A Novel Dietary-Related Model of Esophagitis and Barrett’s Esophagus, a Premalignant Lesion

Naihsuan C. Guy, Harinder Garewal, Hana Holubec, Harris Bernstein, Claire M. Payne, Carol Bernstein, Achyut K. Bhattacharyya, and Katerina Dvorak

Abstract: Barrett’s esophagus (BE) is a premalignant lesion in which columnar epithelium (containing goblet cells) replaces esophageal squamous cells. Previous evidence suggested that hydrophobic bile acids and zinc deficiency each play a role in BE development. We fed wild-type C57BL/6 mice a zinc-deficient diet containing the hydrophobic bile acid, deoxycholic acid for various times up to 152 days. All mice fed this diet developed esophagitis by 69 days on the diet and 63% of the mice on this diet for 88 to 152 days also developed a BE-like lesion. Esophageal tissues showed thickened mucosa, increased proliferation, and increased expression of markers associated with oxidative and nitrosative stress. The newly formed BE-like lesions expressed Mucin-2, a marker of columnar differentiation. They also showed translocation of the p65 subunit of nuclear factor-κB and β-catenin to the nucleus and typical histological changes associated with BE lesions. This mouse model of esophagitis and BE is expected to contribute to a deeper understanding of BE pathogenesis and to strategies for prevention of BE progression to cancer.

Introduction

Barrett’s esophagus (BE) is a premalignant lesion at the distal part of the esophagus that arises as a consequence of chronic gastroesophageal reflux. Heartburn and gastroesophageal reflux disease (GERD) are common medical conditions in Western countries. Approximately 40% of adults complain of heartburn at least monthly (1). BE is estimated to be present in approximately 10 to 12% of patients undergoing endoscopic examination for symptomatic GERD (2). This represents at least 2 million people in the United States (1). Patients with BE have an increased risk of developing esophageal adenocarcinoma (EAC) (3). Multiple epidemiological studies have shown that the incidence of esophageal adenocarcinoma in the United States and Western Europe is rapidly rising (4). EAC has a poor prognosis, with a median survival of less than 1 yr (1).

Histologically, BE is characterized by intestinal metaplasia (IM), a condition where squamous epithelial cells are replaced by metaplastic intestinal-like columnar epithelium containing goblet cells. However, the mechanism of development of BE is poorly understood. Obesity and chronic irritation of the esophageal mucosa by gastric acids, proteases, and bile acids are considered 2 major risk factors for BE development (5–7).

Current animal models used for studying BE and EAC include canine, rabbit, rat, and mouse models, which all require a surgical procedure. The most common rat model uses esophagogastroduodenal anastomosis without concomitant chemical carcinogen treatment, leading to development of columnar-lined esophagus (CLE) including metaplasia, dysplasia, and EAC (8). However, these surgical animal models have significant limitations. They are expensive, technically challenging, time consuming, nonphysiological, and not very efficient. Therefore, it is important to develop an animal model that can acquire CLE by a nonartificial and less invasive method.

Epidemiological studies have indicated the association of zinc deficiency with increased risk of cancer development (9). The total body zinc content in humans is 2 to 4 g, and there is no specialized zinc storage system in the body. Therefore, regular supplementation is essential (10). Approximately 10% of the U.S. population consumes less than half the recommended daily allowance for zinc (10). Interestingly, tumors and glandular metaplasia resembling BE developed in nearly 20% of p53−/− mice after 3 wk on a zinc-deficient diet and a single treatment with N-nitrosomethylbenzylamine (NMBA) and in 14% of
transgenic mice overexpressing cyclin D1 after 4 wk on a zinc-deficient diet and a single treatment with NMBA (11,12).

Clinical studies have shown that obesity, 1 of the risk factors for developing BE, is associated with hypozincemia and defective antioxidant status (5). Also, certain commonly used drugs that lower gastric secretion, such as proton pump inhibitors (PPI) and H2 receptor inhibitors, diuretics, ACE inhibitors, and oral contraceptives decrease zinc absorption (13–16).

Bile is a component of gastroduodenal refluxate (17). Primary bile acids, such as glycocholic acid, taurocholic acid, glycodeoxycholic acid, and glycochenodeoxycholic acid, are the bile acids predominantly present in the esophagus of patients with GERD. It has been shown that PPI therapy causes an overgrowth of gastric bacteria in patients with GERD (18). Such bacteria may deconjugate the primary bile acids to form the more toxic unconjugated bile acids such as deoxycholic acid (DOC) (18). DOC is also present in the refluxate of BE patients (19).

We hypothesized that a diet deficient in zinc and supplemented with a bile acid, such as DOC, can lead to the development of BE-like lesions in mice. Such a novel experimental animal model for studying esophagitis and BE may give insight into the induction of columnar differentiation, proliferation, DNA damage, and oxidative stress in the esophagus. Experimental animal models should provide useful insights into possible molecular mechanisms underlying development of esophagitis and consequently to possible strategies for preventing progression to BE.

### Materials and Methods

#### Animal Model

Forty-eight male C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in the University of Arizona’s Animal Care facility. The animals were housed 2 in a cage and raised under nonsterile microisolator conditions in compliance with the National Institutes of Health’s regulations and guidelines for care and use of laboratory animals. The mice were maintained on a 12-h light–dark cycle with free access to deionized drinking water. The mice were randomized into 4 dietary groups with 12 mice per group. They were fed customized diets prepared by Harlan Teklad (Madison, WI). The diets were fed starting at 5 wk of age until completion of the dietary treatment. The 4 diet groups were a control diet, a zinc-deficient diet (Zn−), a diet supplemented with 0.2% DOC (Sigma D6750) (DOC+), and a zinc-deficient diet supplemented with 0.2% DOC (DOC+Zn−). The vitamin mix used in each of the diets is given in Table 1, and all components of the 4 diets are shown in Table 2. The mice were monitored for clinical signs of ill health. One mouse on the DOC+Zn− diet died after 137 days on the diet, before sacrifice, and was not included in the study.

#### Histopathological Analysis

For histological evaluation, the formalin-fixed, paraffin-embedded mouse tissues were sectioned and stained with hematoxylin and eosin (H&E). Diagnosis of BE was made by the presence of columnar metaplasia containing goblet cells surrounded by squamous epithelium. The pathologist evaluating the tissues was blinded as to the identity of the diets of the mice prior to his histological evaluation and analysis for the presence of BE lesions.

#### Immunohistochemistry

Tissues were evaluated for expression of individual biomarkers by employing standard indirect immunohistochemical techniques using different antibodies. In brief, the tissue sections were deparaffinized and hydrated. Antigen retrieval was performed by immersing the sections in 0.01M citrate buffer (pH 6.1) and heating in a microwave at high power for 2 min and 45 s and then at the defrost setting for 6 min. 8-hydroxy-deoxyguanosine monoclonal antibody

### Table 1. Vitamin Mix From Teklad (CA.40060) in g/kg

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminobenzoic acid</td>
<td>11.0132</td>
</tr>
<tr>
<td>Vitamin C, ascorbic acid, coated (97.5%)</td>
<td>101.6604</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.0441</td>
</tr>
<tr>
<td>Vitamin B12 (0.1% in mannitol)</td>
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</tr>
<tr>
<td>Calcium pantothenate</td>
<td>6.6079</td>
</tr>
<tr>
<td>Choline dihydrogen citrate</td>
<td>349.6916</td>
</tr>
<tr>
<td>Folic acid</td>
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<tr>
<td>Inositol</td>
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</tr>
<tr>
<td>Vitamin K3, menadione</td>
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</tr>
<tr>
<td>Niacin</td>
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</tr>
<tr>
<td>Pyridoxine HCl</td>
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</tr>
<tr>
<td>Riboflavin</td>
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</tr>
<tr>
<td>Thiamin HCl</td>
<td>2.2026</td>
</tr>
<tr>
<td>Vitamin A palmitate (500,000 IU/g)</td>
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</tr>
<tr>
<td>Vitamin D3, cholecalciferol (500,000 IU/g)</td>
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</tr>
<tr>
<td>Vitamin E, DL-tocopheryl acetate (500 IU/g)</td>
<td>24.2291</td>
</tr>
<tr>
<td>Corn starch</td>
<td>466.6878</td>
</tr>
</tbody>
</table>

Mice were kept on their diets and then sacrificed at intervals up to 152 days on diet, except for 3 mice on the DOC+ diet and 4 mice on the control diet, which were terminated after a longer period (225 days) to determine if a lengthier time on the DOC+ diet would give rise to BE (there was no BE in these longer-treated mice). Mice were sacrificed using CO2 asphyxiation.

#### Animal Tissue Preparation

Tissues from the esophagus and stomach were taken and immediately fixed in 10% buffered formalin for 24 h at 4°C and then transferred to 70% ethanol. The tissue samples were then processed for paraffin embedding and sectioned.
(2 μg/ml; QED Bioscience Inc., San Diego, CA) was used for detecting oxidative DNA damage. For this special staining, the slides were immersed in 4N HCl for 20 min and then rinsed in 4 changes of nanopure water and placed in 0.1M Borax for 5 min followed by 2 rinses in nanopure water to open up the chromatin. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min, and then the slides were incubated with 1.5% normal serum (of either goat, mouse, horse, or rabbit, corresponding to the organism in which the secondary antibody was created) for 45 min. The primary antibodies included polyclonal antibodies against Mucin-2 (MUC2; Santa Cruz Biotechnology, Santa Cruz, CA; 1:50 dilution), proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution), nitrotyrosine (Upstate Cell Signaling Solutions, Lake Placid, NY; 1:60 dilution), and nuclear factor-κB (NF-κB) p65 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:400 dilution) and monoclonal antibodies against 8-hydroxy-deoxyguanosine (8-OH-dG; QED Bioscience, San Diego, CA; 1:400 dilution) and monoclonal antibodies against 8- and treated with avidin-biotin complex followed by a chromagen, 3,3′-diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin, dehydrated, and mounted with Cytoseal™ 60 mounting medium (Richard-Allan Scientific, Kalmazoo, MI).

Statistical Evaluations

Histological changes in gastroesophageal morphology were determined in hematoxylin and eosin stained sections from esophageal tissue obtained from each of the terminated mice in each experimental group. A morphologic grading score was established by evaluating degrees of proliferation, keratinization, and esophagitis on a scale of 0 to 4 for each of these categories (with 0 being no significant change from those mice on the control diet, 1 being a small detectable change, 2 being a moderate change, 3 being a substantial change, and 4 being a maximal change) and presence or absence of BE-like lesions evaluated as 0 or 4 for absence or presence of BE, respectively. A composite score was determined as the sum of the scores for the 4 categories. The morphologic score for each dietary group was expressed as a mean ± SD. An unpaired Student t-test was performed, and a P value of <0.05 was considered statistically significant.

To determine whether the frequency of BE occurring during a dietary treatment was significantly different from that in control-fed mice, the 2-sample Wilcoxon rank sum (Mann–Whitney) test was used.

Results

Histopathology

Mice were terminated at intervals after starting on the 4 test diets (control, Zn−, DOC+ and DOC+Zn− diets) to evaluate at what point histological changes began to occur in their esophagus and to determine when, if at all, BE occurred. By 152 days on the diets, mice on the DOC+Zn− and Zn− diets showed a decline in their general health so that the majority of mice were terminated by this point. The 2 mice terminated at the early time of only 41 days on the diets (1 on a DOC+ diet and 1 on a DOC+Zn− diet) had no histopathological changes. At longer times of 69 to 152 days on the diets, the DOC+Zn− diet induced gastroesophageal changes resembling typical histological findings associated with reflux esophagitis and BE in patients (Fig. 1D, 1E, 1F). Of the mice fed the DOC+Zn− diet for times between

<table>
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<tr>
<th>Control</th>
<th>Zn(−)</th>
<th>DOC(+)</th>
<th>Zn(−)DOC(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg white solids, spray dried</td>
<td>200.0000</td>
<td>200.0000</td>
<td>200.0000</td>
</tr>
<tr>
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<td>632.2658</td>
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<td>632.2658</td>
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<tr>
<td>Corn oil</td>
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<tr>
<td>Cellulose</td>
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<td>30.0000</td>
<td>29.9107</td>
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<tr>
<td>Vitamin mix (see Table 1)</td>
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<td>10.0000</td>
<td>10.0000</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.0040</td>
<td>0.0040</td>
<td>0.0040</td>
</tr>
<tr>
<td>Ethoxyquin (antioxidant)</td>
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<td>0.0200</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>2.4752</td>
<td>2.4752</td>
<td>2.4752</td>
</tr>
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<td>Potassium chloride</td>
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</tr>
<tr>
<td>Sodium chloride</td>
<td>0.7781</td>
<td>0.7781</td>
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</tr>
<tr>
<td>Ferrous sulfate</td>
<td>0.2000</td>
<td>0.2000</td>
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<tr>
<td>Manganese sulfate</td>
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</tr>
<tr>
<td>Cupric sulfate</td>
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<tr>
<td>Potassium iodate</td>
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<td>0.0004</td>
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<tr>
<td>Zinc carbonate</td>
<td>0.0893</td>
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<tr>
<td>Sodium deoxycholate</td>
<td>—</td>
<td>—</td>
<td>2.0000</td>
</tr>
</tbody>
</table>

Table 2. Composition of the Mouse Diets in g/kga

a: Abbreviations are as follows: Zn, zinc; DOC, deoxycholic acid.
Figure 1. Histological appearance of the esophagus of mice fed different diets. A: Control diet. B: DOC+ diet. Note thickened epithelium and hyperkeratinization compared to the control. C: Zn− diet. Note some hypertrophy of papillae and hyperkeratinization. D: DOC+Zn− diet. Note development of esophagitis. E: DOC+Zn− diet. Note the presence of metaplastic epithelium with goblet cells of BE-like lesion. [hematoxylin and eosin (H&E); original magnification A to E: ×100]. F: DOC+Zn− diet. Note papillae hypertrophy (green arrow), increased angiogenesis (black arrow), and inflammation (red arrow) (H&E; original magnification: ×200).

88 days and 152 days (Fig. 2A), 63% (5 out of 8) developed BE-like lesions in which normal squamous epithelium was replaced by specialized intestinal metaplastic epithelium containing goblet cells (Fig. 1E). One mouse on the Zn− diet developed BE by 152 days on the diet as indicated in Fig. 2A. All mice fed for between 69 days and 152 days with the DOC+ diet (Fig. 1B), Zn− diet (Fig. 1C), or Zn−DOC+ diet (Fig. 1D) developed basal cell hyperproliferation and/or hyperkeratinization in esophageal tissues. The Zn−DOC+ diet, fed for between 69 and 152 days, induced esophagitis characterized by the development of papillae hypertrophy (Fig. 1F, green arrow), angiogenesis (Fig. 1F, black arrow), and an increased infiltration of inflammatory cells (Fig. 1F, red arrow). We performed immunohistochemical staining of the newly formed BE-like lesions to examine the expression of biomarkers of columnar differentiation, proliferation, oxidative and nitrosative stresses, NF-κB p65, and β-catenin (see following).
Figure 2. A: The mean weights of the mice on each diet between the time they were entered onto the diets (at 5 wk of age) until 152 days on the diets is shown. Arrows below the weight curves indicate the times at which the indicated number of mice on the deoxycholic acid (DOC) + zinc (Zn) − diet (shown in the Fig. as D+Z−) were terminated. The text below these lower arrows also indicates how many of the mice had Barrett’s esophagus (BE) at the time of termination. Arrows above the weight curves indicate the times at which the given number of mice on the control diet (shown by C), the DOC + diet (shown by D+), and the Zn − diet (shown by Z−) were terminated. Only 1 of these latter mice (on the Zn− diet) had a small BE lesion, as indicated. B: Effect of diets shown on the abscissa on gastroesophageal morphology. The mean morphology composite score based on degree of proliferation, keratinization, esophagitis, and the presence of BE-like lesions was determined for each group of mice. Asterisks indicate a statistically significant difference compared to the control group (P < 0.05).

**Dietary Effects on Body Weight and Gastroesophageal Morphology**

Mean body weight for each group of mice, measured during days 0 to 152 of the dietary treatments, is shown in Fig. 2A. Fig. 2A also shows the numbers and times at which mice were terminated, between 42 and 152 days on the diets, by vertical arrows. Fig. 2B shows the mean morphologic composite scores for mice terminated between 69 and 152 days on the diet for mice fed the control diet, the DOC+ diet, the Zn− diet and the DOC+Zn− diet based on the degree
Figure 3. Immunohistochemical analysis of Mucin2 (MUC2) in the esophagus of mice fed different diets. (A–C) A: Control diet. B: deoxycholic acid (DOC) + zinc (Zn)− diet. Note that this tissue section shows esophagitis but no Barrett’s esophagus (BE)-like lesion. C: DOC + Zn− diet. Note that this tissue shows strong MUC2-positive expression in the goblet cells at the base of crypts in a BE-like lesion (original magnification: ×400). Immunohistochemical analysis for proliferating cell nuclear antigen (PCNA) in the esophagus of mice fed different diets. (D–F) D: Control diet. Note that there are relatively few PCNA stained cells, thus indicating the normal baseline of proliferating cells in the esophageal tissue. E: DOC + Zn− diet. Note that this tissue has developed esophagitis and increased PCNA-positive cells throughout the hypertrophied papillae. F: DOC + Zn− diet. Note that this tissue has developed a BE-like lesion. Strong nuclear staining and a significant increase of positive PCNA expression is observed in the metaplastic epithelium of this BE-like lesion (original magnification: ×200).

Columnar Differentiation Markers

As shown in Fig. 1E, there was goblet cell-type metaplasia in BE-like lesions in tissues from mice fed with a DOC + Zn− diet. The lesions were characterized by the appearance of columnar-like epithelium with expression of MUC2 in the goblet cells (Fig. 3C). MUC2-positive cells were not observed in the mice fed the control diet (Fig. 3A) or in the mice fed a DOC + Zn− diet that only developed esophagitis (Fig. 3B).

PCNA

PCNA is an accessory protein for DNA polymerase δ and an indicator of cell cycle progression at the G1/S transition (20). There was minimal PCNA staining within cells of mice fed the control diet (Fig. 3D). In contrast, in mice fed a DOC + Zn− diet, numerous PCNA-positive cells occurred throughout the mucosal tissue displaying esophagitis, especially in the enlarged papillae in the basal layer (Fig. 3E). Strong abundant positive expression of PCNA was also observed in the metaplastic epithelium of BE-like lesions.
in the esophageal tissue of mice fed the DOC+Zn− diet (Fig. 3F).

Oxidative Stress and DNA Damage

8-OH-dG is a modified DNA base that is formed due to attack by hydroxyl radicals that are formed as byproducts and intermediates of aerobic metabolism and during oxidative stress (21). As shown in Fig. 4A, there was minimal 8-OH-dG signal in cells of mice fed the control diet. However, the tissues from mice fed a DOC+Zn− diet resulted in elevated levels of 8-OH-dG in inflamed esophageal tissue (Fig. 4B) and in BE-like lesions (Fig. 4C).

Nitrosative Stress

Antinitrotyrosine antibody was used to detect 3-nitrotyrosine, providing good evidence for the formation of peroxynitrite and other reactive nitrogen species in vivo. As shown in Fig. 4D and 4E, nitrotyrosine was absent or weak in esophageal tissues taken from mice fed the control diet and from mice that were fed the DOC+Zn− diet but that only developed esophagitis. In contrast, nitrotyrosine immunoreactivity was intense in the epithelial cells in BE-like lesions from mice that were fed the DOC+Zn− diet (Fig. 4F).

Positive nuclear nitrotyrosine expression is also present in some cells in the BE-like lesions (Fig. 4F).

Transcription Factor NF-κB p65

As illustrated in Fig. 5A, esophageal epithelium from mice fed the control diet showed only cytoplasmic immunoreactivity of the p65 subunit of NF-κB. However, nuclear translocation of the p65 was observed in regions of esophagitis (Fig. 5B) and BE-like lesion tissues (Fig. 5C) of mice fed the DOC+Zn− diet.

β-Catenin

β-catenin is a key regulator in the E-cadherin-mediated cell adhesion system and acts as an oncoprotein when transported to the nucleus. Fig. 5D shows that β-catenin expression is localized at the cell membrane in the esophageal squamous epithelium of mice fed the control diet and for most cells in mice fed the DOC+Zn− diet and showing esophagitis (Fig. 5E). Loss of membranous β-catenin immunoreactivity with diffuse cytoplasmic staining and nuclear staining were observed in some cells of tissues showing esophagitis (Fig. 5E) and BE-like lesions (Fig. 5F) in tissues of mice fed the DOC+Zn− diet. Focal nuclear staining for β-catenin was...
also observed in some scattered cells in the BE-like lesions (Fig. 5F).

Discussion

EAC is one of the most deadly forms of cancer, with a mortality rate exceeding 90%, and an incidence that is increasing rapidly in the United States and many regions of Western Europe. The major risk factor for the development of EAC is BE (22). Patients with BE are at 30 to 40 times greater risk of developing EAC than individuals in the general population (23). BE is a premalignant lesion of the distal part of the esophagus that arises as a consequence of chronic gastroesophageal reflux. The classic endoscopic feature of BE is the presence of salmon pink mucosa at the distal region of the esophagus. Histologically, BE is defined as the metaplastic conversion of normal esophageal squamous epithelium into columnar intestinalized epithelium with the presence of goblet cells. The molecular mechanisms underlying the development of BE are unclear. However, BE appears to result from chronic irritation by bile acids and gastric acid (7).

Importantly, bile acids have been linked to the development of gastrointestinal (GI) cancers (24). Clinical studies have shown an increased concentration of bile acids in patients with reflux disease and BE (17). Bile acids cause oxidative stress, DNA damage, perturbed mitochondrial respiration, and induction of apoptosis (24–28). In addition, gastric acid and bile acids may alter cell signaling pathways (29–31). Bile acids are implicated in promoting GI cancers by modulating cell signaling pathways, causing epigenetic changes, increasing proliferation, and promoting development of apoptosis resistance on long-term exposure (24). Both human studies and animal models of GI reflux esophagitis have implicated hydrophobic bile acids in the development of the BE lesion and EAC (24).

Zinc is an essential mineral that is found in almost every cell. Zinc is an important component of over 1,000 proteins, including DNA-binding proteins with zinc fingers, copper/zinc superoxide dismutase, and several proteins involved in DNA damage repair such as p53, which is mutated in half of human tumors (32). Zinc directly stimulates the activity of more than 100 enzymes (33). Therefore, insufficient zinc intake can impair antioxidant defenses and compromise DNA-repair mechanisms, making the cell highly susceptible to oxidative DNA damage. In humans, there is a significant inverse association between dietary intake of zinc and incidence of EAC (34). Furthermore, BE lesions develop in about 20% of p53−/− mice or transgenic mice overexpressing cyclin D1 that were on a zinc deficient diet and treated once with the carcinogen NMBA (11).
Mucins are secretory proteins of the GI mucosa expressed in a cell-specific manner in normal GI tissues. MUC2 is a principal secretory mucin abundantly found in the colo-rectum (35). MUC2 is associated predominantly with normal intestinal goblet cells and is highly expressed in colon and gastric tumors (36). MUC2 is also a specific marker for IM in BE (35,37,38). We observed MUC2 positive cells in BE-like lesions but not in normal appearing tissue or in tissue displaying esophagitis. Because MUC2 expression is a marker for goblet cells in human BE (35,37,38), our study indicates that a DOC+Zn− diet induces the development of intestinal-like columnar epithelium containing goblet cells, a BE-like lesion in mice.

We assessed the proliferative responses of mouse tissues by evaluating PCNA immunoreactivity. PCNA is a protein synthesized in early G1 and S phases of the cell cycle. It plays an essential role in cell cycle progression, DNA replication, and DNA repair (20). PCNA staining was increased in mice fed a DOC+Zn− diet, indicating that cell proliferation increased during progression from normal squamous to esophagitis to the BE-like lesion. These results are consistent with findings in surgical animal models (21). Also, PCNA expression in BE-like lesions in our mouse model was similar to the expression pattern in patient BE tissues that we evaluated by Ki-67 immunoreactivity, another marker of proliferation (39).

Accumulating evidence indicates that oxidative stress plays a key role in the pathogenesis of reflux esophagitis, BE, and EAC (40). Oxidative injury is increased, and antioxidant capacity is suppressed in the esophageal mucosa during chronic gastroesophageal reflux (41). Formation of 8-OH-dG is an indicator of oxidative DNA damage. Our dietary model showed increased 8-OH-dG immunoreactivity in BE-like lesions. This result is consistent with our findings in patients with BE that levels of 8-OH-dG are increased in BE tissue compared to normal squamous tissue (42).

The powerful oxidant peroxynitrite anion is formed when the superoxide anion is scavenged by nitric oxide. Peroxynitrite can amplify the inflammatory process (43). Nitrotyrosine, resulting from the reaction of peroxynitrite with tyrosine, serves as a footprint of peroxynitrite (44,45). Nitrotyrosine staining can be used as an indirect measure of peroxynitrite formation in vivo (46). In a surgical rat model for EAC, increased nitrotyrosine staining was found, suggesting that peroxynitrite generation may contribute to esophageal carcinogenesis (44). We investigated nitrotyrosine formation in our dietary model and found intense nitrotyrosine staining in BE-like lesions in mice fed the DOC+Zn− diet. Our findings are consistent with those reported in an experimental model of esophagitis where peroxynitrite formation was a common event in the presence of excess of superoxide anion radicals (43).

NF-κB is a ubiquitous transcription factor that is activated in response to a variety of pathogenic stimuli including endotoxins, oxidative and nitrosative stresses, and proinflammatory cytokines (47). NF-κB is a dimer, which consists of p50 and p65 subunits. NF-κB is normally sequestered in the cytoplasm by the inhibitory molecule IκB (48). Phosphorylation of the p65 subunit, or phosphorylation of IκB and its subsequent degradation, leads to translocation of NF-κB from the cytoplasm to the nucleus and activation of the DNA binding activity of NF-κB (48). NF-κB subsequently upregulates the transcription of proinflammatory genes and genes involved in cell growth, proliferation, apoptosis, angiogenesis, and metastasis (49). Increased levels of NF-κB activation have been found in a number of tumors including EAC (49). NF-κB is translocated to the nucleus in Barrett’s mucosa, probably as a consequence of the inflammation of this mucosa resulting from GERD. We found infiltration of inflammatory cells in esophagitis tissue and BE-like lesions from mice that were fed the DOC+Zn− diet. Nuclear translocation of NF-κB occurred in esophagitis tissue and BE-like lesions from mice fed the zinc-deficient diet supplemented with DOC. Our study demonstrates that zinc deficiency in combination with DOC may play a role in activating NF-κB DNA-binding activity, which may, in turn, contribute to the development of BE.

β-Catenin is an important regulator in the cadherin-mediated cell adhesion system. Nuclear β-catenin expression has been associated with cellular transformation and tumor invasion and metastasis in many cancers including EAC (50–52). β-catenin contributes to carcinogenesis when there is a disruption in the adenomatous polyposis coli (APC)/β-catenin/T-cell factor signal transduction pathway (53,54). In our study, all normal squamous epithelium from the control diet group showed uniform membranous β-catenin immunoreactivity, and no nuclear staining was detected. Normally, β-catenin nuclear translocation is prevented by binding to E-cadherin on the cellular membrane or by the destruction of β-catenin by APC-GSK3 complex formation (53). We found decreased or absent expression of β-catenin in the cellular membrane and an increase in diffuse cytoplasmic staining in esophagitis tissue and BE-like lesions from mice fed the DOC+Zn− diet compared to normal control epithelium. Focal nuclear staining for β-catenin was observed in some cells in BE-like lesions.

In conclusion, our mouse model, employing a diet that mimics inadequate zinc intake and a component associated with a high fat diet, DOC, has led to the development of murine esophagitis and BE-like lesions. Our dietary animal model has many advantages over current surgical animal models. Feeding the mice a diet that mimics inadequate intake of micronutrients, such as zinc, and DOC is a much simpler, noninvasive, cost-effective, and less time-consuming method than surgical alteration. The striking finding in this study is that these dietary modifications induced gastroesophageal changes resembling BE in 5 out of 8 mice (63%) within the period from 88 to 152 days on a DOC+Zn− diet, a significant difference from mice fed a control diet. In addition, in 100% of animals fed with the DOC+Zn− diet, we observed histopathologic changes in areas of the squamous epithelium, which included basal cell hyperplasia, hyperkeratinization, papillae hypertrophy, metaplasia of
the esophagus, increased infiltration of inflammatory cells, and increased angiogenesis. The altered tissues expressed biomarkers for columnar differentiation (MUC2), oxidative stress (8-OH-dG), nitrosative stress (nitryrosine), proliferation (PCNA), and activation of the NF-κB p65 subunit and β-catenin. Proliferation and oxidative stress, along with nuclear translocation of NF-κB and β-catenin, all appeared to be enhanced in a manner comparable to the expression pattern seen in patient biopsies taken from BE lesions (47,49,53–56). Accordingly, the animal model developed here may prove advantageous for the study of the molecular mechanisms underlying development of esophagitis and consequently possible strategies for preventing progression to BE.

The studies shown herein emphasize the deleterious effects of increased hydrophobic bile acids and zinc deficiency. 2 components associated with a Western-style, high-fat, low-micronutrient diet on the esophagus. The observed histopathologic lesions of esophagitis, hyperproliferation, papillae hypertrophy, angiogenesis, and metaplastic columnar epithelium are cellular events on the pathway to neoplasia.

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