Increased Expression of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 in Barrett’s Esophagus and Associated Adenocarcinomas

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Abstract

Barrett’s esophagus is a premalignant condition arising in response to chronic reflux esophagitis. Inducible nitric oxide synthase (iNOS; NOS-2) and cyclooxygenase-2 (COX-2) are mediators of inflammation and regulators of epithelial cell growth. Expression levels of iNOS and COX-2 are high in colorectal adenomas and carcinomas, and COX-2 expression is elevated in gastric cancers. To determine the involvement of iNOS and COX-2 in Barrett’s-associated neoplasia, we measured expression of these genes in metaplastic Barrett’s and esophageal adenocarcinomas. We detected elevated iNOS and COX-2 mRNA levels in Barrett’s mucosa compared with paired gastric control tissues in 16 of 21 (76%) and 17 of 21 (80%) patients, respectively (P < 0.001 for both genes). In esophageal adenocarcinomas, iNOS and COX-2 mRNA levels were increased in four of five and five of five cases, respectively. Furthermore, in 10 of 10 Barrett’s patients, immunohistochemical staining for iNOS and COX-2 expression was strongly positive and higher than in matched gastric controls. Increased COX-2 expression was confirmed by Western blotting. These findings support the hypothesis that iNOS and COX-2 are involved early and often in Barrett’s-associated neoplastic progression.

Introduction

Barrett’s esophagus is the replacement of the normal squamous esophageal epithelium by a specialized columnar lining in response to chronic reflux esophagitis (1). This condition is highly premalignant, being associated with a risk of esophageal adenocarcinoma up to 125-fold greater than the general population (2, 3). The molecular basis of this increased risk is now being elucidated. Generalized genetic instability, evidenced by aneuploidy and chromosomal abnormalities, has been shown to occur early in this process (4, 5). Loss of heterozygosity, inactivation of tumor suppressor genes, and microsatellite instability have all been identified in Barrett’s metaplasia and associated esophageal adenocarcinomas, but the frequency of these alterations in metaplastic mucosa is generally lower than in frank esophageal adenocarcinomas (6). In any case, much remains to be learned about the molecular pathogenesis of Barrett’s-associated neoplastic progression.

iNOS is the enzyme that catalyzes the high output pathway of production of NO from L-arginine, whereas COX-2 is the rate-limiting enzyme for the high output production of prostanooids (prostaglandins and thromboxanes) from arachidonic acid. Increased expression of both iNOS and COX-2 has been demonstrated in intestinal inflammation, in both animal models (7–9) and human diseases (10, 11). Furthermore, we and others have identified high levels of expression of iNOS and COX-2 in Helicobacter pylori-associated gastritis (12, 13). Recently, the importance of COX-2 in GI carcinogenesis has been recognized. Induction of COX-2 expression has been demonstrated in human and rodent colorectal adenomas and carcinomas (20, 21) and, importantly, COX inhibition results in both regression of neoplastic polyps and prevention of their development in individuals with familial adenomatous polyposis, as well as in the murine models of this disease, the min mouse and APC knockout mouse (22–25). In humans, gastric adenocarcinomas have shown increased COX-2 expression with no alteration in levels of COX-1, the constitutive form of cyclooxygenase (24, 25). In contrast, the role of NO and iNOS in human GI neoplasia is more uncertain. Although NO can induce DNA damage (14, 15) and angiogenesis (26), it has been shown to inhibit the growth of epithelial tumor cells (26). NOS activity has been reported to be decreased in colorectal adenomas and carcinomas (27). However, a recent comprehensive study by Amb et al. (28) demonstrated increased Ca-independent iNOS activity and protein expression in both colorectal adenomas and carcinomas.

We and others have determined that iNOS expression in human GI inflammation predominates in the epithelium, and that intestinal epithelial cell lines abundantly express iNOS with cytokine stimulation (10, 29, 30). Although COX-2 expression is abundant in lamina propria mononuclear cells and fibrolasts, it is also readily induced in intestinal epithelial cells in vitro (31). Because Barrett’s epithelium is an intestinoid metaplasia that arises in response to chronic injury (acid reflux), we hypothesized that Barrett’s mucosa would express increased levels of iNOS and COX-2. We also sought to determine whether levels of these gene products would remain elevated in adenocarcinomas arising in Barrett’s esophagus. In this report, we demonstrate frequent overexpression of iNOS and COX-2 mRNA and protein in both Barrett’s metaplastic tissues and frank esophageal adenocarcinomas relative to matching gastric control mucosa.

Materials and Methods

Tissue Samples. All tissues in this study were obtained from endoscopic biopsies or surgical resections from patients at either the University of Maryland or Baltimore Veterans Affairs Medical Centers. Twenty-one paired Barrett’s esophagus and gastric body tissues were included in this study. All cases had histologically confirmed specialized columnar epithelium of Barrett’s esophagus. In addition, five paired tissues from surgical resections for adenocarcinoma arising in known Barrett’s mucosa and adjacent normal esophageal tissue were studied. Tissues were snap-frozen in liquid nitrogen for the mRNA and immunohistochemical and protein expression studies.

RT-PCR Analysis for iNOS and COX-2. Total RNA was isolated using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) and 2 μg of RNA
Fig. 1. RT-PCR analysis of iNOS and COX-2 mRNA expression in Barrett’s esophagus tissues and in associated adenocarcinomas. A, representative gels of transcripts for iNOS (top) and COX-2 (bottom). PCR product sizes in bp are iNOS, 237; COX-2, 305; and β-actin, 496. M, DNA markers; S, stomach; B, Barrett’s mucosa. B, densitometry of iNOS and COX-2 transcripts, standardized to β-actin, from control stomach and Barrett’s mucosa for each patient. *** P < 0.001 versus stomach control by paired Student’s t test. C, transcript abundance of iNOS and COX-2 in esophageal adenocarcinomas. PCR product sizes (in bp) iNOS, 237; COX-2, 756; and β-actin, 496. N, normal esophagus; T, tumor.

from each sample were reverse transcribed using SuperScript II RT (Life Technologies, Inc.) in a total reaction volume of 20 μl. Reverse-transcription product (cDNA; 1 μl) was PCR-amplified with with Ampli-Taq DNA polymerase (Perkin-Elmer/Applied Biosystems, Foster City, CA) and 0.5 pmol each of iNOS or COX-2 forward and reverse primers, with β-actin primers included in the same multiplex PCR reaction as an internal control for efficiency of RT and amount of RNA. Each PCR cycle consisted of a denaturation step (94°C, 1 min), an annealing step (60°C, 1 min), and an elongation step (72°C, 1.5 min). There were a total of 35 cycles, followed by an additional extension step (72°C, 7 min). The primer sequences (F = forward, R = reverse primers) and PCR product sizes were as follows: iNOS, 5’-TCTTGGTCAATGGGAAGCCT-GT-3’ (F) and 5’-CATTGCCAAACGTACTGGTC-3’ (R), 237 bp; two sets of COX-2 primers, 5’-TTCAAATGAGATTGTGGGAAATTGCT-3’ (F) and 5’-GATCATCTCTGCTGAGTATCT-3’ (R), 305 bp plus 5’-CAG-CACCTACGCATCAGTT-3’ (F) and 5’-TCTGGTCAATGGGAAGCCT-GT-3’ (R), 756 bp; and, finally, β-actin, 5’-CCAGAGCAAGAGAGGTATCC-3’ (F) and 5’-CTGTGGTGGTGGAAGCCTGTAG-3’ (R), 436 bp. PCR products were run on 2% agarose gels with 0.5 μg/ml ethidium bromide, and stained bands were visualized under UV light, photographed, digitized with an Agfa Arcus II scanner, and band intensity quantitated using NIH Image version 1.61.

Immunohistochemical Analysis. Sections (5 μm) cut from paraffin-embedded tissues were deparaffinized, and endogenous peroxidase activity was quenched by incubation in 0.3% H₂O₂ in methanol for 30 min. Sections were then microwaved in citrate buffer, pH 6.1, for 30 min for antigen retrieval. Nonspecific binding was blocked with 5% normal horse serum in PBS and the tissue was incubated with monoclonal antibodies to iNOS (antimouse, 1:500 dilution; Transduction Labs, Lexington, KY) or COX-2 (antirat, 1:1000 dilu-
Fig. 2. Immunohistochemical detection of iNOS (A-D) and COX-2 (E-H) in tissues from gastric body (A and E), Barrett's esophagus (B and F), dysplasia (C and G), and carcinoma (D and H). Staining was performed on formalin fixed tissues using specific monoclonal antibodies detected with peroxidase-3,3'-diaminobenzidine (brownish pigment). iNOS staining was absent in the normal gastric body (A) and squamous epithelium (B) in contrast to the staining of the metaplastic appearing glands of the Barrett's mucosa (B) which was especially intense in areas of dysplasia (C) and carcinoma (D). COX-2 protein is present at a low level in the normal gastric body (E) and strongly increased in the lamina propria of Barrett's mucosa (F) and in the epithelium of the dysplastic (G) and malignant glands (H). Tissues incubated with mouse IgG rather than the primary antibodies had absent staining (data not shown) verifying the specificity of the antibody staining.

Western Blot Analysis. Tissues were homogenized in Tris-HCl pH 7.4 buffer containing 0.5% Triton X-100 and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Protein concentration of 14,000 g for 30 min. This was detected using avidin conjugated to horseradish peroxidase. The color was then developed using 3,3'-diaminobenzidine. Mayer's hematoxylin was then added as a counterstain for 5 min.
soluble supernatants from each sample was measured by the method of Bradford (32). Then 100 μg of protein was loaded per lane, separated by SDS-PAGE under reducing conditions, and transferred onto Hybond-PVDF membranes (Amersham Inc., Arlington Heights, IL) by electroblotting. The transfer of protein and equal loading in all lanes was verified using reversible staining with Ponceau S. Membranes were blocked using 5% nonfat dry milk.

COX-2 protein was detected by incubation of blots with a monoclonal antibody to a synthetic peptide from the human COX-2 sequence from Cayman Chemical Company (Ann Arbor, MI) at a dilution of 1:1000 overnight at 4°C, followed by a sheep antimouse secondary antibody conjugated to horseradish peroxidase and determination of enhanced chemiluminescence (Amersham) using exposure to Kodak BioMax MR film.

Results

Increased iNOS and COX-2 mRNA Expression in Barrett’s Esophagus and Associated Adenocarcinoma. iNOS mRNA was readily detectable by RT-PCR in 16 of 21 (76.2%) of Barrett’s tissues, and COX-2 mRNA was similarly expressed in 17 of 21 (81%) of these tissues. As shown in Fig. 1, in all positive cases iNOS and COX-2 levels were increased above those in the gastric control tissues from the same patients, and in no case were gastric levels greater than those in Barrett’s mucosa (P = 0.0004 for iNOS and P = 0.0003 for COX-2; Fisher’s exact test). Fig. 1A demonstrates the increased iNOS and COX-2 mRNA abundance in eight representative patient tissue pairs. Gel densitometry of transcripts (standardized to constitutively expressed β-actin) illustrates the pattern of markedly increased iNOS (P = 0.0002; Student’s t test) and COX-2 (P < 0.0001) mRNA levels in the Barrett’s tissues compared with paired control stomach tissues. Two of the 21 patients with Barrett’s esophagus had dysplasia present and both were positive for both iNOS and COX-2. As shown in Fig. 1C, increased iNOS and COX-2 mRNA expression also occurred in adenocarcinomas arising in Barrett’s mucosa. Expression of both genes was very low to absent in adjacent normal esophagus but readily detectable in four of five patients for iNOS and five of five for COX-2.

We sought to compare the frequency of iNOS and COX-2 expression with that of another growth-related gene previously shown to be abundant in Barrett’s esophagus, transforming growth factor-α (33). The results were very similar: transforming growth factor-α mRNA expression was increased in 17 of 21 cases (81%) of Barrett’s metaplasias and in 5 of 5 Barrett’s-associated esophageal adenocarcinomas versus matched gastric epithelium (data not shown). In contrast, mRNA expression levels of the proinflammatory cytokines tumor necrosis factor-α, interleukin-8, and monocyte chemotactic protein-1 were not increased in Barrett’s tissues compared with gastric controls (data not shown).

Increased iNOS and COX-2 Protein Expression. To confirm the expression of iNOS and COX-2 at the protein level and to assess their cellular sources, immunostaining for iNOS and COX-2 was performed in 10 of the patients, with the following pattern consistently observed (Fig. 2). With iNOS, there was a lack of staining in the gastric body compared with intense staining of metaplastic Barrett’s glands (Fig. 2A and B). There was also no iNOS protein in adjacent squamous epithelium (Fig. 2B). For COX-2 (Fig. 2F), staining localized primarily to lamina propria mononuclear cells and myofibroblasts, with no detectable staining of the Barrett’s epithelium or adjacent squamous esophageal epithelium. Gastric mucosa showed occasional low-level COX-2 staining in the lamina propria (Fig. 2E), but of much less intensity than in the lamina propria of the Barrett’s tissue. Particularly intense epithelial iNOS staining was discovered in areas of esophageal dysplasia and adenocarcinoma (Fig. 2C and D). In dysplastic and malignant esophageal glands, COX-2 uniquely localized to the epithelium, in addition to the lamina propria mononuclear cell staining seen in gastric control and Barrett’s tissues (Fig. 2G and H).

Because we observed COX-2 protein expression in the gastric body by immunohistochemistry, we sought to confirm up-regulation of COX-2 in Barrett’s tissues by Western blot analysis. Fig. 3 demonstrates increased COX-2 protein abundance in Barrett’s metaplasias relative to paired stomach, as well as increased COX-2 protein expression in Barrett’s-associated carcinomas compared with adjacent esophagus.

Discussion

These results suggest, for the first time, that iNOS and COX-2 are involved in Barrett’s-associated esophageal neoplasia. Furthermore, our data suggest that overexpression of these proteins constitutes an early event in the esophageal neoplastic transformation process which occurs at the precancerous metaplastic stage. Overexpression of iNOS and COX-2 was demonstrated in the great majority of metaplastic and dysplastic or cancerous Barrett’s specimens, suggesting that this abnormality is involved in most cases of Barrett’s lesions at diverse stages of neoplastic development. Findings obtained at the mRNA level (by RT-PCR) were strongly corroborated by similar findings at the protein level (by immunoblotting or immunohistochemistry).

Our findings that iNOS and COX-2 were consistently up-regulated in Barrett’s tissues, in contrast to other pro-inflammatory cytokines, suggest that this is a somewhat specific effect and not simply a function of generalized inflammation, per se. Moreover, the persistence of increased iNOS and COX-2 expression in dysplasia and carcinoma implies that induction of these genes may be necessary for maintenance of the malignant phenotype as well. The hypothesis that iNOS and COX-2 are involved in human neoplastic transformation has been well supported by data obtained in tumors of other organ systems. This evidence is particularly strong in the case of COX-2, where up-regulation has been demonstrated in colorectal and gastric cancers (20, 21, 24, 25). Furthermore, pharmacological inhibition of COX-2 activity has proven effective in reducing colonic polyp formation in humans and mouse models as well as in triggering polyp regression (22, 23). Mice doubly null for the COX-2 and APC genes show a marked reduction of polyp formation relative to APC-null mice alone (21). In vitro data suggest that a key underlying mechanism for the proneoplastic effect of COX-2 is potent inhibition of epithelial apoptosis coupled with stimulatory effects on epithelial proliferation (18, 34).
Our data implicate the involvement of iNOS in GI carcinogenesis. Previous studies have demonstrated increased iNOS expression in breast and gynecological malignancies (35, 36), but only recently has iNOS induction been demonstrated in colorectal cancer (28). We and others have identified increased iNOS expression in *H. pylori* gastritis (12, 13, 37), but the present study is the first report, to our knowledge, of iNOS expression in upper GI tract dysplasia or carcinoma. NO has been shown to produce DNA damage and mutation in cell-free systems (14, 15), but specific findings have been difficult to demonstrate in cultured cells.\(^4\) NO can induce wild-type S3 protein expression, which then down-regulates iNOS transcription via a p53 binding site in the iNOS promoter (38). This suggests that regulation of iNOS expression may be one important effect of p53, and that NO may be capable of inducing p53 mutation with chronic stimulation (38, 39).

Recently, peroxynitrite, the reaction product of NO and superoxide, was shown to induce DNA strand breaks in gastric epithelial cells whereas NO alone had no effect (40). In vivo, nitrosamines, which can be formed from NO, have long been implicated in upper GI tract carcinogenesis (41).

It should be noted that both NO and prostaglandins can have important protective effects in the GI mucosa. Although NO inhibition has been associated with amelioration of intestinal inflammation in several chemically-induced models, iNOS null mice develop more severe acetic acid colitis than wild-type controls (42). Moreover, recent data from one of our laboratories (K. T. W.) indicate that selective iNOS inhibition worsens the enterocolitis of IL-10 null mice and acute colitis in rat models. Similarly, COX-2 inhibition exacerba
tes rat models of colitis (9). It is likely that some of the anti-
flammatory effects of NO and prostaglandins may ultimately prove to be deleterious in the transformation to dysplasia and cancer, how-
ever. For example, inhibition of cytokine production by NO and prostaglandins (43, 44) and leukocyte adhesion to vascular endotheli-
um by NO (45) may reduce immune response to tumors. Similarly, induction of apoptosis by NO (39) may ultimately result in increased epithelial turnover and proliferation as may the cytotoxic effects of prostaglandins in the GI epithelium.

The localization of iNOS predominantly to the epithelium in the Barrett’s tissues is consistent with published findings in human inflammatory bowel disease (10) and supports a potential role for NO in the regulation of epithelial cell growth. This role now seems more likely, in light of the particularly intense iNOS staining that we observed in dysplastic and malignant Barrett’s epithelium. Interest-
ingly, in metaplastic Barrett’s mucosa, COX-2 expression was ob-
served primarily in the lamina propria, consistent with our findings in intestinal inflammation and *H. pylori* gastritis; however, upon pro-
gression to dysplasia and carcinoma, a shift of staining to the epitheli-
um was observed, suggesting that COX-2 overexpression in these cells may constitute a relatively late event.

Our findings in the present study have important clinical implica-
tions. iNOS, COX-2, or both genes may prove valuable as biomarkers of cancer risk for metaplastic and dysplastic Barrett’s lesions, as well as prognostic indicators for frank adenocarcinomas. iNOS or COX-2 may form the basis for future novel therapeutic and/or preventive strategies using techniques to inhibit these genes, such as molecular mimicry, pharmacological antagonism, or antisense gene therapy. In addition, our findings suggest that the involvement of iNOS and COX-2 should be investigated in other tumor types and premalignant lesions such as cervical dysplasia, oral leukoplakia, gastric intestinal metaplasia, ulcerative colitis dysplasia, pancreatic hyperplasia, or early premalignant skin lesions.

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