Leukotriene A₄ Hydrolase in Rat and Human Esophageal Adenocarcinomas and Inhibitory Effects of Bestatin

Xiaoxin Chen, Ning Li, Su Wang, Nan Wu, Jungil Hong, Xiaolong Jiao, Mark J. Krasna, David G. Beer, Chung S. Yang

Background: Esophageal adenocarcinoma (EAC) is increasing at the most rapid rate of any cancer in the United States. An esophagogastroduodenal anastomosis (EGDA) surgical model in rats mimics human gastroesophageal reflux and results in EAC. Leukotriene A₄ hydrolase (LTA₄H), a protein overexpressed in EAC in this model, is a rate-limiting enzyme in the biosynthesis of leukotriene B₄ (LTB₄), a potent inflammatory mediator. We used this model and human EAC and non-tumor tissues to elucidate the expression pattern of LTA₄H and to evaluate it as a target for chemoprevention.

Methods: LTA₄H expression was examined by western blotting and immunohistochemistry. The functional role of LTA₄H in carcinogenesis was investigated by use of an LTA₄H inhibitor, bestatin, in the rat EGDA model. All statistical tests were two-sided. Results: LTA₄H was overexpressed in all 10 rat EACs examined, compared with its level in normal rat tissue; it was also overexpressed in four of six human EAC tumor samples, compared with its level in adjacent non-tumor tissue. In tissue sections from 20 EGDA rats and 92 patients (86 with EAC, one with dysplasia, and five with columnar-lined esophagus), LTA₄H was expressed in infiltrating inflammatory cells and overexpressed in the columnar cells of preinvasive lesions and cancers, especially in well-differentiated EACs, as compared with the basal cells of the normal esophageal squamous epithelium. Bestatin statistically significantly inhibited LTB₄ biosynthesis in the esophageal tissues of EGDA rats (without bestatin = 8.28 ng/mg of protein; with bestatin = 4.68 ng/mg of protein; difference = 3.60, 95% CI = 1.59 to 5.61; \( P = .002 \)) and reduced the incidence of EAC in the EGDA rats from 57.7% (15 of 26 rats) to 26.1% (6 of 23 rats) (difference = 31.6%, 95% CI = 0.3% to 56.2%; \( P = .042 \)). Conclusion: LTA₄H overexpression appears to be an early event in esophageal adenocarcinogenesis and is a potential target for the chemoprevention of EAC. [J Natl Cancer Inst 2003;95:1053–61]

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focused on cyclooxygenase and 5-lipoxygenase pathways because their metabolites, prostaglandin E₂ and leukotriene B₄ (LTB₄), respectively, are among the most potent inflammatory mediators. Cyclooxygenase 2 is overexpressed in human EAC (10) and in EGDA-induced rat EAC (11). Cyclooxygenase 2 expression increased substantially when biopsy samples of human preinvasive lesions were exposed to pulses of gastric acid or bile acids in an organ culture system, and this effect was attenuated by NS-398, a selective cyclooxygenase 2 inhibitor (12). NS-398 also induces apoptosis in human EAC cell lines in a cyclooxygenase 2- and prostaglandin E₂-dependent manner (13). Sulindac, a nonselective cyclooxygenase inhibitor, substantially inhibited esophageal adenocarcinogenesis in rat surgical models (11,14).

The 5-lipoxygenase pathway of arachidonic acid metabolism bifurcates into three pathways leading to the production of LTB₄ (leukotriene A₄ hydrolase–dependent), LTC₄/D₄/E₄ (leukotriene C₄ synthase–dependent), and 5-hydroxyeicosatetraenoic acid. Inhibition or knockout of 5-lipoxygenase suppresses inflammation (15–17), cell growth (18), or tumor formation in various animal models of cancers (19,20). Such a chemopreventive effect has been associated with inhibition of LTB₄ biosynthesis (21) and can be stronger than that of a cyclooxygenase inhibitor (22). However, the 5-lipoxygenase pathway has not yet been studied in EAC. In human gastroesophageal reflux disease and CLE, the levels of LTB₄ and prostaglandin E₂ were higher in biopsy samples of esophageal mucosa than in normal human esophageal tissue. Antacid treatment reduced the production of LTB₄ and prostaglandin E₂ (23,24).

Leukotriene A₄ hydrolase (LTA₄H) was identified as an overexpressed protein in the EAC of EGDA rats in a previous proteomic experiment using two-dimensional gel electrophoresis and mass spectrometry (25). Because of the important roles of LTB₄ in inflammation and potentially also in inflammation-associated carcinogenesis, we elucidated the expression pattern of LTA₄H, a rate-limiting enzyme in the biosynthesis of LTB₄, in rat and human EACs, and evaluated it as a target for chemoprevention of EAC. We compared LTA₄H protein expression in rat and human EAC tumor tissues with that in non-tumor esophageal tissues. The EGDA rat model was used to investigate bestatin, an LTA₄H inhibitor, as an agent for chemoprevention.

PATIENTS AND METHODS

Proteomics

Frozen samples of duodenal epithelium, esophageal epithelium, and EAC tumors from two EGDA rats were used for the proteomic experiment. These samples were obtained from a previous experiment in which the animals developed visible EAC 40 weeks after EGDA without iron supplementation (5). Chronic inflammation caused by EGDA-induced reflux of duodenal and gastric contents was present in the esophageal epithelia under microscopic examination. Inflammation at the squamocolumnar junction was very severe.

Two-dimensional gel electrophoresis with 50 μg of protein was performed as described previously (25). The images were analyzed with Phoretix 2D Full software version 4.0 (Nonlinear Dynamics, Durham, NC), so that differing spots and all major unchanged spots were outlined, quantified, and matched on all gels. Protein spots of interest (more than threefold higher in EAC tumor than in duodenal epithelium) were excised, digested in the gel by trypsin, and analyzed with matrix-assisted laser desorption/ionization mass spectrometry on a PerSeptive Voyager DE-RI mass spectrometer (PerSeptive Biosystems, Framingham, MA) in the linear mode. The protein corresponding to the peptide masses detected was determined with an online database (http://www.ExPaSy.ch/).

Western Blot Analysis of LTA₄H

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting were performed as described previously (26). A 20-μg protein sample was loaded per lane. A polyclonal rabbit anti-LTA₄H antiserum (1:1000 dilution; Cayman Chemical, Ann Arbor, MI) was used to detect rat and human LTA₄H by western blotting and immunohistochemistry. This antiserum detects one band of approximately 69 kD on a western blot and recognizes both rat and human LTA₄H because of the high degree of amino acid sequence homology (93%) between the two enzymes (27). Ten sets of frozen rat samples (duodenum, esophagus, and tumor), including the two sets used for proteomics, were obtained from a previous experiment (5). Six pairs of surgically resected human samples (EAC tumor and adjacent non-tumor tissue) were obtained from the Tissue Retrieval Service, Cancer Institute of New Jersey (New Brunswick, NJ). Written informed consent was obtained from all patients, and patient identifiers were coded to protect confidentiality. Hematoxylin and eosin staining confirmed the diagnosis: two well-differentiated EAC (samples 01 and 04), two moderately differentiated EAC (samples 03 and 06), and two poorly differentiated EAC (samples 02 and 05). All samples were glandular adenocarcinoma in nature. All adjacent non-tumor tissues contained large amounts of infiltrating inflammatory cells. Proteins from rat and human tissues were prepared for western blotting from five to 10 frozen sections between two histologically confirmed sections that were carefully scraped into microcentrifuge tubes.

LTA₄H Immunohistochemistry

The avidin–biotin–peroxidase complex method (Elite ABC kit; Vector Laboratories, Burlingame, CA) and polyclonal rabbit anti-LTA₄H antiserum (1:1000 dilution) were used for immunohistochemical staining of LTA₄H in archival formalin-fixed paraffin-embedded tissue sections from 20 EGDA rats and 92 patients. Written informed consent was obtained from all patients, and patient identifiers were coded to protect confidentiality. Each rat tissue section contained a variety of histologic types, including normal esophagus, esophagitis, CLE, dysplasia, and EAC. Among the 92 human esophageal samples, 86 were EAC, one was dysplasia, and five were CLE; 21 of the 92 patients had archival paraffin sections, and 71 had tissue spotted on one tissue microarray (Table 1). In this collection of tissue samples, some patients contributed samples of more than one type of histology (squamous epithelium, CLE, dysplasia, or EAC), six contributed samples of all four types of histology, but no one contributed more than one tissue sample with the same histology.

Histologic diagnosis was made by using established criteria (28). Paraffin sections were treated with antigen-unmasking fluid (BD Pharmingen, San Diego, CA) and then incubated with the primary antibody. The cytoplasm and/or nuclei of cells expressing LTA₄H were stained dark brown. Staining was evaluated and graded by a single pathologist (S. Wang). Staining intensity in epithelial cells in human paraffin sections was rated
Table 1. Histopathology and leukotriene A₄ hydrolase (LTA₄H) immunostaining of human esophageal samples

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of tissue samples</th>
<th>LTA₄H staining intensity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous epithelium†</td>
<td>11</td>
<td>0.4 (0.0 to 0.5)</td>
</tr>
<tr>
<td>Columnar-lined esophagus (CLE)</td>
<td>21</td>
<td>1.7 (1.4 to 1.7)§</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>20</td>
<td>2.2 (1.8 to 2.2)</td>
</tr>
<tr>
<td>Total esophageal adenocarcinoma (EAC)</td>
<td>86</td>
<td>2.0 (1.7 to 2.0)‡</td>
</tr>
<tr>
<td>Well-differentiated EAC</td>
<td>29</td>
<td>2.4 (2.0 to 2.4)§</td>
</tr>
<tr>
<td>Moderately differentiated EAC</td>
<td>43</td>
<td>1.7 (1.4 to 1.7)§</td>
</tr>
<tr>
<td>Poorly differentiated EAC</td>
<td>12</td>
<td>1.7 (1.0 to 1.9)§§</td>
</tr>
<tr>
<td>Mucinous EAC</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*Staining intensity in epithelial cells was scored as follows: 0 = no positive staining or slight staining close to background, 1 = weakly positive staining, 2 = moderately positive staining, 3 = strongly positive staining. Only the mean intensity of LTA₄H staining was shown for mucinous EAC. All the other cases of EACs were glandular adenocarcinoma.
†Staining intensity was expressed as that in the basal cells of esophageal squamous epithelium.
‡With two-sided Student’s t test, CLE, dysplasia, and EAC had statistically significantly higher levels of LTA₄H than the squamous epithelium (P < 0.001). Dysplasia expressed higher levels of LTA₄H than CLE (P = .04). There was no statistically significant difference between CLE and EAC (P = .22) or between dysplasia and EAC (P = .42).
§With two-sided Student’s t test, well-differentiated EAC had a statistically significantly higher level of LTA₄H than moderately differentiated EAC (P = .003) and poorly differentiated EAC (P = .041). There was no statistically significant difference between moderately differentiated EAC and poorly differentiated EAC (P = .92).

Chemoprevention of EGDA-Induced Rat EAC by Bestatin

EGDA was performed on 60 rats, as described above, 54 of which survived the surgery. Rats were given iron dextran (12.5 mg of iron per kg) every 2 weeks intraperitoneally, starting 4 weeks after surgery and continuing for the duration of the experiment. The animals were weighed once every other week. One week after surgery, rats were randomly assigned to two groups. Twenty-seven EGDA rats were given bestatin at a dose of 10 mg/kg, intraperitoneally, three times a week (group B). Ten non-operated rats were included as negative controls (group A). Forty weeks after surgery, these rats were killed; the esophagus was removed, opened longitudinally, and examined for gross abnormalities. Tumor volume (4/3πr³, where r is the radius) was determined by measuring the height, length, and width of all visible tumors and using the average of the three measurements as the diameter. The esophagus was cut longitudinally, fixed in 10% buffered formalin, Swiss-rolled as previously described (29), processed, and embedded in paraffin. Five-micrometer sections were mounted on glass slides and used for pathologic analyses. A small piece of esophagoduodenal junction was frozen and stored at −80°C for future analysis of LTB₄.

Histopathologic analysis was carried out on slides stained with hematoxylin and eosin (slides 1, 10, 20, and 30) by a single individual (S. Wang) who was blinded to the treatment assignments. CLE was characterized by the presence of intestinal columnar epithelium containing a villiform surface, mucous glands, and intestinal-type goblet cells, above the blue prolene suture. Dysplastic lesions were diagnosed by the partial loss of cell polarity and maturation, nuclear atypia, and an increased numbers of mitotic figures. EAC was diagnosed when dysplastic columnar epithelial cells invaded through the basement membrane (28).

LTB₄ Enzyme Immunoassay

Eight pairs of frozen rat samples (upper esophagus and esophagoduodenal junction) from the short-term experiment (bestatin-treated EGDA rats and non–bestatin-treated EGDA rats) and eight frozen rat samples (esophagoduodenal junction) from each group of the bestatin chemoprevention experiment were analyzed for LTB₄. Frozen tissue samples were analyzed immediately after removal from a −80°C freezer. After being homogenized in a buffer containing 0.1 M Tris–HCl (pH 7.1) and 20 mM EDTA, the protein concentration and the LTB₄ concentration were determined. LTB₄ was measured as described by the manufacturer of an enzyme immunoassay kit.
(Cayman Chemical Co., Ann Arbor, MI). The amount of LTB₄ was expressed as nanograms per milligram of protein.

Statistical Analysis

Student’s t test was used to analyze differences in the intensity of LTA₄H immunostaining and the tissue level of LTB₄, which were normally distributed. The results for tumor incidence were analyzed by the χ² test. The Wilcoxon signed rank test was used for the other analyses. Statview, version 4.2 (SAS Institute, Cary, NC) software was used for all statistical tests and calculations, and all statistical tests were two-sided.

RESULTS

Overexpression of LTA₄H in Rat and Human EAC

Fifty micrograms of protein from the tissue samples (EAC tumor and nearby esophageal and duodenal epithelia) of two EGDA rats were separated by two-dimensional gel electrophoresis. After silver staining and laser scanning, the intensities of protein spots on two-dimensional gels were visually estimated and then quantified with imaging software. As we described previously (25), the density of 12 spots was more than threefold higher in both pairs of tumor samples than in the duodenal epithelial samples. Eleven of the 12 spots were identified by mass spectrometry as known proteins in the database, including glucose-regulated protein 94 (Grp94), LTA₄H, Grp78, calnexin, heat shock protein 90β, ER61, transferrin, Rho GDP-dissociation inhibitor, creatine kinase M chain, vitamin D-binding protein, and annexin V. One of the 12 spots was not in the database. We first characterized overexpression of Grp94 in rat and human EAC (25). Because Grp94 inhibits apoptosis by maintaining intracellular calcium homeostasis, our data suggested an association of oxidative stress, Grp94 overexpression, and apoptosis regulation in esophageal adenocarcinogenesis.

In this study, we characterized the enzyme LTA₄H (EC 3.3.2.6; Swiss-Prot accession number P30349; theoretical pI = 5.70; molecular mass = 69,044 d), which has tumor/duodenum density ratios of 6 : 1 and 3.5 : 1 for the two sets of rat samples on the two-dimensional gels. Seven major LTA₄H peptide peaks (1150, 1373, 1476, 1698, 1808, 1943, and 2048 m/z) were identified by mass spectrometry (Fig. 1, A).

Western blotting with a polyclonal antiserum against LTA₄H confirmed the overexpression of this enzyme in EAC tumors compared with the expression in normal duodenal tissue (Fig. 1, B). A similar expression pattern was observed in all 10 sets of rat samples (data not shown). The level of LTA₄H in esophageal tissues was similar to (Fig. 1, B, lane E1 versus lane T1) or lower than (Fig. 1, B, lane E2 versus lane T1) in EAC tumors.

The expression of LTA₄H was examined in six sets of frozen human samples of EAC tumors and adjacent non-tumor esophageal tissues. Overexpression of LTA₄H was observed in four human EACs (samples 01, 02, 04, and 05) as compared with the...
expression in corresponding adjacent non-tumor esophageal tissues. Another two EACs (samples 03 and 06) had a lower level of LTA4H than corresponding adjacent non-tumor tissues (Fig. 1, C).

Expression of Rat and Human LTA4H as Determined Immunohistochemically

LTA4H expression was examined immunohistochemically in paraffin sections of rat and human tissues. Tissue sections from 20 EGDA rats, containing histologically normal tissue and CLE and EAC tissues, were used for this analysis. Consistent staining patterns of LTA4H were found for all tissue types. In the normal rat esophagus, LTA4H was not detected in epithelial cells (Fig. 2, A). In the squamous epithelium with inflammation, LTA4H was strongly detected in the infiltrating inflammatory cells in the stroma (Fig. 2, B) but was not detected in squamous epithelial cells in the basal and parabasal cell layers. LTA4H was detected in columnar cells of CLE (Fig. 2, C) and was strongly detected in EAC tumor cells (Fig. 2, D). When expressed, LTA4H was detected in the cytoplasm and in the nucleus.

In the paraffin sections of human esophageal tissues (92 patients), LTA4H was rarely detected in basal cells of the squamous epithelium but was usually detected in the parabasal cells of the esophageal squamous epithelium adjacent to the cancer tissue (Fig. 3, A). A moderate level of LTA4H was detected in most cells in CLE (Fig. 3, B and C). The level of LTA4H detected in dysplastic lesions (Fig. 3, D) and EAC (Fig. 3, E) was higher than that in CLE. However, in three EACs, little LTA4H was detected in tumor cells but a high level of LTA4H was detected in infiltrating inflammatory cells (Fig. 3, F).

To minimize bias in evaluating the staining intensity of the epithelial cells, polymorphonuclear neutrophils in the blood vessel on the same slide were used as an internal positive control (staining intensity = 3). Tissues that did not show strong LTA4H staining in these cells were regarded as poorly preserved and excluded from analysis. When the staining intensity in epithelial cells was compared among different histologic stages, LTA4H expression in CLE, dysplastic, and EAC tissues was statistically significantly higher than that in basal cells of squamous epithelium (P < .001). A higher level of LTA4H was expressed in dysplastic lesions than in CLE (P = .04). No statistically significant difference was observed in the level of LTA4H in dysplastic and EAC tissues (P = .42; Table 1). Among the six patients who contributed all four types of tissue, LTA4H expression was statistically significantly higher in CLE, dysplastic, and EAC tissue than in basal cells of squamous epithelium (P < .001). No statistically significant difference was observed in the level of LTA4H among CLE, dysplastic, and EAC tissues (P = .109). Because all patients had reflux esophagitis, no normal esophageal squamous epithelium was available. The parabasal cells of the esophageal squamous epithelium expressed a high level of LTA4H (intensity score = 2.18, 95% CI = 1.78 to 2.30).

A higher level of LTA4H was detected in well-differentiated EAC than in moderately differentiated EAC (P = .003) and poorly differentiated EAC (P = .04). No statistically significant difference in the level of LTA4H was observed between moderately and poorly differentiated EAC (P = .92). LTA4H was also highly expressed in two samples of mucinous EAC in this tissue collection (Table 1).

Chemoprevention of EGDA-Induced EAC in Rats by Bestatin

To assess the functional role of LTA4H in esophageal adenocarcinogenesis, bestatin, an LTA4H inhibitor, was tested for its effect on LTB4 biosynthesis in EGDA rats. The level of LTB4 in tissue from the esophagoduodenal junction of rats treated with bestatin (10 mg/kg, intraperitoneally, daily for 7 days) was statistically significantly lower (3.16 ng/mg of protein, 95% CI = 2.14 to 3.67 ng/mg) than that in untreated EGDA rats (7.64 ng/mg of protein, 95% CI = 6.22 to 8.11 ng/mg) (P = .014). The level of LTB4 in the upper esophagus was not statistically significantly different in untreated rats (1.64 ng/mg of protein, 95% CI = 1.29 to 1.82 ng/mg) and rats treated with bestatin (1.58 ng/mg of protein, 95% CI = 0.96 to 1.89 ng/mg) (P = .91). Because EGDA rats always had severe inflammation at the esophagoduodenal junction and little or no inflammation in the upper esophagus, bestatin treatment appeared to inhibit LTA4H activity in chronically inflamed esophageal tissues.

In a long-term chemoprevention study, the EGDA rats were active and healthy. Rats in group A were non-operated controls, rats in group B were assigned to receive EGDA, and rats in group C were assigned to receive EGDA and bestatin treatment. One rat from group B and four rats from group C died before the termination of the experiment. Body weight was slightly lower for EGDA animals (group B) than for the non-operated control animals (group A). Body weight for bestatin-treated EGDA animals (group C) was also lower than that of the EGDA group (group B) and the non-operated control group (group A) (Fig. 4). There was no statistically significant difference between group A and group B (P = .10) and between group B and group C (P = .09). However, group C had a statistically significantly lower body weight than group A (P = .03). At 40 weeks after EGDA, group B had a tumor incidence of 57.7% (15 of 26 rats), which was similar to that in our previous study (5). Treatment with bestatin at a dose of 10 mg/kg intraperitoneally, three times a week, statistically significantly reduced tumor incidence to

![Fig. 2. Expression of leukotriene A4 hydrolase (LTA4H) in rat esophageal tissues detected by immunohistochemistry. Columnar cells of rat columnar-lined esophagus (C) and esophageal adenocarcinoma (D) overexpress LTA4H, compared with the basal cells of the normal esophagus (A) and esophagitis (B). The cytoplasm and/or nuclei of cells expressing LTA4H were stained dark brown. Infiltrating inflammatory cells were also strongly stained. All scale bars = 100 μm.](image)
The enzyme-inhibitory effect, the level of LTB4 in the rat esophageal and dysplasia (staining intensity /H11505/H11505/B) esophageal squamous epithelium. B Columnar-lined esophagus (CLE; staining intensity = 1) and dysplasia (staining intensity = 3). C and D Higher magnification of boxed parts of panel B. E EAC tumor cells (staining intensity = 0) and infiltrating inflammatory cells (staining intensity = 3). Scale bars in A, B, E, and F = 100 μm; scale bars in C and D = 50 μm.

Statistically significantly decreased the level of LTB4 to 4.68 ng/mg of protein (difference between groups B and C = 3.60, 95% CI = 1.59 to 5.61; P = .002) (Table 2).

**DISCUSSION**

In this study, we demonstrate the overexpression of LTA4H in esophageal adenocarcinogenesis and the inhibition of carcinogenesis in a rat model by bestatin, an LTA4H inhibitor. LTA4H is a bifunctional zinc enzyme with epoxide hydrolase and aminopeptidase activities (30). The aminopeptidase activity of LTA4H is generally assumed to process peptides related to inflammation and host defense (31). The epoxide hydrolase activity of LTA4H hydrolyzes the epoxide LTA4 to the diol LTB4, which mainly functions as a chemotactrant and an activator of inflammatory cells in inflammation, immune responses, host defense against infection, platelet-activating factor-induced shock, and lipid homeostasis (32–34). LTB4 acts as an autocrine and paracrine factor to the nearby cells that express the specific LTB4 receptors (BLT1 and BLT2), with BLT1 being mainly involved in chemotaxis and BLT2 being possibly involved in leukocyte degranulation and superoxide production (35). Inhibition of LTA4H by specific inhibitors or gene knockout decreases the effect of inflammatory diseases in animal models (32,36,37). Consequently, LTA4H has long been regarded as an anti-inflammatory target (38).

Besides their involvement in inflammation, LTA4H and LTB4 are also associated with esophageal adenocarcinogenesis. The levels of LTB4 and prostaglandin E2 were substantially higher in human biopsy samples of preinvasive esophageal lesions than in biopsy samples of normal subjects (24). Using immunohistochemistry, we found overexpression of LTA4H in the preinvasive and cancer cells of EGDA rats. We used a staining intensity scoring system to semi-quantify the expression levels of LTA4H in the esophageal tissues of 92 human patients and found that LTA4H was much higher in human CLE, dysplastic, and EAC tissues than in basal cells of the esophageal epithelium. LTA4H overexpression appears to be an early event in esophageal adenocarcinogenesis. Rat esophageal squamous epithelial cells did not express LTA4H (Fig. 2, A), even in the presence of reflux esophagitis (Fig. 2, B). However, LTA4H was expressed in the parabasal cells of human esophageal squamous epithelium (Fig. 3, A). The reason for the difference is not known. One
lung cancers (data not shown). LTB4 was overproduced in human cancers, including colon and other human cancers, including colon and (46,47).

apoptosis in human pancreatic cancer cells (48).

tory cells, LTA4H was still overexpressed in the tumor cells because of high expression of LTA4H in infiltrating inflammatory cells. LTA4H was still overexpressed in the tumor cells compared with the expression in the esophageal squamous epithelial cells (Fig. 2, B and D). Probably also because of high expression of LTA4H in infiltrating inflammatory cells, some human EAC samples (03 and 06) expressed less LTA4H protein than their respective adjacent non-tumor esophageal tissues (Fig. 1, C).

We found by immunohistochemical analysis that LTA4H was also overexpressed in other human cancers, including colon and lung cancers (data not shown). LTB4 was overproduced in human colon cancer tissue (mainly in the epithelial layer) compared with normal colon tissue (39). LTB4 at nanomolar levels stimulated the proliferation of colon cancer cells (40,41); inhibited apoptosis of intestinal epithelial cells by regulating the expression of cyclooxygenase 2, β-catenin, and Bcl-2 (42); enhanced oxidative stress (43,44); and promoted cell spreading (45). Topical application of LTB4 to the skin leads not only to inflammation but also to substantial hyperplasia in the epidermis (46,47). A BLT1 antagonist inhibited proliferation and induced apoptosis in human pancreatic cancer cells (48). Thus, LTA4H and/or LTB4 may play an important role in esophageal adenocarcinogenesis by 1) augmenting inflammation in inflammatory cells through positive feedback mediated by its receptors and downstream signaling kinases (49,50) and 2) stimulating autocrine and paracrine growth of preinvasive and cancer cells. Results of this study and our previous study on cyclooxygenase 2 (11) indicate that aberrant arachidonic acid metabolism appears to play an important role in the development of EAC associated with chronic inflammation (5,6).

LTA4H is expressed in many types of cells in normal human, rat, and mouse tissues, including epithelial cells (e.g., from the gastrointestinal tract, skin, breast, prostate, and kidney and cells from other organs), inflammatory cells, endothelial cells, fibroblasts, muscle cells, liver cells, adipocytes, and neurons (data not shown). This pattern of cellular distribution is consistent with the ubiquitous involvement of LTB4 in inflammatory conditions of many tissues. Under certain stimuli or physiologic or pathologic conditions, LTA4H may be stimulated or inhibited by treatment with cytokines (51,52), treatment with cyclosporin A (53), smoking (54), chorionic gonadotrophin during early pregnancy (55), glomerulonephritis (56), angiotensin-induced hypertension (57), or bacterial infection of the oral mucosa (58). LTA4H expression in the esophagus may be stimulated by exposure of esophageal epithelium to gastroesophageal reflux. Bile acids, an important constituent in the gastroesophageal refluxate, increase LTA4H expression and increase LTB4 biosynthesis in the human EAC cell line SKGT4 (data not shown), in CaCo-2 colon cancer cells (59), and in rat colonic mucosa (60).

To further demonstrate the role of LTA4H in esophageal adenocarcinogenesis, we investigated whether bestatin, an LTA4H inhibitor (30,61), was a chemopreventive agent for EAC in EGDA rats. Bestatin was chosen because it inhibits proliferation and induces apoptosis in cancer cell lines (62–64), inhibits carcinogen-induced stomach tumorigenesis (65), activates E-cadherin-mediated adhesion of breast cancer cells (66), and promotes the immune reaction against cancer (67). The low toxicity of bestatin is important for its use in chemoprevention. In this study, the injection of bestatin did not produce toxicity, although it slightly decreased the body weight of EGDA rats. This result is consistent with the results of a previous study (68) that showed that bestatin at a dose as high as 250 mg/kg given intravenously did not induce any signs of toxicity. In our short-term study, bestatin given at 10 mg/kg intraperitoneally daily for 7 days statistically significantly inhibited LTB4 biosynthesis at the esophagoduodenal junction. In the long-term chemoprevention study in EGDA rats, bestatin inhibited LTB4 production by 44% in rat esophageal tissues and reduced the incidence of EAC by 55% at 40 weeks after EGDA. These results are consistent with the hypothesis that bestatin suppressed the development of EAC by inhibiting the epoxide hydrolase activity of LTA4H; however, other actions might also contribute to its chemopreventive effect (69). Bestatin can also inhibit LTA4H aminopeptidase activity (30), which may play a role in carcinogenesis (70,71). Inhibition of aminopeptidase N/CD13 by bestatin was associated with its immunomodulating effect for cancer therapy (63,64,72,73).

Studies that use our rat EGDA model are relevant to human EAC. We have previously investigated the similarity of rat CLE and EAC to human diseases by studying histopathologic progression of rat esophageal adenocarcinogenesis, mucin-staining features, expression of differentiation markers, and mRNA expression profiles. As with human CLE and EAC (74), most EGDA-induced rat CLE and EACs originated from metaplasia of the multipotential stem cells in the basal cell layer of the esophageal squamous epithelium (Su Y, Chen X, Klein M, Yang CS, Goyal RK: unpublished results). Using proteomics, we have

### Table 2. Chemopreventive effects of bestatin on rat esophageal adenocarcinogenesis (EAC)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>No. of animals</th>
<th>EAC incidence, % (No. of animals with EAC/total No.)†</th>
<th>Tumor volume, cm³ (95% CI)‡</th>
<th>LTB4, ng/mg of protein (95% CI)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Negative control</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>0.68 (0.61 to 0.70)</td>
</tr>
<tr>
<td>B</td>
<td>EGDA</td>
<td>26</td>
<td>57.7 (15/26)</td>
<td>0.34 (0.28 to 0.35)</td>
<td>8.28 (6.51 to 8.91)</td>
</tr>
<tr>
<td>C</td>
<td>Bestatin-treated EGDA</td>
<td>23</td>
<td>26.1 (6/23)</td>
<td>0.16 (0.08 to 0.19)</td>
<td>4.68 (3.34 to 5.15)</td>
</tr>
</tbody>
</table>

*Esophagogastroduodenal anastomosis (EGDA) was performed as described previously (5). Animals were treated with iron dextran intraperitoneally at 12.5 mg of iron per kilogram every 2 weeks, starting 4 weeks after surgery and continuing for the duration of the experiment. Bestatin was freshly dissolved in phosphate-buffered saline (pH 7.2) and administered at 10 mg/kg intraperitoneally three times a week. One rat in group B and four rats in group C died after surgery and before the termination of the experiment. — = none.

†Statistically significant difference between group B and group C by two-sided χ² test (P = .042).

‡Statistically significant difference between group B and group C by two-sided Wilcoxon signed rank test (P = .009). CI = confidence interval.

§Statistically significant differences between groups A and B (P<001) and between groups B and C (P = .002), by two-sided Student’s t test.
identified Grp94 and LTA4H as overexpressed proteins in rat and human EACs. As one of the stress proteins covalently attached to potential tumor-specific antigens, Grp94 may be used to mediate immunotherapy and immunoprevention of EAC (25).

As an important metabolizing enzyme of arachidonic acid, LTA4H appears to be a potential target for the chemoprevention of human EAC. Recent studies of the crystal structure and molecular biology of LTA4H (75,76) suggested that LTA4H epoxide hydrolase activity could be targeted efficiently and specifically by some potent LTA4H epoxide hydrolase inhibitors (38,77). We urge that future studies test the chemopreventive effects of these inhibitors on EAC.

**References**


NOTES
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