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Reduced 15*S*-Lipoxygenase-2 Expression in Esophageal Cancer Specimens and Cells and Upregulation *In Vitro* by the Cyclooxygenase-2 Inhibitor, NS398[1]

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Abstract

Alterations in arachidonic acid metabolism are involved in human carcinogenesis. Cyclooxygenase (COX) and lipoxygenase (LOX) are key enzymes in this metabolism. We analyzed the expression of 15*S*-lipoxygenase-2 (15-LOX-2) mRNA and protein in surgical specimens from normal (*N*=37) and malignant (63) esophageal tissues using *in situ* hybridization and immunohistochemistry (IHC), and in normal (1), premalignant (1), and malignant (5) esophageal cell lines using Northern and Western blotting. 15-LOX-2 was expressed in normal esophageal epithelial cells (EECs) at the highest levels, whereas an SV40-immortalized HET-1A line and three of five esophageal cancer cell lines failed to express it at detectable levels. 15-LOX-2 was detected in 76% (28/37) of the normal esophageal mucosae, but only in 46% (29/63) of the cancer specimens using IHC (*P*<.01). Transient transfection of 15-LOX-2 expression vectors into esophageal cancer cells significantly inhibited the proliferation of 15-LOX-2–negative cancer cells. The COX-2 inhibitor, NS398, induced 15-LOX-2 expression in esophageal cancer cells, which is associated with reduced cell viability. This study demonstrated that 15-LOX-2 expression is lost in esophageal cancers and that the induction of 15-LOX-2 can inhibit cancer cell proliferation. Further investigation of the effects of nonsteroidal anti-inflammatory drugs on 15-LOX-2 expression and apoptosis in esophageal cancer cells may be warranted.

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Keywords: 15-LOX-2, esophageal cancer, NSAIDs, *in situ* hybridization, immunohistochemistry.

Introduction

Esophageal cancer is a significant worldwide health problem. This disease typically is at an advanced pathologic stage at diagnosis and is associated with a very poor prognosis, reflected by an overall 5-year survival rate of less than 10% [1,2]. Relatively less common in the United States than in other countries, esophageal cancer annually accounts for an estimated 13,100 new cases (only 1% of all diagnosed cancers) and 12,600 deaths, and is the seventh leading cause of death from cancer in American men [2]. Cancer statistics [2] have shown that the incidence of esophageal adenocarcinoma is increasing rapidly in the United States, for reasons that are not clear. New approaches to the prevention, early identification, and treatment of esophageal cancer are urgently needed.

Epidemiologic and experimental studies have demonstrated beneficial effects of nonsteroidal anti-inflammatory drugs (NSAIDs) in the prevention of human cancers, especially those in the gastrointestinal tract [3–9]. For example, in studies [4,5,9] of individuals who took NSAIDs regularly, mortality from esophageal cancer was reduced by 40% to 50%. In addition, in animal models of esophageal carcinogenesis, NSAIDs were shown to reduce the frequency and the number of premalignant and malignant lesions [10,11]. More recent data from our and others' laboratories showed that NSAIDs can induce tumor cells to undergo apoptosis [3–8, 12–15]. While inducing apoptosis, the cyclooxygenase-2 (COX-2) inhibitor, NS398, induced the release of cytochrome *C* from the mitochondria, activating caspase-9 and caspase-3 and resulting in the cleavage of poly(ADP-ribose) polymerase [8]. These NSAID effects were associated with the agents' abilities to inhibit COX-2 enzymatic activity and to upregulate the expression of 15*S*-lipoxygenase-1 (15-LOX-1) [14]. Another study [16], however, indicated that the modulation of COX-2 and 15-LOX-1 may not explain all of the effects of NSAIDs in cancer prevention and therapy.

The 15*S*-lipoxygenase-2 (15-LOX-2) enzyme is expressed in skin, cornea, lung, and prostate cells [17]. In

Abbreviations: 15-LOX-2, 15*S*-lipoxygenase-2; AA, arachidonic acid; BrdU, 5-bromo-2′-deoxyuridine; COX-2, cyclooxygenase-2; NSAIDs, nonsteroidal anti-inflammatory drugs

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contrast to its expression in differentiated secretory cells in benign human prostate, *15-LOX-2* expression is reduced in prostate adenocarcinomas [18,19], suggesting that the alteration of this gene plays a role in carcinogenesis and that the modulation of the gene's expression could be a mechanism for chemopreventive strategies. To better understand the role of the 15-LOX-2 enzyme in carcinogenesis and chemoprevention, we first examined the expression of 15-LOX-2 mRNA and protein in surgical specimens from normal and malignant esophageal tissues using *in situ* hybridization (ISH) and immunohistochemistry (IHC), and in normal, premalignant, and cancerous esophageal cell lines using Northern and Western blotting. We also investigated the effects of 15-LOX-2 on cell proliferation using transient transfection assay and, lastly, the effects of NS398 on 15-LOX-2 expression and cell viability in these cell lines.

Materials and Methods

Cell Lines and Surgical Specimens

The normal esophageal epithelial cells (EECs) were derived from benign human esophageal mucosa and were obtained from the Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University (Kyoto, Japan). The SV40-immortalized esophageal cell line, HET-1A, was provided by Dr. Curtis Harris, Laboratory of Human Carcinogenesis, National Cancer Institute (Bethesda, MD) [20]. The esophageal cancer cell lines TE-1, TE-3, TE-7, TE-8, and TE-12 were obtained from the First Department of Pathology, Hiroshima University School of Medicine (Hiroshima, Japan) [7].

Human esophageal tissue specimens were obtained from the Department of Pathology, The University of Texas M.D. Anderson Cancer Center (Houston, TX) and from InnoGenex (San Ramon, CA). Sixteen samples came from esophageal adenocarcinoma patients and 47 samples from squamous cell carcinoma (SCC) patients (a total of 63 tumor samples). There were 37 samples of morphologically normal squamous esophageal mucosae from sectioned margins. Of these 37, there were 33 paired tumor and normal samples. All samples were routinely fixed in 10% buffered formalin, embedded in paraffin, and cut into 4-μm sections. One section from each sample was stained with hematoxylin and eosin for classification.

ISH

A previously described method of nonradioactive ISH was used [21]. Briefly, a 15-LOX-2 cDNA fragment was cloned from the prostate cancer cell line, PC3, using a primer set of 5′-TGCCTCTCGCCATCCAGCT-3′ and 5′-TGTTCCCCTG GGATTTAGATGGA- 3′. The 1-kb polymerase chain reaction (PCR) fragment was then subcloned into pTOPO 2.1 plasmid (Invitrogen, Carlsbad, CA) and sequenced; it exactly matched the previously reported 15-LOX-2 cDNA sequence [17]. For ISH, the plasmid was linearized by *Bam*HI and labeled with digoxigenin-UTP by using T7 polymerase. The binding quality and the specificity of the digoxigenin-labeled antisense riboprobe were verified using negative control sections and compared with the results of immunohistochemical studies.

Immunohistochemistry

The immunohistochemical localization of the 15-LOX-2 protein was performed using a modified avidin–biotin complex (ABC) technique [18]. Tissue sections were deparaffinized in xylene and rehydrated in a series of ethanol solutions (100% to 50%). The endogenous peroxidase activity was blocked by incubation in a 1% methanolic hydrogen peroxide solution for 30 minutes. This procedure was followed by preincubation with 20% normal goat serum to minimize nonspecific binding of the second antibody. The sections were then incubated at 23°C for 4 hours with polyclonal rabbit anti–15-LOX-2 antibody (see Ref. [18]) at a dilution of 1:800 in phosphate-buffered saline (PBS). After being washed three times in PBS, the sections were incubated with biotinylated with goat anti–rabbit IgG (H+L) (Vector Laboratories, Burlingame, CA) for 30 minutes at 23°C and then incubated with the ABC kit (Vector Laboratories) for 30 minutes in darkness. They were then incubated with 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) solution for 20 minutes to visualize the peroxidase complex. The sections were mounted with Aquamount medium under coverslips. Control sections were incubated with the second antibody only.

Review and Scoring of Stained Sections

The sections stained by IHC or ISH for 15- LOX-2 were reviewed by routine microscopy and scored as positive or negative, where positive staining indicates that 10% or more of the epithelial cells were immunopositive. A statistical analysis was performed using the McNemar test to determine the association between the staining results in distant normal and cancerous tissues. The Kendall test is used to determine the accordance between ISH and IHC. *P* values were generated using Statistica version 4.1 for the PowerMac (StatSoft, Tulsa, OK).

Cell Cultures and Treatment

The esophageal cancer TE cells were plated in tissue culture dishes and grown in Dulbecco's modified Eagle's minimal essential medium with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The EEC cells were grown in serum-free keratinocyte growth medium (GIBCO-Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HET-1A cells were grown in LHC-8 medium in tissue culture dishes precoated with a mixture of 18 μg of fibronectin, 18 μl of vitrogen, 180 μl of 10× bovine serum albumin, and 2 ml of LHC basal medium (all from Biofluids, Rockville, MD) at 37°C in a humidified atmosphere of 96.5% air and 3.5% CO_2. To detect cell viability after treatment with NS398, TE-1, TE-3, TE-7, TE-8, and TE12 cells were treated with and without NS398 (50 or 100 μM) for 5 days. The culture medium was replaced once at 72 hours. On day 5, the cells were fixed with 10% trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid, and then the optical

densities were read on an automated spectrophotometric plate reader at a single wavelength of 490 nm. The percentage of growth inhibition was calculated from the equation: % Control = $(OD_t/OD_c)\times 100$, where OD_t and OD_c are the values of optical densities in treated and control cultures, respectively.

RNA Purification and Analysis by Northern Blotting

RNA were extracted from the monolayer cultures when they became about 90% confluent using Tri-reagent (Molecular Research Center, Cincinnati, OH). For the Northern blot analysis, 30 μg of total cellular RNA was fractionated on 1.2% formaldehyde agarose gels, stained with ethidium bromide, and transferred in 10× saline–sodium citrate (SSC) to nylon filters by capillary transfer. The 15-LOX-2–cDNA fragment was released from pTOPO2.1 using *Eco*RI and used as the probe for Northern blotting. The probe was labeled with ^{32}P to a specific activity of approximately 2×10^9 dpm/μg using random hexanucleotides as primers [22]. The filters were prehybridized and hybridized at 68°C in Rapid-Hyb buffer (Amersham-Phamarcia Biotechnology, Arlington Heights, IL) with probes used at 10^7 cpm/filter. The filters were washed with 2× SSC and 0.1% sodium dodecyl sulfate (SDS) for 10 minutes at 23°C, and with 0.1× SSC and 0.1% SDS for 20 minutes at 68°C; then they were placed against a Hyperfilm-MP (Amersham-Pharmacia Biotechnology) for autoradiography.

Protein Extraction and Western Blotting

Cellular proteins were extracted from esophageal cells in a lysis buffer containing 30 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10 mM sodium pyrophosphate, 2 mM orthoranadate, 1% Triton X-100, 1% NP40, 100 mM sodium fluoride, 0.2 mM phenylmethylsulfonyl fluoride, and one tablet of protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). The protein concentration in the samples was measured using the Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA). Protein samples (100 μg) extracted from each cell line were subjected to gel electrophoresis in 10% denaturing polyacrylamide slab gels in the presence of SDS. The proteins were then transferred electrophoretically to a hybond-C nitrocellulose membrane (Amersham-Pharmacia Biotechnology) at 150 V for 2 hours at 4°C. The membrane was subsequently immersed in 0.5% Ponceau S in 1% acetic acid to stain the proteins and to validate that equal amounts of protein were loaded in each lane and transferred efficiently. After incubating the nitrocellulose membranes overnight in a blocking solution containing 5% bovine serum albumin in TBST, the membranes were incubated for 3 hours with rabbit anti–15-LOX-2 antibody (1:1000) and subsequently with a second antibody from the Electrochemiluminescence (ECL) kit from Amersham-Pharmacia Biotechnology, according to the manufacturer's instructions. The membranes were then washed three times in PBS containing 0.1% Tween-20, incubated with ECL solution (Amersham-Pharmacia Biotechnology) for 1 to 2 minutes, and exposed to the X-ray film for detection of chemiluminescence. After that, the membranes were stripped and reprobed with anti–β-actin antibody (Sigma) for verification of equal protein loading.

Inhibition of 5-Bromo-2′-Deoxyuridine (BrdU) Incorporation Through 15-LOX-2 Transfection

The esophageal cancer cell lines TE-1, TE-8, and TE-12 were grown on monolayer culture and then transiently transfected with either pEGFP–15-LOX-2 expression vector or pEGFP empty vector as the control (both vectors were provided by Dr. Dean G. Tang) for 12 hours; 10 μM BrdU was added to the growth medium and the cells were cultured for an additional 8 hours. The cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes to preserve the green fluorescent protein (GFP) and permeabilized in 1% Triton X-100 for 20 minutes at room temperature. The cells were then subjected to BrdU immunostaining as previously described by Tang et al. [23].

Detection of 15-LOX-2 Expression Using Quantitative Real-Time Reverse Transcriptase (RT) PCR

The numbers of 15-LOX-2 mRNA copies in the untreated and treated esophageal cancer cells were determined by real-time quantitative RT-PCR using a Lightcycler fluorescence temperature rapid air cycler (Roche Molecular Biochemicals) as previously described by Shappell et al. [19]. The primers used, 5′-GCC-TCT-CGC-CAT-CCA-GCT-3′ (forward) and 5′-TGC-CGA-GTT-CTC-CTT-CCA-TGA-3′ (reverse), resulted in a 126-bp amplified product.

Results

Expression of 15-LOX-2 in Esophageal Cell Lines

As shown in Figure 1, EECs, established from normal esophagus, expressed 15- LOX-2 at the highest levels.

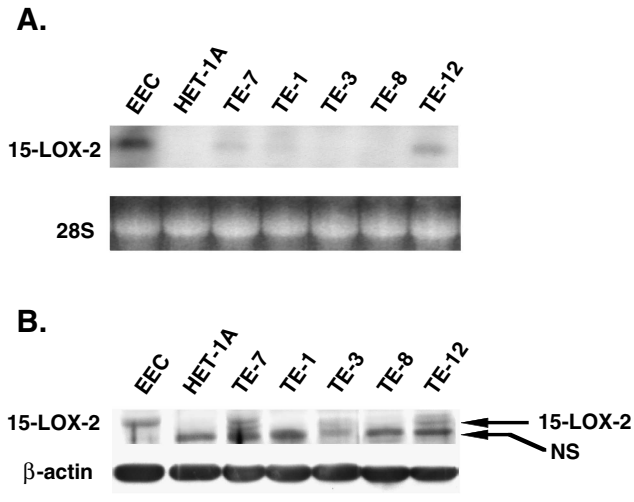

Figure 1. *Analysis of 15-LOX-2 mRNA and protein using Northern (A) and Western (B) blotting. Normal esophageal cells (EECs), SV40-immortalized esophageal epithelial cell line HET-1A, and esophageal cancer cell lines TE-1, TE-3, TE-7, TE-8, and TE-12 were grown in monolayer cultures to about 90% confluence. The total RNA and protein were isolated and subjected to Northern and Western blot analyses, respectively. The experiments were repeated once with similar results.*

Table 1. Differential Expression of 15-LOX-2 in Esophageal Tissues.

Detection method	% (Number of positive/total)		
	Normal	Adenocarcinoma	SCC
ISH	73 (27/37)	19 (3/16)	60 (28/47)*
IHC	76 (28/37)	19 (3/16)	55 (26/47)*

*P<.01 by McNemar test for the 33 paired samples of normal squamous epithelia and SCCs.

In contrast, the esophageal cancer cell lines TE-7 and TE-12 expressed 15-LOX-2 at very low levels. Neither 15-LOX-2 mRNA nor the corresponding protein was detectable in the SV40-immortalized premalignant EEC line HET-1A or in the TE-1, TE-3, or TE-8 cell lines. In the Western blots, all cell lines produced nonspecific bands, except for normal EECs (Figure 1*B*) or other normal epithelial cells (e.g., of the prostate, breast, skin, and bronchus; data not shown). The reason the nonspecific bands appeared solely in tumor lines is unknown; however, it may represent different variants of 15-LOX-2, which have been reported in prostate and lung cancer cells [23,24].

Differential Expression of 15-LOX-2 in Normal and Malignant Esophageal Tissues

As shown in Table 1, 27 of 37 normal squamous epithelium samples from the sectioned margins of esophageal cancers were positive for 15-LOX-2 mRNA, according to the ISH experiments, and 28 of 37 samples were positive for 15-LOX-2 protein, according to immunohistochemical analysis. On the other hand, only 3 of 16 esophageal adenocarcinoma samples and 28 of 47 SCC samples were positive for 15-LOX-2 protein (immunohistochemical analysis), and only 3 of 16 esophageal adenocarcinoma samples and 26 of 47 SCC samples expressed 15-LOX-2 mRNA (ISH). The positive staining usually occurred at the differentiated areas of cancer nests or suprabasal cells of normal mucosa; basal, undifferentiated tumor, and stromal cells were virtually negative (Figure 2). The McNemar test showed P<.01 for both

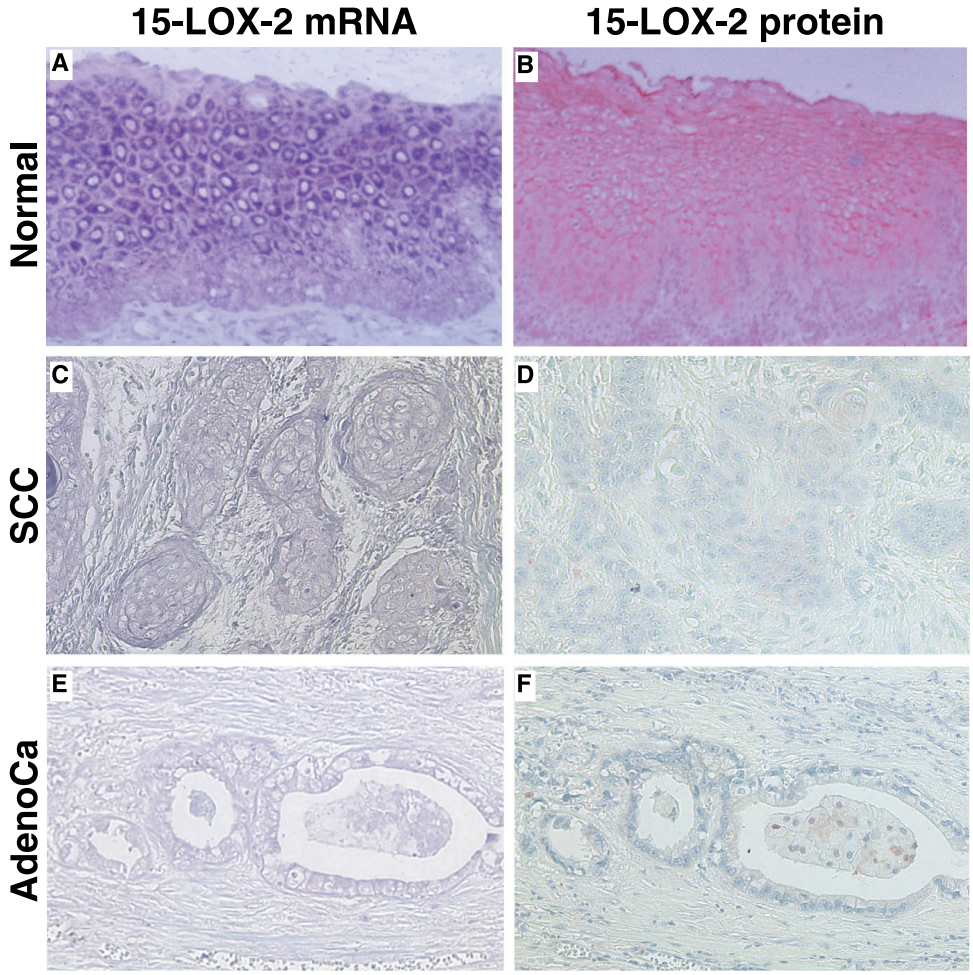

Figure 2. *Differential expression of 15-LOX-2 mRNA and protein in normal and malignant esophageal surgical specimens. 15-LOX-2 mRNA and protein were detected using ISH and IHC, respectively. Consecutive sections of formalin-fixed and paraffin-embedded human esophageal carcinomas and distant normal tissues were hybridized with 15-LOX-2 antisense digoxigenin-labeled cRNA probe, which results in purple to blue staining of the positive signal in the cytoplasm. IHC was performed by using polyclonal rabbit anti–15-LOX-2 antibody, and the AEC was the chromogen, resulting in red staining of the positive signal in the cytoplasm. (A), (C), (D), (E), (F)=200× original magnification; (B)=100× magnification.*

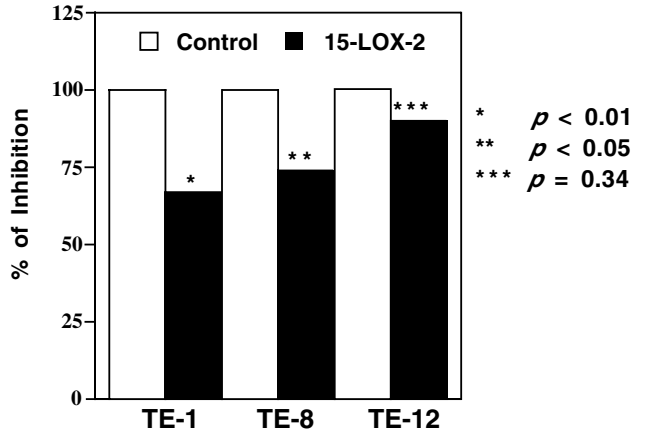

Figure 3. *Inhibition of esophageal cancer cell proliferation by the expression of 15-LOX-2. Esophageal cancer cell lines TE-1, TE-8, and TE-12 were transiently transfected with the control or 15-LOX-2 expression vectors for 12 hours and treated with BrdU for 8 hours. The cells were then stained with anti–BrdU antibody (see Materials and Methods) and more than 200 GFP-positive cells were counted for positive or negative BrdU staining. The percentage of inhibition of BrdU incorporation was calculated from the equation:* $\%\ Inhibition = [1-(N_b/N_g)] \times 100$, *where* N_b *and* N_g *are the numbers of BrdU-positive cells in GPF-positive cells of 15-LOX-2–transfected and control cultures, respectively. The data showed that 15-LOX-2 significantly reduced BrdU incorporation in 15-LOX-2–negative esophageal cancer cells. The experiments were performed in triplicate with similar results.*

the ISH and IHC results between normal and SCC tissues (Table 1). The Kendall test showed that $\tau = 0.88$, SE = 0.03, and $P < .00001$, indicating a strong concordance between our ISH and IHC results.

Inhibition of Esophageal Cancer Cell Proliferation by Restoration of 15-LOX-2 Expression

To evaluate the role of 15-LOX-2 in inhibiting esophageal cancer cell proliferation, we transiently transfected either the GFP reporter expression vector of 15-LOX-2, pEGFP–15-LOX-2, or the empty pEGFP vector as the control into esophageal cancer cell lines TE-1, TE-8, and TE-12 and then treated these cells with BrdU. After BrdU immunostaining, we counted more than 200 GFP-positive cells for positive or negative BrdU staining in both the control and the 15-LOX-2–transfected cell lines. The data (Figure 3) showed that transient transfection of 15-LOX-2 reduced BrdU incorporation by 33% in TE-1, 26% in TE-8, and only 10% in TE-12 (a χ^2 test showed *P* values of .01, .05, and .34, respectively). One of the representative figures is shown in Figure 4. These data demonstrated that 15-LOX-2 inhibited BrdU incorporation more in 15-LOX-2–negative esophageal cancer cells (TE-1 and TE-8) than in 15-LOX-2–positive esophageal cancer cells (TE-12), suggesting that 15-LOX-2 reduces cancer cell proliferation.

Modulation of 15-LOX-2 and Reduced Cell Viability after Treatment with NS398

Our previous studies demonstrated that the NSAID, NS398, could induce esophageal cancer cells to undergo apoptosis, which was associated with COX-2 expression and upregulation of 15-LOX-1 [8,14]. In the present study, we investigated the modulation of 15-LOX-2 using NS398 in esophageal cancer cell lines. NS398 at 100 μM was able to induce 15-LOX-2 mRNA expression, as detected by quantitative real-time RT-PCR (Figure 5). To test whether the upregulation of 15-LOX-2 was associated with cell viability, we grew these cells on a monolayer culture and treated them with NS398 (50 and 100 μM) for 5 days. The cell viability assay showed that the treatment of these cell lines with NS398, especially at the concentration of 100 μM, was associated with upregulation of 15-LOX-2 (Figure 6). As shown in our previous study in TE-8 cells, the reduced cell viability after treatment with 100 μM NS398 resulted from the induction of apoptosis [8,14].

Discussion

Arachidonic acid is converted to prostaglandins, prostacyclin, and thromboxane by COX and to hydroxyeicosatetraenoic acids (HETEs) or leukotrienes by LOX [25]. Three LOXs have been discovered in humans: 5-LOX, 12-LOX, and 15-LOX [18]. 15-LOX-1 and 15-LOX-2, the two known isoenzymes of 15-LOX, differ from each other in tissue distribution and enzyme activity. For example, the tissue distribution of 15-LOX-2 is more limited than that of 15-LOX-1 [17]. 15-LOX-2 converts arachidonic acid exclusively to 15*S*-hydroperoxyeicosatetraenoic acid (15-HPETE), which is reduced by cellular peroxidases to 15*S*-hydroxyeicosatetraenoic acid (15-HETE), whereas 15-LOX-1 metabolizes linoleic acid to 13*S*-hydroxyoctadecadienoic acid. Unlike 15-LOX-1, 15-LOX-2 metabolizes linoleic acid less efficiently than it does arachidonic acid [17].

Our previous study demonstrated that 15-LOX-1 is expressed in normal esophageal tissues and reduced in esophageal cancer tissues. We also found that certain NSAIDs can restore 15-LOX-1 expression in esophageal cancer cells and that the induction of 15-LOX-1 expression is critical to apoptosis induction by these NSAIDs. Both NS398, a COX-2–selective inhibitor, and sulindac, a nonselective COX inhibitor, induced the expression of 15-LOX-1 in a time-dependent manner [14].

The present study is the first to demonstrate that 15-LOX-2 mRNA and protein are expressed in normal esophageal epithelium and cells but downregulated in esophageal cancer cell lines and tissue specimens. The data also showed that 10 of 37 samples of normal esophageal epithelium did not express 15-LOX-2, indicating that the morphological normal epithelium obtained from the sectioned margins of esophageal carcinomas may not have been truly "normal" at the molecular level, although the function of 15-LOX-2 in esophageal epithelium and the cause of its downregulation in esophageal cancer remain unclear. In prostate tissues, the uniform expression of 15-LOX-2 in prostate apical or secretory cells suggests a role for this enzyme in secretion function. A reduced expression of 15-LOX-2 in atrophic prostate glands and prostate adenocarcinoma may thus parallel a reduction of secretory differentiation [18]. The loss

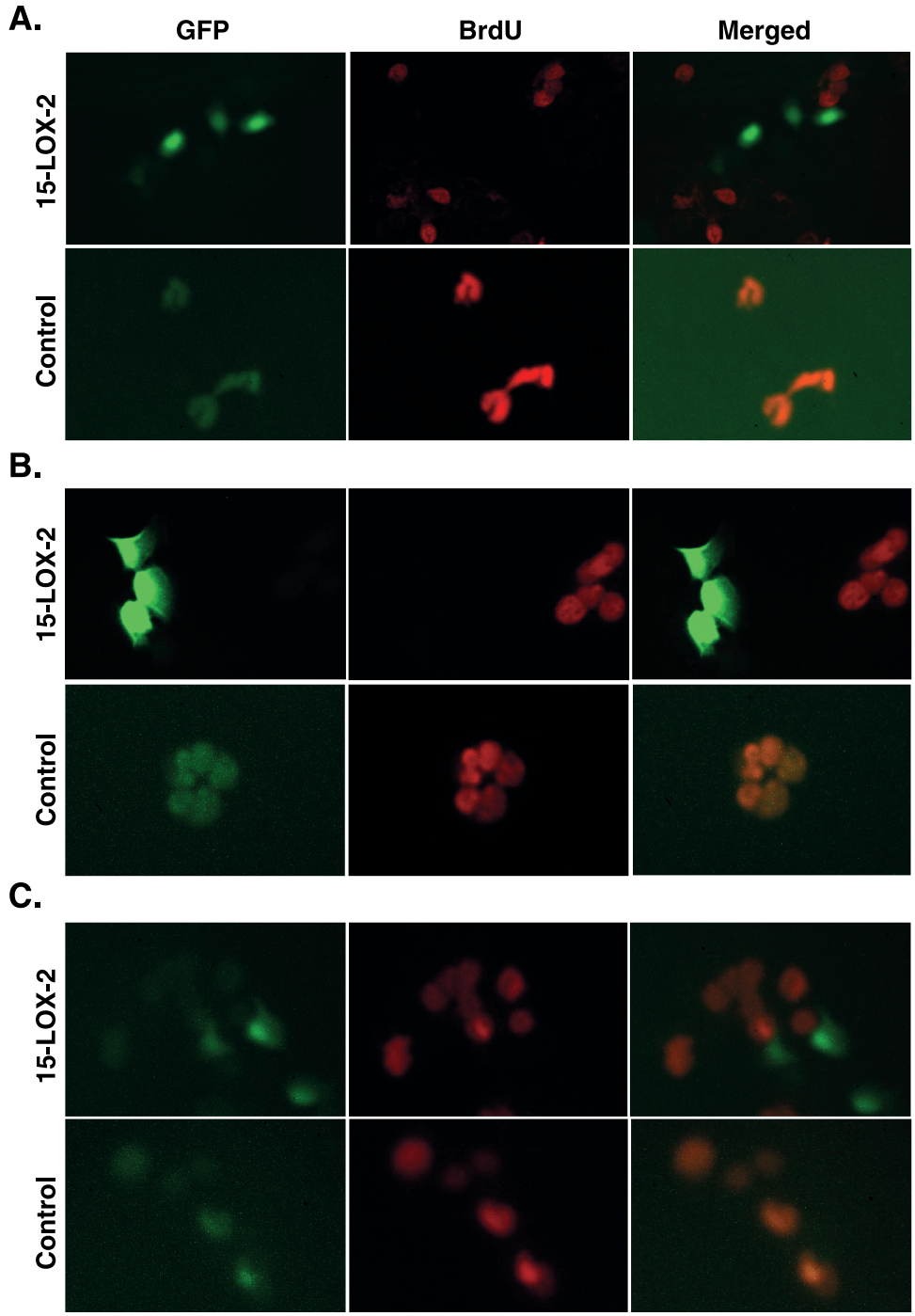

Figure 4. *Inhibition of BrdU incorporation by 15-LOX-2 in esophageal cancer cell lines. 15-LOX-2 expression vectors were transiently transfected into the esophageal cancer cell lines TE-1 (A), TE-8 (B), and TE-12 (C), respectively, for 12 hours and then treated with BrdU for 8 hours (see Materials and Methods). After BrdU immunostaining, more than 200 cells were counted for positive staining of GFP (green), for positive or negative BrdU (red) staining in these cells.*

of 15-LOX-2 expression may alter the metabolism of arachidonic acid and linoleic acid and reduce 15-HETE formation, which may be crucial events in cancer development and progression. A recent study demonstrated that 15-LOX-2 could suppress prostate cancer development by inhibiting cell cycle progression [23]. Our present data indicate that the expression of 15-LOX-2 can significantly reduce BrdU incorporation in 15-LOX-2–negative esophageal cancer cells and so inhibits cell proliferation.

This study also shows that NS398 can induce 15-LOX-2 expression and that this induced expression is associated with reduced cell viability. The molecular mechanism of this event is unclear. The relationships or interactions among COX-2, 15-LOX-1, and 15-LOX-2 are also unknown. Taken together, the data from this study of 15-LOX-2 and our previous studies of COX-2 [7,8] and 15-LOX-1 [14] indicate that COX-2 is overexpressed in esophageal cancer cells and tissues and that the expression of 15-LOX-1 and

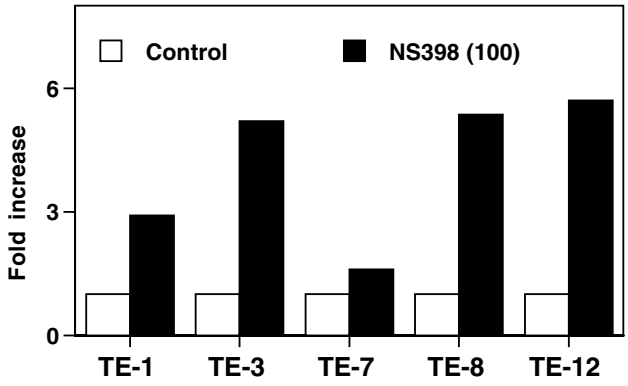

Figure 5. *Induction of 15-LOX-2 expression after treatment with NS398 using quantitative RT-PCR. Esophageal cancer cell lines TE-1, TE-3, TE-7, TE-8, and TE-12 were grown on monolayer and treated with NS398 (100 μM) for 5 days. Afterwards, total cellular RNA was isolated and subjected to real-time RT-PCR analysis. The experiments were repeated once.*

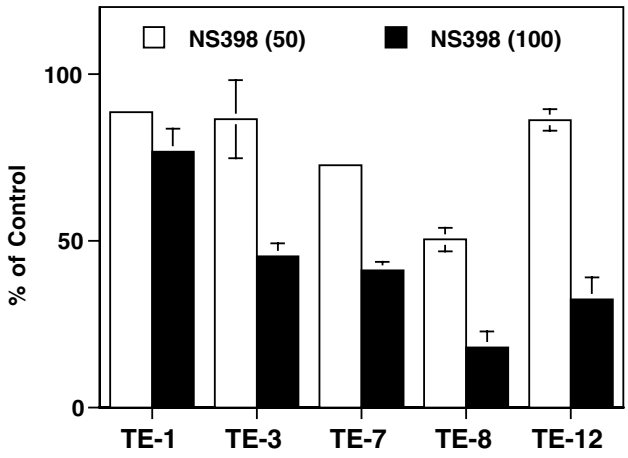

Figure 6. *Cell viability assay. Esophageal cancer cell lines TE-1, TE-3, TE-7, TE-8, and TE-12 were treated with and without NS398 (50 or 100 μM) for 5 days. Culture medium was replaced once at 72 hours. On day 5, the cells were fixed with 10% trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid, and then the optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 490 nm. The percentage of growth inhibition was calculated from the equation: % Control = $(OD_t/OD_c) \times 100$, where OD_t and OD_c are the values of optical densities in treated and control cultures, respectively. The experiments were repeated once.*

15-LOX-2 is reduced. Moreover, certain NSAIDs can induce apoptosis in esophageal cancer cells in association with a reduced COX-2 activity and an upregulation of 15-LOX-1 and 15-LOX-2. Therefore, further studies, including transfection of 15-LOX-1 and 15-LOX-2 expression vectors and antisense COX-2 constructs into esophageal cancer cell lines, may enhance our understanding of the importance of these enzymes in esophageal carcinogenesis.

Acknowledgements

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