Nonsteroidal Anti-Inflammatory Drugs Induce Apoptosis in Esophageal Cancer Cells by Restoring 15-Lipoxygenase-1 Expression

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ABSTRACT

In previous studies, we have found that expression of 15-lipoxygenase-1 (15-LOX-1) and its major product, 13S-hydroxyoctadecadienoic acid, are decreased in human colorectal cancers and that nonsteroidal anti-inflammatory drugs (NSAIDs) can therapeutically induce 15-LOX-1 expression to trigger apoptosis in human colorectal cancer cells. NSAIDs similarly induce apoptosis in esophageal cancer cells, although the mechanisms of these effects remain to be defined. In the present study, we tested whether 15-LOX-1 is down-regulated in human esophageal cancers using paired normal and tumor human surgical samples and whether NSAIDs can up-regulate 15-LOX-1 to restore apoptosis in esophageal cancer cells. We found that: (a) 15-LOX-1 was down-regulated in human esophageal carcinomas; (b) NSAIDs induced 15-LOX-1 expression during apoptosis in esophageal cancer cells; and (c) 15-LOX-1 inhibition suppressed NSAID-induced apoptosis, which was restored by 13S-hydroxyoctadecadienoic acid but not by its parent compound, linoleic acid. These findings demonstrate that 15-LOX-1 is down-regulated in human esophageal carcinomas and that NSAIDs induce apoptosis in esophageal cancer cells via up-regulation of 15-LOX-1. They also support the concept that the loss of the proapoptotic role of 15-LOX-1 in epithelial cancers is not limited to human colorectal cancers.

INTRODUCTION

Apoptosis appears to mediate the chemopreventive effects of NSAIDs in both the esophagus and the colon (1–5). NSAIDs can alter the production of different metabolites of polyunsaturated fatty acids (e.g., linoleic acid and arachidonic acid) by modulating the activity of lipoxygenases and cyclooxygenases (6, 7). We reported previously that the expression of 15-LOX-1, the principle enzyme for metabolizing colonic linoleic acid to 13S-HODE, is down-regulated in human colorectal cancer cells (8). More recently, we showed that NSAIDs induce expression of 15-LOX-1 in colorectal cancer cells irrespective of COX-2 expression and that up-regulation of 15-LOX-1 is critical to the induction of apoptosis by NSAIDs (7, 9). NSAIDs induce apoptosis independently of COX-2 inhibition in colorectal cancer cells (2, 9); we and others have also found that NSAIDs can induce apoptosis independently of COX-2 inhibition in esophageal cancer cells (4, 5). However, the relationship between 15-LOX-1 and esophageal carcinogenesis remains undefined, and the effect of NSAIDs on 15-LOX-1 expression during induction of apoptosis in esophageal cancer cells is unknown. Because of the similarity between the effects of NSAIDs in colon and esophageal cancer cells, we conducted this study, designed to assess: (a) whether expression of 15-LOX-1 is altered in esophageal carcinomas; and (b) whether the effects of NSAIDs on apoptosis in esophageal cancer cells are mediated through up-regulation of 15-LOX-1.

MATERIALS AND METHODS

Materials. Rabbit polyclonal antiserum to recombinant human 15-LOX-1 and standards of recombinant 15-LOX-1 were gifts from Drs. Mary Mulkins and Elliot Sigal (Roche Bioscience, Palo Alto, CA; Ref. 10). Standard solutions of 13S-HODE and linoleic acid were obtained from Cayman Chemical, Inc. (Ann Arbor, MI). We obtained antiprotease mixture tablets from Boehringer Mannheim, Inc. (Indianapolis, IN), 13S-HODE ELISA kits from Oxoid Biomedical Research (Oxford, MI), and 15S-HETE enzyme immunosorbent assay kits from Assay Designs, Inc. (Ann Arbor, MI). CAF was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Human esophageal cancer cell lines SKG-4, SKG-5, and BE-3 were obtained from Dr. David Schrump (National Cancer Institute, Bethesda, MD), and TE-8 was obtained from Dr. E. Tahara (Hiroshima University, Hiroshima, Japan). TE-8 cells were derived from moderately differentiated squamous cell carcinoma, SKG-5 and BE-3 from poorly differentiated adenoscarcinomas, and SKG-4 from a well-differentiated adenoscarcinoma arising in Barrett’s esophagus. We purchased NS-398 from Cayman Chemical, Inc. (Ann Arbor, MI), and sulindac from Sigma Chemical Co. (St. Louis, MO). Other reagents, molecular-grade solvents, and chemicals were obtained from regular commercial manufacturers or as specified below.

Human Esophageal Tissue Samples. Esophageal samples of surgical specimens from 15 subjects who underwent resection for esophageal cancer were procured from the Department of Pathology, University of Texas M. D. Anderson Cancer Center. These specimens contained both the tumor and normal epithelia. All of the samples were routinely fixed in standard 10% buffered formalin, embedded in paraffin, and cut into 4-μm sections. One each of these sections was stained with H&E for classification.

Immunohistochemical Evaluation of 15-LOX-1 Protein Expression. Tissue sections were deparaffinized with xylene and rehydrated in a series of ethanol. The sections were then incubated with 3% hydrogen peroxide in ethanol for 15 min to inactivate endogenous peroxides. Next, the sections were placed in 0.25% trypsin at 37°C for 5 min. Nonspecific antibody-binding sites were blocked using SuperBlock (Pierce, Rockford, IL) for 30 min. Tissue sections were incubated in anti-15-LOX-1 primary antibody solution (1:200) in a humidified chamber at 4°C overnight. The next day, they were washed with PBS and then incubated for 30 min with the secondary antibody solution (1:100 biotinylated anti-rabbit antibody, Vector Lab) and subsequently incubated for the color reaction with 3- amino-9-ethylcarbazole (Sigma Chemical Co.) solution for 20 min to visualize the peroxidase complex. The sections were finally mounted with Aquamount medium under coverslips. Control sections were incubated with normal mouse IgG instead of primary antibodies or with the second antibody only. Staining was evaluated and rated by a pathologist (X. X.) with extensive experience in immunohistochemistry (11). Intensity of staining was rated on a scale of 0–4 (8). The area of maximal intensity was used for grading as long as it comprised ≥10% of the region of interest (esophageal epithelial tissues). Because the staining was generally homogeneous, without tremendous variation in intensity in the regions of interest, we did not quantify the proportions of stained cells.

Cell Cultures. TE-8 cells were cultured and harvested for use in assays to evaluate 15-LOX-1 protein expression, 13-LOX-1, and 15-LOX-1 expression, and apoptosis, as described in the following paragraphs. Cells were grown in...
Table 1 Results of 15-LOX-1 immunohistochemical staining of paired normal and malignant esophageal tissues

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Histological characteristics of normal samples</th>
<th>Positive staining gradea</th>
<th>Histological characteristics of tumor samples</th>
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<tr>
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<td>AdenoCA</td>
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</tbody>
</table>

a Median for normal samples = 1, median for tumor samples = 0; P = 0.00061, Sign test.

b SCC, squamous cell carcinoma; AdenoCA, adenocarcinoma.

Effects of NSAIDs on 15-LOX-1 Expression. In all of the tested esophageal squamous cancers and adenocarcinomas, 15-LOX-1 expression was absent, and sulindac restored 15-LOX-1 expression (data not shown). In the time course experiments, NS-398 and sulindac induced 15-LOX-1 protein expression in a time-dependent manner in TE-8 cells starting at 24 h (Fig. 1b). The induced expression of 15-LOX-1 protein preceded the significant inhibition of cell growth by NSAIDs by >24 h (Fig. 2A).

NSAIDs increased 13-S-HODE but not 15-S-HETE Production in Esophageal Cancer Cells. The induced expression of 15-LOX-1 increased formation of 13-S-HODE in TE-8 cells treated with NS-398 or sulindac, whereas 13-S-HODE was below detectable levels in untreated cells. NS-398 and sulindac increased 15-LOX-1 protein expression from endogenous 13-S-HODE to 24.93 ng/μg protein and 10.25 ng/μg protein (means ± SE), respectively, after 48 h of treatment, and CAF blocked the enzymatic activity of 15-LOX-1 and reduced 13-S-HODE to below detectable levels (Fig. 1c). In contrast, 15-S-HETE levels were very low (less than 0.4 pg/μg protein), and treatment with NS-398 or sulindac resulted in no increase in these levels (data not shown).

15-LOX-1 Inhibition Blocked NSAID-induced Apoptosis. Both NS-398 and sulindac inhibited the growth of TE-8 cells and induced apoptosis, which became evident at 72 h (Fig. 2, A–C and Fig. 3A). At a 2.2 μM concentration, which selectively inhibits enzymatic activity of 15-LOX-1 (7), CAF inhibited the ability of the NSAIDs to induce apoptosis in TE-8 cells (Fig. 2, A–C, and Fig. 3A). Quantitation of sub-G1 fractions of cells and DNAragmentation assays confirmed that apoptosis was induced by NS-398 and sulindac and was inhibited by CAF (Fig. 2C and Fig. 3, A and C).

13-S-HODE Supplementation Restored Apoptosis in TE-8 Cells Treated with NSAIDs and CAF. 13-S-HODE restored apoptosis in TE-8 cells treated with NS-398 + CAF (Fig. 3, A–C). 13-S-HODE (1.35 μM) induced apoptosis in TE-8 cells as confirmed by DNA ladderin and quantitation of sub-G1 fractions (Fig. 3, A and C). In equal concentration (1.35 μM), LA, the parent compound of 13-S-HODE, failed to restore apoptosis in TE-8 cells treated with the NSAIDs + CAF (Fig. 3, A–C). Fig. 3B shows the cellular morphological changes of cell loss and apoptosis, including cytoplasmic and nuclear shrinkage that corroborate these findings.

DISCUSSION

Our current results demonstrate that 15-LOX-1 is down-regulated in esophageal cancers compared with normal tissue, and NSAIDs can
Fig. 1. a, immunohistochemical staining of 15-LOX-1 expression in normal human esophageal tissue and tissue from esophageal tumors. Paraffin-embedded tissue sections were stained by an immunoperoxidase method, as described in “Materials and Methods.” A, a low-magnification (×200) picture of a normal tissue section. B, paired tumor section from the same patient (×200). C, a low-magnification (×200) picture of a normal tissue section from a second patient. D, a low-magnification (×200) of a paired cancer tissue section from the second patient. Tumor epithelium showed markedly decreased staining (red-brown color) compared with that of normal epithelium.

b, effects of NSAIDs on 15-LOX-1 protein expression in esophageal carcinoma cell lines. TE-8 cells were treated with either NS-398 or sulindac; cultured for 12, 24, 48, or 72 h; and then harvested. Expression of 15-LOX-1 protein was analyzed by Western blotting (50 μg of crude protein/sample). NS-398 induced a time-dependent increase in 15-LOX-1 protein expression. Top panel, Lane 1, positive standard control (20 ng recombinant human 15-LOX-1 protein, S) for 15-LOX-1; Lanes 2–5, control experiment with untreated cells at 12, 24, 48, and 72 h; Lanes 6–9, NS-398-treated cells at 12, 24, 48, and 72 h. Sulindac induced similar 15-LOX-1 protein expression in TE-8 cells (Bottom panel). Bottom panel, Lane 1, positive standard control (20 ng recombinant human 15-LOX-1 protein); Lanes 2–5, control experiment with untreated cells at 12, 24, 48, and 72 h; Lanes 6–9, sulindac-treated cells at 12, 24, 48, and 72 h. Sulindac-induced expression was also seen in the control experiment, indicating that sulindac had a similar effect on 15-LOX-1 protein expression.

c, effects of NSAIDs on 13-S-HODE production in TE-8 cells. TE-8 cells were treated with sulindac or NS-398 (NS) with and without CAF, cultured for 48 h, harvested, and then processed for 13-S-HODE measurement as described in “Materials and Methods.” Values shown are the means of triplicate experiments; bars, ± SE. * represents values below the lowest detection level of 5 ng/well.
restore 15-LOX-1 expression in esophageal cancer cells to induce apoptosis in a manner that is dependent on 15-LOX-1 activity. These observations parallel those of our previous work in human colorectal carcinomas (7, 8). This study showed that 15-LOX-1 expression is absent in most human esophageal carcinomas. Similarly, we found that several esophageal carcinoma cell lines lacked expression of 15-LOX-1. These observations also add to the data supporting our hypothesis that 15-LOX-1 down-regulation leads to tumorigenesis by decreasing 13-S-HODE levels and, thus, inhibiting apoptosis. 15-LOX-1 is down-regulated in colorectal cancers, and 13-S-HODE is reduced in colorectal cancers (8); supplemental 13-S-HODE added to colorectal cancer cells restores apoptosis in these cells (8). Furthermore, 15-LOX-1 transfection and subsequent expression inhibit growth of osteosarcoma cells during the expression of 15-LOX-1 (10), and products of 15-LOX-1 induce apoptosis in lymphocytes (12). Additionally, sodium butyrate (13, 14) and interleukin 4 (15, 16) induce 15-LOX-1 and apoptosis or growth inhibition in colorectal cancer cells.

We have also found that NSAIDs can restore 15-LOX-1 expression in esophageal cancer cells, and the induction of 15-LOX-1 expression is critical to apoptosis induction by NSAIDs. Both NS-398, a COX-2 selective inhibitor, and sulindac, a COX-2 nonselective inhibitor, induced the expression of 15-LOX-1 in a time-dependent manner. The NSAID-induced expression of 15-LOX-1 was enzymatically active and increased the formation of 13-S-HODE but not of 15-S-HETE (the product of 15-LOX-1 derived from arachidonic acid), it is clear that the effects of 15-LOX-1 up-regulation on apoptosis are mediated through 13-S-HODE. The selective inhibition of the enzymatic activity of 15-LOX-1 also inhibited 13-S-HODE formation in esophageal cancer cells. This inhibition of 13-S-HODE formation by 15-LOX-1 resulted in blocking apoptosis, as we have confirmed by using various methods to measure apoptosis (e.g., DNA laddering and sub-G₁ cell fractions). Moreover, 13-S-HODE restored apoptosis in cells treated with NS-398 and CAF, whereas identical
concentrations of linoleic acid, the parent compound of 13-S-HODE, failed to exert the same effects. This additionally confirms that the conversion of linoleic acid to 13-S-HODE, which is catalyzed by the enzymatic activity of 15-LOX-1 in a human cell (17), is crucial to the ability of NSAIDs to induce apoptosis in esophageal cancer cells.

These results further extend our previous findings in human colorectal cancers indicating that 15-LOX-1 is down-regulated in a manner related to the loss of apoptosis in cancer cells (8) and that NSAID-induced apoptosis is mediated via 15-LOX-1 up-regulation (7, 9). In esophageal carcinomas, our findings also show the unique role of 15-LOX-1 in promoting apoptosis through the production of 13-S-HODE, thus potentially inhibiting tumorigenesis.

Fig. 3. A, TE-8 cells were given no treatment (control), NS-398 (NS), NS-398 + CAF, NS-398 + CAF + 13-S-HODE, or NS-398 + CAF + LA. Cells were cultured for 72 h, harvested, stained with propidium iodide, and assessed for sub-G1 fractions by flow cytometry. Values shown are the means of triplicate experiments; bars, ± SE. Inhibition of 15-LOX-1 by CAF blocked apoptosis induction by NS-398. 13-S-HODE, but not its parent compound, LA, restored NSAID-induced apoptosis blocked by 15-LOX-1 inhibition in TE-8 cells. B, light-microscopy photos (×200) of TE-8 cells after 72 h of treatment with NS-398, no treatment (control), NS-398 + CAF, NS-398 + CAF + 13-S-HODE, and NS-398 + CAF + LA. C, TE-8 cells were treated as follows: Lane 2, no treatment (control); Lane 3, NS-398 + CAF; Lane 4, NS-398 + CAF + LA; Lane 5, NS-398; and Lane 6, NS-398 + CAF + 13-S-HODE. DNA was extracted 72 h later and analyzed with agarose gel electrophoresis. NS-398-treated cells exhibited the typical DNA fragmentation of apoptosis. Inhibition of 15-LOX-1 by CAF (Lane 3) blocked these changes, whereas 13-S-HODE reestablished apoptosis in these cells (Lane 6). LA had no effects on the blocking of NS-398-induced apoptosis by CAF (Lane 4). Lane 1 represents a standard DNA ladder.
REFERENCES


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Cancer Res 2001;61:4879-4884.

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