier solvent was 1% acetic acid in acetonitrile/water (1:1) and the flow rate was 2–4 ml/min. The molecular mass of sample components was estimated from the *m/z* values of series of ions (15). MALDI-MS was performed on an LDI 1700 time of flight mass spectrometer equipped with a pulsed nitrogen laser (337 nm) (Biomolecular Separations, Reno, NV). Sinapinic acid was used as a matrix and BSA was used as the external standard.

Influence of Milk Components on Cell Viability. The cells were grown to confluency in 96-well plates. At time 0, 100 μ l of medium was replaced by 100 μ l of experimental solution and incubated at 37°C for 30 min. [³H]Thymidine (0.5 μ Ci; 1 Ci = 37 GBq; Amersham) was added and the cells were incubated for 4 h at 37°C. The supernatant was discarded and the cells were washed twice in phosphate-buffered saline, detached by trypsin treatment, and harvested; the radioactivity was measured in a 1205 Betaplate liquid scintillation counter (Wallac). Cell viability was also assayed by trypan blue exclusion.

Transmission Electron Microscopy. Cells were double fixed in glutaraldehyde plus osmium tetroxide, embedded in agar 100, poststained with uranyl acetate and lead, sectioned, and examined by transmission electron microscopy.

DNA Fragmentation. Cells (2×10^6 cells) were lysed and centrifuged at $20,000 \times g$, and the supernatant was extracted with phenol/chloroform. Precipitated oligonucleosome length DNA fragments were loaded on 1.8% agarose gels, electrophoresed with constant current set at 60 mA, and visualized with ethidium bromide (17).

High molecular weight DNA fragments were detected by field-inversion gel electrophoresis (FIGE) as described (17).

RESULTS

Human Milk Induces Apoptosis. Human milk from several donors induced apoptosis in transformed and nontransformed immature cell lines and lymphoid cells but not in mature cells. This effect was first observed in studies with A549 cells, a human lung carcinoma cell line used to investigate bacterial adherence and antimicrobial properties of human milk. Cell viability was reduced by 98% (Table 1), and the cells displayed morphological changes compatible with apoptosis (nuclear condensation, appearance of apoptotic bodies, and cell shrinkage; Fig. 1). Associated with this was the formation of high molecular weight DNA fragments in A549 cells. MDCK cells displayed both high molecular weight DNA fragments and the typical oligonucleosome length DNA fragments (DNA laddering) (data not shown). Human milk produced maximal DNA fragmentation when diluted 1:10. Bovine milk was inactive (data not shown).

Characterization of the Active Component. Human milk was fractionated to identify the component(s) triggering apoptosis. The fractions were tested for effects on the viability of four human cell lines (A549, NCI, A-498, and J 82). The results with A549 cells are shown in Table 1. The cytotoxic activity precipitated with the casein fraction of human milk; no activity remained in the whey fraction. After ion-exchange chromatography of casein, the apoptosis-inducing activity was in fraction VI that eluted only after 100% 1 M NaCl (Fig. 2). This fraction contained proteins with molecular masses of 14, 28, and 100 kDa (Fig. 2 *Inset*). The bands of fraction VI showed complete N-terminal sequence homology with human α -lactalbumin (data not shown). By ESI-MS, the estimated molecular mass of the major component of the active protein fraction (14.088 kDa) was close to the molecular mass of α -lactalbumin calculated from the amino acid sequence (14.078 kDa). The small differences ruled out most known posttranslational modifications and suggested that the major component did not differ in its covalent structure from α -lactalbumin. MALDI-MS showed a major peak close to 14 kDa, consistent with mono-

Table 1. Effects of human milk and human milk fractions on viability of human lung carcinoma cell line A549

	Cell viability, %*	
	TBE†	TT‡
Medium control	92	100 (21,750)
Human milk		
Donor 1	2	0
Donor 2	2	1
Donor 3	4	2
Fraction of milk (donor 1)		
Whey	92	62
Casein	0	0
Casein fraction		
I	96	93
II	88	91
III	74	69
IV	89	111
V	79	73
VI§	0	0
Control		
Bovine milk	100	64
α -Lactalbumin, human	92	80
α -Lactalbumin, bovine	93	82
Lysozyme, human	87	69
Lysozyme, chicken	86	75
Lactoferrin, human	98	¶
MAL, human	0	0

*Equivalent results were obtained with cell lines NCI, A498, and J 82.

†Cell viability was determined by trypan blue exclusion. Mean values from three separate experiments are shown.

‡Cell viability was determined by [³H]thymidine incorporation. cpm of the medium control is shown in parentheses. Viability is measured as (cpm sample $\times$ 100)/(cpm control). Mean values from three separate experiments are shown.

§Fractions I–VI were separated by ion-exchange chromatography. Fraction VI was eluted after 1 M NaCl.

¶Not determined.

||Commercial monomeric α -lactalbumin was multimerized by passage over an ion-exchange column.

meric α -lactalbumin, but also peaks at 28 and 42 kDa, consistent with the di- and trimeric forms.

Monomeric, commercial human or bovine α -lactalbumin had no effect on cell viability (Table 1). However, when monomeric α -lactalbumin was passed over the ion-exchange column, it multimerized and became cytotoxic. Other human milk proteins (lactoferrin and lysozyme) were found to be inactive at a concentration of 5 mg/ml before and after ion-exchange chromatography (Table 1). We concluded that a multimeric form of α -lactalbumin in human milk induces cell death.

Sensitivity of Different Cell Types to MAL. MAL reduced the viability of human epithelial cell lines (A549, NCI, A-498, J 82, Caco-2, HT 29, and 5637), primate kidney cell lines (GMK, Vero), a canine kidney cell line (MDCK), and two mouse cell lines (WEHI 164 and B9) by >90% (Table 2). Embryonic human cells (Hel, HFF) and human peripheral blood lymphocytes were sensitive, while peripheral blood granulocytes demonstrated intermediate sensitivity to MAL. Human epithelial cells from either the upper respiratory tract or the urinary tract and mouse kidney and bladder epithelial cells were resistant to the cytotoxic effect of MAL (Table 2).

MAL Induces Apoptosis. Oligonucleosome-length DNA fragments characteristic of apoptosis were observed in thymocytes treated with 0.3, 0.5, and 0.7 mg of MAL per ml for 4 h (Fig. 3) and in MDCK cells treated with 1 mg of MAL per ml for 24 h (Fig. 3). Fragmentation increased with the concentration of MAL. In A549 cells, MAL produced HMW DNA fragments of the kind that is seen early in thymocyte apoptosis

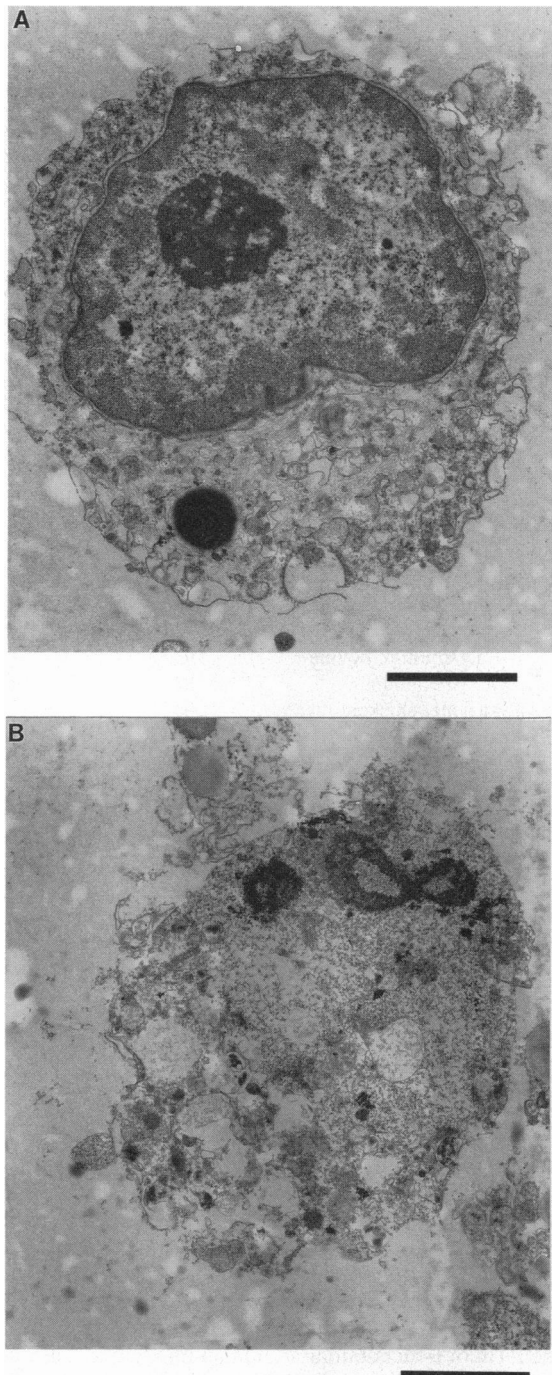


FIG. 1. Apoptosis in MDCK cells exposed to human milk (1:10 dilution; 24 h). (A) Control. (B) Human milk. (Bars = 2.3 μ m.)

(Fig. 3) (17). DNA ladders were not observed in A549 cells even after higher concentrations of MAL (5 mg/ml) or prolongation of the incubation time (48 h) (data not shown).

In many experimental systems, the induction of apoptosis is accompanied by *de novo* mRNA and protein synthesis and is blocked by protein synthesis inhibitors (2, 18). Cycloheximide effectively prevented MAL-induced DNA laddering in MDCK cells and cell death in A549 and MDCK cells (data not shown).

Role of Ca^{2+} in MAL-Induced Apoptosis. The effect of MAL on $[\text{Ca}^{2+}]_i$ in A549 cells and rat thymocytes was assessed by using fura-2 (12) and with the Ca^{2+} -ATPase inhibitor thapsigargin as a positive control (19). Thapsigargin caused a sustained increase in $[\text{Ca}^{2+}]_i$ and DNA fragmentation after 4 h in rat thymocytes (20, 21). In A549 cells, thapsigargin (100

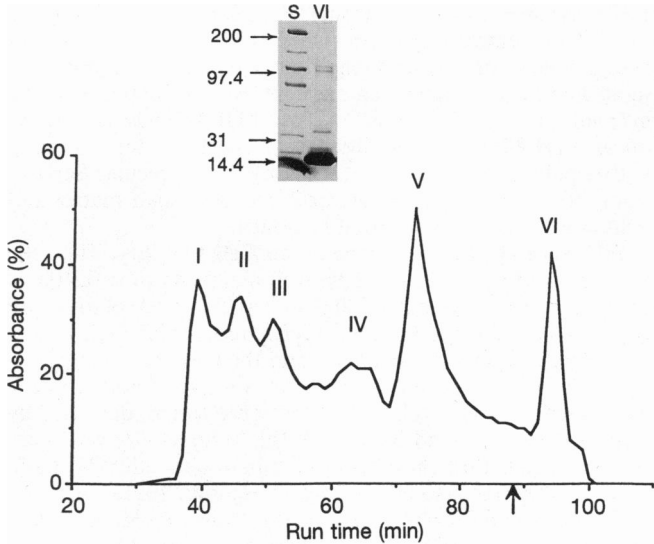


FIG. 2. Casein (100 mg) was fractionated on a DEAE-Trisacryl M ion-exchange column by FPLC. Eluted fractions were pooled as indicated (I–VI). Arrow indicates 100% 1 M NaCl. (Inset) SDS/PAGE of pool VI. Lane S, molecular size markers (kDa) (Bio-Rad).

nM) rapidly induced a sustained increase in $[\text{Ca}^{2+}]_i$ (Fig. 4*A*), and DNA fragmentation occurred after 24 h (Fig. 4*B*).

MAL induced a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ in A549 cells and rat thymocytes after a 30-s exposure (Fig. 4). Cell death was prevented under calcium-free condi-

Table 2. Effects of MAL on viability of different cell types

Cells	Cell viability*			
	Medium		MAL	
	%†	cpm‡ (100%)	%†	cpm‡ (%)
Cell lines				
A549, human lung	95	21,750	0	50 (0)
NCI, human lung	91	28,500	0	15 (0)
A-498, human kidney	84	45,400	5	40 (0)
J 82, human bladder	92	7,800	4	20 (0)
Caco-2, human intestine	98	18,700	0	40 (0)
HT-29, human intestine	99	48,400	6	75 (0)
HTB9, human kidney	98	11,200	4	40 (0)
GMK, monkey kidney	72	14,500	0	50 (0)
Vero, monkey kidney	83	45,400	12	400 (1)
MDCK, dog kidney	95	53,700	4	15 (0)
B9, mouse	100	35,400	0	20 (0)
WEHI, mouse	90	14,400	1	20 (0)
Embryonic cells				
Hel, human lung	67	—	8	—
HFF, human foreskin	90	—	8	—
Nontransformed cells				
Nph epithelium, human	43	—	45	—
Urinary epithelium, human	77	—	75	—
Granulocytes, human	100	—	62	—
Lymphocytes, human	100	—	26	—
Kidney, mouse	46	—	62	—
Bladder, mouse	38	—	48	—
Thymocytes, rat	100	—	0	—

*Cell viability was monitored by $[\text{^3H}]$ thymidine incorporation and trypan blue exclusion. Viability of embryonic and nontransformed cells was assayed only by trypan blue exclusion, since these cell types did not incorporate $[\text{^3H}]$ thymidine.

†% viability as determined by trypan blue exclusion.

‡cpm as determined by $[\text{^3H}]$ thymidine incorporation. Medium control denotes 100%; numbers in parentheses denote (cpm sample $\times$ 100)/(cpm control). Means of three separate experiments are shown.

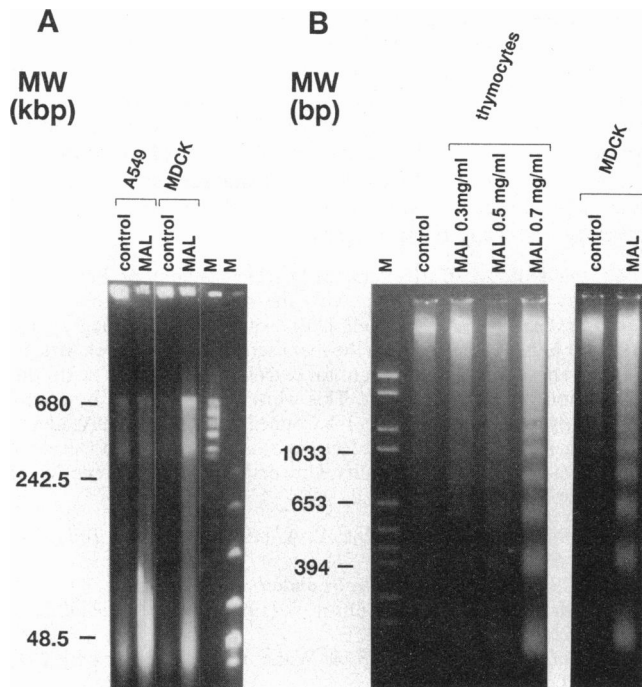


FIG. 3. Chromatin cleavage induced by MAL in A549 and MDCK cells and in rat thymocytes. A549 cells and MDCK cells were incubated with 1 mg of MAL per ml for 24 h. Rat thymocytes were incubated with MAL for 4 h. (A) Chromatin cleavage was analyzed by FIGE. DNA size calibration markers were from Sigma; chromosomes were from *Saccharomyces cerevisiae* (225–2200 kbp) (lane 4) and a mixture of DNA *Hind*III fragments, DNA, and DNA concatemers (0.1–200 kbp) (lane 5). (B) Oligonucleosome fragmentation was analyzed by gel electrophoresis. A mixture of fragments from pBR328 DNA cleaved separately with *Bgl* I and *Hinf* I were used as molecular size markers (lanes M) (Boehringer Mannheim).

tions, suggesting Ca^{2+} to be involved in the apoptotic signal. These results were confirmed by measurement of chromatin cleavage using FIGE. Formation of high molecular weight DNA fragments induced by MAL in MDCK cells was prevented by removal of Ca^{2+} from the medium (Fig. 5).

DISCUSSION

We report here that human milk induces apoptosis in several mammalian cell lines and immature cells. The apoptosis-inducing activity was in the casein fraction of human milk and was characterized as a multimeric form of human α -lactalbumin. MAL induced apoptosis in transformed and nontransformed cell lines, both characterized by an immature phenotype. Embryonic cells and peripheral blood lymphocytes were also sensitive, while mature epithelial cells and cells from solid organs were resistant to the apoptotic effect of this milk factor. The induction of apoptosis by MAL required protein synthesis. MAL caused an increase in $[\text{Ca}^{2+}]_i$ and did not trigger apoptosis under calcium-free conditions, suggesting a crucial role for the calcium ion in the process. In addition to the characteristic apoptotic morphology seen in MAL-sensitive cells, MAL-induced apoptosis in MDCK cells was associated with the appearance of both high molecular weight and oligonucleosome-length DNA fragments typical of apoptosis (2, 17), whereas MAL-treated A549 cells exhibited high molecular weight DNA fragments but no DNA ladders (cf. Fig. 3). Similar differences in apoptotic DNA fragmentation have previously been found in other cell types and may reflect differences in protease and/or nuclease activities between cell types.

The purification of MAL from human milk by anion-exchange chromatography was monitored by the biological activity of each fraction. SDS/PAGE profiles of the cyto-

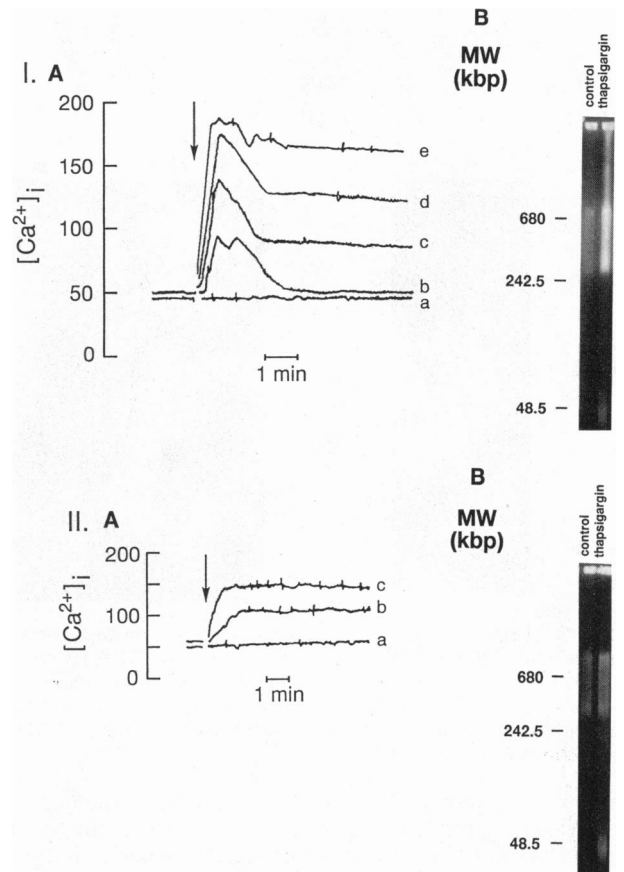


FIG. 4. Effects of MAL and thapsigargin on $[\text{Ca}^{2+}]_i$ in A549 cells and rat thymocytes. (IA) A549 cells: control (trace a); with MAL at 0.5 mg/ml (trace b), 1.0 mg/ml (trace c), and 2.0 mg/ml (trace d); or with 100 nM thapsigargin (trace e). (IB) A549 cells were exposed to 100 nM thapsigargin for 24 h and analyzed for high molecular weight DNA fragmentation; (IIA) Rat thymocytes: control (trace a), with MAL at 1 mg/ml (trace b), or with 100 nM thapsigargin (trace c). (IIB) Rat thymocytes were exposed to 100 nM thapsigargin for 24 h and analyzed for high-molecular weight fragmentation. Traces shown are representative of three separate experiments.

toxic fraction showed a major band at 14 kDa and minor bands in the 30- and 100-kDa regions. The N-terminal sequence of each band was identical to the sequence of α -lactalbumin, but α -lactalbumin itself lacked cytotoxic activity. However, the presence of additional minor bands on SDS/PAGE suggested that the active component may exist in a different aggregation state. By ESI-MS analysis, the molecular masses of the active component (14.088 kDa) and of α -lactalbumin (14.061 kDa) were found to be similar, and the small differences ruled out most known posttranslational modifications. Analysis of the active protein by MALDI-MS showed peaks consistent with monomeric (14 kDa), dimeric (28 kDa), and trimeric (42 kDa) forms of α -lactalbumin, suggesting that the apoptosis-inducing component was possibly a multimeric form of human α -lactalbumin.

The factors that regulate the aggregation state of α -lactalbumin in whole human milk are not understood. Both the bioactivity of whole milk and the size fractionation profile of the α -lactalbumin component indicate that multimers of α -lactalbumin occur naturally. Moreover, we could induce multimerization of monomeric α -lactalbumin by anion-exchange chromatography in the presence of high concentrations of NaCl and recover the apoptosis-inducing activity of whole milk. Several peptides and proteins, including cytokines, bacterial toxins, and viral envelope proteins, induce apoptosis in mammalian cells by receptor-mediated signaling or by

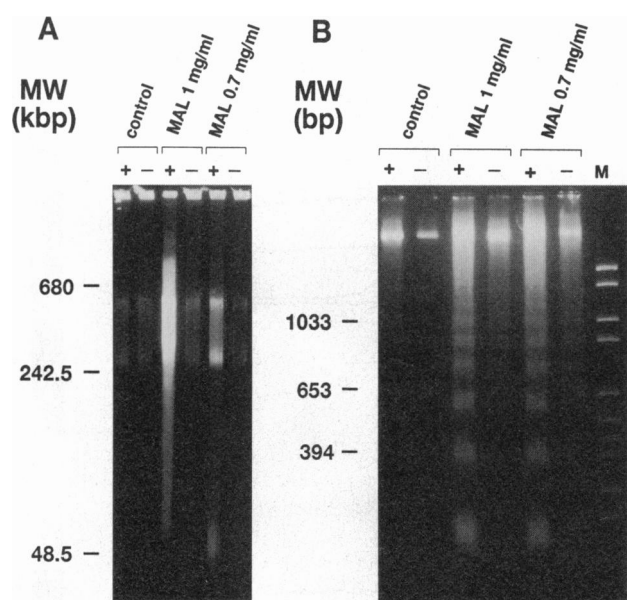


FIG. 5. Effect of removal of extracellular Ca^{2+} on formation of high molecular weight (A) and oligonucleosome (B) DNA fragments in MDCK cells. Cells were incubated in the absence or presence of MAL (1 mg/ml) in complete medium (lanes +) or in Ca^{2+} -free medium (lanes -) and analyzed for DNA fragmentation. Markers were the same as in Fig. 3.

formation of transmembrane pores (21–23). Whether the induction of apoptosis by MAL reflects cross-linking of receptors to which monomeric α -lactalbumin normally binds without any cytotoxic consequence remains to be determined. Also, being in an aggregated state, MAL might possibly form transmembrane pores and evoke apoptosis by allowing the influx of Ca^{2+} and other solutes.

Calcium signals are crucial for induction of apoptosis in thymocytes by many agents, including antibodies to the T-cell receptor–CD3 receptor complex (18). The Ca^{2+} -binding S-100 protein from rat glial cells was recently found to induce apoptosis in PC-12 cells via a sustained increase in $[\text{Ca}^{2+}]_i$ (24). Like the S-100 protein, MAL induced a rapid increase in $[\text{Ca}^{2+}]_i$, and extracellular Ca^{2+} was necessary for induction of apoptosis as shown by the protective effect on DNA fragmentation by removal of Ca^{2+} from the culture medium. As previously found with thymocytes (19, 20), apoptosis could also be induced in A549 cells by thapsigargin, a Ca^{2+} -ATPase inhibitor that produces a sustained increase in $[\text{Ca}^{2+}]_i$. Both the S-100 protein and human α -lactalbumin contain binding sites for Ca^{2+} and Mg^{2+} ions (25), although the mechanism of Ca^{2+} activation by these proteins is still unknown.

Although α -lactalbumin is one of the most abundant proteins in human milk, its functions are not clear. Very recently, homozygous α -lactalbumin-deficient mice were generated (26). Their milk was of such high viscosity that it could not be suckled. However, the state of the pups' gastrointestinal mucosa and gut-associated lymphoid systems was not reported. The potency and selectivity of the apoptosis-inducing activity of MAL lead us to suggest that it may act to restrict certain host cell populations. Cytotoxicity toward thymocytes and peripheral blood lymphocytes, which mostly bear $\alpha\beta$ T-cell receptors, may help explain why the gut mucosa is normally populated by a distinct population of lymphocytes that is largely thymus independent and T-cell receptor $\gamma\delta$ positive (27). This speculation needs to be tested. Also, it has been reported that human milk contains factors that regulate the proliferation and function of lymphocytes (28).

Unlike normal cells, tumor cells may fail to activate the apoptotic pathway. Indeed, a characteristic of most tumor cells

is the resistance to agents that induce apoptosis in nonmalignant cells. In contrast, MAL induced apoptosis in tumor cells but left other cells intact. Since tumor cells are often resistant to the normal apoptosis-inducing signals, our finding may be important in further elucidation of the pathways that lead to apoptosis in tumor cells. Finally, the ability of MAL to induce apoptosis in transformed, but not in mature, epithelial cells may help direct the neonatal mucosal epithelium toward maturity and away from neoplasia.

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- Schwartz, L. M. & Osborne, B. A. (1993) *Immunol. Today* **14**, 582–590.
- Wyllie, A. H. (1980) *Nature (London)* **284**, 555–556.
- Redhead, K., Hill, T. & Mullroy, B. (1990) *FEMS Microbiol. Lett.* **70**, 269–274.
- Gillin, F. D., Reiner, D. S. & Wang, C. S. (1983) *Science* **221**, 1290–1292.
- Fiat, A.-M. & Jollés, P. (1989) *Mol. Cell. Biochem.* **87**, 5–30.
- Matthews, T. H. J., Nair, C. D. G., Lawrence, M. K. & Tyrell, D. A. J. (1976) *Lancet* **25**, 1387–1389.
- Svanborg, C., Aniansson, G., Mestecky, J., Sabharwal, H. & Wold, A. (1991) in *Immunology of Milk and Neonate*, ed. Mestecky, J. (Plenum, New York), pp. 167–171.
- Andersson, B., Dahmén, J., Frejd, T., Leffler, H., Magnusson, G., Noori, G. & Svanborg, C. (1983) *J. Exp. Med.* **158**, 559–570.
- Ofek, I., Mirelman, D. & Sharon, N. (1977) *Nature (London)* **265**, 623–625.
- Håkansson, A., Kidd, A., Wadell, G., Sabharwal, H. & Svanborg, C. (1994) *Infect. Immun.* **62**, 2707–2714.
- Zhivotovsky, B., Nicotera, P., Bellomo, G., Hamson, K. & Orrenius, S. (1993) *Exp. Cell Res.* **207**, 163–170.
- Gryniewicz, G., Poenie, M. & Tsien, R. (1985) *J. Biol. Chem.* **260**, 3440–3450.
- Melander, O. (1947) *Uppsala Läkarfören. Förhandl.* **3–4**, 107–198.
- Simpson, R. J., Moritz, R. L., Begg, G. S., Rubira, M. R. & Nice, E. C. (1989) *Anal. Biochem.* **177**, 221–236.
- Beavis, R. C. & Chait, B. T. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6873–6877.
- Keogh, T., Takigiku, R., Lacey, M. P. & Purdon, M. (1992) *Anal. Chem.* **64**, 1594–1600.
- Zhivotovsky, B., Wade, D., Gahm, A., Orrenius, S. & Nicotera, P. (1994) *FEBS Lett.* **351**, 150–154.
- McConkey, D. J., Hartzell, P., Amador-Pérez, J. F., Orrenius, S. & Jondal, M. (1989) *J. Immunol.* **143**, 1801–1806.
- Jiang, S., Chow, S. C., Nicotera, P. & Orrenius, S. (1994) *Exp. Cell Res.* **212**, 84–92.
- Zhivotovsky, B., Cedervall, B., Jiang, S., Nicotera, P. & Orrenius, S. (1994) *Biochem. Biophys. Res. Commun.* **202**, 122–127.
- Banda, N. K., Bernier, J., Kurahara, D. K., Kunle, R., Haigwood, N., Sekaly, R.-P. & Finkel, T. (1992) *J. Exp. Med.* **176**, 1099–1106.
- Bellomo, G., Perotti, M., Taddei, F., Mirabelli, F., Nicotera, P. & Orrenius, S. (1992) *Cancer Res.* **52**, 1342–1346.
- Jonas, D., Walev, I., Berger, T., Liebefran, M., Palmer, M. & Bhakdi, S. (1994) *Infect. Immun.* **62**, 1304–1312.
- Mariggio, M. A., Fulle, S., Calissano, P., Nicoletti, I. & Fano, G. (1994) *Neuroscience* **60**, 29–35.
- Lindahl, L. & Vogel, H. J. (1984) *Anal. Biochem.* **140**, 394–402.
- Stinnakre, M. G., Vilotte, J. I., Soulier, S. & Mercier, J. C. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 6544–6548.
- Viney, J. L., MacDonald, T. T. & Kilshaw, P. J. (1990) *Eur. J. Immunol.* **20**, 1623–1626.
- Mincheva-Nilsson, I., Hammarström, M.-L., Juto, P. & Hammarström, S. (1990) *Clin. Exp. Immunol.* **79**, 463–469.

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Apoptosis induced by a human milk protein complex

Cellular and structural studies in tumour cells and bacteria

by

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Via lactea (The formation of the Milky Way) by **Jacopo Tintoretto** (1518-1594).
The painting depicts the Greek myth on the formation of the Milky Way. Hera is forced by Zeus to breast-feed Hercules, Zeus child with Alcmene, and milk squirts from Heras breast to become the stars.

At last

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Title:

Apoptosis induced by a human milk protein complex. Cellular and structural studies in tumour cells and bacteria

Abstract:

Human milk contains a vast array of bioactive molecules, with nutritional and protective functions. This thesis describes the effects of a human milk protein complex, MAL, on tumour cells and bacteria. During our studies on the anti-adhesive properties of human milk we observed that a milk fraction killed tumour cells. The morphology of the cells indicated apoptotic cells death and this was verified by DNA fragmentation analysis (*Proc Natl Acad Sci U.S.A* 1995; 92: 8064-8068). The effect of MAL was selective for tumour cells and immature embryonic cells while mature differentiated cells were resistant.

MAL bound to the surface of both sensitive and resistant cells but was internalised only in sensitive cells. MAL was shown to co-localise with mitochondria and this interaction caused a disruption of the mitochondrial membrane potential, which caused release of cytochrome c and caspase activation (*Exp Cell Res* 1999; 249: 260-268). The caspase activation was functional as shown by the cleavage of intracellular substrates. In parallel MAL was also transported via active nuclear transport to the nuclei of sensitive cells. MAL exerted a direct effect on isolated nuclei causing the induction of apoptotic DNA fragmentation without involvement of mitochondrial activation pathways (*Exp Cell Res* 1999; 2246: 451-460).

MAL was purified from the casein fraction of human milk and was shown by N-terminal amino acid sequencing to contain α -lactalbumin. Native α -lactalbumin had no bactericidal or tumouricidal activity, suggesting that the α -lactalbumin of the active fraction had different structural properties. By spectroscopic techniques MAL was shown to have a more flexible tertiary structure with exposure of hydrophobic surfaces like the molten globule form of α -lactalbumin (*J Biol Chem* 1999; 274: 6388-6396). Native α -lactalbumin was inactive, but could be converted to the active form by a folding change combined with a fatty acid to stabilise this fold. These studies verified that α -lactalbumin was the active component and that the conformational change was required for the apoptosis-inducing activity.

MAL was also shown to be bactericidal against *S. pneumoniae*., but not against other gram-negative and other gram-positive bacteria. In *S. pneumoniae* MAL was shown to induce identical DNA fragmentation as seen in tumour cells, suggesting apoptosis-like features of bacterial death. The bacterial death did not involve cytochrome c and caspase-activation, but the bacteria had sequence homologies in three open reading frames of the pneumococcal genome with a caspase-independent effector molecule in eukaryotic cells, apoptosis inducing factor (AIF). These sequences are currently investigated.

Keywords:

Apoptosis, tumour cells, breast-feeding, milk, α -lactalbumin, *S. pneumoniae*, mitochondria, caspases, nuclear uptake.

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Abbreviations

Å	Ångström	kDa	Kilo Dalton
AIDS	Acquired immunodeficiency syndrome	kbp	Kilo base pairs
AIF	Apoptosis inducing factor	MAL	Multimeric α -lactalbumin
ALA	α -lactalbumin	MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight mass spectrometry
ANS	8-Anilinonaphtalene-1-sulfonic acid	MDCK	Madin-Darby canine kidney cells
APAF	Apoptosis protease activating factor	MLS	Mitochondrial localisation sequence
ATP	Adenosyltriphosphate	NaCl	Sodium chloride
BH	Bcl-2 homology region	NAG	<i>N</i> -acetylglucoseamine
bp	Base pairs	NAM	<i>N</i> -acetyl muramic acid
C 18:1	Oleic acid	NLS	Nuclear localisation sequence
C 16:0	Palmitic acid	NMR	Nuclear magnetic resonance
C 14:0	Myristic acid	NOX	NADH oxidase
C 6:0	Caproic acid	NPC	Nuclear pore complex
CAD	Caspase-activated deoxyribonuclease	ORF	Open reading frame
Caspase	Cysteine-dependent <u>aspartate</u> -specific protease	PAGE	Polyacrylamide gel electrophoresis
CD	Circular dichroism	PAK2	p21 activated kinase 2
CD4	Cluster of differentiation 4	PARP	Poly-ADP-ribose polymerase
CD8	Cluster of differentiation 8	PCD	Programmed cell death
CDC	Centers for Disease Control and Prevention	PFA	Paraformaldehyde
Ced	Cell death abnormal	PKC	Protein kinase C
Cpm	Counts per minute	PT	Permeability transition
DATP	Deoxyadenosyltriphosphate	RNA	Ribonucleic acid
DD	Death domain	SIgA	Secretory immunoglobulin A
DED	Death effector domain	SDS	Sodium dodecyl sulfate
DNA	Deoxyribonucleic acids	SREBP	Sterol Regulatory Element Binding Protein
DNA-PK	DNA-protein kinase	TGF- β	Transforming growth factor beta
DR	Death receptor	TIGR	The Institute for Genomic Research
EDTA	Ethylenediaminetetra acetic acid	TNF- α	Tumour necrosis factor alpha
ESI-MS	Electrospray ionisation mass spectrometry	TLC	Thin layer chromatography
FAK	Focal adhesion kinase	UV	Ultra violet
FITC	Fluorescein	WHO	World Health Organization
GduHCl	Guanidine hydrochloric acid		
GTP	Guanosine triphosphate		
HAMLET	Human α -lactalbumin made lethal to tumour cells		
HCl	Hydrochloric acid		
HIV	Human immunodeficiency Virus		
HPLC	High performance liquid chromatography		
hsp	Heat shock protein		
ICAD	Inhibitor of caspase-activated deoxyribonuclease		
ICAM	Intercellular adhesion molecule		
ICE	Interleukin-1 β -converting enzyme		
IgA	Immunoglobulin A		
IL	Interleukin		

List of papers

This thesis is based on the following publications and manuscripts referred to in the text by their Roman numeral:

- I. Håkansson A, Zhivotovsky B, Orrenius S, Sabharwal H, and Svanborg C. Apoptosis induced by a human milk protein. *Proc. Natl. Acad. Sci. USA*. 1995; 92: 8064-8068.
- II. Håkansson A, Andréasson J, Zhivotovsky B, Karpman D, Orrenius S, and Svanborg C. Multimeric α -lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei. *Exp. Cell Res.* 1999; 246: 451-460.
- III. Köhler C*, Håkansson A*, Svanborg S, Orrenius S, and Zhivotovsky B. Protease activation in apoptosis induced by MAL. *Exp. Cell Res.* 1999; 249: 260-268.
- IV. Svensson M, Sabharwal H, Håkansson A, Mossberg A-K, Lipniunas P, Leffler H, Svanborg C, Linse S. Molecular characterization of α -lactalbumin folding variants that induce apoptosis in tumor cells. *J. Biol. Chem.* 1999; 274: 6388-6396.
- V. Håkansson A, Köhler C, Svensson M, Mossberg A-K, Zhivotovsky B, Orrenius S, and Svanborg C. Evidence for apoptosis in bacteria; parallels with mitochondria of eukaryotic cells. (In manuscript)

*These authors share first authorship

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General introduction

*Science is built up of facts like a house is built of stones;
But an accumulation of facts is no more a science than a heap of stones is a house*

Henri Poincaré

The project on which this thesis is based has been an adventure, and will remain so for some time to come. It has proceeded much like a murder investigation might, and I was fortunate to appear at the “scene of the crime” at just the right time. With the help of creative thinking, the clues provided and the “forensic” methodology at hand, a number of the “murderer’s” characteristics have been deciphered. We have also figured out partly how the crime was committed, but additional clues are needed to solve the case. Given the work’s murder-myster flavour, I have decided to write this thesis in a narrative style presenting the results just as they have unfolded. I hope that this will help convey to the reader some of the thrill and joy that this work has produced. I also hope that the text will communicate the sense of adventure that I have felt.

The “magic” of human milk

“...60 million or so years of mammalian evolution – or the perfect hand of the Creator, if you prefer – versus the paltry pay-off of perhaps one and a quarter centuries of laboratory fiddling are finally beginning to be seen for what they are: monumental short-sighted scientific impudence and evolutionary hubris.

James Akaré,

(on society’s slowly evolving awareness of the difference
between breast-feeding and artificial feeding)

According to Greek mythology the stars are divine drops of breast milk. The myth tells that our galaxy, the Milky Way, was formed from milk that squirted from the breast of Hera when she was forced by Zeus to feed Hercules, a son Zeus had conceived outside marriage. In ancient Rome breast milk was considered not only proper nourishment for the infant but also a conveyor of personality and temperament (Wickes, 1953a). Wet-nurses (the mothers close to the Emperor naturally did not breast-feed) were chosen with care and consequently wet-nurses in the Emperor’s service had high rank. Greek boys were breast-fed for a shorter time than girls so as not to become too compassionate. The Emperor Nero was partly forgiven for torching Rome as he had been reared by a drunken nurse, and the aggressiveness of Caligula was explained by the drop of blood placed on the nipple every time he was breast-fed.

During the 19th century breast-feeding rates were low in many parts of the Western world and infant mortality rates were extremely high (Wickes, 1953b). Another decline in breast-feeding frequency was observed at the end of the 1960s concurrently with the development of more specialised infant formula (Zetterström, 1998). The formula invasion was so strong and so persuasive that Lee Forrest Hill commented in 1968: “Formula feeding has become so simple, safe and uniformly successful that breast-feeding no longer seems worth the bother” (Hill, 1968). Today, the beneficial effects of breast-feeding are well documented and its superiority over formula is indisputable. Nevertheless a national survey of physicians in the US, undertaken in the early the 1990s concluded that many general physicians or even pediatricians did not believe that breast-feeding was the most beneficial form of infant feeding or that it protected from infections (Freed *et al.*, 1995).

Nutritional advantages of breast milk.

Breast-feeding provides a well-balanced diet that continuously changes composition according to the age and needs of the individual child (Picciano, 1998; R  ih  , 1985). This has led the World Health Organization to recommend breast milk as the sole source of nutrition for the first 4 to 6 months of life, and that breast-feeding should continue with complementary foods for up to 2 years of age or more (WHO, 1995).

Protein

Human milk has the lowest protein concentration of all mammalian milks (Table 1) (Heine *et al.*, 1991; Kunz & Lonnerdal, 1993; R  ih  , 1985). The protein concentration is higher in the first milk, the colostrum (16 g/l), but levels off during the first days of breast-feeding. Whey proteins predominate in human milk, as compared with over 80% caseins in bovine milk. The major protein in human milk is α -lactalbumin (2.5 g/l) whereas bovine milk contains predominantly α -casein. This makes the amino acid composition of these milks very different with a higher content of cystine and tryptophan in human milk. These amino acids are needed for vital intracellular processes like the production of glutathione and as precursor to serotonin (Davis *et al.*, 1994; R  ih  , 1985).

The differences in protein profiles and concentrations in mammalian milks are important in terms of producing breast milk substitutes. Studies suggest that a high protein intake associated with formula-feeding may cause metabolic maladaptions that persist later in life. Kramer *et al* showed that formula feeding is correlated to obesity in adolescence (Kramer, 1981).

Lipids

Milk lipids are the major source of energy for the infant. Human milk contains approximately 3-4.5% total lipids, the majority being triglycerides (98%), with small amounts of phospholipids and sterols (Jensen, 1996; Jensen *et al.*, 1990) (Table 1). The major difference between human and bovine milk is in the type of fatty acids. The bovine milk has more short-chained saturated fatty acids (C4:0, C10:0, and C14:0), while long-chained and unsaturated (C16:0, C18:1 and C18:2) predominate in human milk. The relatively high concentrations of poly-unsaturated fatty acids (PUFA; linoleic acid and α -linolenic acid) in human milk are essential for the infant's normal development and will be used in the production of precursors for leukotrienes and prostaglandins, and as a major constituent of the cell membranes of the cerebral cortex and the retina (Hamosh, 1998).

Table 1. The composition of human and bovine milk.

Milk fraction	Concentration (g/l)		Milk fraction	Concentration	
	Human milk	Bovine milk		Human milk	Bovine milk
Protein^a			Lipids^b		
Total	8.5-8.9	33-34.5	Total	30-45 g/l	37 g/l
Casein	2.5 (35%)	26-28 (79%)	<u>TG</u>	98%	98%
<u>Whey</u>	6.1-6.4 (65%)	6.8-7.0 (21%)	<u>Phospholipids</u>	1.3%	0.8%
α -Lactalbumin	2.5-2.6	1.2-1.3	<u>Sterols</u>	(0.4%)	(0.3%)
β -Lactoglobulin	-	3.0-3.3	<u>Fatty acids</u>		
Lactoferrin	1.7-2.7	Trace	Short chained	1.3%	9.1%
			(C4-10)		
Lysozyme	0.5	Trace	12:0	3.1%	3.1%
IgA	0.6-1.0	0.03	14:0	5.1%	14.2%
Serum albumin	0.3-0.5	0.3-3.0	16:0	19-25%	42.7%
			16:1	5.7%	3.7%
			18:0	5.8-9.7%	5.7%
Carbohydrates^c			18:1	28-46%	16.7%
Total	77-80	40-50	18:2	6-17%	1.6%
Lactose	55-70	40-50	18:3	0.1-1.4%	1.8%
<u>Oligosaccharides</u>	3.0-6.0	Trace	20:4	0.4-0.7%	Trace
Lacto- N -tetraose	0.5-1.5	Trace			
Lacto- N -fucopentaose I	1.0-1.5	-			
Lacto- N -fucopentaose II	0.5-1.0	-			
NeuAc lactose	0.4-0.8	0.03-0.06			
NeuAc-lacto- N -tetraose	0.3-1.4	Trace			

^a Adapted from (Heine *et al.*, 1991; Lönnerdal, 1985 #70; Råihä, 1985).

^b Adapted from (Jensen, 1996; Jensen *et al.*, 1980; Jensen *et al.*, 1990)

^c Adapted from (Kunz & Rudloff, 1993; Picciano, 1998)

Carbohydrates

Human milk is also a rich source of carbohydrates with higher levels of lactose than in bovine milk (Table 1). The number of complex sugars outnumber by far those in bovine milk, where only a few can be found (Jensen *et al.*, 1990; Picciano, 1998). The complex sugars are free oligosaccharides, glycoproteins or glycolipids. The oligosaccharides in human milk have, with few exceptions, lactose in the reducing end which is elongated by other monosaccharides (Kunz & Rudloff, 1993). They can be classified into seven major groups where fucosyl and sialyl derivatives of lactose and lacto-*N*-tetraose are the major components (Table 1) (Kobata, 1977; Kunz & Rudloff, 1993). The function of these soluble sugars remains poorly characterised. They have been shown to be important, not so much for nutrition as for the defence against microbes (see below).

Anti-microbial properties of milk

Breast-feeding confers protection against infections.

Epidemiological data provide compelling evidence that breast-feeding protect against infections. Breast-fed children have a lower incidence of gastrointestinal infections, respiratory tract infections, meningitis and urinary tract infections, and breast-feeding has been associated with a lower mortality rate from these infections in the developing world (Table 2) (Cunningham *et al.*, 1991; Golding *et al.*, 1997a; Golding *et al.*, 1997b; Hanson, 1998).

Although the protective effect of breast-feeding is more obvious in developing countries, where contaminated water and high exposure increase the spread of pathogens, this is valid in the industrialised world as well. The protective effect is most pronounced against gastrointestinal infections, but strong protection is also observed for lower respiratory tract infections, otitis media and meningitis (Table 2). Protection against urinary tract infections have been suggested in two studies (Mårild *et al.*, 1990; Pisacane *et al.*, 1992), but the protection against the common cold needs further evaluations due to inconsistent results between studies.

The protection provided by breast-feeding has been attributed to two factors: the anti-microbial and immune-modulating factors in the milk, and reduced exposure to pathogens.

Immune modulation

There are reports of hormones and growth factors in human milk with the potential to modulate the infant's immune system (Garofalo & Goldman, 1998). Milk contains

both pro-inflammatory (IL-1 and TNF- α) and anti-inflammatory (IL-10, TGF- β) cytokines. Colony-stimulating factors and chemokines have also been reported. The immune system of the young infant has a reduced ability to produce these cytokines as compared with adult immune cells. By interacting with the mucosa/lymphoid system of the respiratory and gastrointestinal tract, these factors may prime the immune system of the child and potentiate a correct and optimal function (Grosvenor *et al.*, 1993). The epithelial barrier is not fully developed in early infancy and allows the uptake of intact proteins. Growth factors, such as epidermal growth factor, have been suggested to increase the maturation of the gut epithelial barrier, assist in its closing, and protect against invasion of pathogens (Wold & Adlerberth, 1998).

Table 2. The effect of breast-feeding against infection. A compilation of relevant studies.

Conferred protection against:	References
Mortality	(Victora <i>et al.</i> , 1987).
Gastrointestinal infections (enteritis)	(Beaudry <i>et al.</i> , 1995; Dewey <i>et al.</i> , 1995; Howie <i>et al.</i> , 1990; Molbak <i>et al.</i> , 1994; Raisler <i>et al.</i> , 1999; Villalpando & Hamosh, 1998)
Respiratory infections	(Cushing <i>et al.</i> , 1998; Pisacane <i>et al.</i> , 1994; Popkin <i>et al.</i> , 1990; Saarinen, 1982); Wright, 1989 #251]
Otitis media	(Alho <i>et al.</i> , 1990; Aniansson <i>et al.</i> , 1994; Duncan <i>et al.</i> , 1993; Teele <i>et al.</i> , 1989)
Meningitis	(Levine <i>et al.</i> , 1999; Moodley <i>et al.</i> , 1999; Silfverdal <i>et al.</i> , 1997; Takala <i>et al.</i> , 1989)
Urinary tract infections	(Mårild <i>et al.</i> , 1990; Pisacane <i>et al.</i> , 1992; Pisacane <i>et al.</i> , 1990)
Mutiple infections ^a	(Beaudry <i>et al.</i> , 1995; Chandra, 1979; Cunningham, 1979; Dewey <i>et al.</i> , 1995; Gerrard, 1974; Mata, 1984; Narayanan <i>et al.</i> , 1981; Raisler <i>et al.</i> , 1999; Scariati <i>et al.</i> , 1997; Wright <i>et al.</i> , 1998)

^a Including studies where more than one of the above mentioned infections was investigated.

Anti-infective molecules.

In 1961 Hanson demonstrated that milk contains secretory IgA (SIgA) and provided an explanation as to how milk can be a modulator of immuno-competence in the infant (Hanson, 1961). The child ingests approximately 0.5-1 g of SIgA daily. SIgA have been shown to confer protection against several pathogens, for example *Haemophilus influenzae* (Kauppi *et al.*, 1993) and cholera toxin (Glass *et al.*, 1983), as well as a variety of viral infections (Okamoto & Ogra, 1989).

In addition, human milk contains a large number of non-immunologic factors that may confer resistance to infection (Xanthou, 1998) (Table 3). Lactoferrin is an 80 kDa protein that inhibits bacterial growth *in vitro* due to its iron-scavenging effect. The N-terminal peptide lactoferricin B that is formed after pepsin digestion is bactericidal against gram-negative bacteria and has been reported to kill *Candida albicans* (Bellamy *et al.*, 1992; Bellamy *et al.*, 1993; Reiter, 1984; Yamauchi *et al.*, 1993).

Lysozyme lyses bacteria by hydrolysing the β -1,4 linkage between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in the bacterial cell wall. The concentration in human milk is rather high in comparison with other mammalian milks, but the *in vivo* relevance of lysozyme in human milk remains to be defined (Reiter, 1984).

Human milk also contains lactoperoxidase, factors of the complement system and fibronectin that help in the attack of pathogens (Xanthou, 1998) and modified β -lactoglobulin has been shown to inhibit binding of HIV to CD4 positive T cells (Neurath *et al.*, 1996). Milk lipids, especially free fatty acids, lyse viral particles and kill bacteria (Isaacs & Thormar, 1991; Kabara, 1980; Kabara *et al.*, 1972; Sarkar *et al.*, 1973)

Anti-adhesive molecules in milk.

Human milk contains a variety of complex carbohydrates with direct effects against pathogens (Table 3) (Newburg, 1999; Zopf & Roth, 1996). The carbohydrates bind to viral and bacterial lectins and competitively inhibit binding to the corresponding cell-bound glycoconjugates. This results in inhibition of adherence of viruses, bacteria or their toxins to the cells in the mucosa. Human milk mucin was shown to inhibit the binding of S-fimbriated *Escherichia coli* to buccal epithelial cells *in vitro* (Schroten *et al.*, 1993). Lactadherin, a highly glycosylated protein from the fat globule membrane, can protect gastrointestinal cells from rotavirus infection *in vitro* and also protect breast-fed infants from symptomatic rotavirus infections (Newburg *et al.*, 1998). Glycosaminoglycans have been found to inhibit the binding of HIV gp120 to CD4 positive T cells (Newburg *et al.*, 1995) and are together with gangliosides associated with protection against RSV infection in infants. (Portelli *et al.*, 1998).

Studies of the urinary tract have identified cell surface glycoconjugates as the receptors for attaching bacteria (Leffler & Svanborg-Edén, 1980). Soluble oligosaccharide epitopes were later shown to inhibit infection in an experimental urinary tract infection model (Svanborg-Edén *et al.*, 1982). Respiratory tract bacteria, such as *Streptococcus pneumoniae* also attach via carbohydrate receptors belonging to the lactoseries of oligosaccharide sequences (Andersson *et al.*, 1983). *S. pneumoniae* was shown to use the disaccharide GlcNAc β 1-3Gal β , a minimal receptor sequence in their binding to nasopharyngeal cells. Milk contains these sugars in free form and human milk and carbohydrate fractions from human milk block adherence to nasopharyngeal cells (Andersson *et al.*, 1986). Similar oligosaccharide sequences are recognized by other bacteria and have been shown to block *Escherichia coli*, *Campylobacter jejuni*, *Pseudomonas aeruginosa* and *Candida albicans* binding to epithelial cells (Cravioto *et al.*, 1991; Kunz & Rudloff, 1993; Newburg, 1999). There is also a

Table 3. Molecules in human milk with anti-microbial effects

Molecule	Effect	References
IgA	Anti-adhesive, Neutralising	(Glass <i>et al.</i> , 1983; Kauppi <i>et al.</i> , 1993)
Lactoferrin	Bactericidal against gram-negative bacteria	(Bellamy <i>et al.</i> , 1992; Bellamy <i>et al.</i> , 1993; Yamauchi <i>et al.</i> , 1993)
	Immunomodulatory	(Mattsbj-Baltzer <i>et al.</i> , 1996; Wold & Adlerberth, 1998)
Lysozyme	Lyses bacterial cell wall	(Reiter, 1984)
Lactoperoxidase	Directly and indirectly bactericidal	(Reiter, 1984)
Lipids	Lyse bacteria and viruses	(Isaacs & Thormar, 1991; Kabara, 1980; Kabara <i>et al.</i> , 1972; Sarkar <i>et al.</i> , 1973)
Oligosaccharides	Receptor analogues Inhibit bacterial toxins or microbial lectin ligands	(Andersson <i>et al.</i> , 1986; Cravioto <i>et al.</i> , 1991; Newburg, 1999)
Glycolipids	Inhibit the actions of toxins, such as cholera toxins, shiga toxin	(Newburg <i>et al.</i> , 1995)
Lactadherin	Block rotavirus infection	(Newburg <i>et al.</i> , 1998)

neutralising effect of oligosaccharide sequences on bacterial toxins such as *E. coli* labile toxin. Accordingly, milk components can be used to dissect the specificity of host-microbial interactions.

Other functions of human milk.

There is epidemiological evidence that breast-feeding protects against tumour development in children. An initial report from Davis *et al* showed that the overall incidence of tumours in children up to the age of 15 was lower in children who had been breast-fed than among those who had been formula-fed (Davis *et al.*, 1988). This effect was especially pronounced for lymphomas. A number of case-control studies have since been showing a clear protection against development of Hodgkin's disease but with variable results for non-Hodgkin's lymphoma, acute lymphoblastic leukaemia or other cancer forms ((Davis, 1998) and references therein). Only one study has investigated the duration of breast-feeding in relation to tumour disease (Mathur *et al.*, 1993); it showed a protection against all cancers with the strongest protection against lymphomas. The cases were breast-fed 8 months compared with 10 months for controls. The difference was even more pronounced when exclusive breast-feeding was investigated. Here cases were exclusively breast-fed for an average of 3.2 months compared with 4.6 months for controls.

Breast-feeding has also been reported to protect against development of other immunological diseases, such as allergy (Ahmed & Fuchs, 1997; Saarinen & Kajosaari, 1995), infantile diabetes mellitus (Gerstein, 1994; Mayer *et al.*, 1988) and inflammatory gastrointestinal disease (Koletzko *et al.*, 1989). These results imply that factors in milk may help optimise the function of the immune system and regulate cell populations to avoid tumour development in the rapidly growing neonatal intestinal tract.

Serendipity strikes

In the fields of observation, chance favours the prepared mind

Louis Pasteur

To understand the molecular mechanism of *S. pneumoniae* attachment to host cells an adherence assay had been developed where scraped nasopharyngeal epithelial cells were used as a target for bacterial binding (Andersson *et al.*, 1983). In this model system milk and milk fractions could be introduced and evaluated for their inhibitory effect (Figure 1). In addition to the inhibitory effect of oligosaccharides from milk the group had also reported that components from human milk casein had anti-adhesive activity for *S. pneumoniae* (Aniansson *et al.*, 1990).

In the urinary tract bacterial virulence factors, especially the P-fimbriae of *E. coli*, are highly associated with the severity of infection (Svanborg *et al.*, 1994). In the respiratory tract the situation is more complex. *S. pneumoniae* are frequently carried by healthy individuals but the same strain may also cause infection. The transition from colonisation to infection is poorly understood but may relate to host factors rather than bacterial factors (Andersson *et al.*, 1981). Infection with respiratory viruses often precedes bacterial infection in infants, and it has been suggested that this predispose for bacterial superinfection. (Chonmaitree *et al.*, 1986; Clements *et al.*, 1993; Giebink *et al.*, 1980; Henderson *et al.*, 1992; Ruuskanen & Heikkinen, 1994). To investigate viral-bacterial interactions we wanted to investigate changes in respiratory epithelial cells and attachment following viral infection.

My initial task was to adopt the adherence assay to allow viral infections. We chose to use the lung carcinoma cell line A549, which is permissive for adenovirus infection, and showed that the binding of *S. pneumoniae* was increased following viral infection (Håkansson *et al.*, 1994). To investigate the specificity of binding we used the anti-adhesive fractions from human milk. The casein fraction caused a total inhibition of binding to the A549 cells, but also changed the morphology of the cells when inspecting them under the microscope (Figure 1). The cells shrunk, the nuclei were condensed and the cells looked unhealthy, suggesting that the cells were dying. I investigated the viability of the cells by uptake of trypan blue, a dye that is excluded from living cells, and by thymidine incorporation into DNA of dividing cells.

After treatment with the milk fraction A549 cells failed to incorporate any thymidine and most of them were stained with trypan blue (Paper I). In contrast, scraped nasopharyngeal epithelial cells showed a viability of approximately 50% both before and after treatment, suggesting that these cells were resistant to killing by this milk fraction. Furthermore, the morphology did not change in these cells

(Figure 1). These results taken together, indicated that the milk fraction killed lung carcinoma cells while healthy epithelial cells were resistant to this effect.

The active fraction from human milk casein was named MAL, and was shown to contain a folding variant of the human milk protein α -lactalbumin. The purification and characterisation of this fraction will be presented below (see: Purification and characterisation of the active fraction).

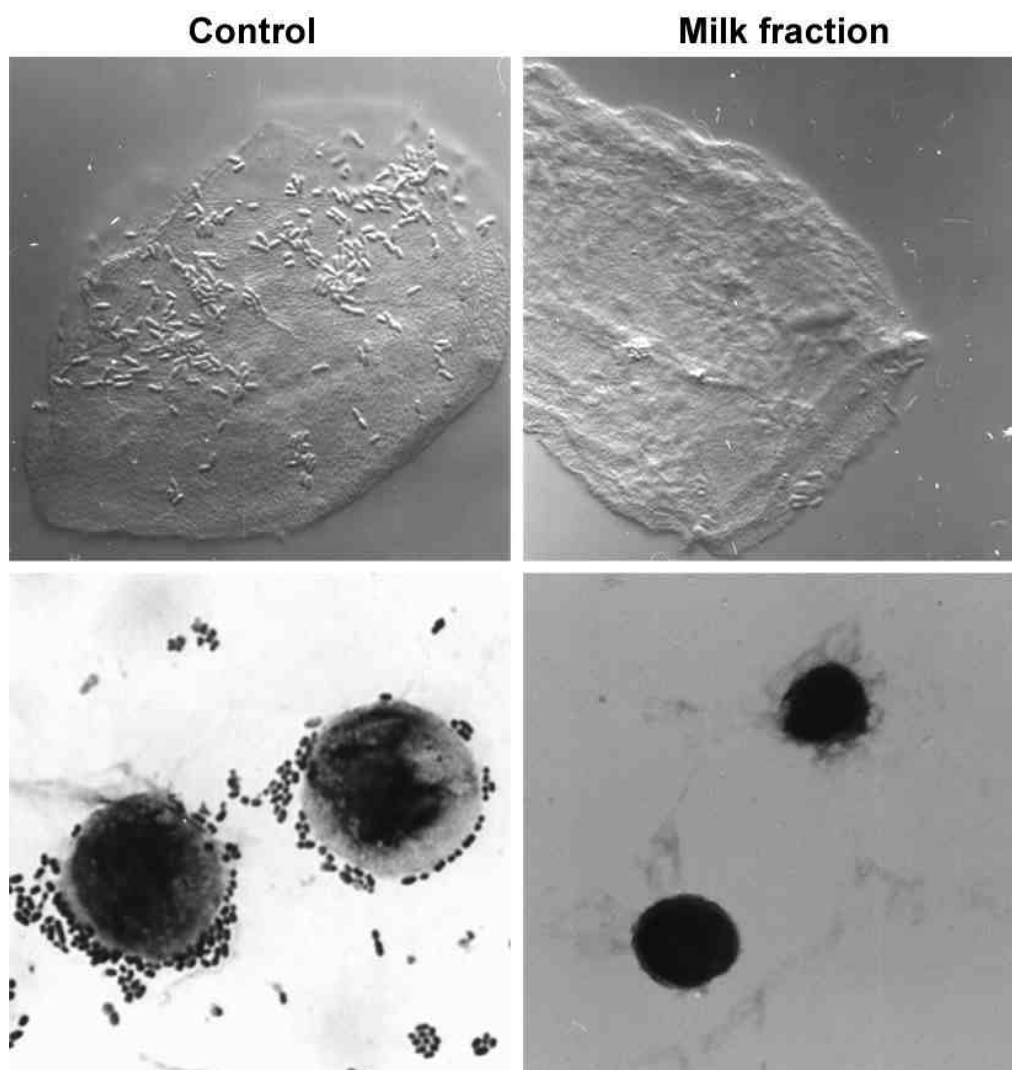


Figure 1 – Adherence of *Streptococcus pneumoniae* to nasopharyngeal epithelial cells (top) and A549 lung carcinoma cells (bottom). Cells were preincubated with milk fraction for 30 minutes before addition of bacteria. The adherence was scored by interference contrast microscopy for nasopharyngeal cells and after staining with May-Grünwald staining reagents for the A549 cells. The micrograph is taken at 40x magnification in a Nikon Diaphot light microscope (Nikon, Japan).

Cell death

*On the basis of our search of the above mentioned dictionaries,
we conclude that the second p is not silent in apoptosis.*

Peter Katsikis and William Leben (Katsikis & Leben, 1995)

The above quotation was part of a continual controversy in the journal *Nature* a few years ago on the pronunciation of the word apoptosis. However, this is not the major problem with the terminology surrounding cell death. The literature on cell death initially presented two conceptually different pathways, one involving the activation of a genetic “suicide” program induced by physiologic events (apoptosis) and the other describing the passive destruction of a cell from noxious stimuli (necrosis) (Kerr *et al.*, 1972). As will be seen the definition of these terms has become more and more difficult based on the accumulation of biochemical and cell biological data in different model systems (Vaux, 1999). As yet there is no need to abandon the original distinction, but to bear in mind that apoptosis and necrosis are not absolute entities.

Cell death – a short history

Cell death in advanced stages of tissue breakdown has been known since the days of Galen and had been called necrosis from the Greek word for dying. However, the notion of cells as structural elements of all living things was not postulated until 1838 by Schleiden and 1839 by Schwann while investigating plants and animals, respectively (Mazzarello, 1999). The first report of naturally occurring cell death was by Voigt (1842) who described a morphology of dying cartilaginous cells very much like what would later be known as apoptosis (Clarke & Clarke, 1996; Majno & Joris, 1995). In his 1858 “Cellular pathology” Virchow introduced the term necrobiosis, which he defined as the spontaneous death of cells as a natural part of their lives.

The introduction of histologic stains in the 1870s had a great impact on cell death research. Physiologic cell death was described by Flemming in 1885 using ovarian follicles. In this model he saw that the epithelial lining of regressing follicles had a distinct morphology that he named chromatolysis, in reference to the typical broken up nuclei of the cells. Identical results were presented by Franz Nissen the same year for regressing mammary tissue.

In 1914 Ludwig Gräper argued that there must be a mechanism to counterbalance mitosis in a tissue. He seconded Flemming’s chromatolysis as the mechanism and indicated that the debris from that type of elimination was taken up

by neighbouring cells. The latter idea had been sparked in 1883 by Metchnikoff, who found phagocytes in the vicinity of dying cells and by Mayer who argued that these cells were there to get rid of the debris. Gräper's paper postulates the importance of this mechanism for morphogenesis of organs in foetal development as well as in endocrine organs later in life, and thus it was an early first grasp of a physiologic mechanism of cell death.

The importance and characteristics of physiologic cell death during embryogenesis was masterly reviewed by Glücksmann in 1951 (Glücksmann, 1951). Dying embryonal cells showed the same morphology in most species, and differed from that induced by noxious stimuli. Twenty years later this morphology was associated not only with embryonic cell death but also with a number of other physiological and pathological circumstances, such as turn-over of adult tissue, as a result of hormone deprivation of endocrine organs and in tumour regression (Kerr *et al.*, 1972). Kerr *et al.* named this type of cell death apoptosis from the Greek word, for leaves falling from a tree or petals from a flower. They suggested that apoptosis was an active, inherently programmed phenomenon of controlled deletion of cells that counteracted mitosis in the regulation of cell populations.

Cell death morphology

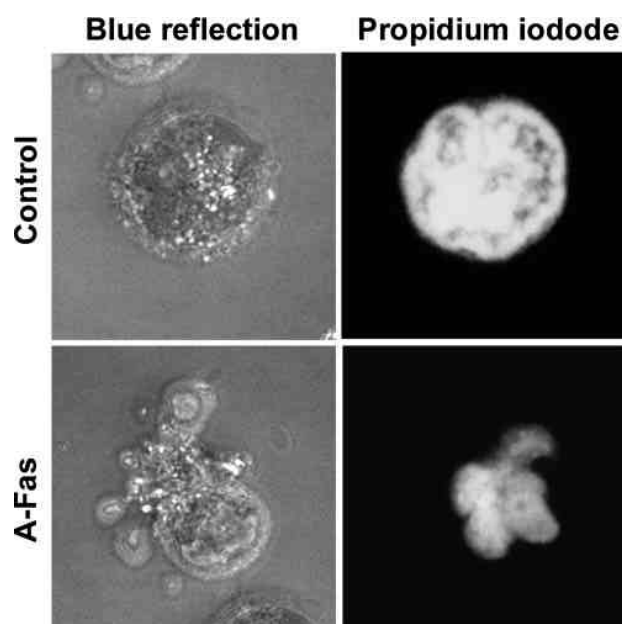
Necrosis

The morphology of necrotic death has been described for a variety of stimuli with ischemia being the best studied model (Rubin & Farber, 1998; Wyllie *et al.*, 1980). The ischemic cells show early cellular swelling, marginal clumping of nuclear chromatin, dilatation of endoplasmic reticulum and mild dispersal of ribosomes. With continued stimulation the cells soon reach an irreversible stage where mitochondrial swelling can be observed that will proceed to lysis of organellar and plasma membranes, with dissolution of ribosomes and lysosomes. The chromatin often appears compacted (pyknosis) and the DNA may be clumped in small discrete masses (karyorhexis). Finally a nuclear ghost will be observed after lysis of the nucleus (karyolysis). The mechanisms involved in this type of cell death are thought to be a result of ion-pump failure from a drop in ATP levels (Eguchi *et al.*, 1997; Leist *et al.*, 1997). This will result in a Ca^{2+} influx and lysis of membranes causing hydrolysis of proteins by lysosomal enzymes. Also the release of lysosomal endonucleases can result in an unspecific DNA fragmentation pattern.

Apoptosis

Apoptotic cells show a different morphology (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Early apoptotic cells show chromatin aggregated in large compacted granular masses margined to the nuclear membrane, cytoplasm condensation and disappearance of microvilli. Cells separate themselves from their neighbours, and organelles are packed together with structures intact, in the decreasing cytoplasmic volume. The plasma membrane will start budding with a final release of membrane-enclosed vesicles with nuclear and organellar material (apoptotic bodies) that are rapidly digested by neighbouring cells or tissue macrophages (Figure 2). The phenomenon is not easily observed *in vivo* since apoptosis usually affects individual cells scattered in the tissues and as these cells are so efficiently eliminated. Most cell remnants will be observed inside neighbouring cells. In cell culture systems where phagocytosis is absent the cells will undergo secondary changes with an end stage morphology resembling necrosis.

Figure 2. Apoptotic morphology of Jurkat T cells after stimulation with anti-Fas antibodies. Jurkat cells were treated with anti-Fas antibodies for 3 hours. The cells were fixed in cold 70% ethanol for 30 min and 25 µg/ml of propidium iodide was added. The cells were then inspected in confocal microscopy (Bio-Rad MRC-1024 confocal equipment attached to a Nikon Eclipse E800 microscope). The left panel depicts a light microscopy image taken with a blue reflection filter and the right panel show the DNA staining with propidium iodide. The anti-Fas treated cells displayed apoptotic morphology with nuclear condensation and apoptotic body formation.



One of the biochemical markers for apoptosis is the specific DNA fragmentation profile that is readily detected by gel electrophoresis. Chromatin is initially cleaved with formation of high molecular weight fragments (approx. 700, 300 and 50 kbp) (Zhivotovsky *et al.*, 1994), postulated to be caused by the topology of the DNA (Lagarkova *et al.*, 1995). This fragmentation proceeds into an oligonucleosome-length DNA fragmentation pattern (DNA “laddering”) associated with the cleavage of DNA between the nucleosomes giving rise to fragments consisting of oligomers of 200 bp (Wyllie, 1980).

Our “funny-looking” cells examined

The morphologic appearance of the cells exposed to MAL suggested that the cells might have died from apoptosis (Figure 1). This was confirmed in transmission electron microscopy showing a typical morphology of apoptosis with chromatin condensation and marginalisation of DNA to the nuclear envelope, cell shrinkage and membrane blebbing (Paper I).

Further studies on the mechanisms were performed in collaboration with the laboratory of Professor Sten Orrenius, a pioneer in the area of apoptosis research, at Karolinska Institute, Stockholm. A549 cells and rat thymocytes were analysed for high molecular weight and oligonucleosome-length DNA fragmentation (Paper I). Incubation with MAL caused the formation of high molecular weight DNA fragments in both cells. Oligonucleosome-length fragmentation was observed only in the rat thymocytes (Paper I). Other mammalian milks, such as bovine milk, goat milk and kangaroo milk did not show any activity. These results indicated that MAL induced apoptosis rather than necrosis.

MAL's impact on tumour cells.

A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli that evoked the change

Sir Rupert Willis (Willis, 1952)

The effect of MAL was initially described using the A549 lung carcinoma cell line. Subsequent experiments demonstrated that MAL had broad activity against a variety of cell lines; results are summarised in Table 4. The experiments distinguished four groups of cells with varying sensitivity:

1) Cell lines of lymphoid origin were highly sensitive to MAL with 0.3-0.5 mg/ml required to kill 50% of the cells in 6 hours (LD_{50}). These cells readily underwent apoptosis, with cell shrinkage, membrane blebbing and apoptotic body formation and they always displayed DNA fragmentation proceeding to oligonucleosome-length fragments. Human cells and cells of murine origin were equally sensitive, suggesting no species specificity. Healthy peripheral lymphocytes (group 4) were more sensitive than other differentiated cells with an LD_{50} of 2 mg/ml, but still the healthy lymphoid cells were more resistant than the tumour cells of lymphoid origin with more than four times higher concentrations required to kill the non-transformed cells.

2) Eighteen carcinomas of different origin have been tested. As the carcinomas died more slowly the LD_{50} was measured after 24 hours. They required a median MAL-concentration of 1.0 mg/ml. The cells shrunk and showed nuclear condensation, but rarely showed apoptotic body formation. Upon MAL-treatment the carcinomas displayed only high molecular weight DNA fragmentation, suggesting that in these cells MAL did not activate the required endonuclease activity. The cells did not lack the enzyme responsible for this DNA fragmentation as they displayed oligonucleosome-length DNA fragmentation in response to other stimuli. The lack of oligonucleosome-length DNA fragmentation is common in many tumour cell lines undergoing apoptosis (McConkey *et al.*, 1996). It should be noted that adenocarcinomas of the breast were as sensitive as tumours of other origin, suggesting no inherent resistance to milk proteins. One cell line, MDCK, is derived from healthy kidney tissue and is non-transformed. This cell line was sensitive to MAL, but was the only one of epithelial origin to display oligonucleosome-length DNA fragmentation.

3) Two embryonic or immature cell lines from embryonic tissue (human endothelial lung cells and human foreskin fibroblasts) were tested for MAL-induced cell death. The viability decreased to 50% at a concentration of approximately 2.5 mg/ml. Immature cells of lymphoid origin (thymocytes from rat) were as sensitive as the lymphoid tumour cells.

4) Non-transformed healthy cells were resistant to MAL. Epithelial cells remained fully viable after 24 hours of incubation with high concentrations of MAL. Granulocytes were also resistant. Primary cultures of human renal tubular epithelial cells (HRTEC) from kidney were resistant to the apoptosis-inducing effect of MAL (Figure 3). The cells survived physiologic concentrations, but died with a necrotic morphology when exposed to more than 4 mg/ml of MAL.

These experiments demonstrated that tumour cells were sensitive to MAL, but that healthy cells (especially of epithelial origin) did not undergo apoptosis in response to MAL. Immature and embryonic cells fell into a group with intermediate resistance. These results posed two important questions for further study:

1. What is the mechanism of MAL-induced apoptosis in sensitive cells?
2. What is the difference in terms of MAL activity between resistant, healthy cells and sensitive tumour cells?

Table 4. The sensitivity of cells of different origin for MAL induced apoptosis

Cells	LD ₅₀ ^a
Carcinomas	1.0 (0.75-1.75)
A549, human lung carcinoma (type II)	1.25
NCI, human bronchial carcinoma	1.0
HTB9, human kidney	- ^b
A-498, human kidney	1.0
GMK, monkey kidney	1.0
Vero, monkey kidney	1.75
MDCK, dog kidney	0.75
J 82, human bladder transitional carcinoma	0.75
Caco-2, human intestine	1.0
HT-29, human intestine	1.25
MCF-7, human breast adenocarcinoma	1.25
SK-BR-3, human breast adenocarcinoma	1.25
MBD-231, human breast adenocarcinoma	1.25
T47d, human breast ductal carcinoma,	1.25
MBD-175VII, human breast ductal carcinoma	1.5
PC-3, human prostate carcinoma	1.0
DU-145, human prostate carcinoma	1.25
U37, human glioma	2.0
BT4C, mouse glioma	0.5
Sarcoma	0.75
WEHI, mouse fibrosarcoma	0.75
Lymphoid cell lines	0.45 (0.3-0.5)
Jurkat, human lymphocytic leukaemia (T)	0.4
L1210, mouse prolymphocytic leukaemia	0.3
B9, mouse lymphocytic leukaemia	0.4
HL-60, human promyelocytic leukaemia	0.5
K562, human myelogenous leukaemia	0.5
U-937, human promyelocytic leukaemia	0.5
Embryonic cells	1.5 (0.5-2.5)
Hel, human endothelial lung	2.5
HFF, human foreskin fibroblast	- ^c
Thymocytes, rat	0.5
Primary cultures	3.8 (2.0->5.0)
Urinary tract epithelium, human	- ^c
Nasopharyngeal epithelium, human	- ^c
HRTEC, renal tubular epithelium, human	4.5
Granulocytes, human peripheral	>5
Lymphocytes, human peripheral	2.0

^a The concentration of MAL required to kill 50% of the cells. Numbers in bold denote median values (range). The LD₅₀ was measured with L1210 cells as reference cells in the experiments. The cell lines studied early in the investigation (HTB9, A-498, GMK, Vero, MDCK, J82, Caco-2 and HT-29) were not run with reference cells.

^b Tested only at a concentration of 5 mg/ml. At this concentration HTB9 cells showed 0% viability,

^c Tested only at a concentration of 5 mg/ml. At this concentration HFF cells showed 8% viability, and urinary and nasopharyngeal epithelia were resistant.

Mechanism of MAL-induced apoptosis

If there is no life there is hope

Anders Håkansson

Investigating MAL-induced cell death

Four cell types were chosen to investigate the mechanism of MAL-induced apoptosis (Figure 3). The mouse leukaemia cell line L1210 was used as a model system for lymphoid tumours, and was replaced by the Jurkat cell line used when human cells were required. The human lung carcinoma A549 was used as a model for carcinomas and HRTEC cells were used as a model for mature, differentiated cells.

Sensitivity to MAL-induced apoptosis.

The concentrations and kinetics of cell death were monitored by trypan blue exclusion and DNA fragmentation (Paper II).

The L1210 and Jurkat cells showed similar kinetics and concentration dependence of cell death and DNA fragmentation induced by MAL, with an LD₅₀ of 0.2-0.4 mg/ml for L1210 cells and 0.25-0.5 mg/ml for Jurkat cells (Figure 3). At this concentration high molecular weight DNA fragmentation was detected already after 1 hour. At 3 hours oligonucleosome-length DNA fragmentation was seen, with maximal intensity at 6 hours. With continued incubation the cells underwent secondary lysis and oligonucleosome-length DNA fragments were not detected. The oligonucleosome-length DNA fragmentation was always more pronounced in the L1210 cells than in Jurkat cells.

The A549 carcinoma cells had a higher LD₅₀ (0.7-1.2 mg/ml) and a slower response. Treatment with the LD₅₀ concentration caused a decrease in viability with concomitant formation of high molecular weight DNA fragments visible by gel electrophoresis at 6 hours. The amount of DNA fragments increased with time and maximal fragmentation was observed at 24 hours.

The HRTEC cells were resistant to MAL-concentrations above the physiologic range. At 5 mg/ml 50% of the cells had died at 24 hours but no apoptotic morphology or DNA fragmentation was detected (Paper II). The cells have intact apoptotic machinery that can be activated by other stimuli (Karpman *et al.*, 1998).

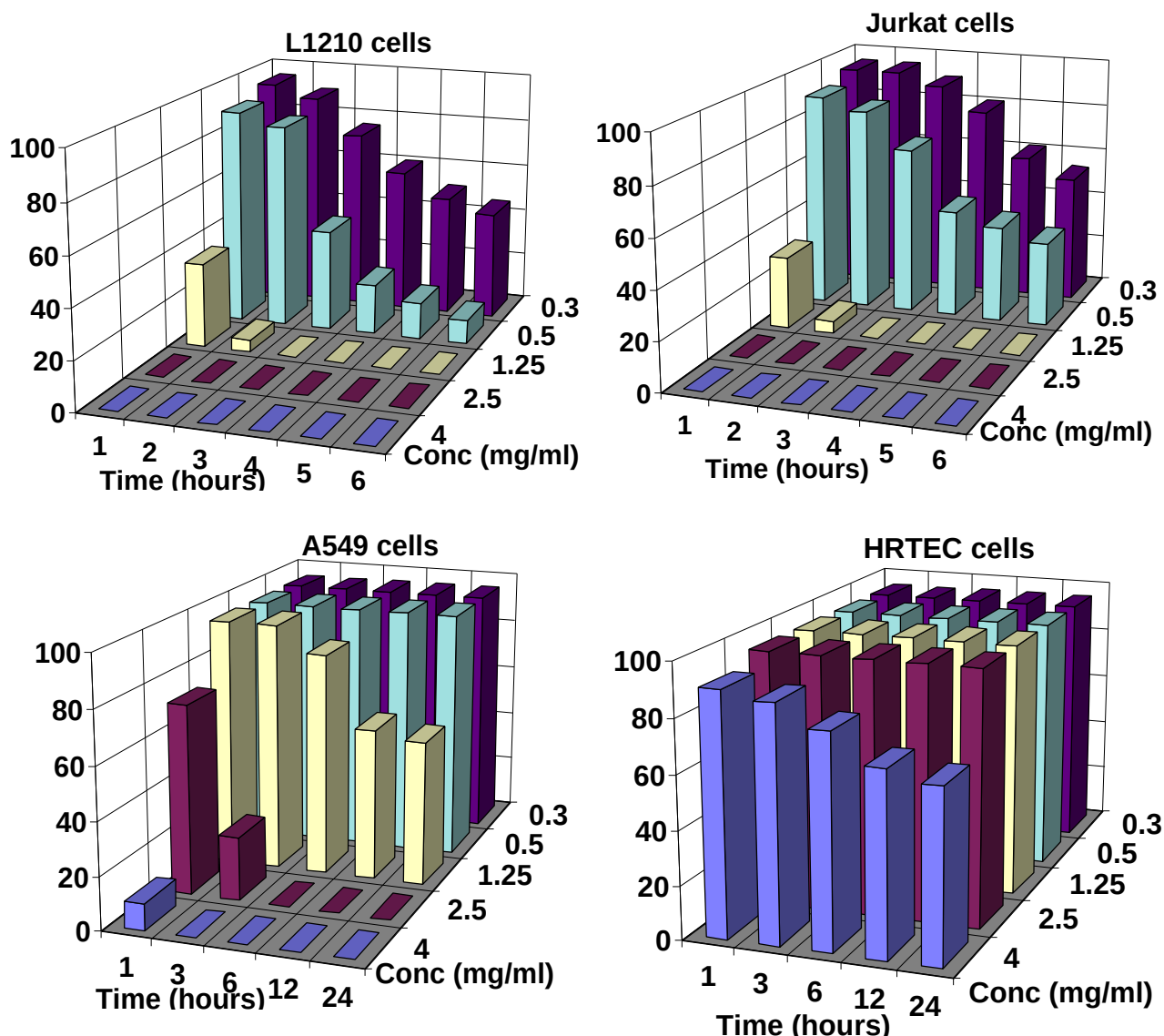


Figure 3 – Dose and time kinetics of MAL-induced cell death. Cells were incubated with different concentrations of MAL for different times and the viability was scored by trypan blue exclusion. The viability is depicted on the Y-axis in per cent.

Association of MAL with the cells.

Possible cellular targets of MAL were investigated by subcellular localisation studies in sensitive and resistant cells (Paper II). The cells were incubated with biotinylated or radiolabelled protein. Localisation of biotinylated protein was inspected after counterstaining with FITC-streptavidin by confocal microscopy and radiolabelled protein associated with subcellular fractions was quantified in a γ -counter.

MAL was shown to bind to the surface of all cell types within 5-10 minutes with maximal binding seen at 30 minutes (Figure 4). MAL bound to the surface of the cells with a patchy distribution in confocal microscopy and the amount of binding was similar between the cell types using radiolabelled protein.

The four cell types showed different staining patterns for the remaining time points. In the most sensitive cells (Jurkat and L1210) a pattern of cytoplasmic granular fluorescence was detected already at 30 minutes and at all later time points in an increasing number of cells (Figure 4). In parallel nuclear accumulation of protein was detected with over 70% of the nuclei stained at 6 hours. The nuclear accumulation was not simultaneous in all cells in the population, but occurred more rapidly in some cells than in others. At the later time points, where most of the cells displayed nuclear localisation, there were still cells with mainly surface binding. These results may reflect that differences in membrane translocation and intracellular transport are dependent on cell cycle phase. Radiolabelled MAL was shown to rapidly associate with the nuclear fraction. At 1 hour 60% of cell associated radioactivity was detected in this fraction with the remaining radioactivity associated mainly with the fraction containing plasma membrane and mitochondria. At 6 hours the majority of MAL was associated with the nuclear fraction. This confirmed the distribution of the protein with biotinylated MAL.

The staining pattern of the A549 cells resembled the Jurkat cells, but cellular uptake and transport was slower. Granular fluorescence was detected at 3 hours and nuclear accumulation was first observed at 6 hours (Figure 4). At 24 hours when maximal DNA fragmentation was detected approximately 50% of the nuclei had been stained. In subcellular fractionation experiments the accumulation of radioactivity to the nuclear fraction resembled that of the fluorescent protein. Nuclear association of radioactivity was low initially and started to increase at 6 hours. At 24 hours 50% of cell associated radioactivity was detected in the nuclear fraction. The rest of the radioactivity was associated with the fraction containing mitochondrial and plasma membrane or with the cytosolic fraction.

The HRTEC cells did not show any protein internalisation by confocal microscopy at any time point; only surface binding was detected (Figure 4). On the other hand, radioactivity was measured in the cytoplasmic fraction in subcellular fractionation experiments after 6 and 24 hours. The discrepancy between the methods may be explained as the cytosolic fraction will contain radioactivity that dissociated from the other fractions during homogenisation and centrifugation.

These studies demonstrated a difference in the intracellular distribution of the protein between sensitive and resistant cells. MAL bound to the surface of all cells but was distributed to different intracellular locations only in sensitive cells. This suggested that binding, internalisation and intracellular transport of the protein may be important for its apoptosis-inducing activity. A search for targets for MAL was therefore initiated.

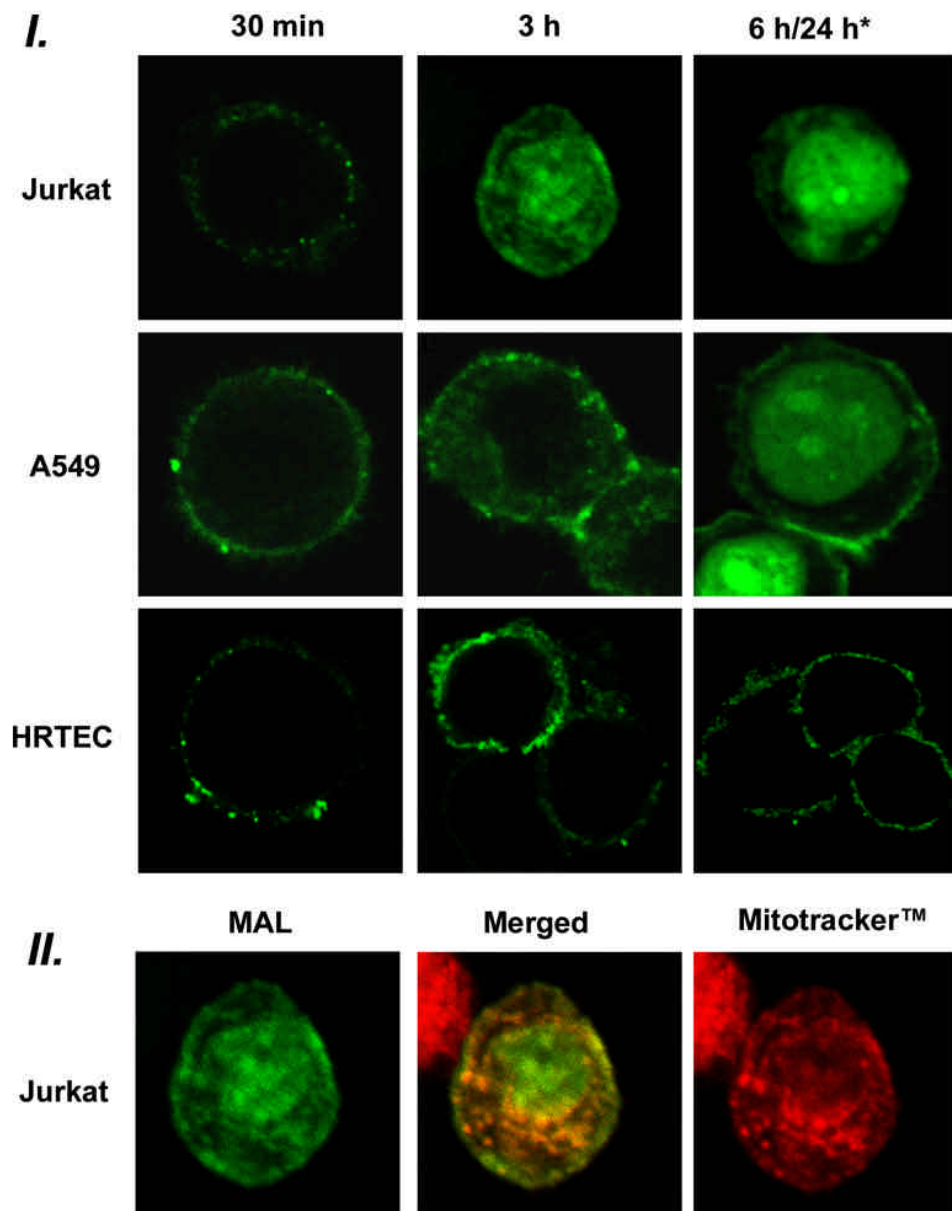


Figure 4 – Subcellular distribution of biotinylated MAL in sensitive and resistant cells.

Panel I. Cells were incubated with biotinylated MAL, fixed with 4% PFA and permeabilised with 0.1% saponin. After counterstaining with FITC-conjugated streptavidin cells were inspected by confocal microscopy. *Cells were examined after 30 minutes, and 3 hours and finally at 6 (Jurkat cells) or 24 hours (A549 and HRTEC cells).

Panel II. Jurkat cells were incubated with biotinylated MAL for 3 hours, fixed with 4% PFA and permeabilised with 0.1% saponin. After counterstaining with FITC-conjugated streptavidin and Mitotracker-Red the cells were inspected by confocal microscopy. Co-localisation of MAL with mitochondria was observed.

Cell surface receptors

MAL was shown to bind to all cell types by confocal microscopy (Figure 4, 5). The binding was quantified by flow cytometry. There was no difference in the amount of biotinylated protein binding to the different cell types, confirming the results with radiolabelled protein (Paper II). This suggested that binding could not explain the difference in sensitivity between resistant and sensitive cells. Still binding may be important as it precedes internalisation of the protein and sensitive and resistant cells may differ in receptor expression.

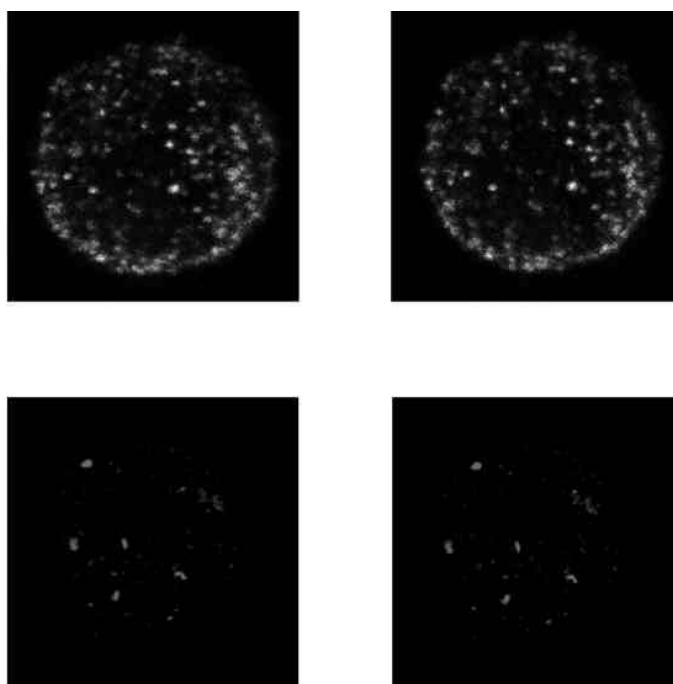


Figure 5 – Stereo view of MAL-binding to A549 cells. A549 cells were incubated with biotinylated MAL (top) or α -lactalbumin (bottom) for 30 minutes, washed and then FITC-streptavidin was added. Twenty-five consecutive Z-sections were collected using the MRC-1024 Confocal equipment (Bio-Rad, Hemel-Hampsted, UK) and they were projected with 20 degrees difference in orientation. The surface binding of MAL was patchy and non-homogenous. α -Lactalbumin bound poorly.

Limited attention has thus far been paid to the characterisation of MAL's cell surface receptor(s). Some preliminary experiments have, however, been performed.

1. Tunicamycin was used to inhibit the N-linked glycosylation of proteins on the cells. PDMP is a ceramide analogue that was used to inhibit the glycosylation of a variety of glycolipid species on the cell surface (Radin & Inokuchi, 1991). Neither

tunicamycin nor PDMP treatment influenced binding of biotinylated MAL in flow cytometry analysis, suggesting that cell surface glycoconjugates are not involved (Table 5).

2. Trypsin, pepsin and proteinase K were used as crude tools to interfere with cell surface proteins. Treatment with all three reagents decreased the binding substantially suggesting that the receptor-function is protein-dependent (Table 5).

Table 5 – Binding of MAL to A549 cells

Cell treatment ^b	Binding (ratio treated/streptavidin) ^a	
	MAL	ALA
None	8.7	1.2
PDMP	8.2	-
Tunicamycin	10.6	-
Trypsin	1.3	-
Proteinase K	1.4	-
Pepsin	3.8	-

^a Biotinylated protein was incubated with A549 cells for 30 minutes. The cells were counterstained with FITC-streptavidin and analysed by flow cytometry. Binding is given as the ratio between the fluorescence of MAL/ALA treated cells and the streptavidin control.

^b Cells were grown in the presence of 50 µg/ml PDMP or 5 µM tunicamycin before binding studies were performed. Cells were also treated with 0.25% trypsin, 2.5 mg/ml pepsin or 2.5 mg/ml of proteinase K for 10 minutes at 37 °C before adding biotinylated protein.

The initiation of cell death commonly involves ligation of receptors belonging to the death receptor family (Ashkenazi & Dixit, 1998; Ashkenazi & Dixit, 1999). The most studied of these receptors is Fas (APO-1 / CD95), which regulates death in the immune system (Debatin *et al.*, 1997). Beside Fas the family of proteins presently constitutes six functional members (TNF-receptor I, death receptors DR3 (Apo 3), DR4 (TRAIL-receptor 1), DR5 (TRAIL-receptor 2) and DR6 and three decoy receptors that have a non-functional intracellular domain (Ashkenazi & Dixit, 1998; Ashkenazi & Dixit, 1999). Induction of apoptosis through ligation of these receptors is thought to involve clustering of the intracellular death domains (DD) followed by the recruitment and activation of caspases, through adaptor molecules (FADD, TRADD) binding to the DD of the receptor (Muzio *et al.*, 1998).

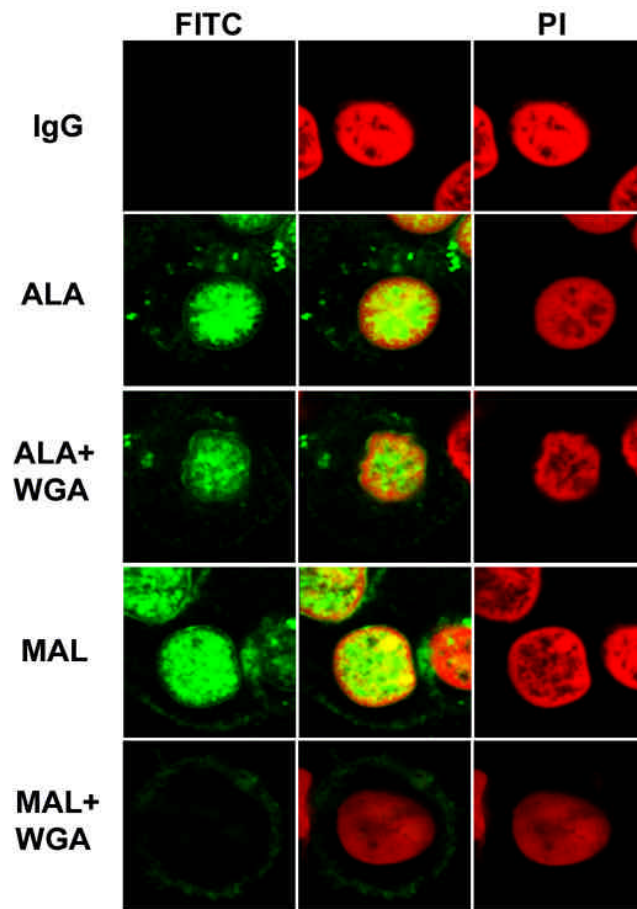


Figure 7 – Nuclear transport of MAL and α -lactalbumin. A549 cells were treated with 40 μ g/ml of digitonin for 5 minutes and washed. Biotinylated MAL or α -lactalbumin was added and nuclear localisation was inspected after counterstaining with FITC-streptavidin. Wheat-germ agglutinin was used as an inhibitor of active nuclear transport. The left row show MAL- or α -lactalbumin-staining (green) and the right row shows DNA staining with propidium iodide (red) with a merged picture in the centre. Nuclear uptake of MAL, but not α -lactalbumin, was inhibited by WGA.

We found no involvement of Fas in MAL-induced apoptosis (Paper III). Jurkat cells were readily killed by ligation of Fas, but the addition of antibodies that block Fas-mediated killing did not affect MAL-induced apoptosis in these cells. A549 cells and L1210 cells were killed by MAL but were not affected by the addition of activating anti-Fas antibodies.

Translocation over the plasma membrane

The granular fluorescence seen in the initial localisation experiments suggested that either MAL was in membrane bound vesicles or that it associated with intracellular structures. Since vesicular uptake may be cytoskeleton-dependent we used the drugs cytochalasin B and colchicin to disrupt the process. No effect was seen on the intracellular uptake of radiolabelled protein. This suggested that uptake was independent of cytoskeleton mediated mechanisms.

Interaction with mitochondria.

The granular fluorescence might also be caused by a co-localisation with mitochondria. This was tested in experiments with MAL and Mitotracker™ in Jurkat and A549 cells. MAL was seen to co-localise with mitochondria in both cell types during the course of its transport into the cell (Figure 4, panel II) (Paper III).

Mitochondria play an important role in apoptotic cell death, by releasing a variety of molecules involved in or activating the execution phase of apoptosis (Liu *et al.*, 1996; Susin *et al.*, 1999b). The mechanism of release of factors, such as cytochrome c, from mitochondria is elusive but three major pathways have been suggested. Release have been postulated to occur as a consequence of permeability transition (PT) that results in disruption of the mitochondrial transmembrane potential (Cai *et al.*, 1998; Crompton, 1999; Green & Reed, 1998; Kroemer *et al.*, 1997; Marchetti *et al.*, 1996; Susin *et al.*, 1998). This is achieved through the formation of large multiprotein-channels, so-called permeability transition pores which involve proteins from both the inner (ANT, adenine nucleotide translocator and Cyp-D, cyclophilin D) and outer (VDAC, voltage dependent anion channel) mitochondrial membrane. The sudden permeability increase of the inner mitochondrial membrane will cause swelling of the mitochondrial matrix and a disruption of the mitochondrial outer membrane leading to release of factors from the interstitial space. The requirement of PT for cytochrome c release is not uniformly accepted as there are reports where cytochrome c occurs in the cytoplasm before the disruption of the mitochondrial membrane potential (Bossy-Wetzel *et al.*, 1998; Eskes *et al.*, 1998; Kluck *et al.*, 1997; Minamikawa *et al.*, 1999; Vander Heiden *et al.*, 1997; Yang *et al.*, 1997a). Release is thought to result from a selective disruption of the mitochondrial outer membrane

that result from matrix swelling in the absence of PT (Vander Heiden *et al.*, 1997) or from pore formation of pro-apoptotic Bcl-2 family members (discussed below) (Manon *et al.*, 1997; Rosse *et al.*, 1998).

Apoptosis-inducing factor (AIF) is released simultaneously with cytochrome c, and translocates to the nucleus where it causes chromatin condensation and high molecular weight DNA fragmentation (Kroemer *et al.*, 1997; Susin *et al.*, 1999b). In addition adenylate kinase, caspase-2, -3, and -9 as well as heat shock proteins hsp-10 and -60 are released from mitochondria upon apoptosis-induction (Kohler *et al.*, 1999; Krajewski *et al.*, 1999; Samali *et al.*, 1999; Single *et al.*, 1998; Susin *et al.*, 1999a). The heat shock proteins have been shown to accelerate the maturation of caspase-3 (Xanthoudakis *et al.*, 1999). The role of these factors in apoptosis induction and the role of mitochondrial location of caspases remain to be determined.

MAL caused a decrease in the mitochondrial membrane potential in Jurkat cells after 15 minutes and within 30 minutes in A549 cells as probed by the fluorescent dye TMRE in confocal microscopy and flow cytometry. This was followed by detection of cytochrome c in post-mitochondrial extracts at 1 hour in Jurkat cells (Paper III). MAL had a direct effect on isolated mitochondria and caused a rapid release of cytochrome c preceded by swelling of the mitochondrial matrix and depolarisation of the mitochondrial membrane. This effect was seen using isolated mitochondria from both transformed and non-transformed cells (rat liver cells), suggesting that the difference in sensitivity between cell types is not explained at the mitochondrial level.

Caspase activation

Caspases are involved in the execution of apoptosis by cleaving intracellular substrates. This inactivates intracellular proteins that are protecting the cells from apoptosis, and activates other molecules needed in the apoptotic process. The interest in proteases during apoptosis was sparked by the finding that the gene *ced-3* involved in programmed cell death of the nematode *C. elegans* had high sequence homology with the mammalian enzyme interleukin-1 β -converting enzyme (Figure 16) (Miura *et al.*, 1993; Yuan *et al.*, 1993). Since then 13 additional mammalian caspases have been found. They are cysteine proteases but have the rather unusual specificity of cleaving preferentially after Asp (D) residues (Alnemri *et al.*, 1996; Kidd, 1998; Thornberry & Lazebnik, 1998). The caspases have been divided into different groups based both on function and cleavage specificity (Garcia-Calvo *et al.*, 1998; Thornberry *et al.*, 1997). Group I caspases (caspase-1 (ICE), -4 and -5) have a consensus cleavage recognition site of WEHD and have a proposed role in maturation of pro-inflammatory cytokines. They have no clear role in apoptosis even

though overexpression of caspase-1 induces apoptosis in target cells (Miura *et al.*, 1993). Group II caspases (2, 3 and 7) have a consensus substrate specificity of DEXD. Two of them (3 and 7) have a short pro-domain and are thought to act as effector enzymes in the cleavage of substrates. Caspase-2 has a longer prodomain and may be involved in the interaction with death domains. The group III caspases (6, 8, and 9) have longer pro-domains, cleave preferentially (I/V/L)EXD and are shown to be upstream initiator caspases that set the cascade going. Caspase-6 has been shown also to have effector functions.

Caspases are present in the cytoplasm (some are also in mitochondria and caspase-2 can be found in the nucleus (Colussi *et al.*, 1998) as latent pro-forms (Thornberry & Lazebnik, 1998). The activation of initiator caspases is thought to involve auto-processing into the active conformation. This can be achieved through oligomerisation in the presence of death receptors as described above or via activation of the apoptosome complex through release of apoptogenic factors from mitochondria (Adrain *et al.*, 1999; Liu *et al.*, 1996; Muzio *et al.*, 1998; Saleh *et al.*, 1999; Yang *et al.*, 1998). Upon release, cytochrome c will interact with the WD repeat region of Apoptosis protease activating factor 1 (APAF-1) (Zou *et al.*, 1997), leading to oligomerisation of APAF-1 that allows the protein to interact with procaspase-9 (Adrain *et al.*, 1999; Li *et al.*, 1997; Saleh *et al.*, 1999). This binding then leads to the autocatalytic activation of caspase-9 in the presence of dATP. The initiator caspases can then activate the effector caspases and initiate the caspase cascade.

Activation of caspases results in the cleavage of a number of intracellular substrates (Figure 6). These include inhibitors of effector molecules, inhibitors of apoptosis, proteins involved in cytoskeletal integrity, apoptotic body formation, DNA integrity and structure or having other functions. The cleavage of these substrates will favour disassembly of the cell and prepare it for elimination (Brown *et al.*, 1997; Cheng *et al.*, 1997; Clem *et al.*, 1998; Fadeel *et al.*, 1999; Gueth-Hallonet *et al.*, 1997; Martin *et al.*, 1995a; Rosen & Casciola-Rosen, 1997).

In MAL-treated cells, caspase activity was detected at the same time as the release of cytochrome c in Jurkat and A549 cells (Paper III). Caspase group II activity (mostly due to caspase-3) peaked at 2 to 3 hours in both Jurkat and A549 cells, while the caspase group III activity peaked earlier consistent with their role as initiator caspases. The activity of caspase group III was also lower.

Three intracellular protein targets were studied in cells undergoing MAL-induced apoptosis (Paper III). Lamin B is a nuclear, structural protein, and the only substrate known so far to be cleaved by caspase-6. PARP is an enzyme in the nucleus involved in genome surveillance and DNA repair and is cleaved primarily by caspase-3. α -Fodrin is also cleaved by caspase-3, but it is located in the cytoplasm.

PARP and α -fodrin were efficiently cleaved in the cells after 6 (Jurkat) and 11 hours (A549 cells), whereas the cleavage of lamin B was less efficient but still detected.

The MAL-induced caspase activity could be blocked by the general caspase inhibitor zVAD-fmk. Addition of zVAD-fmk to MAL-treated Jurkat cells blocked the formation of oligonucleosome-length DNA fragments. The caspase-3 specific inhibitor DEVD-cmk did not block MAL-induced DNA fragmentation. These results may relate to studies showing that this specific inhibitor is less stable in cells (Kitanaka & Kuchino, 1999).

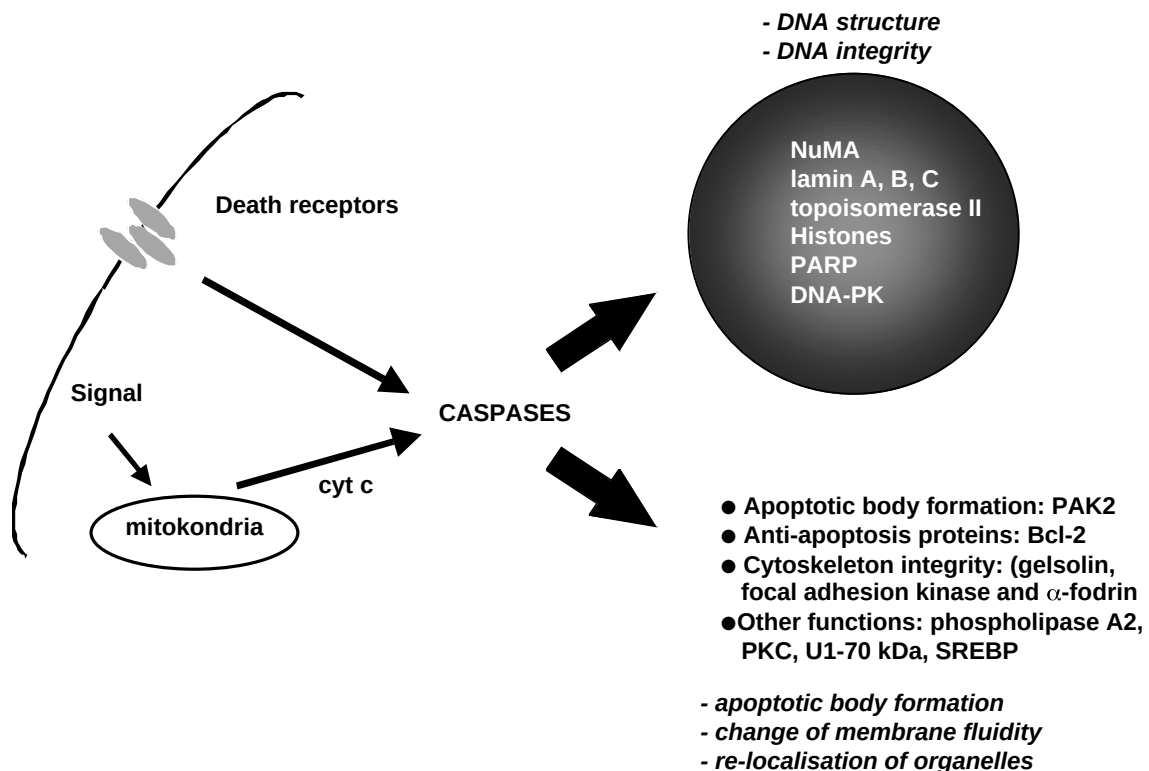


Figure 6. Caspase activation. After activation of caspases through ligation of death receptors and via release of apoptogenic factors from mitochondria the effector caspases (2, 3 and 7) cleave cytoplasmic and nuclear target proteins to destroy cellular architecture and efficiently prepare the cell for elimination.

Nuclear accumulation of MAL

MAL was shown to accumulate in nuclei of sensitive cells. Isolated nuclei from sensitive and resistant cells were treated with 0.2 and 0.4 mg/ml of MAL and DNA fragmentation was investigated by gel electrophoresis after 1 hour (Paper II). Nuclei of all cells displayed high and low molecular weight DNA fragmentation in a pattern consistent with apoptosis.

MAL did not bind to or cleave chromosomal DNA *in vitro* suggesting that endonucleases resident in the nucleus were activated in the presence of MAL. A vast number of DNAses have been implicated in the apoptotic process but there is still no consensus on the nature of endonuclease activity in apoptosis or if different endonucleases may be involved in different model systems (Barry & Eastman, 1993; Fernandes & Cotter, 1993; Gaido & Cidlowski, 1991; Giannakis *et al.*, 1991; Gottlieb *et al.*, 1995; Kawabata *et al.*, 1993; Montague *et al.*, 1997; Peitsch *et al.*, 1993; Ribeiro & Carson, 1993; Shiokawa *et al.*, 1994). The caspase-activated deoxyribonuclease (CAD, DFF40) is the best characterised endonuclease in apoptosis execution (Enari *et al.*, 1998; Liu *et al.*, 1997). This endonuclease is activated by caspases through the cleavage of the inhibitor protein ICAD. CAD is then transported into the nucleus and cleave DNA into oligonucleosome-length fragments (McIlroy *et al.*, 1999; Sakahira *et al.*, 1998).

In whole nuclei MAL was seen to co-localise with DNA, but only in certain areas (Figure 7). The rim of the nucleus immediately inside the nuclear membrane was devoid of MAL whereas MAL predominated in the centre of the nucleus, where DNA content was lower. In gel-shift assays with MAL and DNA no binding of DNA was observed, suggesting that MAL may interact inside the nucleus with molecules other than DNA.

The mechanism of MAL-uptake into the nucleus was investigated in cells permeabilised with digitonin. Digitonin will specifically permeabilise the plasma membrane, leaving the nuclear membrane intact. This model can be used to investigate the nuclear transport mechanisms. The nucleus is connected to the cytoplasmic compartment via nuclear pores, consisting of roughly 100 polypeptides that constitute the nuclear pore complex (NPC) with a mass of about 125,000 kDa. Small proteins (<20-40 kDa) can passively diffuse through the pores, whereas larger proteins require active transport. One mechanism of active nuclear import involves the interaction of nuclear localisation sequences (NLS) with two cytoplasmic proteins: importin α and β (Gorlich, 1998; Gorlich & Mattaj, 1996; Hicks & Raikhel, 1995; Jans & Hübner, 1996; Yoneda, 1997). The NLS will bind to importin α , and will enable the binding to importin β that will dock to the nucleoporins in the NPC. The complex is then translocated through the pores by an energy-dependent mechanism that requires the GTPase Ran. The interaction of the import complex with the nucleoporins can be blocked using wheat-germ agglutinin (WGA). There are also other transport pathways reported that do not use this machinery (Adam, 1999).

Both MAL and ALA were taken up into the nuclei of digitonin-permeabilised A549 cells (Figure 7). WGA inhibited MAL transport into the nucleus but had no effect on ALA transport, suggesting that MAL was taken up into the cells via active

nuclear uptake whereas the native protein passively diffused in (Paper II). When comparing nuclear transport of the different cell types, MAL was found to localise to the nucleus of all cells and induce DNA fragmentation in digitonin-permeabilised cells. This suggested that no differences in the nuclear transport machinery existed between the cell types.

The role of calcium

Calcium can be involved in both the induction and inhibition of apoptosis. A transient increase in intracellular Ca^{2+} has been shown to retard apoptosis in ageing neutrophils, whereas a sustained increase in intracellular Ca^{2+} has been associated with the induction of apoptosis in several models. Glucocorticoid-induced apoptosis of thymocytes results in a Ca^{2+} elevation in the cytosol that leads to apoptosis (McConkey *et al.*, 1989). The effects can be mimicked by Ca^{2+} ionophores or treatment with the endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin, that inhibits the uptake of Ca^{2+} back into the sarcoplasmic reticulum (Jiang *et al.*, 1994; Lam *et al.*, 1994; Wyllie *et al.*, 1984).

Calcium is involved in many aspects of apoptosis signalling, including activation of Ca^{2+} -dependent proteases, endonucleases, and transglutaminases, interference with signal transduction, regulation of gene transcription and change in chromatin structure to enable endonuclease access (McConkey *et al.*, 1994). Recently evidence for a direct Ca^{2+} -mediated effect on mitochondria was presented. Calcineurin, a Ca^{2+} -activated phosphatase, which induces apoptosis, was recently found to dephosphorylate Bad, one of the Bcl-2 proteins (see below) causing its transition from the cytoplasm to the mitochondria (Wang *et al.*, 1999). This translocation was responsible for the release of cytochrome c.

The importance of Ca^{2+} in apoptosis, and the fact that α -lactalbumin is a Ca^{2+} -binding protein that alters its properties depending on Ca^{2+} saturation, made us investigate the role of Ca^{2+} in MAL-induced apoptosis (Paper I). The cells were loaded with the fluorescent probe Fura-2AM that undergoes an absorption shift upon binding Ca^{2+} (380 nm instead of 300 nm excitation wavelength) and the fluorescence was measured after treatment with MAL. Thapsigargin was used as a positive control.

All cells showed an increase in $[\text{Ca}^{2+}]_i$ after the addition of MAL, but the response differed between the sensitive and resistant cells (Figure 8). In the L1210 and A549 cells there was a rapid transient increase in the intracellular Ca^{2+} concentration within 20 seconds of exposure to MAL. The $[\text{Ca}^{2+}]_i$ then decreased and reached a platform level two- to four-fold above the baseline concentration that was maintained throughout the experiment. The resistant HRTEC cells showed a totally

different pattern. 1 mg/ml did not induce an alteration in $[Ca^{2+}]_i$. At higher concentrations a transient increase was observed that went back to baseline levels within two minutes of MAL-exposure. These results indicated a difference after exposure to MAL in the calcium flow between sensitive and resistant cells, suggesting a potential role for Ca^{2+} in MAL-induced apoptosis.

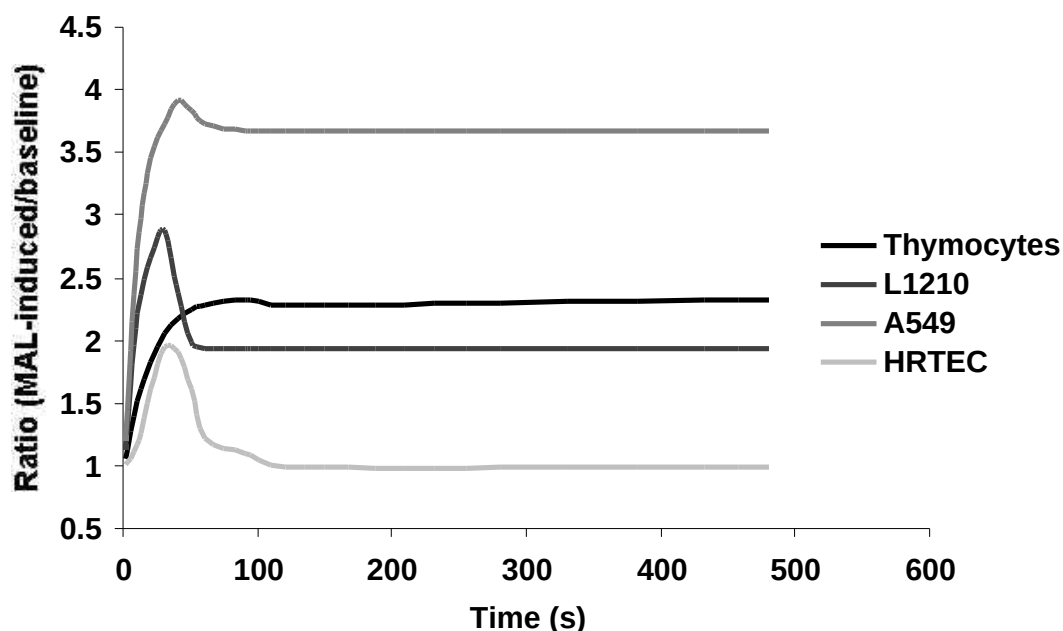


Figure 8. Intracellular Ca^{2+} concentration after MAL-treatment. The cells were treated with 1 mg/ml of MAL with the exception of HRTEC cells that were treated with 2 mg/ml. MAL was added to the cells as time zero and the Ca^{2+} concentration was monitored for 8 minutes. The results are given as the ratio between the Ca^{2+} concentration in MAL-treated cells and the baseline concentration in untreated cells. The baseline concentration was 45, 22, 50, and 50 nM for thymocytes, L1210, A549 and HRTEC cells, respectively.

To investigate further the role of calcium in MAL-induced apoptosis the cells were incubated under conditions where different calcium pathways were blocked. In Ca^{2+} -free media the MAL-induced increase in $[Ca^{2+}]_i$ and DNA fragmentation was blocked, suggesting an important role for Ca^{2+} in MAL-induced apoptosis (Paper I). The results also suggested that the influx of Ca^{2+} was from the extracellular space rather than the intracellular stores. The L-type Ca^{2+} -channel inhibitors verapamil and nifedipine caused partial inhibition of DNA fragmentation in L1210 and A549 cells. Chelators were efficient in blocking the DNA fragmentation induced by MAL in isolated nuclei, which is consistent with the Ca^{2+} and Mg^{2+} -dependence of many endonucleases involved in apoptosis, but could not be used on whole cells due to

adverse effects on cell viability. These results suggest that Ca^{2+} is involved somewhere in the course of MAL-induced apoptosis.

Getting rid of “evidence”

Apoptotic cells in tissue are recognised and phagocytosed before they lyse. A number of molecules on the macrophage surface have been associated with the elimination of apoptotic cells (Figure 9), but their ligands on apoptotic cells have, with a few exceptions, not been determined. On the apoptotic cell phospholipid asymmetry changes, with phosphatidylserine (PS) flipping to the outer leaflet of the membrane, is the most studied phenomenon (Fadok *et al.*, 1992; Martin *et al.*, 1995b). The receptor for PS on the macrophage is not known.

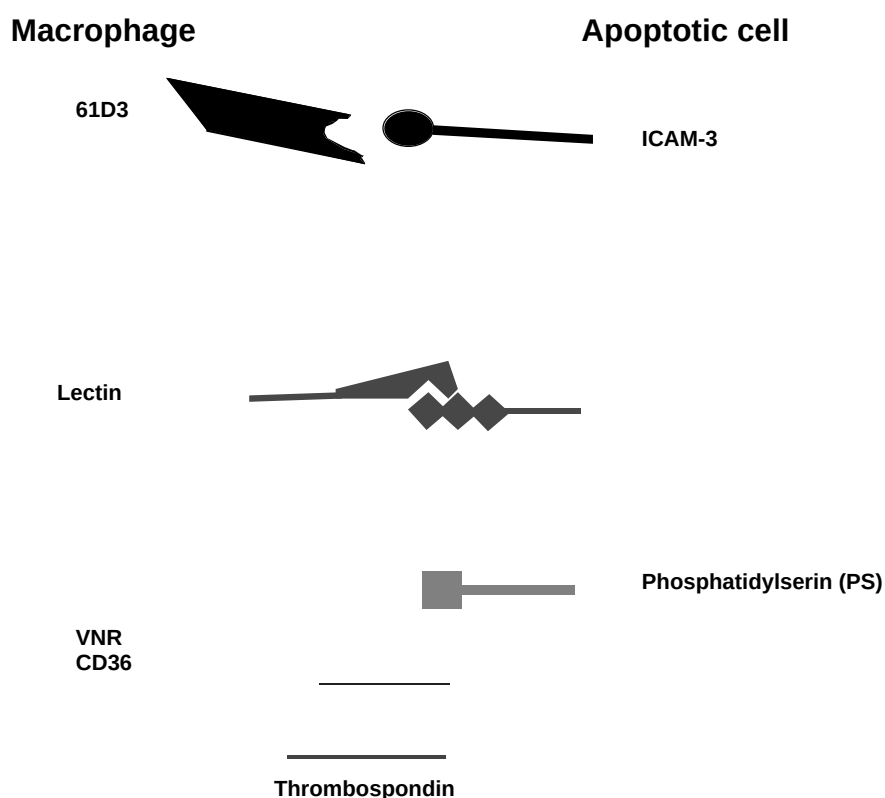


Figure 9. Structures involved in the recognition of apoptotic cells by macrophages. The vitronectin receptor together with CD36 is one putative receptor on macrophages that associates with thrombospondin to make a bridge favouring the binding to apoptotic cells (Savill *et al.*, 1990; Savill *et al.*, 1992). CD14 is another molecule suggested as a candidate receptor on macrophages (Devitt *et al.*, 1998). Surprisingly the interaction with apoptotic cells does not induce any inflammation. Also the scavenger receptors and complement receptors have been suggested in different model systems (Mevorach *et al.*, 1998; Moynault *et al.*, 1998; Oka *et al.*, 1998; Shiratsuchi *et al.*, 1999). Carbohydrates (Duvall *et al.*, 1985), ICAM-3 and PS have been suggested as recognition molecules for macrophages on the surface of the apoptotic cell (Moffatt *et al.*, 1999).

MAL-induced apoptosis causes PS exposure on the outer surface of the cells detected after 1 hour in 20% of the L1210 and Jurkat cells and increased to over 65% at 6 hours. In A549 cells the exposure is observed at a later time point with 15% at 3 hours and 60% at 24 hours.

Regulation of apoptosis

The apoptotic cascade is tightly regulated by several factors with apoptosis-inhibiting and promoting functions. This regulation makes up a complicated network and failure may lead to development of disease. Excessive apoptosis has been demonstrated in neurodegenerative and autoimmune diseases and in the course HIV infection. Inversely, too little apoptosis is associated with tumour development. We have investigated two molecules that are commonly involved in tumour development and progression and whose function is related to sites where MAL has shown activity (mitochondria and nucleus).

The Bcl-2 family of proteins

Bcl-2 was initially observed at the breakpoint of the common t(14;18) translocation in follicular lymphoma (Bakhshi *et al.*, 1985; Cleary & Sklar, 1985; Tsujimoto *et al.*, 1985). The cells were seen to survive longer, rather than proliferate more rapidly than normal cells. Molecular characterisation indicated that the Bcl-2 protein was overexpressed as it came under the influence of the IgG heavy-chain promoter.

Bcl-2 is the founder of a growing family of proteins, where both apoptosis-preventing (Bcl-2, Bcl-X_L, Bcl-w, Bfl-1, Bcl-11, Mcl-1, A1, and Boo) and apoptosis-promoting (Bax, Bak, Bcl-X_S, Bok, Bad, Bid, Bik, Blk, Hrk, Bim, Bnip3 and Diva) proteins can be found (Adams & Cory, 1998; Korsmeyer, 1999; Kroemer, 1997; Reed *et al.*, 1998). Bcl-2 is located to the mitochondrial, nuclear and endoplasmic reticulum membranes (Hockenbery *et al.*, 1990) and is highly homologous to the anti-apoptosis regulator Ced-9 in *C. elegans* (Hengartner & Horvitz, 1994a). It has been suggested that the levels of pro- and anti-apoptosis proteins decide whether or not a cell will be responsive to apoptosis stimuli. The proteins have structural similarities in certain domains called BH 1-4 (Bcl-2 homology domains) (Chittenden *et al.*, 1995; Green & Reed, 1998; Yin *et al.*, 1994; Zha *et al.*, 1996), where the anti-apoptosis effect seems to require an intact BH4 domain (Cheng *et al.*, 1997; Huang *et al.*, 1998) and the apoptosis-promoting function seems to reside in BH3 (Green & Reed, 1998; Wang *et al.*, 1998; Zha *et al.*, 1996).

The Bcl-2-family members regulate the apoptosis induction mainly at the mitochondrial level, but functions outside the mitochondria have been suggested (Distelhorst & McCormick, 1996; Kuo *et al.*, 1998; Lam *et al.*, 1994; Marin *et al.*, 1996). Bcl-2 inhibits the release of cytochrome c from mitochondria and Bax has the opposite effect. The most recent explanation is that both proteins interact with VDAC, a constituent of the permeability transition pore (Shimizu *et al.*, 1999). In liposome experiments Bax was seen to promote the release of cytochrome c when interacting with VDAC in the liposome membrane, whereas Bcl-2 could block this release. This suggest that Bcl-2 regulates permeability transition (Kirsch *et al.*, 1999; Narita *et al.*, 1998; Yang *et al.*, 1997b). Both Bcl-2 and Bax are known to have structural similarities with certain bacterial toxins, especially cholera toxin and cholicins (Schendel *et al.*, 1997), and both proteins can form pores with selectivity for different ions (Antonsson *et al.*, 1997; Lam *et al.*, 1998; Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997). The apoptosis-promoting Bcl-2 family proteins may therefore specifically release cytochrome c through pore-formation in the membrane which will be inhibited by the heterodimerisation with anti-apoptotic proteins.

Bcl-2 can block apoptosis induced by microinjection of cytochrome c suggesting that a protective effects is exerted even after apoptogenic components have escaped the mitochondria (Brustugun *et al.*, 1998). Bcl-X_L, but not Bcl-2 have been found to interact with APAF-1 and Ced-4 suggesting a role in preventing apoptosis by binding and neutralising molecules required for caspase-activation (Chinnaiyan *et al.*, 1997a; Chinnaiyan *et al.*, 1997b; Hu *et al.*, 1998a; Inohara *et al.*, 1998; Pan *et al.*, 1998). Interestingly overexpression of Bcl-2 can block both apoptosis and necrosis in response to several different stimuli (Kane *et al.*, 1995; McConkey, 1998; Reed *et al.*, 1998).

Bcl-2-involvement in MAL-induced apoptosis was investigated using the human myelogenous cell line K562, overexpressing Bcl-2 in stable clones. This cell line has recently been shown to have a sustained increase in the expression of another Bcl-2 family protein, Bcl-X_L, as a consequence of the Philadelphia chromosome (Amarante-Mendes *et al.*, 1998; Benito *et al.*, 1996). This has made the Bcl-2-dependent features hard to discern. Still MAL-treatment of this cell line resulted in apoptosis with an LD₅₀ of 0.5 mg/ml, similar to another lymphoid cell line, Jurkat. The cell line was resistant to other apoptotic stimuli, such as epotoside and staurosporin, which was not the case in the Jurkat cells. This suggested that Bcl-X_L overexpression did not inhibit MAL-induced apoptosis.

When testing clones overexpressing Bcl-2 these cells were as sensitive as non-transfected cells or the vector control. This suggests that Bcl-2 or Bcl-X_L did not inhibit MAL-induced apoptosis. There are few pathways presented so far that are not

inhibited by Bcl-2, and considering that 50% of tumours (especially lymphomas) have an increased ratio in Bcl-2/Bax-expression, this feature of the protein is very interesting.

p53 – the guardian of the genome

The p53 protein was initially isolated through its upregulation in cells infected with SV40 and its co-precipitation with SV40 large and small T antigens (Lane & Crawford, 1979; Linzer & Levine, 1979). p53 is mutated in over 50% of tumours resulting in a resistance to radiation (Cheng & Haas, 1990; Chiba *et al.*, 1990; Hainaut *et al.*, 1998; Hollstein *et al.*, 1991; Lowe *et al.*, 1994). p53 deficient mice were shown to develop spontaneous tumours and thymocytes from these mice were highly resistant to radiation-induced apoptosis (Clarke *et al.*, 1993; Donehower *et al.*, 1992). A familial syndrome with massive cancer incidence has since been detected by Li and Fraumeni and was found to be associated with mutations in the p53 gene rendering the gene product inactive (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). The mechanism of p53 mediated apoptosis was identified in experiments showing that DNA damage upregulated the protein and that this was associated with cell cycle arrest. The current view is that p53 stops the cell cycle upon DNA damage and activates the DNA repair system. If the damage is too intense p53 will initiate apoptosis (Lane, 1993).

To study the p53 involvement in MAL-induced apoptosis we analysed the p53 status of some of our cells. The HL-60, U-937 and K562 cells used did not express detectable amounts of p53. The K562 and HL-60 cells lack p53-expression and U-937 cells have a point mutation leading to an aberrant splicing of mRNA, with no functional protein produced (Ehinger *et al.*, 1996; Ehinger *et al.*, 1995). These cells were as sensitive as the remaining lymphoid cell lines, suggesting that p53 did not participate in MAL-induced apoptosis.

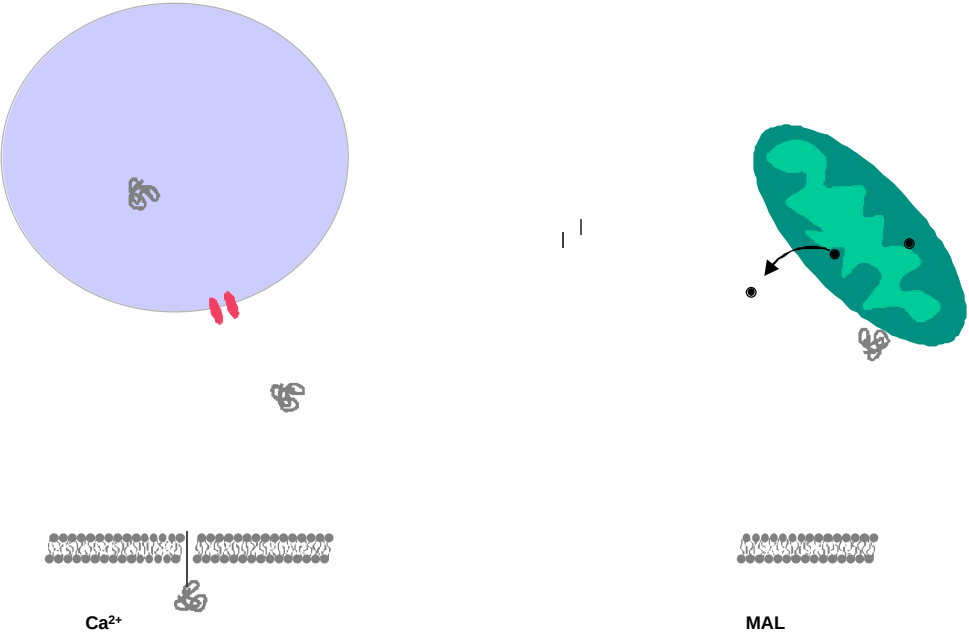
An integrated summary

This section has presented evidence that MAL induces apoptosis in tumour and immature cells but that non-transformed epithelial cells and other mature differentiated cells are resistant. It has also described our attempts to explain the mechanism of MAL-induced apoptosis. A putative model for MAL induced apoptosis is presented in Figure 10 (The encircled numbers below refer to the numbering in the figure)

1. The protein bound to the surface of all cells and the amount of binding *per se* did not explain the difference in sensitivity (①). The receptor(s) for MAL have not been isolated but preliminary experiments suggested that binding was protein-dependent as protease treated cells did not bind MAL. The induction of MAL-induced apoptosis did not involve Fas, a common regulator of apoptosis in immune cell populations.
2. Cell surface binding of MAL was associated with a rapid and sustained increase in intracellular calcium levels in sensitive cells (②). In the resistant cells a transient increase occurred, but intracellular calcium levels quickly returned to basal concentrations. DNA fragmentation and death was inhibited under Ca^{2+} -free conditions after treatment with MAL. This is consistent with a role of Ca^{2+} in MAL-induced apoptosis.
3. In sensitive cells MAL was shown to translocate across the membrane and to appear in the cytoplasm as a granular fluorescence. This staining pattern was explained by a co-localisation with mitochondria (③). In whole cells and isolated mitochondria MAL reduced the mitochondrial membrane potential (④) and triggered the release of cytochrome c (⑤). Release of cytochrome c was associated with activation of caspases (⑥) in MAL-treated cells, observed both through the cleavage of caspase-3 and through cleavage of fluorescent substrates. The caspase activation was functional as determined by the cleavage of cytoplasmic and nuclear target substrates that could be blocked by caspase inhibitors (⑦).
4. The mitochondrial localisation occurred in parallel with nuclear accumulation of protein (⑧). The transport into the nucleus was through active nuclear uptake and could be blocked by WGA. At 6 hours in Jurkat and L1210 cells and at 24 hours in A549 cells the majority of nuclei were stained with MAL. Co-localisation studies of MAL and DNA in confocal microscopy revealed that MAL was located mainly to the centre of the nucleus and DNA more in the periphery, with little overlapping fluorescence. MAL was not shown to bind DNA *in vitro* suggesting that the protein interacted with other molecules in the nucleus. MAL was shown to directly induce DNA fragmentation in isolated nuclei suggesting that no mitochondrial activity was required for the effect.

The implications of these findings are currently being studied. It is possible that the nuclear uptake triggers the cytoplasmic activation in a mitochondrial loop as described for caspase activation through death receptors. It is also possible that MAL induces a caspase response through its interaction with mitochondria that will enable the transport of MAL to the nucleus where MAL may exert additional activity. Finally, MAL may have several targets in the cell and activate parallel pathways independent of each other.

Fig 10



Purification and characterisation of the active component.

*Thou com'st in such a questionable shape
That I will speak to thee. I'll call thee Hamlet*

William Shakespeare, Hamlet act I, scene 4

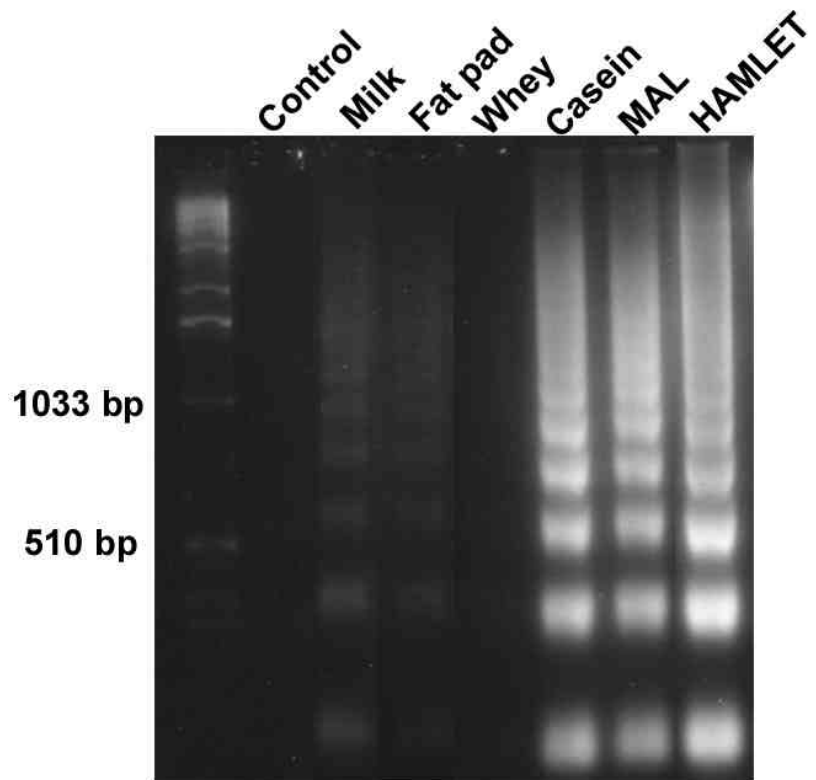
Initial purification strategies

Most of the studies reported here were performed with MAL, the fraction purified from human milk casein by ion exchange chromatography (Paper I, IV). This fraction was obtained from milk that had been centrifuged to remove the fat pad, and separated into casein and whey by acid precipitation (Lönnerdal & Forsum, 1985; Mellander, 1947). The caseins are characterised by ester-bound phosphate, and have low solubility at pH 4-5 with maximum precipitation at 4°C (Räihä, 1985). The whey and casein fractions were lyophilised and tested for effects on cell viability and DNA fragmentation (Figure 11). Activity was found in the casein fraction, while whey had no activity even at very high protein concentrations.

Casein was fractionated further by anion exchange chromatography using a NaCl gradient previously developed for the separation of whey proteins (paper I, IV) (Kunz & Lönnerdal, 1990). A stepwise gradient to 30% yielded 5 peaks, but none of the peaks contained the activity. The active component (MAL) had stuck to the column matrix and eluted after 1 M NaCl as a sharp peak containing all the activity of the casein fraction (Figure 11). Using polyacrylamide gel electrophoresis (PAGE) this fraction was shown to contain several protein bands with masses ranging from 14 kDa to 100 kDa with some material still left in the well. Gel filtration on G-50 Sephadex matrix (Pharmacia Biotech, Stockholm) resulted in two peaks designated K and L. The L fraction ran at 14 kDa and was inactive, but the K fraction contained all biological activity and the same number of bands by PAGE as the initial fraction, indicating that little separation or purification had occurred.

The protein bands were subjected to N-terminal amino acid sequencing by Edman degradation and showed sequence homology to human alpha-lactalbumin, suggesting that the main constituent of the active fraction was α -lactalbumin (Paper I, IV) (Simpson *et al.*, 1989).

Figure 11 – DNA fragmentation in L1210 cells. The cells were exposed to 1:2 dilution of whole milk, 1 mg/ml of fat pad, 10 mg/ml of whey, 1 mg/ml of casein and 0.3 mg/ml each of MAL or HAMLET and prepared for conventional gel electrophoresis. DNA fragments were separated on 1.8% agarose gels.



Characterisation of the α -lactalbumin in the active fraction.

Covalent structure.

α -Lactalbumin isolated from the whey fraction of milk was inactive, suggesting a structural difference compared to the α -lactalbumin in the active fraction. The difference in biologic activity may reflect covalent structure differences like glycosylation and post-translational modifications. These aspects were subsequently investigated.

1. The active fraction was not glycosylated as determined by carbohydrate analysis by HPLC with a Dionex detector.
2. Electrospray ionisation mass spectrometry (ESI-MS) ruled out common post-translational modifications like phosphorylation and methylation. MALDI-TOF mass spectrometry revealed the presence of peaks consistent with 14, 28 and 42 kDa.

It was thus concluded that the active fraction had a retained covalent structure compared to native α -lactalbumin from whey (paper I, IV). Since the active casein-fraction was characterised by the presence of oligomers it was named MAL, for multimeric α -lactalbumin.

MAL's secondary and tertiary structure

The secondary and tertiary structure of MAL was investigated by spectroscopic techniques (paper IV). Native α -lactalbumin purified from human whey and pH 2 treated α -lactalbumin in the molten globule state (see below) were included as controls. By far-UV CD spectroscopy the native α -lactalbumin had a typical double dip at wavelengths 208 and 222 measuring absorbance to α -helix structures, with β -sheet information in between (215 nm). The pH 2-treated α -lactalbumin showed the identical pattern confirming that there was no change in secondary structure. MAL did not differ significantly from native or pH 2-treated α -lactalbumin.

The tertiary structure of the protein was examined by near UV CD spectroscopy, reflecting the mobility of the aromatic tyrosine and tryptophan side chains (Figure 12, panel I). The native α -lactalbumin showed a minimum at 270 nm and a maximum peak at 294 nm consistent with tyrosine and tryptophan absorption, respectively. The pH 2-treated α -lactalbumin showed a loss of signal due to the flexibility of the side chains in the less rigid tertiary structure. The MAL signal resembled the pH 2-treated protein more than the native state. Fluorescence spectroscopy with and without the hydrophobic dye ANS confirmed the flexible tertiary structure of MAL, indicating that the protein exposed hydrophobic surfaces just like the pH 2-treated form of the protein (Figure 12, panel II). We concluded that the active fraction contained a folding variant of α -lactalbumin similar to the molten globule form, and proposed that this folding variant was the active component.

α -Lactalbumin

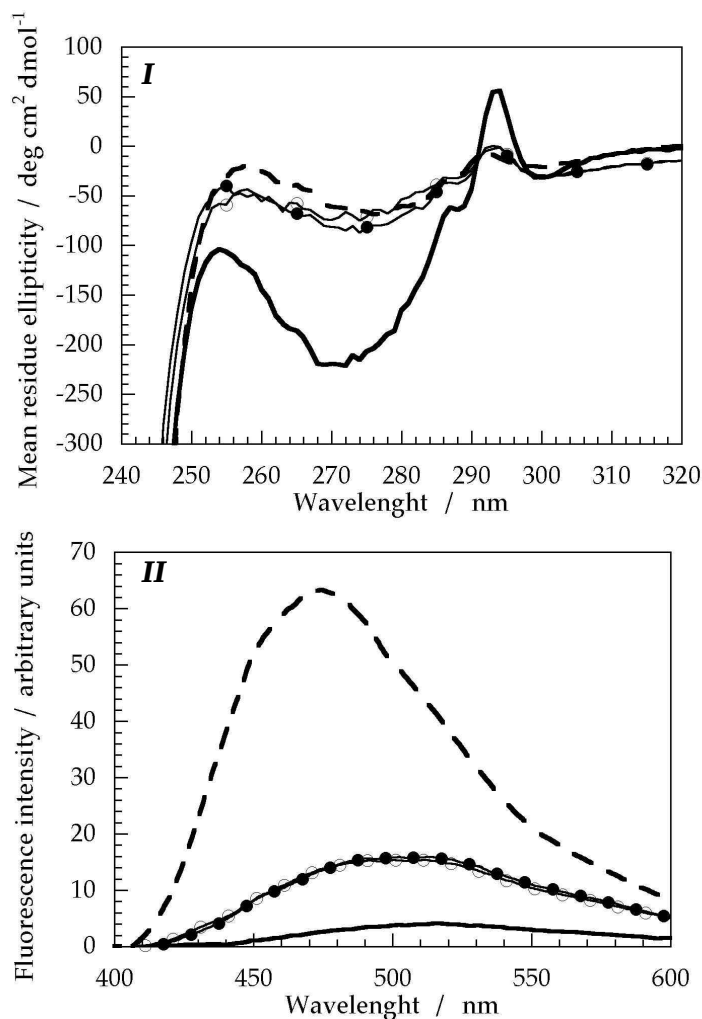
α -Lactalbumin is present in all mammalian milks but the concentrations vary from trace amounts to approximately 1-2 g/l in human milk (McKenzie & White, 1991). Most species have one variant of the protein whereas others (cow, pig and horse) have two or three variants differing in glycosylation or having amino acid substitutions. Recently a variant of human α -lactalbumin was identified that showed glycosylation on the unusual amino acid triplet Asn-Ile-Cys on approximately 1% of the protein molecules (Giuffrida *et al.*, 1997)..

Genetic organisation

The human protein has 123 amino acids and a molecular mass of 14 kDa. The amino acid sequence was determined by Findlay and Brew (Findlay & Brew, 1972) with corrections made from the DNA sequencing studies by Hall *et al* (Hall *et al.*, 1982).

Figure 12 – Panel I: Circular dichroism spectra in the near UV region for native α -lactalbumin (solid line), the pH 2 induced molten globule form (dashed line), MAL(O) and HAMLET (●).

Panel II: ANS titration of native α -lactalbumin (solid line), the pH 2 induced molten globule form (dashed line), MAL (O) and HAMLET (●). The figure shows spectra obtained with 1.5 μ M ANS.



The entire *ala* gene is 2.3 kbp and contains four exons and three introns and the gene has a number of features in common with other mammalian genes (Hall *et al.*, 1987). The gene has been assigned to chromosome 12q13 by hybridisation experiments with rodent/ human somatic cell hybrids and subsequent *in situ* hybridisation to obtain the regional assignment (Davies *et al.*, 1987). This is in the same area as the lysozyme gene, suggesting that gene duplication has occurred, which has later been verified (Brew & Campbell, 1967; Hall *et al.*, 1982; McKenzie, 1996; McKenzie & White, 1991; Peters *et al.*, 1989).

Structure

The crystal structure of α -lactalbumin was first determined for material from the baboon (Acharya *et al.*, 1989; Smith *et al.*, 1987), and the high resolution (1.7 Å) structure of the human protein was determined by Acharya *et al.* (Acharya *et al.*, 1991). α -Lactalbumin has two regions (α and β) that are separated by a deep cleft. The α -region consist of four α -helices formed by residues 1-37 and 85-123 (Kuwajima, 1996; McKenzie & White, 1991; Peng *et al.*, 1995) (for details see Figure 13 A, B). The β -region has an anti-parallel β -sheet, and the structure has four disulphide bonds (Figure 13 B) (Acharya *et al.*, 1991; Acharya *et al.*, 1989).

Ca²⁺-binding

α -Lactalbumin is a metalloprotein with high affinity for Ca^{2+} (Hiraoka *et al.*, 1980). The strong Ca^{2+} -binding loop, referred to as the Ca^{2+} -binding elbow, is located in the β -domain with a dissociation constant (K_D) for Ca^{2+} in the order of 10^{-8} - 10^{-6} M (Figure 13 C) (Aramini *et al.*, 1992; Kronman *et al.*, 1981; McKenzie & White, 1991; Murakami *et al.*, 1982; Qasba & Kumar, 1997; Schaer *et al.*, 1985; Van Ceunebroeck *et al.*, 1985). Mutations that disrupt the loop have been shown to inhibit binding of the Ca^{2+} ion and removal of Ca^{2+} as in apo- α -lactalbumin makes the protein less stable, suggesting that the Ca^{2+} ion is important for stability and also for correct folding of the protein (Berliner *et al.*, 1991; Ikeguchi *et al.*, 1986; Vanderheeren *et al.*, 1996).

α -Lactalbumin can also interact with a number of mono-, and divalent ions both at the strong Ca^{2+} binding site and at other sites of the molecule (Figure 13 C) (Aramini *et al.*, 1996; Berliner & Koga, 1987; Chandra *et al.*, 1998; Gerken, 1984; Hiraoka & Sugai, 1985; McKenzie & White, 1991; Murakami & Berliner, 1983; Musci & Berliner, 1985; Schaer *et al.*, 1985; Veprintsev *et al.*, 1996).

Biologic activity

Lactose synthase was identified in mammary tissue in the 1960s; it was found to consist of two protein fractions where neither had any activity alone (Brodbeck & Ebner, 1966). The B protein was α -lactalbumin and the A protein was a galactosyl transferase (UDP-galactose: *N*-acetylglucosamine- β -4-galactosyl transferase; EC 2.4.1.38), belonging to a group of glycosyltransferases that have been found on cell surfaces and Golgi membranes (Strous, 1986). Its normal function is to couple galactose into $\beta(1\rightarrow4)$ -linkage with *N*-acetylglucosamine in the glycosylation of certain glycoproteins (Ram & Munjal, 1985).

When lactation is initiated α -lactalbumin alters the substrate specificity of the galactosyltransferase from *N*-acetylglucosamine to glucose, thus forming lactose (Ram & Munjal, 1985). The proteins interact on the inner Golgi membrane and require Mg^{2+} and Zn^{2+} . α -Lactalbumin works as a specifier protein and by interacting with the galactosyl transferase the affinity of glucose is increased 1000-fold (Figure 13 C) (Bell *et al.*, 1976; Khatra *et al.*, 1974). Mice deficient in α -lactalbumin produce a highly viscous milk due to the lack of lactose, and they are unable to feed their offspring (Stinnakre *et al.*, 1994). This highlights the importance of lactose as an osmotic molecule involved in driving water out in the milk.

The α -lactalbumin molten globule

α -Lactalbumin is the most employed protein in protein folding studies (Kuwajima, 1996). When a protein is synthesised it folds to the native conformation based on the information included in the sequence (Anfinsen, 1973). According to the current view many proteins fold through a series of intermediate states with increasing native-like features and decreased Gibbs energy (Baldwin, 1995; Dobson, 1995; Kim & Baldwin, 1982; Kuwajima, 1989; Privalov, 1996; Ptitsyn, 1973; Ptitsyn, 1995). A stable intermediate folding state of α -lactalbumin was observed under low pH or heat and was named the molten globule state (Dolgikh *et al.*, 1981; Ohgushi & Wada, 1983).

Cooperative versus non-cooperative folding of α -lactalbumin

During *in vitro* folding of α -lactalbumin the molten globule will appear within milliseconds (Matthews, 1993). This seems to be non-cooperative transition (Griko *et al.*, 1994; Pfeil, 1998; Pfeil *et al.*, 1986; Schulman & Kim, 1996; Schulman *et al.*, 1997; Shimizu *et al.*, 1993; Yutani *et al.*, 1992) although others have reported a cooperative transition to occur under certain conditions (Kuwajima, 1977; Kuwajima *et al.*, 1976; Ptitsyn & Uversky, 1994; Schulman & Kim, 1996; Uversky & Ptitsyn, 1996). The molten globule is then transformed into the native form, by a complex process, which involves the attainment of native side-chain packing and hydrogen bond formation. This transition shows a classical co-operative phase transition by calorimetric measurements and occurs more slowly (Balbach *et al.*, 1996; Kuwajima, 1977; Pfeil *et al.*, 1986). The folding intermediates of α -lactalbumin can be studied at low pH (The A-state), heat (the T-state), moderate concentration of chaotropic agents such as urea or Gdn-HCl, reduction of disulphide bonds or by removing Ca^{2+} (Apo-state) (Creighton, 1995; Dolgikh *et al.*, 1981; Kronman *et al.*, 1964; Kronman *et al.*, 1967; Kuwajima *et al.*, 1985). This has led to the following characteristics:

The molten globule has a core that is packed only a little more loosely than the native form (Dolgikh *et al.*, 1985; Dolgikh *et al.*, 1981; Kataoka *et al.*, 1997; Kronman *et al.*, 1967; Pfeil, 1987; Ptitsyn, 1995) with retained secondary structure as determined by far-UV CD spectra (Dolgikh *et al.*, 1981). In contrast the near-UV CD spectra (measuring the aromatic side chains) are dramatically reduced, suggesting the absence of rigid tertiary structure (Figure 12) (Dolgikh *et al.*, 1985; Dolgikh *et al.*, 1981; Kuwajima *et al.*, 1985; Kuwajima *et al.*, 1976). The apo-form of human α -lactalbumin resembles closely the molten globule state but has more of less tertiary structure still intact, depending on the ionic strength (Hiraoka & Sugai, 1985; Kuwajima *et al.*, 1986; Segawa & Sugai, 1983). The molten globule exposes hydrophobic surfaces as shown

Fig 13 AB

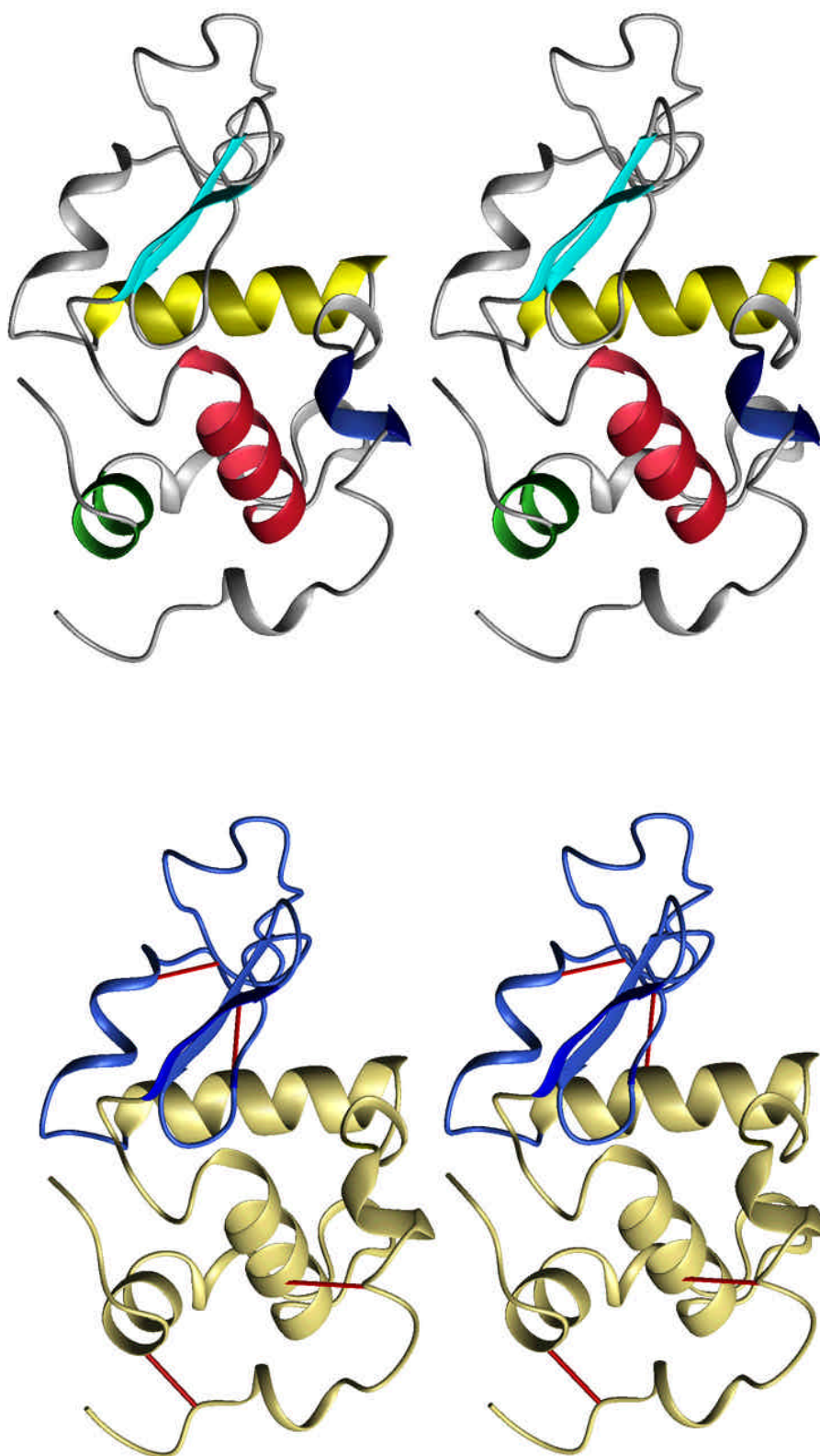


Fig 13 CD

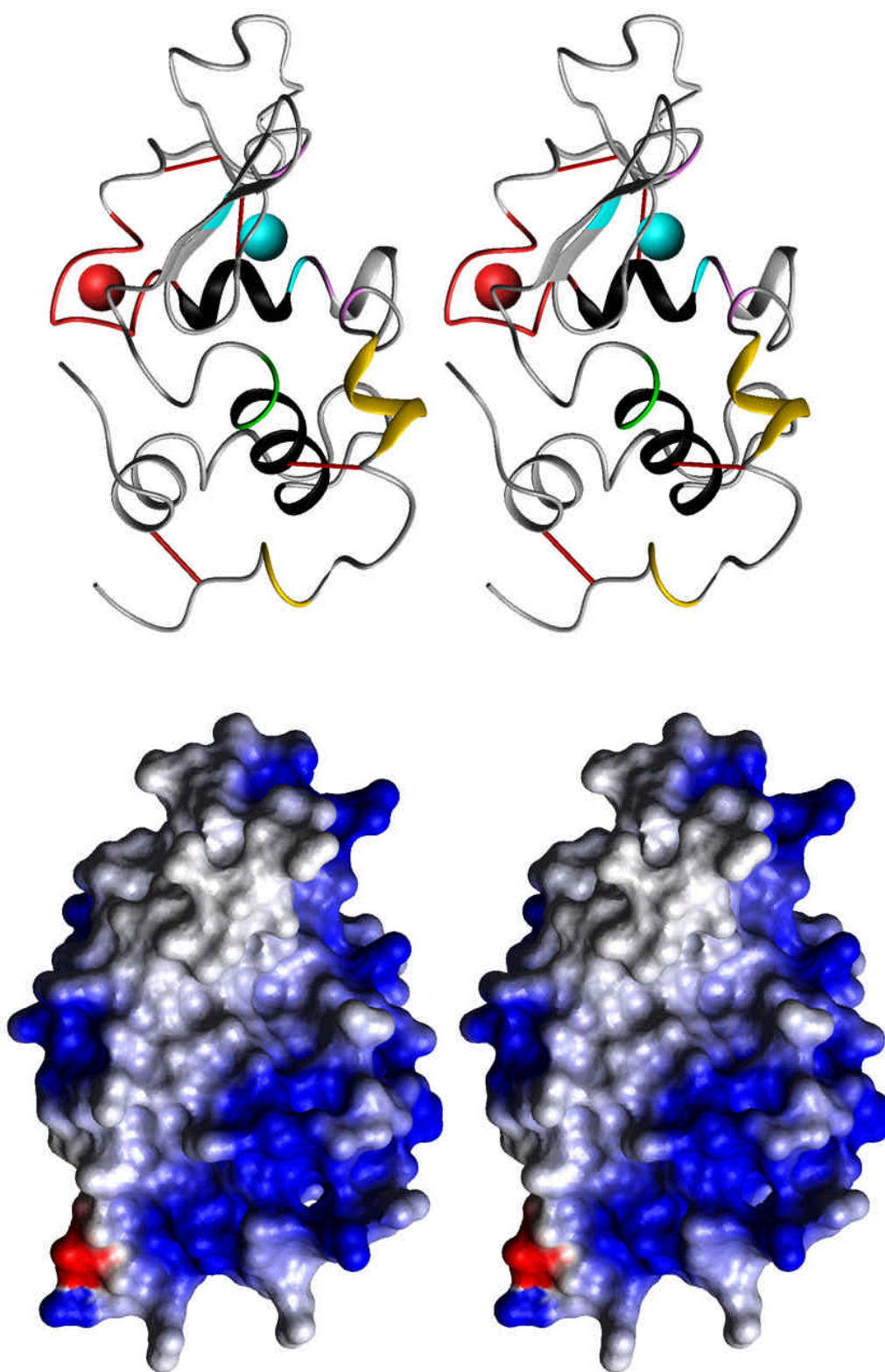


Figure 13. Stereoview structures of human α -lactalbumin. Stereoviews were produced by left and right images with off-set by -10 and 10° , respectively.

(A) Ribbon structure showing the α -helices: A (residues 5-11, in green), B (residues 23-34, in red), C (residues 86-99, in yellow), D (residues 105-109, in blue) and the anti-parallel β -sheet (residues 40-50, in cyan). The structure also shows all 3_{10} helices (in gray).

(B) Ribbon structure of the α domain (residues 1-37, 86-123, in gold) and β domain (residues 38-85, in blue). The picture also depicts the four disulfide bonds in red (6-120, bottom left, 28-111 bottom right, 61-77 top left and 73-91 top right). The 6-120 and 28-111 bonds are located in the α -domain, the 61-77 bond in the β -domain and the 73-91 bond interconnects the two domains (Acharya *et al.*, 1991; Acharya *et al.*, 1989)

(C) Ribbon structure of the functional domains of α -lactalbumin.

Red: The disulfide bonds.

Orange: The Ca^{2+} -binding elbow (orange loop) consist of a 3_{10} helix and an α -helix with a short turn region separating the two helices (Acharya *et al.*, 1991; Acharya *et al.*, 1989; Pike *et al.*, 1996; Stuart *et al.*, 1986). It is flanked by two disulfide bridges making this part of the molecule less flexible (Qasba & Kumar, 1997). The bound Ca^{2+} ion is co-ordinated by a constellation of seven oxygen groups that form a distorted pentagonal bipyramid. Five of the groups are contributed by the side chain carboxylates of Asp82, 87 and 88 or carbonyl oxygens of Lys79 and Asp84. The remaining two oxygens are supplied by two water molecules (Pike *et al.*, 1996; Stuart *et al.*, 1986).

Cyan: The residues shown in cyan (Glu49 and Lys 94) make out the distinct Zn^{2+} binding site together with Glu 116.

Green: The green residues (His 32 and Phe 31) are located in the cleft region and are essential for lactose synthase activity as they are involved in glucose binding (Grobler *et al.*, 1994; Malinovskii *et al.*, 1996; Pike *et al.*, 1996).

Gold: The aromatic cluster (Gln117, Trp118, marked in gold) located adjacent to the lower reaches of the cleft is important for the interaction with galactosyltransferase (Grobler *et al.*, 1994; Prieels *et al.*, 1979) The other region is the flexible loop (residues 105-110, marked in gold to the right) that flanks the aromatic cluster and comprises the other part of the recognition site for galactosyltransferase.

Violet: The violet residues are internal structures of the hydrophobic box region (C helix, β -sheet, residues 60, 95, 103, 104) (Kuwajima, 1989; Schulman *et al.*, 1997; Shimizu *et al.*, 1996). This domain is thought to be important for proper folding of the protein.

Black: The black residues depict helix B and C that have also been implicated as a hydrophobic core region (D'Silva & Lala, 1999; Peng *et al.*, 1995; Shimizu *et al.*, 1996). Other parts not marked in this picture have also been suggested to be involved in stability of the molten globule (Baum *et al.*, 1989; Demarest *et al.*, 1998; Fontana *et al.*, 1997; Pike *et al.*, 1996; Polverino de Laureto *et al.*, 1995; Schulman & Kim, 1996; Song *et al.*, 1998; Uchiyama *et al.*, 1995; Wu & Kim, 1998).

(D) Surface plot of the α -lactalbumin structure. The blue regions are negatively charged side chains and the red are positively charged. White areas depict hydrophobic residues.

The figures were produced using the freeware MolMol version 2.6.1 (Koradi *et al.*, 1996) with coordinates from X-ray crystallography (Acharya *et al.*, 1991).

by the binding of ANS in fluorescence spectroscopy (Figure 12, 13 C, D) (Lala & Kaul, 1992; D'Silva & Lala, 1999; Peng *et al.*, 1995; Shimizu *et al.*, 1996). It is suggested that the protected hydrophobic regions are folding nuclei from which native folding is initiated. Their location and the importance of folding nuclei is a matter of debate (for details see Figure 13 C, D). The hydrophobicity of the molten globule is also suggested to explain the interaction of α -Lactalbumin with membranes (Banuelos & Muga, 1995; Banuelos & Muga, 1996a; Banuelos & Muga, 1996b; Berliner & Koga, 1987; Cawthorn *et al.*, 1997; Cawthorn *et al.*, 1996; Grishchenko *et al.*, 1996; Lala *et al.*, 1995; Montich & Marsh, 1995).

Conversion of native inactive α -lactalbumin to the apoptosis-inducing form requires a folding change (Svensson *et al.*, Submitted)

The results on the structural characterisation of MAL suggested that α -lactalbumin was retained in a molten globule-like conformation and that this conformation was required for the apoptosis-inducing activity. As human milk contains an abundance of highly bioactive molecules we could not rule out the possibility that a minor contaminant was the true active factor. We therefore analysed MAL and the ion-exchange matrix for the presence of additional molecules. Lipid extraction of MAL yielded approximately 10% lipids corresponding to those in whole milk casein. The lipids were found to be inactive at the concentration detected in an active amount of MAL, suggesting that the lipids alone were not responsible for the activity. The results indicated a possible role of lipid as a co-factor in MAL.

If the apoptosis-inducing activity was a feature of the molten globule-like conformation it should be possible to convert native α -lactalbumin to the apoptosis-inducing form. α -Lactalbumin from whey was therefore subjected to conditions favouring formation of the molten-globule in the absence and presence of lipid. After addition of EDTA to remove the tightly bound Ca^{2+} -ion, α -lactalbumin was subjected to ion-exchange chromatography on a clean matrix or on a matrix conditioned with lipids from casein. Native α -lactalbumin was run as a control.

When native and EDTA-treated protein was passed over a clean matrix under conditions identical for the protein purification of MAL the eluting material remained in the native conformation and was unable to induce apoptosis. In contrast, the EDTA-treated α -lactalbumin was retained on a casein-conditioned matrix and eluted with high salt identical to the elution profile of MAL. This material had the same activity as MAL and showed spectroscopic characteristics suggestive of a

molten globule-like conformation. Most of the native α -lactalbumin eluted early in the fractionation, and a small peak (20-30% material) eluting with high salt had no activity against the tumour cells. This suggested that the active conformation could be produced on a column in the presence of lipid from casein. The conversion required that the protein was in the molten globule state; natively folded protein could not be converted.

Sequential runs of EDTA-treated α -lactalbumin over the column resulted in a decrease in the yield of protein, suggesting that the lipids were consumed and taken up as an integral part of the complex. Analysis of lipid before and after the sequential runs revealed a consumption primarily of free fatty acids. Ion-exchange matrices were conditioned with individual free fatty acids from casein (C14:0, C16:0, C18:0 and C18:1). C18:1 was the only fatty acid capable of retaining EDTA-treated α -lactalbumin on the column. The eluted material had the same activity as MAL based on activity against L1210 leukaemia cells and had the same tertiary structure as MAL (Figure 12).

These results prove that the active material consists of α -lactalbumin, and that the active form is stabilised in a molten globule-like conformation in a complex with C18:1 fatty acid (Svensson *et al.*, Submitted). The material after conversion was named HAMLET for Human Alpha-lactalbumin Made Lethal to Tumour cells. The implications of this finding are rather novel and interesting, in so far as one peptide chain can be observed to change conformation and thus also change function. There are examples where proteins have been shown to have different functions depending on changes in cellular localisation, cell type, oligomerisation, substrates or co-factors (Chaput *et al.*, 1988; Faik *et al.*, 1988; Gurney *et al.*, 1986; Jeffery, 1999; Sun *et al.*, 1999; Watanabe *et al.*, 1996; Xu *et al.*, 1996). In these examples structural differences have not been reported. The differences are due merely to the presence of different receptors at different locations or the use of different active sites of the molecule in the presence or absence of co-factors or multimerisation. The prion protein is the only example of a folding change causing change in biologic activity (Prusiner, 1998). The cellular form of this protein PrP^c is produced in neural tissue. The biologic function of this protein is still not determined but recent studies suggest that it acts as a survival protein in nerve cells (Kuwahara *et al.*, 1999). Under certain circumstances the protein changes its conformation to the scrapie form PrP^{sc}. The conversion uses the scrapie form of the protein as a template and involves an as of yet unidentified co-factor thought to be a protein. The resulting conformation has a structure with increased β -sheet formation that can easily aggregate and has been shown to induce apoptosis in target cells (Prusiner, 1998)

Bacteria as a model system for apoptosis!?

“...-favouring the good and rejecting the bad?
I can see no limit to this power, in slowly and beautifully adapting
each form to the most complex relation of life.

Charles Darwin, Origin of the species, 1859

(On evolution and natural selection)

MAL and HAMLET-induced bacterial death

MAL was first identified as a subfraction of human milk casein with antibacterial effects on *Streptococcus pneumoniae*, the most common cause of otitis media in small infants. At 0.3 mg/ml MAL, or HAMLET, rapidly killed 10^7 *S. pneumoniae* in 30 min. The effect was more pronounced for pneumococci than for group A streptococci and *H. influenzae* and there was no effect on other gram-positive (*S. aureus*, *S. epidermidis*, *E. fecalis*) or gram-negative bacteria (*E. coli*, *M. catharralis*, *P. aeruginosa*) (Håkansson *et al.*, Submitted). Further experiments were made with MAL and HAMLET in parallel, but results will only be presented for HAMLET, as this fraction is more well characterised.

In a “Friday afternoon” experiment I tested whether the death of bacteria might be accompanied by similar changes in DNA structure as seen in tumour cells. *S. pneumoniae* were treated with HAMLET for 2 hours and the DNA was subjected to field-inverse and conventional gel electrophoresis. I found that HAMLET induced the formation of high molecular weight DNA fragmentation in bacteria with identical sizes as seen in eukaryotic cells undergoing MAL-induced apoptosis (Figure 14). No oligonucleosome length fragments were observed. DNA fragmentation was not due to the direct activation of known bacterial DNAses as shown in control experiments in mutants with inactivated endo- or exonuclease (Figure 15). This suggested that bacteria can undergo apoptosis-like changes in response to HAMLET.

Bacterial “apoptosis”

Activation of mitochondrial effectors in bacteria.

The fact that HAMLET was killing both tumour cells and bacteria suggested that similar molecular targets for HAMLET existed in pro- and eukaryotes. This was consistent with evolutionary theory postulating that bacteria are the evolutionary origin of mitochondria (Gray *et al.*, 1999; Margulis, 1975), that co-ordinate some

aspects of apoptosis in eukaryotic cells. It has been proposed that the aerobic eubacteria invaded the previously anaerobic archeabacteria about 1,500 million years ago, and eventually became a manifest organelle in the evolving eukaryotic cell (Gray *et al.*, 1999; Margulis, 1975). The closest existing eubacterial relatives are the members of the rickettsial subdivision of the α -proteobacteria, a group of obligate intracellular parasites. The sequence of the most mitochondria-like genome, that of *Rickettsia prowazekii*, was recently published (Andersson *et al.*, 1998).

A number of questions arose: Did bacterial death involve mechanisms associated with those seen in mitochondria-dependent apoptosis? Was the bacterial cell death induced by cytochrome c? Could caspase-like activity be detected? What was the nature of the endonuclease? Could any sequence homologies be found with known mammalian proteins involved in apoptosis? The data presented here are a first attempt to characterise these events.

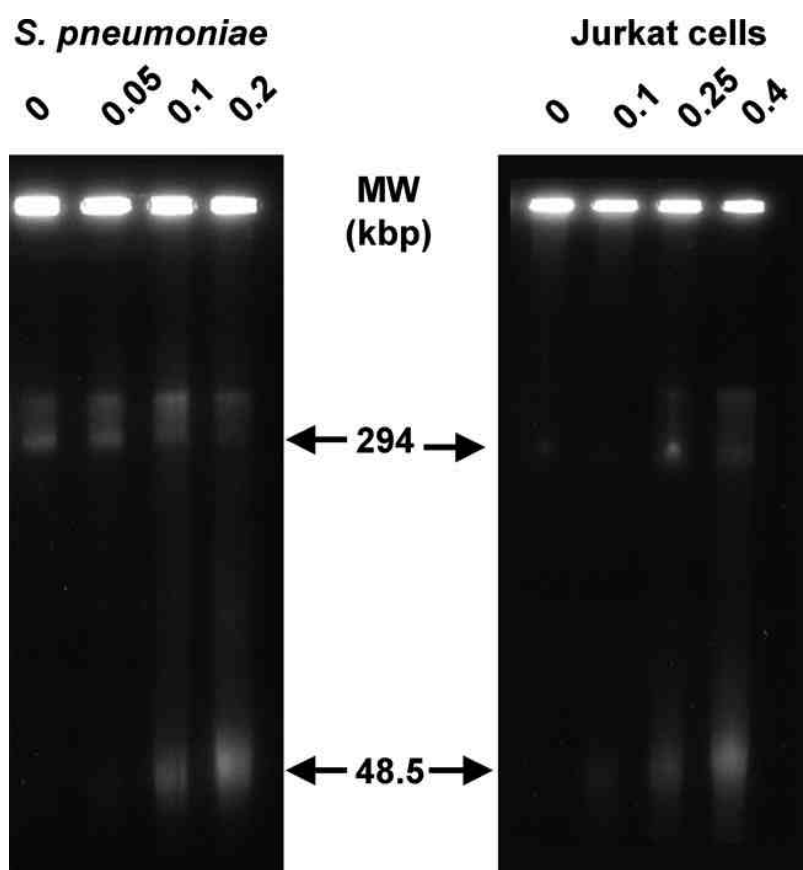


Figure 14 – High molecular weight DNA fragmentation in *S. pneumoniae* and Jurkat cells after treatment with HAMLET.

S. pneumoniae were incubated with HAMLET for 2 hours and Jurkat cells for 6 hours at 37°C and prepared for high molecular weight DNA fragmentation. Both bacteria and cells displayed fragments of 700, 300 and 50 kbp after treatment with MAL.

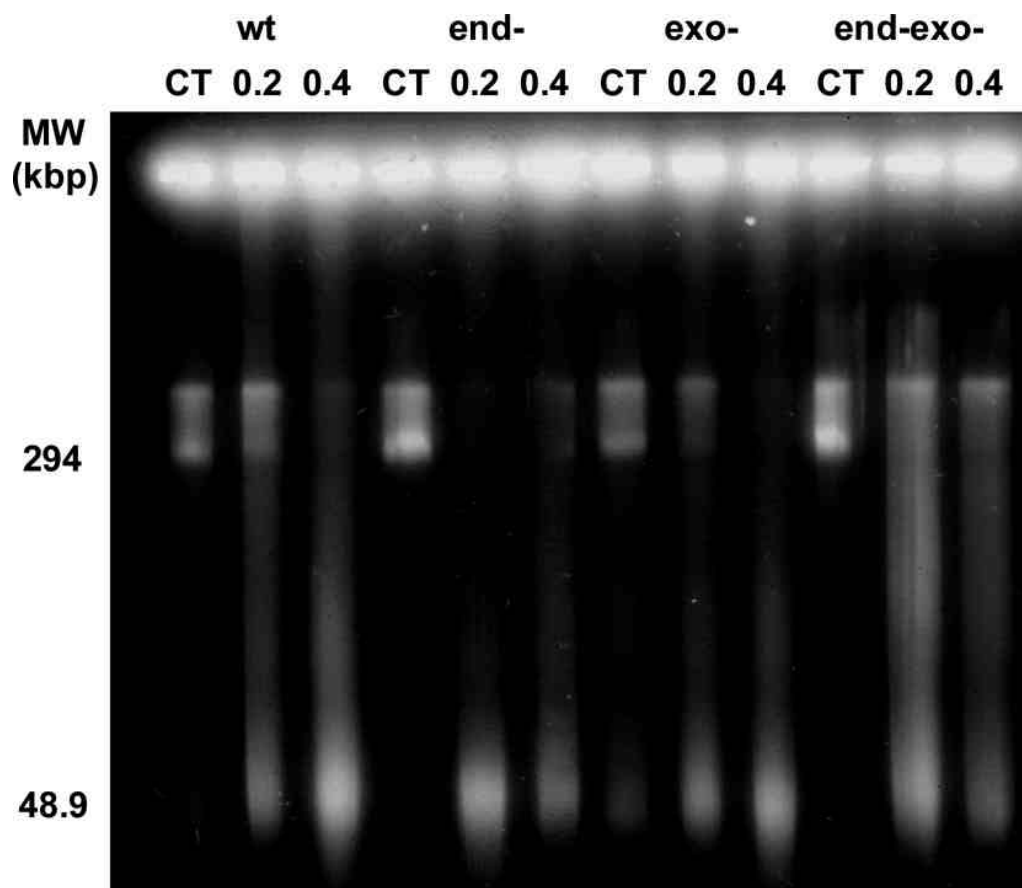


Figure 15 – High molecular weight DNA fragmentation in *S. pneumoniae* strains lacking EndA, ExoA or both nucleases. Three different nuclease activities have thus far been characterised in *S. pneumoniae*. The *endA* gene encodes an endonuclease that is thought to be involved in cleaving incoming DNA in genetic transformation. Mutant bacteria lacking the activity cannot be transformed (Lacks & Greenberg, 1967; Lacks *et al.*, 1975; Puyet *et al.*, 1990). The *exoA* gene encodes an exonuclease responsible for cleaving terminal nucleotides and producing single stranded DNA (Lacks & Greenberg, 1967; Puyet *et al.*, 1989). The function of this enzyme is still not known. The third nuclease is a restriction endonuclease designated *DpnI*. This endonuclease recognises and cleaves at the tetranucleotide sequence 5'-GATC-3' but only in methylated DNA (Lacks & Greenberg, 1975; Lacks & Greenberg, 1977). This strain therefore has unmethylated DNA at this site. There is a variant *DpnII*, with the same specificity but working on unmethylated DNA. This enzyme is not present in bacteria producing *DpnI* activity and the DNA in these bacteria is methylated by a DNA methylase (*DpnA*) (Mannarelli *et al.*, 1985).

Mutants lacking either *endA* or *exoA*, genes or both were kindly provided by Dr. Sanford Lacks, New York, USA, and tested for sensitivity to MAL-induced killing and DNA fragmentation. There was no difference in the concentration of MAL required to kill the bacteria, or in the DNA fragmentation pattern, excluding the role of *endA* and *exoA* in the DNA fragmentation induced upon MAL-treatment.

Binding of HAMLET to S. pneumoniae

HAMLET was shown to target mitochondria in tumour cells and bound rapidly to the surface of isolated mitochondria (Paper V). Binding was optimal at 4°C. Internalisation of the protein into mitochondria at higher temperatures was verified by fixation and permeabilisation before addition of FITC-streptavidin. The binding co-localised with Mitotracker, a mitochondria-specific dye produced by Molecular Probes. The molecular specificity of the reagent will not be divulged by the company.

The kinetics and specificity of HAMLET-binding was similar in *S. pneumoniae*. HAMLET bound within 5 minutes at 4°C and binding was maximal after 20 minutes of incubation. No binding was detected at higher temperatures suggesting internalisation of the protein. Another property of the bacteria was the ability to bind Mitotracker™. While HAMLET binding was specific for *S. pneumoniae* Mitotracker™ was found to interact with several bacterial species (see above). This suggested that there are similarities between mitochondria and bacteria and that HAMLET targets molecules that are shared between mitochondria and *S. pneumoniae*.

Cytochrome c

HAMLET induced the release of cytochrome c in sensitive cells within 1 hour of treatment. Cytochrome c-dependent aerobic respiration is a common feature of aerobic bacteria (Bartsch, 1968). *S. pneumoniae* is a facultative anaerobe that can live under aerobic conditions. The aerobic metabolism is not well studied in *S. pneumoniae* and cytochrome c has not been identified. As antibodies to human cytochrome c did not recognise any protein in the bacterial extracts, cytochrome c was measured by spectrophotometry. Cytochrome c has different spectral properties depending on whether the heme group is oxidised or reduced (Bartsch, 1968; Brown *et al.*, 1993; Thony-Meyer, 1997). When making a subtraction measurement with reduced cytochrome c in the reference cyvette and the oxidised form in the measurement cyvette a typical spectrum is obtained (Paper V). Peak γ - and α -absorbances are seen at 415-416 nm and 550 nm, respectively, with the first peak being the most pronounced (Bartsch, 1968). When analysing pneumococcal extracts before and after treatment with HAMLET we found that bacteria grown on blood agar under aerobic conditions showed a peak at 416 nm and a small peak at 550 nm, indicative of the presence of cytochrome c. Bacteria grown in broth (anaerobic conditions) did not, however, show any presence of cytochrome c specific spectra whether or not treated with HAMLET. As both bacteria grown on blood agar and in broth are equally sensitive to HAMLET and as HAMLET in both cases induce DNA fragmentation, we concluded that cytochrome c was not involved in this activation.

Caspase activity

HAMLET induced caspase activation of both group II and III caspases in the sensitive cells. Bacteria have not been reported to contain caspases. Extracts from HAMLET-treated bacteria were tested for caspase-like activity using cleavage of the fluorescent substrates as the end points. Ac-DEVD-amc (caspase group II) and Ac-IETD-amc (caspase group III) were cleaved by extracts from HAMLET-activated human cells, but no activity was seen at any time point in the bacterial extracts. The same was observed when using Ac-YVAD-amc (caspase group I) on bacterial extracts, suggesting that no caspase-like activity was present.

A bacterial AIF?

As none of these activation pathways appeared to be relevant for *S. pneumoniae* a search for other relevant sequence homologies was initiated. The *S. pneumoniae* genome has been sequenced by TIGR (The Institute for Genomic Research, Rockville, MD, USA), but the sequence is preliminary and has not been published. Sequence blast searches (Altschul *et al.*, 1997) can still be performed at the web-site of the National Center for Biotechnology Information (NCBI) at <http://www.ncbi.nlm.nih.gov>.

Searches of sequence homologies between the pneumococcal genome and human genes involved in apoptosis showed high homology between three open reading frames (ORFs) in the pneumococcal genome (in contiguous fragment 8, 37 and 42) and the human apoptosis-inducing factor (AIF). AIF has recently been cloned and described by Kroemer's lab (Lorenzo *et al.*, 1999; Susin *et al.*, 1999b). The protein has a molecular mass of 57 kDa and is homologous with bacterial oxidoreductases. AIF is a flavo-protein and exhibits oxidoreductase activity, but it does not require this activity for its apoptosis-inducing activity. The molecule is transported to the nucleus where it induces chromatin condensation and high molecular DNA fragmentation. This activity is caspase-independent. AIF has no inherent endonuclease activity and its effects inside the nucleus have not been characterised. The normal function of the protein inside the mitochondria is not known.

One of the OFRs from the pneumococcal genome (contig. 42) was identical to the pneumococcal NADH oxidase. The other two sequences are not characterised. NADH oxidase mutant strains were kindly provided by Dr. Marie-Claude Trombe, Toulouse, France, and were tested for sensitivity to HAMLET-induced killing and DNA fragmentation. There was no difference between the mutant and the parent strains suggesting that NADH oxidase was not the initiator molecule in HAMLET-induced apoptosis. We are currently mutating the other two sequences.

Evolution of apoptosis

The need to control cell death has been seen mainly as a feature of multicellular organisms, required for renewal, development and specialisation of tissue and organ systems. It therefore came as somewhat of a surprise when it was reported that unicellular eukaryotes such as free-living slime mould *Dichyatuim discoideum* or *Achantamoeba* showed morphology and DNA fragmentation similar to dying mammalian cells (Ameisen, 1996; Christensen *et al.*, 1998; Cornillon *et al.*, 1994; Olie *et al.*, 1998; Welburn & Murphy, 1998)

Similarities in the apoptosis execution machinery between bacteria and human cells have not been reported. Prokaryotes have been found to have primitive forms of programmed cell death or rather of terminal differentiation (Hochman, 1997). Certain bacteria can form multicellular aggregates in which single cells develop special functions that differ from other bacterial cells in the population as a response to environmental stresses (Shapiro, 1998). Examples are heterocyst formation in *Anabena* bacteria, fruiting bodies of *Myxobacteria*, the swarm behavior of *Proteus* (Shapiro, 1998). Our studies are the first to suggest that bacteria use mechanisms that are similar to those seen in eukaryotic apoptosis.

A programmed event for regulation of cell death in prokaryotes may either have evolved to regulate the life and death of the bacterial population or for other purposes. The life of *S. pneumoniae* is rather odd in comparison with many other bacterial species. In culture the bacteria will grow into stationary phase, and will then start to activate an autolysin, thereby committing suicide (Tomasz, 1968; Trombe *et al.*, 1992). This action is calcium-dependent, and results in the release of DNA from the interior of the bacteria. Pneumococci efficiently take up DNA from the environment, and become competent for DNA uptake in the presence of Ca^{2+} during the early logarithmic growth phase due to the synthesis of competence factor (Morrison & Baker, 1979; Trombe, 1993; Trombe *et al.*, 1994). For *S. pneumoniae* the fragmentation of DNA before dying may be an evolutionary advantage, as the DNA from one bacterium will be used by other bacteria in the population. This behaviour depicts seemingly altruistic behaviour by sacrificing certain individuals for the good of others.

Apoptosis or apoptosis-like morphology have been described in cells of mammals, plants (Greenberg, 1996), *Drosophila melanogaster* (McCall & Steller, 1997), *C. elegans* (Horvitz, 1999), and yeast (Jurgensmeier *et al.*, 1997; Manon *et al.*, 1997).. There is evidence for a systematic evolution and increased complexity of effector molecules and regulators of apoptosis. This stepwise increase in complexity is shown in Figure 16.

	DR ^b	Caspase ^c	cyt c ^d	bcl-2F ^e	nuc1 ^f	AIF ^g
Metazoans ^a						
Human	DR1-6 (Fas, TNFR Trail)	caspase 1-14	+	+	+	+
<i>D. melanogaster</i>	rip? rid? rpr?	drICE DC1 DC2	+?	-?	?	+?
<i>C. elegans</i>	-?	ced-3	-	ced-9	nuc-1	+?
Eukaryote						
Yeast	-	-	-	-	-	+?
Plant cells	?	?	-	-?	-	+?
Eubacteria						
Unicellular eukaryotes	-	-	-	-	-	+?
<i>R. prowasekii</i>	-	-	-	-	-	+?
<i>S. pneumoniae</i>	-	-	-	-	-?	+?

Figure 16 – Phylogenetic distribution of molecules involved in initiation and execution of apoptosis. The strongest evidence for a gene-directed mechanism of programmed cells death has come from studies of the nematode worm *Caenorhabditis elegans* (Ellis & Horvitz, 1986; Hengartner *et al.*, 1992). This model system has provided a basis for the understanding of basic mechanisms of apoptosis-induction in metazoans and have been important in the study of evolutionary conservation of molecules involved in apoptosis. The characteristics of programmed cells death of *Drosophila melanogaster* is less complete.

^a Metazoans denotes multicellular animals, ^b DR = death receptors. Death receptors are a rather late invention that can be detected in mammals but have no clear counterpart at any other evolutionary level (Aravind *et al.*, 1999). *D. melanogaster* may be an exception as three proteins with partial death domain homology have been found. However, these have not yet been verified as functional death domain homologues. ^c Casp = caspases. The characterisation of caspases was initiated when it was shown that the Ced-3 protein of *C. elegans* had homology with human interleukin-1 β -converting enzyme (Yuan *et al.*, 1993) and overexpression of Ced-3 in human fibroblasts led to the induction of apoptosis in these cells (Miura *et al.*, 1993). Three caspases, drICE, DC-1 and DC-2 are found in *D. melanogaster* (Fraser *et al.*, 1997; Song *et al.*, 1997) but caspases have not been found in unicellular eukaryotes such as yeast, which still die with apoptosis-like morphology and high molecular weight DNA fragmentation (Olie *et al.*, 1998). In plant cells cystein protease activity has been associated with apoptosis but no distinct caspase activity has been detected (Solomon *et al.*, 1999). ^d cyt c = cytochrome c (the letters mark the involvement of cytochrome c in programmed cells death of the organisms, not the presence of cytochrome c in the cells), Cytochrome c is also a rather late regulatory molecule in apoptosis. It does not interact with ced-4 in *C. elegans* and has not been associated with apoptosis in the worm (Hu *et al.*, 1998b). There is some evidence for an involvement of cytochrome c in *D. melanogaster*, but its precise role in the caspase activation is unknown (Varkey

et al., 1999). Ced-4 was more recently shown to be homologous to the human adaptor protein APAF-1 (apoptosis protease activating factor 1) (Zou *et al.*, 1997). No functional homologous proteins have thus far been found in *D. melanogaster*. ^eBcl-2F = Bcl-2 family members, Ced-9 mutations in *C. elegans* were shown to be lethal, and gain of function mutations lead to survival of all cells, suggesting an anti-cell death effect of the protein (Hengartner *et al.*, 1992). This protein is homologous to Bcl-2, that has later been shown to be an important regulator of the cell death machinery (Hengartner & Horvitz, 1994a; Hengartner & Horvitz, 1994b). Bcl-2 can substitute for Ced-9 in *C. elegans* and vice versa (Vaux *et al.*, 1992). Bcl-2 proteins are also latecomers absent in yeast and bacteria (Aravind *et al.*, 1999; Hengartner *et al.*, 1992). There is still no homologies found in *D. melanogaster* (McCall & Steller, 1997), however such molecules would be expected. There is no evidence for bcl-2 proteins in plant cells (Vaux & Korsmeyer, 1999) and overexpression of bcl-X_L in plant cells did not protect them from apoptosis (Mittler & Lam, 1996) ^fnucl = nucleases described to be involved in apoptosis, and ^g AIF = apoptosis-inducing factor There is so far little information on the presence of AIF in different systems. Sequence homologies are found in most levels of evolution, including bacteria, but since the protein's active site is unknown no functional homologues besides the mouse have been determined (Lorenzo *et al.*, 1999).

The evolutionary tree was created from information retrieved at The Tree of Life website (<http://phylogeny.arizona.edu/tree>).

Our findings argue that rudimentary cell death programs may have existed far back in evolution, but that new regulatory and effector molecules have evolved in the eukaryotic cells. The “new” features include caspase-dependent mechanisms, which have taken over as the most important regulatory events in metazoan cells. Some features of apoptosis, for example cell shrinkage, nuclear condensation, mitochondrial effects and high molecular weight DNA fragmentation are observed in plant cells or yeast cells where no caspases have been detected and they can still be induced in human cells when caspase activation is blocked, suggesting the existence of older mechanisms in such cells (Borner & Monney, 1999; Bortner & Cidlowski, 1999; Kawahara *et al.*, 1998; Lavoie *et al.*, 1998).

In the bacteria a candidate pathway is that involving AIF, whose homology with proteins far back in evolution posits a potential role for this molecule as an early players in the first regulated apoptosis program. *S. pneumoniae* were found to resemble HAMLET-treated cells with high molecular weight DNA fragmentation and some indications of cell shrinkage. Bacteria may indeed provide a simpler model system to identify the HAMLET induced apoptosis pathways among the complex pathways seen in mammalian cell apoptosis.

Ett bröstmjölksprotein som dödar tumörceller

(Swedish summary for a lay audience)

Denna avhandling beskriver den tumörcell- och bakteriedödande effekten av ett proteinkomplex från bröstmjolk. Proteinkomplexet, en veckningsvariant av humant α -laktalbumin, inducerar apoptos (programmerad celldöd) i tumörceller och visar sig ha liknande effekter även mot bakterier.

Amning förser inte bara barnet med optimal näring utan har också andra biologiska funktioner. Amning skyddar mot infektioner genom ett flertal komponenter som kan döda eller oskadliggöra bakterier och virus i luftvägarna och mag-tarmkanalen. Ammade barn löper även en lägre risk att drabbas av diabetes, inflammatoriska tarmsjukdomar, allergier och vissa typer av cancer i barndomen.

Under våra studier av mjölksens anti-bakteriella effekter fann vi att mjölken även kunde döda tumörceller genom apoptos, den fysiologiska typ av celldöd som är viktig vid normala processer i kroppen där gamla eller felaktiga celler ska elimineras. Apoptos är särskilt viktig under fosterutvecklingen för att eliminera överflödiga celler och för att forma kroppens organ och extremiteter, men även i vuxen vävnad krävs reglering av cellpopulationers sammansättning. Apoptos är en aktiv process som kräver energi och som utförs på ett förutbestämt och kontrollerat sätt enligt ett nedärvt genetiskt program som finns i de flesta eukaryota celler. Apoptotiska celler har en annorlunda morfologi än nekrotiska celler. Vid apoptos minskar cellens volym, dess organeller förpackas, kärnans kromatin kondenseras och bryts ner, och cellen spricker slutligen upp i membranomslutna vesikler (apoptotiska kroppar) med cellulärt material som äts upp av närliggande celler eller professionella makrofager. Till skillnad från vid nekros aktiveras inte immunförsvaret.

MAL-behandlade tumörceller uppvisade typiska apoptos-förändringar genom att cellerna minskade i volym och kromatinet kondenserades. Samtidigt inducerades den för apoptos typiska DNA fragmenteringen med högmolekylära fragment med en storlek av 50-700 kpb åtföljt av vidare nerbrytning till oligomera fragment om 200 bp.

MAL dödade tumörceller, omogna och embryonala celler men hade ingen aktivitet mot friska icke-transformerade epitelceller. Lymfoida tumörceller uppvisade störst känslighet (0.25-0.5 mg/ml krävdes för att döda 50% av cellerna efter 6 timmar), medan carcinom krävde något mer protein för att dö (ca 1 mg/ml, 24 timmar). Friska epitelceller var resistenta mot apoptosinduktion av MAL.

För att undersöka mekanismerna för MAL-inducerad celldöd och för att försöka förstå skillnaden mellan tumörceller och friska celler valdes två leukemicellinjer (L1210, Jurkat), ett carcinom (A549) samt friska, icke-transformerade epitelceller (HRTEC) för vidare studier. Lokaliseringsstudier visade att proteinet först binder till cellernas yta, men mängden protein på cellytan korrelerade inte till cellernas känslighet för apoptos. Däremot sågs en skillnad i upptag mellan känsliga och resistenta celler. Känsliga celler hade dels en granulär infärgning av cytoplasman, dels fick ca 70% av cellerna färgning av MAL i cellkärnan. De resistenta cellerna uppvisade bara ytbindning och inget protein kunde lokaliseras inuti cellerna. Vi analyserade sedan hur MAL i cytoplasman, respektive i kärnan kunde orsaka apoptos.

Den granulära fluorescensen förklarades av att MAL band till mitokondrier i känsliga celler. Mitokondrier har nyligen visat sig spela en central roll för apoptosförloppet. Apoptosstimuli som når mitokondrierna orsakar vanligen s.k. permeability transition (PT), dvs öppnandet av megakanaler mellan mitokondriens inre och yttre membran. Detta leder till att potentialen över membranen försvinner och mitokondrien svullnar varvid yttermembranen spricker och släpper ut apoptos-inducerande faktorer. En sådan faktor är cytokrom c, som vanligtvis är involverad i elektrontransportkedjan på mitokondriens innermembran som en del av den syreberoende ATP produktionen. Cytokrom c bildar sedan ett komplex med APAF-1 och pro-caspase-9 i cytoplasman, vilket leder till caspase-aktivering. Caspaser är en grupp cysteinproteaser som dels bryter ner molekyler som skyddar cellen mot apoptos, eller bevarar cellens form och arkitektur, dels aktiverar faktorer som krävs för utförandet av apoptosprocessen, såsom DNA-nedbrytande enzymer. MAL-behandlade celler uppvisade snabbt en minskad potential över mitokondriemembranen följt av en frisättning av cytokrom c. Resultaten tyder på att MAL kan ha en direkt effekt på mitokondrienivå i känsliga celler.

Caspas-aktivitet kunde detekteras efter 1 timme, och nådde en aktivitetstopp vid ca 2-3 timmar. Caspasernas visade sig klyva substrat inuti cellen, såsom lamin B som är ett strukturellt protein i kärnan, PARP (poly-ADP-ribose polymerase) som ansvarar för övervakning av DNA integritet samt α -fodrin som är en cytoplasmatisk beståndsdel i cellens cytoskelett.

MAL verkade också direkt på cellkärnor. Först transporterades MAL till kärnan via aktiv nukleär transport. Därefter band MAL starkt till komponenter i kärnan men inte direkt till DNA. Dock visade sig MAL påverka DNA-integriteten på kärnnivå. MAL-behandlade isolerade kärnor uppvisade samma fragmenteringsmönster som apoptotiska celler vilket tyder på att MAL kan inducera apoptosliknande förändringar direkt i kärnan.

MAL isolerades från kasein genom jonbytkromatografi. N-terminal sekvensering påvisade oligomera band av α -laktalbumin, men den nativa formen av proteinet hade ingen tumörcelldödande effekt. Jämförelser mellan den aktiva och inaktiva formen visade inga skillnader i den kovalenta strukturen, men den apoptos-inducerande formen av α -laktalbumin hade en annorlunda tredimensionell konformation. När-UV och fluorescence-spektroskopi visade att den aktiva fraktionen har en mer flexibel struktur och exponerar mer hydrofoba ytor, och att den liknar en känd form av proteinet, den s.k. molten globule state. Denna är en intermediär form som existerar då proteinet veckar sig och som också kan bildas vid lågt pH, i närvaro av denaturerande ämnen såsom urea och då proteinets Ca^{2+} jon tas bort. Den aktiva komponenten skilde sig dock i sin stabilitet från molten globule och vi har föreslagit att detta beror på proteinets inbindning av en fettsyra från mjölken. Vi har också kunnat konvertera nativt α -laktalbumin till den tumörinducerade formen genom att ta bort kalcium och tillsätta fettsyra som stabilisator. Den så tillverkade molekylen har vi gett namnet HAMLET, Human α -laktalbumin made lethal to tumour cells.

MAL och HAMLET dödade också vissa bakterier, speciellt *Streptococcus pneumoniae*. MAL och HAMLET dödade 10^7 bakterier på 30 minuter vid en koncentration av 0.3 mg/ml. Andra streptokocker och *Haemophilus influenzae*, är också känsliga medan ingen effekt kunde uppvisas mot andra grampositiva (*S. aureus*, *S. epidermidis*, *E. fecalis*) eller gramnegativa (*E. coli*, *M. catharralis*, *P. aeruginosa*) bakterier. De cellulära förändringarna hos bakterierna liknade de humana apoptotiska cellerna. Bakterierna klöv sitt DNA efter MAL-behandling och till vår förvåning fann vi att fragmentstorleken var identisk med den man ser vid apoptos i tumörceller. Detta tyder på att apoptos-liknande processer kan induceras också i bakterier.

Parallellen mellan effekten på tumörceller och bakterier blir speciellt intressant då man beaktar släktskapen mellan bakterier och mitokondrier. Eukaryota celler tros ha bildats genom en symbios mellan syretoleranta eubakterier och de evolutionärt äldre archeabakterierna. Det är inte otänkbart att genetiska program som styr apoptosprocessen härrör från tiden som prokaryot organism.

För att testa denna hypotes undersökte vi likheter mellan mitokondrier och *S. pneumoniae*. MAL binder till ytan av både mitokondrier och *S. pneumoniae* och hos båda ses en co-lokaliering med mitokondriespecifika reagenser. Till skillnad från tumörceller så frisatte inte bakterierna cytokrom c och ingen caspase-aktivitet kunde detekteras. Detta tyder på att bakterier kan ha kvar andra äldre evolutionära mekanismer. DNA sekvensanalys av *S. pneumoniae* visade homologi med apoptosis-

inducing factor (AIF), ett flavo-protein som finns i mitokondrien och som frisätts tillsammans med cytokrom c vid induktion av apoptos. AIF kan direkt inducera kromatinkondensering och högmolekylär DNA fragmentering i isolerade cellkärnor utan att aktivera caspaser. AIF visade sekvenshomologi med tre gener i pneumokockernas genom. En av dessa gener, NADH oxidase, var inte involverad i apoptos av bakterierna då MAL fortfarande inducerade död och DNA fragmentering i bakterier som saknar detta protein. De andra två generna kommer att undersökas framöver.

Varför finns komponenter med tumörcelldödande och bakteriedödande effekter i mjölk? Epidemiologiska studier har visat att amning skyddar mot uppkomst av tumörer (speciellt lymfom) och mot infektioner. Barnets immunförsvar är inte fullt utvecklat vid födseln och barnet får därför ett tillskott av antibakteriella och immunmodulerande faktorer med moderns mjölk. Likaså är barnets mukosaytor och lymfoida system i tarmen snabbt växande vävnader, vilka kan behöva reglerande faktorer med mjölken för att undvika fel som kan leda till uppkomst av cancer eller andra sjukdomar.

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*If you don't want to look forward, and don't dare to look back,
then you have to look out*

Tage Danielsson (free translation)

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You sit on the stairs to a small house in front of a just as small garden in the middle of Lund. It is Saturday, it is early Summer and the flowers have just sprung into bloom. The last version of your manuscript is stained with tea that was recently pored into 18th century tea bowls of Chinese porcelain. An air of enthusiasm and science wraps the garden. You have just finished the last ginger cookie on the saucer and in your mouth whirls the distinct taste only Gothenburg Ginger Cookie Bakery can produce. You discuss, you scribble down ideas, you plan, and you write or talk about life. In the middle of everything Catharina runs off to the Rhododendron bush and cuts a few branches off, at the same time mentioning something about two chairs she found in an antique shop and just have to buy, suddenly being right back with a scientific idea. You discuss some more and scribble down the thoughts as they are formulated. –But couldn't it be like this? What if we did like that? The strangest of ideas are allowed and welcome, and make science feel challenging and fun. The telephone is ringing again but she is not answering it, or rather yes she is in the end. While you sit leaned against the sun-heated wall waiting for the call to end you cannot but feel fortunate that you are doing what you do.

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References

- Acharya, K. R., Ren, J. S., Stuart, D. I., Phillips, D. C., and Fenna, R. E. (1991) Crystal structure of human alpha-lactalbumin at 1.7 Å resolution. *J Mol Biol* 221: 571-81
- Acharya, K. R., Stuart, D. I., Walker, N. P., Lewis, M., and Phillips, D. C. (1989) Refined structure of baboon alpha-lactalbumin at 1.7 Å resolution. Comparison with C-type lysozyme. *J Mol Biol* 208: 99-127
- Adam, S. A. (1999) Transport pathways of macromolecules between the nucleus and the cytoplasm. *Curr Opin Cell Biol* 11: 402-6
- Adams, J. M., and Cory, S. (1998) The Bcl-2 protein family: arbiters of cell survival. *Science* 281: 1322-6
- Adrain, C., Slee, E. A., Harte, M. T., and Martin, S. J. (1999) Regulation of Apoptotic Protease Activating Factor-1 Oligomerization and Apoptosis by the WD-40 Repeat Region. *J Biol Chem* 274: 20855-20860
- Ahmed, T., and Fuchs, G. J. (1997) Gastrointestinal allergy to food: a review. *J Diarrhoeal Dis Res* 15: 211-23
- Alho, O. P., Koivu, M., Sorri, M., and Rantakallio, P. (1990) Risk factors for recurrent acute otitis media and respiratory infection in infancy. *Int J Pediatr Otorhinolaryngol* 19: 151-61
- Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) Human ICE / CED-3 protease nomenclature. *Cell* 87: 171
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389-402
- Amarante-Mendes, G. P., Naekyung Kim, C., Liu, L., Huang, Y., Perkins, C. L., Green, D. R., and Bhalla, K. (1998) Bcr-Abl exerts its antiapoptotic effect against diverse apoptotic stimuli through blockage of mitochondrial release of cytochrome C and activation of caspase-3. *Blood* 91: 1700-5
- Ameisen, J. C. (1996) The origin of programmed cell death. *Science* 272: 1278-9
- Andersson, B., Dahmén, J., Freijd, T., Leffler, H., Magnusson, G., Noori, G., and Svanborg-Edén, C. (1983) Identification of a disaccharide unit of a glycoconjugate receptor for pneumocci attaching to human pharyngeal epithelial cells. *J Exp Med* 158: 559-70
- Andersson, B., Eriksson, B., Falsen, E., Fogh, A., Hanson, L. Å., Nylén, O., Peterson, H., and Svanborg-Edén, C. (1981) Adhesion of *Streptococcus pneumoniae* to human pharyngeal epithelial cells in vitro: Differences in adhesive capacity among strains isolated from subjects with otitis media, septicemia or meningitis or from healthy donors. *Infect Immun* 32: 311-7
- Andersson, B., Porras, O., Hanson, L. Å., Lagergård, T., and Svanborg-Edén, C. (1986) Inhibition of attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by human milk and receptor oligosaccharides. *J Infect Dis* 153: 232-7
- Andersson, S. G., Zomorodipour, A., Andersson, J. O., Sicheritz-Ponten, T., Alsmark, U. C., Podowski, R. M., Naslund, A. K., Eriksson, A. S., Winkler, H. H., and Kurland, C. G. (1998) The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* 396: 133-40
- Anfinsen, C. B. (1973) Principles that govern the folding of protein chains. *Science* 181: 223-30
- Aniansson, G., Alm, B., Andersson, B., Håkansson, A., Larsson, P., Nylén, O., Peterson, H., Rignér, P., Svanborg, M., Sabharwal, H., and Svanborg, C. (1994) A prospective cohort study on breast-feeding and otitis media in Swedish infants. *Pediatr Infect Dis J* 13: 183-8
- Aniansson, G., Andersson, B., Lindstedt, R., and Svanborg, C. (1990) Anti-adhesive activity of human casein against *Streptococcus pneumoniae* and *Haemophilus influenzae*. *Microb Pathog* 8: 315-23

- Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J. J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R., and Martinou, J. C. (1997) Inhibition of Bax channel-forming activity by Bcl-2. *Science* 277: 370-2
- Aramini, J. M., Drakenberg, T., Hiraoki, T., Ke, Y., Nitta, K., and Vogel, H. J. (1992) Calcium-43 NMR studies of calcium-binding lysozymes and alpha-lactalbumins. *Biochemistry* 31: 6761-8
- Aramini, J. M., Hiraoki, T., Grace, M. R., Swaddle, T. W., Chiancone, E., and Vogel, H. J. (1996) NMR and stopped-flow studies of metal ion binding to alpha-lactalbumins. *Biochim Biophys Acta* 1293: 72-82
- Aravind, L., Dixit, V. M., and Koonin, E. V. (1999) The domains of death: evolution of the apoptosis machinery. *Trends Biochem Sci* 24: 47-53
- Ashkenazi, A., and Dixit, V. M. (1998) Death receptors: signaling and modulation. *Science* 281: 1305-8
- Ashkenazi, A., and Dixit, V. M. (1999) Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* 11: 255-60
- Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L., and Korsmeyer, S. J. (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* 41: 899-906
- Balbach, J., Forge, V., Lau, W. S., van Nuland, N. A., Brew, K., and Dobson, C. M. (1996) Protein folding monitored at individual residues during a two- dimensional NMR experiment. *Science* 274: 1161-3
- Baldwin, R. L. (1995) The nature of protein folding pathways: the classical versus the new view. *J Biomol NMR* 5: 103-9
- Banuelos, S., and Muga, A. (1995) Binding of molten globule-like conformations to lipid bilayers. Structure of native and partially folded alpha-lactalbumin bound to model membranes. *J Biol Chem* 270: 29910-5
- Banuelos, S., and Muga, A. (1996a) Interaction of native and partially folded conformations of alpha-lactalbumin with lipid bilayers: characterization of two membrane-bound states. *FEBS Lett* 386: 21-5
- Banuelos, S., and Muga, A. (1996b) Structural requirements for the association of native and partially folded conformations of alpha-lactalbumin with model membranes. *Biochemistry* 35: 3892-8
- Barry, M. A., and Eastman, A. (1993) Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch Biochem Biophys* 300: 440-50
- Bartsch, R. G. (1968) Bacterial cytochromes. *Annu Rev Microbiol* 22: 181-200
- Baum, J., Dobson, C. M., Evans, P. A., and Hanley, C. (1989) Characterization of a partly folded protein by NMR methods: studies on the molten globule state of guinea pig alpha-lactalbumin. *Biochemistry* 28: 7-13
- Beaudry, M., Dufour, R., and Marcoux, S. (1995) Relation between infant feeding and infections during the first six months of life. *J Pediatr* 126: 191-7
- Bell, J. E., Beyer, T. A., and Hill, R. L. (1976) The kinetic mechanism of bovine milk galactosyltransferase. The role of alpha-lactalbumin. *J Biol Chem* 251: 3003-13
- Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K., and Tomita, M. (1992) Antibacterial spectrum of lactoferrin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J Appl Bacteriol* 73: 472-479
- Bellamy, W., Wakabayashi, H., Takase, M., Kawase, K., Shimamura, S., and Tomita, M. (1993) Killing of *Candida albicans* by lactoferricin B, a potent antimicrobial peptide derived from the N-terminal region of bovine lactoferrin. *Med Microbiol Immunol* 182: 97-105

- Benito, A., Silva, M., Grillot, D., Nunez, G., and Fernandez-Luna, J. L. (1996) Apoptosis induced by erythroid differentiation of human leukemia cell lines is inhibited by Bcl-XL. *Blood* 87: 3837-43
- Berliner, L. J., and Koga, K. (1987) Alpha-lactalbumin binding to membranes: evidence for a partially buried protein. *Biochemistry* 26: 3006-9
- Berliner, L. J., Meinholtz, D. C., Hirai, Y., Musci, G., and Thompson, M. P. (1991) Functional implications resulting from disruption of the calcium-binding loop in bovine alpha-lactalbumin. *J Dairy Sci* 74: 2394-402
- Borner, C., and Monney, L. (1999) Apoptosis without caspases: an inefficient molecular guillotine? *Cell Death Differ* 6: 497-507
- Bortner, C. D., and Cidlowski, J. A. (1999) Caspase independent/dependent regulation of K(+), cell shrinkage, and mitochondrial membrane potential during lymphocyte apoptosis. *J Biol Chem* 274: 21953-21962
- Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *Embo J* 17: 37-49
- Brew, K., and Campbell, P. N. (1967) The characterization of the whey proteins of guinea-pig milk. The isolation and properties of alpha-lactalbumin. *Biochem J* 102: 258-64
- Brodbeck, U., and Ebner, K. E. (1966) Resolution of a soluble lactose synthetase into two protein components and solubilization of microsomal lactose synthetase. *J Biol Chem* 241: 762-4
- Brown, S., Colson, A. M., Meunier, B., and Rich, P. R. (1993) Rapid screening of cytochromes of respiratory mutants of *Saccharomyces cerevisiae*. Application to the selection of strains containing novel forms of cytochrome-c oxidase. *Eur J Biochem* 213: 137-45
- Brown, S. B., Bailey, K., and Savill, J. (1997) Actin is cleaved during constitutive apoptosis. *Biochem J* 323: 233-7
- Brustugun, O. T., Fladmark, K. E., Doskeland, S. O., Orrenius, S., and Zhivotovsky, B. (1998) Apoptosis induced by microinjection of cytochrome c is caspase-dependent and is inhibited by Bcl-2. *Cell Death Differ* 5: 660-8
- Cai, J., Yang, J., and Jones, D. P. (1998) Mitochondrial control of apoptosis: the role of cytochrome c. *Biochim Biophys Acta* 1366: 139-49
- Cawthern, K. M., Narayan, M., Chaudhuri, D., Permyakov, E. A., and Berliner, L. J. (1997) Interactions of alpha-lactalbumin with fatty acids and spin label analogs. *J Biol Chem* 272: 30812-6
- Cawthern, K. M., Permyakov, E., and Berliner, L. J. (1996) Membrane-bound states of alpha-lactalbumin: implications for the protein stability and conformation. *Protein Sci* 5: 1394-405
- Chandra, N., Brew, K., and Acharya, K. R. (1998) Structural evidence for the presence of a secondary calcium binding site in human alpha-lactalbumin. *Biochemistry* 37: 4767-72
- Chandra, R. K. (1979) Prospective studies of the effect of breast feeding on incidence of infection and allergy. *Acta Paediatr Scand* 68: 691-4
- Chaput, M., Claes, V., Portetelle, D., Cludts, I., Cravador, A., Burny, A., Gras, H., and Tartar, A. (1988) The neurotrophic factor neuroleukin is 90% homologous with phosphohexose isomerase. *Nature* 332: 454-5
- Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. (1997) Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* 278: 1966-8
- Cheng, J., and Haas, M. (1990) Frequent mutations in the p53 tumor suppressor gene in human leukemia T-cell lines. *Mol Cell Biol* 10: 5502-9

- Chiba, I., Takahashi, T., Nau, M. M., D'Amico, D., Curiel, D. T., Mitsudomi, T., Buchhagen, D. L., Carbone, D., Piantadosi, S., Koga, H., and et al. (1990) Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. Lung Cancer Study Group. *Oncogene* 5: 1603-10
- Chinnaiyan, A. M., Chaudhary, D., O'Rourke, K., Koonin, E. V., and Dixit, V. M. (1997a) Role of CED-4 in the activation of CED-3. *Nature* 388: 728-9
- Chinnaiyan, A. M., O'Rourke, K., Lane, B. R., and Dixit, V. M. (1997b) Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* 275: 1122-6
- Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G., and Lutz, R. J. (1995) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *Embo J* 14: 5589-96
- Chonmaitree, T., Howie, V. M., and Truant, A. L. (1986) Presence of respiratory viruses in middle ear fluids and nasal wash specimens from children with acute otitis media. *Pediatrics* 77: 698-702
- Christensen, S. T., Leick, V., Rasmussen, L., and Wheatley, D. N. (1998) Signaling in unicellular eukaryotes. *Int Rev Cytol* 177: 181-253
- Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* 362: 849-52
- Clarke, P. G., and Clarke, S. (1996) Nineteenth century research on naturally occurring cell death and related phenomena. *Anat Embryol (Berl)* 193: 81-99
- Cleary, M. L., and Sklar, J. (1985) Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. *Proc Natl Acad Sci U S A* 82: 7439-43
- Clem, R. J., Cheng, E. H., Karp, C. L., Kirsch, D. G., Ueno, K., Takahashi, A., Kastan, M. B., Griffin, D. E., Earnshaw, W. C., Veluona, M. A., and Hardwick, J. M. (1998) Modulation of cell death by Bcl-XL through caspase interaction. *Proc Natl Acad Sci U S A* 95: 554-9
- Clements, D. A., Henderson, F. W., and Neebe, E. C. (1993) in *Fifth International Symposium on Recent Advances in Otitis Media* (Lim, D., ed), pp. 27-29, Decker Periodicals
- Colussi, P. A., Harvey, N. L., and Kumar, S. (1998) Prodomain-dependent nuclear localization of the caspase-2 (Nedd2) precursor. A novel function for a caspase prodomain. *J Biol Chem* 273: 24535-42
- Cornillon, S., Foa, C., Davoust, J., Buonavista, N., Gross, J. D., and Golstein, P. (1994) Programmed cell death in Dictyostelium. *J Cell Sci* 107: 2691-704
- Cravioto, A., Tello, A., Villafan, H., Ruiz, J., del Vedovo, S., and Neeser, J. R. (1991) Inhibition of localized adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. *J Infect Dis* 163: 1247-55
- Creighton, T. (1995) Disulphide-coupled protein folding pathways. *Philos Trans R Soc Lond B Biol Sci* 348: 5-10
- Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 341: 233-249
- Cunningham, A. S. (1979) Morbidity in breast-fed and artificially fed infants. II *J Pediatr* 95: 685-9
- Cunningham, A. S., Jelliffe, D. B., and Jelliffe, E. F. (1991) Breast-feeding and health in the 1980s: a global epidemiologic review. *J Pediatr* 118: 659-66
- Cushing, A. H., Samet, J. M., Lambert, W. E., Skipper, B. J., Hunt, W. C., Young, S. A., and McLaren, L. C. (1998) Breastfeeding reduces risk of respiratory illness in infants. *Am J Epidemiol* 147: 863-70

- D'Silva, P. R., and Lala, A. K. (1999) Hydrophobic photolabeling as a new method for structural characterization of molten globule and related protein folding intermediates. *Protein Sci* 8: 1099-103
- Davies, M. S., West, L. F., Davis, M. B., Povey, S., and Craig, R. K. (1987) The gene for human alpha-lactalbumin is assigned to chromosome 12q13. *Ann Hum Genet* 51: 183-8
- Davis, M. K. (1998) Review of the evidence for an association between infant feeding and childhood cancer. *Int J Cancer Suppl* 11: 29-33
- Davis, M. K., Savitz, D. A., and Graubard, B. I. (1988) Infant feeding and childhood cancer. *Lancet* 2: 365-8
- Davis, T. A., Nguyen, H. V., Garcia-Bravo, R., Fiorotto, M. L., Jackson, E. M., Lewis, D. S., Lee, D. R., and Reeds, P. J. (1994) Amino acid composition of human milk is not unique. *J Nutr* 124: 1126-32
- Debatin, K. M., Beltinger, C., Bohler, T., Fellenberg, J., Friesen, C., Fulda, S., Herr, I., Los, M., Scheuerpflug, C., Sieverts, H., and Stahnke, K. (1997) Regulation of apoptosis through CD95 (APO-I/Fas) receptor-ligand interaction. *Biochem Soc Trans* 25: 405-10
- Demarest, S. J., Fairman, R., and Raleigh, D. P. (1998) Peptide models of local and long-range interactions in the molten globule state of human alpha-lactalbumin. *J Mol Biol* 283: 279-91
- Devitt, A., Moffatt, O. D., Raykundalia, C., Capra, J. D., Simmons, D. L., and Gregory, C. D. (1998) Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* 392: 505-9
- Dewey, K. G., Heinig, M. J., and Nommsen-Rivers, L. A. (1995) Differences in morbidity between breast-fed and formula-fed infants. *J Pediatr* 126: 696-702
- Distelhorst, C. W., and McCormick, T. S. (1996) Bcl-2 acts subsequent to and independent of Ca²⁺ fluxes to inhibit apoptosis in thapsigargin- and glucocorticoid-treated mouse lymphoma cells. *Cell Calcium* 19: 473-83
- Dobson, C. M. (1995) Finding the right fold. *Nat Struct Biol* 2: 513-7
- Dolgikh, D. A., Abaturon, L. V., Bolotina, I. A., Brazhnikov, E. V., Bychkova, V. E., Gilmanshin, R. I., Lebedev Yu, O., Semisotnov, G. V., Tiktopulo, E. I., Ptitsyn, O. B., and et al. (1985) Compact state of a protein molecule with pronounced small-scale mobility: bovine alpha-lactalbumin. *Eur Biophys J* 13: 109-21
- Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S., and Ptitsyn, O. B. (1981) Alpha-Lactalbumin: compact state with fluctuating tertiary structure? *FEBS Lett* 136: 311-5
- Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356: 215-21
- Duncan, B., Ey, J., Holberg, C. J., Wright, A. L., Martinez, F. D., and Taussig, L. M. (1993) Exclusive breast-feeding for at least 4 months protects against otitis media. *Pediatrics* 91: 867-72
- Duvall, E., Wyllie, A. H., and Morris, R. G. (1985) Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology* 56: 351-8
- Eguchi, Y., Shimizu, S., and Tsujimoto, Y. (1997) Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res* 57: 1835-40
- Ehinger, M., Bergh, G., Olofsson, T., Baldetorp, B., Olsson, I., and Gullberg, U. (1996) Expression of the p53 tumor suppressor gene induces differentiation and promotes induction of differentiation by 1,25-dihydroxycholecalciferol in leukemic U-937 cells *Blood* 87: 1064-74
- Ehinger, M., Nilsson, E., Persson, A. M., Olsson, I., and Gullberg, U. (1995) Involvement of the tumor suppressor gene p53 in tumor necrosis factor- induced differentiation of the leukemic cell line K562 *Cell Growth Differ* 6: 9-17

- Ellis, H. M., and Horvitz, H. R. (1986) Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* 44: 817-29
- Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391: 43-50
- Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A., and Martinou, J. C. (1998) Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg²⁺ ions. *J Cell Biol* 143: 217-24
- Fadeel, B., Hassan, Z., Hellstrom-Lindberg, E., Henter, J. I., Orrenius, S., and Zhivotovsky, B. (1999) Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of human myeloid leukemia cells. *Leukemia* 13: 719-28
- Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148: 2207-16
- Faik, P., Walker, J. I., Redmill, A. A., and Morgan, M. J. (1988) Mouse glucose-6-phosphate isomerase and neuroleukin have identical 3' sequences. *Nature* 332: 455-7
- Fernandes, R. S., and Cotter, T. G. (1993) Activation of a calcium magnesium independent endonuclease in human leukemic cell apoptosis. *Anticancer Res* 13: 1253-9
- Findlay, J. B., and Brew, K. (1972) The complete amino-acid sequence of human alpha-lactalbumin. *Eur J Biochem* 27: 65-86
- Fontana, A., Polverino de Laureto, P., De Filippis, V., Scaramella, E., and Zamboni, M. (1997) Probing the partly folded states of proteins by limited proteolysis. *Fold Des* 2: R17-26
- Fraser, A. G., McCarthy, N. J., and Evan, G. I. (1997) drICE is an essential caspase required for apoptotic activity in *Drosophila* cells. *Embo J* 16: 6192-9
- Freed, G. L., Clark, S. J., Sorenson, J., Lohr, J. A., Cefalo, R., and Curtis, P. (1995) National assessment of physicians' breast-feeding knowledge, attitudes, training, and experience. *Jama* 273: 472-6
- Gaido, M. L., and Cidlowski, J. A. (1991) Identification, purification, and characterization of a calcium-dependent endonuclease (NUC18) from apoptotic rat thymocytes. NUC18 is not histone H2B. *J Biol Chem* 266: 18580-5
- Garcia-Calvo, M., Peterson, E. P., Leiting, B., Ruel, R., Nicholson, D. W., and Thornberry, N. A. (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem* 273: 32608-13
- Garofalo, R. P., and Goldman, A. S. (1998) Cytokines, chemokines, and colony-stimulating factors in human milk: the 1997 update. *Biol Neonate* 74: 134-42
- Gerken, T. A. (1984) Amino group environments and metal binding properties of carbon-13 reductively methylated bovine alpha-lactalbumin. *Biochemistry* 23: 4688-97
- Gerrard, J. W. (1974) Breast-feeding: second thoughts. *Pediatrics* 54: 757-64
- Gerstein, H. C. (1994) Cow's milk exposure and type-2 diabetes mellitus: a critical overview of the clinical literature. *Diabetes Care* 17: 13-19
- Giannakis, C., Forbes, I. J., and Zalewski, P. D. (1991) Ca²⁺/Mg²⁺-dependent nuclease: Tissue distribution, relationship to inter-nucleosomal DNA fragmentation and inhibition by Zn²⁺. *Biochem. Biophys. Res. Com.* 181: 915-920
- Giebink, G. S., Berzins, I. K., Marker, S. C., and Schiffman, G. (1980) Experimental otitis media after nasal inoculation of *Streptococcus pneumoniae* and Influenzae A virus in chinchillas. *Infect Immun* 30: 445-50

- Giuffrida, M. G., Cavaletto, M., Giunta, C., Neuteboom, B., Cantisani, A., Napolitano, L., Calderone, V., Godovac-Zimmermann, J., and Conti, A. (1997) The unusual amino acid triplet Asn-Ile-Cys is a glycosylation consensus site in human alpha-lactalbumin. *J Protein Chem* 16: 747-53
- Glass, R. I., Svennerholm, A. M., Stoll, B. J., Khan, M. R., Hossain, K. M., Huq, M. I., and Holmgren, J. (1983) Protection against cholera in breast-fed children by antibodies in breast milk. *N Engl J Med* 308: 1389-92
- Glücksmann, A. (1951) Cell deaths in normal vertebrate ontogeny. *Biol Rev Camb Philos Soc* 26: 59-86
- Golding, J., Emmett, P. M., and Rogers, I. S. (1997a) Does breast feeding protect against non-gastric infections? *Early Hum Dev* 49 Suppl: S105-20
- Golding, J., Emmett, P. M., and Rogers, I. S. (1997b) Gastroenteritis, diarrhoea and breast feeding. *Early Hum Dev* 49 Suppl: S83-103
- Gorlich, D. (1998) Transport into and out of the cell nucleus. *Embo J* 17: 2721-7
- Gorlich, D., and Mattaj, I. W. (1996) Nucleocytoplasmic transport. *Science* 271: 1513-8
- Gottlieb, R. A., Giesing, H. A., Engler, R. L., and Babior, B. M. (1995) The acid deoxyribonuclease of neutrophils: a possible participant in apoptosis-associated genome destruction. *Blood* 86: 2414-8
- Gray, M. W., Burger, G., and Lang, B. F. (1999) Mitochondrial evolution. *Science* 283: 1476-81
- Green, D. R., and Reed, J. C. (1998) Mitochondria and apoptosis. *Science* 281: 1309-12
- Greenberg, J. T. (1996) Programmed cell death: a way of life for plants. *Proc Natl Acad Sci U S A* 93: 12094-7
- Griko, Y. V., Freire, E., and Privalov, P. L. (1994) Energetics of the alpha-lactalbumin states: a calorimetric and statistical thermodynamic study. *Biochemistry* 33: 1889-99
- Grishchenko, V. M., Kalinichenko, L. P., Deikus, G. Y., Veprintsev, D. B., Cawthorn, K. M., Berliner, L. J., and Permyakov, E. A. (1996) Interactions of alpha-lactalbumins with lipid vesicles studied by tryptophan fluorescence. *Biochem Mol Biol Int* 38: 453-66
- Grobler, J. A., Wang, M., Pike, A. C., and Brew, K. (1994) Study by mutagenesis of the roles of two aromatic clusters of alpha-lactalbumin in aspects of its action in the lactose synthase system. *J Biol Chem* 269: 5106-14
- Grosvenor, C. E., Picciano, M. F., and Baumrucker, C. R. (1993) Hormones and growth factors in milk. *Endocr Rev* 14: 710-28
- Gueth-Hallonet, C., Weber, K., and Osborn, M. (1997) Cleavage of the nuclear matrix protein NuMA during apoptosis. *Exp Cell Res* 233: 21-4
- Gurney, M. E., Heinrich, S. P., Lee, M. R., and Yin, H. S. (1986) Molecular cloning and expression of neuroleukin, a neurotrophic factor for spinal and sensory neurons. *Science* 234: 566-74
- Hainaut, P., Hernandez, T., Robinson, A., Rodriguez-Tome, P., Flores, T., Hollstein, M., Harris, C. C., and Montesano, R. (1998) IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. *Nucleic Acids Res* 26: 205-13
- Hall, L., Craig, R. K., Edbrooke, M. R., and Campbell, P. N. (1982) Comparison of the nucleotide sequence of cloned human and guinea-pig pre-alpha-lactalbumin cDNA with that of chick pre-lysozyme cDNA suggests evolution from a common ancestral gene. *Nucleic Acids Res* 10: 3503-15
- Hall, L., Emery, D. C., Davies, M. S., Parker, D., and Craig, R. K. (1987) Organization and sequence of the human alpha-lactalbumin gene. *Biochem J* 242: 735-42
- Hamosh, M. (1998) Protective function of proteins and lipids in human milk. *Biol Neonate* 74: 163-76
- Hanson, L. A. (1998) Breastfeeding provides passive and likely long-lasting active immunity. *Ann Allergy Asthma Immunol* 81: 523-33; quiz 533-4, 537

- Hanson, L. Å. (1961) Comparative immunological studies of the immunoglobulins of human milk and serum. *Int Arch Allergy Appl Immunol* 20: 65-79
- Heine, W. E., Klein, P. D., and Reeds, P. J. (1991) The importance of alpha-lactalbumin in infant nutrition. *J Nutr* 121: 277-83
- Henderson, F. W., Collier, A. M., Sanyal, M. A., Watkins, J. M., Fairclough, A. L., Clyde, W. A., and Denny, F. W. (1992) A longitudinal study of respiratory viruses and bacteria in the etiology of acute otitis media with effusion. *N Engl J Med* 306: 1377-83
- Hengartner, M. O., Ellis, R. E., and Horvitz, H. R. (1992) *Caenorhabditis elegans* gene ced-9 protects cells from programmed cell death. *Nature* 356: 494-9
- Hengartner, M. O., and Horvitz, H. R. (1994a) *C. elegans* cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* 76: 665-76
- Hengartner, M. O., and Horvitz, H. R. (1994b) Programmed cell death in *Caenorhabditis elegans*. *Curr Opin Genet Dev* 4: 581-6
- Hicks, G. R., and Raikhel, N. V. (1995) Protein import into the nucleus: An integrated view. *Annu Rev Cell Dev Biol* 11: 155-89
- Hill, L. F. (1968) A salute to La Leche League International. *J Pediatr* 73: 161-162
- Hiraoka, Y., Segawa, T., Kuwajima, K., Sugai, S., and Murai, N. (1980) alpha-Lactalbumin: a calcium metalloprotein. *Biochem Biophys Res Commun* 95: 1098-104
- Hiraoka, Y., and Sugai, S. (1985) Equilibrium and kinetic study of sodium- and potassium-induced conformational changes of apo-alpha-lactalbumin. *Int J Pept Protein Res* 26: 252-61
- Hochman, A. (1997) Programmed cell death in prokaryotes. *Crit Rev Microbiol* 23: 207-14
- Hockenbery, D., Nunez, G., Millman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348: 334-6
- Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) p53 mutations in human cancers. *Science* 253: 49-53
- Horvitz, H. R. (1999) Genetic control of programmed cell death in the nematode *Caenorhabditis elegans*. *Cancer Res* 59: 1701s-1706s
- Howie, P. W., Forsyth, J. S., Ogston, S. A., Clark, A., and Florey, C. D. (1990) Protective effect of breast feeding against infection. *BMJ* 300: 11-6
- Hu, Y., Benedict, M. A., Wu, D., Inohara, N., and Nunez, G. (1998a) Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci U S A* 95: 4386-91
- Hu, Y., Ding, L., Spencer, D. M., and Nunez, G. (1998b) WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation. *J Biol Chem* 273: 33489-94
- Huang, D. C., Adams, J. M., and Cory, S. (1998) The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *Embo J* 17: 1029-39
- Håkansson, A., Kidd, A., Wadell, G., Sabharwal, H., and Svanborg, C. (1994) Adenovirus infection enhances in vitro adherence of *Streptococcus pneumoniae*. *Infect Immun* 62: 2707-14
- Håkansson, A., Mossberg, A. K., Sabharwal, S., Svensson, M., Linse, S., Lazou, I., Lönnerdal, B., and Svanborg, C. (Submitted) A folding variant of alpha-lactalbumin with bactericidal activity against *Streptococcus pneumoniae*.
- Ikeguchi, M., Kuwajima, K., and Sugai, S. (1986) Ca²⁺-induced alteration in the unfolding behavior of alpha-lactalbumin. *J Biochem (Tokyo)* 99: 1191-201
- Inohara, N., Gourley, T. S., Carrio, R., Muniz, M., Merino, J., Garcia, I., Koseki, T., Hu, Y., Chen, S., and Nunez, G. (1998) Diva, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death. *J Biol Chem* 273: 32479-86

- Isaacs, C. E., and Thormar, H. (1991) The role of milk-derived antimicrobial lipids as antiviral and antibacterial agents. *Adv Exp Med Biol* 310: 159-65
- Jans, D. A., and Hübner, S. (1996) Regulation of protein transport to the nucleus: Central role of phosphorylation. *Physiol Rev* 76: 651-85
- Jeffery, C. J. (1999) Moonlighting proteins. *Trends Biochem Sci* 24: 8-11
- Jensen, R. G. (1996) The lipids in human milk. *Prog Lipid Res* 35: 53-92
- Jensen, R. G., Clark, R. M., and Ferris, A. M. (1980) Composition of the lipids in human milk: a review. *Lipids* 15: 345-55
- Jensen, R. G., Ferris, A. M., Lammi-Keefe, C. J., and R.A., H. (1990) Dairy foods. Lipids of bovine and human milks: A comparison. *J Dairy Sci* 73: 223-40
- Jiang, S., Chow, S. C., Nicotera, P., and Orrenius, S. (1994) Intracellular Ca²⁺ signals activate apoptosis in thymocytes: studies using the Ca(2+)-ATPase inhibitor thapsigargin. *Exp Cell Res* 212: 84-92
- Jurgensmeier, J. M., Krajewski, S., Armstrong, R. C., Wilson, G. M., Oltersdorf, T., Fritz, L. C., Reed, J. C., and Oltie, S. (1997) Bax- and Bak-induced cell death in the fission yeast *Schizosaccharomyces pombe*. *Mol Biol Cell* 8: 325-39
- Kabara, J. J. (1980) Lipids as host-resistance factors of human milk. *Nutr Rev* 38: 65-73
- Kabara, J. J., Swieczkowski, D. M., Conley, A. J., and Truant, J. P. (1972) Fatty acids and derivatives as antimicrobial agents. *Antimicrob Agents Chemother* 2: 23-8
- Kane, D. J., Ord, T., Anton, R., and Bredesen, D. E. (1995) Expression of bcl-2 inhibits necrotic neural cell death. *J Neurosci Res* 40: 269-75
- Karpman, D., Håkansson, A., Perez, M. T., Isaksson, C., Carlemalm, E., Caprioli, A., and Svanborg, C. (1998) Apoptosis of renal cortical cells in the hemolytic-uremic syndrome: *in vivo* and *in vitro* studies. *Infect Immun* 66: 636-44
- Kataoka, M., Kuwajima, K., Tokunaga, F., and Goto, Y. (1997) Structural characterization of the molten globule of alpha-lactalbumin by solution X-ray scattering. *Protein Sci* 6: 422-30
- Katsikis, P. D., and Leben, W. R. (1995) Apoptosis. *Nature* 374: 670
- Kauppi, M., Saarinen, L., and Kayhty, H. (1993) Anti-capsular polysaccharide antibodies reduce nasopharyngeal colonization by *Haemophilus influenzae* type b in infant rats. *J Infect Dis* 167: 365-71
- Kawabata, H., Anzai, N., Masutani, H., Hirama, T., Yoshida, Y., and Okuma, M. (1993) Detection of Mg(2+)-dependent endonuclease activity in myeloid leukemia cell nuclei capable of producing internucleosomal DNA cleavage. *Biochem Biophys Res Commun* 191: 247-54
- Kawahara, A., Ohsawa, Y., Matsumura, H., Uchiyama, Y., and Nagata, S. (1998) Caspase-independent cell killing by Fas-associated protein with death domain. *J Cell Biol* 143: 1353-60
- Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26: 239-57
- Khatra, B. S., Herries, D. G., and Brew, K. (1974) Some kinetic properties of human-milk galactosyl transferase. *Eur J Biochem* 44: 537-60
- Kidd, V. J. (1998) Proteolytic activities that mediate apoptosis. *Annu Rev Physiol* 60: 533-73
- Kim, P. S., and Baldwin, R. L. (1982) Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. *Annu Rev Biochem* 51: 459-89
- Kirsch, D. G., Doseff, A., Chau, B. N., Lim, D. S., de Souza-Pinto, N. C., Hansford, R., Kastan, M. B., Lazebnik, Y. A., and Hardwick, J. M. (1999) Caspase-3-dependent Cleavage of Bcl-2 Promotes Release of Cytochrome c. *J Biol Chem* 274: 21155-21161
- Kitanaka, C., and Kuchino, Y. (1999) Caspase-independent programmed cell death with necrotic morphology. *Cell Death Differ* 6: 508-15

- Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275: 1132-6
- Kobata, A. (1977) in *The glycoconjugates*. Horowitz, M. I., and Pigman, W., eds) Vol. I, Academic Press, London, pp. 423-40
- Kohler, C., Gahm, A., Noma, T., Nakazawa, A., Orrenius, S., and Zhivotovsky, B. (1999) Release of adenylate kinase 2 from the mitochondrial intermembrane space during apoptosis. *FEBS Lett* 447: 10-2
- Koletzko, S., Sherman, P., Corey, M., Griffiths, A., and Smith, C. (1989) Role of infant feeding practices in development of Crohn's disease in childhood. *BMJ* 298: 1617-8
- Koradi, R., Billeter, M., and Wuthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J Mol Graph* 14: 51-5, 29-32
- Korsmeyer, S. J. (1999) BCL-2 gene family and the regulation of programmed cell death. *Cancer Res* 59: 1693s-1700s
- Krajewski, S., Krajewska, M., Ellerby, L. M., Welsh, K., Xie, Z., Deveraux, Q. L., Salvesen, G. S., Bredesen, D. E., Rosenthal, R. E., Fiskum, G., and Reed, J. C. (1999) Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. *Proc Natl Acad Sci U S A* 96: 5752-7
- Kramer, M. S. (1981) Do breast-feeding and delayed introduction of solid foods protect against subsequent obesity? *J Pediatr* 98: 883-7
- Kroemer, G. (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat Med* 3: 614-20
- Kroemer, G., Zamzami, N., and Susin, S. A. (1997) Mitochondrial control of apoptosis. *Immunol Today* 18: 44-51
- Kronman, M. J., Andreotti, R., and Vitols, R. (1964) Inter- and intramolecular interactions of alpha-lactalbumin. II. Aggregation reactions at acid pH. *Biochemistry* 3: 1152-60
- Kronman, M. J., Holmes, L. G., and Robbins, F. M. (1967) Inter- and intramolecular interactions of alpha-lactalbumin. 8. The alkaline conformational change. *Biochim Biophys Acta* 133: 46-55
- Kronman, M. J., Sinha, S. K., and Brew, K. (1981) Characteristics of the binding of Ca²⁺ and other divalent metal ions to bovine alpha-lactalbumin. *J Biol Chem* 256: 8582-7
- Kunz, C., and Lonnerdal, B. (1993) Protein composition of rhesus monkey milk: comparison to human milk. *Comp Biochem Physiol Comp Physiol* 104: 793-7
- Kunz, C., and Lönnerdal, B. (1990) Human-milk proteins: analysis of casein and casein subunits by anion-exchange chromatography, gel electrophoresis, and specific staining methods. *Am J Clin Nutr* 51: 37-46
- Kunz, C., and Rudloff, S. (1993) Biological functions of oligosaccharides in human milk. *Acta Paediatr* 82: 903-12
- Kuo, T. H., Kim, H. R., Zhu, L., Yu, Y., Lin, H. M., and Tsang, W. (1998) Modulation of endoplasmic reticulum calcium pump by Bcl-2. *Oncogene* 17: 1903-10
- Kuwahara, C., Takeuchi, A. M., Nishimura, T., Haraguchi, K., Kubosaki, A., Matsumoto, Y., Saeki, K., Yokoyama, T., Itoharu, S., and Onodera, T. (1999) Prions prevent neuronal cell-line death. *Nature* 400: 225-6
- Kuwajima, K. (1977) A folding model of alpha-lactalbumin deduced from the three-state denaturation mechanism. *J Mol Biol* 114: 241-58
- Kuwajima, K. (1989) The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. *Proteins* 6: 87-103
- Kuwajima, K. (1996) The molten globule state of alpha-lactalbumin. *Faseb J* 10: 102-9

- Kuwajima, K., Harushima, Y., and Sugai, S. (1986) Influence of Ca^{2+} binding on the structure and stability of bovine alpha-lactalbumin studied by circular dichroism and nuclear magnetic resonance spectra. *Int J Pept Protein Res* 27: 18-27
- Kuwajima, K., Hiraoka, Y., Ikeguchi, M., and Sugai, S. (1985) Comparison of the transient folding intermediates in lysozyme and alpha-lactalbumin. *Biochemistry* 24: 874-81
- Kuwajima, K., Nitta, K., Yoneyama, M., and Sugai, S. (1976) Three-state denaturation of alpha-lactalbumin by guanidine hydrochloride. *J Mol Biol* 106: 359-73
- Lacks, S., and Greenberg, B. (1967) Deoxyribonucleases of *Pneumococcus*. *J Biol Chem* 242: 3108-20
- Lacks, S., and Greenberg, B. (1975) A deoxyribonuclease of *Diplococcus pneumoniae* specific for methylated DNA. *J Biol Chem* 250: 4060-66
- Lacks, S., and Greenberg, B. (1977) Complementary specificity of restriction endonucleases of *Diplococcus pneumoniae* with respect to DNA methylation. *J Mol Biol* 114: 153-68
- Lacks, S., Greenberg, B., and Neuberger, M. (1975) Identification of a deoxyribonuclease implicated in genetic transformation of *Diplococcus pneumoniae*. *J Bacteriol* 123: 222-32
- Lagarkova, M. A., Iarovaia, O. V., and Razin, S. V. (1995) Large-scale fragmentation of mammalian DNA in the course of apoptosis proceeds via excision of chromosomal DNA loops and their oligomers. *J Biol Chem* 270: 20239-41
- Lala, A. K., and Kaul, P. (1992) Increased exposure of hydrophobic surface in molten globule state of alpha-lactalbumin. Fluorescence and hydrophobic photolabeling studies. *J Biol Chem* 267: 19914-8
- Lala, A. K., Kaul, P., and Ratnam, P. B. (1995) Membrane-protein interaction and the molten globule state: interaction of alpha-lactalbumin with membranes. *J Protein Chem* 14: 601-9
- Lam, M., Bhat, M. B., Nunez, G., Ma, J., and Distelhorst, C. W. (1998) Regulation of Bcl-xL channel activity by calcium. *J Biol Chem* 273: 17307-10
- Lam, M., Dubyak, G., Chen, L., Nunez, G., Miesfeld, R. L., and Distelhorst, C. W. (1994) Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes. *Proc Natl Acad Sci U S A* 91: 6569-73
- Lane, D. P. (1993) Cancer. A death in the life of p53. *Nature* 362: 786-7
- Lane, D. P., and Crawford, L. V. (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature* 278: 261-3
- Lavoie, J. N., Nguyen, M., Marcellus, R. C., Branton, P. E., and Shore, G. C. (1998) E4orf4, a novel adenovirus death factor that induces p53-independent apoptosis by a pathway that is not inhibited by zVAD-fmk. *J Cell Biol* 140: 637-45
- Leffler, H., and Svanborg-Edén, C. (1980) Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. *FEMS Microbiol Lett* 8: 127-34
- Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., and Nicotera, P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* 185: 1481-6
- Levine, O. S., Farley, M., Harrison, L. H., Lefkowitz, L., McGeer, A., and Schwartz, B. (1999) Risk factors for invasive pneumococcal disease in children: a population-based case-control study in North America. *Pediatrics* 103: E28
- Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479-89

- Linzer, D. I., and Levine, A. J. (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells *Cell* 17: 43-52
- Liu, X., Naekyung Kim, C., Yang, J., Jemmerson, R., and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. *Cell* 86: 147-57
- Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 89: 175-84
- Lorenzo, H. K., Susin, S. A., Penninger, J., and Kroemer, G. (1999) Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. *Cell Death Differ* 6: 516-524
- Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. (1994) p53 status and the efficacy of cancer therapy *in vivo*. *Science* 266: 807-10
- Lönnerdal, B., and Forsum, E. (1985) Casein content of human milk. *Am J Clin Nutr* 41: 113-20
- Majno, G., and Joris, I. (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* 146: 3-15
- Malinovsky, V. A., Tian, J., Grobler, J. A., and Brew, K. (1996) Functional site in alpha-lactalbumin encompasses a region corresponding to a subsite in lysozyme and parts of two adjacent flexible substructures. *Biochemistry* 35: 9710-5
- Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Jr., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A., and et al. (1990) Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 250: 1233-8
- Mannarelli, B. M., Balganes, T. S., Greenberg, B., Springhorn, S. S., and Lacks, S. A. (1985) Nucleotide sequence of the Dpn II DNA methylase gene of *Streptococcus pneumoniae* and its relationship to the dam gene of *Escherichia coli*. *Proc Natl Acad Sci U S A* 82: 4468-72
- Manon, S., Chaudhuri, B., and Guerin, M. (1997) Release of cytochrome c and decrease of cytochrome c oxidase in Bax- expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. *FEBS Lett* 415: 29-32
- Marchetti, P., Castedo, M., Susin, S. A., Zamzami, N., Hirsch, T., Macho, A., Haeflner, A., Hirsch, F., Geuskens, M., and Kroemer, G. (1996) Mitochondrial permeability transition is a central coordinating event of apoptosis. *J Exp Med* 184: 1155-60
- Margulis, L. (1975) Symbiotic theory of the origin of eukaryotic organelles; criteria for proof. *Symp Soc Exp Biol* : 21-38
- Marin, M. C., Fernandez, A., Bick, R. J., Brisbay, S., Buja, L. M., Snuggs, M., McConkey, D. J., von Eschenbach, A. C., Keating, M. J., and McDonnell, T. J. (1996) Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca²⁺. *Oncogene* 12: 2259-66
- Martin, S. J., O'Brien, G. A., Nishioka, W. K., McGahon, A. J., Mahboubi, A., Saido, T. C., and Green, D. R. (1995a) Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. *J Biol Chem* 270: 6425-8
- Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R. (1995b) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 182: 1545-56
- Mata, L. (1984) The importance of breast-feeding for optimal child health and well being. *Man Clin Mutat* 3 Suppl: 29-33
- Mathur, G. P., Gupta, N., Mathur, S., Gupta, V., Pradhan, S., Dwivedi, J. N., Tripathi, B. N., Kushwaha, K. P., Sathy, N., Modi, U. J., and et al. (1993) Breastfeeding and childhood cancer. *Indian Pediatr* 30: 651-7
- Matthews, C. R. (1993) Pathways of protein folding. *Annu Rev Biochem* 62: 653-83

- Mattsby-Baltzer, I., Roseanu, A., Motas, C., Elverfors, J., Engberg, I., and Hanson, L. A. (1996) Lactoferrin or a fragment thereof inhibits the endotoxin-induced interleukin-6 response in human monocytic cells. *Pediatr Res* 40: 257-62
- Mayer, E. J., Hamman, R. F., Gay, E. C., Lezotte, D. C., Savitz, D. A., and Klingensmith, G. J. (1988) Reduced risk of IDDM among breast-fed children. *Diabetes* 37: 1625-32
- Mazzarello, P. (1999) A unifying concept: the history of the cell theory. *Nat Cell Biol* 1: E13-E15
- McCall, K., and Steller, H. (1997) Facing death in the fly: genetic analysis of apoptosis in *Drosophila*. *Trends Genet* 13: 222-6
- McConkey, D. J. (1998) Biochemical determinants of apoptosis and necrosis. *Toxicol Lett* 99: 157-68
- McConkey, D. J., Nicotera, P., Hartzell, P., Bellombo, G., Wyllie, A. H., and Orrenius, S. (1989) Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca²⁺ concentration. *Arch Biochem Biophys* 269: 365-70
- McConkey, D. J., Nicotera, P., and Orrenius, S. (1994) Signalling and chromatin fragmentation in thymocyte apoptosis. *Immunol Rev* 142: 343-63
- McConkey, D. J., Zhivotovsky, B., and Orrenius, S. (1996) Apoptosis - Molecular mechanisms and biomedical implications. *Mol Aspects Med* 17: 1-115
- McIlroy, D., Sakahira, H., Talanian, R. V., and Nagata, S. (1999) Involvement of caspase 3-activated DNase in internucleosomal DNA cleavage induced by diverse apoptotic stimuli [In Process Citation] *Oncogene* 18: 4401-8
- McKenzie, H. A. (1996) alpha-Lactalbumins and lysozymes. *EXS* 75: 365-409
- McKenzie, H. A., and White, F. H., Jr. (1991) Lysozyme and alpha-lactalbumin: structure, function, and interrelationships. *Adv Protein Chem* 41: 173-315
- Mellander, O. (1947) On chemical and nutritional differences between casein from human and from cow's milk. *Uppsala Läkarfören. Förhandl.* 3-4: 107-98
- Mevorach, D., Mascarenhas, J. O., Gershov, D., and Elkon, K. B. (1998) Complement-dependent clearance of apoptotic cells by human macrophages. *J Exp Med* 188: 2313-20
- Minamikawa, T., Williams, D. A., Bowser, D. N., and Nagley, P. (1999) Mitochondrial permeability transition and swelling can occur reversibly without inducing cell death in intact human cells. *Exp Cell Res* 246: 26-37
- Minn, A. J., Velez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M., and Thompson, C. B. (1997) Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature* 385: 353-7
- Mittler, R., and Lam, E. (1996) Sacrifice in the face of foes: pathogen-induced programmed cell death in plants. *Trends Microbiol* 4: 10-5
- Miura, M., Zhu, H., Rotello, R., Hartwig, E. A., and Yuan, J. (1993) Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* 75: 653-60
- Moffatt, O. D., Devitt, A., Bell, E. D., Simmons, D. L., and Gregory, C. D. (1999) Macrophage recognition of ICAM-3 on apoptotic leukocytes. *J Immunol* 162: 6800-10
- Molbak, K., Gottschau, A., Aaby, P., Hojlyng, N., Ingholt, L., and da Silva, A. P. (1994) Prolonged breast feeding, diarrhoeal disease, and survival of children in Guinea-Bissau. *BMJ* 308: 1403-6
- Montague, J. W., Hughes, F. M., Jr., and Cidlowski, J. A. (1997) Native recombinant cyclophilins A, B, and C degrade DNA independently of peptidylprolyl cis-trans-isomerase activity. Potential roles of cyclophilins in apoptosis. *J Biol Chem* 272: 6677-84

- Montich, G. G., and Marsh, D. (1995) Interaction of alpha-lactalbumin with phosphatidylglycerol. Influence of protein binding on the lipid phase transition and lipid acyl chain mobility. *Biochemistry* 34: 13139-45
- Moodley, J. R., Coetzee, N., and Hussey, G. (1999) Risk factors for meningococcal disease in Cape Town. *S Afr Med J* 89: 56-9
- Morrison, D. A., and Baker, M. F. (1979) Competence for genetic transformation in pneumococcus depends on synthesis of a small set of proteins. *Nature* 282: 215-7
- Moynault, A., Luciani, M. F., and Chimini, G. (1998) ABC1, the mammalian homologue of the engulfment gene ced-7, is required during phagocytosis of both necrotic and apoptotic cells. *Biochem Soc Trans* 26: 629-35
- Murakami, K., Andree, P. J., and Berliner, L. J. (1982) Metal ion binding to alpha-lactalbumin species. *Biochemistry* 21: 5488-94
- Murakami, K., and Berliner, L. J. (1983) A distinct zinc binding site in the alpha-lactalbumins regulates calcium binding. Is there a physiological role for this control? *Biochemistry* 22: 3370-4
- Musci, G., and Berliner, L. J. (1985) Probing different conformational states of bovine alpha-lactalbumin: fluorescence studies with 4,4'-bis[1-(phenylamino)-8-naphthalenesulfonate]. *Biochemistry* 24: 3852-6
- Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) An induced proximity model for caspase-8 activation. *J Biol Chem* 273: 2926-30
- Mårild, S., Jodal, U., and Hanson, L. A. (1990) Breastfeeding and urinary-tract infection. *Lancet* 336: 942
- Narayanan, I., Prakash, K., and Gujral, V. V. (1981) The value of human milk in the prevention of infection in the high-risk low-birth-weight infant. *J Pediatr* 99: 496-8
- Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., and Tsujimoto, Y. (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc Natl Acad Sci U S A* 95: 14681-6
- Neurath, A. R., Jiang, S., Strick, N., Lin, K., Li, Y. Y., and Debnath, A. K. (1996) Bovine beta-lactoglobulin modified by 3-hydroxyphthalic anhydride blocks the CD4 cell receptor for HIV. *Nat Med* 2: 230-4
- Newburg, D. S. (1999) Human milk glycoconjugates that inhibit pathogens. *Curr Med Chem* 6: 117-27
- Newburg, D. S., Linhardt, R. J., Ampofo, S. A., and Yolken, R. H. (1995) Human milk glycosaminoglycans inhibit HIV glycoprotein gp120 binding to its host cell CD4 receptor. *J Nutr* 125: 419-24
- Newburg, D. S., Peterson, J. A., Ruiz-Palacios, G. M., Matson, D. O., Morrow, A. L., Shults, J., Guerrero, M. L., Chaturvedi, P., Newburg, S. O., Scallan, C. D., Taylor, M. R., Ceriani, R. L., and Pickering, L. K. (1998) Role of human-milk lactadherin in protection against symptomatic rotavirus infection. *Lancet* 351: 1160-4
- Ohgushi, M., and Wada, A. (1983) 'Molten-globule state': a compact form of globular proteins with mobile side-chains. *FEBS Lett* 164: 21-4
- Oka, K., Sawamura, T., Kikuta, K., Itokawa, S., Kume, N., Kita, T., and Masaki, T. (1998) Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc Natl Acad Sci U S A* 95: 9535-40
- Okamoto, Y., and Ogra, P. L. (1989) Antiviral factors in human milk: implications in respiratory syncytial virus infection. *Acta Paediatr Scand Suppl* 351: 137-43
- Olie, R. A., Durrieu, F., Cornillon, S., Loughran, G., Gross, J., Earnshaw, W. C., and Golstein, P. (1998) Apparent caspase independence of programmed cell death in Dictyostelium. *Curr Biol* 8: 955-8

- Pan, G., O'Rourke, K., and Dixit, V. M. (1998) Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J Biol Chem* 273: 5841-5
- Peitsch, M. C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H. R., Mannherz, H. G., and Tschoop, J. (1993) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *Embo J* 12: 371-7
- Peng, Z. Y., Wu, L. C., Schulman, B. A., and Kim, P. S. (1995) Does the molten globule have a native-like tertiary fold? *Philos Trans R Soc Lond B Biol Sci* 348: 43-7
- Peters, C. W., Kruse, U., Pollwein, R., Grzeschik, K. H., and Sippel, A. E. (1989) The human lysozyme gene. Sequence organization and chromosomal localization. *Eur J Biochem* 182: 507-16
- Pfeil, W. (1987) Is thermally denatured protein unfolded? The example of alpha-lactalbumin. *Biochim Biophys Acta* 911: 114-6
- Pfeil, W. (1998) Is the molten globule a third thermodynamic state of protein? The example of alpha-lactalbumin. *Proteins* 30: 43-8
- Pfeil, W., Bychkova, V. E., and Ptitsyn, O. B. (1986) Physical nature of the phase transition in globular proteins. Calorimetric study of human alpha-lactalbumin. *FEBS Lett* 198: 287-91
- Picciano, M. F. (1998) Human milk: nutritional aspects of a dynamic food. *Biol Neonate* 74: 84-93
- Pike, A. C., Brew, K., and Acharya, K. R. (1996) Crystal structures of guinea-pig, goat and bovine alpha-lactalbumin highlight the enhanced conformational flexibility of regions that are significant for its action in lactose synthase. *Structure* 4: 691-703
- Pisacane, A., Graziano, L., Mazzarella, G., Scarpellino, B., and Zona, G. (1992) Breast-feeding and urinary tract infection. *J Pediatr* 120: 87-9
- Pisacane, A., Graziano, L., and Zona, G. (1990) Breastfeeding and urinary tract infection. *Lancet* 336: 50
- Pisacane, A., Graziano, L., Zona, G., Granata, G., Dolezalova, H., Cafiero, M., Coppola, A., Scarpellino, B., Ummarino, M., and Mazzarella, G. (1994) Breast feeding and acute lower respiratory infection. *Acta Paediatr* 83: 714-8
- Polverino de Laureto, P., De Filippis, V., Di Bello, M., Zambonin, M., and Fontana, A. (1995) Probing the molten globule state of alpha-lactalbumin by limited proteolysis. *Biochemistry* 34: 12596-604
- Popkin, B. M., Adair, L., Akin, J. S., Black, R., Briscoe, J., and Flieger, W. (1990) Breast-feeding and diarrheal morbidity. *Pediatrics* 86: 874-82
- Portelli, J., Gordon, A., and May, J. T. (1998) Effect of compounds with antibacterial activities in human milk on respiratory syncytial virus and cytomegalovirus *in vitro*. *J Med Microbiol* 47: 1015-8
- Prieels, J. P., Bell, J. E., Schindler, M., Castellino, F. J., and Hill, R. L. (1979) Involvement of histidine-32 in the biological activity of alpha-lactalbumin. *Biochemistry* 18: 1771-6
- Privalov, P. L. (1996) Intermediate states in protein folding. *J Mol Biol* 258: 707-25
- Prusiner, S. B. (1998) Prions. *Proc Natl Acad Sci U S A* 95: 13363-83
- Ptitsyn, O. B. (1973) Stages in the mechanism of self-organization of protein molecules. *Dokl Akad Nauk SSSR* 210: 1213-5
- Ptitsyn, O. B. (1995) Molten globule and protein folding. *Adv Protein Chem* 47: 83-229
- Ptitsyn, O. B., and Uversky, V. N. (1994) The molten globule is a third thermodynamical state of protein molecules. *FEBS Lett* 341: 15-8
- Puyet, A., Greenberg, B., and Lacks, S. A. (1989) The *exoA* gene of *Streptococcus pneumoniae* and its product, a DNA exonuclease with apurinic endonuclease activity. *J Bacteriol* 171: 2278-86
- Puyet, A., Greenberg, B., and Lacks, S. A. (1990) Genetic and structural characterization of *endA*. *J Mol Biol* 213: 727-38

- Qasba, P. K., and Kumar, S. (1997) Molecular divergence of lysozymes and alpha-lactalbumin. *Crit Rev Biochem Mol Biol* 32: 255-306
- Radin, N. S., and Inokuchi, J.-I. (1991) Use of PDMP for the study of glycosphingolipid functions. *TIGG* 3: 200-213
- Raisler, J., Alexander, C., and O'Campo, P. (1999) Breast-feeding and infant illness: a dose-response relationship? *Am J Public Health* 89: 25-30
- Ram, B. P., and Munjal, D. D. (1985) Galactosyltransferases: physical, chemical, and biological aspects. *CRC Crit Rev Biochem* 17: 257-311
- Reed, J. C., Jurgensmeier, J. M., and Matsuyama, S. (1998) Bcl-2 family proteins and mitochondria. *Biochim Biophys Acta* 1366: 127-37
- Reiter, B. (1984) in *IDF* Vol. 191, pp. 2-35, Prague
- Ribeiro, J. M., and Carson, D. A. (1993) Ca²⁺/Mg(2+)-dependent endonuclease from human spleen: purification, properties, and role in apoptosis. *Biochemistry* 32: 9129-36
- Rosen, A., and Casciola-Rosen, L. (1997) Macromolecular substrates for the ICE-like proteases during apoptosis. *J Cell Biochem* 64: 50-4
- Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., and Borner, C. (1998) Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature* 391: 496-9
- Rubin, E., and Farber, J. L. (1998) *Pathology*, 3rd Ed., Lippincott-Raven, Philadelphia
- Ruuskanen, O., and Heikkinen, T. (1994) Viral-bacterial interaction in acute otitis media. *Pediatr Infect Dis J* 13: 1047-9
- Räihä, N. C. R. (1985) Nutritional proteins in milk and protein requirement of normal infants. *Pediatrics* 75 (Suppl): 136-141
- Saarinén, U. M. (1982) Prolonged breast feeding as prophylaxis for recurrent otitis media. *Acta Paediatr Scand* 71: 567-71
- Saarinén, U. M., and Kajosaari, M. (1995) Breastfeeding as prophylaxis against atopic disease: prospective follow-up study until 17 years old. *Lancet* 346: 1065-9
- Sakahira, H., Enari, M., and Nagata, S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391: 96-9
- Saleh, A., Srinivasula, S. M., Acharya, S., Fishel, R., and Alnemri, E. S. (1999) Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J Biol Chem* 274: 17941-5
- Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., and Orrenius, S. (1999) Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells. *Embo J* 18: 2040-8
- Sarkar, N. H., Charney, J., Dion, A., and D., M. (1973) Effect of human milk on the tumor mammary virus. *Cancer Res.* 33: 626-629
- Savill, J., Dransfield, I., Hogg, N., and Haslett, C. (1990) Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature* 343: 170-3
- Savill, J., Hogg, N., Ren, Y., and Haslett, C. (1992) Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest* 90: 1513-22
- Scariati, P. D., Grummer-Strawn, L. M., and Fein, S. B. (1997) A longitudinal analysis of infant morbidity and the extent of breastfeeding in the United States. *Pediatrics* 99: E5
- Schaer, J. J., Milos, M., and Cox, J. A. (1985) Thermodynamics of the binding of calcium and strontium to bovine alpha-lactalbumin. *FEBS Lett* 190: 77-80

- Schendel, S. L., Xie, Z., Montal, M. O., Matsuyama, S., Montal, M., and Reed, J. C. (1997) Channel formation by antiapoptotic protein Bcl-2. *Proc Natl Acad Sci U S A* 94: 5113-8
- Schlesinger, P. H., Gross, A., Yin, X. M., Yamamoto, K., Saito, M., Waksman, G., and Korsmeyer, S. J. (1997) Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. *Proc Natl Acad Sci U S A* 94: 11357-62
- Schroten, H., Plogmann, R., Hanisch, F. G., Hacker, J., Nobis-Bosch, R., and Wahn, V. (1993) Inhibition of adhesion of S-fimbriated E. coli to buccal epithelial cells by human skim milk is predominantly mediated by mucins and depends on the period of lactation. *Acta Paediatr* 82: 6-11
- Schulman, B. A., and Kim, P. S. (1996) Proline scanning mutagenesis of a molten globule reveals non-cooperative formation of a protein's overall topology. *Nat Struct Biol* 3: 682-7
- Schulman, B. A., Kim, P. S., Dobson, C. M., and Redfield, C. (1997) A residue-specific NMR view of the non-cooperative unfolding of a molten globule. *Nat Struct Biol* 4: 630-4
- Segawa, T., and Sugai, S. (1983) Interactions of divalent metal ions with bovine, human, and goat alpha-lactalbumins. *J Biochem (Tokyo)* 93: 1321-8
- Shapiro, J. A. (1998) Thinking about bacterial populations as multicellular organisms. *Annu Rev Microbiol* 52: 81-104
- Shimizu, A., Ikeguchi, M., Kobayashi, T., and Sugai, S. (1996) A synthetic peptide study on the molten globule of alpha-lactalbumin. *J Biochem (Tokyo)* 119: 947-52
- Shimizu, A., Ikeguchi, M., and Sugai, S. (1993) Unfolding of the molten globule state of alpha-lactalbumin studied by ¹H NMR. *Biochemistry* 32: 13198-203
- Shimizu, S., Narita, M., and Tsujimoto, Y. (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 399: 483-7
- Shiokawa, D., Ohyama, H., Yamada, T., Takahashi, K., and Tanuma, S. (1994) Identification of an endonuclease responsible for apoptosis in rat thymocytes. *Eur J Biochem* 226: 23-30
- Shiratsuchi, A., Kawasaki, Y., Ikemoto, M., Arai, H., and Nakanishi, Y. (1999) Role of class B scavenger receptor type I in phagocytosis of apoptotic rat spermatogenic cells by Sertoli cells. *J Biol Chem* 274: 5901-8
- Silfverdal, S. A., Bodin, L., Hugosson, S., Garpenholt, O., Werner, B., Esbjorner, E., Lindquist, B., and Olcen, P. (1997) Protective effect of breastfeeding on invasive *Haemophilus influenzae* infection: a case-control study in Swedish preschool children. *Int J Epidemiol* 26: 443-50
- Simpson, R. J., Moritz, R. L., Begg, G. S., Rubira, M. R., and Nice, E. C. (1989) Micropreparative procedures for high sensitivity sequencing of peptides and proteins. *Anal Biochem* 177: 221-36
- Single, B., Leist, M., and Nicotera, P. (1998) Simultaneous release of adenylate kinase and cytochrome c in cell death. *Cell Death Differ* 5: 1001-3
- Smith, S. G., Lewis, M., Aschaffenburg, R., Fenna, R. E., Wilson, I. A., Sundaralingam, M., Stuart, D. I., and Phillips, D. C. (1987) Crystallographic analysis of the three-dimensional structure of baboon alpha-lactalbumin at low resolution. Homology with lysozyme. *Biochem J* 242: 353-60
- Solomon, M., Belenghi, B., Delledonne, M., Menachem, E., and Levine, A. (1999) The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *Plant Cell* 11: 431-44
- Song, J., Bai, P., Luo, L., and Peng, Z. Y. (1998) Contribution of individual residues to formation of the native-like tertiary topology in the alpha-lactalbumin molten globule. *J Mol Biol* 280: 167-74
- Song, Z., McCall, K., and Steller, H. (1997) DCP-1, a *Drosophila* cell death protease essential for development. *Science* 275: 536-40

- Srivastava, S., Zou, Z. Q., Pirollo, K., Blattner, W., and Chang, E. H. (1990) Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature* 348: 747-9
- Stinnakre, M. G., Vilotte, J. L., Soulier, S., and Mercier, J. C. (1994) Creation and phenotypic analysis of alpha-lactalbumin-deficient mice. *Proc Natl Acad Sci U S A* 91: 6544-8
- Strous, G. J. (1986) Golgi and secreted galactosyltransferase. *CRC Crit Rev Biochem* 21: 119-51
- Stuart, D. I., Acharya, K. R., Walker, N. P., Smith, S. G., Lewis, M., and Phillips, D. C. (1986) Alpha-lactalbumin possesses a novel calcium binding loop. *Nature* 324: 84-7
- Sun, Y. J., Chou, C. C., Chen, W. S., Wu, R. T., Meng, M., and Hsiao, C. D. (1999) The crystal structure of a multifunctional protein: phosphoglucose isomerase / autocrine motility factor / neuroleukin. *Proc Natl Acad Sci U S A* 96: 5412-7
- Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M. C., Alzari, P. M., and Kroemer, G. (1999a) Mitochondrial release of caspase-2 and -9 during the apoptotic process. *J Exp Med* 189: 381-94
- Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999b) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397: 441-6
- Susin, S. A., Zamzami, N., and Kroemer, G. (1998) Mitochondria as regulators of apoptosis: doubt no more. *Biochim Biophys Acta* 1366: 151-65
- Svanborg, C., Agace, W., Hedges, S., Lindstedt, R., and Svensson, M. (1994) Bacterial adherence and mucosal cytokine production. *Ann NY Acad Sci* : 162-81
- Svanborg-Edén, C., Freter, R., Hagberg, L., Hull, R., Hull, S., Leffler, H., and Schoolnik, G. (1982) Inhibition of experimental ascending urinary tract infection by an epithelial cell-surface receptor analogue. *Nature (London)* 298: 560-2
- Svensson, M., Håkansson, A., Mossberg, A. K., S., L., and Svanborg, C. (Submitted) Human alpha-lactalbumin made lethal to tumor cells.
- Takala, A. K., Eskola, J., Palmgren, J., Ronnberg, P. R., Kela, E., Rekola, P., and Makela, P. H. (1989) Risk factors of invasive *Haemophilus influenzae* type b disease among children in Finland. *J Pediatr* 115: 694-701
- Teele, D. W., Klein, J. O., and Rosner, B. (1989) Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. *J Infect Dis* 160: 83-94
- Thony-Meyer, L. (1997) Biogenesis of respiratory cytochromes in bacteria. *Microbiol Mol Biol Rev* 61: 337-76
- Thornberry, N. A., and Lazebnik, Y. (1998) Caspases: enemies within. *Science* 281: 1312-6
- Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem* 272: 17907-11
- Tomasz, A. (1968) Biological consequences of the replacement of choline by ethanolamine in the cell wall of *Pneumococcus*: choline formation, loss of transformability, and loss of autolysis. *Proc Natl Acad Sci U S A* 59: 86-93
- Trombe, M. C. (1993) Characterization of a calcium porter of *Streptococcus pneumoniae* involved in calcium regulation of growth and competence. *J Gen Microbiol* 139: 433-9
- Trombe, M. C., Clave, C., and Manias, J. M. (1992) Calcium regulation of growth and differentiation in *Streptococcus pneumoniae*. *J Gen Microbiol* 138: 77-84

- Trombe, M. C., Rieux, V., and Baille, F. (1994) Mutations which alter the kinetics of calcium transport alter the regulation of competence in *Streptococcus pneumoniae*. *J Bacteriol* 176: 1992-6
- Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C. M. (1985) Involvement of the bcl-2 gene in human follicular lymphoma. *Science* 228: 1440-3
- Uchiyama, H., Perez-Prat, E. M., Watanabe, K., Kumagai, I., and Kuwajima, K. (1995) Effects of amino acid substitutions in the hydrophobic core of alpha-lactalbumin on the stability of the molten globule state. *Protein Eng* 8: 1153-61
- Uversky, V. N., and Ptitsyn, O. B. (1996) All-or-none solvent-induced transitions between native, molten globule and unfolded states in globular proteins. *Fold Des* 1: 117-22
- Van Ceunebroeck, J. C., Hanssens, I., Joniau, M., and Van Cauwelaert, F. (1985) Thermodynamics of the Ca²⁺ binding to bovine alpha-lactalbumin. *J Biol Chem* 260: 10944-7
- Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* 91: 627-37
- Vanderheeren, G., Hanssens, I., Meijberg, W., and Van Aerschot, A. (1996) Thermodynamic characterization of the partially unfolded state of Ca(2+)-loaded bovine alpha-lactalbumin: evidence that partial unfolding can precede Ca²⁺ release. *Biochemistry* 35: 16753-9
- Varkey, J., Chen, P., Jemmerson, R., and Abrams, J. M. (1999) Altered cytochrome c display precedes apoptotic cell death in *Drosophila*. *J Cell Biol* 144: 701-10
- Vaux, D. L. (1999) Caspases and apoptosis - biology and terminology. *Cell Death Differ* 6: 493-4
- Vaux, D. L., and Korsmeyer, S. J. (1999) Cell death in development. *Cell* 96: 245-54
- Vaux, D. L., Weissman, I. L., and Kim, S. K. (1992) Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. *Science* 258: 1955-7
- Veprintsev, D. B., Permyakov, E. A., Kalinichenko, L. P., and Berliner, L. J. (1996) Pb²⁺ and Hg²⁺ binding to alpha-lactalbumin. *Biochem Mol Biol Int* 39: 1255-65
- Victoria, C. G., Smith, P. G., Vaughan, J. P., Nobre, L. C., Lombardi, C., Teixeira, A. M., Fuchs, S. M., Moreira, L. B., Gigante, L. P., and Barros, F. C. (1987) Evidence for protection by breast-feeding against infant deaths from infectious diseases in Brazil. *Lancet* 2: 319-22
- Villalpando, S., and Hamosh, M. (1998) Early and late effects of breast-feeding: does breast-feeding really matter? *Biol Neonate* 74: 177-91
- Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. (1999) Ca²⁺-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* 284: 339-43
- Wang, K., Gross, A., Waksman, G., and Korsmeyer, S. J. (1998) Mutagenesis of the BH3 domain of BAX identifies residues critical for dimerization and killing. *Mol Cell Biol* 18: 6083-9
- Watanabe, H., Takehana, K., Date, M., Shinozaki, T., and Raz, A. (1996) Tumor cell autocrine motility factor is the neuroleukin / phosphohexose isomerase polypeptide. *Cancer Res* 56: 2960-3
- Welburn, S. C., and Murphy, N. B. (1998) Prohibitin and RACK homologues are up-regulated in trypanosomes induced to undergo apoptosis and in naturally occurring terminally differentiated forms. *Cell Death Differ* 5: 615-22
- WHO. (1995) Nutrition. Information and attitudes among health personnel about early infant-feeding practices. *Wkly Epidemiol Rec* 70: 117-20
- Wickes, I. G. (1953a) A history of infant feeding. Part I: Primitive peoples. Ancient works. Renaissance writers. *Arch Dis Child* 28: 151-8
- Wickes, I. G. (1953b) A history of infant feeding. Part III: Eighteenth and nineteenth century writers. *Arch Dis Child* 28: 332-40

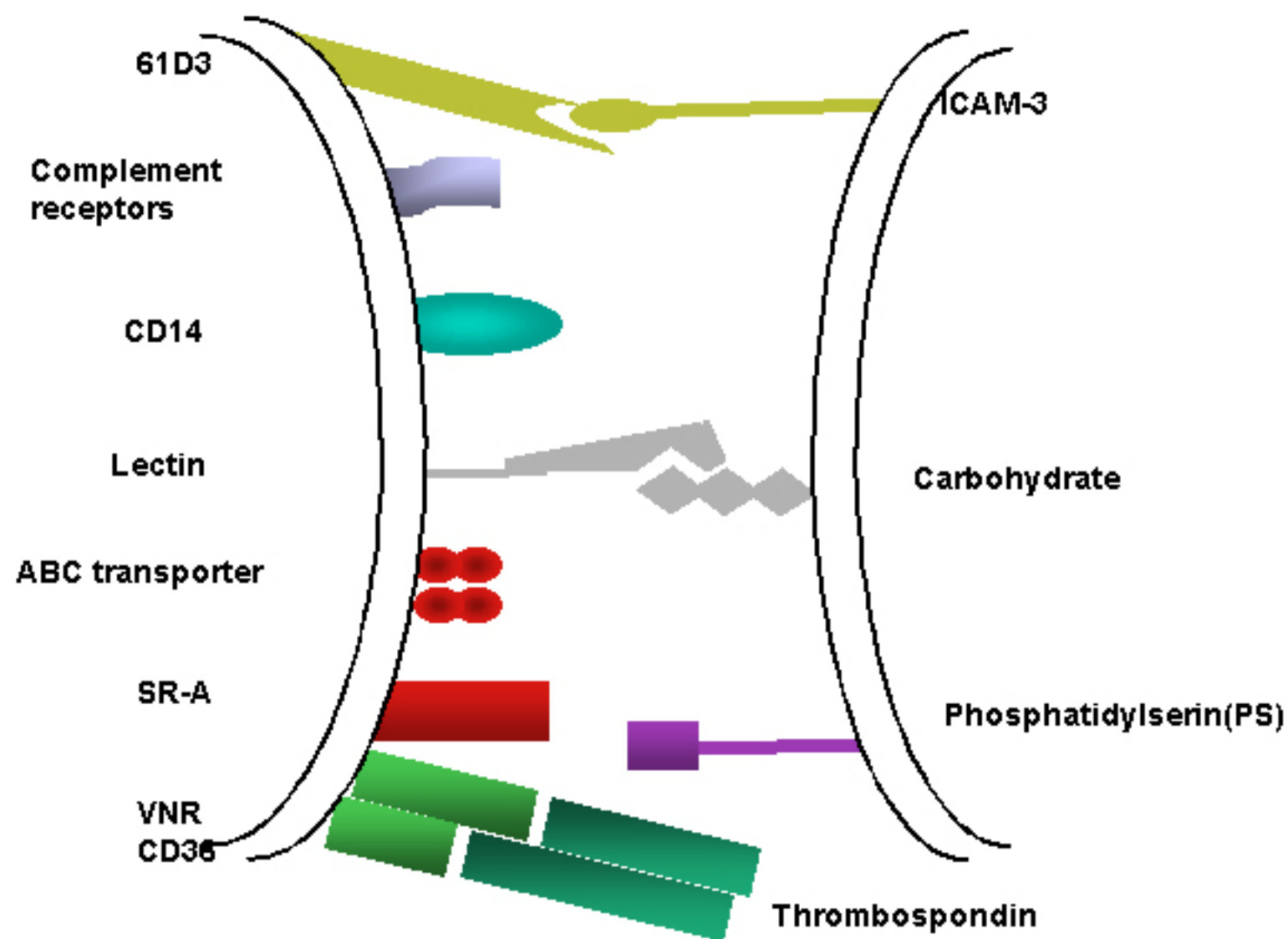
- Willis, R. A. (1952) *The spread of tumours in the human body.*, Butterworth & Co, London
- Wold, A. E., and Adlerberth, I. (1998) Does breastfeeding affect the infant's immune responsiveness? *Acta Paediatr* 87: 19-22
- Wright, A. L., Bauer, M., Naylor, A., Sutcliffe, E., and Clark, L. (1998) Increasing breastfeeding rates to reduce infant illness at the community level. *Pediatrics* 101: 837-44
- Wu, L. C., and Kim, P. S. (1998) A specific hydrophobic core in the alpha-lactalbumin molten globule. *J Mol Biol* 280: 175-82
- Wyllie, A. H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 284: 555-6
- Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* 68: 251-306
- Wyllie, A. H., Morris, R. G., Smith, A. L., and Dunlop, D. (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol* 142: 67-77
- Xanthou, M. (1998) Immune protection of human milk. *Biol Neonate* 74: 121-33
- Xanthoudakis, S., Roy, S., Rasper, D., Hennessey, T., Aubin, Y., Cassady, R., Tawa, P., Ruel, R., Rosen, A., and Nicholson, D. W. (1999) Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. *Embo J* 18: 2049-56
- Xu, W., Seiter, K., Feldman, E., Ahmed, T., and Chiao, J. W. (1996) The differentiation and maturation mediator for human myeloid leukemia cells shares homology with neuroleukin or phosphoglucose isomerase. *Blood* 87: 4502-6
- Yamauchi, K., Tomita, M., Giehl, T. J., and Ellison, R. T. (1993) Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect Immun* 61: 719-28
- Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997a) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked [see comments] *Science* 275: 1129-32
- Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997b) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275: 1129-32
- Yang, X., Chang, H. Y., and Baltimore, D. (1998) Essential role of CED-4 oligomerization in CED-3 activation and apoptosis. *Science* 281: 1355-7
- Yin, X. M., Oltvai, Z. N., and Korsmeyer, S. J. (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature* 369: 321-3
- Yoneda, Y. (1997) How proteins are transported from the cytoplasm to the nucleus. *J Biochem* 121: 811-7
- Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993) The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* 75: 641-52
- Yutani, K., Ogasahara, K., and Kuwajima, K. (1992) Absence of the thermal transition in apo-alpha-lactalbumin in the molten globule state. A study by differential scanning microcalorimetry. *J Mol Biol* 228: 347-50
- Zetterström, R. (1998) Foreword. *Biol Neonate* 74: 80-83
- Zha, H., Aime-Sempe, C., Sato, T., and Reed, J. C. (1996) Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. *J Biol Chem* 271: 7440-4

- Zhivotovsky, B., Wade, D., Gahm, A., Orrenius, S., and Nicotera, P. (1994) Formation of 50 kbp chromatin fragments in isolated liver nuclei is mediated by protease and endonuclease activation. *FEBS Lett* 351: 150-4
- Zopf, D., and Roth, S. (1996) Oligosaccharide anti-infective agents. *Lancet* 347: 1017-21
- Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90: 405-13



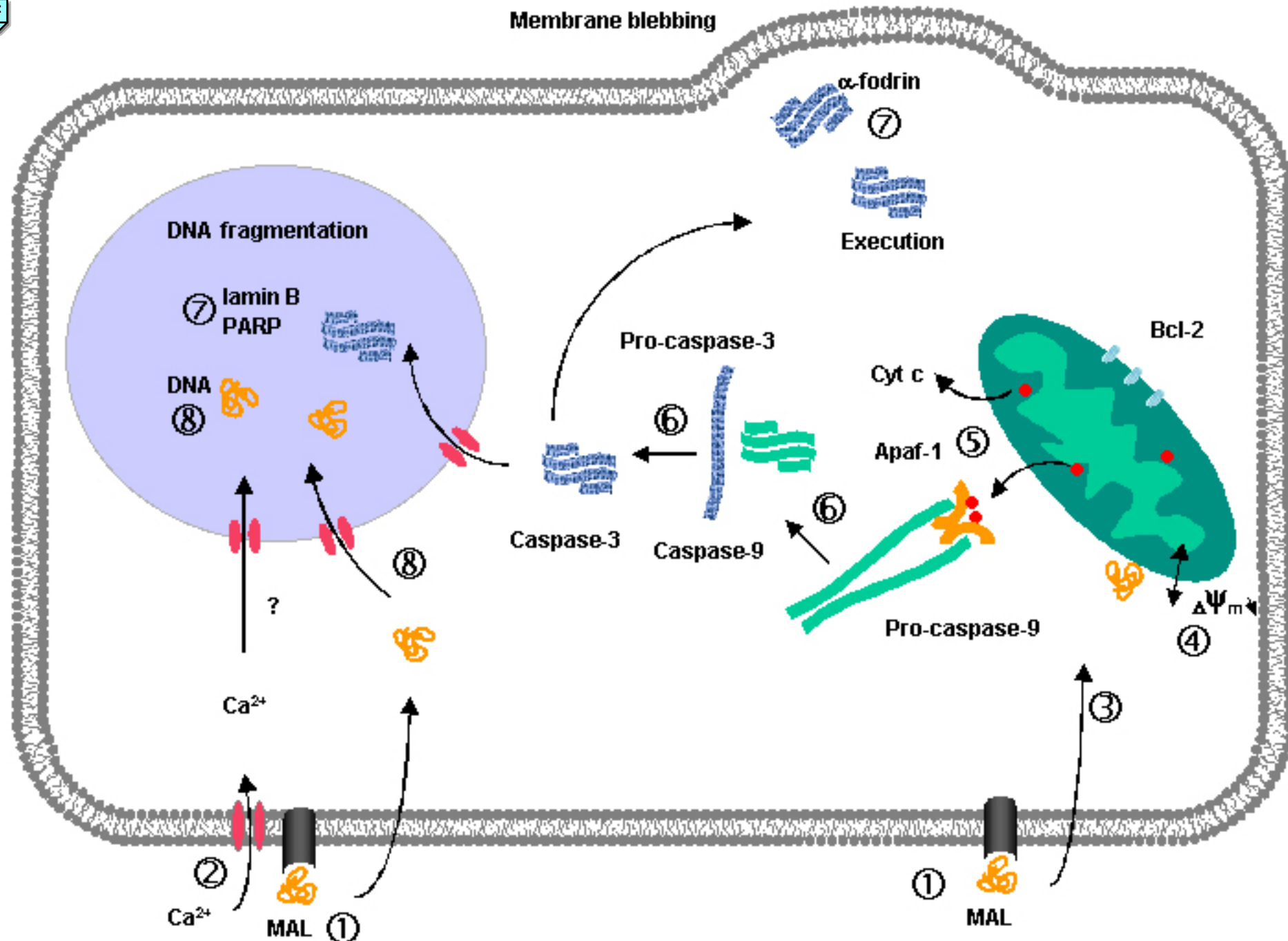
Macrophage

Apoptotic cell





Membrane blebbing



Multimeric α -Lactalbumin from Human Milk Induces Apoptosis through a Direct Effect on Cell Nuclei

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A fraction from human milk containing spf-multimer α -lactalbumin (MAL) induces apoptosis in tumor cells and immature cells but spares mature cells. The mechanism of apoptosis induction and the molecular basis for the difference in susceptibility between tumor cells and healthy cells have not been defined. In this study we examined the interaction of MAL with different cellular compartments, using confocal microscopy and subcellular fractionation. MAL was shown to accumulate in the nuclei of sensitive cells rather than in the cytosol, the vesicular fraction, or the ER–Golgi complex. Nuclear uptake occurred rapidly in cells that were susceptible to the apoptosis-inducing effect, but not in nuclei of resistant cells. Nuclear uptake was through the nuclear pore complex and was critical for the induction of DNA fragmentation, since inhibition of nuclear uptake with WGA rescued digitonin-permeabilized cells from induction of DNA fragmentation. Ca^{2+} was required for MAL-induced DNA fragmentation but nuclear uptake of MAL was independent of Ca^{2+} . This way MAL differs from most previously described agents in that it crosses the plasma membrane and cytosol, and enters cell nuclei where it induces DNA fragmentation through a direct effect at the nuclear level. © 1999 Academic Press

Key Words: apoptosis; human milk; tumor; nuclear targeting; α -lactalbumin.

INTRODUCTION

Apoptotic cell death is characterized by loss of cytoplasmic material, nuclear changes with marginalization of chromatin, and the formation of apoptotic bodies [1–3]. The reduction in cell viability is accompanied by stepwise chromatin fragmentation with initial formation of high-molecular-weight (HMW) DNA fragments (50–300 kbp) followed by oligonucleosome-length DNA fragments consisting of oligomers of approximately 200

bp [4–8]. Apoptosis inducing agonists often bind to specific cell surface receptors and activate transmembrane signaling events that trigger a cascade of cytoplasmic and nuclear changes. There are also agents that bypass this sequential activation and instead trigger the apoptosis cascade through targeting of molecules directly in the cytoplasm and/or at the nuclear level. One example is Granzyme B, a serine protease in the cytoplasmic granules of cytolytic T lymphocytes that enters perforin-exposed cells and activates the caspase cascade in the cytoplasm. Intracellular granzyme B is then translocated to the nuclei of target cells and directly cleaves nuclear substrates, such as DNA-PK, poly(ADP-ribose) polymerase, and NuMA, the cleavage of which is a well known event in apoptosis [9, 10].

The transport of macromolecules from the cytoplasm into the nucleus is highly regulated [11–13]. Nuclear pore complexes (NPCs) are the sites of exchange of macromolecules between the cytoplasm and nucleoplasm. The NPCs allow passive diffusion of molecules smaller than 20–40 kDa but larger proteins are delayed. Entry of large molecules or complexes into the nucleus requires active transport to be efficient and is commonly carrier-mediated. The specificity for the carrier may be determined by nuclear targeting or nuclear localization sequences (NLS) that characterize proteins with the ability to enter the nucleus.

We recently described a protein complex in human milk that induces apoptosis in tumor cells and immature cells, but spares other cells [14]. The active complex was initially isolated from casein by ion-exchange chromatography and was shown by N-terminal amino acid sequencing, mass spectrometry, and gel filtration to contain multimers of α -lactalbumin (MAL). The monomeric form of α -lactalbumin, that occurs in human milk whey, did not induce apoptosis. The molecular basis for this difference in activity between MAL and α -lactalbumin (ALA) has not been defined. Furthermore, the difference in sensitivity between tumor cells

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and nonmalignant mature cells has not been explained at the molecular level.

Here we examined the cellular distribution of MAL in sensitive and resistant cells, and compared it to the inactive monomeric form of α -lactalbumin. The results suggested that MAL was transported into cell nuclei of cells that were susceptible to its apoptosis-inducing effect and suggests an alternative mechanism of apoptosis induction where an exogenous molecule that targets cell nuclei can induce the nuclear changes seen in apoptotic cells through a direct effect at the nuclear level.

MATERIALS AND METHODS

Reagents. FITC-conjugated swine anti-rabbit and rabbit anti-human antibodies and FITC-conjugated streptavidin were from Dakopatts a/s (Glostrup, Denmark). The anti-DNA antibodies were kindly provided by the Clinical Immunology Laboratory, Lund University Hospital, Lund, Sweden. DEAE-Trisacryl M was from BioSeptra (Villeneuve la Garenne, France). The Biotin Labeling kit was from Boehringer Mannheim GmbH (Germany) and ^{125}I was from Amersham International plc (Amersham, England). Flowcheck and Flowset fluorospheres were from Coulter Inc. (Hialeah, FL). SeaKem GTG agarose and SeaPlaque GTG low-melting-temperature agarose were from SeaKem, FMK Bioproducts (Rockdale, USA). 2-Mercaptoethanol, Dulbecco's modified Eagle's medium, fetal calf serum (FCS), gentamicin, L-glutamine, nonessential amino acids, penicillin/streptomycin solution, RPMI 1640, and sodium pyruvate were from Gibco/BRL, Life Technology Ltd. (Paisley, Scotland, UK). Dimethylsulfoxide, Hepes, Tween-20, and Tris(hydroxymethyl) aminomethane were from Kebo Lab (Stockholm, Sweden). Heparin, and PD-10 column were from Pharmacia Biotech (Stockholm, Sweden). α -Lactalbumin, antipain, bovine serum albumin, ethylenediaminetetraacetate (EDTA), ethylene glycol-bis(*b*-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), lactoperoxidase, leupeptin, *N*-lauroylsarcosine, nifedipine, phenylmethylsulfonyl fluoride (PMSF), propidium iodide, proteinase K, Triton X-100, trypsin, and verapamil were from Sigma Chemicals Inc. (St. Louis, MO). BAPTA/AM was from Calbiochem (CA, USA). Digitonin and 1,4-dithiothreitol (DTT) were from ICN (CA, USA). Trypan blue was from Chroma Gesellschaft, Schmid & Co (Stuttgart, Germany).

Purification of MAL from human milk. Human milk was thawed, centrifuged to remove fat, and separated into casein and whey by acid precipitation [15]. The casein precipitate was harvested by centrifugation, washed three to five times in distilled water, and lyophilized. The lyophilized casein was dissolved in 1 mM Tris-HCl, pH 8.5, and was fractionated using an ion-exchange column packed with DEAE-Trisacryl M attached to an FPLC system (BioLogic automated system, Bio-Rad Laboratories, Stockholm, Sweden) using a NaCl gradient. The MAL complex eluted with 1 M NaCl. The eluate was desalted by dialysis (membrane cutoff 3.5 kDa) against distilled water for at least 48 h and with four changes of water and lyophilized. MAL, the fraction eluting from the ion-exchange column, contained >75% monomeric α -lactalbumin by weight and less than 20% multimer. We were not able to isolate the active species due to equilibrium between the different molecular species of the protein.

Protein labeling. MAL, ALA, BSA, and human IgG were biotinylated according to the manufacturer's instructions. For labeling with ^{125}I , 25 μl of protein (25 μg) dissolved in PBSA-T buffer (30 mM Na_2HPO_4 , 10 mM KH_2PO_4 , 120 mM NaCl, pH 7.4, with 0.1% Na-Azide, 0.05% Tween-20) was incubated with 2 μl of ^{125}I (0.2 mCi), 2 μl of lactoperoxidase (2.5 mg/ml), and 2 μl of H_2O_2 (1:2000 in PBS; 30 mM Na_2HPO_4 , 10 mM KH_2PO_4 , and 117 mM NaCl) for 2 min at room

temperature. The reaction was stopped by the addition of 500 μl of PBSA-T. The labeled protein was purified on a PD-10 column. Fractions (500 μl) in PBSA-T were eluted and fractions containing radioactivity were stored at -20°C . The labeled proteins eluted in fractions 7 and 8 and had a total activity of approximately 2×10^8 cpm.

Cell lines and primary cultures. The murine lymphoblastoid leukemia cell line L1210 (ATCC CLL 219) was cultured in suspension at 37°C , 5% CO_2 , in cell culture flasks (Falcon, Becton Dickinson Labware, New Jersey) in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1:100 dilution of nonessential amino acids, 1 mM sodium pyruvate, 50 $\mu\text{g}/\text{ml}$ of gentamicin, and 50 μM 2-mercaptoethanol. At a density of 7×10^5 cells/ml the cells were harvested by centrifugation at 320g and the pellet was resuspended in PBS or RPMI depending on the experiment.

The human lung carcinoma cell line A549 (ATCC CLL 185) grew as monolayers attached to the bottom of cell culture flasks (Falcon) under the same condition as described above but in medium without 2-mercaptoethanol. Human renal tubular epithelial cells (HRTEC) were isolated from the kidney of a 3-year-old boy [16]. The cells were cultured in Dulbecco's modified medium with the addition of 15% FCS, 2 mM glutamine, 100 $\mu\text{g}/\text{ml}$ of penicillin, 100 U of streptomycin, and 50 U/ml of heparin. Confluent monolayers of A549 and HRTEC cells were detached by addition of versene (140 mM NaCl, 2.4 mM KCl, 8 mM Na_2HPO_4 , 1.6 mM KH_2PO_4 , and 0.5 mM EDTA, pH 7.2) for 10 min at 37°C and were harvested by centrifugation at 320g. The pelleted cells were resuspended in PBS or RPMI depending on experiment.

Cell viability and DNA fragmentation. The cell suspensions (900 μl , 2×10^6 cells/ml) were mixed with MAL (100 μl) in 24-well plates, incubated at 37°C in 5% CO_2 , and the cells were harvested by aspiration. Cell aliquots were tested for viability by trypan blue exclusion. Remaining cells were analyzed for DNA fragmentation [14].

HMW DNA fragments were analyzed in cells embedded in low melting point agarose gel treated with proteinase K. Samples were run by electrophoresis at 180 V in 1% agarose gels in $0.5 \times \text{TBE}$ (45 mM Tris, 1.25 mM EDTA, 45 mM boric acid, pH 8.0), at 12°C , with the ramping rate changing from 0.8 s to 30 s for 24 h, using a forward to reverse ratio of 3:1. Oligonucleosome-length fragments were analyzed in ethanol-precipitated supernatants from lysed cells run on 1.8% agarose gels overnight applying a 40-V constant voltage. DNA was visualized under ultraviolet light (305 nm) after staining with ethidium bromide and photographed using Polaroid 55 positive-negative film.

Cell surface binding and intracellular distribution of biotinylated protein. L1210, A549, and HRTEC cell suspensions (4×10^6 cells/ml, 95 μl) were incubated at room temperature with 5 μl of biotinylated MAL, ALA, or BSA (5 mg/ml) and then washed in PBS with centrifugation at 320g for 10 min to remove unbound protein. To detect surface bound protein, cells were incubated for 30 min at room temperature with FITC-conjugated streptavidin (1:100 in PBS, 100 μl), washed in PBS, suspended in 300 μl of PBS, and analyzed by flow cytometry on a Coulter Epics Profile II flow cytometer (Coulter), equipped with a 488-nm argon laser. Green fluorescence was detected with a 525-nm band pass filter, and the PMT voltage was initially set to 1250 V and adjusted for day to day variation using calibration with Flowcheck and Flowset fluorospheres (Coulter). The binding was calculated as the quotient between the fluorescence intensity of the sample and the fluorescence intensity of the streptavidin control.

To detect intracellular protein, cells exposed to biotinylated protein were permeabilized with saponin to allow entry of FITC-conjugated streptavidin. Cells harvested by centrifugation at 320g were fixed by suspension in phosphate-buffered paraformaldehyde (4%) [17] for 5 min at room-temperature, washed in PBS, and permeabilized with 0.1% saponin in PBS. After washing in 0.1% saponin, FITC-conjugated streptavidin (1:100 in 0.1% saponin, 100 μl) was

added and the cells were incubated for 30 min at room temperature. The cells were washed twice in PBS-saponin and once in PBS, mounted on a glass slide, and analyzed in a Bio-Rad 1024 laser scanning confocal equipment (Bio-Rad Laboratories, Hemel-Hempstead, UK) attached to a Nikon Diaphot inverted microscope (Nikon, Japan).

Cell surface binding and intracellular distribution of radiolabeled MAL. L1210, A549, or HRTEC cell suspensions (4×10^6 cells/ml, 100 μ l) were incubated with radiolabeled protein (MAL, ALA, or IgG) for 30 min. Unbound protein was removed by washing three times in PBS. Radioactivity in different samples was quantitated in an Epics γ -counter (Coulter).

Subcellular fractionation was according to Graham *et al.* and Trent *et al.* [18, 19]. L1210, A549 or HRTEC cell suspensions (4×10^6 cells/ml, 500 μ l) were incubated at 37°C with 1×10^6 cpm of ^{125} I-labeled MAL, ALA, or IgG, washed three times with ice-cold PBS, suspended in 500 μ l of cold homogenization buffer (10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, and 2 mM $CaCl_2$ for L1210 cells and 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, for A549 and HRTEC cells, both buffers containing 0.1 mM PMSF, 10 μ g/ml of antipain, and 10 μ g/ml of leupeptin), and left on ice for 15 min. The cells were disrupted in a Dounce homogenizer (Thomas, Philadelphia, PA) with pestle size 415. Sucrose was added to a final concentration of 250 mM, the homogenate was centrifuged at 1000g for 10 min at 4°C, and the pellet was washed once by centrifugation in homogenization buffer. The supernatants from both centrifugations were further centrifuged at 12,000g for 30 min at 4°C (pellet 2). The supernatant was centrifuged at 150,000g for 1 h at 4°C (pellet 3). The radioactivity of the pellets and remaining supernatant from the differential centrifugation was measured in a γ -counter.

The purity of the fractions was determined by quantitation of specific markers [20]. DNA and RNA were detected with ethidium bromide. Sample (1 vol) was mixed with 1 vol of heparin solution (25 μ g/ml) and 1 ml of PBS or with 1 vol of heparin, 1 vol of RNase A (50 μ g/ml), and 1 vol of PBS. The samples were incubated at 37°C for 20 min and 1 vol of ethidium bromide was added (25 μ g/ml). Fluorescence was measured after 60 s incubation using an excitation wavelength of 360 nm and an emission wavelength of 580 nm. PBS served as blank, 1 vol of homogenate in 4 vol of PBS served as background correction factor, and the fluorescence was compared to a DNA standard (25 μ g/ml of λ DNA). Catalase activity was measured by mixing sample (0.5 ml) with H_2O_2 (0.5 ml), for 3 min at 4°C. H_2SO_4 (0.1 ml) was added to stop the reaction, $KMnO_4$ (0.7 ml) was added, and the optical density of the sample was measured at 480 nm within 1 min. Alkaline phosphatase activity was detected by mixing 50 μ l of sample with 200 μ l of assay mixture (5 ml of 16 mM *p*-nitrophenylphosphate solution, 5 ml of 50 mM sodium borate buffer (pH 9.5), and 20 μ l of 1 M $MgCl_2$) and incubated at 37°C for 60 min. The absorbance was measured at 410 nm.

The first pellet (pellet 1 or nuclear fraction) contained nuclei, large mitochondria, and large sheets of plasma membrane. The second pellet contained mitochondria, plasma membrane, and cytoplasmic organelles. The third pellet contained small vesicles, endoplasmic reticulum, and the supernatant contained cytosolic proteins.

Studies of active nuclear uptake. Active nuclear uptake was studied in digitonin-permeabilized cells [21, 22]. Briefly, 5×10^5 cells were incubated in 100 μ l of nuclear transport buffer (NTB; 20 mM Hepes, pH 7.3, 110 mM KAc, 5 mM NaAc, 2 mM DTT, 1 mM EGTA), with leupeptin, antipain (1 μ g/ml each), and digitonin (40 μ g/ml) for 5 min at room temperature. Cells were diluted in NTB and harvested by centrifugation at 320g for 10 min.

Biotinylated ALA, MAL, or IgG (5 μ l, 5 mg/ml) was added to digitonin-permeabilized cells in a total volume of 100 μ l NTB supplemented with 1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase, incubated at 30°C, and washed twice in NTB with centrifugation at 320g for 10 min. The cells were treated

with 0.2% Triton-X 100 in NTB for 6 min, washed, and incubated for 30 min with FITC-conjugated streptavidin (1:100 dilution, 100 μ l), washed in NTB and inspected in a Nikon Microphot microscope with epifluorescence equipment or by laser scanning confocal microscopy in a Bio-Rad MRC-1024 instrument. Inhibition of uptake across the nuclear pore was studied in digitonin-permeabilized cells preincubated with 50 μ g/ml of wheat-germ agglutinin (WGA). The role of Ca^{2+} was examined after preincubation of the digitonin-permeabilized cells with 0.5 mM EDTA, 0.5 mM BAPTA/AM or 10 μ M verapamil for 20 min at room-temperature and exposed to biotinylated protein.

For DNA fragmentation experiments digitonin-permeabilized L1210, A549, or HRTEC cells (2×10^6 /ml) were incubated at 37°C with MAL or ALA (0.4 mg/ml) and treated as above. Inhibition of DNA fragmentation was studied by preincubating the nuclei for 20 min at room temperature with 50 μ g/ml of WGA before addition of MAL.

Effect of MAL on isolated nuclei. L1210, A549, and HRTEC nuclei were isolated from homogenized cells. Pellet 1 from the homogenization was washed twice in nuclear buffer (25 mM Hepes, pH 7.0, 120 mM KCl, 2 mM K_2HPO_4 , 0.3 mM sucrose) for 10 min at 500g and suspended in nuclear buffer.

L1210, A549, or HRTEC nuclei in suspension were placed in 24-well plates (900 μ l), mixed with MAL, ALA, or BSA (100 μ l in nuclear buffer), incubated at 37°C for 1 or 2 h, harvested by aspiration, and analyzed for DNA fragmentation. Inhibition of DNA fragmentation was studied by preincubating the nuclei for 10 min at room temperature with 50 μ g/ml of WGA, 500 μ M EDTA, 500 μ M EGTA, 10 μ M BAPTA/AM, or 10 μ M verapamil before addition of MAL.

RESULTS

Difference in sensitivity to MAL-induced apoptosis between tumor cell lines and primary cells in culture. Three cell types were selected based on differences in sensitivity to MAL-induced apoptosis (Fig. 1A). The mouse leukemia L1210 cells were highly sensitive with 50% of the cells killed after 6 h exposure to 0.5 mg/ml of MAL. The A549 lung carcinoma cells were intermediately sensitive and required 24 h exposure to 1.25 mg/ml of MAL in order for 50% of the cells to lose their viability. The primary kidney epithelial HRTEC cells remained fully viable after 24 h exposure to 4 mg/ml of MAL. Loss of viability in L1210 and A549 cells was accompanied by DNA fragmentation but DNA fragments were not formed in the HRTEC cells (Fig. 1B). The monomeric form of the protein, ALA (5 mg/ml), had no effect on cell viability and did not induce DNA fragmentation in the L1210, A549, or HRTEC cells.

Interaction of MAL with the surface of sensitive and resistant cells. The surface binding of biotinylated MAL, ALA, or IgG (0.25 mg/ml) to L1210, A549 and HRTEC cells was examined by confocal microscopy (Fig. 2). Both MAL and ALA bound to the three cell types and were distributed in patches separated by unstained areas (Fig. 2). By flow cytometry an increase in surface-bound protein was detected after 10 min and a maximum was reached after 30 min incubation with MAL. There was no quantitative difference in cell surface binding of MAL between the three cell types (binding $\pm$ SD = 10.1 ± 1.4 (L1210), 11.5 ± 2.4 (A549), and

8.5 ± 2.8 (HRTEC)). There was no difference in efficiency of cell surface binding between MAL and ALA (binding $\pm$ SD = 8.1 ± 1.1 (L1210), 8.8 ± 2.1 (A549), and 6.2 ± 3.0 (HRTEC)).

Cell surface binding of ^{125} I-labeled MAL and ALA increased with the concentration of MAL to reach saturation after 30 min. There was no difference in cell surface binding of ^{125} I-labeled MAL or ALA between the three cell types (Fig. 3). The total amount of cell bound ^{125} I-labeled ALA was, however, lower than for MAL ($P < 0.01$ for all cell types).

These experiments suggested that the differential susceptibility of L1210, A549, and HRTEC cells to MAL-induced apoptosis was not due to differences in MAL surface binding. Furthermore, both the active and the inactive forms of α -lactalbumin bound efficiently to the surface of the three cell types.

Localization of MAL to different intracellular compartments. Cells were examined by confocal microscopy after exposure to biotinylated MAL, ALA, or BSA, fixed, and permeabilized with saponin to allow entry of FITC-streptavidin (Fig. 2). MAL was found in the nuclei of L1210 and A549 cells, but not HRTEC cells.

Nuclear staining was first detected in circa 10% of the L1210 cells after 2 h incubation with 0.25 mg/ml of MAL. After 6 h more than 70% of the nuclei stained brightly. Cytoplasmic staining was not observed in those cells. Nuclear uptake of MAL occurred more slowly in the A549 cells. After 6 h about 15% of A549 cell nuclei stained, and by 24 h about 50% of the A549 nuclei stained brightly. Prior to the detection of nuclear staining, MAL was observed as granular fluorescence evenly distributed throughout the cytoplasm of A549 cells (Fig. 2). Nuclear uptake was not observed in the HRTEC cells exposed to biotinylated MAL.

The distribution of ^{125} I-labeled MAL and the kinetics of uptake into the different cellular compartments are shown in Fig. 4. About 80% of the total radioactivity associated with the L1210 cells was recovered in the nuclear fraction after 6 h. The A549 cells had taken about 50% of ^{125} I-labeled MAL into the nuclear fraction after 24 h. In the HRTEC cells only 16% of the total cell associated radioactivity was found in the nuclear fraction after 24 h of incubation. The amount of MAL in the other subcellular fractions did not differ between the cell types (Fig. 4)

There was a marked difference in cellular distribution between MAL and ALA. The monomeric protein was mainly recovered from the cytosol and not from the nuclei (Fig. 5). The cytosolic content of ALA was 80, 75, and 60% of that of MAL in the respective cell type. The nuclear uptake of ^{125} I-labeled ALA did not differ between the cell types.

The cellular localization studies with biotinylated and ^{125} I-labeled MAL suggested that MAL was trans-

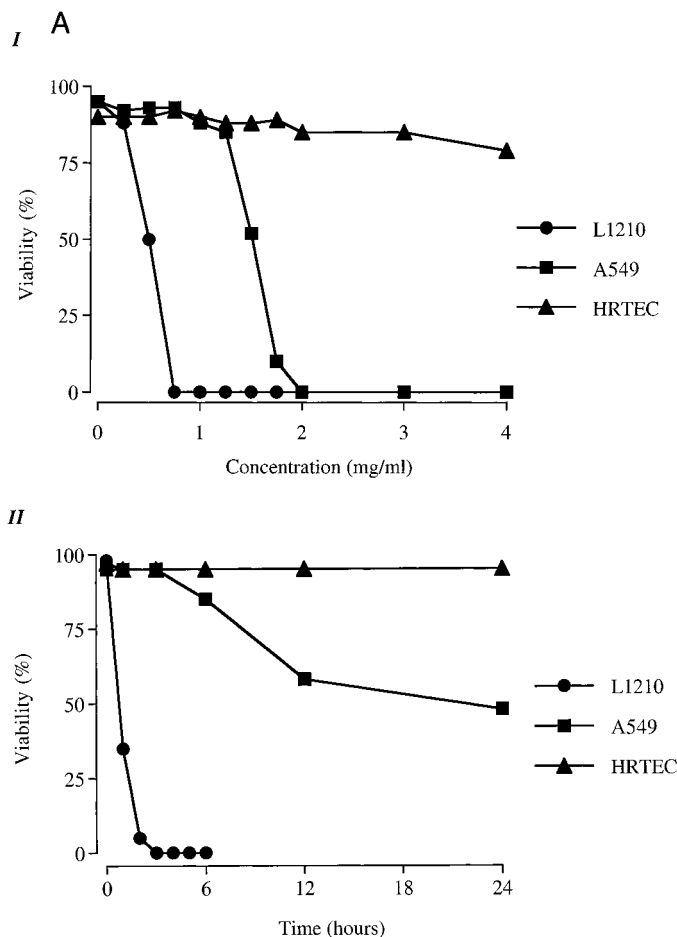


FIG. 1. MAL induces apoptosis in the L1210 and A549 cell lines but not in HRTEC primary cultures. (A) Viability as determined by trypan blue exclusion of cells exposed to MAL in relation to time and concentration. (I) The viability of L1210, A549, and HRTEC cells after exposure to different concentrations of MAL for 6 h (L1210) or 24 h (A549 and HRTEC). The MAL-concentration required to kill 50% of the cells was 0.5 mg/ml for L1210 cells and 1.25 mg/ml for A549 cells. HRTEC cells remained fully viable. (II) The time dependent killing of L1210, A549, and HRTEC cells after exposure to 1.25 mg/ml of MAL. (B) Chromatin cleavage induced by MAL in L1210 and A549 cells was analyzed by field-inverse gel electrophoresis and oligonucleosomal fragmentation was detected by conventional gel electrophoresis. DNA fragmentation was not detected in HRTEC cells. The amount of active multimer in the MAL-fraction was $\leq 20\%$ by weight.

ported into the nuclei and that the nuclear uptake of MAL into different cells was proportional to their sensitivity to MAL-induced cell death. Nuclear uptake of ALA was much less efficient than that of MAL.

Interaction of MAL with isolated nuclei. Nuclei were purified from L1210, A549, and HRTEC cells and incubated with different concentrations of MAL (0.2 and 0.4 mg/ml). The formation of HMW and oligonucleosome-length DNA fragments was examined. While some degree of spontaneous DNA fragmentation oc-

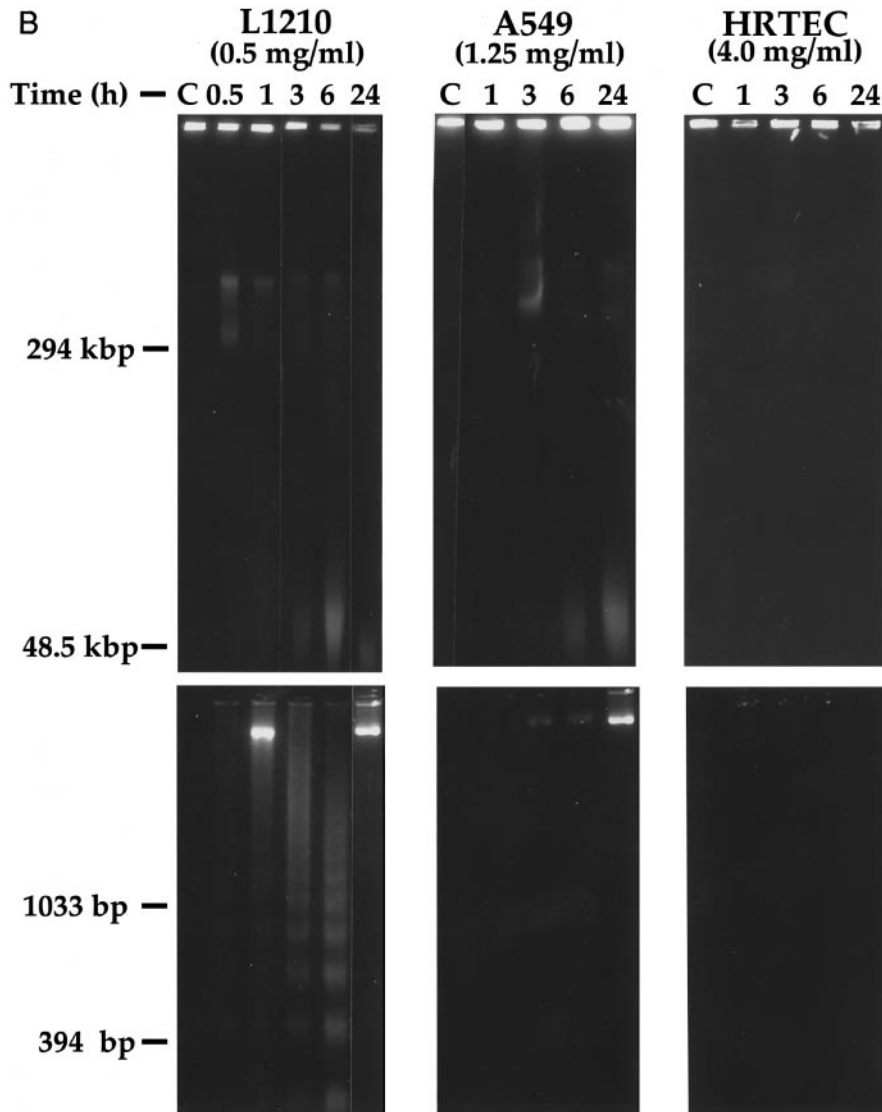


FIG. 1—Continued

curred in unstimulated nuclei from the three cell types, MAL (0.4 mg/ml) enhanced the formation of HMW DNA fragments after 1 h and of oligonucleosome length fragments after 2 h of incubation (Fig. 6). MAL was, however, not found to possess endonuclease activity when tested on purified DNA from the sensitive cells (data not shown). ALA did not induce DNA fragmentation in isolated nuclei.

There was no difference in sensitivity to MAL-induced DNA fragmentation between nuclei from the three cell types. These results demonstrated that MAL triggered the induction of DNA fragmentation in the absence of activated cytoplasm and suggested that the differential sensitivity to MAL was determined by the nuclear targeting process rather than by the effect on the nuclei per se.

Uptake of MAL and DNA fragmentation in nuclei of digitonin-permeabilized cells. L1210, A549, and HRTEC cells were permeabilized with digitonin and exposed to biotinylated MAL, ALA, or IgG. MAL was imported directly into the nuclei of digitonin-treated cells with maximum levels reached after 20 min (Fig. 7). HMW DNA fragments were observed after 1 h and oligonucleosomal fragments after 2 h incubation with 0.4 mg/ml of MAL (Fig. 6). ALA entered nuclei of digitonin-permeabilized cells as predicted by its molecular mass (14 kDa) but did not induce DNA fragmentation. There was no nuclear uptake of IgG.

Role of the nuclear pore complex in the uptake of MAL. Active transport over the nuclear pore was examined in digitonin-treated cells, using wheat-germ

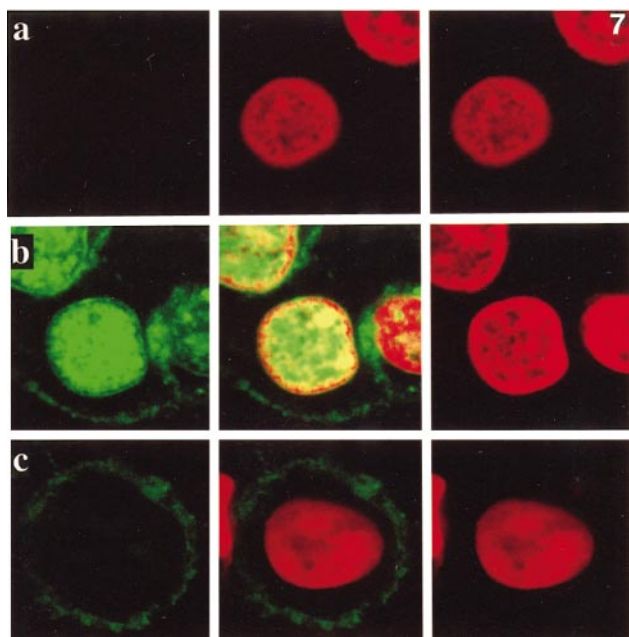
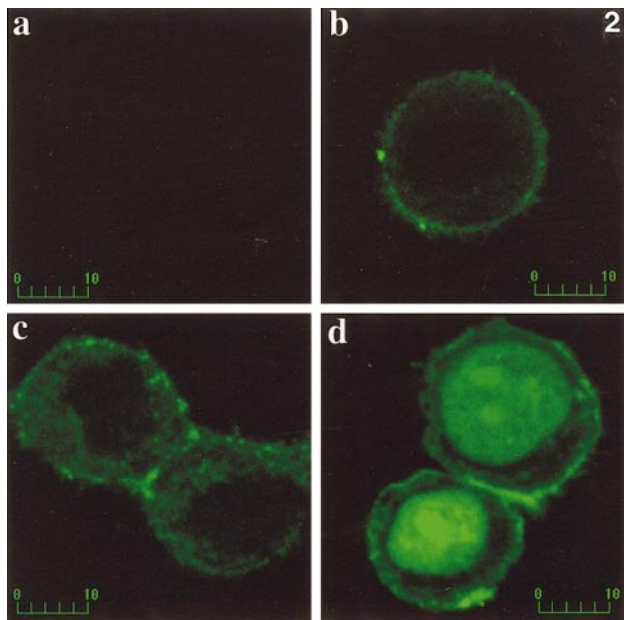


FIG. 2. Interaction of MAL with different cellular compartments in A549 cells. (a) Streptavidin control. (b) Cell surface binding detected after 30 min incubation. The morphology and intensity of MAL cell surface binding was similar for the three cell types and for ALA. (c) Cytoplasmic, granular fluorescence was detected after 3 h incubation with MAL. (d) Nuclear accumulation was maximal after 24 h incubation. The nuclear accumulation was more rapid in the L1210 cells. Nuclear accumulation of MAL was not observed in the HRTEC cells. The scale bar units are in micrometers.

FIG. 7. Nuclear uptake of MAL in digitonin-permeabilized A549 cells, as visualized by confocal microscopy. The cells were incubated with biotinylated protein for 20 min, washed, and counterstained with FITC-conjugated streptavidin (green). There was no nuclear staining in the IgG-treated cells (a), but strong nuclear staining of cells exposed to MAL (b). Nuclear uptake of MAL was inhibited after

agglutinin (WGA) which inhibits the transport of importin–protein complexes through the nuclear pore. Digitonin-permeabilized A549 cells were preincubated with 50 $\mu\text{g}/\text{ml}$ of WGA for 20 min, washed, and exposed to biotinylated MAL, IgG, or ALA (Fig. 6). WGA completely blocked the nuclear uptake of MAL, but had no effect on ALA that diffuses freely over the nuclear membrane.

Preincubation of isolated nuclei from A549 cells or digitonin-permeabilized A549 cells with 50 $\mu\text{g}/\text{ml}$ of WGA inhibited the MAL-induced DNA fragmentation as compared to control nuclei (Fig. 6).

Role of Ca^{2+} in the nuclear uptake of MAL and in MAL-induced DNA fragmentation. MAL-induced apoptosis was previously shown to require extracellular Ca^{2+} [14]. This suggested that Ca^{2+} might influence the nuclear uptake mechanism and/or the induction of DNA fragmentation once MAL reaches the cell nuclei. L1210, A549, and HRTEC cells were pretreated with an inhibitor of Ca^{2+} uptake (verapamil), with extracellular Ca^{2+} -chelators (EDTA and EGTA) and with an intracellular Ca^{2+} -chelator (BAPTA/AM). ^{125}I -labeled MAL was added to the cells, and the nuclear uptake was examined after 6 h in L1210 cells and after 24 h in A549 and HRTEC cells. No effect on the nuclear uptake of MAL was observed.

The MAL-induced DNA fragmentation was subsequently examined in control nuclei and nuclei incubated with the Ca^{2+} inhibitors. EDTA and BAPTA/AM completely blocked the MAL-induced DNA fragmentation in nuclei isolated from L1210 and A549 cells. These results demonstrate that Ca^{2+} is required for MAL-induced DNA fragmentation in isolated nuclei, but that the nuclear uptake of MAL is Ca^{2+} -independent.

DISCUSSION

Epidemiological studies have provided evidence that breast-feeding protects against cancer [23–25]. Breast-fed children have a lower incidence, especially of lymphomas, but also of malignancies in general than bottle-fed children, and the frequency decreases with the length of breast-feeding [25]. This association implies

pretreatment of the cells with WGA (c). Nuclear DNA was counterstained with 25 $\mu\text{g}/\text{ml}$ of propidium iodide (red). The integrity of the nuclear membrane of digitonin-permeabilized cells was tested using sera containing anti-nuclear antibodies. After incubation and washing in NTB, the cells were incubated with FITC-conjugated anti-human IgG antibodies (1:100 dilution in NTB) for 30 min at room temperature, washed, and inspected by confocal microscopy. At a concentration of 40 $\mu\text{g}/\text{ml}$ of digitonin no nuclear staining was detected. In cells treated with Triton-X 100, which permeabilizes the nuclear membrane, bright nuclear staining was observed after the addition of anti-nuclear antibodies.

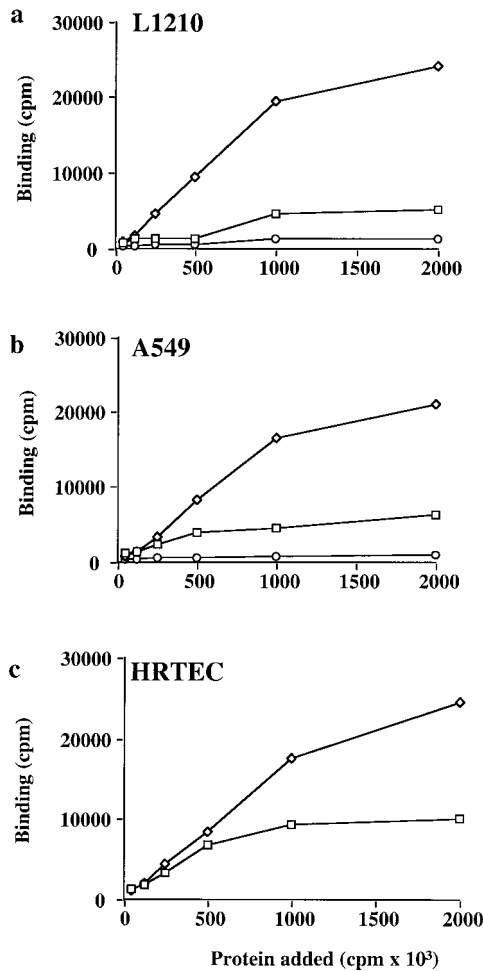


FIG. 3. Cell associated radioactivity in L1210, A549, and HRTEC cells after 30 min exposure to increasing concentrations of radiolabeled MAL ($\diamond$), ALA ($\square$), or IgG ($\circ$).

that milk contains molecules that influence tissue development.

Tumor cells and mature nonmalignant cells differ in sensitivity to MAL, an α -lactalbumin complex in human milk. The tumor cells undergo apoptosis, while healthy, nonmalignant cells survive and continue to proliferate in the presence of MAL. This study compared the subcellular distribution of MAL in sensitive and resistant cells. By confocal microscopy and subcellular fractionation, MAL was shown to accumulate in nuclei of cells that were sensitive to its apoptosis effect but not in resistant cells. Nuclear uptake occurred before or at the onset of DNA fragmentation and required active transport through the nuclear pore complex. MAL had a direct effect on nuclei; it was able to induce DNA fragmentation in isolated nuclei in the absence of activated cytoplasm. Inhibition of MAL transport through the nuclear pore rescued the nuclei from MAL-mediated DNA fragmentation. The results

demonstrate that MAL can target the cell nucleus and that the nuclear targeting mechanisms are more readily available in cells that are sensitive to MAL-induced apoptosis than in resistant cells. MAL differs from most previously described apoptosis-inducing agents in that it induces apoptosis through a direct effect at the nuclear level. Similar intranuclear targeting of apoptosis-inducing agents have been seen only for granzyme B in perforin-treated target cells and for glucocorticoids in thymocytes and certain T-cell lines [9, 10, 26].

α -Lactalbumin is the major protein of human milk whey. In its monomeric form it is a 14-kDa protein with one high affinity Ca^{2+} binding site. We have previously reported that the monomeric, native form of α -lactal-

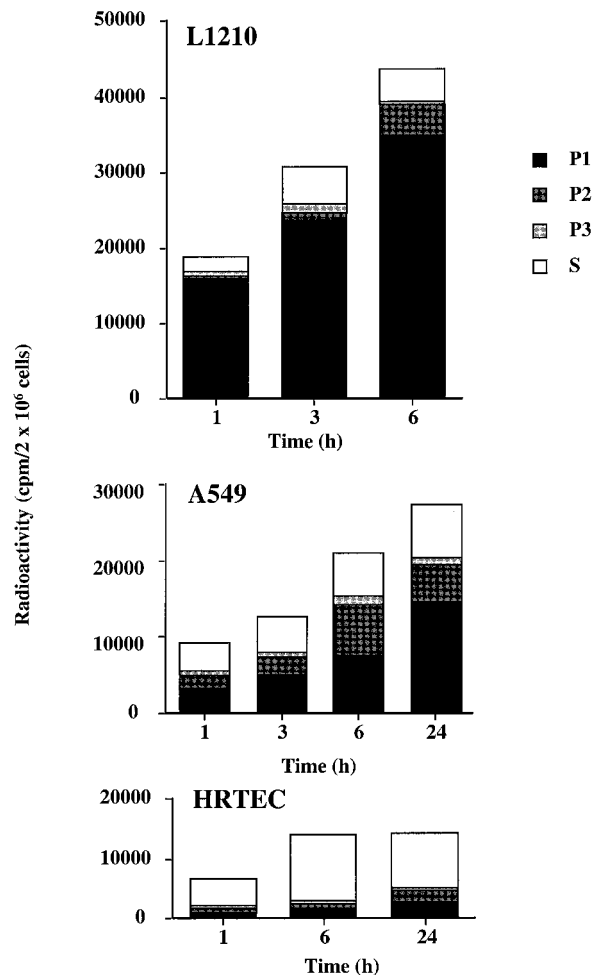


FIG. 4. Localization of ^{125}I -labeled MAL to different subcellular compartments in L1210, A549, and HRTEC cells. Cells were exposed to 10^6 cpm of radiolabeled MAL and homogenized, and radioactivity associated with each subcellular fraction was quantitated in a γ -counter. The nuclear fraction contained mostly nuclei; pellet 2 contained plasma-, Golgi-, and ER-membranes and mitochondria; pellet 3 contained small vesicles; and the supernatant contained the cytosol.

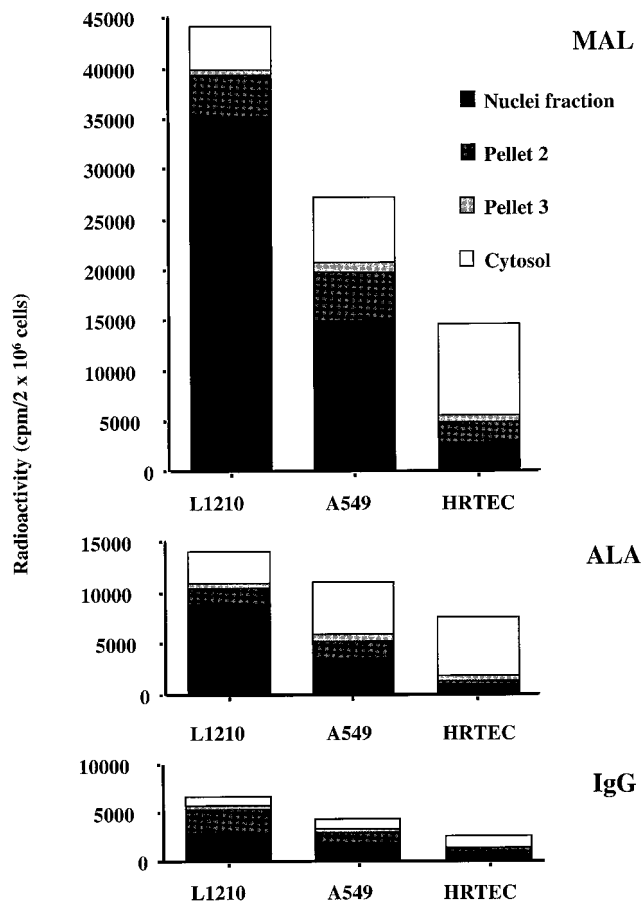


FIG. 5. Difference in subcellular distribution between MAL and ALA. L1210, A549, and HRTEC cells were exposed to 10^6 cpm of radiolabeled proteins for 6, 24, and 24 h, respectively. The cells were homogenized and subjected to differential centrifugation, and the radioactivity of each subcellular fraction was determined. The recovery from the nuclear fraction (pellet 1) was 34,800 cpm (78%), 14,200 cpm (51%), and 2,300 cpm (16%) for the L1210, A549, and HRTEC cells, respectively. The recovery from pellet 2 was 4500 cpm (10%), 5100 cpm (19%), and 2100 cpm (15%). The recovery from pellet 3 was 800 cpm (2%), 1400 cpm (5%), and 1100 cpm (8%) and the recovery from the cytosol was 4500 cpm (10%), 6800 cpm (25%), and 8600 cpm (61%), respectively.

bumin does not induce apoptosis [14]. In this study the monomer was found to bind to the cytoplasmic membrane of sensitive and resistant cells, and to be taken up into the cytoplasm. Like monomeric α -lactalbumin, MAL bound to the surface of sensitive and resistant cells but in addition MAL entered the nuclei of the cells in which it induced apoptosis. This difference in cellular localization may relate to differences in protein folding between the monomeric and the multimeric forms of the protein. Recent studies have shown that MAL consists of α -lactalbumin multimers in the molten globule state complexed with fatty acid. We are currently investigating how changes in protein folding from the native monomer to the molten globule multimer influence the transport over the nuclear mem-

brane, so as to explain the nuclear uptake of the multimer.

For some exogenous apoptosis-inducing molecules, like Fas-ligand or TNF, the binding to their respective cell surface receptors is crucial for apoptosis induction. Ligand-receptor interaction triggers transmembrane and/or intracellular signaling pathways leading to apoptosis [27–29]. Consequently, cells that lack cell surface receptors for these agonists are resistant to their

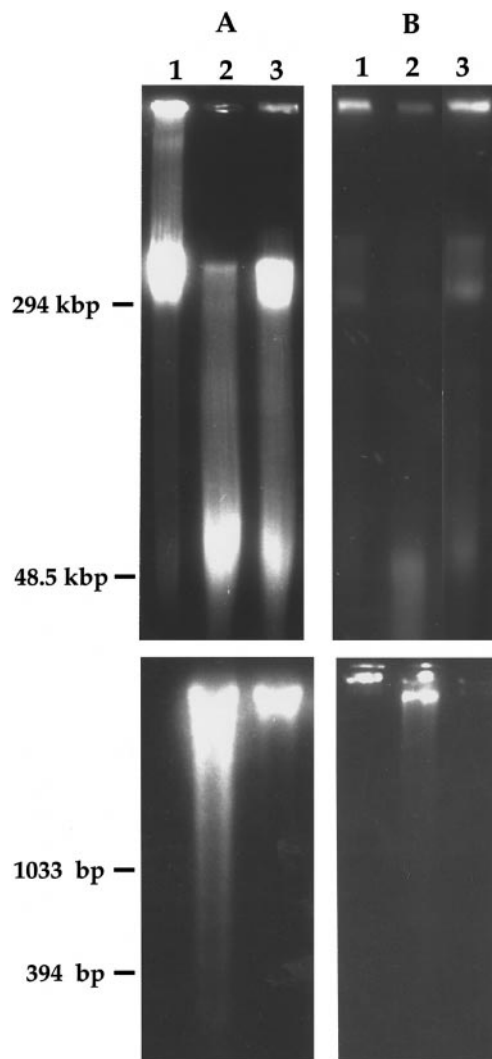


FIG. 6. Chromatin cleavage in isolated nuclei (A) or digitonin-treated A549 cells (B) after 2 h incubation with 0.4 mg/ml of MAL. Transport over the nuclear pore was inhibited by preincubation of the isolated nuclei or the digitonin-treated cells with 50 μ g/ml of WGA. Chromatin cleavage was analyzed by field-inverse gel electrophoresis (top) and oligonucleosomal fragmentation was detected by conventional gel electrophoresis (bottom). Lanes A1 and B1 show limited DNA fragmentation in untreated nuclei or digitonin-permeabilized cells. Lanes A2 and B2 show nuclei or digitonin-treated cells exposed to MAL and lanes A3 and B3 show nuclei or digitonin-treated cells exposed to MAL after preincubation with 50 μ g/ml of WGA.

apoptosis-inducing effects. In this study there was no difference in cell surface binding of MAL to sensitive and resistant cells, suggesting that receptors for MAL were present on all cells. By confocal microscopy MAL was shown to bind in patches, suggesting either that MAL bound as preformed aggregates or that the bound MAL accumulated in certain areas of the membrane through capping or other mechanisms influencing receptor distribution. Cell surface binding was rapid, saturatable at high MAL concentrations, and it was also specific as defined by competition between labeled and unlabeled MAL (data not shown). There was no difference in cell surface binding between MAL and the monomeric inactive form of the protein. These results suggest that MAL differs from agonists like TNF and Fas-ligand in that cell surface binding does not itself determine if apoptosis will be induced.

There was also no evidence that the cytoplasmic uptake differed between sensitive and resistant cells. Uptake of ^{125}I -labeled MAL into the cytoplasm occurred with similar kinetics in the L1210, A549, and HRTEC cells, and the amount of MAL in the cytoplasm was similar in the three cases. There was, however, a direct link between the susceptibility to MAL-induced apoptosis and the nuclear accumulation of MAL in the three cell types. MAL was detected after 2 h in nuclei of sensitive L1210 cells (20%, 0.5 mg/ml) and the fraction of cells having MAL-containing nuclei increased to about 80% after 6 h. In A549 cells nuclear uptake and DNA fragmentation required almost threefold more protein and still it occurred more slowly. Finally, nuclear uptake was low or absent in HRTEC cells.

Evidence for a direct nuclear effect of MAL was obtained in experiments with isolated nuclei and digitonin-permeabilized cells. MAL entered the nuclei and stimulated the formation of HMW and oligonucleosome-length DNA fragments in isolated nuclei more rapidly than in intact cells. The monomeric form of the protein was able to enter the isolated nuclei and to pass into nuclei of digitonin-permeabilized cells, but did not induce DNA fragmentation. Interestingly, there was no difference in sensitivity to MAL between the nuclear fractions isolated from the three cell types, suggesting that sensitive and resistant cells differ in the transport of MAL into the nucleus and not in the susceptibility to MAL once it reaches the nuclear compartment.

The uptake of MAL into the nucleus was via the nuclear pore complex as shown by inhibition studies using WGA, a lectin that binds to glycosylated regions of the nucleoporins and sterically hinders transport of the importin-protein complex through the nuclear pore. WGA treatment blocked MAL uptake into the nuclei of digitonin-treated cells and inhibited MAL-induced DNA fragmentation. Classical nuclear localization sequences often include clusters of basic amino

acids that share little or no sequence homology [11, 30]. When conducting a sequence analysis of the monomeric form of α -lactalbumin we did not find known nuclear targeting motifs and the monomer did not target the cell nucleus. It is likely, therefore that MAL carries structural modifications, not present in ALA, that confer affinity for the nuclear pore and the ability to activate DNA fragmentation in the nucleus.

Lactalbumins are Ca^{2+} binding proteins with one high affinity and one low affinity Ca^{2+} -binding site [31]. MAL was previously shown to increase intracellular Ca^{2+} levels. This Ca^{2+} flux was required for apoptosis to occur; removal of extracellular Ca^{2+} inhibited MAL-induced apoptosis [14]. In this study we examined if the Ca^{2+} dependence reflected an effect on nuclear uptake of MAL and/or the induction of DNA fragmentation. Pretreatment of MAL-sensitive cells with Ca^{2+} chelators had no effect on the nuclear localization of MAL but was found to inhibit MAL-mediated DNA fragmentation in isolated nuclei. These observations suggested that apoptosis occurs as a result of at least two converging mechanisms: a Ca^{2+} -independent transport of MAL into the nucleus and a Ca^{2+} dependent activation step in the nuclear compartment.

Rapidly growing tissues need control mechanisms that select for maturity and drive the cells toward differentiation rather than malignancy. This study describes a naturally occurring molecular complex in human milk, that may have a surveillance function in tissue maturation. Such molecules should be explored as cancer therapeutics. They may be predicted to have the advantage over currently available compounds in that they target tumor cells and immature cells rather than healthy cells and in that they kill these cells through apoptosis rather than necrosis, thus minimizing tissue destruction and the side effects that accompany many current treatments.

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REFERENCES

1. Schwartz, L. M., and Osborne, B. A. (1993). Programmed cell death, apoptosis and killer genes. *Immunol. Today* 14, 582-590.
2. McConkey, D. J., Zhivotovsky, B., and Orrenius, S. (1996). Apoptosis—Molecular mechanisms and biomedical implications. *Mol. Aspects Med.* 17, 1-115.

3. Kerr, J. F., Winterford, C. M., and Harmon, B. V. (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 73, 2013–26.
4. Arends, M. J., Morris, R. G., and Wyllie, A. H. (1990). Apoptosis: The role of endonuclease. *Am. J. Pathol.* 136, 593–608.
5. Zhivotovsky, B., Gahm, A., Ankarcrona, M., Nicotera, P., and Orrenius, S. (1995). Multiple proteases are involved in thymocyte apoptosis. *Exp. Cell Res.* 221, 404–412.
6. Kumar, S. (1995). ICE-like proteases in apoptosis. *Trends Biochem. Sci.* 20, 198–202.
7. Yasuhara, N., Eguchi, Y., Tachibana, T., Imamoto, N., Yoneda, Y., and Tsujimoto, Y. (1997). Essential role of active nuclear transport in apoptosis. *Genes Cells* 2, 55–64.
8. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD. *Nature* 391, 43–50.
9. Froelich, C. J., Hanna, W. L., Poirier, G. G., Duriez, P. J., D'Amours, D., Salvesen, G. S., Alnemri, E. S., Earnshaw, W. C., and Shah, G. M. (1996). Granzyme B/perforin-mediated apoptosis of Jurkat cells results in cleavage of poly(ADP-ribose) polymerase to the 89-kDa apoptotic fragment and less abundant 64-kDa fragment. *Biochem Biophys Res Commun.* 227, 658–65.
10. Andrade, F., Roy, S., Nicholson, D., Thornberry, N., Rosen, A., and Casciola Rosen, L. (1998). Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. *Immunity* 8, 451–60.
11. Jans, D. A., and Hübner, S. (1996). Regulation of protein transport to the nucleus: Central role of phosphorylation. *Physiol. Rev.* 76, 651–685.
12. Yoneda, Y. (1997). How proteins are transported from the cytoplasm to the nucleus. *J. Biochem.* 121, 811–817.
13. Görlich, D., and Mattaj, I. W. (1996). Nucleocytoplasmic transport. *Science* 271, 1513–1518.
14. Håkansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., and Svanborg, C. (1995). Apoptosis induced by a human milk protein. *Proc. Natl. Acad. Sci. USA* 92, 8064–8068.
15. Melander, O. (1947). *Uppsala Läkarfören. Förhandl.* 3-4, 107–198.
16. Karpman, D., Håkansson, A., Perez, M.-T. R., Isaksson, C., Carlemalm, E., Caprioli, A., and Svanborg, C. (1998). Apoptosis of renal cortical cells in the hemolytic uremic syndrome: *In vivo* and *in vitro* studies. *Infect. Immunol.* 66, 636–644.
17. Sander, B., Andersson, J., and Andersson, U. (1991). Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol. Rev.* 119, 65–92.
18. Graham, J. (1992). Isolation of subcellular organelles and membranes. In "Centrifugation, a Practical Approach," Ed: Rickwood, D. Oxford IRL Press, Washington, DC pp. 161–182.
19. Trent, D. F., Seither, R. L., and Goldman, D. (1991). Compartmentation of intracellular folates. *Biochem. Pharmacol.* 42, 1015–1019.
20. Graham, J., and Ford, T. C. (1992). Marker enzymes and chemical assays for the analysis of subcellular fractions. In "Centrifugation, a Practical Approach," Ed: Rickwood, D. Oxford IRL Press, Washington, DC pp. 309–333.
21. Adam, S. A., Sterne Marr, R., and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111, 807–816.
22. Shannon Moore, M., and Blobel, G. (1992). The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic fractions. *Cell.* 69, 939–950.
23. Newcomb, P. A., Storer, B. E., Longnecker, M. P., Mittendorf, R., Greenberg, E. R., Clapp, R. W., Burke, K. P., Willett, W. C., and MacMahon, B. (1994). Lactation and a reduced risk of pre-menopausal breast cancer. *N. Engl. J. Med.* 330, 81–87.
24. Siskind, V., Schofield, F., Rice, D., and Bain, C. (1989). Breast cancer and breastfeeding: Results from an Australian case-control study. *Am. J. Epidemiol.* 130, 229–236.
25. Davis, M. K., Savitz, D. A., and Graubard, B. I. (1988). Infant feeding and childhood cancer. *Lancet* 2, 365–368.
26. Cidlowski, J. A., King, K. L., Evans Storms, R. B., Montague, J. W., Bortner, C. D., and Hughes, F. M., Jr. (1996). The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. *Recent Prog. Horm. Res.* 51, 457–90; discussion 490–491.
27. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R., and Ferguson, T. A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 270, 1189–1192.
28. Tepper, C. G., Jayadev, S., Liu, B., Bielawska, A., Wolff, R., Yonehara, S., Hannun, Y. A., and Seldin, M. F. (1995). Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. *Proc. Natl. Acad. Sci. USA* 92, 8443–8447.
29. Zheng, L., Fisher, G., Miller, R. E., Peschon, J., Lynch, D. H., and Leonardo, M. J. (1995). Induction of apoptosis in mature T cells by tumor necrosis factor. *Nature* 377, 348–351.
30. Garcia-Bustos, J., Heitman, J., and Hall, M. N. (1991). Nuclear protein localization. *Biochim. Biophys. Acta* 1071, 83–101.
31. Ren, J., Stuart, D. I., and Acharya, K. R. (1993). Alpha-lactalbumin possesses a distinct zinc binding site. *J. Biol. Chem.* 268, 19292–19298.

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Conversion of α -lactalbumin to a protein inducing apoptosis

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In this study α -lactalbumin was converted from the regular, native state to a folding variant with altered biological function. The folding variant was shown to induce apoptosis in tumor cells and immature cells, but healthy cells were resistant to this effect. Conversion to HAMLET (human α -lactalbumin made lethal to tumor cells) required partial unfolding of the protein and a specific fatty acid, C18:1, as a necessary cofactor. Conversion was achieved with α -lactalbumin derived from human milk whey and with recombinant protein expressed in *Escherichia coli*. We thus have identified the folding change and the fatty acid as two key elements that define HAMLET, the apoptosis-inducing functional state of α -lactalbumin. Although the environment in the mammary gland favors the native conformation of α -lactalbumin that serves as a specifier in the lactose synthase complex, the conditions under which HAMLET was formed resemble those in the stomach of the nursing child. Low pH is known to release Ca^{2+} from the high-affinity Ca^{2+} -binding site and to activate lipases that hydrolyze free fatty acids from milk triglycerides. We propose that this single amino acid polypeptide chain may perform vastly different biological functions depending on its folding state and the *in vivo* environment. It may be speculated that molecules like HAMLET can aid in lowering the incidence of cancer in breast-fed children by purging of tumor cells from the gut of the neonate.

Proteins adopt a series of conformations during their synthesis and transport through human cells. According to the classical view, this process is determined by the amino acid sequence, and thermodynamics force the protein to adopt the conformation with the lowest free energy. During the last decade this classical view has been challenged, because proteins have been shown to adopt several stable conformations, depending on which kinetic pathway is followed (1). The high-energy barriers between the different conformations can be overcome with the help of chaperones (2, 3), but in many systems, the factors that cause or relieve such kinetic barriers remain to be specified.

Stable conformational variants of a protein may expose distinct functional regions and thus differ in biological activity. The most striking example is the prion protein that can change from the normal α -helix-rich to a β -sheet-rich, disease-causing isoform (4, 5). In a previous study we proposed α -lactalbumin as a second example of a protein that can acquire different functions depending on its folding state (6). An α -lactalbumin complex from acid-precipitated human milk casein was shown to induce apoptosis in tumor cells and immature cells, but not in mature, differentiated cells (7). Surprisingly, native α -lactalbumin (Fig. 1A) isolated from its traditional source, human milk whey, did not induce apoptosis. The difference in activity between the two forms of the protein was not caused by a change in secondary structure, but the active complex was found to have undergone a change in tertiary structure, with a conformational switch toward a molten globule-like state (6).

If folding variants of α -lactalbumin differ in biologic activity, it should be possible to convert native α -lactalbumin to the active, apoptosis-inducing form by changing its conformation. In the present study, we prove that α -lactalbumin, indeed, can be converted from the native conformation to the apoptosis-

inducing form and define the requirements for this structural and functional change. HAMLET (human α -lactalbumin made lethal to tumor cells) is shown to consist of partially unfolded α -lactalbumin that has integrated a cofactor, which stabilizes the conformation. The cofactor has been identified as a specific fatty acid.

Materials and Methods

Purification of Human α -Lactalbumin. Native α -lactalbumin was purified from human milk whey by ammonium sulfate precipitation followed by phenyl-Sepharose chromatography (8) and size-exclusion chromatography on a Sephadex G-50 column. The purity of the protein was controlled by SDS/PAGE and agarose gel electrophoresis and by spectroscopic techniques.

Recombinant α -lactalbumin was purified from *Escherichia coli* (BL21 DE3 pLysS), carrying the vector pALA with the entire human α -lactalbumin gene inserted between the *Nde*I (site 100) and *Eco*RI (site 499) sites of the pAED4 vector (pALA was a kind gift from P. S. Kim, Howard Hughes Medical Institute, Cambridge, MA), after induction with isopropyl β -D-thiogalactoside (1 mM). Inclusion bodies were isolated from 1 liter of culture medium, dissolved in 40 ml of buffer (8 M urea/10 mM Tris-HCl/10 mM reduced glutathione, pH 8.0), and applied to a DEAE cellulose column. The protein was eluted with 10 mM Tris/7 M urea/1 M NaCl/1 mM CaCl_2 , pH 8.0. The protein was reduced with 10 mM reduced glutathione added dropwise (2 ml/h) to 500 ml of folding buffer (10 mM Tris-HCl/1 mM CaCl_2 /100 mM KCl/10 mM reduced glutathione/1 mM oxidized glutathione/20% glycerol, pH 8.0, at room temperature) (9). When folding was complete, 10 mM EDTA was added, the folding suspension was applied to a phenyl-Sepharose column, and α -lactalbumin was eluted with 1 mM CaCl_2 as described (8). The native fold was confirmed by 8-anilino-1-naphthalene-1-sulfonic acid (ANS) fluorescence and near-UV CD spectroscopy, with the characteristic 270-nm tyrosine minimum and the 294-nm tryptophan maximum (Fig. 1C).

Apo α -lactalbumin was generated from 25 mg of native α -lactalbumin dissolved at 1.8 mM in Tris (10 mM Tris-HCl, pH 8.5) by adding 3.5 mM EDTA to remove bound Ca^{2+} . The conformational change was confirmed by near-UV CD and ANS spectroscopy. The near-UV CD spectrum showed the characteristic loss of signal in the tyrosine and tryptophan regions, and the apo α -lactalbumin-bound ANS, as shown by increased intensity and an intensity maximum, shifted to 480 nm (Fig. 1C and D).

Spectroscopic Analyses. CD spectra were obtained by using a JASCO J-720 spectropolarimeter with a JASCO PTC-343 Pel-

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Abbreviations: HAMLET, human α -lactalbumin made lethal to tumor cells; ANS, 8-anilino-1-naphthalene-1-sulfonic acid.

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tier-type thermostated cell holder. Quartz cuvettes were used with 1-cm path length, and spectra were recorded between 320 and 240 nm. The wavelength step was 1 nm, the response time was 4 s, and the scan rate was 10 nm per minute. Six scans were recorded and averaged for each spectrum. Baseline spectra were recorded with pure buffer in each cuvette and subtracted from the protein spectra.

ANS fluorescence emission spectra were recorded at 25°C on a Perkin–Elmer LS-50B spectrometer by using a quartz cuvette with 1-cm excitation path length, between 400 and 600 nm (step, 1 nm), with excitation at 385 nm. Both the excitation and emission bandpass were set to 5 nm.

Stock solutions were prepared by dissolving lyophilized samples in 10 mM potassium phosphate buffer at pH 7.5. The concentrations were determined by amino acid analysis after acid hydrolysis, and spectra were recorded on aliquots diluted in 10 mM potassium phosphate buffer at pH 7.5.

¹H NMR Spectra. ¹H NMR spectra were recorded by using an Omega 500 spectrometer at 500 MHz in D₂O with 0.15 M NaCl at 37°C for 1–2 mM solutions of protein. Lyophilized HAMLET or native or apo α -lactalbumin (15 mg) was dissolved in 500 μ l of D₂O, and the pH was set to 7.0 by using NaOD. Oleic acid (4 mg) was dissolved in 75 μ l of ethanol-d₆, and 10 μ l was added to 500 μ l of D₂O. Apo α -lactalbumin for ¹H NMR was generated by dissolving α -lactalbumin in doubly distilled water containing 10-fold molar excess of EGTA at pH 8.0. The sample was applied to a G-25 gel-filtration column after an aliquot of saturated NaCl (calcium-depleted) and eluted by doubly distilled water. The sample was passed through the saturated NaCl to reduce binding of EGTA to the protein, and EGTA-free protein was eluted in the water.

Anion-Exchange Chromatography. α -Lactalbumin in the native or apo state was subjected to ion-exchange chromatography as described (6). Briefly, the column (14 cm $\times$ 1.6 cm) was packed with DEAE-Trisacryl M (BioSeptra, Villeneuve-la-Garonne, France) attached to a Bio-Logic chromatography system (Bio-Rad) and eluted with a NaCl gradient (buffer A: 10 mM Tris-HCl, pH 8.5; buffer B: buffer A containing 1 M NaCl). The eluted protein fractions were desalted by dialysis (Spectra/Por; Spectrum Medical Industries, Laguna Hills, CA; membrane cut-off, 3.5 kDa) against distilled water, with at least four changes of water, and lyophilized.

Casein Conditioning of the Ion-Exchange Matrix. Casein was isolated from human milk as described (6). The casein (50 mg) was dissolved in 10 ml of 10 mM Tris-HCl (pH 8.5), applied to the column, and run as described. This procedure yielded apoptosis-inducing material (6), and the matrix thereafter was denoted as casein-conditioned. α -Lactalbumin in the native or in the apo state was applied to the casein-conditioned matrix (25 mg dissolved in 10 ml of 10 mM Tris-HCl, pH 8.5, with or without EDTA) and eluted as described above.

Identification of the Cofactor on the Casein-Conditioned Column Matrix. To elute protein cofactors, the casein-conditioned matrix was sequentially washed with 10 mM EDTA, 4 M urea, and 20% ethanol. Eluted protein fractions were analyzed by SDS/PAGE. This procedure yielded residual α -lactalbumin, but no other proteins were detected.

Lipids were eluted from the ion-exchange matrix using organic solvents (10). Casein-conditioned matrix (2 ml) was dissolved in chloroform/methanol/water (1:2:0.8 vol/vol/vol) and incubated at 37°C for 1.5 h. Chloroform/water (1:1 vol/vol) was added, the solution was mixed thoroughly, the two phases were separated overnight, and the organic phase was collected and dried under nitrogen. Lipids were dissolved in chloroform

(3 mg/30 μ l) and 10 μ l was applied in triplicates to silica gel glass plates (1 mg on each lane) and developed by using petroleum ether/diethyl ether/acetic acid/methanol (80:20:1:2 vol/vol/vol/vol). The lipids were visualized by iodine vapor, and defined lipid species were scraped off the plates and eluted from the silica gel with chloroform/methanol (2:1, 1:1, 1:2 vol/vol). The fatty acids were analyzed further on a Varian gas chromatograph (model 3500) equipped with a split/splitless injector and a flame ionization detector. The fatty acids were separated on a fused silica capillary column (0.25-mm inner diameter) (Chrompack, Stockholm). The column temperature was programmed from 140°C to 240°C at 8°C per min. Chromatograms were evaluated by using a Varian integrator model 4290.

Fatty Acid Conditioning of the Ion-Exchange Matrix. Ten milligrams of palmitic acid (16:0), steric acid (18:0), myristic acid (14:0), or oleic acid (18:1) was dissolved in 500 μ l of 99.5% ethanol by sonication (3 min using a Branson 2200 bath sonicator; Branson). After addition of 10 ml of 10 mM Tris-HCl, pH 8.5, the different lipid solutions were applied to four separate, newly packed DEAE-Trisacryl M matrices. Native or apo α -lactalbumin isolated from milk or *E. coli* was applied to each matrix and eluted with NaCl, as described above.

Bioassays of Apoptosis. The L1210 (ATCC, CCL 219), A549 (ATCC, CCL 185), A-498 (ATCC, HTB 44) and Jurkat (European Cell Culture Collection, no. 88042803) cell lines were cultured as described (7). The cells were harvested by centrifugation (200 $\times$ g for 10 min), resuspended in cell culture medium, and seeded into 24-well plates (Falcon/Becton Dickinson) at a density of 2×10^6 per well. The different molecular forms of α -lactalbumin, lipid extracts, or individual fatty acids were dissolved in cell culture medium, without FCS, and added to the cells (final volume, 1 ml per well). Plates were incubated at 37°C in 5% CO₂ atmosphere with addition of 100 μ l of FCS to each well after 30 min. Cell culture medium served as a control. Cell viability and DNA fragmentation were determined after 6 h of incubation, as described (7).

Subcellular Localization Studies. L1210 cells (2×10^6 cells/ml, 490 μ l) were incubated at 25°C with 12 μ l of the biotinylated protein preparations, and surface-bound protein was visualized after counterstaining with FITC-conjugated streptavidin. Intracellular protein was detected after fixation in 4% paraformaldehyde and permeabilization with 0.1% saponin to allow entry of FITC-conjugated streptavidin. Cells were analyzed in Bio-Rad 1024 laser-scanning confocal equipment attached to a Nikon Eclipse E800 microscope.

Results

Apo α -Lactalbumin Can Be Converted to a Folding Variant with Apoptosis-Inducing Activity. Whey-derived or recombinant α -lactalbumin was used as starting material in experiments aiming to convert the inactive, native protein to the active form (Fig. 1B). As a first step, the native proteins were partially unfolded by EDTA treatment, and the conformational change to the apo state was confirmed by UV CD and ANS fluorescence spectroscopy (Fig. 1C and D).

Apo α -lactalbumin then was subjected to ion-exchange chromatography on a column previously exposed to human milk casein. Eluted material was tested for its ability to kill tumor cells and to induce DNA fragmentation and was examined by spectroscopic techniques. Apo α -lactalbumin bound strongly to the ion-exchange matrix and eluted only after 1 M NaCl (Fig. 2A). Eluted protein remained in a partially unfolded conformation as shown by loss of signal in the near-UV CD spectrum and increased binding of ANS (Fig. 2C and D), and it induced apoptosis with DNA fragmentation in tumor cells (Fig. 2E).

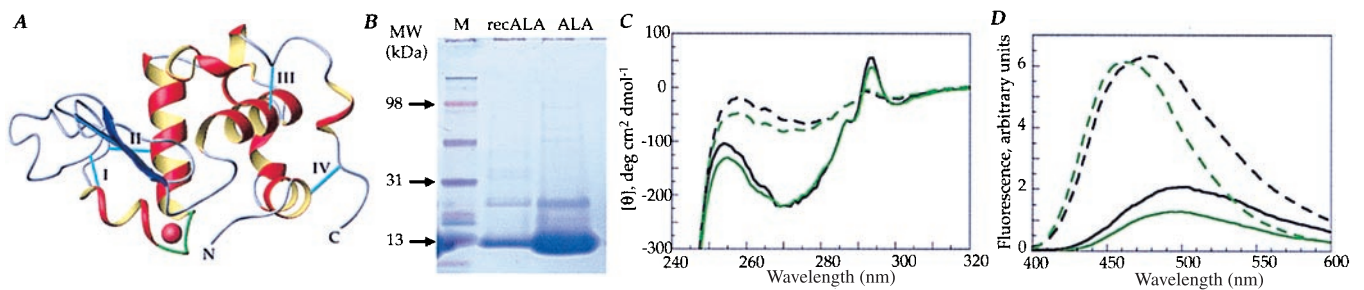


Fig. 1. α -Lactalbumin has been studied extensively as a model of protein-folding intermediates (12–15). On lowering the pH the acidic side chains are protonated and the protein adopts the A state or molten globule state (16–18). A similar partially unfolded state, the apo state, is formed at neutral pH if the Ca^{2+} ion is removed (19, 20). Although these conformations essentially have retained secondary structure, they have fluctuating tertiary structure (18), exposed hydrophobic surfaces, and tryptophan residues accessible to solvent. (A) Three-dimensional structure of native human α -lactalbumin. α -Lactalbumin (14 kDa) is shown with four α -helices (red and yellow, residues 1–34 and 86–123) and an antiparallel β -sheet (blue, residues 38–82). The high-affinity Ca^{2+} -binding site (green) is coordinated by the side chain carboxylates of Asp-82, Asp-87, and Asp-88, the carbonyl oxygens of Lys-79 and Asp-84, and two water molecules. Four disulfide bonds (cyan) are indicated with roman numerals: I, 61–77; II, 73–91; III, 28–111; and IV, 6–120. Crystal structure coordinates are from Acharya *et al.* (11), and the structure was created with MOLMOL 2.6.1 (21). (B) SDS/PAGE of whey-derived and recombinant α -lactalbumin; SDS/PAGE on 4–20% polyacrylamide precast gels in a Bio-Rad Mini Protean II cell. Lanes: 1, molecular mass standard (Multimark Multicolored Standard; NOVEX, San Diego); 2, recombinant α -lactalbumin (14 and 30 kDa); 3, whey-derived α -lactalbumin (14 and 30 kDa). (C) Near-UV CD spectra of α -lactalbumin. Native whey-derived (solid, black line) or recombinant (solid, green line) α -lactalbumin showed the characteristic 270-nm tryptophan maximum and the 294-nm tyrosine minimum. The EDTA-treated whey-derived (dashed, black line) and recombinant (dashed, green line) apo α -lactalbumin controls showed the characteristic loss of signal in the tyrosine and tryptophan region. (D) ANS fluorescence spectra of α -lactalbumin. Whey-derived (solid, black line) or recombinant (solid, green line) α -lactalbumin in the native state did not bind ANS, but after EDTA treatment (whey: dashed, black line; recombinant: dashed, green line) ANS binding increased and the intensity maximum shifted to 480 nm.

α -Lactalbumin in the native conformation was used as a negative control. It could not be activated by these procedures (Fig. 2).

Does Conversion Require a Cofactor? The conversion of apo α -lactalbumin to the apoptosis-inducing form involved a cofactor from

casein. This was first suspected when conversion could not be achieved on a clean column matrix (Fig. 2B). Furthermore, the eluted protein differed from the previously described partially denatured form of α -lactalbumin as Ca^{2+} -depleted or acid-denatured protein revert to the native state when solvent con-

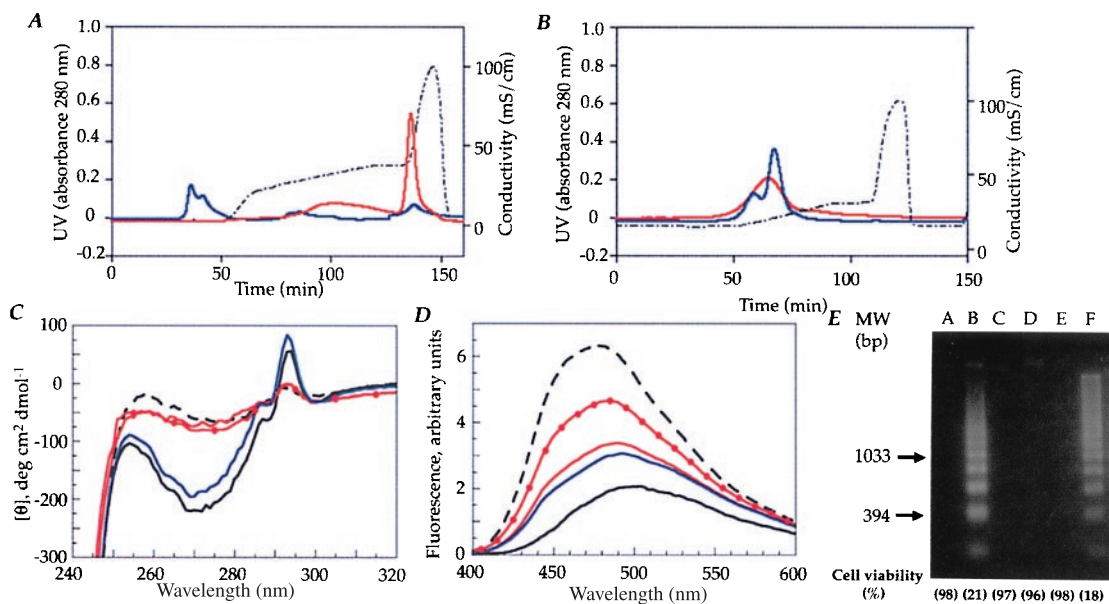


Fig. 2. Conversion of α -lactalbumin to the apoptosis-inducing form requires partial unfolding of the protein and the presence of a cofactor. (A) Apo α -lactalbumin was subjected to ion-exchange chromatography on a column previously exposed to human milk casein. More than 95% of the protein was retained on the column and eluted with 1 M NaCl (solid, red line). Native α -lactalbumin was not retained (solid, blue line). The NaCl gradient is shown by the dotted line. (B) Apo α -lactalbumin (solid, red line) and native α -lactalbumin (solid, blue line) eluted in the void volume when subjected to ion-exchange chromatography on a clean column. (C) CD spectra of the apo α -lactalbumin eluate (solid, red line) from the casein-conditioned matrix did not differ from the apo α -lactalbumin control (dashed, black line). The active fraction from casein was used as a control (solid, red line with solid circle). Native α -lactalbumin had similar spectra before (solid, black line) and after (solid, blue line) elution from the column. (D) ANS spectra of the apo α -lactalbumin eluate (solid, red line) from the casein-conditioned matrix did not differ from the apo α -lactalbumin control (dashed, black line). The native control did not bind ANS before (solid, black line) or after elution from the casein-conditioned matrix (solid, blue line). The active fraction from casein was used as a control (solid, red line with solid circle). (E) Loss of viability and DNA fragmentation of L1210 cells. Lanes: A, cell culture medium; B, the active fraction from human milk casein (0.2 mg/ml); C, native α -lactalbumin (1.0 mg/ml); D, void peak vol from the clean matrix (1.0 mg/ml); E, native α -lactalbumin eluate from the casein-conditioned matrix (1.0 mg/ml); and F, apo α -lactalbumin eluate from the casein-conditioned matrix (0.2 mg/ml). The proteins in lanes B and F were active.

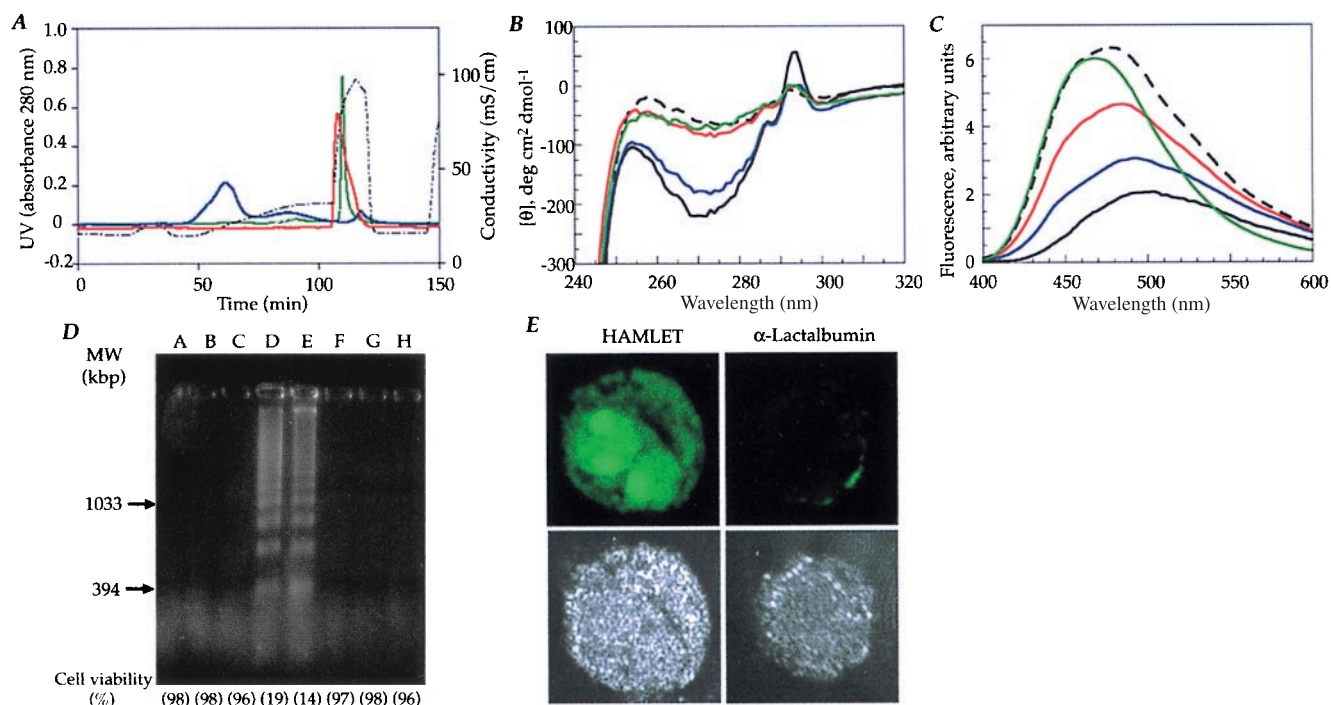


Fig. 3. C18:1 is the fatty acid needed to convert apo α -lactalbumin to the apoptosis-inducing form. (A) Whey-derived or recombinant α -lactalbumin was subjected to ion-exchange chromatography by using a matrix preconditioned with C18:1 fatty acid. Whey-derived α -lactalbumin was added to the column in its native (solid, blue line) or apo state (solid, red line). The apo α -lactalbumin bound to the C18:1-conditioned matrix and eluted as a sharp peak after 1 M NaCl. Native α -lactalbumin bound poorly to the matrix, with $>50\%$ in the void. Recombinant apo α -lactalbumin bound to the C18:1-conditioned matrix (solid, green line) and eluted after 1 M NaCl. (B) Near-UV CD spectra of proteins eluting from the C18:1-conditioned matrix. The spectrum of HAMLET (solid, red line) and recombinant HAMLET (solid, green line) strongly resembled the apo α -lactalbumin control (dashed, black line). Native α -lactalbumin before (solid, black line) and after (solid, blue line) passage over the column had native properties. (C) ANS fluorescence spectra of material eluted from the C18:1-conditioned column. HAMLET (solid, red line) and recombinant HAMLET (solid, green line) resembled the apo α -lactalbumin control (dashed, black line). The native α -lactalbumin eluate off the C18:1-conditioned column (solid, blue line) and the native α -lactalbumin control (solid, black line) showed low ANS binding. (D) DNA fragmentation and loss of cell viability in L1210 cells. Lanes: A, cell culture medium; B, whey-derived native α -lactalbumin (1.0 mg/ml); C, recombinant, native α -lactalbumin (1.0 mg/ml); D, HAMLET (0.2 mg/ml); E, recombinant HAMLET (0.2 mg/ml); F, native α -lactalbumin eluate off a C18:1-conditioned column (5 mg/ml); G, lipids extracted from casein-conditioned matrix (0.05 mg/ml); and H, 18:1 fatty acid (0.025 mg/ml). Material in lanes D and E induced apoptosis. (E) Subcellular distribution of HAMLET in L1210 cells. Cell surface binding of HAMLET was detected after 30 min, followed by translocation into the cytoplasm and accumulation in the cell nuclei (Upper). Native α -lactalbumin bound weakly to the cell surface and did not enter the cells (Upper). The cellular outline is shown in blue reflection mode (Lower).

ditions are brought back to normal, but the converted protein was preserved in a partially unfolded state even at neutral pH and in the presence of Ca^{2+} . We also observed that the conversion efficiency of the casein-conditioned column gradually decreased with repeated runs. Thus, a cofactor from casein appeared to be required to maintain the protein in its altered state with apoptosis-inducing activity.

Identification of the Cofactor. The cofactor was identified by chemical extraction of the casein-conditioned matrix under conditions suitable for proteins or lipids. Solvents known to elute proteins (1 M NaCl, 10 mM EDTA, 4 M urea, or 20% ethanol) released only residual α -lactalbumin; no other proteins were detected. Organic solvents (chloroform, methanol), on the other hand, released lipids from the column matrix. Individual lipid species were identified by GC/MS as C18:1, C16:0, and C14:0 fatty acids (data not shown).

New column matrices then were conditioned with each of the fatty acids or with C18:0 and exposed to whey-derived or recombinant α -lactalbumin in the native or apo states. Fractions eluting after 1 M NaCl were tested for apoptosis-inducing activity. α -Lactalbumin from human milk whey and recombinant protein was shown to convert to the active complex only on the C18:1 fatty acid-preconditioned column and only when applied in the apo form. The proteins were retained on the C18:1-

conditioned column, and each eluted as a sharp peak after 1 M NaCl, with a yield of about 90% (Fig. 3A). By near-UV CD and ANS spectroscopy (Fig. 3B and C), the whey-derived and recombinant activated complexes strongly resembled the apo α -lactalbumin control, and both complexes induced apoptosis in L1210 cells (Fig. 3D). The C18:1-converted apo α -lactalbumin from human milk whey was named HAMLET, and the converted recombinant apo α -lactalbumin was named recombinant HAMLET.

Conditioning of the ion-exchange matrix with the other fatty acids (C18:0, C16:0, and C14:0) did not convert apo α -lactalbumin to HAMLET, and native α -lactalbumin could not be activated on the C18:1-conditioned column. Most of the material eluted in the void volume with a small peak after 1 M NaCl that showed native three-dimensional structure and lacked apoptosis-inducing activity (Fig. 3D). These results identified the C18:1 fatty acid as the responsible cofactor required to convert α -lactalbumin to HAMLET.

There was no direct cellular effect of the C18:1 fatty acid or of lipid extracts from the casein-conditioned matrix at concentrations found in HAMLET (Fig. 3D). Furthermore, simple mixing experiments with C18:1 fatty acid and apo α -lactalbumin showed that the mixtures had lower activity than HAMLET at similar protein and lipid concentrations.

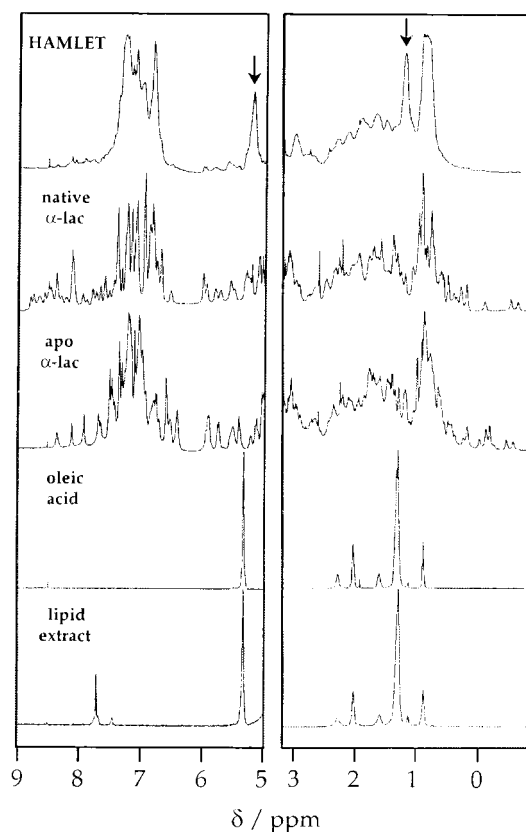


Fig. 4. ^1H NMR spectra of HAMLET, native α -lactalbumin, apo α -lactalbumin, oleic acid, and a lipid extract from HAMLET. The aromatic and methylated regions are shown *Left* and *Right*, respectively. Virtually identical spectra were obtained for whey-derived and recombinant α -lactalbumin. The broad lines and lack of out-shifted methyl signals suggest that HAMLET is in a partially unfolded state that is significantly different from the native form of the protein, which displays narrow lines and a large shift dispersion. Furthermore, signals arising from oleic acid are much broader in the HAMLET spectrum (arrows) than in the oleic acid spectrum.

^1H NMR Spectroscopy. ^1H NMR spectra of the native α -lactalbumins were characteristic of folded and well-ordered proteins with narrow lines and significant shift dispersion, a large number of sharp signals in the aromatic region (around 7 ppm), and several out-shifted methyl signals (between 0.7 and -0.6 ppm) (Fig. 4). The spectra of the apoproteins displayed narrow lines and significant shift dispersion, with significant variations relative to the native state in the chemical shifts of a large number of resonances (Fig. 4).

There were marked differences between HAMLET and native α -lactalbumin. The spectrum of HAMLET showed broader lines and little shift dispersion. The lines in the aromatic region were more clustered, with no outshifted methyl signals below 0.7 ppm (Fig. 4). The spectrum of HAMLET did not change between low and physiological salt concentrations. Despite the similarities seen by optical spectroscopy, there were, by NMR, distinct spectral differences between HAMLET and the apo form of α -lactalbumin, suggesting additional conformational changes because of the interaction with the lipid cofactor. Although C18:1 fatty acid was detected in the spectrum of HAMLET (as indicated by arrows), the signals were much broader compared with the signals of free oleic acid, suggesting that the fatty acid has become an integral part of HAMLET.

HAMLET Induces Apoptosis in Tumor Cell Lines and Targets Cell Nuclei. When isolated from human milk casein the α -lactalbumin folding variant has broad activity against transformed cells of both

human and animal origin (7). HAMLET was shown to induce apoptosis in several human and murine tumor cell lines, including the Jurkat and L1210 leukemia cell lines, the A549 lung carcinoma line, and the A-498 kidney carcinoma line. Native α -lactalbumin had no effects on these cells.

The apoptosis-inducing activity of the converted fractions was compared by using the L1210 mouse leukemia cell line. The loss of cell viability and induction of DNA fragmentation were used as end points (Figs. 2*E* and 3*D*). The L1210 cells died rapidly when exposed to whey-derived or recombinant HAMLET (200 $\mu\text{g}/\text{ml}$), and DNA fragmentation was induced. The L1210 cells survived exposure to native α -lactalbumin both before and after the protein had been eluted from the ion-exchange column.

By confocal microscopy we observed striking differences in subcellular localization and nuclear uptake depending on the folding state of α -lactalbumin. Whey-derived and recombinant HAMLET bound to the cell surface, passed through the cytoplasm to the nucleus, and accumulated in the cell nucleus (Fig. 3*E*). Native α -lactalbumin bound weakly to the cell surface but was not seen to translocate into the cytoplasm or to reach the cell nuclei (Fig. 3*E*).

Discussion

The present study demonstrated that α -lactalbumin can alter its biological function depending on the conformational state. We also showed that a specific fatty acid, C18:1, was a necessary cofactor. We thus have identified the folding change and the fatty acid as the two key elements that define HAMLET, the apoptosis-inducing functional state of α -lactalbumin.

The conversion to HAMLET was achieved first by changing the conformation of α -lactalbumin from the native to a partially unfolded state. EDTA treatment was chosen because it releases calcium and opens up the three-dimensional structure of the protein, and the conformational change was confirmed by spectroscopic techniques. By near-UV CD spectroscopy native α -lactalbumin had a minimum at 270 nm arising from tyrosine residues and a maximum at 294 nm arising from tryptophan residues. The EDTA-treated protein showed the characteristic loss of signal, indicating partial unfolding with less restrained tyrosines and tryptophans. Removal of Ca^{2+} made the protein more hydrophobic as probed in ANS fluorescence spectroscopy, and HAMLET had increased ANS fluorescence intensity and a shift to a shorter wavelength compared with the native protein.

The second requirement for the stable conversion of native α -lactalbumin was the presence of the C18:1 fatty acid on the ion-exchange matrix. In the absence of this cofactor, the apo form of the protein was unstable and reverted to the native, inactive form at neutral pH and in the presence of Ca^{2+} . The cofactor initially was identified by GC/MS in a lipid extract from the spent ion-exchange matrix. Several fatty acids present in casein eluted as part of the active complex, but only the unsaturated form of the C:18 fatty acid was able to precondition the column matrix for conversion of apo α -lactalbumin to HAMLET. Other structurally related fatty acids like the C18:0, the C16:0, and the C14:0 fatty acids were inactive, suggesting that only the unsaturated C18:1 could act as the necessary cofactor. It may be speculated that the increased hydrophobicity of the apo α -lactalbumin-folding variant enhances binding of C18:1 to exposed hydrophobic regions of the molecule, but the molecular details of these interactions remain to be defined.

α -Lactalbumin is the most abundant protein in human milk (22). The nursing child ingests around 2 g of α -lactalbumin, which travels from the mammary gland through the gastrointestinal tract of the baby. The environmental conditions in the mammary gland favor the native state of the protein with the tightly bound Ca^{2+} ion, and α -lactalbumin functions as a specifier protein in lactose synthesis (23). Through lactose, the water

content of milk is controlled. This is crucial for successful breast-feeding, and it has been shown that α -lactalbumin knock-out mice produce extremely viscous milk and fail to nurse their offspring (24).

Quite different environmental conditions meet α -lactalbumin in the stomach of the breast-fed child. The low pH is known to favor the release of Ca^{2+} (16–18), and casein is precipitated. The acid lipase hydrolyses triglycerides, and fatty acids are released (25, 26). By chance, we happened to purify the apoptosis-inducing form of α -lactalbumin from human milk after precipitation of casein at low pH, thus mimicking the conditions in the stomach of the breast-fed child (7, 27). When these conditions were reproduced *in vitro*, α -lactalbumin was shown to change to the partially unfolded state and to form HAMLET in complex with the obligatory C18:1 fatty acid.

The purification of proteins from biological fluids has a long history of uncertainty because of the potential for contamination. We used two approaches to exclude that the activity of our complex was due to some contaminating component other than the predominant protein. First, we used α -lactalbumin from human milk whey rather than from casein, showed that it was inactive, and then activated it by partial unfolding and exposure to oleic acid. Second, we expressed the native protein in *E. coli* and showed that the product was inactive in the native state and that it could be converted equally to the active form through the same procedures. Thus, we feel confident that contamination with other biologically active milk ingredients does not explain our results.

There are obvious parallels between α -lactalbumin and the prion system. Both proteins have multiple activities depending on their folding state, and both require a cofactor for the functional transition. The prion protein first changes to a molten globule-like state and then proceeds to a nonreversible β -sheet-rich form (4, 5), which is the disease-causing isoform. Like the prion protein, α -lactalbumin changes its fold to a molten globule-like state but stays in this intermediate form rather than altering its secondary structure to a β -sheet-rich state. The prion protein

has been postulated to require a second protein called “protein X” for its functional switch, but despite many attempts, this cofactor has not been identified (28, 29). In the α -lactalbumin system, we demonstrate that a specific fatty acid can promote switching and/or stabilize the active complex. In the α -lactalbumin system the cofactor was not a protein but a C18:1 fatty acid.

Breast-feeding has been proposed to protect both mother and child against cancer. The overall incidence of childhood cancer is reduced in breast-fed children, with an especially strong epidemiological association for lymphomas (crude odds ratio, 8.9) (30). These differences imply that human milk furnishes the baby with molecules that have a beneficial effect on tissue development and homeostasis, but the mechanisms have not been investigated. It may be speculated that molecules such as HAMLET can have a protective function and that HAMLET is one of several naturally occurring surveillance molecules that purge unwanted cells from the local tissues, thereby driving the intestinal mucosa toward maturity. By inducing apoptosis, HAMLET may reduce the pool of potentially malignant cells that could serve as nuclei for future tumor development and explain the reduced frequency of cancer in breast-fed individuals. Further studies are required to understand the *in vivo* relevance of these findings.

Taken together, these observations suggest that proteins may respond to different environments by changing their fold and that this process allows a single polypeptide chain to exert vastly different and beneficial biologic functions in different tissue compartments.

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- Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Hawkins, P. N., Dobson, C. M., Radford, S. E., Blake, C. C., et al. (1997) *Nature (London)* **385**, 787–793.
- Herrmann, J. M., Malkus, P., & Schekman, R. (1999) *Trends Cell Biol.* **9**, 5–7.
- Martin, J., & Hartl, F. U. (1997) *Curr. Opin. Struct. Biol.* **7**, 41–52.
- Safar, J., Roller, P., Gajdusek, D., & Gibbs, C. J. (1993) *J. Biol. Chem.* **268**, 20276–20284.
- Safar, J., Roller, P., Gajdusek, D., & Gibbs, C. J. (1994) *Biochemistry* **33**, 8375–8383.
- Svensson, M., Sabharwal, H., Hakansson, A., Mossberg, A. K., Lipniunas, P., Leffler, H., Svanborg, C., & Linse, S. (1999) *J. Biol. Chem.* **274**, 6388–6396.
- Håkansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., & Svanborg, C. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 8064–8068.
- Lindahl, L., & Vogel, H. J. (1984) *Anal. Biochem.* **140**, 394–402.
- Chaudhuri, T. K., Horii, K., Yoda, T., Arai, M., Nagata, S., Terada, T. P., Uchiyama, H., Ikura, T., Tsumoto, K., Kataoka, H., et al. (1999) *J. Mol. Biol.* **285**, 1179–1194.
- Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–916.
- Acharya, K. R., Ren, J. S., Stuart, D. I., Phillips, D. C., & Fenna, R. E. (1991) *J. Mol. Biol.* **221**, 571–581.
- Kuwajima, K. (1989) *Proteins* **6**, 87–103.
- Dobson, C. M. (1991) *CIBA Found. Symp.* **161**, 167–181.
- Wu, L. C., & Kim, P. S. (1998) *J. Mol. Biol.* **280**, 175–182.
- Kataoka, M., Kuwajima, K., Tokunaga, F., & Goto, Y. (1997) *Protein Sci.* **6**, 422–430.
- Wu, L. C., Schulman, B. A., Peng, Z. Y., & Kim, P. S. (1996) *Biochemistry* **35**, 859–863.
- Schulman, B. A., Redfield, C., Peng, Z. Y., Dobson, C. M., & Kim, P. S. (1995) *J. Mol. Biol.* **253**, 651–657.
- Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S., & Ptitsyn, O. B. (1981) *FEBS Lett.* **136**, 311–315.
- Pfeil, W. (1987) *Biochim. Biophys. Acta* **911**, 114–116.
- Kuwajima, K. (1996) *FASEB J.* **10**, 102–109.
- Koradi, R., Billeter, M., & Wuthrich, K. (1996) *J. Mol. Graph.* **14**, 29–32, 51–55.
- Heine, W. E., Klein, P. D., & Reeds, P. J. (1991) *J. Nutr.* **121**, 277–283.
- Brodbeck, U., & Ebner, K. E. (1966) *J. Biol. Chem.* **241**, 762–764.
- Stinnakre, M. G., Vilotte, J. L., Soulier, S., & Mercier, J. C. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 6544–6548.
- Sarles, J., Moreau, H., & Verger, R. (1992) *Acta. Paediatr. Scandinavica* **81**, 511–513.
- Bernback, S., Blackberg, L., & Hernell, O. (1990) *J. Clin. Invest.* **85**, 1221–1226.
- Maffei, H. V., & Nobrega, F. J. (1975) *Gut* **16**, 719–726.
- Kaneko, K., Zulianello, L., Scott, M., Cooper, C. M., Wallace, A. C., James, T. L., Cohen, F. E., & Prusiner, S. B. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 10069–10074.
- Telling, G. C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F. E., DeArmond, S. J., & Prusiner, S. B. (1995) *Cell* **83**, 79–90.
- Davis, M. K., Savitz, D. A., & Graubard, B. I. (1988) *Lancet* **2**, 365–368.

A folding variant of human α -lactalbumin induces mitochondrial permeability transition in isolated mitochondria

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A human milk fraction containing multimeric α -lactalbumin (MAL) is able to kill cells via apoptosis. MAL is a protein complex of a folding variant of α -lactalbumin and lipids. Previous results have shown that upon treatment of transformed cells, MAL localizes to the mitochondria and cytochrome *c* is released into the cytosol. This is followed by activation of the caspase cascade. In this study, we further investigated the involvement of mitochondria in apoptosis induced by the folding variant of α -lactalbumin. Addition of MAL to isolated rat liver mitochondria induced a loss of the mitochondrial membrane potential ($\Delta\Psi_m$), mitochondrial swelling and the release of cytochrome *c*. These changes were Ca^{2+} -dependent and were prevented by cyclosporin A, an inhibitor of mitochondrial permeability transition. MAL also increased the rate of state 4 respiration in isolated mitochondria by exerting an uncoupling effect. This effect was due to the presence of fatty acids in the MAL complex because it was abolished completely by BSA. BSA delayed, but failed to prevent, mitochondrial swelling as well as dissipation of $\Delta\Psi_m$, indicating that the fatty acid content of MAL facilitated, rather than caused, these effects. Similar results were obtained with HAMLET (human α -lactalbumin made lethal to tumour cells), which is native α -lactalbumin converted *in vitro* to the apoptosis-inducing folding variant of the protein in complex with oleic acid. Our findings demonstrate that a folding variant of α -lactalbumin induces mitochondrial permeability transition with subsequent cytochrome *c* release, which in transformed cells may lead to activation of the caspase cascade and apoptotic death.

Keywords: α -lactalbumin; cytochrome *c*; mitochondria; mitochondrial permeability transition; tumour cells.

Diverse physiological and pathological stimuli can induce apoptosis by distinct pathways that converge in a common programme of cell suicide. Upon apoptotic triggering, a unique family of cysteine aspartate proteases, caspases, becomes activated [1]. In many models of apoptosis, caspase activation requires the release of apoptogenic factors, such as cytochrome *c*, from the mitochondrial intermembrane space. Once released into the cytosol, cytochrome *c* binds to Apaf-1, and together with pro-caspase-9 and dATP, forms the so-called apoptosome complex [2]. This association leads to the activation of pro-caspase-9 which, in turn, initiates the caspase cascade by activating pro-caspase-3. Although cytochrome *c* release has been observed in many experimental models of

apoptosis, the precise mechanism responsible for its movement across the outer mitochondrial membrane remains unclear. Different hypotheses have been proposed [3]. Cytochrome *c* may be released via specific channels in the outer mitochondrial membrane or the release may be a consequence of mitochondrial swelling leading to the rupture of the outer mitochondrial membrane. Mitochondrial swelling may be due to opening of the mitochondrial permeability transition (MPT) pore, a polyprotein complex formed at the contact sites between the inner and the outer mitochondrial membranes, resulting in loss of the mitochondrial transmembrane potential, $\Delta\Psi_m$ [4]. In several models of apoptosis, MPT pore inhibitors, such as cyclosporin A (CsA), prevent dissipation of $\Delta\Psi_m$ and concomitantly prevent apoptosis [5].

We have previously shown that multimeric α -lactalbumin (MAL), a human milk fraction containing a protein complex of α -lactalbumin and lipids [6], induces apoptosis in tumour cells and immature cells, but spares differentiated nontransformed cells [7]. However, the exact mechanisms of apoptosis induction by MAL are unknown. Previous data have shown that release of cytochrome *c* and activation of the caspase cascade are early events during MAL-induced killing [8]. Co-localization of MAL with mitochondria suggested that MAL may induce apoptosis via a direct interaction with mitochondria, which leads to the release of cytochrome *c* into the cytosol. The apoptosis-inducing component of MAL is believed to be a folding variant of α -lactalbumin because native α -lactalbumin does not induce apoptosis and the α -lactalbumin in MAL is present in the so-called molten globule-like state [6].

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Abbreviations: AK-2, adenylate kinase-2; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; HAMLET, human α -lactalbumin made lethal to tumour cells; $\Delta\Psi_m$, mitochondrial membrane potential; MAL, multimeric α -lactalbumin; MPT, mitochondrial permeability transition; RuRed, ruthenium red; TMRE, tetramethylrhodamine ethyl ester; TPP^+ , tetraphenylphosphonium; Ub0, ubiquinone 0.

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To further support this, we recently showed that HAMLET (human α -lactalbumin made lethal to tumour cells), which is native α -lactalbumin converted *in vitro* into the molten globule-like state, was as effective as MAL at inducing apoptosis [9]. Both MAL and HAMLET contain fatty acids, which are believed to be important for the stabilization of the molten globule-like folding state of the protein. In order to investigate the role of mitochondria in apoptosis triggered by this folding variant of α -lactalbumin, the effects of MAL and HAMLET were examined using isolated rat liver mitochondria as a model system. Here we show that MAL, as well as HAMLET, induces MPT in isolated mitochondria resulting in a loss of $\Delta\Psi_m$, mitochondrial swelling and release of proteins from the mitochondrial intermembrane space. We have also shown that the fatty acids in the α -lactalbumin complexes facilitated MPT induction by mild uncoupling of mitochondria.

MATERIALS AND METHODS

Preparation of mitochondria

Liver mitochondria were isolated from male Wistar rats (150–200 g) by differential centrifugation in buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM Hepes and 1 mM EDTA, pH 7.4 (MSH) [10]. The mitochondrial fraction was washed and resuspended (90–100 mg protein·mL⁻¹) in MSH buffer without EDTA. The protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL, USA) with BSA as a standard.

Mitochondrial swelling

Swelling of isolated rat liver mitochondria was estimated spectrophotometrically as a decrease in absorbance measured at 540 nm [11]. Mitochondria (0.5 mg protein·mL⁻¹) were suspended in buffer A (0.25 M sucrose, 10 mM Tris/Mops, 2.5 mM KH₂PO₄, 5 mM succinate and 2 μ M rotenone, pH 7.4) and pre-incubated in a thermostated cuvette (30 °C) in the presence of 20 nmoles of Ca²⁺ per mg of protein. Mitochondrial swelling was triggered by different concentrations of MAL or HAMLET. Mitochondrial swelling was also assessed in the presence of inhibitors [1 μ M CsA, 50 μ M ubiquinone 0 (Ub0), 10 μ M ruthenium red (RuRed)] as well as in the presence of 50 μ M EGTA or 0.05% BSA.

Release of intermembrane proteins from isolated mitochondria

Mitochondrial swelling was induced as described above and 5 min later mitochondria were pelleted by centrifugation (10 000 g 15 min). The post-mitochondrial supernatants were concentrated $\approx$ 10-fold using a vacuum lyophilizer, diluted with 4 $\times$ Laemmli's loading buffer without glycerol and subjected to Western blot analysis. Briefly, samples were boiled for 4 min and the proteins were separated by electrophoresis on 15% SDS/polyacrylamide gels. The proteins were then electroblotted onto a nitrocellulose membrane, 0.45 μ m (Sartorius AG, Göttingen, Germany), blocked overnight in a high-salt buffer (50 mM Tris/base, 500 mM NaCl, pH 7.4) containing 5% dried milk and 1% BSA, and probed with antibodies against cytochrome *c* (1 : 2500) or adenylate kinase-2 (1 : 2500). Primary antibody binding was detected using secondary goat antimouse (1 : 10 000) or a goat antirabbit (1 : 10 000) conjugated with horseradish peroxidase and visualized by enhanced chemiluminescence as described in

the manufacturer's instructions (Amersham, Buckinghamshire, UK).

Loss of $\Delta\Psi_m$ in isolated mitochondria

Estimation of $\Delta\Psi_m$ in isolated mitochondria was performed using an electrode sensitive to the cation tetraphenylphosphonium (TPP⁺). Energized mitochondria rapidly accumulate TPP⁺ from the incubation buffer compatible with the formation of a membrane potential. Mitochondria (1.5 mg protein·mL⁻¹) were incubated at 25 °C in 2 mL of a buffer containing 125 mM KCl, 2.5 mM KH₂PO₄, 5 mM Tris, 5 mM succinate and 2.5 μ M TPP⁺, pH 7.5. Rotenone (2 μ M) was added immediately after the addition of mitochondria to the incubation medium. Mitochondria were loaded with 40 nmoles of Ca²⁺ per mg of protein and the loss of $\Delta\Psi_m$ was monitored upon addition of MAL or HAMLET in the absence or presence of 0.05% BSA, 1 μ M CsA or 500 μ M ATP.

Mitochondrial respiration

Oxygen consumption in isolated rat liver mitochondria was measured using a Clark-type oxygen electrode (Yellow Spring Instrument Co., OH, USA) at 25 °C. The incubations contained 1.5 mg protein·mL⁻¹ in a buffer consisting of 125 mM KCl, 5 mM Tris, 2.5 mM KH₂PO₄, pH 7.4. Mitochondria were energized with 5 mM succinate in the presence of 5 μ M rotenone. State 3 respiration was then initiated by the addition of 500 nmol ADP. The rate of uncoupled respiration was determined after addition of 3 μ M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to the incubation mixture. The effect of different concentrations of MAL and oleic acid (dissolved in ethanol) on oxygen consumption was determined in the absence or presence of 0.05% BSA. Mitochondria with a respiratory control ratio (defined as the rate of respiration in the presence of ADP divided by the rate obtained following the expenditure of ADP) > 4 were used in the experiments.

RESULTS

MAL-induced swelling of Ca²⁺-loaded rat liver mitochondria and release of intermembrane space proteins

Within minutes of adding MAL to Ca²⁺-loaded mitochondria, large amplitude swelling was induced that was concentration dependent (Fig. 1A). In our experiments, the concentration of Ca²⁺ was not high enough to induce swelling in the absence of MAL. However, the presence of Ca²⁺ was obligatory, because EGTA, a calcium chelator, and RuRed, an inhibitor of mitochondrial Ca²⁺ uptake, prevented MAL-induced swelling (Fig. 1B). Inhibitors of the MPT pore, such as CsA and Ub0 [12], also completely prevented the swelling of mitochondria (Fig. 1B), indicating that MAL-induced swelling was associated with MPT induction.

Mitochondrial swelling was accompanied by the release of cytochrome *c* and adenylate kinase-2 (AK-2) from the mitochondrial intermembrane space (Fig. 1C). This release was inhibited by CsA, Ub0, EGTA and RuRed, suggesting that MPT was required for the release of these proteins from the mitochondria.

Loss of $\Delta\Psi_m$ induced by MAL in isolated mitochondria

Addition of MAL to Ca²⁺-loaded mitochondria caused a biphasic decrease in $\Delta\Psi_m$ (Fig. 1D). The first rapid phase was

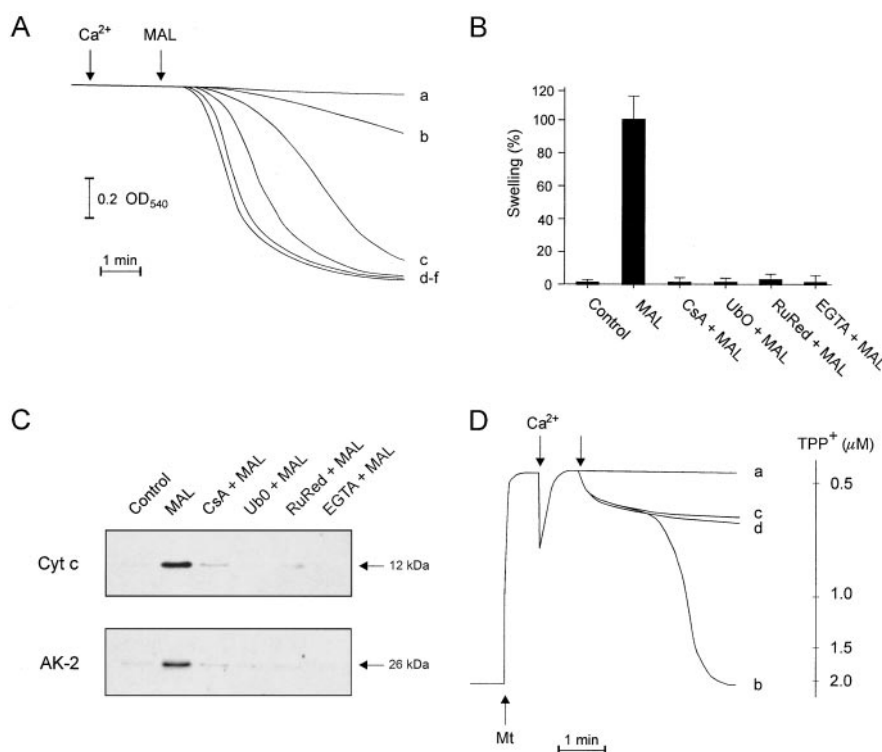


Fig. 1. MAL induces swelling of isolated rat liver mitochondria (A and B), release of cytochrome *c* and adenylate kinase-2 from the intermembrane space (C), and a decrease in mitochondrial membrane potential ($\Delta\Psi_m$) (D). (A) Mitochondria ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) were pre-incubated with 20 nmoles of Ca^{2+} per mg of mitochondrial protein at 30°C . Swelling of mitochondria was induced by addition of different concentrations of MAL and monitored continuously as changes in A_{540} . Curve a: control; curves b–f: 2.5, 5, 10, 20, 40 $\mu\text{g}\cdot\text{mg protein}^{-1}$, respectively. (B) Mitochondrial swelling was induced by MAL ($10 \mu\text{g}\cdot\text{mg protein}^{-1}$), as described in (A), in the absence or presence of 1 μM CsA, 50 μM UbO, 10 μM RuRed or 50 μM EGTA. The swelling is expressed in per cent, where the change in absorbance induced by MAL represents 100%. (C) Mitochondria ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) were loaded with 20 nmoles of Ca^{2+} per mg of protein at 30°C . Untreated or MAL-treated ($10 \mu\text{g}\cdot\text{mg protein}^{-1}$) mitochondria were incubated for 5 min in the absence or presence of 10 μM CsA, 50 μM UbO, 10 μM RuRed or 50 μM EGTA. After incubation, mitochondria were pelleted and the supernatants were subjected to Western blot analysis as described in Materials and methods. The membranes were probed with antibodies against cytochrome *c* and AK-2. (D) MAL-induced loss of $\Delta\Psi_m$ in isolated mitochondria estimated using a TPP^+ -sensitive electrode. Mitochondria ($1.5 \text{ mg}\cdot\text{mL}^{-1}$) were incubated as described in Materials and methods. TPP^+ , 2.5 μM ; Ca^{2+} , 40 nmoles $\cdot\text{mg protein}^{-1}$; MAL, 40 $\mu\text{g}\cdot\text{mg protein}^{-1}$. Curve a: control; curve b: MAL; curves c, d, MAL and 1 μM CsA or 500 μM ATP, respectively.

followed by a larger, time-dependent second phase of membrane depolarization that resulted in a complete collapse of $\Delta\Psi_m$. Inhibitors of MPT, such as CsA and ATP, blocked the second phase of the decrease in $\Delta\Psi_m$, but failed to prevent the initial shift. The first phase of the decrease in $\Delta\Psi_m$ was also observed when mitochondria were incubated with MAL in the absence of Ca^{2+} (data not shown). These data suggested that, in contrast to the second phase, the first decrease in $\Delta\Psi_m$ was not a consequence of MPT.

Stimulation of state 4 respiration induced by MAL

In the experiments described above, $\Delta\Psi_m$ was supported by the mitochondrial respiratory chain via oxidation of succinate. The CsA-insensitive decrease in $\Delta\Psi_m$ upon addition of MAL could be due to either inhibition of respiration or uncoupling of mitochondria. In order to investigate this, mitochondrial oxygen consumption was measured. Addition of MAL to mitochondria accelerated state 4 respiration in a concentration-dependent manner (Fig. 2A). This resulted in a decrease in the respiration control ratio, which was more prominent at higher concentrations of MAL. An increase in the rate of state 4 respiration indicated that MAL enhanced the proton permeability of the inner mitochondrial membrane and thereby induced mild

uncoupling of mitochondria. MAL caused only a slight decrease in the rate of respiration in the presence of ADP (state 3) or the protonophore CCCP, suggesting that neither mitochondrial ATPase nor the components of the respiratory chain were affected. Acceleration of respiration was not a consequence of MPT, because it occurred in the absence of Ca^{2+} and was not prevented by CsA (data not shown).

The involvement of fatty acids in MAL-induced effects on respiration, loss of $\Delta\Psi_m$ and mitochondrial swelling

Mild uncoupling of mitochondria may be due to the fatty acid content of MAL. Lipid extraction of MAL yielded $\approx 5\text{--}10\%$ (w/w) lipids (unpublished data). Oleic acid (18 : 1) is the most abundant fatty acid in human milk [13] and also the major fatty acid in MAL. Pre-incubation of mitochondria with BSA, which sequesters fatty acids, prevented the increase in state 4 respiration as well as the decrease in the respiration control ratio induced by MAL (Fig. 2B). Addition of the corresponding amount of oleic acid to mitochondria induced a BSA-inhibitable increase in state 4 respiration and a decrease in the respiration control ratio (Fig. 2B). These results indicated that the uncoupling of respiration was due to fatty acids present in MAL.

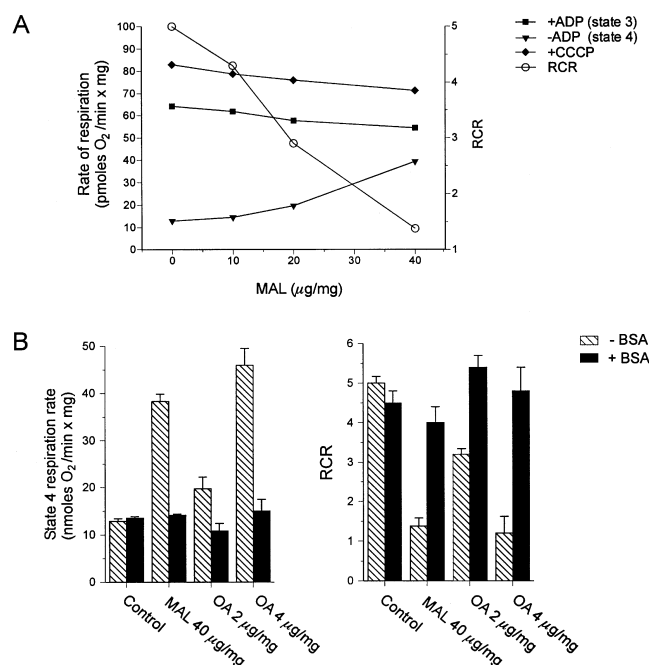


Fig. 2. Effect of MAL on mitochondrial respiration. Respiration of rat liver mitochondria ($1.5 \text{ mg} \cdot \text{mL}^{-1}$) was evaluated as described in Materials and methods. State 3 mitochondrial respiration was induced by the addition of 500 nmoles ADP and mitochondria were uncoupled using $3 \mu\text{M}$ CCCP. (A) Rates of respiration (state 3, state 4 and uncoupled) and respiratory control ratio (RCR) in isolated mitochondria treated with different concentrations of MAL. (B) Effect of BSA (0.05%) on MAL- and oleic acid (OA)-induced alteration of state 4 respiration rate and respiratory control ratio in isolated mitochondria.

The effect of BSA on MAL-induced swelling and loss of $\Delta\Psi_{\text{m}}$ in isolated mitochondria is illustrated in Fig. 3. BSA prevented the first decline in $\Delta\Psi_{\text{m}}$ upon addition of MAL (Fig. 3B), which was insensitive to CsA, indicating that the initial decrease in $\Delta\Psi_{\text{m}}$ was due to partial uncoupling of mitochondria by fatty acids. However, MAL was still able to induce swelling of mitochondria and collapse $\Delta\Psi_{\text{m}}$ in the presence of BSA, although BSA delayed these events slightly (Fig. 3A).

Induction of MPT by HAMLET

HAMLET is prepared *in vitro* by subjecting EDTA-treated α -lactalbumin to ion-exchange chromatography on a matrix conditioned with oleic acid [9]. In order to investigate whether HAMLET had similar effects as MAL on isolated mitochondria, the MPT-inducing capacity of HAMLET was examined. HAMLET preparations contain low amounts of EDTA ($\approx 10 \mu\text{M}$), which is known to suppress the induction of MPT by binding Ca^{2+} in the incubation buffer. Taking this into consideration, experiments with HAMLET were performed in the presence of 1 mM Mg^{2+} , which saturated EDTA and overcame its effect on MPT induction. Similar to MAL, HAMLET induced swelling and biphasic dissipation of $\Delta\Psi_{\text{m}}$, which resulted in the release of proteins from the intermembrane space (Fig. 4). These events were a consequence of MPT induction because CsA, Ub0 and RuRed inhibited swelling and the second phase decrease in $\Delta\Psi_{\text{m}}$. HAMLET also exhibited similar effects on mitochondrial respiration as MAL, and BSA abolished its uncoupling effect (data not shown). BSA delayed,

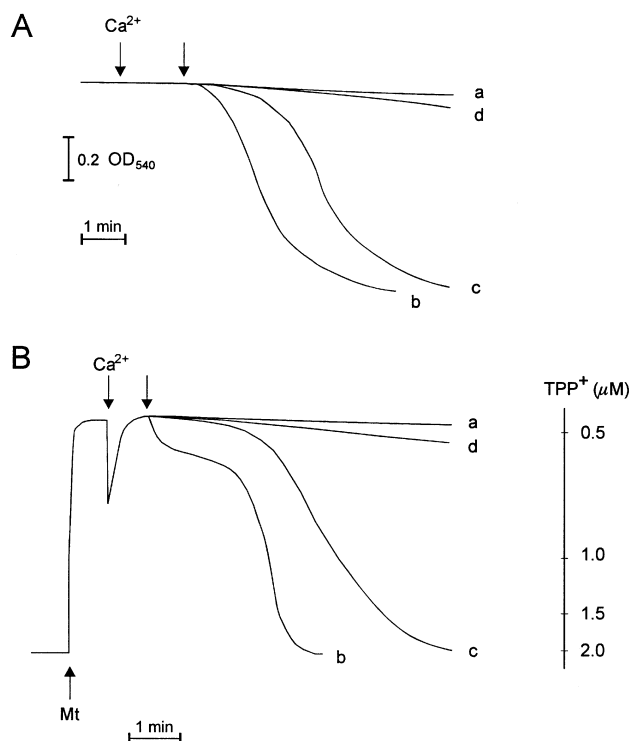


Fig. 3. The effect of BSA on MAL-induced swelling of isolated mitochondria and the decrease in $\Delta\Psi_{\text{m}}$. (A) Rat liver mitochondria ($0.5 \text{ mg} \cdot \text{mL}^{-1}$) were pre-incubated with 20 nmol of Ca^{2+} per mg of protein at 30°C . Mitochondrial swelling was induced by the addition of MAL ($10 \mu\text{g} \cdot \text{mg}^{-1}$) and monitored continuously as changes in A_{540} . Curve a: control; curve b: MAL; curve c: MAL and 0.05% BSA; curve d: 0.05% BSA. (B) Mitochondria ($1.5 \text{ mg} \cdot \text{mL}^{-1}$) were loaded with Ca^{2+} ($40 \text{ nmol} \cdot \text{mg}^{-1}$) and the decrease in $\Delta\Psi_{\text{m}}$ was induced by the addition of MAL ($40 \mu\text{g} \cdot \text{mg}^{-1}$). Curve a: untreated mitochondria; curve b: MAL; curve c: MAL and 0.05% BSA; curve d: 0.05% BSA.

but did not abolish, the HAMLET-induced decrease in $\Delta\Psi_{\text{m}}$, again demonstrating that the fatty acids present in the α -lactalbumin complexes were not responsible for opening of the MPT pore.

DISCUSSION

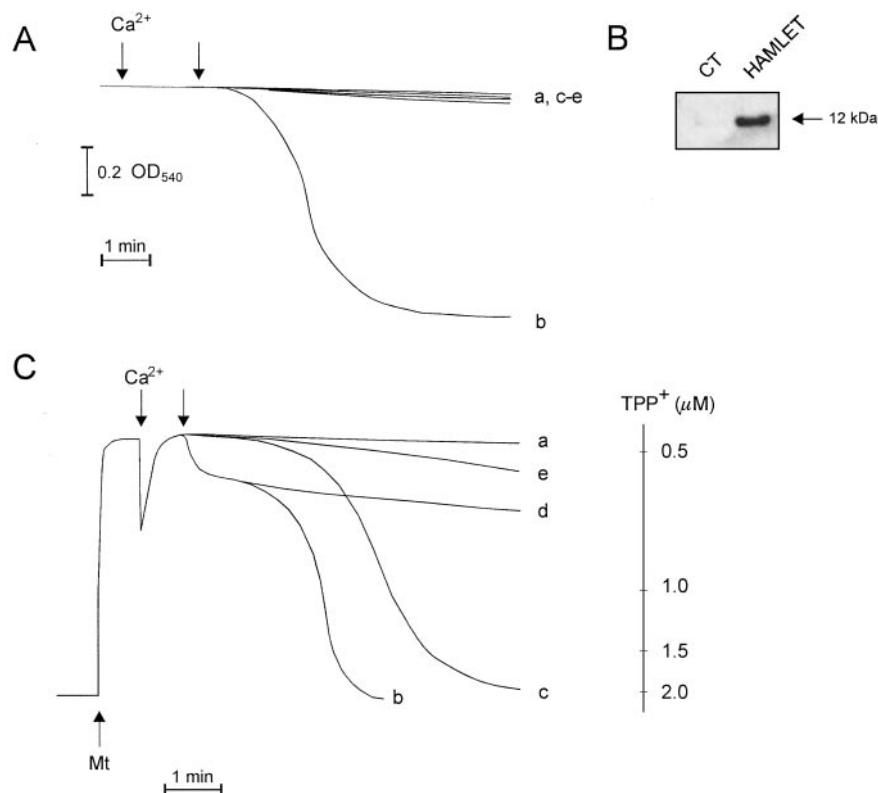
A previous study reported that cytochrome *c* release and caspase activation are involved in MAL-induced apoptosis in transformed cells [8]. MAL was internalized into the cytoplasm and co-localized with mitochondria. The results presented in this study show that MAL may induce apoptosis via a direct interaction with mitochondria, resulting in an opening of the MPT pore with the subsequent release of mitochondrial constituents, including cytochrome *c*, from the intermembrane space.

Although the precise mechanism of release of cytochrome *c* during apoptosis is unclear, in many experimental models this event is associated with a collapse of $\Delta\Psi_{\text{m}}$ due to Ca^{2+} -dependent MPT pore opening [14]. Opening of the MPT pores renders the mitochondrial inner membrane nonselectively permeable to small molecules ($< 1.5 \text{ kDa}$). As a consequence, water and solutes enter the mitochondrial matrix via osmosis, which leads to swelling and rupture of the outer mitochondrial membrane. Although Ca^{2+} is the obligatory pore activator, oxidative stress, adenine nucleotide depletion and a decrease in

Fig. 4. HAMLET induces MPT in isolated mitochondria.

(A) Rat liver mitochondria ($0.5 \text{ mg} \cdot \text{mL}^{-1}$) were pre-incubated with 20 nmol of Ca^{2+} per mg of protein at 30°C . Mitochondrial swelling was induced by addition of HAMLET ($20 \text{ } \mu\text{g} \cdot \text{mg protein}^{-1}$) and monitored continuously as a change in A_{540} . Curve a: control; curve b: HAMLET; curves c–e: HAMLET and $1 \text{ } \mu\text{M}$ CsA; $50 \text{ } \mu\text{M}$ Ub0; and $10 \text{ } \mu\text{M}$ RuRed, respectively.

(B) HAMLET-induced release of cytochrome *c* from isolated mitochondria. Mitochondria ($0.5 \text{ mg} \cdot \text{mL}^{-1}$) were pre-incubated with 20 nmol of Ca^{2+} per mg of protein at 30°C . After 5 min incubation, untreated or HAMLET-treated ($20 \text{ } \mu\text{g} \cdot \text{mg protein}^{-1}$) mitochondria were pelleted and the supernatants were subjected to Western blot analysis as described in Materials and methods. The membrane was probed with an antibody against cytochrome *c*. (C) Effect of BSA and CsA on HAMLET-induced loss of $\Delta\Psi_m$. Mitochondria ($1.5 \text{ mg} \cdot \text{mL}^{-1}$) were loaded with Ca^{2+} ($40 \text{ nmol} \cdot \text{mg protein}^{-1}$) and the decrease in $\Delta\Psi_m$ was induced by addition of HAMLET ($60 \text{ } \mu\text{g} \cdot \text{mg protein}^{-1}$). Curve a: control; curve b: HAMLET; curve c: HAMLET and 0.05% BSA; curve d: HAMLET and $1 \text{ } \mu\text{M}$ CsA; curve e: 0.05% BSA.



$\Delta\Psi_m$ can significantly increase the sensitivity of MPT to Ca^{2+} [15].

As mentioned above, MAL is a fraction of human milk containing a complex of a folding variant of α -lactalbumin and lipids. In contrast, HAMLET is not a fraction of human milk, but native α -lactalbumin converted *in vitro* to the apoptosis-inducing folding variant of the protein. While native α -lactalbumin is unable to induce apoptosis (or MPT when added to isolated mitochondria), HAMLET and MAL kill tumour cells via apoptosis with similar kinetics.

MAL added to isolated rat liver mitochondria induced swelling of mitochondria, followed by the release of cytochrome *c* and AK-2 into the medium. The MPT inhibitors CsA and Ub0 abolished this release. CsA is a classical inhibitor of MPT, while Ub0 was recently shown to interact with the MPT pore and inhibit its opening [12]. Both swelling and the release of cytochrome *c* and AK-2 from MAL-treated mitochondria were prevented by chelation of Ca^{2+} , or inhibition of Ca^{2+} entry into mitochondria by RuRed, suggesting that these events are Ca^{2+} -dependent. Similar results were obtained with HAMLET, further supporting the concept that the folding variant of α -lactalbumin is the active component in MAL.

Investigation of the effect of the folding variant of α -lactalbumin on mitochondria revealed that, in addition to MPT induction, this complex also affected mitochondrial respiration. Both MAL and HAMLET induced mild uncoupling of mitochondria, which resulted in a concentration-dependent acceleration of mitochondrial respiration (state 4) and a marked decrease in the respiration control ratio. It should be stressed that alteration of mitochondrial respiration occurred without Ca^{2+} loading and was not prevented by CsA, hence MPT was not involved. However, the activation of state 4 respiration and decrease in respiration control ratio values were completely restored by BSA.

The dual effects of the folding variant of α -lactalbumin became even more evident when estimating the changes in $\Delta\Psi_m$. Addition of this complex to Ca^{2+} -loaded mitochondria induced a concentration-dependent rapid shift in $\Delta\Psi_m$ followed by a time-dependent collapse of $\Delta\Psi_m$. CsA, as well as ATP, prevented the second and more severe phase of decreased $\Delta\Psi_m$, but not the first shift, which was apparently brought about by fatty acids because it was prevented completely by BSA. However, BSA failed to rescue the mitochondria from MPT and was only able to delay the collapse of $\Delta\Psi_m$ (as well as swelling), suggesting that fatty acids in the α -lactalbumin complexes sensitize mitochondria towards permeability transition.

The lipid component of MAL and HAMLET is believed to be important for maintaining α -lactalbumin in the active apoptosis-inducing folding state, the so-called molten globule-like state [6], which is an intermediate state between the native and denatured form of the protein. This folding variant of α -lactalbumin has a native-like secondary structure, but a less well-defined tertiary structure.

The ability of free fatty acids, such as oleic acid and palmitic acid, to uncouple mitochondria, and thereby cause an increased rate of respiration, has been well documented [16]. In our experiments, the addition of a corresponding amount of oleic acid to mitochondria induced similar effects as MAL and HAMLET on both state 4 respiration and respiration control ratio in a BSA-inhibitable manner. When added to Ca^{2+} -loaded mitochondria, the fatty acid itself induced a rapid partial decrease in $\Delta\Psi_m$ (data not shown) but not the secondary collapse seen upon addition of MAL or HAMLET; hence, oleic acid did not induce opening of the MPT pore under our experimental conditions. The fatty acids in both MAL and HAMLET only facilitated pore opening, which is in agreement with other studies showing that a partial uncoupling of mitochondria favours MPT induction [17,18].

In conclusion, our results demonstrate that a folding variant of α -lactalbumin induces MPT in isolated mitochondria. Although the relevance of this finding for MAL/HAMLET-induced apoptosis requires further investigation, it seems likely that the MPT-mediated mitochondrial pathway contributes to caspase activation and apoptosis also in intact cells treated with these agents.

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REFERENCES

1. Cryns, V. & Yuan, J. (1998) Proteases to die for. *Genes Dev.* **12**, 1551–1570.
2. Zou, H., Li, Y., Liu, X. & Wang, X. (1999) An apaf-1-cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem.* **274**, 11549–11556.
3. Reed, J.C. (1997) Cytochrome *c*: can't live with it – can't live without it. *Cell* **91**, 559–562.
4. Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* **341**, 233–249.
5. Marzo, I., Brenner, C. & Kroemer, G. (1998) The central role of the mitochondrial megachannel in apoptosis: evidence obtained with intact cells, isolated mitochondria, and purified protein complexes. *Biomed. Pharmacother.* **52**, 248–251.
6. Svensson, M., Sabharwal, H., Håkansson, A., Mossberg, A.K., Lipniunas, P., Leffler, H., Svanborg, C. & Linse, S. (1999) Molecular characterization of α -lactalbumin folding variants that induce apoptosis in tumor cells. *J. Biol. Chem.* **274**, 6388–6396.
7. Håkansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H. & Svanborg, C. (1995) Apoptosis induced by a human milk protein. *Proc. Natl Acad. Sci. USA* **92**, 8064–8068.
8. Köhler, C., Håkansson, A., Svanborg, C., Orrenius, S. & Zhivotovsky, B. (1999) Protease activation in apoptosis induced by MAL. *Exp. Cell Res.* **249**, 260–268.
9. Svensson, M., Håkansson, A., Mossberg, A.-K., Linse, S. & Svanborg, C. (2000) Conversion of α -lactalbumin to a protein inducing apoptosis. *Proc. Natl Acad. Sci. USA* **97**, 4221–4226.
10. Löttscher, H.R., Winterhalter, K.H., Carafoli, E. & Richter, C. (1980) Hydroperoxide-induced loss of pyridine nucleotides and release of calcium from rat liver mitochondria. *J. Biol. Chem.* **255**, 9325–9330.
11. Nicolli, A., Basso, E., Petronilli, V., Wenger, R.M. & Bernardi, P. (1996) Interactions of cyclophilin with the mitochondrial inner membrane and regulation of the permeability transition pore, and cyclosporin A-sensitive channel. *J. Biol. Chem.* **271**, 2185–2192.
12. Fontaine, E., Ichas, F. & Bernardi, P. (1998) A ubiquinone-binding site regulates the mitochondrial permeability transition pore. *J. Biol. Chem.* **273**, 25734–25740.
13. Jensen, R.G. (1996) The lipids in human milk. *Prog. Lipid Res.* **35**, 53–92.
14. Yang, J.C. & Cortopassi, G.A. (1998) Induction of the mitochondrial permeability transition causes release of the apoptogenic factor cytochrome *c*. *Free Rad. Biol. Med.* **24**, 624–631.
15. Halestrap, A.P., Woodfield, K.Y. & Connern, C.P. (1997) Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. *J. Biol. Chem.* **272**, 3346–3354.
16. Rottenberg, H. & Hashimoto, K. (1986) Fatty acid uncoupling of oxidative phosphorylation in rat liver mitochondria. *Biochemistry* **25**, 1747–1755.
17. Bernardi, P. (1992) Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J. Biol. Chem.* **267**, 8834–8839.
18. Scorrano, L., Petronilli, V. & Bernardi, P. (1997) On the voltage dependence of the mitochondrial permeability transition pore – a critical appraisal. *J. Biol. Chem.* **272**, 12295–12299.

Bladder cancers respond to intravesical instillation of HAMLET (human α -lactalbumin made lethal to tumor cells)

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We studied if bladder cancers respond to HAMLET (human α -lactalbumin made lethal to tumor cells) to establish if intravesical HAMLET application might be used to selectively remove cancer cells *in vivo*. Patients with nonmuscle invasive transitional cell carcinomas were included. Nine patients received 5 daily intravesical instillations of HAMLET (25 mg/ml) during the week before scheduled surgery. HAMLET stimulated a rapid increase in the shedding of tumor cells into the urine, daily, during the 5 days of instillation. The effect was specific for HAMLET, as intravesical instillation of NaCl, PBS or native α -lactalbumin did not increase cell shedding. Most of the shed cells were dead and an apoptotic response was detected in 6 of 9 patients, using the TUNEL assay. At surgery, morphological changes in the exophytic tumors were documented by endoscopic photography and a reduction in tumor size or change in tumor character was detected in 8 of 9 patients. TUNEL staining was positive in biopsies from the remaining tumor in 4 patients but adjacent healthy tissue showed no evidence of apoptosis and no toxic response. The results suggest that HAMLET exerts a direct and selective effect on bladder cancer tissue *in vivo* and that local HAMLET administration might be of value in the future treatment of bladder cancers.

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Key words: bladder cancer; apoptosis; therapeutics; α -lactalbumin; protein folding

Bladder cancers are common and remain a challenge, despite significant therapeutic advances. The prevalence is about 1/4000, making this the fourth most common malignancy in the United States and the fifth in Europe.¹ Surgery or combinations of surgery and cytostatic drugs are used successfully, but therapy-resistant tumors still cause significant morbidity and mortality.¹ Superficial papillary nonmuscle invasive tumors may be removed by transurethral resection and the short-term prognosis is excellent but the patients need life-long follow-up because of a high recurrence rate combined with a risk for dedifferentiation.² Invasive tumors are eventually treated by radical cystectomy and some patients receive adjuvant systemic chemotherapy but these tumors have a poor prognosis with 5-year survival rates of 40% or less.³ Focal or multifocal “cancer *in situ*” (CIS) is restricted to the urothelium initially, but may also progress and require cystectomy. A variety of topical treatments are used to prevent or delay cystectomy, and the drugs used for intravesical instillation include Thiotepa, Epirubicin, Mitomycin and Bacille-Calmette-Guerin (BCG).^{4,5} BCG treatment is efficient and results in a recurrence free interval of at least 2 years in about 70% of the patients with superficial bladder cancers.⁶ BCG causes significant side effects, however, including granulomatous prostatitis, local discomfort, dysuria and may require systemic antituberculous therapy. The incidence of life-threatening complications of BCG therapy is relatively low (0.4%),⁷ but a significant number of deaths have been reported, especially in immunosuppressed patients. The problems with BCG therapy nowadays are more due to noneffectiveness than to toxicity, however.

HAMLET (human α -lactalbumin made lethal to tumor cells) is a complex of α -lactalbumin and oleic acid that kills tumor cells *in vitro*.^{8,9} To form HAMLET, α -lactalbumin is first unfolded by the removal of the tightly bound Ca^{2+} ion, which coordinates the native conformation of the protein. The partially unfolded protein is subsequently bound to oleic acid on an ion-exchange matrix,

and the complex is eluted with high salt.¹⁰ The biological properties of HAMLET are defined by the partially unfolded state of α -lactalbumin,^{8,11} and by the fatty acid, as neither the protein alone nor the fatty acid show this tumoricidal activity. The tumor cells die by an apoptosis-like mechanism but healthy, differentiated cells, are resistant to the lethal effects of HAMLET.^{9,12} The anti-tumor activity of HAMLET is maintained also *in vivo*, as shown in experimental animal models and clinical studies. Local HAMLET infusion was shown to delay the development of tumors and to prolong survival in a human glioblastoma xenograft model.¹³ In a randomized, placebo-controlled study, topical HAMLET administration was shown to efficiently remove or reduce human skin papillomas.¹⁴ These observations suggested that topical HAMLET administration might be useful in cancer patients.

In vitro studies have shown that kidney and bladder carcinoma cell lines undergo a rapid death response to HAMLET.¹⁵ In our study, we used an intravesical instillation protocol to investigate if HAMLET triggers apoptosislike death in bladder cancer cells, *in vivo*. This approach was based on the established practice of local instillation of antitumor substances such as BCG bacteria in patients with bladder cancer.⁷

Methods

Patients

Nine male patients awaiting transurethral surgery for newly diagnosed or recurrent superficial bladder cancer were invited to participate in the study and received HAMLET instillations (Table I). Seven patients had nonmuscle invasive tumors diagnosed by cystoscopy and urine cytology and 2 patients had CIS diagnosed by mucosal biopsies and urine cytology. Patient P1 had multiple solid tumors, patients P2, P3 and P5–P7 had newly diagnosed papillary tumors and P1 and P4 had recurrences of previously diagnosed and treated nonmuscle invasive tumors. Patient P8 had a recurrence after BCG treatment of multifocal CIS and patient P9 had a newly diagnosed CIS, which had not been treated with BCG. Patients P2, P5–P9 and P9 were previously healthy.

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TABLE 1 - CLINICAL STATUS AND RESPONSE TO HAMLET

Patient age (years)	Diagnosis	Exposure time (h)	Shed cells			Outcome			Benign adjacent tissue	
			Fold increase	% TUNEL positive	Cytology	Remaining tumour		TUNEL	Histology	TUNEL
						Morphology	Histology			
P1 (82)	Multiple, solid (r)	5.6	15	13	Atypical cells	Dry, not bleeding	TA grade 2	Pos.	Moderate focal dysplasia	Neg.
P2 (38)	Solitary, papillary (p)	8.2	100	45	No data	Tumor necrosis	T1 grade 2	Pos.	Sample missing	n.s.
P3 (72)	Solitary, papillary (p)	12.4	1.2	0	Cancer grade 2	Partial tumor necrosis	TA grade 2	Neg.	n.s.; Mild urothelial hyperplasia but no atypic cells	Neg.
P4 (84)	Solitary, papillary (r)	8.5	30	n.s.	Atypical cells	Surface atrophy	TA grade 1	Pos.	Normal urothelium	Neg.
P5 (73)	Solitary, papillary (p)	10.3	30	65	Cancer grade 2	Tumor necrosis	TA grade 2	n.s.	Sample missing	n.s.
P6 (61)	Solitary, papillary (p)	21.2	180	4	Susp. cancer grade 1	Superficial tumor atrophy	TA grade 1	Neg.	n.s.; Normal urothelium	Neg.
P7 (64)	Solitary, solid (p)	4.7	2.8	41	Cancer grade 3	Surface atrophy	TA grade 2	Pos.	Sample missing	n.s.
P8 (62)	3/4 biopsies positive for CIS	10.6	6	71	Cancer grade 3	Not relevant	1/4 biopsies positive		n.s.; Normal urothelium	
P9 (80)	3/4 biopsies positive for CIS	8.3	7	61	Cancer grade 3	Not relevant	No remaining tumor		One fragment with atypic cells, remaining biopsies normal	

n.s., no sample available; (p), primary diagnosed tumors; (r), recurrence of previously diagnosed tumor.

Study design

At inclusion, the patients were subjected to cystoscopy to assess the tumor size and to document the lesions with endoluminal photography (Nikon, Tokyo, Japan). During the week preceding scheduled surgery, intravesical instillations of HAMLET were given daily for 5 days. The patients were asked to avoid fluid intake for 4 hr before, and immediately after the instillation of HAMLET, to delay the dilution effect. To avoid urinary tract infection, low dose, per oral antibiotic prophylaxis was used (Ciprofloxacin or Trimetoprim). At the time of surgery, cystoscopy with endoluminal photography was repeated and the tumor size and morphology were reassessed. The instillations were performed in the outpatient clinic where the patients were under close surveillance to detect any subjective or objective side effects of the HAMLET administration.

HAMLET was instilled into the bladder through a soft polyethylene catheter (Lowfric[®] Ch 12, Astra Zeneca, Södertälje, Sweden). Prior to instillation, the bladder was completely emptied and the urine was collected for preinstillation analysis. HAMLET (30 ml, 25 mg/ml) was deposited in the bladder, the catheter was removed, and the patients were encouraged to postpone voiding to maximize the exposure to HAMLET. The HAMLET concentration (25 mg/ml) was based on the skin papillomas study,¹⁴ where a concentration of 10 mg/ml of HAMLET was found to be efficient. We calculated that 25 mg/ml would be required to reach a similar concentration in the bladder, as newly produced urine would be diluting the instilled HAMLET concentration.

Control instillations were performed in 5 patients. Three of them were included in the HAMLET study. C1 = P5 (native α -lactalbumin) and C2 = P7 (PBS) were given control instillations before HAMLET treatment and C3 = P9 (NaCl) after completed HAMLET instillations. Two patients were subjected only to control instillations with NaCl before scheduled surgery. C4 had a recurrence with a single small papillary tumor and C5 was a female patient with a newly diagnosed large papillary tumor.

IRB approval was obtained from the Medical Ethics Committee of the Lund Medical Faculty, Lund, Sweden, and informed consent was obtained from each patient (LU 454-00).

HAMLET

HAMLET was prepared from human milk α -lactalbumin, as described.¹² The starting material was excess milk from the hospital milk bank of the quality required for feeding of premature babies (HIV and Hepatitis B negative, microbial burden was ≤ 1 CFU/mg protein). The milk was stored at -20°C until used. Briefly, α -lactalbumin was purified from milk by ammoniumsulphate precipitation followed by hydrophobic interaction chromatography. EDTA treatment was used to unfold the protein and oleic acid was incorporated on an ion-exchange matrix, forming HAMLET. For intravesical instillation, HAMLET was dissolved in PBS (25 mg/ml, 30 ml) under sterile conditions.

Urine cytology

To examine if voided tumor cells showed a cell death response to HAMLET, urine samples were obtained prior to and after each HAMLET instillation and cells in uncentrifuged urine were counted by light microscopy (Nikon Eclipse E800, Tokyo, Japan) using a Bürker chamber. Cell viability was determined by trypan blue exclusion. Cell morphology was determined after hematoxylin- and eosin staining. Apoptotic cells were identified by the TUNEL assay (Roche, Basel, Switzerland) and examined by fluorescence microscopy using the LSM META 510 software package (Carl Zeiss, Jena, Germany). Representative cells were photographed using a CCD camera (Diagnostic Instruments, MI). In addition, the urine samples were examined at the Department of Pathology at Lund University Hospital. All of the patients were subjected to urine cytology, which was used as a diagnostic tool. For urine cytology, cells concentrated from 30 ml urine are used. Cell shedding in response to HAMLET was examined in uncentrifuged urine. Urine cytology is based on 30 ml of urine, and cells are cen-

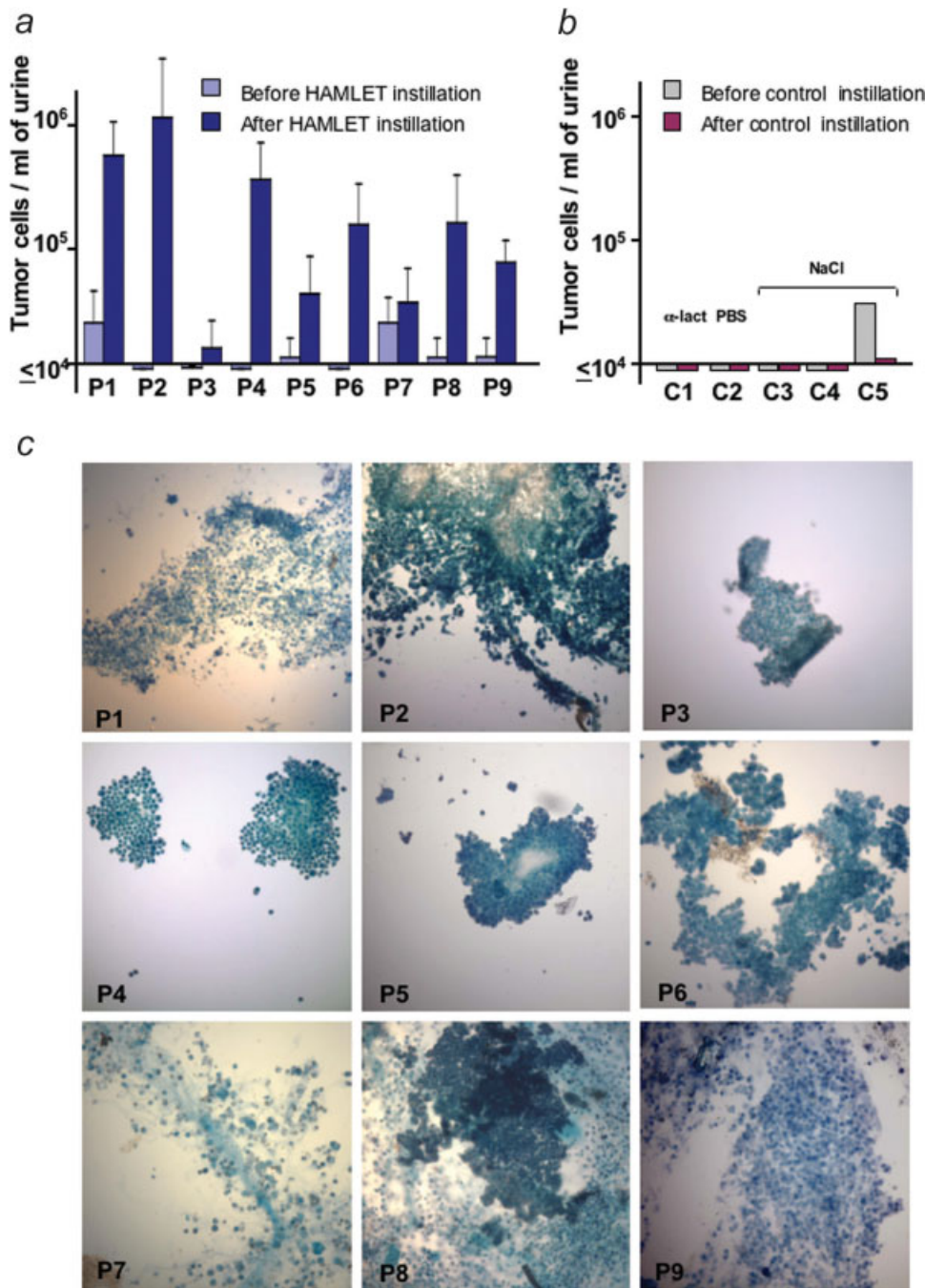


FIGURE 1 – Shedding of tumor cells following intravesical instillation of HAMLET (30 ml, 25 mg/ml). Urine samples were obtained from each patient ($n = 9$) before and about 2 hr after each HAMLET instillation (88 samples). (a) Number of cells in the urine of each patient, before or after HAMLET instillation (individual means $\pm$ SDs of 5 daily instillations). (b) Patients who received PBS, NaCl or α -lactalbumin instillations showed no increase in cell shedding (number of cells excreted after one instillation). C1 = P5 received one instillation with native α -lactalbumin and C2 = P7 received one instillation of PBS one day before the HAMLET instillations. C3 = P9 received one instillation of NaCl (0.9%) after the HAMLET instillation period. C4 and C5 were not exposed to HAMLET but received one instillation each of NaCl before their scheduled surgery. (c) Cells and tissue aggregates in urine after HAMLET instillation. Light microscopy of trypan blue stained, unfixed cells. The aggregates were not included in the numbers in (a).

trifuged onto appropriate glass slides. Cells excretion in response to HAMLET was examined in uncentrifuged urine.

Tissue biopsies

Tumor biopsies were collected at surgery, fixed in 4% paraformaldehyde in PBS for 24 hr, treated with 10, 20 and 25% sucrose in PBS solution, embedded in Tissue-Tec (Sakura Finetek, Torrance, CA, USA) and frozen in propane alcohol chilled with dry ice. Biopsies were stored in -80°C until use. Serial 10- μm sections were cut on a Microm HM500M (Microm Microtech, Francheville, France). Apoptotic cells were detected by the TUNEL assay. Paraffin-embedded tissue samples from benign adjacent tissue were obtained from pathology.

Statistical analysis. The analysis of cell shedding used the Mann-Whitney U test (InStat 3, GraphPad Software, San Diego, CA, USA).

Results

Patients with superficial bladder cancers were subjected to intravesical HAMLET instillations on 5 consecutive days, during the week before transurethral surgery. The patients did not report any symptoms of the HAMLET instillations, other than those caused by the catheterization procedure *per se*. The study examine if bladder tumor cells respond *in vivo* to topical HAMLET instillation but was not a treatment trial. This was not a treatment trial,

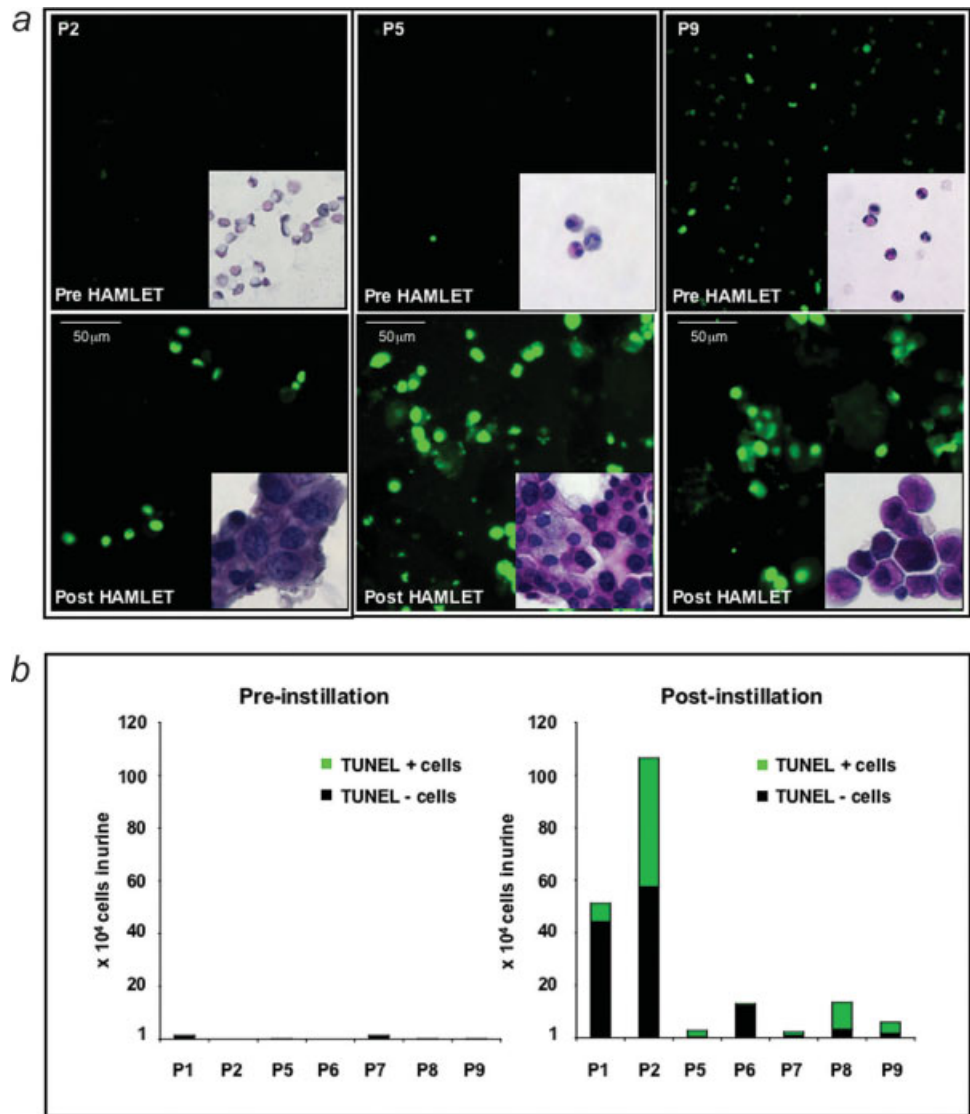


FIGURE 2 – Shed apoptotic cells as defined by TUNEL staining. (a) Overview of pre- and postinstillation samples from 3 representative patients. Cells excreted from patients were cyto-centrifuged onto poly-L-lysine coated glass slides, stained with hematoxylin-eosin and examined by the TUNEL assay. Lower right panels show the morphology of shed cells by hematoxylin-eosin staining (magnification $\times 40$). In the preinstillation samples occasional TUNEL positive neutrophils were observed. In the postinstillation samples, the TUNEL positive cells were shed tumor cells. Patient numbers are in the upper left corners. (b) Frequency of shed TUNEL positive tumor cells in each patient. Pre- and postinstillation samples were not available for TUNEL staining in P3 and P4.

but a study examining if bladder tumor cells respond *in vivo* to topical HAMLET instillation by undergoing apoptosis.

HAMLET triggers shedding of tumor cells

The HAMLET instillations caused massive shedding of cells into the urine in all patients except P3 (Fig. 1a). The mean number of single cells increased from levels below $10^4/\text{ml}$ to a mean of $2.9 \times 10^5 \pm 1.3 \times 10^5$ in the postinstillation samples ($p < 0.0001$, Mann Whitney). In addition, most of the posturine samples contained large cell aggregates (Fig. 2c). The number of cells in these aggregates could not be quantified but they added considerably to the total number of shed cells, as there were no aggregates in the preinstillation samples. The increase in cell shedding occurred daily, during the 5 days of instillation ($p < 0.01$, compared to the preinstillation cell numbers), and cell shedding occurred in all patients except patient P3 (Table I). Before HAMLET instillation, the spontaneous shedding of tumor cells was $< 10^4/\text{ml}$ of urine in most of the patients (Table I), and cell aggregates or tissue fragments were not detected.

To examine if cell shedding was caused by HAMLET, 5 patients received control instillations with buffer or α -lactalbumin (Fig. 1b). There was no increase in cell shedding after intravesical instillation of buffer or native α -lactalbumin, and aggregates were

not observed in the urine samples of those patients (Fig. 1b, $p > 0.05$). As prophylactic antibiotic treatment was given to both HAMLET treated and control patients, the influence of this variable on cell excretion could be excluded.

TUNEL staining of shed cells

After HAMLET instillation, more than 90% of the shed cells and cells aggregates were dead, as determined by trypan blue exclusion (Fig. 1c). Apoptotic changes in the shed cells were detected using the TUNEL assay (Fig. 2a). In the preinstillation samples, tumor cells were scarce but some samples from patients P5 and P9 contained a few apoptotic neutrophils. After HAMLET instillation, TUNEL positive tumor cells were detected in 6 patients (4 with papillomas and 2 with CIS). The highest proportion of TUNEL positive cells was found in patients P2, P5, P7 and P9 (Fig. 3b, Table I).

The morphology of the shed cells is shown in Figure 4a. Many cells had large amorphous nuclei, little cytoplasm and condensed chromatin suggesting that they originated from the tumors. Other cells showed no definitive tumor characteristics, but were multinucleated or with degenerative changes. TUNEL staining was in the nuclei of the shed cells (Fig. 4b). There was no significant

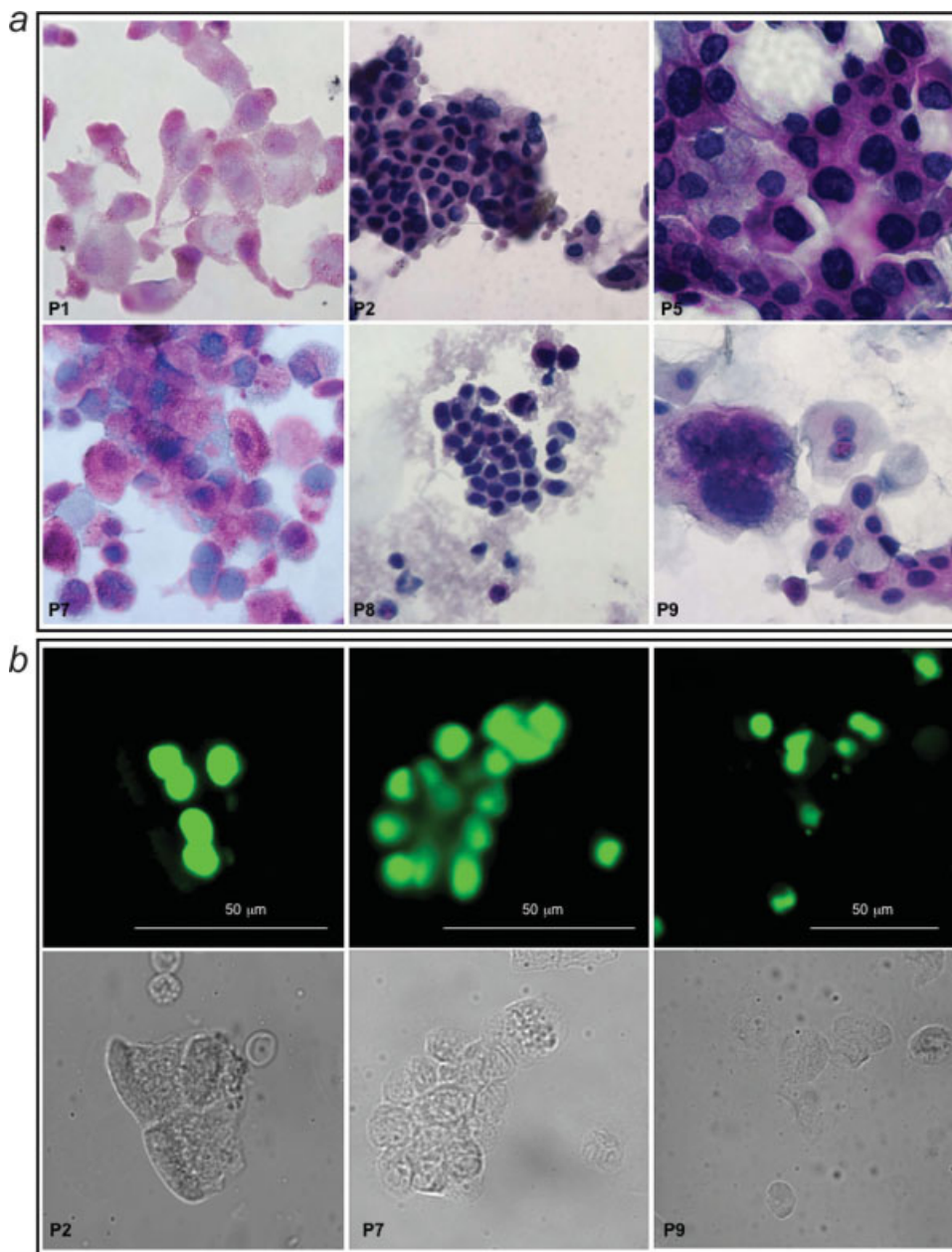


FIGURE 3 – Morphology of shed tumor cells after HAMLET instillation. (a) Hematoxylin-eosin staining of tumor cells from 6 patients. Patient numbers are in the lower left corner. (b) TUNEL staining and morphology of shed tumor cells from patients P2, P7 and P9, with the corresponding light microscopy image.

increase in the shedding of squamous or transitional epithelial cells with normal morphology.

Effects of HAMLET on tumor size and morphology

The response of the papillary tumors to HAMLET was examined by endoscope and documented by photography (Fig. 4). The clinical outcome is summarized in Table I. Patients P2 and P5 (TA grade 2) showed a nearly complete resolution of the tumor after the HAMLET instillations. Most of the papillary structure had been lost and the base of the tumors appeared fractured. Patient P3 (TA grade 2) carried a papillary tumor on the left bladder wall, which was too large to be captured in 1 photograph (Fig. 4). The HAMLET instillations caused a reduction in tumor size of about 50% and the tumor was fractured and appeared fragile. Patient P4 had 2 small papillary tumors on the left bladder neck (TA grade 1). HAMLET caused a minor reduction in tumor size but a marked change in tumor character with surface atrophy (Fig. 4). Patient P1 (TA grade 2, not shown) showed no apparent reduction in tumor size but a change in tumor character from brit-

tle and bleeding on contact to “dry.” Patient P6 had a fairly large papillary tumor on the right side of the bladder (TA grade 1, not shown) that showed signs of peripheral atrophy after HAMLET exposure. Patient P7 had 2 solid tumors that did not change appearance significantly after HAMLET exposure (not shown).

Apoptotic cells in tumor biopsies but not in adjacent healthy tissue

Biopsies were obtained from the tumors in connection with surgery, and the presence of tumor cells in the biopsies was confirmed by pathology. Sections without diathermia damage and with an intact epithelial lining were selected for analysis. Apoptotic cells were detected by the TUNEL assay (Fig. 5, Table I). TUNEL positive tumor areas were detected in 6 of 9 patients (Fig. 5a); 4 of the patients had papillomatous tumors (P1–P4 and P6) and 2 had CIS (P8, P9). Patient P5 did not deliver a sample, as most of the tumor was lost due to cell shedding. TUNEL positive areas were not found in patients P3 and P6 and those patients lacked TUNEL positive cells in urine (Table I).

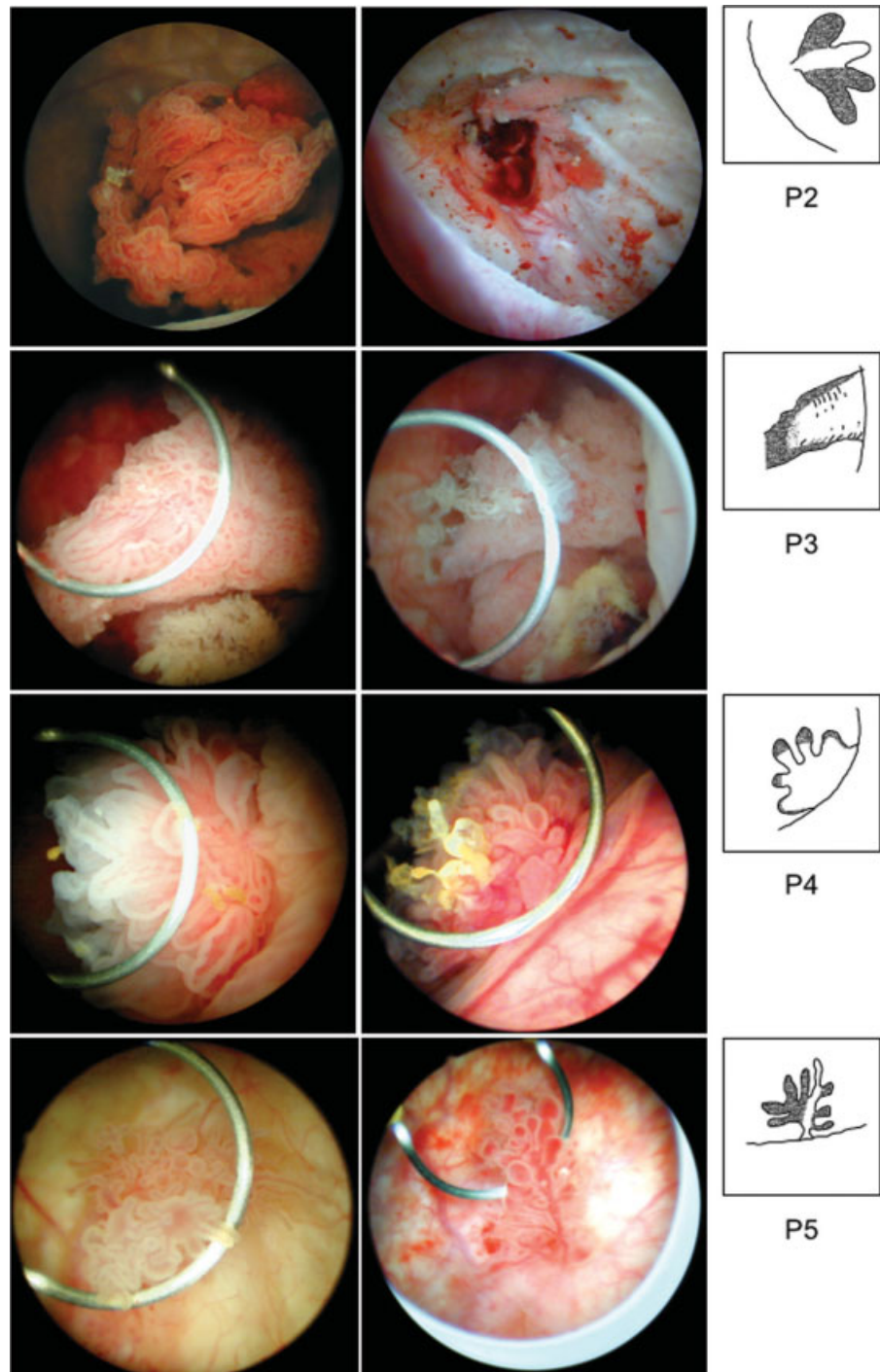


FIGURE 4 – Macroscopic changes in papillary tumors after HAMLET exposure. The tumor is shown before (left panels) and after (right panels) the 5 HAMLET instillations. Changes in tumor size in individual patients are illustrated by the drawings next to each pair of photographs. The diathermia loop is 5-mm wide. Patient numbers are below each schematic.

In 4 patients, biopsies were also obtained from benign tissues distant from the tumor. The pathologist confirmed that tumor cells were absent from those samples. TUNEL positive cells were not detected in this urothelium (Fig. 5b, Table I).

Discussion

Our study shows that HAMLET triggers tumor cell death *in vivo* in patients with bladder cancers. The tumors were exposed to HAMLET topically, by intravesical instillations on 5 consecutive days during the week preceeding scheduled surgery and the tissue response was evaluated at surgery. Rapid shedding of dead

tumor cells occurred after each HAMLET instillation, but control instillations with inactive α -lactalbumin or salt solutions did not trigger shedding. The HAMLET instillations caused a reduction in tumor size in four papillomas, and a slight change in character in an additional two. Biopsies from the remaining tumor showed abundant apoptotic changes, but there was no evidence of apoptosis in healthy benign tissues surrounding the tumor. We conclude that HAMLET triggers tumor cell death *in vivo*.

HAMLET kills tumor cells and embryonal cells *in vitro*, but healthy differentiated cells survive high concentrations of HAMLET.¹² This relative selectivity is a surprising and potentially very useful property and has been confirmed in a limited

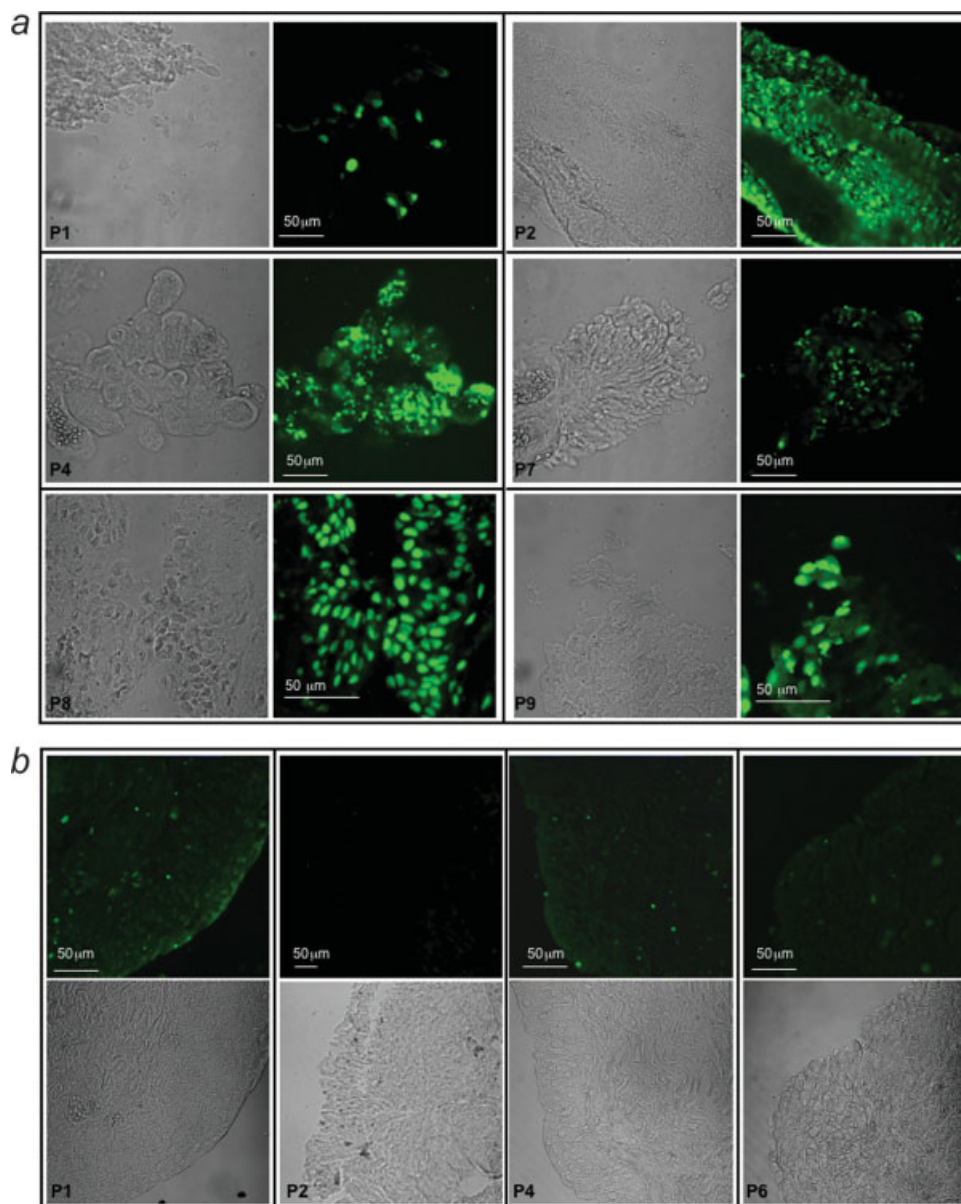


FIGURE 5 – Apoptotic response to HAMLET *in vivo* as shown by TUNEL staining of tumor biopsies. (a) Tissue sections were obtained at surgery, after 5 HAMLET instillations. Tissue sections are shown by light microscopy (left panels) and by fluorescence microscopy, with TUNEL positive cells (right panels). (b) Lack of apoptosis in tissue sections from healthy tissues adjacent to the tumor. The TUNEL-stained image is shown in the upper panels and light microscopy images in the lower panels. The patient number are shown in the lower left corner.

number of *in vivo* studies. Patients with skin papillomas, who received HAMLET topically during 3 weeks, did not report adverse effects or show signs of irritation in the skin surrounding the lesions.¹⁴ In a rat xenograft model of human glioblastoma, tumor cell death was observed after injection of HAMLET into the brain, but there was no apoptotic response in healthy brain tissue surrounding the tumor and no evidence of toxicity after injection of HAMLET into healthy rat brains.¹³ The present study provided further evidence in support of selectivity. There was an apoptotic response in tumor biopsies from HAMLET-treated patients, but there was no evidence of apoptosis in the biopsies from benign bladder tissue in the same patients. HAMLET thus appears to trigger a death response preferentially in malignant bladder cells, although the influence of variables such as the glucosaminoglycan layer has not been considered in our study. There is little concern that HAMLET would cause systemic toxicity. HAMLET is inactivated as the fatty acid is lost and the protein reverts to serum, because of the loss of the fatty acid and reversion of the protein to the native state, which is inactive in the apoptosis assay. The fatty acid is “stolen” by other high-affinity. Serum albumin is one of the fatty acid bind-

ing proteins that successfully compete with α -lactalbumin for oleic acid, like albumin. Furthermore, studies of breast-fed children have shown that the absorption of α -lactalbumin across the intestinal mucosa is harmless, and thus the presence of α -lactalbumin in the systemic circulation is considered physiological.

Topical tumor therapy is widely used in urologic oncology. BCG treatment has been shown to reduce papillary tumor recurrences by up to 40%.⁷ The therapeutic effect has been attributed to the local production of inflammatory mediators including TNF.¹⁶ Several differences between BCG and HAMLET were noticed. HAMLET appears to mainly act directly on cancer cells *in vivo*, and not through the recruitment of immune-effector cells. In the skin papilloma study, immunocompetent and immunocompromised individuals showed a similar response, suggesting that HAMLET does not act through specific immunity. Furthermore, the effect of HAMLET was more rapid than BCG, as seen by tumor cell excretion within a few hours after HAMLET instillation. Finally, the patients did not develop symptoms from the instillations, other than the irritation because of frequent catheterizations. Ongoing studies in a murine bladder cancer model have confirmed

that tumor progression is limited by intravesical instillations of HAMLET (Mossberg *et al.*, in preparation). Thus, topical HAMLET administration represents a new and potentially useful

approach to bladder cancer therapy. Controlled trials are needed to evaluate the potential of HAMLET as a topical agent in bladder cancer patients.

References

1. Sengupta N, Siddiqui E, Mumtaz FH. Cancers of the bladder. *J R Soc Health* 2004;124:228–9.
2. Soloway MS, Sofer M, Vaidya A. Contemporary management of stage T1 transitional cell carcinoma of the bladder. *J Urol* 2002;167:1573–83.
3. Stein JP, Lieskovsky G, Cote R, Groshen S, Feng AC, Boyd S, Skinner E, Bochner B, Thangathurai D, Mikhail M, Raghavan D, Skinner DG. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. *J Clin Oncol* 2001;19:666–75.
4. Malmstrom PU. Advances in intravesical therapy of urinary bladder cancer. *Expert Rev Anticancer Ther* 2004;4:1057–67.
5. Schenkman E, Lamm DL. Superficial bladder cancer therapy. *Sci World J* 2004;4(Suppl 1):387–99.
6. Hudson MA, Herr HW. Carcinoma in situ of the bladder. *J Urol* 1995;153:564–72.
7. Lamm DL. Efficacy and safety of bacille Calmette-Guerin immunotherapy in superficial bladder cancer. *Clin Infect Dis* 2000;31(Suppl 3):S86–90.
8. Svensson M, Mossberg AK, Pettersson J, Linse S, Svanborg C. Lipids as cofactors in protein folding: stereo-specific lipid-protein interactions are required to form HAMLET (human α -lactalbumin made lethal to tumor cells). *Protein Sci* 2003;12:2805–14.
9. Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborg C. Apoptosis induced by a human milk protein. *Proc Natl Acad Sci USA* 1995;92:8064–8.
10. Svensson M, Håkansson A, Mossberg AK, Linse S, Svanborg C. Conversion of α -lactalbumin to a protein inducing apoptosis. *Proc Natl Acad Sci USA* 2000;97:4221–26.
11. Svensson M, Fast J, Mossberg AK, Durringer C, Gustafsson L, Hallgren O, Brooks CL, Berliner L, Linse S, Svanborg C. α -Lactalbumin unfolding is not sufficient to cause apoptosis, but is required for the conversion to HAMLET (human α -lactalbumin made lethal to tumor cells). *Protein Sci* 2003;12:2794–804.
12. Svensson M, Hakansson A, Mossberg AK, Linse S, Svanborg C. Conversion of α -lactalbumin to a protein inducing apoptosis. *Proc Natl Acad Sci USA* 2000;97:4221–6.
13. Fischer W, Gustafsson L, Mossberg AK, Gronli J, Mork S, Bjerkvig R, Svanborg C. Human α -lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival. *Cancer Res* 2004;64:2105–12.
14. Gustafsson L, Leijonhufvud I, Aronsson A, Mossberg AK, Svanborg C. Treatment of skin papillomas with topical α -lactalbumin-oleic acid. *N Engl J Med* 2004;350:2663–72.
15. Svanborg C, Agerstam H, Aronson A, Bjerkvig R, Durringer C, Fischer W, Gustafsson L, Hallgren O, Leijonhufvud I, Linse S, Mossberg AK, Nilsson H *et al.* HAMLET kills tumor cells by an apoptosis-like mechanism—cellular, molecular, and therapeutic aspects. *Adv Cancer Res* 2003;88:1–29.
16. Schamhart DH, de Boer EC, de Reijke TM, Kurth K. Urinary cytokines reflecting the immunological response in the urinary bladder to biological response modifiers: their practical use. *Eur Urol* 2000;37(Suppl 3):16–23.

Qualitative Analysis of Cancer Patients' Experiences Using Donated Human Milk

Susanne M. Rough, MS, RD, Pauline Sakamoto, MS, PHN, RN, Caroline H. Fee, MA, and Clarie B. Hollenbeck, PhD

Abstract

This represents the first published account from the patient's perspective of the use of human milk as cancer therapy. Purposive sampling was used to select a sample of 10 participants. Five were patients and 5 were family proxies. Individual interviews were conducted using confirmatory interviewing technique to obtain individual perspectives on the motivation for cancer patients to take donated human milk. Human milk therapy improved the quality of life (QOL) measures in the physical, psychological, and spiritual domains for most patients interviewed. The patients continued their use of human milk despite cost, taste, and discouragement from the conventional medical community. The study results support the theory that QOL may be more important to cancer patients than cancer outcomes and may improve patient medical care overall. These interviews offer information to cancer patients, their practitioners, and donor milk banks on outcomes and symptom relief from this therapy. *J Hum Lact.* 25(2):211-219

Keywords: cancer, human milk, donor milk banking, research, quality of life

Research supports that human milk provides not only a superior food for human infants but protective factors as well. While investigating the effect of human milk on bacterial adherence to a human lung cancer cell line

at Lund University in Sweden, Hakansson et al¹ made the serendipitous discovery that *in vitro*, human milk causes apoptosis, or programmed cell death, in several varieties of cancer cells. A few human *in vivo* research studies using a modified human milk protein complex realized similar effects on human cancer cells.^{2,3} (Human skin papilloma cells were studied in 1 case. Although human skin papilloma infections are usually benign, they may be premalignant.) Because of this knowledge, some people with cancer are requesting human milk. Between 1999 and the present, cancer patients have requested donor human milk from the San Jose Mother's Milk Bank (SJMMB) as a complement or alternative to their conventional biomedical cancer treatment. However, no information has been collected from these patients regarding their experience with the human milk therapy.

Cancer trials generally measure tumor response, overall survival, toxicity, and level of disease-free status. Complementary and alternative medicine (CAM) is most often used for conditions such as cancer for which no complete cure is offered by conventional medicine. When patients consider CAM, they are

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usually seeking quality of life even when a clinical cure is not expected. The purpose of the present qualitative study is to interview cancer patients who have taken human milk as part of their therapy, to determine their motivation for using human milk, to learn about the effect donor milk had on their quality of life, and to observe how taking donor milk fits into their overall treatment plan. We also asked about their use of other CAM therapies and their communication with their health care team regarding human milk therapy.

Methods

Research Subjects, Sampling, Setting

Because the goal of this study was description and exploration of human milk as a therapy for cancer, purposive sampling⁴ was used to access subjects or their family members who were able and willing to describe their experience with human milk therapy. Subjects were limited to cancer patients, aged 18 to 80 years, who had requested and were receiving donor milk from SJMMB from 1999 through 2005. Thirty-nine patients who received donor milk were contacted by US mail and asked to participate. Four letters were undeliverable, 1 patient refused to participate, and 23 did not respond to the request. All 23 were contacted again by mail with no new responses. Of the 11 recipients or family members who responded with signed consent forms, 1 became too ill to continue the study, leaving a final total of 10 participants from the SJMMB.

Personal interviews were conducted between the primary researcher and the human milk recipients. If the recipient was no longer alive, interviews were conducted with a family member or primary caregiver as proxy. Each interview lasted approximately 1 to 1.5 hours. One interview was conducted with each participant. If a person-to-person interview was not feasible, an interview was conducted by phone. All responses were included in the study analysis.

Instrument Design

No gold standard exists for quality of life (QOL) testing. Standard generic, general cancer-specific, and site-specific instruments were evaluated, but none were appropriate for human milk therapy administered to a diverse cancer population. The structured interview is useful when no previous data have been collected regarding a specific therapy, and it was thought to be the most appropriate data collection method for this study.

A unique instrument specific to human milk therapy was developed to focus the interview and to ensure consistency between interviews.

Instruments are organized into scales⁵ that measure a different aspect or domain of health-related QOL. Lindley⁶ reported that the QOL domains most influenced in patients with cancer are symptoms and side effects, social functioning, physical functioning, and psychological status. Economic factors and spirituality are often included. For this study, the investigators sought a multidimensional assessment by including the psychological, social, and physical domains. Prompting questions in the psychological domain included the effectiveness of the human milk treatments as perceived by the patient. Social domain questions assessed the patient's communication with health care providers, perceived barriers to use of this treatment, cost for the milk, duration and dose of human milk treatment, and sources for locating information on human milk therapy. Physical domain questions assessed the patient's symptoms before, during, and after taking the human milk therapy and the patient's response to all therapies. Demographic information was gathered for comparison to other health-related QOL measures, and the patient's stage and type of cancer before, during, and after human milk therapy were recorded from patient recall. The instrument was limited to 24 questions to avoid fatiguing the participant before completion.

The instrument was pilot tested before use with 3 cancer patients not participating in the study, 2 health care providers, 2 donor milk bank directors, and 2 nutritional science professors. Feedback from the group was used to revise the questionnaire.

Research Procedures

The initial interview question was broadly phrased to allow the patients to identify what they considered important about their cancer and treatment choices. Each participant was encouraged to explain his or her motivation for requesting human milk as CAM. The content and direction of the interviews were determined by the participant's responses, but a consistency among all interviews was established and maintained by a standard set of prompting questions. Although a combination of note taking and tape recording was used to record participants' responses, the researchers relied most on extensive notes taken during the interviews.

Confirmatory interviewing technique⁶ was used. At relevant points throughout each interview, the interviewer read a summary of the participant's key

Table 1. Comparison of Patients' Stage of Cancer Before and After Initiating Human Milk Therapy (HMT)

	Age, y	Gender	Daily Dose, oz	Duration of HMT During Study Period, mo	Other Therapies Used Along With HMT	Cancer Type	Cancer Stage at Initiation of HMT ^a	Cancer Stage at Conclusion of HMT ^a
A ^b	61	F	8-12 ^c	24	Vitamin C, shark's cartilage, milk thistle, sodium butyrate, turmeric, chemotherapy	Breast	Moderate	Advanced
B ^d	80	F	12-16	2	None	Lung	Advanced	Death
C ^b	53	M	2 ^c	62	Vitamin C, herbs, acupuncture, chiropractic, homeopathy, naturopathy, vitamins, minerals	Prostate	Early	Remission
D ^b	60	F	8-12 ^c	10	Herbs, supplements, music, prayer, acupuncture, essiac tea, bird's nest soup, chemotherapy, radiation	Lung	Moderate	Advanced
E ^d	81	M	7 ^c	1	None	Lung	Advanced	Death
F	70	M	3.5 ^c	37	None	Prostate	Early	Remission
G ^d	86	F	0-3.5	2	Wheatgrass, herbs from friends	Lung	Advanced	Death
H ^d	44	M	0-12	2	Radiation	Lymphoma	Advanced	Death
I ^d	54	M	8-12	22	Dietary changes, UCLA trial medication	Colon	Advanced	Death
J	68	F	8-12	8	Vitamins, minerals, chemotherapy, radiation	Breast	Moderate	Remission

^a Self-reported by patient or proxy.^b Received milk from sources other than San Jose Mother's Milk Bank.^c Consumed raw milk.^d Used proxy.

concepts to determine whether the notes accurately reflected the participant's experiences and to see whether the participant wanted to make any additional points or changes. In this way, the interviewer could clarify unclear areas without biasing the participants' answers. The interviews did not seek to prove or disprove a set theory. The questionnaire and the data collection protocol were approved by the San Jose State University Institutional Review Board for use of human subjects.

Analysis Method

Categories were defined for reporting results, and relevant themes were combined. Data were analyzed descriptively and are presented using frequency, median, and range. Nonnumerical data were analyzed conceptually by the primary researcher, who analyzed the themes and content and identified core concepts from the responses. Ten responses for each of the 24 questions were recorded and analyzed for trends and core concepts. Similar responses were tallied for each question, and personal comments or differences that did not fit a pattern were maintained in the narrative

format. When responses overlapped more than 1 category, they were included in the most relevant category.

Results

Participants' Characteristics

Of the 39 recipients of donor human milk, 11 recipients or proxies agreed to participate, but 1 relapsed before the interview took place and the patient declined to be interviewed. Five of the 10 respondents were patients who answered the interview on their own behalf, whereas 5 of the 10 were family or friends answering for a deceased recipient. Participants were equally divided between male and female and ranged in age from 44 to 86 years with a mean age of 65.7 years. All participants received some higher education, and half were postgraduates. Eight participants were Caucasian, 1 was Asian, and 1 was Middle Eastern. The primary cancer was lung ($n = 4$), followed by breast ($n = 2$), prostate ($n = 2$), colon ($n = 1$), and lymphoma ($n = 1$) (Table 1). Patients' perceived cancer symptoms are reported in Table 2.

Table 2. Perceived Cancer Symptoms Before and After Initiating Human Milk Therapy (HMT)

<i>Patient</i>	<i>Symptoms Before HMT</i>	<i>Symptoms After HMT</i>
A ^a	Patient had chemotherapy symptoms: tiredness, nausea, diarrhea. Patient was allergic to cow's milk.	Patient had no nausea, diarrhea, or tiredness and was not allergic to human milk.
B ^b	Patient had difficulty swallowing and eating because of size of tumor.	Patient had no difficulty swallowing human milk. Human milk was easier to digest, improved her QOL, and offered more nutrients. She tolerated liquids better than regular food.
C ^a	Patient had elevated PSA levels.	PSA levels dropped 1 month after initiating human milk therapy. No other supplements had changed during this time.
D ^a	Patient had trouble swallowing, was very sick and unable to work, and had no appetite.	Patient felt stronger and stayed in remission; cough decreased and respiratory function improved. Patient was able to exercise more to offset fatigue, had increased appetite, and gained weight. Milk donated by family and friends gave patient positive feeling.
E ^b	Patient was not a (cow) milk drinker.	Patient was energized by human milk. His energy was not depleted by cancer, and his spirits improved.
F	Patient practiced watchful waiting.	Patient continued watchful waiting.
G ^b	Data were unavailable.	Data were unavailable.
H ^b	Patient had no appetite, couldn't tolerate food, and was losing weight.	Patient requested and drank milk without coaxing after a couple days. Patient's stomach felt better; kept milk down. Patient didn't push milk away like other food.
I ^b	Patient had weight loss and diarrhea.	Patient had fewer colds and fever and less "gray" appearance. Weight was maintained.
J	Chemotherapy made patient feel miserable: nauseous, weak, wheezy.	Patient felt stronger and found it easier to get through chemotherapy. She had fewer colds. She feels well and cancer is gone.

Abbreviations: QOL, quality of life; PSA, prostate specific antigen.

^a Received milk from sources other than San Jose Mother's Milk Bank.

^b Used proxy.

Patient Progress

Two patients were in the early stages of their cancer when they started the human milk treatment, 3 were at moderate stages of cancer, and 5 were in advanced stages of cancer. Three of the patients were in remission, 2 advanced in their stage of cancer, and 5 had died. Of the 5 who died, 3 died within 1 year of initiating the human milk treatment, and 2 died within 2 months (Table 1).

Source of Information

All of the patients in this study acquired information about human milk therapy from Internet research. Four had some connection to a person in a human lactation-related field. One patient learned of the treatment through his prostate cancer support group. Two patients were also under the care of alternative therapy clinics that included human milk as part of their CAM treatment plan. The Web research was conducted by spouses and other family members and led them to studies conducted done by Hakansson et al¹ and Svensson et al⁷ as well as in vitro studies by Fischer et al² and Gustafsson et al.³

Dose and Duration

Prescriptions were obtained from a physician of choice by each patient to initiate human milk treatment.

No optimal dose has been determined experimentally, so doses varied from 59 to 355 mL (2-12 oz) per day. Patients received total quantities between 3 and 174 L (100 and 5891 oz) over the course of treatment, which varied from 6 to more than 338 weeks, amounting to a total of 584 L (19 733 oz or 154 gallons) of human milk for the entire study group. Dose and duration varied between participants but were related to stage of cancer at initiation of human milk treatment. The 5 patients who died during treatment were at terminal stages of cancer when they initiated human milk treatment, and they received the smallest overall quantity of milk for less than 3 months. Four patients continue to take human milk for prophylactic purposes. Two of the patients who are in remission and are taking the milk for prophylactic purposes have received the largest overall quantity of human milk for the longest period of time.

The exact dose of human milk consumed by these patients is not known. Four patients reported reducing their dose because of the cost of the milk. At \$3 US per ounce processing fee (plus shipping), human milk is relatively expensive, and 2 patients commented that it was the most expensive supplement they took. (This is the price participants paid for milk from the SJMMB. The processing fee range for milk banks that are part of Human Milk Bank Association of North America is

\$3-\$5 per ounce plus shipping.) Only 1 patient was able to bill his insurance, and 1 paid for the milk with disability payments.

All participants received human milk from the SJMMB, but 3 also received milk from other donors. The amount from other donors varied based on availability. One participant commented that it added spiritual value when the milk was a gift. Human milk from additional donors is not included in this study. Two recipients were refused milk from 5 other sources including other milk banks. The reason given was that human milk is not a proven cancer treatment.

Time of day for consuming the milk varied among participants, with 3 not taking it on a regular schedule, 4 taking it on an empty stomach at night, 1 taking it on an empty stomach in the morning, 1 taking it between meals, and 1 taking it after dinner.

Other Therapies

Seven participants took other CAM therapies in addition to the human milk, including vitamin C ($n = 2$) and acupuncture ($n = 2$). Other therapies included shark's cartilage, bird's nest soup, milk thistle, turmeric, wheatgrass, chiropractic, homeopathy, naturopathy, music, herbs, and vitamins. Two participants were being treated by alternative clinics, which planned a complementary regimen compatible with the chemicals in the conventional medical treatment and based on their laboratory results and symptoms. The clinics recommended a holistic plan including exercise to enhance the chemotherapy, music, prayer, appetite stimulants, hydration tips, and soothing supplements, with a stated goal of achieving peace of mind.

Although 5 of the 10 participants were receiving both conventional biomedicine and CAM treatment, 5 patients opted out of conventional treatment: 2 because of "watchful waiting" during the early stage of their cancer; 2 because radical surgery was the only other option, and 1 because she expected a decrease in QOL from conventional biomedicine. Two surviving patients planned to continue all multiple therapies because something was helping them improve, although they were not sure which treatment it was. One patient asked, "How much does belief help?"

Barriers to Use of Human Milk

Although all cancer patients informed their medical practitioners of their desire to take human milk, some met resistance from a medical professional who felt the therapy was clinically unproven or refused to look

at the *in vitro* research study results. One patient anticipated resistance from her radiologist and chose not to tell him. When the cancer patients met resistance, they reported finding a more supportive practitioner to write their prescription. They wondered aloud how the milk could hurt them or what they had to lose by eating a food safe enough for infant consumption. Some family members were skeptical but were aware of others taking the same treatment and did not interfere with this novel treatment. Four of the 5 terminal patients did not meet resistance. The patients reported that their practitioner supported the human milk therapy because they knew their patient was terminal and felt that this could not hurt them and might help.

Other potential barriers to the use of human milk were the cost, media censure, and taste. The average cost/processing fee of human milk from the SJMMB was \$3 per ounce, and shipping costs were applied to shipments more than 30 miles from the SJMMB. Some participants were criticized for taking milk away from babies. One participant discontinued drinking the milk because of the "awful" taste; 2 masked the taste of the pasteurized milk, describing it as an oily undertaste and a slightly cooked taste. Another patient said the human milk had a gamey taste and held her nose and swallowed the milk while reminding herself that it was healing. She also masked the taste with chocolate. Participants who did not like the taste of pasteurized milk signed waivers to receive raw milk. Five who received raw milk from the milk bank or from other sources reported that raw milk tastes delicious, sweet, and lively. One participant described the milk as thick but did not notice the taste. One participant did not like milk otherwise but liked the human milk in a smoothie. Two recipients could drink the human milk but reported being allergic to other milk. One participant's milk consumption was limited to when a particular caregiver was present.

Expectations

When asked to recall their expectations at the initiation of human milk therapy and to determine whether these expectations had been met, 6 cancer patients reported that they had no expectations. Two hoped that the therapy would strengthen and support their immune system, and they felt that it had. One had hoped for a reversal and that did not happen, and 2 thought it was a long shot and did not expect that it would cure their cancer, which it did not. For 3 patients it offered hope,

either that things would change or that their digestion would improve. One patient was looking for anything that helped. Donor milk was seen as a last-ditch effort when nothing else worked and was worth trying for a “one in a million chance of success.” One patient expected it to taste good and was not prepared for the taste she encountered.

Quality of Life Effects

When considering the more radical, invasive treatments, which might have prolonged her survival, 1 patient opted for the human milk therapy, because it had the potential to improve her QOL even though it was perceived to be an unproven cancer treatment. Another patient noted that although his cancer had not improved, it also had not progressed, and there were no other options. Two other patients noted that although human milk did not cure their cancer, their energy did increase. One patient believed that human milk would speed up and enhance the healing process. Another expected that the human milk therapy would improve his cancer stage, whereas others believed that the milk maintained their health and cited examples of human milk taken for prophylactic use or for stomach ulcers.

Patients' Assessment of Efficacy of Treatment

Only 1 patient felt that the human milk therapy had no health impact and would not use human milk treatment again unless testing proved its efficacy. The others had no expectation, considered the treatment experimental, and delayed their expectations while watchfully waiting. One participant would take human milk again if the taste was improved, and 1 participant described the human milk therapy recipients as desperate people looking for desperate measures—anything that helps. Another participant did not feel desperate, but he “did not want to die” either. He felt that if there is “a possible million to one chance that it might help,” a patient might want to try it. He proposed the unanswerable question: “Even if the human milk did not cure the cancer, did it stop it from progressing?” Without evidence, “the only benefit (of human milk therapy) is psychological. People are looking for hope. This is a pathway for hope and gives the patient something positive when most things about cancer are negative.”

Discussion

Human milk, according to cancer patients in this study, provided a well-tolerated, energizing therapy

that gave them hope for an improved QOL. Physical improvements listed by patients as positive effects of the treatment were numerous: increased energy, immune support, increased strength, improved respiratory function, and improved appetite. Three participants noted that human milk offered nutritional support and additional calories and that it eased the side effects chemotherapy had on their digestive system. The spiritual function of human milk therapy was mentioned when 1 patient asked, “How much does belief help?” Patients in this study agreed with results found by Shapira et al that “some patients may trade survival advantages for better QOL when presented with the choice of alternative outcomes.”^{8(p252)} These cancer patients chose to take donor human milk in an attempt to improve their medical care and QOL. Because this is not a randomized, controlled trial, the effects may not be attributable to human milk therapy.

In all cases, Internet research led to the research by Hakansson et al¹ and Svensson et al⁷ as well as *in vitro* studies by Fischer et al² and Gustafsson et al³; this research is provocative and suggests that treatment with modified human milk proteins has the potential for success as a cancer therapy. Information about CAM therapies is readily available on the Internet, but it is difficult for readers to separate reputable sources⁹ from unproven treatments. Human milk is a natural food for all infants and has a long history of safe use with premature and medically challenged infants. These factors may have accounted for its recommendation, despite a dearth of large-scale, *in vivo*, scientific studies.

Six patients in this study reported using multiple therapies in addition to human milk. Five of the patients did not use conventional cancer treatment while taking the human milk therapy. Verhoef et al¹⁰ found that 5% of cancer patients abandon conventional therapy for alternative methods. Terminally ill patients are reported to be more likely to take unproven, untested substitutes.¹¹ For 5 of the terminally ill patients in this study, conventional therapies had been exhausted or had unacceptable side effects or significant risks. Patients used CAM because their particular condition¹¹ could not be treated effectively with conventional medical treatment. Some of the patients in this study reported that the conventional treatment would have decreased their QOL, and their priority was QOL over the possible benefits of conventional treatment with negative side effects.

All participants were honest with their practitioner about taking the human milk therapy, unlike a previous study¹² which reported that CAM use is only disclosed

to physicians by 46% of cancer patients. The 100% disclosure rate in this study is likely because a prescription is required for receipt of human milk from donor milk banks.

Five of the 10 recipients met resistance from medical professionals when seeking a prescription, especially from oncologists and surgeons. The resistance was selective and determined by the stage of cancer. When patients in earlier stages of cancer met resistance, they were told that human milk therapy is an unproven treatment. Terminally ill patients' choice to take human milk was supported by medical professionals who stated that the therapy might help and could not hurt.

Participants in this study reported being criticized by physicians and a newscaster for taking human milk destined for premature infants. This appears to be an unsubstantiated opinion. The milk given to adults is mature milk (milk from mothers of older babies) and does not affect the supply for premature babies. Priority for distribution of donor human milk is determined by the Human Milk Bank Association of North America (HMBANA) guidelines.¹³ According to these guidelines, premature babies or term infants have priority over adult cancer patients in receipt of donor milk.

The total quantity of 584 L delivered to the 10 recipients in this study from September 1999 to December 2005 amounts to less than 2% of the total 28 389 L (958 936 oz) delivered to all recipients from SJMMB during this same period (data from the SJMMB annual report 2005). More important, the SJMMB average amounts represent total amounts requested and delivered and do not reflect the amount of milk available. The average amount of milk received by adult cancer patients from all 11 HMBANA member milk banks in 2005 would be less than 4.0% to 4.5% of donor milk distributed in that year (listed as an adult illness in data from the HMBANA annual report 2005. Adult illness includes cancer and immune deficiencies).

Six cancer patients in the present study said that the cost/processing fee for the milk including shipping was a reason for reducing the dose or seeking alternate sources of human milk. Eisenberg et al¹⁴ found that 58% of CAM users paid for alternative therapy entirely out of pocket. Even though 9 recipients could not bill their insurance for the processing fee for the donor milk, they continued to use this therapy. Previous studies^{9,14,15} also suggested that cost is not a deterrent to CAM use. The findings in this study suggest that some cancer patients were determined to use human milk therapy despite the high cost, lack of insurance

coverage, or discouragement by the conventional medical community. Barrett¹⁶ reported a national trend toward coverage of CAM therapies by insurers, mainly because of consumer demand. The insurers' main concern¹⁶ is lack of research on efficacy and lack of standards of practice. Randomized trials of human milk therapy are needed to evaluate the effectiveness of this potential therapy for various types of cancer. If human milk therapy is shown to be effective, insurance companies should include this therapy in their cancer treatment and QOL programs.

Study Limitations

There are some limitations to this study. The study is retrospective and subjective and depends on the patient's or proxy's memory and on the mode of questioning. Although the sample size is limited, the qualitative data provided by participants provide useful insights. QOL measurement and open-ended questioning generate large amounts of data. Combining this data in a concise way may have affected the interpretation of the results, and the process of tabulating subjective responses may introduce human errors.

Although the amount of milk delivered to the patients is known, the amount of milk consumed and the amount shared or received from other sources are not certain. As a result, no quantitative measurement of milk consumption is included in the study results. The amounts presented in this study may or may not be accurate, so it is difficult to draw conclusions based on them. Some patients took other treatments concurrently with the human milk, and all conventional cancer treatments varied. Because multiple therapies were taken, no results can be specifically attributed to human milk therapy.

Family proxies were needed for half of the responses in this study. According to the findings of Tang and McCorkle,¹⁷ cancer patients and their caregivers agree moderately well ($r > 0.60$) on the patient's QOL, and proxies can be considered reliable alternates as sources of data for terminal cancer patients on objective questions, whereas they showed moderate to poor agreement on subjective questions. Two of the 5 patients lived with the family proxy, and the other 3 proxies were in frequent contact with the patient. Given this close contact, their answers were likely to be in strong agreement for observable questions. The QOL answers in this study are subjective, and it is possible that they do not perfectly represent the patients' responses, because data obtained from a proxy may or

may not reflect the feelings the patient would have expressed. These study results may not be generalizable to other populations of cancer patients using human milk therapy.

Conclusions

The subjects in this study were willing to experiment with this unproven treatment despite the relatively high processing fee for the human milk, lack of insurance coverage for the fee, an unpleasant taste to some, and discouragement from the conventional medical establishment for others. Although the patients did not initially request the human milk for its life-enhancing effects, they found that it eased the side effects of cancer treatment and the ill effects of their condition. Dosage was reduced by some participants because of the high processing fee for the milk, possibly diluting the effect a higher dose or longer duration might have had on their results.

Human milk was found by cancer patients in this study to be well tolerated and nutrient dense. Some found that it eased their difficulties swallowing and was soothing to the gastrointestinal tract. This finding is very interesting and worth pursuing in future studies.

Participants reported that human milk reduced the nausea, weakness, and general lethargy that they experienced after initiating chemotherapy. Patients reported an improved level of function and physical improvements, such as increased energy, immune support, increased strength, improved respiratory function, and increased appetite, as a result of taking human milk therapy. Their recovery was full of uncertainty, and they sometimes felt like things were out of their control. This treatment option offered them hope and a sense of control and responsibility in their treatment plan.

Satisfaction with one's QOL may be more important than cancer outcomes and may improve a patient's medical care overall. Participants in this study who were in moderate to advanced stages of multiple cancers reported improvements in their QOL measures in the physical, psychological, and spiritual domains compared with what they experienced before taking the human milk treatment. Patients in early stages of cancer or in remission continued to take the human milk for prophylactic purposes because they felt it had improved their general well-being. In these cases, the human milk therapy was associated with a perception of improved QOL for the cancer patients.

Larger samples are needed from randomized trials that test the effectiveness of human milk in the treatment of human cancers. If human milk therapy is shown to be effective, insurance companies should include this therapy in their cancer treatment and QOL programs. Under HMBANA guidelines, premature and term infants are given priority over adult cancer patients for receipt of donor human milk. Currently, interested milk banks are able to meet the needs of cancer patients requesting donor human milk from available sources. It is unknown at this time whether other sources could be tapped to meet increased demands for human milk.

Three recipients in this study supplemented their human milk supply with milk from nursing mothers in their community (HMBANA does not recommend use of milk from unscreened donors). It is possible that human milk is more widely used than is known through milk bank data. Surveys of area clinics and social organizations are needed to determine a more representative number of total requests for human milk by cancer patients. More data would reveal a trend of the total number of requests for human milk and would clarify whether total requests for human milk are increasing, decreasing, or staying the same. This larger pool of data would offer a clearer picture of cancer patients' value for human milk therapy and would confirm whether there is a need for large-scale, *in vivo* scientific studies on the effects of human milk therapy on cancer patients' QOL.

References

1. Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborg C. Apoptosis induced by a human milk protein. *Proc Natl Acad Sci U S A*. 1995;92:8064-8068.
2. Fischer W, Gustafsson L, Mossberg AK, et al. Human alpha-lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival. *Cancer Res*. 2004;64:2105-2112.
3. Gustafsson L, Leijonhufvud I, Aronsson A, Mossberg AK, Svanborg C. Treatment of skin papillomas with topical alpha-lactalbumin-oleic acid. *N Engl J Med*. 2004;350:2663-2672.
4. Caspi O, Koithan M, Criddle M. Alternative medicine or "alternative" patients: a qualitative study of patient-oriented decision-making processes with respect to complementary and alternative medicine. *Med Decis Making*. 2004;24:64-79.
5. Litwin M, Fitzpatrick JM, Fossa S, Newling DWW. Defining an international research agenda for quality of life in men with prostate cancer. *Prostate*. 1999;41:58-67.
6. Lindley C. Quality of life measurements in oncology. *Pharmacotherapy*. 1992;12:346-352.
7. Svensson M, Durringer C, Hallgren O, Mossberg AK, Hakansson A, Svanborg C. HAMLET—a complex from human milk that induces

- apoptosis in tumor cells but spares healthy cells. *Adv Exp Med Biol.* 2002;503:125-132.
8. Schapira MM, Lawrence WF, Katz DA, McAuliffe TL, Nattinger AB. Effect of treatment on quality of life among men with clinically localized prostate cancer. *Med Care.* 2001;39:243-253.
 9. Cassileth BR, Deng G. Complementary and alternative therapies for cancer. *Oncologist.* 2004;9:80-89.
 10. Verhoef MJ, Hilsden RJ, O'Beirne M. Complementary therapies and cancer care: an overview. *Patient Educ Couns.* 1999;38:93-100.
 11. Spence M, Ribeaux P. Complementary and alternative medicine: consumers in search of wellness or an expression of need by the sick? *Psychology and Marketing.* 2004;21:113-139.
 12. Boon H, Stewart M, Kennard MA, et al. Use of complementary/alternative medicine by breast cancer survivors in Ontario: prevalence and perceptions. *J Clin Oncol.* 2000;18:2515-2521.
 13. Human Milk Bank Association of North America. *Guidelines for the Establishment and Operation of a Donor Human Milk Bank.* Raleigh, NC: HMBANA; 2005:24.
 14. Eisenberg DM, Davis RB, Ettner SL, et al. Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. *JAMA.* 1998;280:1569-1575.
 15. Harris P, Finlay I, Cook A, Thomas KJ, Hood K. Complementary and alternative medicine use by patients with cancer in Wales: a cross sectional survey. *Complement Ther Med.* 2003;11:249-253.
 16. Barrett B. Complementary and alternative medicine: what's it all about? *Wis Med J.* 2001;100:20-26.
 17. Tang ST, McCorkle R. Use of family proxies in quality of life research for cancer patients at the end of life: a literature review. *Cancer Invest.* 2002;20:1086-1104.



OPEN ACCESS

ORIGINAL ARTICLE

Prevention and treatment of colon cancer by peroral administration of HAMLET (human α -lactalbumin made lethal to tumour cells)

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ABSTRACT

Background Most colon cancers start with dysregulated Wnt/ β -catenin signalling and remain a major therapeutic challenge. Examining whether HAMLET (human α -lactalbumin made lethal to tumour cells) may be used for colon cancer treatment is logical, based on the properties of the complex and its biological context.

Objective To investigate if HAMLET can be used for colon cancer treatment and prevention. *Apc*^{Min/+} mice, which carry mutations relevant to hereditary and sporadic human colorectal tumours, were used as a model for human disease.

Method HAMLET was given perorally in therapeutic and prophylactic regimens. Tumour burden and animal survival of HAMLET-treated and sham-fed mice were compared. Tissue analysis focused on Wnt/ β -catenin signalling, proliferation markers and gene expression, using microarrays, immunoblotting, immunohistochemistry and ELISA. Confocal microscopy, reporter assay, immunoprecipitation, immunoblotting, ion flux assays and holographic imaging were used to determine effects on colon cancer cells.

Results Peroral HAMLET administration reduced tumour progression and mortality in *Apc*^{Min/+} mice. HAMLET accumulated specifically in tumour tissue, reduced β -catenin and related tumour markers. Gene expression analysis detected inhibition of Wnt signalling and a shift to a more differentiated phenotype. In colon cancer cells with APC mutations, HAMLET altered β -catenin integrity and localisation through an ion channel-dependent pathway, defining a new mechanism for controlling β -catenin signalling. Remarkably, supplying HAMLET to the drinking water from the time of weaning also significantly prevented tumour development.

Conclusions These data identify HAMLET as a new, peroral agent for colon cancer prevention and treatment, especially needed in people carrying APC mutations, where colon cancer remains a leading cause of death.

INTRODUCTION

Colon cancers remain a major therapeutic challenge. Although the 5-year survival rate is extremely favourable after early detection and treatment of localised tumours, most colorectal cancers are either locally or distantly invasive at the time of diagnosis, limiting treatment options and lowering survival.¹ About 160 000 cases of colorectal cancer are diagnosed each year and 60 000 patients die of the disease in the USA alone.^{2–3} Worldwide, the number of deaths related to colon cancer has been estimated

Significance of this study**What is already known about this subject?**

- Colon cancer accounts for about 600 000 deaths annually, worldwide.
- More than 80% of sporadic and all hereditary colorectal tumours show a loss of function, owing to truncating mutations in the APC gene.
- Mice carrying the *Apc*^{Min/+} allele with a nonsense point mutation at nucleotide 2549 spontaneously develop multiple intestinal polyps. *Apc*^{Min/+} mice have therefore been extensively used as a model of familial and sporadic colorectal cancer.
- Inhibition of Wnt/ β -catenin signalling has therefore been proposed as a therapeutic approach in colon cancers.
- Human α -lactalbumin made lethal to tumour cells (HAMLET) is a complex of α -lactalbumin and oleic acid with broad antitumour activity.

What are the new findings?

- Peroral HAMLET administration acts therapeutically, by reducing the tumour burden and prolonging survival.
- Supplying HAMLET in the drinking water from weaning acts prophylactically and inhibits tumour development.
- HAMLET accumulates in intestinal tumours rather than in adjacent, healthy tissue.
- HAMLET suppresses β -catenin-dependent transcription.
- The effects of HAMLET were ion channel-dependent, defining a new mechanism modulating β -catenin signalling and survival in colon cancer cells.

How might it impact on clinical practice in the foreseeable future?

- Treatment of established colon cancer remains a major challenge. In addition, efficient preventive measures other than surgery are not available to families genetically prone to this disease. Here we identify HAMLET as a new, efficient therapeutic agent active against colon cancer. The results also suggest a new approach to colon cancer prevention.

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by the WHO at about 600 000. The familial forms of colon cancer (familial adenomatous polyposis

Colon cancer

coli (APC)) are caused by a loss of APC function, often owing to truncating mutations in the gene,⁴ and more than 80% of all sporadic colorectal tumours show similar mutations. Tumour formation is initiated by a second, somatic mutation of the wild-type APC allele and with time, the tumours will accumulate additional mutations, affecting, for example, KRAS and p53.⁴ The APC point mutation is sufficient for tumour initiation, however, and mice carrying the *Apc*^{Min/+} allele with a nonsense point mutation at nucleotide 2549 spontaneously develop multiple intestinal polyps. *Apc*^{Min/+} mice have therefore been extensively used as a model of familial and sporadic colorectal cancer.⁵

APC acts as a tumour suppressor by operating as a scaffold protein in the β -catenin destruction complex. Together with axin, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3), the APC complex phosphorylates the oncoprotein β -catenin for subsequent ubiquitination and proteasomal degradation. APC mutations reduce β -catenin proteolysis and enhance its nuclear translocation,^{6,7} resulting in overactivation of the Wnt/ β -catenin signalling pathway. Increased nuclear translocation of β -catenin and interactions with TCF/LEF (T-cell factor/lymphoid enhancer binding factor) family proteins stimulate β -catenin-dependent gene expression, including *cyclin D1*, *VEGF* and *COX-2*, thereby increasing proliferation and growth.⁸

HAMLET (human α -lactalbumin made lethal to tumour cells) consists of partially unfolded α -lactalbumin and oleic acid and is the first member of a new family of tumouricidal unfolded protein-lipid complexes.⁹ HAMLET kills many types of tumour cells in vitro¹⁰ and this tumouricidal activity is maintained in vivo.^{11–13} Tumour cells are broadly sensitive to HAMLET, reflecting shared molecular features resulting from oncogenic transformation.¹⁴ In lung carcinoma cells, the sensitivity to HAMLET's tumouricidal effect reflected the level of c-Myc expression, and shRNA silencing of c-Myc or Ras pathway members conferred resistance to HAMLET. Furthermore, HAMLET sensitivity was reduced by shRNAs targeting glycolytic enzymes like hexokinase 1,¹⁴ and by metabolomic analysis a rapid metabolic paralysis was detected in tumour cells after HAMLET treatment.

Examining whether HAMLET may be used for colon cancer treatment is logical, based on the properties of the complex and its biological context. The constituents of HAMLET are present in human milk, where the complex is formed after unfolding of the protein and hydrolysis of oleic acid from milk triglycerides. At low pH, HAMLET may be assembled, for passage through the gastrointestinal tract of the breast-fed child. In vitro studies have shown that HAMLET is protease resistant,¹⁵ suggesting that by surviving the harsh gastrointestinal environment HAMLET may reach and kill cells that are sensitive to its effects, including colon cancer progenitors. This study investigated whether HAMLET acts as a colon cancer therapeutic agent, using peroral administration in the *Apc*^{Min/+} mouse model. In view of the stepwise tumour development taking place in this model, we also explored if HAMLET acts prophylactically in genetically susceptible mice. The results clearly demonstrate prophylactic and therapeutic effects of HAMLET, as well as effects on the Wnt/ β -catenin signalling pathway.

METHODS

HAMLET production

HAMLET was produced as described.¹⁶ Briefly, native α -lactalbumin was purified from human milk by hydrophobic interaction chromatography. The protein was unfolded with EDTA, subjected to ion-exchange chromatography on a matrix preconditioned with oleic acid and eluted with a high salt

concentration solution. HAMLET was lyophilised after purification and kept frozen until use.

In vivo treatment with HAMLET

Apc^{Min/+} mice were obtained from Jackson Laboratories. For the HAMLET therapeutic protocol, 8–10-week-old male mice were orally gavaged with 10 mg of HAMLET in 400 μ l phosphate-buffered saline (PBS), twice daily for 10 days. Mice were not given water or food for 5 h before HAMLET administration. Food and water were provided 30 min after HAMLET oral administration. Sham-treated mice were gavaged with 400 μ l PBS. Mice were killed 5 weeks after the end of HAMLET treatment and intestinal tissue samples were collected for further analysis. A similar treatment was used in the survival study groups where mice were observed until 40 weeks of age.

For HAMLET prophylaxis, 5-week-old *Apc*^{Min/+} mice were provided with HAMLET (10 mg/day) in the drinking water for 10 weeks and killed at 15 weeks of age. The HAMLET dosage of 10 mg/400 μ l twice daily was selected based on positive results in previous clinical studies, where a concentration of 10 mg/ml was effective in topical application on skin papillomas¹³ and 25 mg/ml for topical instillation in the urinary bladder where most of the substance is eliminated by voiding.¹²

All animal protocols and procedures were reviewed and approved by the institutional animal experimental ethics committee at the Lund District Court, Sweden.

Tumour enumeration and sample collection

Mice were killed by CO₂ inhalation. The entire gastrointestinal tract was removed and kept in ice-cold PBS for dissection. The stomach and colon were not included in the analysis owing to low tumour incidence. The small intestine was cut into three equal-length segments. To remove intestinal contents, each segment was flushed with ice-cold PBS with the help of a gavage needle attached to a syringe. Each segment was kept on a filter paper and longitudinally opened. Tumour numbers and size were determined using a dissecting microscope (Olympus).

In vivo HAMLET uptake

After 5 h of starvation, mice were orally gavaged with 10 mg HAMLET in 400 μ l PBS. After 2, 4 and 6 h of HAMLET administration, mice were killed with CO₂ inhalation. To detect HAMLET in the intestinal lumen, 5 cm intestinal segments from the upper or lower small intestine were harvested and washed with 500 μ l of ice-cold PBS. Sodium dodecyl sulphate lysis buffer (2%) was added in the intestinal washing, heated (5 min, 95°C) and centrifuged at high speed for 10 min. Supernatant was collected and used for western blotting for the detection of HAMLET using anti- α -lactalbumin antibodies (GeneTex, Inc).

For the detection of HAMLET in tumour tissue, intestinal segments were flushed thoroughly (5 ml PBS, $\times 3$) to remove luminal contents. Segments were longitudinally opened and again washed in PBS (5 ml PBS, $\times 3$). Tumour and healthy tissue were further used for western blot detection of HAMLET.

Methylene blue staining

The opened intestinal segments were spread flat between sheets of filter paper and fixed overnight in 10% neutral buffered formalin. Formalin-fixed sections were transferred to 70% ethanol and stained with 0.2% methylene blue. Stained sections were rinsed in deionised water and imaged by dissecting microscope.

Cell transfection and luciferase assay

For reporter gene assay, DLD1 cells were seeded in 96-well plates and a Cignal TCF/LEF Reporter (Luciferase) kit (SABiosciences) was used, according to manufacturer's recommendations.

Holographic microscopy

For phase holographic imaging, cells were cultured overnight in a tissue culture flask and treated with HAMLET. Images were captured at different time points from the same spot on the flask. The HoloMonitor M2 digital holographic microscope (Phase Holographic Imaging AB, Lund, Sweden) records the three-dimensional structure of cells by their exposure to 0.8 mW HeNe laser (633 nm) using interfering wave fronts induction. The interference pattern was recorded as a hologram on a digital sensor.

Statistics

All in vitro experiments were repeated at least three times. Data are expressed as means $\pm$ SEM. The differences between control and treatment groups were determined by analysis of variance followed by *t* tests. Differences in survival were evaluated by Kaplan–Meier analysis with a log-rank (Mantel–Cox) test.

Additional methods

Additional methods are described in the online supplementary methods.

RESULTS

HAMLET reduces intestinal tumour burden and increases survival

HAMLET was administered perorally to tumour-bearing *Apc^{Min/+}* mice (8–10 weeks of age) and tumour development was assessed in tissues obtained 5 weeks after the end of treatment (figure 1A). Sham-fed mice receiving PBS as gavage were used as controls. Peroral HAMLET administration caused a significant reduction in the number of small intestinal tumours and in tumour size compared with sham-treated mice (figure 1B–E). In intestinal segments opened longitudinally, the total number of polyps was reduced by about 58% ($p < 0.0001$) (figure 1E). A reduction in polyp number and size was also evident in methylene blue-stained intestinal segments (figure 1C) and in haematoxylin and eosin-stained 'Swiss roll' sections (figure 1D). Intestinal villus hyperplasia was noticed in untreated mice but treated mice showed normal villi (see online supplementary figure S1). Importantly, the peroral HAMLET-treated *Apc^{Min/+}* mice showed significantly improved survival compared with sham-treated mice ($p = 0.0103$) (figure 1F). The frequency of

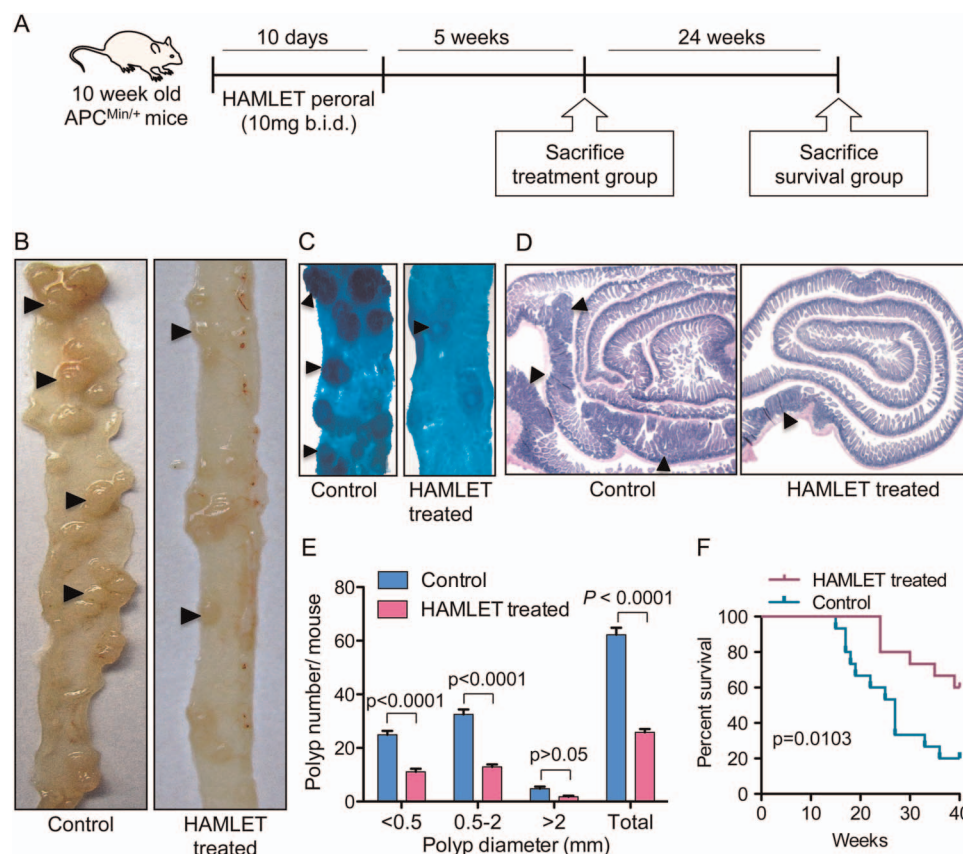


Figure 1 Human α -lactalbumin made lethal to tumour cells (HAMLET) treatment reduces intestinal tumour progression and increases survival. (A) *Apc^{Min/+}* mice were treated orally with 10 mg of HAMLET, twice daily for 10 days. The mice were killed 5 weeks after the end of treatment or observed for survival until 40 weeks of age. (B) Dissection photomicrographs of small intestinal segments showing tumours (arrowheads) in HAMLET-treated or control mice. (C) Methylene blue stained whole mounts of intestinal segments (arrowheads, tumours). (D) Haematoxylin and eosin stained intestinal Swiss roll sections showing smaller and fewer polyps in HAMLET-treated *Apc^{Min/+}* mice than in controls. (E) Quantification of the therapeutic effect on tumour number and size. A significant reduction of intestinal tumour load ($p < 0.0001$ for total tumour count) was seen in HAMLET-treated mice ($n=10$) compared with sham-treated mice ($n=10$) (two-way analysis of variance test). Error bars represent $\pm$ SEM. (F) Kaplan–Meier survival analysis. The end point was reached when mice were moribund or at 40 weeks. HAMLET-treated mice ($n=15$) showed improved survival compared with sham-treated mice ($n=15$). $p=0.0103$, log-rank (Mantel–Cox) test.

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colonic polyps is usually low in *Apc^{Min/+}* mice.¹⁷ As the frequency in this study was <1 lesion per mouse, no reliable comparisons between HAMLET-treated and sham-fed mice were made.

Reduced levels of β -catenin pathway proteins in treated tumours

To examine if HAMLET modifies the Wnt/ β -catenin signalling pathway, β -catenin and β -catenin-regulated proteins were quantified in tumour biopsy specimens (figure 2A). β -Catenin staining was markedly reduced in HAMLET-treated tumour tissues compared with controls, and this difference was confirmed by western blotting on tumour tissue homogenates (figure 2B).

Furthermore, the expression of proteins downstream from β -catenin was also affected (figure 2A). Cyclin D1 staining was reduced in HAMLET-treated mice and this effect was confirmed by western blotting ($p=0.003$). VEGF and COX-2 immunostaining and western blotting showed a similar decrease (figure 2A,B), and the results were confirmed by ELISA of tissue homogenates (figure 2D,E). Finally, a marked reduction in the frequency of Ki-67-positive cells ($p=0.001$) suggested that HAMLET inhibits tumour cell proliferation (figure 2A,C).

HAMLET targets tumour cells in vivo and is resistant to gastric enzymes

The presence of HAMLET in tumour tissue was visualised in sections obtained 4 h after HAMLET administration. HAMLET was shown to accumulate in the tumours (figure 2F), with strong nuclear staining in individual tumour cells. In contrast, healthy cells adjacent to the tumour did not take up HAMLET. Differences in HAMLET content between tumours and healthy tissue were confirmed by western blotting (figure 2G).

To examine if HAMLET resists proteolytic degradation in vivo, gastrointestinal contents harvested after HAMLET gavage were subjected to western blotting, using α -lactalbumin-specific antibodies (figure 2H). Bands of 14 kDa were detected in the contents of the stomach and distal small intestine, 6 h after oral administration, suggesting that the HAMLET complex remains intact in the gastrointestinal tract.

HAMLET modifies β -catenin structure and localisation in colon cancer cells

To further examine how HAMLET affects β -catenin and Wnt signalling, we used the human colon cancer cell line DLD1, carrying a homozygous mutation that inactivates the APC tumour suppressor.¹⁸ The susceptibility to HAMLET was first assessed by real-time holographic imaging (figure 3A). A time-dependent decrease in cell area ($p<0.0001$) and an increase in maximum thickness were seen ($p<0.0001$). The rapid reduction in viability was confirmed as a drop in ATP levels ($p<0.01$) and Presto blue staining ($p<0.001$) (figure 3B).

A reduction in β -catenin, cyclin D1 and VEGF levels was detected by western blot analysis (figure 3C). By confocal microscopy, a rapid change in β -catenin distribution was also observed. Nuclear β -catenin staining was lost and cytoplasmic staining was reduced ($p<0.0001$) (figure 3D). To examine whether this loss of nuclear β -catenin influences gene expression, TCF/LEF reporter activity was quantified using the TOP-flash dual luciferase assay. A dose-dependent reduction in luciferase levels was detected ($p<0.05$) (figure 3E).

In parallel, β -catenin was shown to accumulate at the cytoplasmic membrane (figure 3D,F). As β -catenin binds to the intracellular domain of E-cadherin,¹⁹ HAMLET-treated cells were double-stained. Specific colocalisation of β -catenin and E-cadherin

was detected at the cytoplasmic membrane in HAMLET-treated cells but not in control cells (figure 3F). By E-cadherin pull-down, an increase in bound β -catenin was detected, confirming this interaction (see online supplementary figure S2). E-cadherin expression remained unchanged after HAMLET treatment (see online supplementary figure S3).

Significant β -catenin fragmentation was also detected in HAMLET-treated DLD1 cells (figure 4A,B). As caspase-3-dependent β -catenin fragmentation has been reported,²⁰ caspase-3 activation was quantified (see online supplementary figure S4). Rapid activation of caspase-3 was detected and inhibition by zVAD confirmed the involvement in β -catenin fragmentation (figure 4C). The calpain inhibitor leupeptin had no effect. HAMLET did not significantly affect p-GSK $3\alpha/\beta$ which phosphorylates β -catenin for proteasomal degradation²¹ (see online supplementary figure S5). Zhao *et al*²² recently reported that AMP-activated protein kinase (AMPK) responds to the loss of ATP by phosphorylating β -catenin. We therefore examined if HAMLET alters AMPK expression, but found no change after 3 h (see online supplementary figure S6). Treatment with α -lactalbumin alone showed no effect on cellular ATP levels in DLD1 cells (see online supplementary figure S7A). Moreover, α -lactalbumin or oleic acid treatment failed to induce any significant differential expression of genes in DLD1 cells (see online supplementary figure S7B).

Ion fluxes triggered by HAMLET explain effects on β -catenin

Ion channels support oncogenic transformation,²³ and in other tumour cells, we have observed that specific ion fluxes activated by HAMLET explain many aspects of the tumouricidal response (Storm *et al*, unpublished). To further examine the mechanism of β -catenin fragmentation, DLD1 cells were pretreated with the ion channel inhibitors amiloride or barium chloride (BaCl_2). Both inhibitors blocked β -catenin fragmentation (figure 4C), the activation of caspase-3 (figure 4D) and the loss of nuclear β -catenin (figure 4E). In comparison, zVAD only affected nuclear β -catenin levels in about 30% of the cells, suggesting that while ion fluxes may activate caspase-dependent mechanisms, caspase-independent mechanisms are also involved (see online supplementary figure S8). Furthermore, cell death was prevented by the ion channel blockers ($p<0.001$) but not by zVAD (figure 4F). The activation of ion fluxes by HAMLET was confirmed in DLD1 cells preloaded with Ca^{2+} or K^+ fluorophores (figure 4G).

The effects of HAMLET on cell viability and the fragmentation of β -catenin were confirmed in SW480 colon cancer cells carrying the APC mutations (see online supplementary figure S9).

HAMLET acts as a prophylactic agent against intestinal cancer

To examine if HAMLET may be used prophylactically, the drinking water of *Apc^{Min/+}* mice was supplemented with HAMLET (10 mg/mouse) daily, from the time of weaning until 15 weeks of age (figure 5A). A marked reduction in tumour development was seen compared with controls receiving non-supplemented drinking water. A significant reduction in the number and size of polyps was seen ($p<0.0001$) (figure 5B–D). Haematoxylin and eosin staining of 'Swiss roll' sections confirmed these differences (see online supplementary figure S10). In tumour biopsies (figure 5E), β -catenin staining was reduced by HAMLET as was cyclin D1 and COX-2 staining, confirming effects seen in the therapeutic group (figure 2).

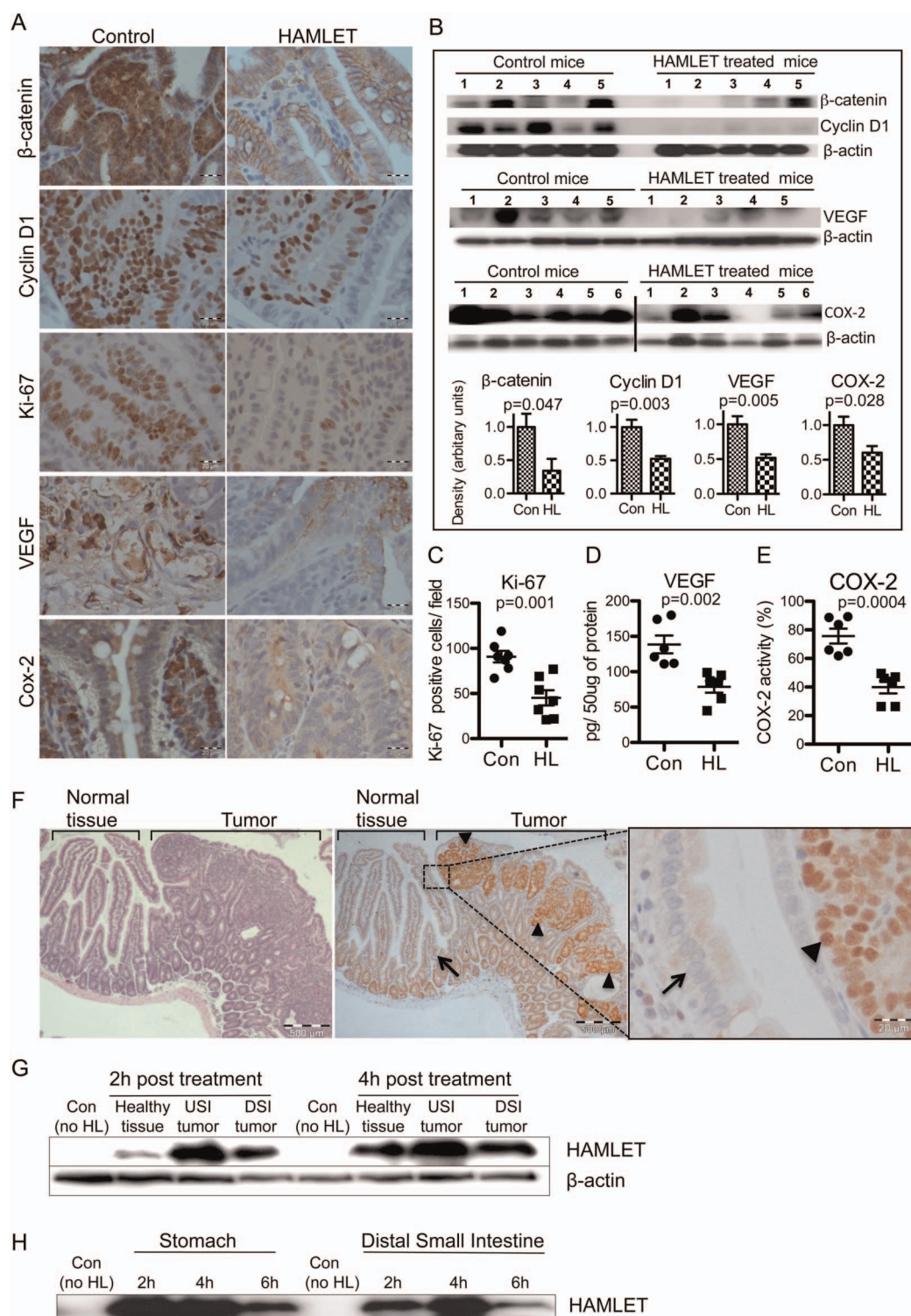


Figure 2 Selective human α -lactalbumin made lethal to tumour cells (HAMLET) uptake and reduced β -catenin pathway proteins and proliferation markers. (A) Representative immunostained sections demonstrating reduced β -catenin, cyclin D1, Ki-67, VEGF and COX-2 levels in tumours from HAMLET-treated compared with sham-treated mice (scale bars, 20 μ m). (B) Western blots confirming significant reduction of β -catenin ($p=0.047$), cyclin D1 ($p=0.003$), VEGF ($p=0.005$) and COX-2 ($p=0.028$). Histograms show the mean densitometry values for the blots of each protein (t test, $n=5-6$). (C) Quantification of Ki-67 positive cells (t test, $n=5$). (D, E) Quantification of VEGF and COX-2 activity in tumour lysates by ELISA shows significant reduction (t test, $n=6$). (F) Representative immunostaining showing HAMLET uptake (arrowheads, intense HAMLET staining) by tumour tissue, 4 h after HAMLET treatment (left and middle scale bars, 500 μ m; right, 20 μ m). Neighbouring healthy tissue (arrow) shows low HAMLET uptake. Some crypt cells show weak nuclear HAMLET staining, suggesting a low degree of uptake by these rapidly proliferating, healthy cells. (G) Quantification of HAMLET uptake by western blots of small intestinal lysates. Small intestinal tumour tissues show higher HAMLET content than the healthy tissue. β -actin was the loading control. USI, upper small intestine; DSI, distal small intestine. (H) Stability of HAMLET in the intestine. After oral HAMLET gavage (2, 4 and 6 h), luminal contents were collected from the stomach and small intestinal segments for western blotting using anti- α -lactalbumin antibody. In all panels, error bars represent $\pm$ SEM.

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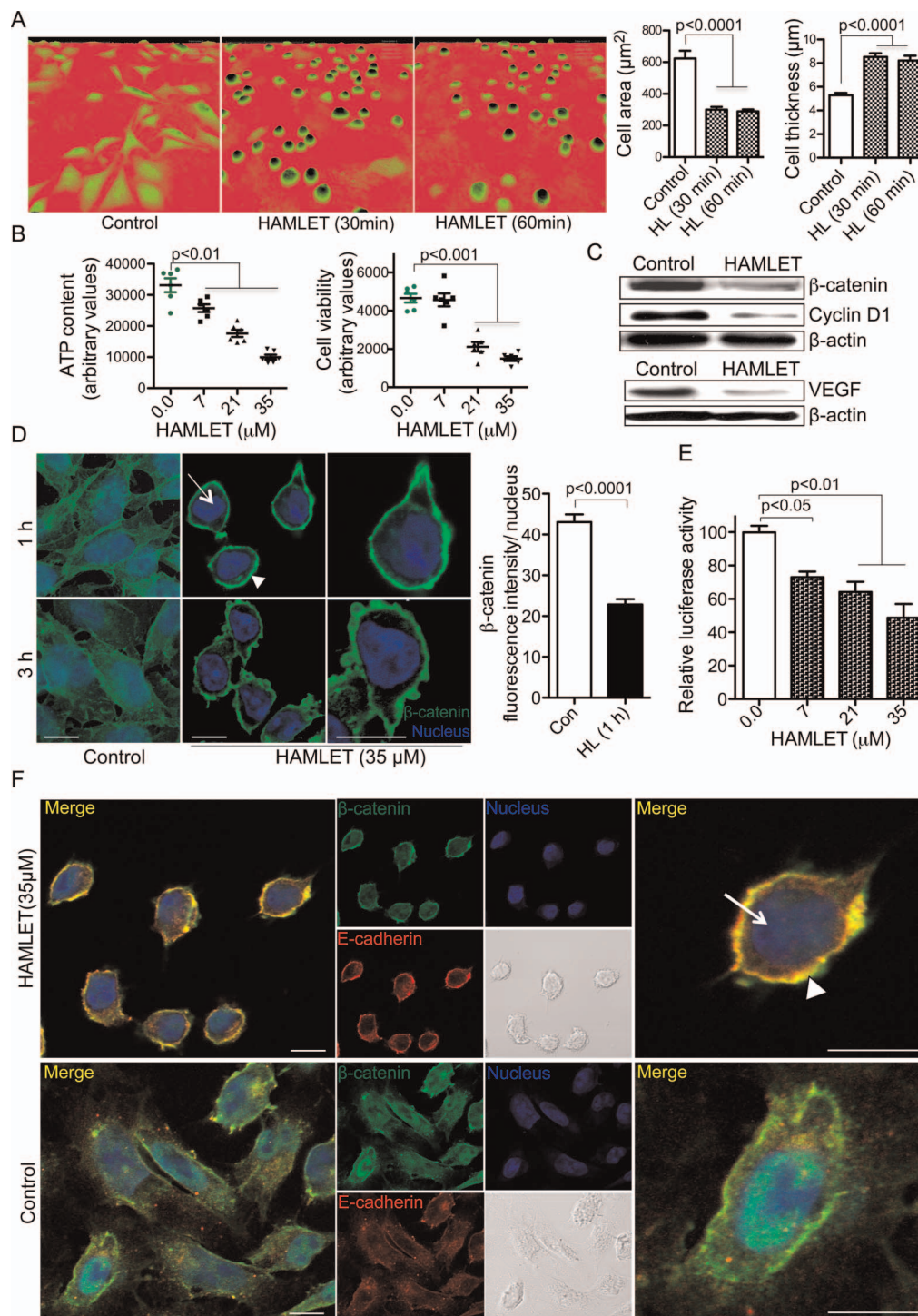


Figure 3 Effects of human α -lactalbumin made lethal to tumour cells (HAMLET) on Wnt/ β -catenin pathway in vitro. APC mutated, DLD1 human colon cancer cells were exposed to HAMLET. (A) Time-dependent morphological changes, detected by phase contrast holographic microscopy. Significant reduction in surface area ($p < 0.0001$) and increase in thickness ($p < 0.0001$) are quantified (one-way analysis of variance (ANOVA) test, $n=3$). (B) Dose-dependent loss of viability after HAMLET treatment (3 h), quantified by ATP measurements ($p < 0.01$) and Presto blue ($p < 0.001$) (one-way ANOVA test, $n=3$). (C) Decrease in β -catenin, cyclin D1 and VEGF protein levels after HAMLET treatment (3 h, 35 μM). Western blot, with β -actin as loading control. (D) DLD1 cell monolayers were treated with HAMLET (35 μM , 1 and 3 h), fixed and immunostained for β -catenin (green). Nuclei were counterstained with DRAQ5 (blue). HAMLET significantly reduced nuclear β -catenin staining in treated cells ($p < 0.0001$) (t test, $n=3$). The uniform cytoplasmic staining was replaced by strong membrane staining (scale bars, 10 μm). (E) TCF/LEF (T-cell factor/lymphoid enhancer binding factor) reporter activity quantified by TOP-flash dual luciferase assay. A dose-dependent reduction in luciferase activity was detected after 3 h of HAMLET treatment ($p < 0.01$ and $p < 0.05$) (one-way analysis of variance test, $n=3$). (F) HAMLET enhances colocalisation of β -catenin and E-cadherin on the cell membrane. Cells exposed to HAMLET (35 μM , 3 h) were double stained for β -catenin (green), E-cadherin (red) and DRAQ5 (blue) as a nuclear staining agent. Both β -catenin and E-cadherin were intensely colocalised at the cell membrane with increase in β -catenin staining (scale bars, 10 μm). In all panels, error bars represent $\pm$ SEM.

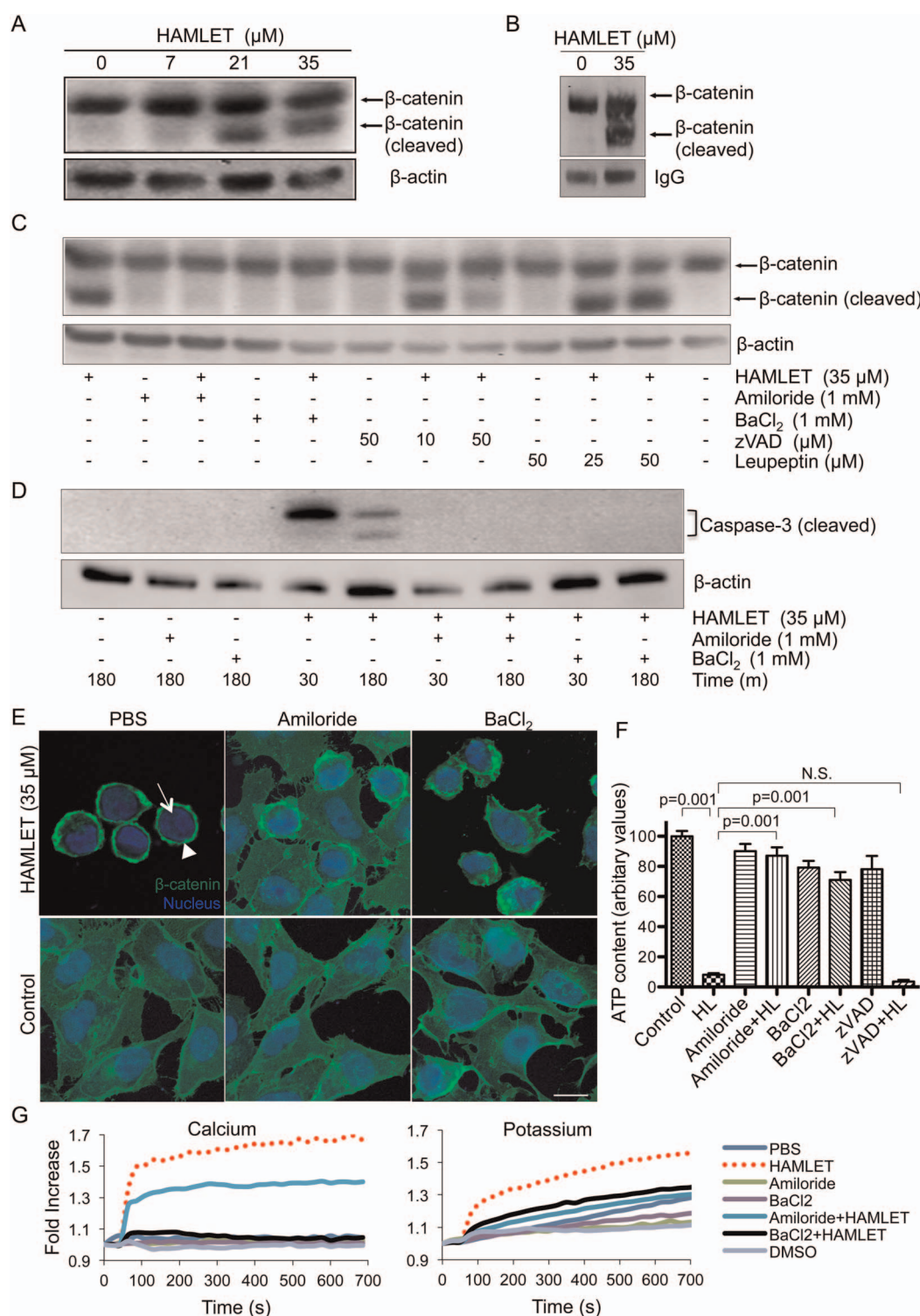


Figure 4 Ion channel-dependent degradation and nuclear translocation of β -catenin in human colon cancer cells DLD1. (A) Dose-dependent degradation of β -catenin in DLD1 cells after human α -lactalbumin made lethal to tumour cells (HAMLET) treatment (3 h). β -actin was the loading control. (B) Immunoprecipitation of HAMLET-treated cells (35 μ M, 1 h). β -catenin was pulled down and subjected to western blot. Cleavage of β -catenin was seen after HAMLET treatment. (C) DLD1 cells were pretreated with ion channel inhibitors (amiloride and BaCl₂), caspases (zVAD) or calpains (leupeptin) as indicated and subsequently HAMLET-treated (35 μ M, 1 h). Ion channel inhibitors completely, and zVAD partially, abrogated HAMLET-induced β -catenin cleavage but not leupeptin. (D) Western blot demonstrating ion channel-dependent caspase activation by HAMLET. Ion channel inhibitors (amiloride and BaCl₂) completely abrogated HAMLET-induced caspase-3 activation. (E) HAMLET-induced (35 μ M, 1 h) loss of nuclear β -catenin staining (green) was prevented by pretreatment with ion channel inhibitors amiloride and BaCl₂. Nuclei are stained blue with DRAQ5 (scale bars, 10 μ m). (F) Ion channel inhibitors prevent HAMLET-induced cell death ($p < 0.001$) (one-way analysis of variance test, $n = 3$). (G) HAMLET induced calcium and potassium fluxes in fluorophore-loaded DLD1 cells that were abrogated by amiloride and BaCl₂. Error bars represent $\pm$ SEM.

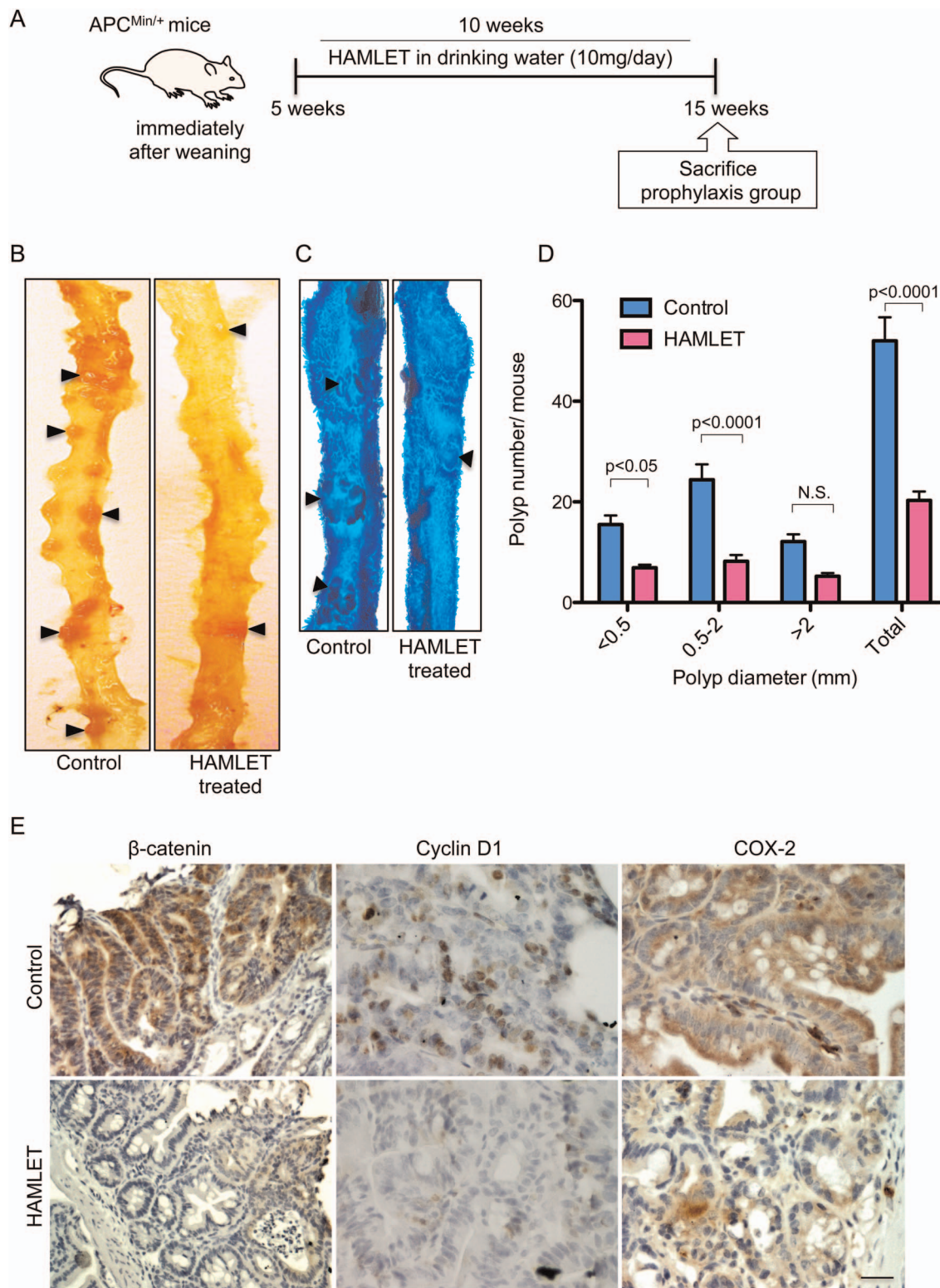


Figure 5 Human α -lactalbumin made lethal to tumour cells (HAMLET) prophylaxis reduces intestinal tumour development. (A) *ApC*^{Min/+} mice were provided with HAMLET (10 mg/day) supplemented in the drinking water for 10 weeks and killed at 15 weeks of age. (B) Dissection photomicrographs of small intestinal segments showing prevention of tumour development (arrowheads) in HAMLET-treated mice (n=10) compared with sham-treated mice (n=10). (C) Methylene blue-stained whole mounts of intestinal segments shows reduced tumour (arrowheads) load in HAMLET-treated mice. (D) Quantification of the tumour number and size after HAMLET prophylaxis. HAMLET-treated mice (n=10) showed significant reduction (p<0.0001 for total tumour count) compared with sham-treated mice (n=10) (two-way analysis of variance test). Error bars represent $\pm$ SEM. (E) Representative immunostained sections demonstrating reduced β -catenin, cyclin D1 and COX-2 levels in HAMLET-treated tumours compared with mock-treated tumours (scale bars, 20 μ m).

Gene expression profiling in tumours remaining after prophylaxis/treatment

To examine how HAMLET modifies the gene expression profile *in vivo*, tumours recovered from HAMLET-treated or control mice were characterised by whole-genome transcriptional profiling (figure 6). After HAMLET prophylaxis, marked differences in gene expression were seen (figure 6A). Using a stringent cut-off point of false discovery rate (FDR)-adjusted p value <0.05 and $\log_2(\text{FC}) > 1$, 224 unique genes (125 upregulated and 99 downregulated) were differentially expressed.

By gene set enrichment analysis (GSEA, figure 6B and see online supplementary figure S11), HAMLET was shown to decrease the expression of genes in the β -catenin signalling pathway ($p=0.002$). DNA-replication genes were also inhibited ($p=0.002$), (figure 6B).

Gene sets enriched in HAMLET-treated tumours included oxidative phosphorylation ($p=0.001$) and glycolysis ($p=0.013$) as well as glutathione metabolism ($p=0.001$), (figure 6C). Importantly, a coordinated upregulation of almost all genes necessary for glycolysis was seen after HAMLET treatment, with the most significant being fructose-1,6-bisphosphatase, glucose-6-phosphatase and lactate dehydrogenase. In addition, a scatterplot analysis indicated a coordinated increase in a large number of genes involved in oxidative phosphorylation ($p<0.001$) (figure 6D), an increased conversion of glucose into pyruvate and increased ATP synthesis, suggesting a departure from the 'Warburg phenotype' in tumour cells surviving HAMLET treatment. Furthermore, a number of glutathione S-transferases and enzymes were also more highly expressed, as were detoxifying compounds that protect against oxidative stress by conjugation of glutathiones. Upregulated genes in the oxidative phosphorylation machinery included both ATPases, NADH dehydrogenases and cytochrome c oxidase, indicating a general increase of activity in this pathway.

HAMLET was also identified as a regulator of several transcription factors (figure 6E). CTNNB1 (β -catenin) was negatively regulated, with six downstream transcripts showing decreased expression (TNFRSF11B, SPP1, SERPINE1, MMP7, IGFBP5 and CYP51A1), consistent with the GSEA. Transcription factors regulating the defence against reactive oxygen species were upregulated (NFE2L2, NR113 and NR112). HAMLET also stimulated HNF4, which drives the expression of lipolytic enzymes that might be needed to metabolise oleic acid in HAMLET-treated cells (figure 6E).

Significant effects of HAMLET on gene expression were also seen in the therapeutic group compared with sham-fed controls (see online supplementary figure S12A). The most highly expressed genes were involved in glucose metabolism, including a large fraction of key enzymes responsible for glycolytic fluxes (see online supplementary figures S12B–D and 13, ingenuity p value 10^{-9} and GSEA FDR q value 0.05). In addition, negative regulators of the Wnt signalling pathway were enriched (q values 0.032). Wnt inhibitory factor 1 was upregulated twofold as was SOX17 (sex determining region Y-box 17), which modulates Wnt signalling through binding to Wnt3A.

We conclude that peroral prophylactic and therapeutic effects of HAMLET are accompanied by a well-defined series of stable changes in gene expression, affecting Wnt signalling and β -catenin, as well as glycolysis, oxidative phosphorylation and lipid metabolism in tumour tissues.

DISCUSSION

Colorectal cancer is the second leading cause of cancer-related deaths in the USA and more than 160 000 cases are diagnosed

annually.^{2,3} A significant number of people inherit the susceptibility to colon cancer.⁴ Despite repeated, often invasive diagnostic and therapeutic procedures, many develop the disease.⁴ In this study, the prophylactic and therapeutic effects of HAMLET against colon cancer, together with a clear demonstration of inhibition of β -catenin signaling, are therefore of great potential significance. *Apc^{Min/+}* mice were used as a model of human disease, and spontaneous, age-related tumour development was drastically reduced after peroral HAMLET prophylaxis. In addition, the number and size of intestinal tumours as well as associated mortality were reduced by peroral HAMLET administration to mice with established tumours. These effects were accompanied by changes in Wnt/ β -catenin signaling, and gene expression analysis suggested that surviving tumour cells had been routed to a more benign phenotype with reduced Wnt signalling and increased aerobic glycolysis. HAMLET thus offers a new approach to colon cancer treatment, acting by removing established tumour cells and preventing tumour development in the intestinal mucosa. We propose that peroral HAMLET administration should be tested clinically, especially in families bearing APC gene mutations.

Aberrant activation of Wnt/ β -catenin signalling is fundamental to the pathogenesis of colon cancer, and molecular control of this pathway has become a major therapeutic focus.^{8,24} In normal cells, proteasomal β -catenin degradation proceeds through the formation of a destruction complex, limiting nuclear translocation and transcription of β -catenin-dependent genes. In colon cancer cells, β -catenin degradation is impaired and nuclear translocation is enhanced, leaving the Wnt-signalling pathway overactive and the cells tumour prone. Extracellular Wnt inhibitors, including the secreted frizzled-related proteins, have been pursued as potential therapeutic agents,²⁵ and small molecular antagonists of the protein–protein interaction between β -catenin and transcription TCFs have shown encouraging preclinical results.²⁶ In addition, numerous epidemiological studies have detected benefits of non-steroidal anti-inflammatory drugs²⁷ with a molecular mechanism recently linked to inhibition of nuclear accumulation of β -catenin.²⁸ In response to HAMLET, β -catenin was proteolytically cleaved and the nuclear β -catenin content was markedly reduced. The remaining polypose tissue from HAMLET-treated mice showed a reduction in staining of β -catenin, VEGF and COX-2 as well as proliferation markers cyclin D1 and Ki-67. These findings identify HAMLET as a new Wnt pathway modifier, acting by reducing nuclear β -catenin accumulation and β -catenin-dependent gene expression.

Rapid activation of ion fluxes has recently been proposed as a unifying mechanism of tumour cell death in response to HAMLET (Storm *et al*, unpublished). Inhibitors blocking Na^+ and K^+ fluxes were shown to rescue tumour cells from death and in addition, the inhibitors blocked HAMLET internalisation, changed gene expression, mitogen-activated protein kinase (MAPK) signalling and the stress response to HAMLET (Storm *et al*, unpublished). In this study, HAMLET-dependent ion channel activation was shown also to control the change in β -catenin structure and function, caused by HAMLET. These effects were executed, in part, via caspase-3 activation but the fragmentation and the relocation of β -catenin from the nuclei to the cell periphery required ion channel activation. Caspase-3-dependent β -catenin fragmentation has previously been described in colon cancer cells, based on inhibition by zVAD.²⁹ Furthermore, HAMLET has been shown to activate caspase-3 in tumour cells³⁰ but the functional importance of this response has been unclear, as caspase inhibition does not prevent the cytotoxic response to HAMLET. Importantly, HAMLET treatment did not alter the levels of phosphorylated or total GSK3, indicating that

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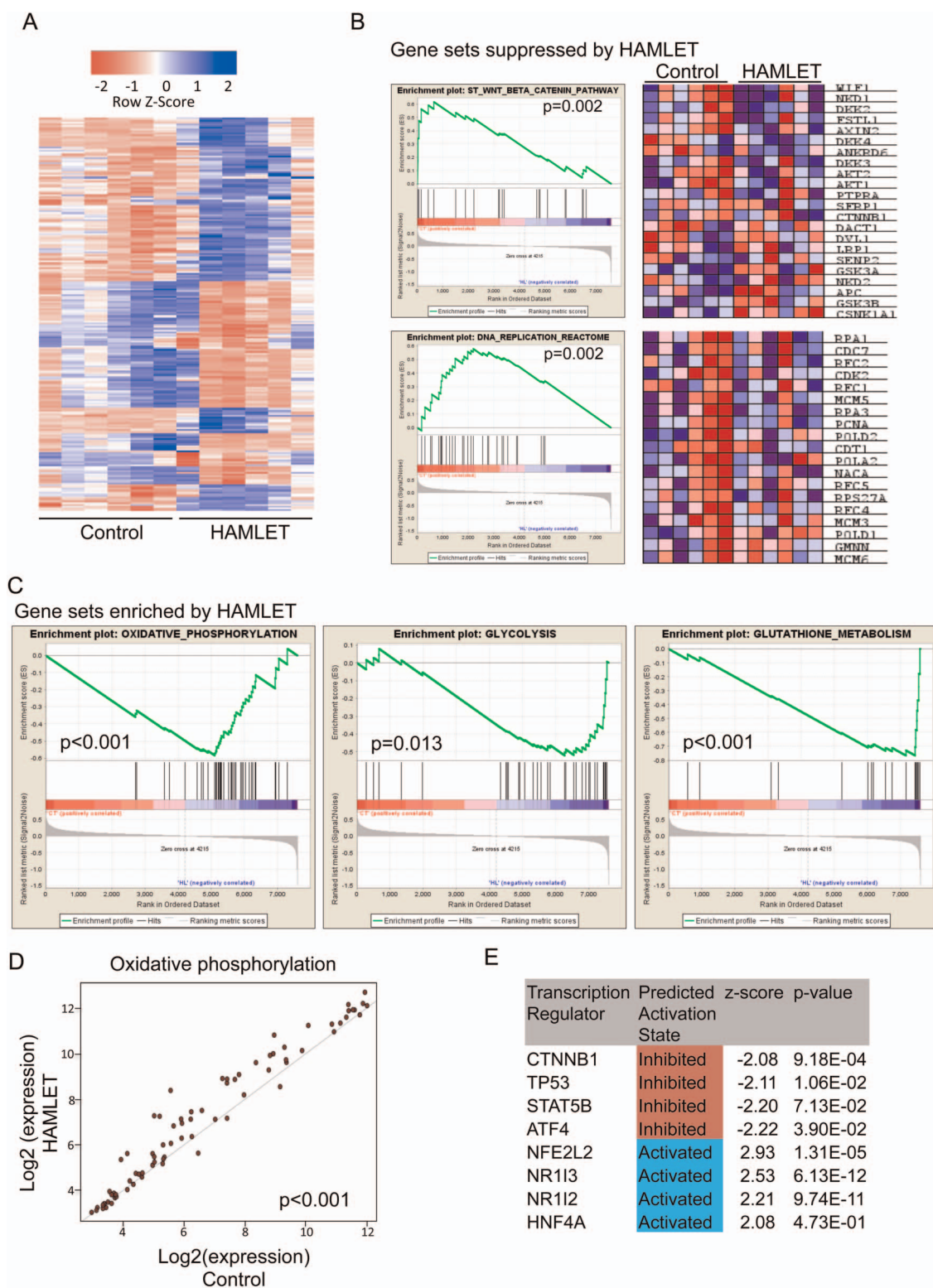


Figure 6 Whole-genome profiling identifying distinct transcriptional events in tumours after human α -lactalbumin made lethal to tumour cells (HAMLET) prophylaxis. (A) Heat map of differentially expressed genes (log₂ fold change >1 and false discovery rate (FDR)-adjusted p value <0.05, n=224) between HAMLET and mock treated tumours, indicating that there are distinct differences between the two groups. (B) Gene sets depleted by HAMLET treatment included β -catenin and DNA replication pathways. Heat maps shows expression of individual genes for each tumour. See also online supplementary figure S8. (C) Expression of genes involved in oxidative phosphorylation (p<0.001), glycolysis (p=0.013) and glutathione metabolism (p<0.001) were enriched after HAMLET treatment. (D) Scatterplot visualisation of genes in the oxidative phosphorylation pathway showing a coordinated increase in expression of almost all genes after HAMLET treatment (p<0.001). (E) Ingenuity Pathway Analysis highlighting suppressed and enhanced transcription factors. β -Catenin (CTNNB1) signalling is inhibited in HAMLET-treated tumours.

HAMLET is not modulating downstream components of the Wnt pathway by this mechanism. These findings suggest that in addition to the effects on β -catenin signalling, HAMLET triggers a Wnt-independent, ion channel-dependent mechanism controlling β -catenin in colon cancer cells. In addition, we suggest that caspase-3 functions as an effector of β -catenin fragmentation in HAMLET-treated tumour cells.

Gene expression analysis detected short-term and long-term effects of HAMLET in vivo consistent with the effects seen at protein level. After HAMLET prophylaxis, surviving tumours showed a marked reduction in β -catenin-dependent gene expression. Aerobic glycolysis was enhanced, consistent with a suppression of the Warburg phenotype, which was identified by shRNA screening as one of the essential determinants of HAMLET sensitivity in tumour cells.¹⁴ Furthermore, glutathione signalling was activated, indicating an oxidative stress response. A similar but less complete change in gene expression was seen in the therapeutic group, where the expression of Wnt signalling inhibitors was enhanced combined with a general stimulation of genes involved in glycolysis.

Gene expression analysis of the tissue samples from the prophylactic group detected a reduction in β -catenin gene expression, suggesting that the β -catenin-driven tumour development might be delayed compared with development in the sham-fed controls. In the therapeutic group that received HAMLET after tumours were established, no decrease in β -catenin expression was seen but an increase in Wnt signalling inhibitors was detected, compared with fully developed tumours. This may suggest that different mechanisms account for the shift in β -catenin function in cells surviving HAMLET challenge during prophylaxis or treatment. These studies suggest that HAMLET drives a gradual loss of the malignant phenotype and a gain of more normal cellular functions. The mechanism is not clear, but we speculate that an enrichment of more differentiated cells may result from purging by HAMLET of the most malignant and HAMLET-sensitive cells. In addition, a redirection of the cellular machinery towards a more differentiated phenotype may occur.

The lack of selectivity for tumour tissue is a major concern in colon cancer treatment and side effects are often severe. HAMLET is formed from beneficial human milk constituents and has previously been shown to preferentially kill tumour cells rather than healthy, differentiated cells. Our study provides further in vivo evidence for HAMLET's tumour selectivity. By comparing adjacent areas of tumour tissues and healthy tissues in individual *Apc^{Min/+}* mice, HAMLET was shown to reach intestinal polyps and to be internalised selectively by tumour cells. There was no evidence of toxicity or inflammatory changes in healthy tissue, suggesting that side effects to peroral HAMLET treatment may be limited. Furthermore, these observations are of particular interest, as they suggest that the *APC* mutation alone does not render intestinal cells sensitive to HAMLET. While all cells in these mice carry the *Apc^{Min/+}* mutation, it is well established that colorectal tumour development involves the sequential accumulation of oncogenic mutations, including *KRAS*, *c-myc* and *p53*. The point at which tumours become sensitive to HAMLET may reveal at which point of tumour development cells become sensitive to the cytotoxic and therapeutic effects of HAMLET. The selective uptake of HAMLET into tumour tissue and reduction in malignancy is encouraging for the development of future therapeutic tools against colon cancer.

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Contributors MP and CS conceived the study and designed the experiments. MP conducted most of the in vivo experiments and analysed the data. MP and AN performed in vitro experiments, western blotting, immunostaining and microscopy. SH helped in E-cadherin immunostaining and confocal microscopy. PS performed the transcriptomic analysis of tumour tissues. MP, AN and CS analysed the in vitro data. CS and MP wrote the manuscript with contributions from PS. All authors read and agreed on the final manuscript.

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Competing interests This study received no financial support from any commercial organisations. CS is a shareholder in HAMLET Pharma, a currently inactive company holding the patent rights to HAMLET.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

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REFERENCES

- Gangadhar T, Schilsky RL. Molecular markers to individualize adjuvant therapy for colon cancer. *Nat Rev Clin Oncol* 2010;7:318–25.
- Siegel R, Ward E, Brawley O, et al. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin* 2011;61:212–36.
- Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* 2009;361:2449–60.
- Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;319:525–32.
- Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990;247:322–4.
- Clevers H. Wnt/ β -catenin signaling in development and disease. *Cell* 2006;127:469–80.
- Moon RT, Bowerman B, Boutros M, et al. The promise and perils of Wnt signaling through β -catenin. *Science* 2002;296:1644–6.
- Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling. *Cell* 2000;103:311–20.
- Pettersson-Kastberg J, Aits S, Gustafsson L, et al. Can misfolded proteins be beneficial? The HAMLET case. *Ann Med* 2009;41:162–76.
- Svanborg C, Agerstam H, Aronson A, et al. HAMLET kills tumor cells by an apoptosis-like mechanism—cellular, molecular, and therapeutic aspects. *Adv Cancer Res* 2003;88:1–29.
- Fischer W, Gustafsson L, Mossberg AK, et al. Human alpha-lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival. *Cancer Res* 2004;64:2105–12.
- Mossberg AK, Wullt B, Gustafsson L, et al. Bladder cancers respond to intravesical instillation of HAMLET (human alpha-lactalbumin made lethal to tumor cells). *Int J Cancer* 2007;121:1352–9.
- Gustafsson L, Leijonhufvud I, Aronsson A, et al. Treatment of skin papillomas with topical alpha-lactalbumin-oleic acid. *N Engl J Med* 2004;350:2663–72.
- Storm P, Aits S, Puthia MK, et al. Conserved features of cancer cells define their sensitivity to HAMLET-induced death; c-Myc and glycolysis. *Oncogene* 2011;30:4765–79.
- Gustafsson L, Aits S, Onnerfjord P, et al. Changes in proteasome structure and function caused by HAMLET in tumor cells. *PLoS One* 2009;4:e5229.
- Svensson M, Hakansson A, Mossberg AK, et al. Conversion of alpha-lactalbumin to a protein inducing apoptosis. *Proc Natl Acad Sci USA* 2000;97:4221–6.
- Koehl GE, Spitzner M, Ousingsawat J, et al. Rapamycin inhibits oncogenic intestinal ion channels and neoplasia in *Apc^{Min/+}* mice. *Oncogene* 2010;29:1553–60.
- Ilyas M, Tomlinson IP, Rowan A, et al. Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc Natl Acad Sci USA* 1997;94:10330–4.

Colon cancer

- 19 Aberle H, Butz S, Stappert J, *et al.* Assembly of the cadherin-catenin complex *in vitro* with recombinant proteins. *J Cell Sci* 1994;107:3655–63.
- 20 Steinhilber U, Badock V, Bauer A, *et al.* Apoptosis-induced cleavage of beta-catenin by caspase-3 results in proteolytic fragments with reduced transactivation potential. *J Biol Chem* 2000;275:16345–53.
- 21 Ikeda S, Kishida M, Matsuura Y, *et al.* GSK-3beta-dependent phosphorylation of adenomatous polyposis coli gene product can be modulated by beta-catenin and protein phosphatase 2A complexed with Axin. *Oncogene* 2000;19:537–45.
- 22 Zhao J, Yue W, Zhu MJ, *et al.* AMP-activated protein kinase (AMPK) cross-talks with canonical Wnt signaling via phosphorylation of beta-catenin at Ser 552. *Biochem Biophys Res Commun* 2010;395:146–51.
- 23 Kunzelmann K. Ion channels and cancer. *J Membr Biol* 2005;205:159–73.
- 24 Segditsas S, Tomlinson I. Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* 2006;25:7531–7.
- 25 Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 2003;116:2627–34.
- 26 Lepourcelet M, Chen YN, France DS, *et al.* Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. *Cancer Cell* 2004;5:91–102.
- 27 Thun MJ, Namboodiri MM, Heath CW Jr. Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med* 1991;325:1593–6.
- 28 Greenspan EJ, Madigan JP, Boardman LA, *et al.* Ibuprofen inhibits activation of nuclear [beta]-catenin in human colon adenomas and induces the phosphorylation of GSK-3[beta]. *Cancer Prev Res (Phila)* 2011;4:161–71.
- 29 Fukuda K. Apoptosis-associated cleavage of beta-catenin in human colon cancer and rat hepatoma cells. *Int J Biochem Cell Biol* 1999;31:519–29.
- 30 Hallgren O, Aits S, Brest P, *et al.* Apoptosis and tumor cell death in response to HAMLET (human alpha-lactalbumin made lethal to tumor cells). *Adv Exp Med Biol* 2008;606:217–40.



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