PERGAMON

Leukemia Research 27 (2003) 859–863

Leukemia Research

www.elsevier.com/locate/leukres

Case report

Sustained complete remission of CLL associated with the use of a Chinese herbal extract: case report and mechanistic analysis

Traci E. Battle [a,b], Hugo Castro-Malaspina [c], John G. Gribben [a,b], David A. Frank [a,b,*]

[a] *Department of Medical Oncology, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115, USA*
[b] *Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*
[c] *Memorial Sloan-Kettering Cancer Center, New York, NY, USA*

Received 3 October 2002; accepted 20 December 2002

Abstract

We report the case of a man with chronic lymphocytic leukemia (CLL) who, in the absence of cytotoxic chemotherapy, began taking a Chinese herbal extract. Shortly thereafter, he experienced a steady decline of his lymphocytosis and adenopathy, and he remains in remission now over 10 years later. The herbal extract inhibited the survival of primary CLL cells under in vitro culture conditions. However, it did not inhibit the activation of Akt or MAP kinase, nor did it inhibit the serine phosphorylation of STAT1. Thus, through an as yet unknown mechanism, this extract appears to exert pro-apoptotic effects in CLL.

Keywords: Apoptosis; Signal transduction; Complementary therapy; Non-Hodgkin's lymphoma; Kinase; Protein phosphorylation

1. Introduction

The patient, a 57-year-old man, presented to his primary physician in December 1990 with flu like symptoms and fatigue, and was found to have a white blood cell (WBC) count of 39,400, with greater than 90% lymphocytes, hemoglobin 13.1, and platelet count of 280,000. A blood smear showed a preponderance of mature appearing lymphocytes. Bone marrow biopsy revealed an infiltration of lymphocytes representing 64% of the cellularity. Mildly enlarged cervical, axillary, and inguinal lymph nodes were detected on examination, and CT scan of the chest and abdomen revealed mildly enlarged axillary and paraortic lymph nodes. Immunophenotyping revealed a population of CD19+ cells which were monoclonal for kappa light chain, and weakly expressed CD5. PCR analysis demonstrated a clonal immunoglobulin heavy chain rearrangement.

In July 1991, the patient had a WBC count of 60,000. At that time, he consulted with a practitioner of Chinese medicine, who began to provide him with a mixture of what the patient described as twigs, roots, and bark. The patient was told to boil these in water, allow the mixture to cool and concentrate by evaporation, then strain and drink daily. Subsequently, the patient's WBC count and absolute lymphocyte count (ALC) steadily decreased (Fig. 1), and his lymphadenopathy resolved. By March 1992, the WBC count was 10,000 with 13% lymphocytes. In March 1993, the WBC count was 5300 with 25% lymphocytes. At that time, bone marrow biopsy showed 16% lymphocytes. Flow cytometry at this time no longer revealed a clonal population of lymphocytes consistent with chronic lymphocytic leukemia (CLL). The patient traveled extensively for his job. A notation in a medical record indicated that when he was unable to have access to the herbal extract for 2 months, his WBC count rose, although documentation of the values was not available.

The patient subsequently left the country for 4 years. When he returned in March 1997, he was taking the extract only weekly. His WBC count in March 1997 was 6300 with 24% lymphocytes. He maintained normal hematologic values through his last visit in September 2000, without ever having received standard cytotoxic chemotherapy. At that time, he had no peripheral adenopathy, and there was no evidence of a clonal population of lymphocytes in his peripheral blood by either flow cytometry or PCR analysis for immunoglobulin gene rearrangements. It was at this visit that he provided a sample of his herbal extract. The patient subsequently left the country again, and discontinued the

Abbreviations: ALC, absolute lymphocyte count; CLL, chronic lymphocytic leukemia; FBS, fetal bovine serum; STAT, signal transducer and activator of transcription; WBC, white blood cell

* Corresponding author. Tel.: +1-617-632-4714; fax: +1-617-632-6356.
E-mail address: david_frank@dfci.harvard.edu (D.A. Frank).

0145-2126/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0145-2126(03)00009-2

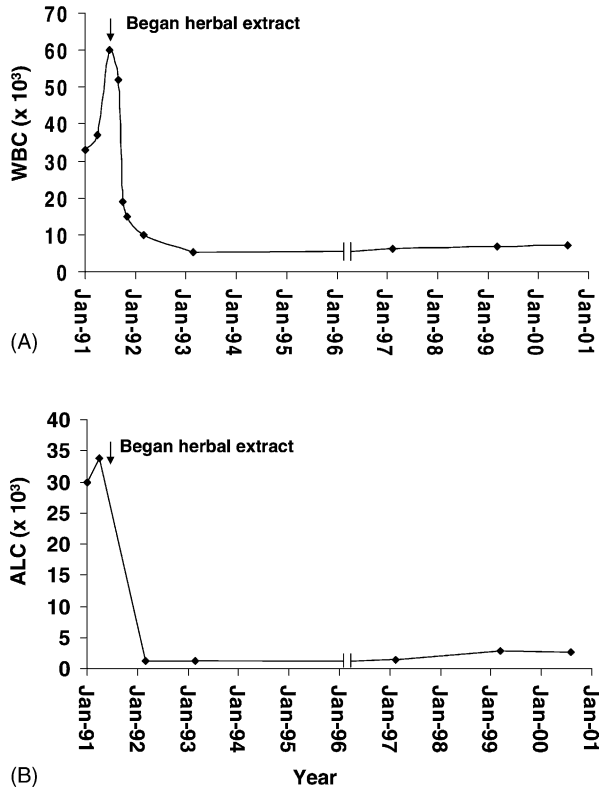

Fig. 1. (A) Total white blood cell (WBC) count and (B) absolute lymphocyte count (ALC) of the patient prior to and after initiation of herbal extract. The breaks in the curves represent the time the patient was abroad and without the extract, in which he was reported to have experienced a rise in his WBC and ALC.

herbal extract. He has remained in a hematologic complete remission for an additional one and one-half years.

2. Materials and methods

2.1. Preparation of extracts

The patient prepared his herbal extract as described in Section 1. In September 2000, he provided approximately 10 ml of this material for evaluation. It was a thick, dark brown, cloudy liquid with little odor. The material was sterilized by passage through a 0.22 μM filter, aliquoted, and frozen at −20 °C until use. Black tea and green tea (Lipton) were brewed according to the manufacturer's directions, cooled, filter sterilized, and used as described.

2.2. CLL cell in vitro survival assay

CLL cells were obtained from the peripheral blood of untreated patients after obtaining informed consent. Mononuclear cells were separated by density centrifugation, and frozen prior to use. For survival assays, cells were thawed, and viable cell count was determined. Cells were cultured in RPMI/1640 containing 10% fetal bovine serum (FBS). Only cells with greater than 97% viability were used for survival assays. Cells were resuspended at a density of 10×10^6 cells/ml and cultured in the presence of herbal extract, black tea, or green tea at the dilutions indicated. Cell density was determined by counting cells using a hemocytometer at the time of culture initiation and every 24 h thereafter. Viability was determined by trypan blue exclusion.

2.3. Analysis of activation of cellular signaling pathways

CLL cells were cultured in RPMI/1640 containing 10% fetal bovine serum. SVR hemangiosarcoma cells [1] were cultured in low glucose DMEM containing 10% FBS. Cells were incubated with the herbal extract for 3 h (CLL) or 1 h (SVR) at 37 °C and then harvested for Western blot analysis. Cells were lysed in buffer containing 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.5% NP40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2 mg/ml of pepstatin. Equal amounts of protein were resolved on 7% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Blots were incubated with an antibody that recognizes the serine phosphorylated form of STAT1 (Ser 727) [2], the phosphorylated form of Akt (Ser 473), or the phosphorylated form of p44/42 MAP kinase (Thr202/Tyr204) (Cell Signaling Laboratories, Beverly, MA) using 1:10,000 (Ser-P-STAT1) or 1:5000 (P-Akt and P-MAPK) dilution of the antibody in Tris-buffered saline/0.05% Tween 20 (TBST). The blots were stripped and re-probed with antibodies that recognize the phosphorylated and unphosphorylated forms of STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA, SC-346), Akt, or p44/42 MAP kinase (Cell Signaling Laboratories, Beverly, MA) using a 1:20,000 (STAT1) or 1:5000 (Akt and MAPK) dilution of the antibodies. Blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Calbiochem, La Jolla, CA) and detection was performed using the Renaissance chemiluminescent ECL kit (NEN Dupont, Boston, MA).

2.4. PCR amplification of V(D)J transcripts

RNA was extracted from 2×10^6 B cells using the RNeasy Total RNA kit (Qiagen, Valencia, CA). mRNA was reverse transcribed using the Super-Script Preamplification System for first strand cDNA synthesis (Life Technologies, Carlsbad, CA). VH–DJH–CH regions were amplified using sense primers specific for the sequences of the different VH families together with antisense primers specific for the CH 1-sequence and the Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). Each reaction consisted of 30 cycles (1 min denaturation at 94 °C, 1 min annealing at 58 °C, 1 min extension at 72 °C, and 10 min extension at 72 °C). The PCR cDNA products were purified, ligated into pCR-Blunt II-TOPO vector (Zero Blunt TOPO Cloning kit; Invitrogen, Carlsbad, CA), and transfected in TOP10 One

Shot competent cells (Invitrogen). Bacterial colonies were selected for sequencing and 30 clones were sequenced. No two colonies had the same IgH sequence, indicating the absence of a clonal product.

3. Results

Given the strong temporal correlation between the patient's initiation of this herbal 'tea', and the clinical and molecular remission he experienced from his CLL, we examined whether this extract affected the survival of CLL cells during in vitro culture. CLL cells from two different untreated patients were cultured in serum-containing medium in the presence or absence of the herbal extract. Given the small quantity of the extract available, it was necessary to test the unfractionated material rather than to attempt a partial separation of components. Thus, to control for the effect of solute concentration, black and green tea, each of which has been purported to display anticancer activity [3,4], were also used as controls. Primary CLL cells have a very low growth fraction, and show no proliferation in in vitro culture. However, when cultured for up to 48 h, the viability of the cells remains intact. By contrast, cells cultured in the presence of the herbal extract showed a decreased viability at 24 and 48 h of culture (Fig. 2). CLL cell viability was affected minimally in the presence of black or green tea.

To determine the mechanism by which the herbal extract inhibited the viability of CLL cells, we focused on three pathways which may play a role in the biology of CLL. STAT transcription factors are serine phosphorylated in CLL cells, but not in normal B cells [2], and may be involved in promoting the survival of these cells [5–7]. However, treatment of primary CLL cells with the herbal extract had no effect on either STAT1 expression, or its phosphorylation on serine 727 (Fig. 3A). Two other signaling cascades known to promote cellular survival are the phosphoinositide 3-kinase (PI3K)/Akt pathway [8], and the Mek/MAP kinase pathway [9]. Although both of these pathways may play a role in the survival of CLL cells, their level of activation in primary CLL cells is relatively low, limiting the ability to detect their inhibition. To assess the ability of the herbal extract to inhibit these pathways, the endothelial-derived cell line SVR, which displays prominent activation of both Akt and MAP kinase, was utilized [1]. Neither the PI3K/Akt pathway nor the Mek/MAP kinase pathway were inhibited by the herbal tea extract at the concentrations examined (Fig. 3B).

4. Discussion

Although natural products have been the source of many medically important drugs, and herbal remedies are gaining increasing popular acceptance, the identification of clinically useful compounds from plant sources is a difficult undertaking. The complete clinical and molecular remission of CLL in the absence of therapy is an extremely unusual event, and likely occurs in well under 1% of cases [10]. Its association with the use of an herbal extract raises the question of whether a substance in this material can mediate an antitumor effect. If so, several potential mechanisms could be considered. One possibility is that a substance in the herbal extract was aiding in the generation of an immune response to the patient's CLL. Several observations would argue against this possibility. First, CLL cells are believed to be relatively non-immunogenic, and sophisticated vaccine

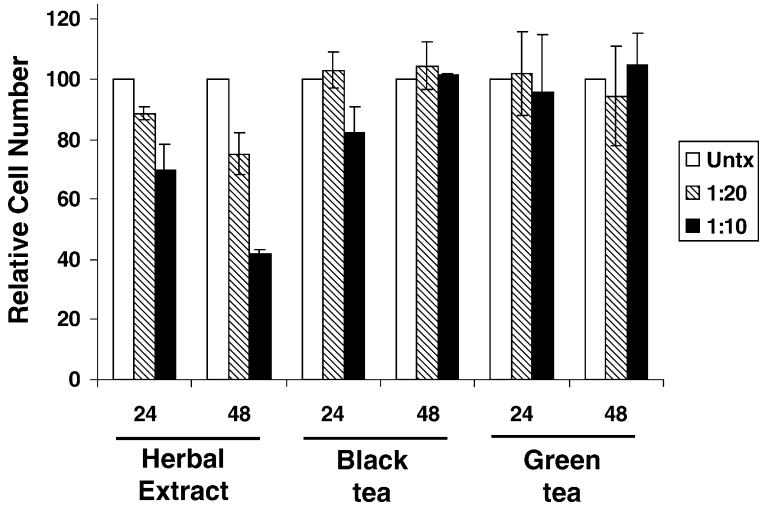

Fig. 2. CLL viability in the presence of herbal extract, black tea, or green tea. CLL cells from two separate patients were cultured for 48 h in the presence of herbal extract, black tea, or green tea at the dilutions indicated. Cell viability was determined by trypan blue exclusion every 24 h. The relative cell number was determined by comparing the number of cells in the treated sample to that of the untreated (Untx) cells. Results are expressed as the mean ± standard error of mean.

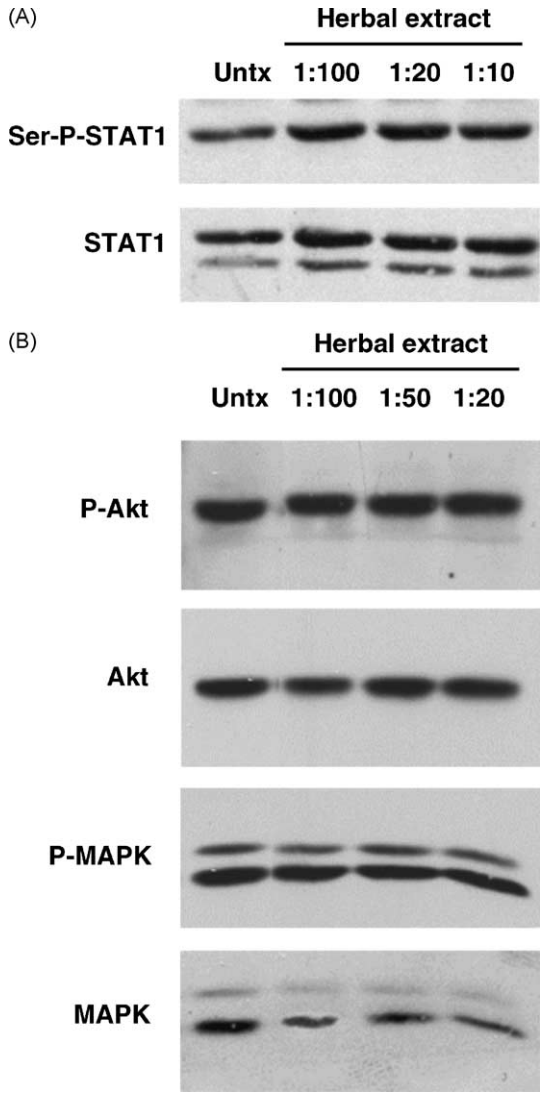

Fig. 3. Western blot analysis of the effect of herbal extract on cellular signaling pathways. **(**A) CLL cells and **(**B) SVR cells were incubated with or without (Untx) herbal extract at the dilutions indicated, prior to harvesting for Western blot analysis. Blots were probed with antibodies specific for the serine phosphorylated form of STAT1, phosphorylated form of Akt, or the phosphorylated form of MAPK. Blots were stripped and re-probed with antibodies that recognize both the unphosphorylated and phosphorylated forms of each protein. STAT1 has two isoforms of 91 kDa (STAT1α) and 84 kDa (STAT1β); only STAT1α contains the site of serine phosphorylation. MAPK has two isoforms of 44 kDa (Erk1) and 42 kDa (Erk2), both of which can become phosphorylated.

strategies are required to generate immune recognition of CLL cells [11]. Second, a decrease in the total WBC count and total lymphocyte count occurred almost concurrently with the initiation of the herbal extract. Most immune based strategies, such as donor lymphocyte infusion or vaccination, in humans are associated with a delay of several months before a therapeutic effect can be detected [12]. Finally, the observation that the tumor response stopped with temporary discontinuation of therapy would also argue against an immune based effect. More likely, the herbal extract acted directly as an inhibitor of CLL survival, as supported by the in vitro experiments.

Given the apparent absence of side effects experienced by this patient, and the normal hematopoiesis maintained while taking this herbal medication for a period of 9 years, such an active agent must be fairly potent and have a high therapeutic index. Polyphenols present in teas and herbal extracts have been described to have pro-apoptotic actions [4]. The small amount of material provided for laboratory evaluation precluded an analysis of active components or a detailed mechanistic evaluation of the inhibitory effect on CLL cell survival. Nonetheless, three potential intracellular mediators, STAT1, PI3K/Akt, and Mek/MAP kinase were not modulated by the herbal extract. If more material becomes available, it would be worthwhile to focus on proteins which regulate survival and apoptosis in CLL cells, such as members of the bax and bcl-2 families. Targeting such molecules could explain the decreased survival of CLL cells in the absence of apparent clinical toxicity. In addition, there is precedence for modulation of bcl-2 family members, for example by phosphorylation, by plant-derived anticancer agents such as taxotere [13,14].

In summary, this patient experienced a sustained complete clinical and molecular remission of CLL, in the absence of any cytotoxic chemotherapy. This remission was associated with the long-term use of an herbal extract which was found to decrease survival of primary CLL cells in vitro. While the mechanism of action is currently not understood, this observation may provide an important first step towards the development of a new class of agents for use in this otherwise incurable disease.

Acknowledgements

The authors thank Dr. Agnes Pasquier-Castro for essential clinical contributions. This work was supported by NIH grants CA93053, CA79547 and CA 81534, and the family and friends of Amanda Hendricks.

Contributions. T.E. Battle contributed to the concept and design, data analysis and interpretation, drafting of the article, provided critical revisions, gave final approval and helped with data collection and assembly. H. Castro-Malaspina provided critical revisions to the article, gave final approval and provided study materials. J.G. Gribben contributed data analysis and interpretation, provided critical revisions, gave final approval and helped collect and assemble data. D.A. Frank contributed to the concept and design, data analysis and interpretation, drafting of the paper, provided critical revisions and gave final approval.

References

[1] Arbiser JL, Moses MA, Fernandez CA, Ghiso N, Cao Y, Klauber N, et al. Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. Proc Natl Acad Sci 1997;94:861–6.

[2] Frank DA, Mahajan S, Ritz J. B lymphocytes from patients with chronic lymphocytic leukemia contain STAT1 and STAT3 constitutively phosphorylated on serine residues. J Clin Invest 1997;100:3140–8.
[3] Chung JY, Huang C, Meng X, Dong Z, Yang CS. Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: structure–activity relationship and mechanisms involved. Cancer Res 1999;59: 4610–7.
[4] Yang GY, Chen L, Lee MJ, Landau JM. Inhibition of growth and induction of apoptosis in human cancer cells by tea polyphenols. Carcinogenesis 1998;19:611–6.
[5] Lin TS, Mahajan S, Frank DA. STAT signaling in the pathogenesis and treatment of leukemias. Oncogene 2000;19:2496–504.
[6] Battle TE, Frank DA. The role of STATs in apoptosis. Curr Mol Med 2002;2:381–92.
[7] Frank DA, Mahajan S, Ritz J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. Nature Med 1999;5:444–7.
[8] Sun M, Wang G, Paciga JE, Feldman RI, Yuan Z-Q, Ma X-L, et al. AKT1/PKBa kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. Am J Pathol 2001;159:431–7.
[9] Ballif BA, Blenis J. Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Diff 2001;12:397–408.
[10] Ribera JM, Vinolas N, Urbano-Ipizua A, Gallart T, Montserrat E, Rozman C. “Spontaneous” complete remissions in chronic lymphocytic leukemia: report of three cases and review of the literature. Blood Cells 1987;12:471–9.
[11] Wierda WG, Cantwell MJ, Woods SJ, Rassenti LZ, Prussak CE, Kipps TJ. CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. Blood 2000;96:2917–24.
[12] Collins RHJ, Shpilberg O, Drobyski WR, Porter DL, Giralt S, Champlin R, et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. J Clin Oncol 1997;15:433–44.
[13] Berchem GJ, Bosseler M, Mine N, Avalosse B. Nanomolar range docetaxel treatment sensitizes MCF-7 cells to chemotherapy induced apoptosis, induces G2M arrest and phosphorylates bcl-2. Anticancer Res 1999;19:535–40.
[14] Kolfschoten GM, Hulscher TM, Duyndam MC, Pinedo HM, Boven E. Variation in the kinetics of caspase-3 activation, Bcl-2 phosphorylation and apoptotic morphology in unselected human ovarian cancer cell lines as a response to docetaxel. Biochem Pharmacol 2002;63:733–43.