

# Nonsteroidal Anti-Inflammatory Drugs Induce Apoptosis in Esophageal Cancer Cells by Restoring 15-Lipoxygenase-1 Expression<sup>1</sup>

Imad Shureiqi, Xiaochun Xu, Dongning Chen, Reuben Lotan, Jeffrey S. Morris, Susan M. Fischer, and Scott M. Lippman<sup>2</sup>

Departments of Clinical Cancer Prevention [I. S., X. X., D. C., S. M. L.], Gastrointestinal Medical Oncology [I. S.], Biostatistics [J. S. M.], and Carcinogenesis [S. M. F.], and Division of Cancer Prevention [R. L.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

## ABSTRACT

In previous studies, we have found that expression of 15-lipoxygenase-1 (15-LOX-1) and its main product, 13-S-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 13-S-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<sup>3</sup> 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 13-S-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 be-

tween 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 13-S-HODE and linoleic acid were obtained from Cayman Chemical, Inc. (Ann Arbor, MI). We obtained antiprotease mixture tablets from Boehringer Mannheim, Inc. (Indianapolis, IN), 13-S-HODE ELISA kits from Oxford Biomedical Research (Oxford, MI), and 15-S-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 SKGT-4, SKGT-5, and BE-3 were obtained from Dr. David Schrupp (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, SKGT-5 and BE-3 from poorly differentiated adenocarcinomas, and SKGT-4 from a well-differentiated adenocarcinoma 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- $\mu$ m 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 ethanols. 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 antirabbit 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 Aqua mount 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  $\geq$ 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-S-HODE and 15-S-HETE levels, and apoptosis, as described in the following paragraphs. Cells were grown in

Received 3/9/01; accepted 4/16/01.

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<sup>1</sup> Supported in part by the State of Texas Tobacco Settlement Funds and in part by National Cancer Institute, NIH, Department of Health and Human Services grants 1K07 CA86970 and CA16672. S. M. Lippman holds the Margaret and Ben Love Professorship in Clinical Cancer Care.

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Clinical Cancer Prevention, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 236, Houston, TX 77030-4095. Phone: (713) 745-3672; Fax: (713) 794-4679; E-mail: slippman@mdanderson.org.

<sup>3</sup> The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; 15-LOX-1, 15-lipoxygenase-1; 13-S-HODE, 13-S-hydroxyoctadecadienoic acid; COX-2, cyclooxygenase-2; 15-S-HETE, 15-S-hydroxyeicosatetraenoic acid; CAF, caffeic acid; NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; LA, linoleic acid.

RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin (Life Technologies, Inc., Grand Island, NY). When cells reached 60–80% confluence, they were treated once with NSAIDs in concentrations that have been shown previously to induce apoptosis (7), either 120  $\mu\text{M}$  of NS-398 (COX-2-selective NSAID) or 150  $\mu\text{M}$  of sulindac (COX-2-nonselective NSAID) in  $\leq 0.5\%$  DMSO. They were then cultured for times ranging from 12 to 72 h (the precise time points are specified below within each experiment description; Ref. 9). The presence of 0.5% DMSO did not affect cell growth in repeated experiments (data not shown). At the time of treatment, the concentration of FBS was reduced to 1%.

Next, we used CAF at a concentration of 2.2  $\mu\text{M}$  to inhibit 15-LOX-1, which we examined with respect to NSAID-induced apoptosis. We have established previously the specificity of this concentration of CAF for inhibiting 15-LOX-1 in colorectal cancer cells (7). TE-8 cells were treated with either NS-398 or sulindac, with and without CAF. For additional assessment of whether the effects of 15-LOX-1 inhibition resulted from loss of 13-S-HODE production, 1.35  $\mu\text{M}$  of 13-S-HODE or linoleic acid was added to cells treated with NS-398 or sulindac + CAF as described previously (9).

**Western Blot Analysis of 15-LOX-1 Protein.** Esophageal cells (TE-8, SKGT-4, SKGT-5, and BE-3) were grown in RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin (Life Technologies, Inc., Grand Island, NY), and then treated with and without sulindac (150  $\mu\text{M}$ ) when reaching 60–80% confluence. Cells were cultured for 72 h after treatment, then were lysed, sonicated, and kept frozen at  $-70^\circ\text{C}$  until analysis. For the time course treatment experiments, TE-8 cells were cultured for 12, 24, 48, or 72 h after being treated with NS-398 (120  $\mu\text{M}$ ) or sulindac (150  $\mu\text{M}$ ) and were similarly harvested and stored until the time of analyses. Equal aliquots of protein from each sample were subjected to 8% SDS-PAGE under reducing conditions. After being transferred, blots were probed with 15-LOX-1 primary antibody and processed by the ECL method, as described previously (7).

**Immunoassay Quantitation of Endogenous 13-S-HODE and 15-S-HETE Production.** TE-8 cells were cultured for 48 h after treatment with NS-398 or sulindac and then lysed. 13-S-HODE and 15-S-HETE were extracted as described previously (9). Endogenous levels of 13-S-HODE and 15-S-HETE were measured by following the manufacturer protocol specified in commercial 13-S-HODE ELISA and 15-S-HETE enzyme immunosorbent assay kits.

**Assessments of Apoptosis.** Apoptosis was evaluated by several methods: DNA gel electrophoresis, microscopic examination to identify morphological changes associated with apoptosis, and flow-cytometric cell-cycle distribution analysis to determine sub- $G_1$  fractions. For DNA gel electrophoresis, cells were harvested 72 h after treatment and then lysed. DNA was extracted from an equal number of cells, precipitated, subjected to electrophoresis on 1% agarose gels, and examined after being stained with ethidium bromide, as described previously (7). Phase-contrast microscopy was used to assess gross evidence of apoptosis. Cells also were stained with propidium iodide for determination of cell-cycle distribution by flow-cytometric analyses, as described previously (9); the resulting data were used to calculate subdiploid DNA peaks (sub- $G_1$  fractions) as a measure of apoptosis.

**Statistical Analyses.** Data from immunohistochemical staining of paired normal and cancerous tissues were compared for the frequency of positive staining in the normal and tumor tissues and for the staining intensity using Sign test. Data were analyzed using SAS software (SAS Institute, Cary, NC).

## RESULTS

**15-LOX-1 Expression Was Reduced in Esophageal Squamous and Adenocarcinomas.** 15-LOX-1 expression was detected in 14 of 15 (93%) of the normal tissues, in contrast to only 2 of 15 (13%) of the tumors (Table 1 and Fig. 1a). This difference in the frequency of positive staining between normal and tumor tissues was statistically significant ( $P = 0.0002$ ). Furthermore, the overall staining intensity was higher in the normal tissue (median = 1) than it was in the tumor (median=0,  $P = 0.00061$ ; Table 1). The staining was cytoplasmic (Fig. 1a), and none was observed in the negative controls (data not shown). Similarly, all of the screened esophageal squamous and adenocarcinoma cell lines showed no expression of 15-LOX-1 protein (data not shown).

Table 1 Results of 15-LOX-1 immunohistochemical staining of paired normal and malignant esophageal tissues

| Sample No. | Histological characteristics of normal samples | Positive staining grade <sup>a</sup> | Histological characteristics of tumor samples | Positive staining grade <sup>a</sup> |
|------------|--|--------------------------------------|---|--------------------------------------|
| 1          | Normal squamous                                | 3                                    | SCC <sup>b</sup>                              | 0                                    |
| 2          | Normal squamous                                | 0                                    | AdenoCA <sup>b</sup>                          | 0                                    |
| 3          | Normal squamous                                | 1                                    | AdenoCA                                       | 0                                    |
| 4          | Normal squamous                                | 1                                    | AdenoCA                                       | 0                                    |
| 5          | Normal squamous                                | 1                                    | AdenoCA                                       | 0                                    |
| 6          | Normal squamous                                | 1                                    | SCC   | 0                                    |
| 7          | Normal squamous                                | 1                                    | AdenoCA                                       | 0                                    |
| 8          | Normal squamous                                | 2                                    | AdenoCA                                       | 1                                    |
| 9          | Normal squamous                                | 2                                    | SCC   | 0                                    |
| 10         | Normal squamous                                | 1                                    | AdenoCA                                       | 0                                    |
| 11         | Normal squamous                                | 2                                    | SCC   | 1                                    |
| 12         | Normal squamous                                | 2                                    | SCC   | 0                                    |
| 13         | Normal squamous                                | 2                                    | SCC   | 0                                    |
| 14         | Normal squamous                                | 2                                    | SCC   | 0                                    |
| 15         | Normal squamous                                | 1                                    | AdenoCA                                       | 0                                    |

<sup>a</sup> Median for normal samples = 1, median for tumor samples = 0;  $P = 0.00061$ , Sign test.

<sup>b</sup> 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 levels of endogenous 13-S-HODE to 24.93 ng/ $\mu\text{g}$  protein and 10.25 ng/ $\mu\text{g}$  protein (means  $\pm$  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/ $\mu\text{g}$  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  $\mu\text{M}$  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- $G_1$  fractions of cells and DNA-fragmentation 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  $\mu\text{M}$ ) induced apoptosis in TE-8 cells as confirmed by DNA laddering and quantitation of sub- $G_1$  fractions (Fig. 3, A and C). In an equal concentration (1.35  $\mu\text{M}$ ), 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

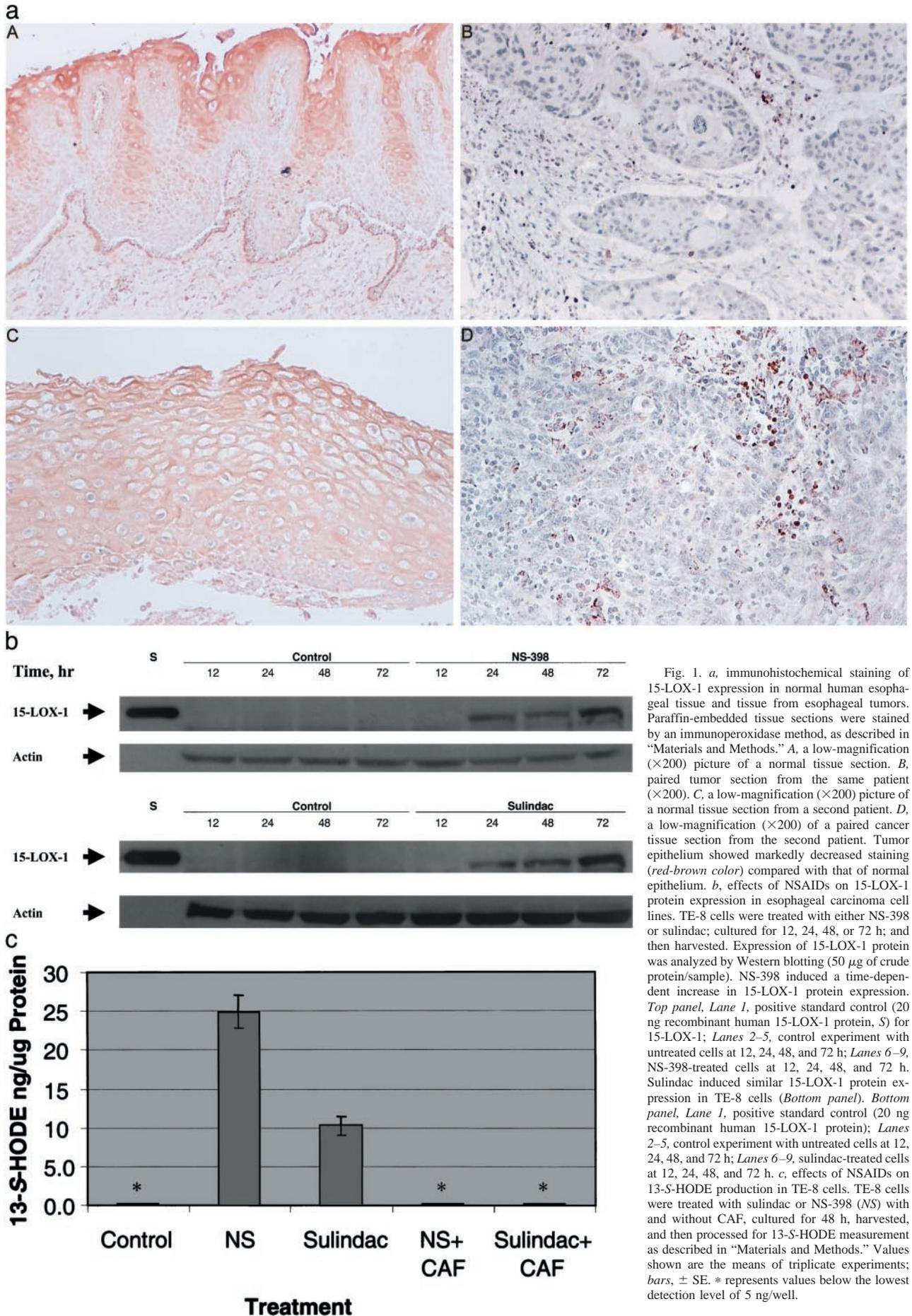


Fig. 1. *a*, immunohistochemical staining of 15-LOX-1 expression in normal human esophageal tissue and tissue from esophageal tumors. Paraffin-embedded tissue sections were stained by an immunoperoxidase method, as described in "Materials and Methods." *A*, a low-magnification ( $\times 200$ ) picture of a normal tissue section. *B*, paired tumor section from the same patient ( $\times 200$ ). *C*, a low-magnification ( $\times 200$ ) picture of a normal tissue section from a second patient. *D*, a low-magnification ( $\times 200$ ) 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  $\mu$ g 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); Lane 5 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. *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,  $\pm$  SE. \* represents values below the lowest detection level of 5 ng/well.



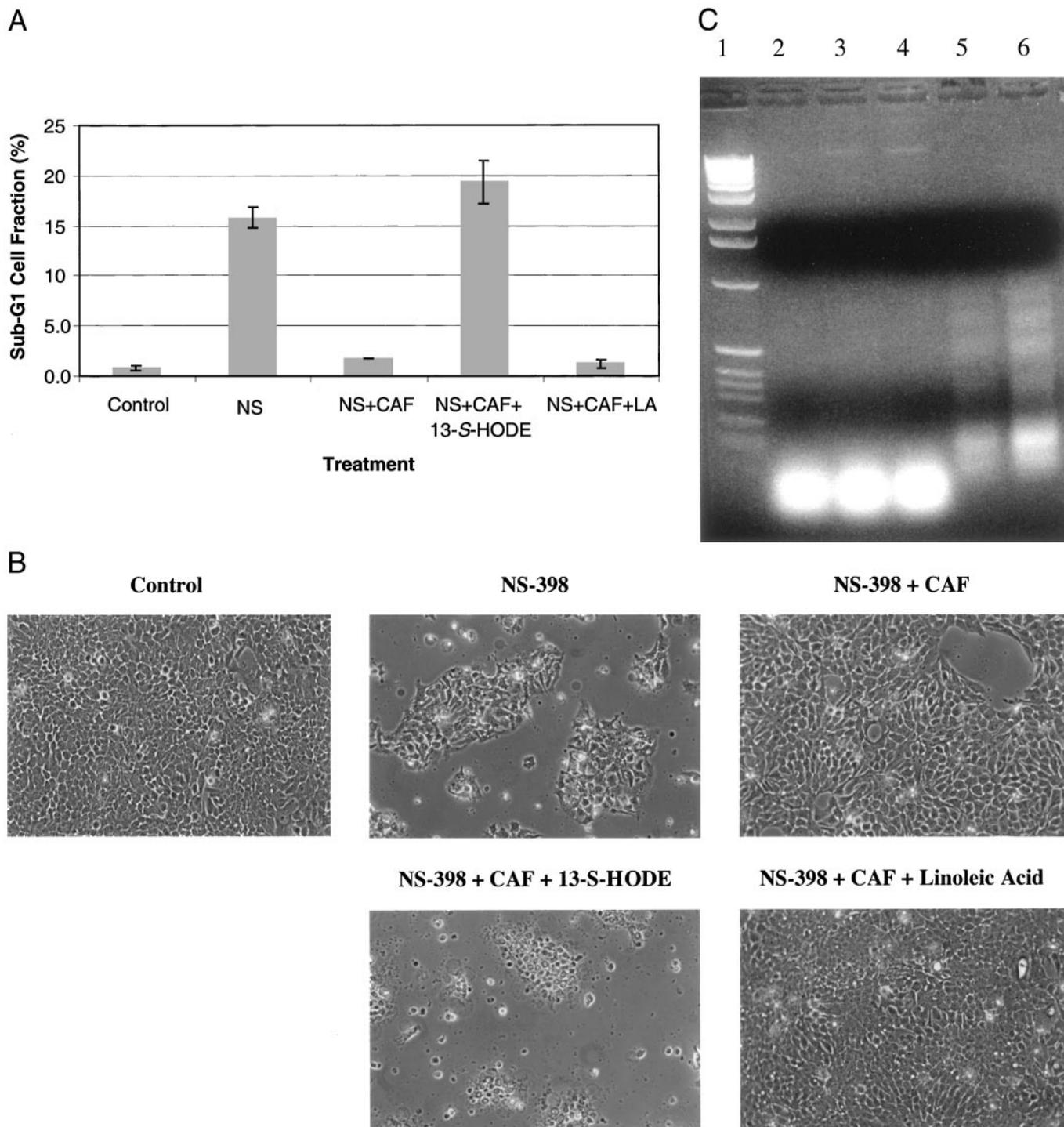


Fig. 3. *A*, TE-8 cells were given no treatment (*control*), NS-398 (*NS*), NS-398 + CAF, NS-398 + CAF + 13-S-HODE, or NS-398 + CAF + LA. Cells were cultured for 72 h, harvested, stained with propidium iodide, and assessed for sub-G<sub>1</sub> fractions by flow cytometry. Values shown are the means of triplicate experiments; bars,  $\pm$  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 ( $\times 200$ ) 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.

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 colo-

rectal 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. In contrast,

other lipoxygenases, such as 5-LOX and 12-LOX, block apoptosis and promote cell proliferation through the formation of HETE products, thereby promoting tumorigenesis (18, 19).

Additional studies are needed to elucidate the mechanisms of NSAID-induced 15-LOX-1 expression in cancer cells. The signal transduction pathway by which 13-S-HODE might induce apoptosis and differentiation remains to be defined; one plausible explanation is that 13-S-HODE activates peroxisome proliferator-activated receptor- $\gamma$ , which in turn triggers apoptosis and cell differentiation (20, 21). The concept that 15-LOX-1 down-regulation is mechanistically related to the loss of apoptosis in cancer cells and that NSAIDs restore 15-LOX-1 expression to induce apoptosis in cancer cells appears to be applicable to organs other than the colon. Additional research efforts are needed to develop interventions that specifically target this pathway, as a means of preventing and treating tumorigenesis.

Our current findings on the role of 15-LOX-1 in human esophageal cancer and its response to NSAIDs are the first reported in this or any system other than colorectal cancer. These results expand our understanding of the biology of esophageal carcinogenesis and identify a novel molecular target for the study of chemoprevention in this site.

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*Cancer Res* 2001;61:4879-4884.

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