Overexpression of 5-Lipoxygenase and Cyclooxygenase 2 in Hamster and Human Oral Cancer and Chemopreventive Effects of Zileuton and Celecoxib

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ABSTRACT

Purpose: Previous studies have suggested an important role of aberrant arachidonic acid metabolism, especially the cyclooxygenase (Cox) pathway, in oral carcinogenesis. However, it is unknown whether the 5-lipoxygenase (5-Lox) pathway contributes to oral carcinogenesis, and whether combination of inhibitors of both pathways may have synergistic or additive effects of chemoprevention.

Experimental Design: 5-Lox expression was examined in 7,12-dimethylbenz[a]anthracene (DMBA)–induced hamster and human oral cancer tissues by immunohistochemistry, and Cox2 expression was investigated in hamster oral tissues using in situ hybridization. Zileuton (a specific 5-Lox inhibitor) and celecoxib (a specific Cox2 inhibitor), either alone or in combination, were investigated for their chemopreventive effects on the DMBA-induced hamster model at the post-initiation stage through topical application.

Results: 5-Lox was overexpressed during oral carcinogenesis in hamsters and humans, as well as Cox2 in the hamster tissues. In a chemoprevention study using the post-initiation DMBA model, incidence of hamster oral squamous cell carcinoma was reduced from 76.9% (20 of 26) to 45.8% (11 of 24, P < 0.05) and 32.1% (9 of 28, P < 0.01) by 3% and 6% topical zileuton, respectively; and to 57.6% (15 of 26, P > 0.05) and 50% (12 of 24, P < 0.05) by 3% and 6% topical celecoxib, respectively. When used in combination, celecoxib and zileuton (3% of each) had an additive inhibitory effect on the incidence of squamous cell carcinoma (36%, 9 of 25, P < 0.01). Other pathologic variables and the levels of leukotriene B4 and prostaglandin E2 of the hamster tissues were reduced as well.

Conclusions: The results clearly showed that both 5-Lox and Cox2 played important roles in oral carcinogenesis. Zileuton and celecoxib prevented oral carcinogenesis at the post-initiation stage through their inhibitory effects on arachidonic acid metabolism.

INTRODUCTION

Oral cancer is a common neoplasm worldwide, particularly in developing countries. In the United States, oral cancer incidence and mortality rates have been increasing in recent years, especially among young males (1). Approximately 28,260 new cases and 7,230 deaths are expected in 2004 (2). Unfortunately, survival of oral cancer patients has not improved significantly despite recent advances in radiotherapy and chemotherapy. The 5-year survival rate remains around 50% during the past years. Even the surviving patients are usually left with severe functional compromise and may develop a second cancer within a few years (2, 3). Therefore, it is important to understand the pathogenesis of and design preventive strategies for this disease.

Aberrant arachidonic acid metabolism has been suggested to play an important role in human oral carcinogenesis (4). Aspirin use was associated with a lower risk of the cancers of the upper aerodigestive tract, including oral cancer (5). Cyclooxygenase 2 (Cox2) was barely detectable in the normal epithelium but up-regulated in hyperplasia and further overexpressed in dysplasia and squamous cell carcinoma (SCC; refs. 6, 7). Cox2 may be up-regulated by exposure to tobacco and areca nut constituents (8). This event preceded alterations of other biomarkers related to apoptosis and angiogenesis and was associated with poor clinical prognosis of premalignant lesions. Cox2 inhibitors retarded the growth of human oral cancer cells in both prostaglandin E2 (PGE2)–dependent and -independent manners (9), and suppressed 4-nitroquinoline-1-oxide-induced tongue cancer in rats as well (10). Previous studies on a “complete” 7,12-dimethylbenz[a]anthracene (DMBA)–induced hamster cheek pouch model suggested chemopreventive effects of nonsteroidal anti-inflammatory drugs (e.g., ibuprofen, indomethacin, and aspirin) on oral cancer. However, the results were not conclusive because of small sample sizes and contradictory outcomes (11–16).

In addition to the alterations of the Cox2 pathway, the level of leukotriene B4 (LTB4), a metabolite of the 5-lipoxygenase (5-Lox) pathway, was found to be 10- to 30-fold higher in hamster and human SCC than in normal tissues (17). 5-Lox metabolites, such as 5-hydroeicosatetraenoic acid, LTB4, and cysteinyl leukotrienes, are known to recruit and activate inflammatory cells, increase vascular permeability and induce contraction of smooth muscles (18). 5-Lox knockout mice were more...
resistant to inflammation or certain inflammation-associated diseases (e.g., atherosclerosis) and more susceptible to infections (19). 5-Lox was found to be overexpressed in human prostate (20), pancreatic (21), colon (22), bladder (23), testicular (24), and esophageal cancers (25). In colon cancer, 5-Lox overexpression was also negatively associated with clinical prognosis, especially for patients at the Dukes’ B stage (22). Inhibition of the 5-Lox pathway was antiproliferative and proapoptotic in cancer cells (26). Chemopreventive effects of 5-Lox pathway inhibitors have been shown in animal models of lung (27), skin cancer (28), pancreatic (29, 30), and esophageal cancers (25). However, it is unknown whether the 5-Lox pathway plays an important role in oral carcinogenesis.

In this study, we showed overexpression of 5-Lox and Cox2 in cancers of DMBA-treated hamster cheek pouch and human oral cavity. Zileuton (a specific 5-Lox inhibitor) and celecoxib (a specific Cox2 inhibitor), either alone or in combination, were topically applied on the DMBA-treated hamster oral mucosa, to test their chemopreventive effects on oral carcinogenesis at the post-initiation stage.

MATERIALS AND METHODS

5-Lipoxygenase Immunohistochemistry. The avidin-biotin-peroxidase complex method (Elite ABC kit; Vector Laboratories, Burlingame, CA) and a monoclonal mouse anti-5-Lox antibody (5 μg/mL; RDI, Inc., Parsippany, NJ) were used for immunohistochemical staining on archival formalin-fixed, paraffin-embedded tissue sections of hamster and human oral cancer. The paraffin sections were pretreated with antigen unmasking fluid (BD PharMingen, San Diego, CA) before being incubated with the primary antibody. Omission of the first antibody and preadsorption with recombinant 5-Lox protein (RDI) were used as negative controls to confirm the specificity of the antibody for immunohistochemistry.

Table 1

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NOTE. Diagnosis was based on the current WHO pathological criteria of oral cancer (32). NA indicates that certain histology not available for that case. Staining intensity in epithelial cells was scored follows: –, no positive staining; ±, indefinite positive staining; +, weakly positive staining; ++, moderately positive staining; ++++, strongly positive staining.

Hamster oral mucosa samples, 10 nontreated and 20 DMBA-treated, were obtained from a previous experiment in our lab (31). The DMBA-treated samples were exposed to DMBA (0.5%, 100 μL in mineral oil) for 6 weeks (thrice per week through topical application) and to mineral oil for another 18 weeks. Archival tissue sections of 20 cases of human oral cancer were obtained from the pathology archive of the Beijing Hospital for Stomatology (Table 1). Written informed consent was obtained from all patients, and patient identifiers were coded to protect confidentiality. Two tissue microarrays of human oral cancer (CC27-01 and CC34-11; Cybrdi, Inc., Gaithersburg, MD) were also examined for 5-Lox expression in SCC and other histologic types of oral cancer. In total, there were 73 spots representing 53 individual oral cancer cases of different pathologic types, according to the established criteria (32).

5-Lox staining was evaluated and graded by a pathologist (S.W.). Staining intensity in epithelial cells on human paraffin sections was rated on a scale of “−” as no positive staining, “±” as indefinite staining, “+” as weakly positive staining, “++” as moderately positive staining, and “+++” as strongly positive staining. The area of maximal intensity was used for grading as long as it comprised >10% of the region of interest (oral epithelial tissues). Polymorphonuclear neutrophils were used as internal positive controls of staining (intensity designated as +), because these cells were known to express high levels of 5-Lox (33).

Cyclooxygenase 2 In situ Hybridization. Several commercial Cox2 antibodies were tested on the hamster tissues and failed to generate consistent staining patterns. Therefore, in situ hybridization was used to analyze the expression of Cox2 on paraffin sections of hamster oral mucosa. Digoxigenin-labeled antisense and sense cRNA probes (kindly provided by Dr. Xiaochun Xu, M.D. Anderson Cancer Center) were prepared via a digoxigenin-RNA labeling and detection kit (Boehringer...
Mannheim, Mannheim, Germany), and used for analysis as described previously (25).

**Chemoprevention of Hamster Oral Carcinogenesis by Zileuton and Celecoxib.** The animal study was approved by the Animal Care committee of Rutgers University (protocol 91-024). Male Syrian golden hamsters (6 weeks old; Harlan, Indianapolis, IN) were housed four per cage. All animals were given lab chow and tap water ad libitum. After 1 week of acclimatization, the left pouch of 158 hamsters was topically treated with 100 μL of 0.5% DMBA in mineral oil (Sigma, St. Louis, MO) with a paintbrush thrice every week for 6 weeks. Hamsters were then randomly assigned to six groups for topical treatment with the following agents for 18 weeks: mineral oil, 3% and 6% zileuton (Abbott Laboratories, Abbott Park, IL), 3% and 6% celecoxib (LKT Laboratories, Inc., St. Paul, MN), and a combination of zileuton and celecoxib (3% of each; Table 2). Zileuton and celecoxib were dissolved in mineral oil at the concentrations, and 100 μL of the solution were applied topically on the left buccal pouch thrice every week. Fifteen hamsters were used as the negative control (group A). The hamsters were monitored for their body weights once every other week and sacrificed by CO2 asphyxiation at week 24.

The whole cheek pouch was excised and flattened on a transparency. Visible tumors in the oral cavity were counted, and the length, width, and height of each tumor were measured for calculation of the tumor volume: \( V = \frac{4}{3} \pi r^3 \) (where \( r \) was the average radius of the three diameter measurements in mm). One piece without visible tumor was frozen immediately in liquid nitrogen for future analysis of PGE2 and LT4. The remaining tissue was cut into four pieces of approximately equal width, fixed in 10% PBS buffered formalin, Swiss-rolled, and processed routinely. Thirty sections (5 μm) of each sample were cut, and the 1st, 15th, and 30th slides were H&E stained for histopathologic analysis. Basal cell hyperplasia, dysplasia, SCC, and papillomas were diagnosed with established criteria (31, 32).

Enzyme Immunoassay of Leukotriene B4 and Prostaglandin E2. Frozen samples of the hamster oral mucosa were analyzed immediately after being taken out of a −80°C freezer. After pulverization and homogenization in a buffer containing zileuton and indomethacin, the tissue samples were aliquoted for protein concentration determination and organic extraction. The organic extract was dried with nitrogen and reconstituted in the enzyme immunoassay buffer for determination of LT4 and PGE2 (Cayman Chemical Co., Ann Arbor, MI). The tissue levels of LT4 and PGE2 were expressed as nanograms per milligram protein.

**Statistical Considerations and Analysis.** A contingency table \( \chi^2 \) test was used to analyze the association of 5-Lox immunohistochemical staining intensity with histology in human oral tissue samples. The tumor incidence was compared by the \( \chi^2 \) test. The dose-response analysis on zileuton and celecoxib was done with Mantel-Haenszel’s \( \chi^2 \) test. One-way ANOVA test was used to compare body weight, the number of visible tumors, and the numbers of oral lesions using the SAS software. The tumor volume was analyzed with Wilcoxon signed rank test. Student’s \( t \) test was used for statistical analysis of the levels of LT4 and PGE2. An isobolographic analysis was used to test the combination effect between zileuton and celecoxib (34). We assumed the expected response with combination, \( g(\text{zileuton } 3\%, \text{ celecoxib } 3\%) \), was a monotone, nondecreasing and continuous function. Synergistic, additive or antagonistic effect was defined if \( g(\text{zileuton } 3\%, \text{ celecoxib } 3\%) \) was higher than, equal to, or lower than \( g(\text{zileuton } 6\%, \text{ celecoxib } 0) \) and \( g(\text{zileuton } 0, \text{ celecoxib } 6\%) \), respectively. Here \( g \) was defined as the incidence of SCC in our animal model.
RESULTS

Overexpression of 5-Lipoxygenase and Cyclooxygenase 2 in DMBA-Induced Hamster Oral Carcinogenesis. With immunohistochemistry, 5-Lox was detected positive in only a few cells in the stroma of normal hamster oral mucosa (Fig. 1A). After DMBA treatment, 5-Lox was expressed in histologically normal mucosa (Fig. 1B) and further up-regulated in hyperplasia (Fig. 1C), dysplasia (Fig. 1D), and SCC (Fig. 1E). This expression pattern indicated that 5-Lox overexpression was an early event in DMBA-induced oral carcinogenesis.

In the normal hamster oral mucosa, Cox2 was barely detectable in the epithelial cells (Fig. 1F). In DMBA-treated samples, however, a low level of Cox2 was observed in some histologically normal areas (Fig. 1G). Cox2 was obviously up-regulated in hyperplasia (Fig. 1H), dysplasia (Fig. 1I), and SCC (Fig. 1J). It seemed that hyperplasia and dysplasia even expressed higher levels of Cox2 than SCC. This expression pattern was similar to that in human samples as reported in the literature (6, 7), indicating that Cox2 overexpression was an early event in oral carcinogenesis.

Overexpression of 5-Lipoxygenase in Human Oral Cancers. Using immunohistochemistry, we first examined the expression of 5-Lox in 20 cases of human oral cancer samples (Table 1). In the normal tissues adjacent to the tumor, only two of nine cases had indefinite staining in the epithelial cells (Fig. 2A). In the basal and parabasal layers of the oral epithelium, 5-Lox was overexpressed in hyperplasia (Fig. 2B), dysplasia (Fig. 2C), and SCC (Fig. 2D). The staining score was significantly associated with the pathologic stages (P < 0.0001; Table 1).

To confirm the expression pattern of 5-Lox in SCC and other histologic types of human oral cancer, two tissue microarrays were obtained for 5-Lox immunohistochemistry. 5-Lox was expressed in all the 29 cases of SCC, with the staining intensity of +, ++, and +++ in 8, 20, and 1 cases, respectively. In the other histologic types of human oral cancer (1 malignant tongue fibrous histiocytoma, 1 tongue adenosquamous carcinoma, 2 tongue pleomorphic adenoma, 1 tongue benign lymphoepithelioma, 4 tongue and parotid malignant myoepithelioma, 1 parotid acinic cell carcinoma, 3 parotid mucoepidermoid carcinoma, 8 parotid adenocarcinoma, 2 parotid adenoid cystic carcinoma, and 1 parotid adenolymphoma), 5-Lox expression was enhanced in most of these samples except benign lymphoepithelioma (Fig. 2E-P).

Chemopreventive Effects of Zileuton and Celecoxib. The DMBA-treated hamster oral mucosa (group B) significantly lowered the body weight from week 12 to 24 as compared with the nontreated group (group A, Fig. 3; P < 0.05). This may be a result of lower food intake due to DMBA-induced inflammation or tumor development. Overall, all the animals were healthy. Treatment with zileuton (groups C and D), celecoxib (groups E and F), or the combination (group G), had no major effect on the body weight, as compared with the positive control (group B).

At week 24, 6% zileuton (group D), 6% celecoxib (group F), and the combination (group G) significantly decreased the incidence of visible tumors (84.6%, 23 of 26, group B), to 46.4% (13 of 28, P < 0.01), 54.2% (13 of 24, P < 0.05), and 44% (11 of 25, P < 0.01), respectively (Table 2). In a similar fashion, the average number of tumors, the tumor volume, and the number of papillomas, were also inhibited by zileuton, celecoxib, or the combination. Microscopic examination showed that treatments with 3% zileuton (group C), 6% zileuton (group D), 3% celecoxib (group E), 6% celecoxib (group F), and the combination (group G), reduced the incidence of SCC (76.9%, 20 of 26, group B) to 45.8% (11 of 24, P < 0.05), 32.1% (9 of 28, P < 0.0001), 57.6% (15 of 26, P > 0.05), 50% (12 of 24, P < 0.05), and 36% (9 of 25, P < 0.001), respectively. Zileuton or celecoxib alone inhibited the incidence of SCC in a dose-dependent manner (P < 0.001 and P < 0.05, respectively).

The incidence of SCC was also used to evaluate the combination effect between zileuton and celecoxib. Because the combination of celecoxib and zileuton was not more effective than 6% zileuton or 6% celecoxib alone, it suggested an additive effect of zileuton and celecoxib (3% of each) on oral carcinogenesis in our animal model.

Effects of Zileuton and Celecoxib on Leukotriene B4 and Prostaglandin E2. Both levels of LTB4 (P < 0.05) and PGE2 (P < 0.001) in the hamster oral tissue significantly increased in DMBA-treated hamsters (group B) compared with the negative control (group A). Zileuton, at the doses of 3% (group E) or 6% (group F), significantly decreased the level of LTB4 (P < 0.01 and P < 0.05, respectively). Three percent celecoxib (group C, P < 0.01), but not 6% celecoxib (group D), significantly reduced the level of PGE2 in hamster oral tissues. Combination of zileuton and celecoxib (3% of each, group G) significantly decreased the levels of both LTB4 and PGE2 (P < 0.0001).

DISCUSSION

This study clearly showed that 5-Lox and Cox2 were overexpressed in oral cancer of both hamsters and humans. Zileuton and celecoxib, alone or in combination, prevented the development of DMBA-induced oral cancer in hamster cheek pouch at the post-initiation stage, and such chemopreventive effects were correlated with their inhibitory effects on arachidonic acid metabolism.

As an important arachidonic acid–metabolizing enzyme, 5-Lox was markedly up-regulated in the stromal inflammatory cells and epithelial cells at the early stages of oral squamous cell carcinogenesis (Fig. 1). In several other histologic types of human oral cancer, 5-Lox was also found to be overexpressed (Fig. 2). Zileuton was effective against DMBA-induced hamster oral carcinogenesis and seemed even more effective than celecoxib (Table 2). Consistent with this finding, topically applied zileuton was more effective than Cox inhibitors in suppressing the inflammation of the mouse dermatitis models induced by topical phorbol ester or arachidonic acid (35, 36). Oral administration of zileuton or other inhibitors of the 5-Lox pathway has been shown to be chemopreventive in animal models of pancreatic cancer (29), lung cancer (27), skin cancer (28), and esophageal adenocarcinoma (25). These findings suggested that the 5-Lox pathway of arachidonic acid metabolism played an important role in inflammation-associated carcinogenesis, including oral cancer.

Cox2 has been known to play an important role in the initiation and post-initiation stages of carcinogenesis (37, 38). Overexpression of Cox2 has been well documented in human oral cancer (6, 7). In this study, we found Cox2 overexpression even in DMBA-treated, yet histologically normal, hamster oral mucosa (Fig. 1G). During the progression of oral carcinogenesis,
Cox2 was markedly overexpressed at the premalignant and malignant stages (Fig. 1H, I, and J). Using the DMBA-induced hamster cheek pouch model, we showed that celecoxib prevented oral carcinogenesis in a dose-dependent manner. This confirmed the results of previous studies on the 4-nitroquinoline-1-oxide-induced tongue cancer model with rats (10). Altogether these studies showed that Cox2 overexpression was an early event in oral carcinogenesis, and Cox2 inhibitors could be

![Fig. 1 Overexpression of 5-Lox (A-E) and Cox2 (F-J) in hamster oral mucosa. 5-Lox was detected by immunohistochemistry and Cox2 in situ hybridization. Normal hamster oral mucosa did not show positive staining of 5-Lox (A) and Cox2 (F) in epithelial cells. In DMBA-treated histologically normal oral mucosa, 5-Lox (B) and Cox2 (G) were overexpressed in epithelial cells. In hyperplasia, 5-Lox (C) and Cox2 (H) expression in the epithelial cells were further enhanced. At the stages of dysplasia (D and I) and carcinoma (E and J), 5-Lox (D and E) and Cox2 (I and J) were also overexpressed as compared with normal oral mucosa. Besides the epithelial cells, some inflammatory cells in the stroma also expressed 5-Lox and Cox2 at these pathological stages. Bars, 50 μm for 5-Lox staining or 100 μm for Cox2 staining.](image)
used at the premalignant stage for the prevention of oral cancer through topical application or oral administration.

Both 5-Lox and Cox2 play important roles in inflammation and inflammation-associated carcinogenesis. The 5-Lox and Cox2 pathways are activated together during inflammation, and blocking one pathway may activate the other (39, 40). In a phorbol ester–induced mouse dermatitis model, 5-Lox was mainly involved in the priming event, whereas Cox2 was involved in subsequent oxidative stress (41). Our results clearly showed that 5-Lox and Cox2 were up-regulated at the early stages of oral carcinogenesis. We showed an additive chemopreventive effect on the incidence of SCC by zileuton and celecoxib in our animal model. Similar additive effects of the inhibitors of the 5-Lox and Cox pathways have been shown in animal models of lung (26), pancreatic (30), and esophageal cancer (25). Zileuton (3% and 6%) and celecoxib (3%) reduced the levels of LTB4 and PGE2 in hamster oral tissues, respectively; 6% celecoxib did not (Table 2). It was possible that the hamster oral mucosa used for this analysis might contain different histology. Zileuton and celecoxib may also be chemopreventive through 5-Lox- and Cox2-independent effects. As a hydroxyurea compound, zileuton is an iron chelator (42). A Cox2-specific inhibitor, NS398, inhibited the growth of human oral SCC cells by both Cox2-dependent and -independent mechanisms (7).

Topical application was used for administration of zileuton and celecoxib in this study. As reported previously, celecoxib was anti-inflammatory and chemopreventive when given both systemically and topically in a UV-induced skin carcinogenesis model (43, 44). Topical celecoxib in polymer film inhibited tumorigenesis of inoculated human oral cancer cells when applied at the site of inoculation (45). In this study, rather high concentrations of zileuton and celecoxib allowed local permeation into keratinized squamous epithelium of hamster oral mucosa. Because human oral cavity is covered by keratinized, nonkeratinized, and specialized epithelia with varying permeability to these agents, we believe the doses of topical chemopreventive agents need to be adjusted according to the locations of premalignant lesions. Systemic absorption of the topically applied agents from the gut may also contribute, most likely to a minor extent, to the chemopreventive effects of zileuton and celecoxib in this study. The doses we used (3% or 6% in 100 μL mineral oil thrice every week) were equivalent to...
~ 20 or 40 ppm if these agents were given in the diet *ad libitum*. A 200-g hamster eats about 20 g of diet every day. Such doses in diet are probably too low to exert any significant effect on carcinogenesis.

In summary, this study identified both 5-Lox and Cox2 as chemopreventive targets of oral cancer. Their specific inhibitors when applied topically prevented DMBA-induced hamster oral carcinogenesis through their inhibitory effects on arachidonic acid metabolism. Considering the recent withdrawal of Vioxx due to cardiovascular side effects, we believe further studies are needed to translate the animal studies into a safe and efficacious strategy for long-term use in the prevention of human oral cancer.

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