Increased 5-lipoxygenase expression and induction of apoptosis by its inhibitors in esophageal cancer: a potential target for prevention

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Arachidonic acid (AA) is the major precursor of several classes of signal molecules and the alteration of its metabolism is involved in human carcinogenesis. For instance, 5-lipoxygenase (5-LOX) converts AA to hydroxyeicosatetraenoic acids or leukotrienes (LTs), which are able to enhance proliferation, increase survival and suppress the apoptosis of human cells. To determine the potential use of 5-LOX inhibitors in the prevention of esophageal cancer, we first analyzed the 5-LOX expression in esophageal tissue samples using immunohistochemistry and then examined the effect of the 5-LOX inhibitors AA861 and REV5901 on cell viability and apoptosis in esophageal cancer cell lines. 5-LOX expression was present in 79% (127/161) of esophageal cancer but in only 13% (4/32) of normal esophageal mucosa. 5-LOX was also expressed in all the eight esophageal cancer cell lines. Moreover, 5-LOX inhibitors caused a dose- and time-dependent reduction of cell viability, which was due to the induction of apoptosis and associated with LTB4 suppression. Our data also showed that both LTB4, a product of 5-LOX and LTB4 receptor antagonist U-75302 were able to prevent AA861 and REV5901 on induction of apoptosis. The present study demonstrated that 5-LOX protein expression is increased in esophageal cancer and that 5-LOX inhibitors can induce esophageal cancer cells to undergo apoptosis, suggesting that 5-LOX may be an effective target in the prevention of esophageal cancer.

Introduction

Esophageal cancer is a significant health problem worldwide because of its poor prognosis and the increasing incidence of esophageal adenocarcinoma (1–4). The most common histological types of primary esophageal cancer are squamous cell carcinoma (SCC) and adenocarcinoma, which have overlapping risk factors, gene alterations, and identical treatment and prognosis in the clinic (2–4). Tobacco smoke is the most important risk factor for both esophageal SCC and adenocarcinoma; gastroesophageal reflux resulting in Barrett’s esophagus is another important risk factor for adenocarcinoma (5–7). Over the past two decades, a large body of knowledge of the molecular alterations associated with esophageal cancer has been generated, which has greatly improved our understanding of this deadly disease. Further investigation should be focused on its prevention, early identification and treatment by incorporating this existing knowledge.

Arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid, is the major precursor of several classes of signal molecules. Alteration of AA metabolism is thought to be involved in human carcinogenesis, because some of the AA metabolites enhance cell proliferation and increase cell survival but suppress apoptosis (8–10). AA is converted to prostaglandins, prostacyclins and thromboxanes by cyclooxygenase (COX) and to hydroxyeicosatetraenoic acids (HETEs) or leukotrienes (LTs) by lipoxygenase (LOX). Previous studies, including some studies by our group, demonstrated that certain COX-2 inhibitors, such as NS398, decreased cell viability in COX-2-positive esophageal cancer cell lines but not in a COX-2-negative cell line (9,11). The percentage of apoptosis induced by NS398 was associated with the level of COX-2 expression. The caspase inhibitor and PGE2 can block the effect of NS398 on induction of apoptosis. However, other studies have reported that some COX-2 inhibitors, such as celecoxib, have COX-independent effects on different cancer cells (12,13). There were two major arguments supporting the COX-independent effects of COX-2 inhibitors: the dose used was usually much higher than that needed to inhibit COX-2 enzymatic activity, and the drugs were also effective in cancer cells that do not express COX-2 (14). Recently, it was reported that the products of 5-LOX were able to enhance proliferation and increase the survival of human cells (10), and that the 5-LOX protein expression is increased in different human cancers, such as breast and prostate cancers (15–17). Thus, to determine the 5-LOX protein level in esophageal cancer and to test the use of its inhibitors in the prevention or treatment of esophageal cancer, we first analyzed the 5-LOX expression in esophageal tissue samples using immunohistochemistry and then examined the effect of its inhibitors AA861 and REV5901 on cell viability and apoptosis in esophageal cancer cell lines.

Materials and methods

Tissue samples

Specimens were obtained from 161 cases of esophageal cancer at Anhui Medical University, China (n = 81), and The University of Texas MD Anderson Cancer Center (n = 80). Thirty-two distant normal squamous mucosa specimens were also obtained. The samples were routinely fixed in 10% buffered formalin, embedded in paraffin and cut into 4-μm-thick sections. All sections were stained with hematoxylin and eosin for classification. Our Institutional Review Board (IRB) approved the protocol for the use of the patient samples for the study.
Immunohistochemistry

Immunohistochemical localization of the 5-LOX protein was performed using an avidin-biotin complex technique as described previously (18) with a monoclonal anti-5-LOX antibody at a dilution of 1:50 (Clone # 3; BD Bioscience, San Jose, CA). The stained sections were reviewed and scored using an Olympus microscope. The sections were then signed as having positive or negative staining. Positive staining was defined as ≥10% of the epithelial cells stained positively. Statistical analysis was performed using the χ2-test or Fisher’s exact test to determine the association between normal and tumor tissues. P-values were generated using the Statistica software program (version 4.01 for Macintosh, StatSoft, Tulsa, OK).

Cell lines and treatments

The esophageal SCC cell lines TE-1, TE-3, TE-7, TE-8 and TE-12 were obtained from Eiichi Tahara (First Department of Pathology, Hiroshima University School of Medicine, Japan), while the esophageal adenocarcinoma cell lines SKGT-4 and SKGT-5, and Barrett’s esophagus cell line BE3 were obtained from David Schrump (Surgery Branch, National Cancer Institute, Bethesda). These cell lines were plated in tissue culture dishes and grown in Dulbecco’s modified Eagle’s minimal essential medium with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 95% air and 5% CO2. To examine the effect of the 5-LOX inhibitors AA861 and REV901, the cells were plated in regular medium and incubated for 24 h. The medium was then replaced with either control medium or medium containing AA861 at 30, 60 or 120 μM or REV901 at 5, 10 or 20 μM (both from Cayman Chemical, Ann Arbor, MI). These drugs were dissolved in dimethyl sulfoxide (DMSO) and diluted into the medium before each experiment. The medium was completely replaced with fresh medium every 72 h. At the end of the experiment, the cells were fixed with 10% trichloroacetic acid, stained with 0.4% sulforhodamine B and then washed with PBS (Sigma Chemical Co., St Louis, MO) for normalization and equal protein loading. The percentage of growth inhibition was calculated using the equation: % control = 1 – (ODt/ODc) × 100, where ODt and ODc are the optical densities in treated and control cultures, respectively.

Protein extraction and western blotting

Cellular proteins were isolated in lysis buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin and 50 mM Tris–HCl (pH 8.0). The samples were then stored on ice for 10 min and centrifuged at 13 000 g for 30 min. The protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer’s protocols. Samples containing 50 μg of protein extracted from esophageal cancer cell lines were subjected to gel electrophoresis in 10% polyacrylamide slab gels in the presence of SDS. The proteins were then transferred electrophoretically to a Hybond-C nitrocellulose membrane (Amersham, Arlington Heights, IL) at 125 V for 2 h at 4°C. The membrane was subsequently immersed in 0.5% Ponceau S in 1% acetic acid to stain the proteins and to confirm that equal amounts of protein were loaded in each lane and transferred efficiently. After incubation overnight in a blocking solution containing 5% bovine skin milk powder in 10 mM phosphate-buffered saline (PBS, pH 7.4), the nitrocellulose membrane was incubated for 3 h with an anti-5-LOX antibody (BD Bioscience, Ann Arbor, MI) at a dilution of 1:250. The membrane was then washed with PBS buffer to remove the excess unbound antibodies and incubated for 1 h with a horse anti-mouse antibody (Amersham) at a dilution of 1:5000. After incubation, the membrane was washed three times in PBS containing 0.1% Tween-20, incubated with an enhanced chemiluminescence solution (Amersham) for 1-2 min, and then exposed to an X-ray film for the detection of a positive signal. Afterward, the membrane was reblotted with an anti-β-actin antibody (Sigma Chemical Co., St Louis, MO) for normalization and equal protein loading.

DNA extraction and gel electrophoretic analysis of DNA fragmentation

Soluble DNA was extracted after 2 days of treatment with 60 μM AA861 or 10 μM REV901. The cells floating in the medium were collected after centrifugation, while the cells that remained attached to the dish were detached by scraping. The cells were then centrifuged into a pellet and resuspended in Tris–EDTA buffer (pH 8.0). The plasma membrane of the cell was lysed in 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100 on ice for 15 min. Next, the lysate was centrifuged at 12 000 g for 15 min to separate soluble (nucleoprotein) DNA from pelleted (intact genomic) DNA. Soluble DNA was treated with RNase A (50 μg/ml) at 37°C for 1 h, and then proteinase K (100 μg/ml) in 0.5% SDS at 50°C for 2 h. The residual material was extracted with phenol/chlorform, precipitated in ethanol, electrophoresed on a 1.8% agarose gel and stained with ethidium bromide. The gels were then photographed in the dark using UV illumination.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay

A TUNEL assay was performed using a commercial kit (APO-BRIDU Apoptosis Kit; Phoenix Flow Systems, San Diego, CA). The esophageal cancer cells were treated with the control medium or a medium containing 60 μM AA861, 10 μM REV95901, 0.5 μM LTβ or 1 μM U-73502 (all from Cayman) alone, or in combination for 2 days. Both the floating and attached cells were collected, labeled with fluorescein dye and stained with propidium iodide according to a protocol provided by the manufacturer. The cells were then analyzed by flow cytometry using a FACScan flow cytometer (Epics Profile; Coulter Corp., Hialeah, FL).

ELISA detection of LTB4 production

The esophageal cancer cell lines TE-1, TE-3, SKGT-4 and SKGT-5 were plated in monolayer cultures for 16 h and then treated with or without AA861 (60 μM) or REV95901 (10 μM) for 2 days. The conditioned media were then taken for the detection of LTβ using an ELISA assay kit (Cayman) according to the manufacturer’s instructions.

RT-PCR

For RT-PCR analysis, 2 μg of total cellular RNA, purified by Tri-reagent (MRC, Cincinnati, OH) from esophageal cancer cell lines, was subjected to cDNA synthesis using a reverse transcription kit (Roche Molecular Biochemicals, Indianapolis, IN) at 42°C for 50 min. PCR was then performed on the cDNA product in each tube using different primer sets. Primers for 5-LOX were (sense) 5'-TAGTGGCCTGAAAGACCA-3' and (antisense) 5'-CCTAGCGAAGTTAACAC-3'; these primers amplified a 261 bp PCR product. Primers for β-actin were (sense) 5'-TCTTCTGCGTTTTCCGTAG-3' and (antisense) 5'-ACTCCAGCCGGCTTACCAC-3'; these primers amplified a 203 bp PCR product. The PCR was performed using Roche high fidelity kit in a thermal cycler (PE thermal cycler 9700 from Applied Biosystems, Foster City, CA). The reaction was initiated with a 5-min incubation at 95°C, followed by 35 amplification cycles (95°C for 30 s, 58°C for 1 min, and 72°C for 1 min) and a final extension step (72°C for 10 min and storage at 4°C). Equal volumes of the PCR product from each sample were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed.

Results

Increased expression of 5-LOX protein in esophageal cancer

Using immunohistochemistry, we analyzed the 5-LOX expression in formalin-fixed, paraffin-embedded tissue sections from 161 esophageal cancer cases (81 from Anhui Medical University in China and 80 from MD Anderson Cancer Center). The Chinese cases consisted of only SCC but the Anderson cases consisted of both SCC (27 cases) and adenocarcinoma (53 cases). 5-LOX expression was detected in 4/32 cases (13%) of the normal epithelium and in 127/161 cases (79%) of the esophageal cancer. The difference in 5-LOX expression between normal and tumor tissues was statistically significant (P < 0.00001). We did not find any difference in 5-LOX expression between SCC and adenocarcinoma, or between Chinese and Anderson cases (Table I). 5-LOX protein was expressed in cancerous epithelial cells and

| Table I. Expression of 5-LOX in esophageal cancer tissue specimens |
|----------------------|-------------------------|----------------|
| 5-LOX expression     | Positive (%)            | Negative (%) |
| Chinese cases SCC    | 69                      | 12           |
| Anderson cases SCC   | 21                      | 6            |
| ADC                  | 37                      | 16           |

SCC, squamous cell carcinoma; ADC, adenocarcinoma.

There is no statistical difference between SCC and ADC or Chinese and Anderson SCCs.
also in some of the stromal cells; however, normal esophageal squamous epithelial cells were mostly negative for 5-LOX expression (Figure 1). Furthermore, we correlated the expression of 5-LOX with clinicopathological data and observed that the 5-LOX expression was statistically significantly associated with the gender of the patients, tumor differentiation, lymph node metastasis and tumor size in Chinese cases. We did not observe such associations in the MD Anderson cases (Table II).

**Decreased cell viability after treatment with 5-LOX inhibitors in esophageal cancer cell lines**

All the esophageal cancer cell lines were grown in a monolayer culture in medium supplemented with 10% FBS. The level of 5-LOX protein expression in esophageal cancer cell lines was determined by western blotting. The results showed that TE-1, TE-8, TE-12 and SKGT-4 had a high level expression of the 5-LOX protein; TE-3, TE-7 and BE3 had an intermediate level of expression; and SKGT-5 had a very low level of expression (Figure 2). To determine the effect of the 5-LOX inhibitors AA861 and REV5901 on the survival of these cells, we chose TE-1, TE-3, SKGT-4 and SKGT-5 based on their 5-LOX expression level. We grew these cell lines in monolayer cultures and treated them with AA861 at a concentration of 30, 60 or 120 μM or REV5901 at 5, 10 or 20 μM for 1, 3 or 5 days. Although the 3-day treatment showed the effect of these drugs on cell viability, the 5-day treatment with the highest dose reached the maximal effect, and AA861 was more effective

### Table II. Association of 5-LOX expression with clinicopathological parameters from esophageal cancer patients

<table>
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SCC, squamous cell carcinoma; ADC, adenocarcinoma.

*a*Compared with well differentiated and moderately differentiated tumor.

*b*Compared with moderately differentiated and poorly differentiated tumor.
than REV5901 in these cells (Figure 3). Furthermore, we found a good correlation between sensitivity of these cells to these drugs according to their level of 5-LOX expression (Figure 2). For instance, TE-1 and SKGT-4, which had a high level of 5-LOX expression, were more sensitive to the treatment, whereas SKGT-5, which had a very low level of 5-LOX expression, was the least sensitive to the treatment (Figure 3). Similar trends were observed when we evaluated the production and suppression of LTB4 levels in these cells after treatment with AA861 and Rev5901 (Figure 4).

Decreased cell viability due to induction of apoptosis by 5-LOX inhibitors

To determine whether the reduced cell viability by AA861 and REV5901 in these esophageal cancer cell lines was due to induction of apoptosis, we performed DNA fragmentation and TUNEL assays. We chose the intermediate dose of the drugs and the 2-day treatment schedule. Figures 5 and 6 show that these drugs induced these cells to undergo apoptosis and that the percentage of apoptosis was associated with the level of 5-LOX expression.

Induction of apoptosis by 5-LOX inhibitors correlated with suppression of LTB4 production

To examine the role of LTB4, the product of 5-LOX and the LTB4 receptor (LTB4R) in mediating the effects of these two drugs on induction of apoptosis in esophageal cancer cells, we measured LTB4 levels in these cells before and after treatment with AA861 (60 µM) and Rev5901 (10 µM). The results are shown in Figure 4, which demonstrates that these two drugs could suppress the LTB4 production in esophageal cancer cells. In addition, we performed RT–PCR analysis of LTB4 mRNA in these four esophageal cancer cell lines. The results showed LTB4 mRNA was present in all four cell lines and the drug treatment caused no appreciable changes in its expression (Figure 7). Furthermore, we used LTB4 and LTB4 receptor antagonist U-75302 to block the induction of apoptosis by these two drugs. The data indicate that both LTB4 and its receptor antagonist U-75302 were able to block the apoptosis induced by both AA861 and REV5901. But U-75302 alone induced an apoptosis at a rate of 6–13% in these cells (control cells showed an apoptosis rate between 3 and 6%) (Figure 6A and B).

Discussion

AA can be converted by 5-LOX to 5-HPETE, and then through 5-HETE to LTA4, all of which show certain levels of biological activity in humans (8,19). However, LTA4 can be further hydrolased to LTB4 by LTA4 hydrolase. LTB4 binds to LTB4 receptor and then takes part in its biological actions (19,20). Several studies have demonstrated that LTB4 can enhance proliferation and suppress apoptosis in human cancer cells (10,20), and that it is important as a chemoattractant in the fields of immunology and hematology (19). The present study is the first detailed investigation on the effect of the 5-LOX inhibitors AA861 and Rev5901 on esophageal cancer cell lines. Our data demonstrate that the 5-LOX protein expression was increased in both esophageal SCC and adenocarcinoma cells when compared with the normal epithelial cells. All the eight esophageal cancer cell lines expressed 5-LOX, the inhibitors of which caused a dose- and time-dependent
reduction in cell viability and induced apoptosis, which was associated with the level of 5-LOX expression and LTB4 production in these cell lines. Further study showed that the induction of apoptosis by these drugs can be blocked by both LTB4, a product of 5-LOX, and an LTB4 receptor antagonist, suggesting that the inhibition of the molecules in the 5-LOX signaling pathway, including 5-LOX, LTB4 receptor and LTA4 hydrolase inhibitors, should be further evaluated as chemopreventive or therapeutic agents for esophageal cancer.

5-LOX expression has been reported to be increased in cancers of the pancreas, breast, prostate and esophagus (15–17, 21). Zhi et al. (21) using a cDNA microarray technique demonstrated that 5-LOX was differentially expressed in normal esophageal epithelial and SCC cells. Their study showed an increased expression of 5-LOX in SCC cells in 77% (20/26) of the cases, which is consistent with our results. A recent study showed that 5-LOX was overexpressed in human esophageal adenocarcinoma as well as in rat esophageal

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Fig. 5. DNA ladder formation in esophageal cancer cells induced by AA861 and REV5901. The cells were incubated with DMSO (0), 60 μM AA861 (1) or 10 μM REV5901 (2) for 2 days. Both floating and adherent cells were collected, and soluble DNA from each cell fraction was extracted and subjected to electrophoresis on a 1.8% agarose gel. The gels were stained with ethidium bromide and photographed. M, molecular weight marker.

Fig. 6. TUNEL assay. (A) Esophageal cell lines were treated with DMSO (as controls), or AA861 (60 μM), REV5901 (10 μM) or LTB4 (0.5 μM) alone, or in combination for 2 days. Both floating and adherent cells were collected, labeled with Br-dUTP and stained with propidium iodide. The cells were then analyzed by flow cytometry using a FACSscan flow cytometer and the data were presented as the percentage of apoptosis. These experiments were repeated once more. (B) Esophageal cell lines were treated with DMSO (as controls) or AA861 (60 μM), REV5901 (10 μM) or U-75302 (1 μM) alone, or in combination for 2 days.
been shown to suppress proliferation and induce apoptosis in cancer cells through both LTB4R-1 inhibition and PPAR-γ activation (26).

A recent study further demonstrated that LTA₄ hydrolyase is overexpressed in esophageal cancer. This protein is a rate-limiting enzyme in the biosynthesis of LTB₄ (19), the inhibition of which suppressed LTB₄ production, which was associated with a reduced incidence of esophageal adenocarcinoma in an animal model (20). Recently, the chemopreventive agent curcumin was shown to affect AA metabolism by blocking the phosphorylation of cPLA2, decreasing the expression of COX-2 and inhibiting the catalytic activities of 5-LOX (27). Taken together, the present study and prior evidence suggest that the importance of 5-LOX overexpression and inhibition of the 5-LOX signaling pathway has the potential to become a target for prevention and treatment of esophageal cancer. Although the underlying mechanisms by which these inhibitors induce apoptosis of esophageal cancer cells are not clear, a further investigation of 5-LOX inhibitors in esophageal cancer should be undertaken in the future.

Acknowledgements

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References


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