Topical Sulindac Combined With Hydrogen Peroxide in the Treatment of Actinic Keratoses

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ABSTRACT

Background: Actinic keratoses (AKs) are a precancerous condition of the skin that have the potential to become squamous cell cancer (SCC). Sulindac is a Food and Drug Administration (FDA)-approved nonsteroidal anti-inflammatory drug (NSAID) that has been shown to have clinically significant anticancer effects. Malignant cells may have a different response to oxidative stress than normal cells.

Objective: To establish a role of increased reactive oxygen species (ROS) in the mechanism of cancer killing by sulindac in the presence of an oxidizing agent. To assess the tolerability and efficacy of the combination of gels containing sulindac and hydrogen peroxide in the treatment of patients with AKs.

Methods: Cell culture studies were performed using a skin SCC cell line and normal human epidermal keratinocytes. After treatment with sulindac and an oxidizing agent, cell viability, and intracellular ROS levels were measured. An open-label clinical trial was performed using sulindac and hydrogen peroxide gels daily for 3 weeks on AKs involving the upper extremities.

Results: In SCC cells, a combination of sulindac and an oxidizing agent lead to 400 to 500% increases in intracellular ROS, which resulted in significant cell death. In sharp contrast, normal keratinocytes did not show increases in ROS levels and were not killed. A clinical trial using the combination of sulindac and hydrogen peroxide therapy in 5 patients with AKs revealed that 60% of the treated AKs responded and 50% showed no residual AK on histopathology specimens after skin biopsy.

Limitations: The small number of patients and the lack of a placebo group.

Conclusion: Increased levels of ROS appear to be important in the selective killing of cancer cells in the presence of sulindac and oxidizing agents. Further studies are necessary to define the role of the combination of sulindac and oxidizing agent therapy in patients with AKs and skin cancer.

INTRODUCTION

Sulindac is a Food and Drug Administration (FDA)-approved nonsteroidal anti-inflammatory drug (NSAID) that has been prescribed for more than 20 years for the treatment of pain associated with inflammation and stiffness due to arthritis and other inflammatory conditions.[1] Sulindac has also been shown to have clinically significant anticancer effects. For over a decade, sulindac has been of interest as a clinical treatment for adenomatous colorectal polyps and colon cancer,[2] especially in patients with familial adenomatous polyposis.[3] Sulindac induces apoptosis of colon cancer cells, although the mechanism is not fully understood.[4] Recent scientific studies suggest that malignant cells have a different response to oxidative stress than do normal cells.[5] It has been suggested that because cancer cells are more metabolically active, they produce higher levels of reactive oxygen species (ROS) and are more vulnerable to further oxidative stress by ROS-generating agents, such as peroxides.[6] Recently, using colon cancer cells, it was reported that sulindac led to an enhancement of the anticancer activity of bortezomib, a proteasome inhibitor, by a mechanism involving oxidative stress and the generation of intracellular ROS.[7] Similarly, when sulindac was used in combination with an arsenic drug, a synergistic enhancement in the killing of lung cancer cells occurred.[8] Furthermore, sulindac has been shown to significantly potentiate the tumor growth inhibitor activity of doxorubicin in a human lung cancer xenograft model.[9]

Actinic keratoses (AKs) are a precancerous condition of the skin that has the potential to become squamous cell cancer similar to adenomatous polyps of the colon that can lead to colon cancer if left untreated.[10] In this article, the authors establish a role of increased ROS in the mechanism of cancer killing by sulindac in the presence of peroxide. Furthermore, the authors present the results of a proof-of-concept clinical study that was designed to assess the tolerability and efficacy of the combination of sulindac and hydrogen peroxide gels in subjects with AKs of the upper extremities.

METHODS

Materials

Sulindac and peroxides were obtained from Sigma-Aldrich company (St. Louis, Mo). Sulindac and hydrogen peroxide were individually formulated into topical gels containing deionized water, SD-40 alcohol, hydroxyethyl cellulose, xanthan gum, glycerin, Carbopol®, sodium citrate, and triethanolamine.

Cells

A skin squamous cell carcinoma (SCC-25) cell line (ATCC #CRL-1628) was maintained in Dulbecco's MEM (Eagle) supplemented with 10% fetal bovine serum. The normal human epidermal keratinocytes (HEK) were derived from human neonatal foreskin (Dermatology clinic; University of Miami). The HEK cells were maintained in equal volumes of defined keratinocyte SFM with growth supplements (Gibco Cat # 10744-010) in combination with Medium-154 (Cascade Biologics Cat # 154-500) containing human keratinocyte growth supplements.

Experimental Design

The cells were treated with sulindac (500 µM) for 24 hours prior to exposure to tert-butyl hydroperoxide (TBHP) or hydrogen peroxide for 2 hours. Because of its increased stability, TBHP was routinely used in the cell culture experiments. Cell suspensions containing sulindac, or the control with no sulindac, were plated in 96-well microtiter plates containing a total of 6 X 10^4 cells per well. The plates were incubated for 24 hours at 37°C in a 5% CO_2 incubator, washed and fresh culture medium containing the indicated final concentration of TBHP (without sulindac) was added to the cells for 2 hours.

Cell Viability Assay

Cell viability was determined by the CellTiter 96® Aqueous One Solution Cell Proliferation (MTS) Assay (Promega Corp, Madison, Wis). The amount of MTS that is metabolized was measured by absorbance at 490 nm using a colorimetric microtiter plate reader (SpectraMax® Plus^{384}; Molecular Devices, Sunnyvale Calif.). Absorbance is directly proportional to the number of viable cells.

ROS Levels

Intracellular ROS levels were measured with a fluorescent microscope after using 2′, 7′-dichlorodihydrofluorescein diacetate dye, (H_2DCFDA): a membrane permeable dye that passively diffuses into cells and increased levels of ROS cleave the acetate groups resulting in emission of green fluorescence (Invitrogen Corp., Carlsbad, Calif). The relative change in fluorescence was determined by counting the number of fluorescent cells in the treated group compared to the untreated control and adjusting for the percentage of viable cells.

Clinical Study

The clinical study was approved by the institutional review board and informed consent was obtained from all subjects prior to enrollment. Patients were included if they had 3 clinically recognizable AKs, greater than 4 mm in size, involving the upper extremities. This was an open, uncontrolled study using sulindac (1% or 5%) gel followed by the application of hydrogen peroxide (25%) gel directly to 2 AKs (1 AK served as an untreated control). The AKs were treated daily for a duration of 3 weeks and participants were seen for evaluation on week 0, week 1, week 2, week 3, and week 6 to assess efficacy and safety. At baseline and at each follow-up visit, the treated AKs were photographed and measured. At the end of the study, skin biopsies of the treated AKs were performed and histopathology examination was done by a dermatopathologist.

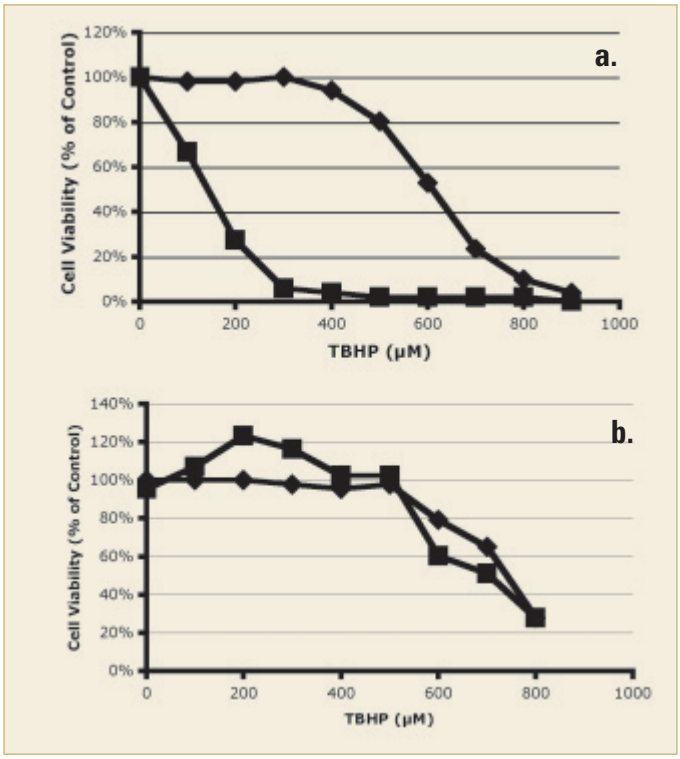

FIGURE 1. Effect of sulindac on A) squamous cell carcinoma (SCC) skin cells and B) normal human epidermal keratinocytes (HEK) skin cells exposed to varying concentrations of tert-butyl hydroperoxide (TBHP). Cells were incubated without sulindac (♦) or with 500 µM sulindac (■) for 24 hours prior to the addition of TBHP for 2 hours.

RESULTS

Sulindac Causes Selective Killing of Skin Cancer Cells in the Presence of an Oxidizing Agent *in Vitro*

Initial experiments by the authors investigated the effect of sulindac on the viability of skin squamous cell cancer (SCC) cells under conditions of oxidative stress caused by TBHP. The combination of sulindac and TBHP enhanced the killing of the skin cancer cells (Figure 1A). The decrease in viability of SCC cells treated with 500µM sulindac was statistically significant (P <.02) at concentrations of TBHP from 200µM to 600µM. In the presence of 200-400µM TBHP, nearly 100% of the untreated SCC cells were viable in contrast to cells treated with sulindac alone, which had markedly diminished viability (less than 10-30% viability). In contrast to the results seen with the SCC cells, normal HEK cells exhibited no enhanced sensitivity to killing by oxidative stress when treated with sulindac (Figure 1B). In fact, the data suggested that the normal HEK cells were mildly protected from oxidative damage after

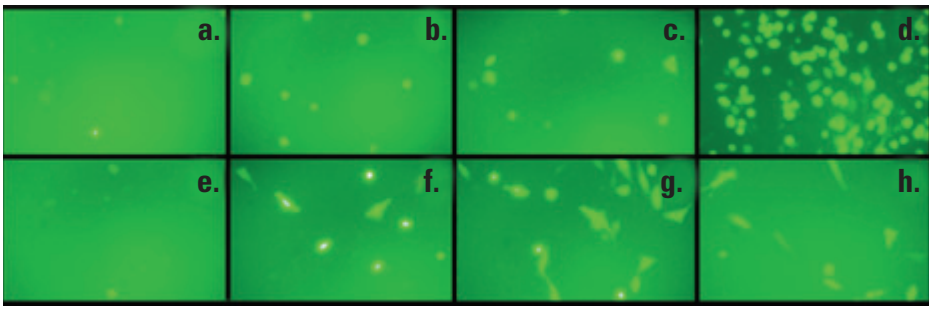

FIGURE 2. Intracellular reactive oxygen species (ROS) levels in squamous cell carcinoma (SCC) skin **(a-d)** and normal skin human epidermal keratinocytes (HEK) **(e-h)** cells. Untreated cells **(a and e)**; cells treated with sulindac alone **(b and f)**; cells treated with tert-butyl hydroperoxide (TBHP) alone **(c and g)**; cells treated with sulindac and TBHP **(d and h)**. All panels contained similar cell densities and the green fluorescence signal was generated and quantified with a fluorescent microscope.

treatment with sulindac at the lower concentrations of TBHP. Similar results were also obtained when TBHP was replaced with H_2O_2 (600-1000μM, data not shown).

Sulindac Increases the Levels of Intracellular ROS in Skin SCC Cells After Oxidative Stress

The ROS levels were determined in SCC skin and normal HEK cells after treatment with sulindac and TBHP as described in methods. Figure 2 shows the results using skin SCC cells (Figures 2a-d) and normal skin HEK cells (Figures 2e-h). All panels contained similar cell densities and as described in the methods, the green fluorescence is an indication of ROS levels. Figure 2a shows the low levels of background fluorescence of SCC cells not treated with either sulindac or TBHP. Figure 2b shows SCC cells treated with sulindac alone (500 μM) and Figure 2c shows SCC cells treated with TBHP alone (200 μM). In both cases there is very limited increase in fluorescence in the SCC cells, which indicate that intracellular ROS levels in these cells remain low in the presence of either of these agents. In sharp contrast, SCC cells that were treated with the combination of sulindac followed by TBHP (Figure 2d) had very high levels of intracellular fluorescence (400-500% increase) compared to the untreated controls. These data show that sulindac combined with TBHP leads to markedly increased levels of intracellular ROS in SCC cells.

Similar experiments were done using normal skin HEK cells. In contrast to results obtained with the SCC cells, normal skin HEK cells had slightly higher levels (50-100% increase) of intracellular fluorescence in the presence of sulindac or TBHP alone (compare Figure 2b to Figure 2f and Figure 2c to Figure 2g). The most striking difference between SCC cells and normal HEK cells was observed when cells were treated with the combination of sulindac followed by TBHP (compare Figure 2, panels d and h). With normal cells, (as seen in panel h) intracellular fluorescence did not increase as compared to SCC cells where, as noted above, there was a very large increase in fluorescence (Fiqure 2d).

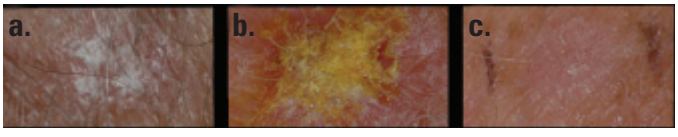

FIGURE 3. A study patient with an actinic keratosis (AK) on the upper extremity treated with the combination of sulindac and hydrogen peroxide gels. **a)** Entry (no treatment), **b)** after 3 weeks (yellow crust), and **c)** after 6 weeks (no evidence of AK after skin biopsy).

Proof of Concept Clinical Study on Actinic Keratoses

In total, 9 patients were recruited into the study and 5 subjects who completed the study were deemed evaluable. From this group of patients, there were a total of 10 AKs that were treated with the combination of sulindac and hydrogen peroxide gels. Six of the 10 AKs that received treatment had a measurable reduction in size or were not visible as shown by clinical photography. In addition, skin biopsies at the end of treatment revealed no residual involvement after histopathology examination of 5 of the 6 AKs that responded to the treatment. During treatment, all of the treated AKs exhibited a very mild inflammatory response that was characterized by the appearance of a vivid yellow-colored crust, the yellow color not involving the normal surrounding skin (Figure 3). Participants rated local tolerability as "good" throughout the study. One patient developed a mild papular skin eruption on her extremities that resolved after the discontinuation of the treatment. There were no other study-related adverse events.

DISCUSSION

These laboratory studies have revealed that the combination of sulindac and TBHP (or H_2O_2) significantly enhances the killing of SCC cells. This effect occurs at concentrations of sulindac and TBHP that individually have little or no activity directed against cancer cells. Sulindac appears to render the cancer cells more sensitive to oxidative stress, since when sulindac is combined with TBHP, an oxidizing agent, the intracellular levels of ROS rise significantly (4 to 5 fold increase) and the cancer cells undergo death. Of importance, a similar effect on normal skin HEK cells was not observed. Mitochondrial dysfunction is probably playing a role in the mechanism for the selective killing of cancer cells. There appears to be a loss of the mitochondrial membrane potential in cultured cancer cells under the experimental conditions described here (unpublished data) which may lead to apoptotic cell death.[11] Although the increased levels of ROS appear to play an important role in the selective killing of the cancer cells, further studies need to be performed to define the precise mechanism of action. It is important to note that other nonsteroidal anti-inflammatory drugs such as acetyl salicylic

acid, ibuprofen, and diclofenac had no anticancer activity under our conditions (unpublished data). This finding suggests that the anticancer activity is related to a unique structural feature of sulindac and is not dependent on cyclo-oxygenase inhibition. Our results using normal HEK skin cells may be related to the finding that sulindac, given orally or applied topically, can protect mice against skin damage and genetic changes leading to cell transformation, resulting from UVB exposure.[12]

Given the laboratory findings, a proof-of-concept clinical trial was initiated to determine if a topical formulation of sulindac in combination with hydrogen peroxide could provide benefit in patients with AKs. Since the medication was delivered directly to the AKs, it was hoped that efficacy would result with a diminished capacity for adverse systemic effects. This effect was anticipated because the total daily topical dose of sulindac (15 mg or 30 mg) that was applied to the skin of each patient in the trial was at least 10-fold less than the daily recommended oral dose (300 mg) for treating arthritic conditions. Furthermore, the systemic absorption of topically administered sulindac was expected to be minimal since the skin contains a strong barrier that should prevent the penetration of substances such as sulindac. Hydrogen peroxide has been used as a medicinal product for over a century and its germicidal power is well known to be due to its oxidizing effect. Over-the-counter teeth bleaching products, such as Crest White Strips and Rembrandt Whitening gels, contain hydrogen peroxide concentrations ranging from 6% up to 36% and are available to consumers for home or professional use.

The results of this preliminary clinical trial suggest that the combination of sulindac and peroxide gels was well tolerated in patients with AKs involving the extremities. This is the first clinical trial that has used this combination therapy for treating AKs. The results revealed that 60% of the treated AKs responded to therapy by exhibiting a decrease in size or becoming not visible to the naked eye. In addition, 50% of the treated AKs showed no residual AK on histopathology specimens after skin biopsy at the end of the study. Furthermore, sulindac alone had no effect on two AKs that were treated during the trial (personal observation), suggesting that the combination is essential for a response. It is important to note that there were some limitations to the study, such as the lack of a placebo-controlled trial and the small numbers of patients that were deemed evaluable. Further studies are necessary to better define the role of this therapy in patients with AKs and skin cancer.

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DISCLOSURE

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REFERENCES

1. Green D, Ts'ao CH, Rossi EC. Sulindac. *Lancet.* 1977;1:804.
2. Rao CV, Rivenson A, Simi B, et al. Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. *Cancer. Res* 1995;55:1464–1472.
3. Labayle E, Fischer D, Vielh P, et al. Sulindac causes regression of rectal polyps in familial adenomatous polyposis. *Gastroenterology.* 1991;101:635-639.
4. Jung B, Barbier V, Brickner H, et al. Mechanisms of sulindac-induced apoptosis and cell cycle arrest. *Cancer Lett.* 2005;219:15-25.
5. Hileman EO, Liu J, Albitar M, et al. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemother Pharmacol.* 2004;53:209-219.
6. Lopez-Lazaro M. Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy. *Cancer Lett.* 2007;252:1-8.
7. Minami, T, Adachi, M, Kawamura, R, et al. Sulindac enhances the proteosome inhibitor bortezomib-mediated oxidative stress and anticancer activity. *Clin Cancer Res.* 2005;11:5248-5256.
8. Jin, HO, Yoon SI, Seo SK, et al. Synergistic induction of apoptosis by sulindac and arsenic trioxide in human lung cancer A549 cells via reactive oxygen species-dependent down-regulation of survivin. *Biochem Pharmacol.* 2006;72:1228-1236.
9. O'Connor R, Heenan M, Connolly L, et al. Increased anti-tumour efficacy of doxorubicin when combined with sulindac in a xenograft model of an MRP-1-positive human lung cancer. *Anticancer Res.* 2004;24:457-464.
10. Mostow EN, Johnson TM. Malignant transformation from actinic keratoses to squamous cell carcinomas. *Arch Dermatol.* 1992;128:560-1.
11. Pelicano, H, Feng, L, Zhou, Y, et al. Inhibition of mitochondrial respiration. A novel strategy to enhance drug induced apoptosis in human leukemia cells by a ROS mediated mechanism. *J Biol Chem.* 2003;278:37832-37839.
12. Athar M, An KP, Tang X, et al. Photoprotective effects of sulindac against ultraviolet B-induced phototoxicity in the skin of SKH hairless mice. *Toxicol Appl Pharmacol.* 2004;195:370-378.

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