Increased Acid Exposure in Patients With Gastroesophageal Reflux Disease Influences Cyclooxygenase-2 Gene Expression in the Squamous Epithelium of the Lower Esophagus

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Hypothesis: Although genetic changes associated with the progression to Barrett esophagus and adenocarcinoma have been identified, changes in gene expression associated with gastroesophageal reflux disease have not been reported. We examined expression levels of several genes important in carcinogenesis and compared expression levels with alterations in esophageal acid exposure.

Patients, Design, and Setting: Prospective analysis of 61 patients initially seen with reflux symptoms at a private academic hospital.

Interventions: Paired esophageal biopsy specimens of squamous epithelium 3 cm above the squamocolumnar junction. All patients had 24-hour pH monitoring performed.

Main Outcome Measures: Cyclooxygenase (COX) 1, COX-2, thymidylate synthase, human telomerase reverse transcriptase (hTERT), Bcl-2 protein, survivin protein, secreted protein acidic and rich in cysteine (SPARC), tetraspan (TSPAN), and caudal-type homeobox transcription factor 2 (CDX2) messenger RNA expression analysis was performed on snap-frozen, microdissected tissue using a quantitative reverse transcriptase–polymerase chain reaction method. Linear regression and the Pearson product moment correlation were used to relate gene expression to parameters of the 24-hour pH record.

Results: Expression levels of COX-2 correlated positively with the 24-hour pH score ($r=0.25, P=.05$). There was no correlation between the expression of other tested genes and esophageal acid exposure. There was also no significant increase in COX-2 expression in patients with esophagitis or in those who used nonsteroidal anti-inflammatory drugs.

Conclusions: To our knowledge, these data provide among the first reported correlation of genetic changes and increased esophageal acid exposure in patients with gastroesophageal reflux symptoms. The changes in gene expression occur before any metaplastic changes in the tissue are apparent, and may in the future be useful in predicting which patients will progress through a metaplasia-dysplasia carcinoma sequence.

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It is clearly established that both the intestinal metaplasia associated with Barrett esophagus and its subsequent progression to esophageal adenocarcinoma are associated with pathologic gastroesophageal reflux (GER).¹ Although both endogenous and exogenous factors likely play a role in the metaplasia to carcinoma process, GER, and its resulting alterations in esophageal mucosa, is by far the most important factor. Gastroesophageal reflux associated with the development of intestinal metaplasia is generally both severe and long-standing, and is characterized by profound alterations in the esophageal luminal environment, including frequent and wide fluctuations in luminal pH and high exposure to inflammatory components such as bile salts and proteolytic enzymes.²³ Indeed, a critical look at the ambulatory pH and bile tracings of a patient with long segment Barrett esophagus makes one wonder how the cells even survive! Further influences on cellular function are added daily in the form of dietary nitrates,⁴ alcohol, tobacco smoke,⁵ and a wide variety of drug therapies.

A large body of research over the past decade has outlined many of the genetic changes that are important in the metaplastic-dysplastic-carcinoma progression of esophageal adenocarcinoma.⁶ Implied genes, including those involved in cell signaling, cell cycle control, cell adhesion, and apoptosis are well character-
ized and their expression levels are known to be altered in esophageal carcinogenesis.7 Much less is known, and little attention has been given, to the luminal factors that cause these changes, particularly at the earliest stages.8 In an attempt to identify alterations in gene expression prior to the development of metaplasia, we compared expression levels of several known genes with the degree of acid exposure in the lower esophagus found on 24-hour esophageal pH monitoring.

**METHODS**

**SUBJECTS**

The study population consisted of 61 patients (32 men and 29 women) with symptoms of GER who underwent upper endoscopy and biopsy performed between January 9, 2002, and April 8, 2003. The mean age of these patients was 49 years (age range, 23-76 years). Approximately 38 (62%) of the 61 patients had positive 24-hour pH studies, and 11 (18%) of the 61 patients had endoscopic esophagitis.

**PROCEDURES**

All patients underwent endoscopy and had paired biopsy specimens obtained from 3 cm above the gastroesophageal junction. Biopsy specimens were snap-frozen in liquid nitrogen and stored at –80°C. Tissue samples were examined by a pathologist to ensure that all samples contained only squamous mucosa. Total RNA was isolated by using a single-step guanidinium isothiocyanate method using the Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ) according to the manufacturer’s instructions, and complementary DNAs (cDNA) were prepared from each sample as previously described.9

Quantitation of cyclooxygenase (COX) 1, COX-2, thymidylate synthase, human telomerase reverse transcriptase (hTERT), Bcl-2 protein, survivin, secreted protein acidic and rich in cysteine (SPARC), tetraspan (TSPAN), and cadal-type homeobox transcription factor 2 (CDX2) cDNA and an internal reference cDNA (β-actin) using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System TaqMan; Perkin-Elmer Applied Biosystems, Foster City, Calif) as described previously.10,11 The polymerase chain reaction (PCR) mixture consisted of 1.2 µmol/L each of the primers; 200 µmol/L probe; 0.03 µmol/L AmpliTaq Gold Polymerase; 200 µmol/L each of deoxycytidinonine 5'-triphosphate (dATP), deoxyuridin 5'-triphosphate (dCTP), deoxyguanosine 5'-triphosphate (dGTP), and thymidine 5'-triphosphate (dTTP); 3.5 mmol/L magnesium chloride; and 1 × TaqMan Buffer A, which contains a reference dye, to a final volume of 25 µl (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50°C for 10 seconds, 95°C for 10 minutes, followed by 46 cycles at 93°C for 15 seconds, and 60°C for 1 minute. Colon, liver, and lung RNAs (all Strategene, La Jolla, Calif) were used as control calibrators on each plate. Primers and probe sequences used are available from us on request. For each sample, parallel QRT-PCRs were performed for the gene of interest and the β-actin reference cDNA to normalize for input cDNA. The ratio between the values obtained provided relative gene expression levels for the gene locus investigated.

Twenty-four-hour pH monitoring was performed by positioning a glass pH electrode (Mui Scientific, Toronto, Ontario) 5 cm above the manometrically measured upper border of the lower esophageal sphincter. The electrode was connected to a digital recording device (Microdigitrapper; Synectics Medical, Irving, Tex), and the pH was continually monitored for 24 hours. The patients’ diets were limited to foods having a pH in the range of 3 to 7. The stored data were transferred to a computer and analyzed using a standard software package (Multigram; Gastrosoft, Irving, Tex) according to our standard protocol. The following parameters were measured: total percentage of time in which the pH was less than 4, percentage of time the pH was less than 4 when the subject was upright, percentage of time the pH was less than 4 when the subject was supine, total number of GER episodes longer than 5 minutes, time of the longest GER episode, and a composite score based on these parameters (DeMeester score).

**DATA ANALYSIS**

Statistical analysis was performed by converting both gene expression levels and pH monitoring results into normally distributed data. Acid exposure data were transformed by using the natural logarithm, while gene expression data were transformed using the cubic root of the natural logarithm. The associations between gene expression levels and acid exposure scores were analyzed using scatterplots; Pearson product moment correlations were calculated. A multivariate linear regression model was used to further analyze the data; variables significant at a 0.20 level were retained. Partial Pearson product moment correlations were then calculated. Transformed gene expression data were also compared in patients with and without evidence of endoscopic or histologic esophagitis and in patients who used or did not use aspirin. Histologic esophagitis was defined by the presence of intraepithelial eosinophils. These comparisons were performed using the t test.

The clinical characteristics of the study population are summarized in Table 1. At the time the biopsy specimen was obtained, only 9 of the 61 patients gave a history of using an nonsteroidal anti-inflammatory drug (NSAID). Twenty-three patients (38%) had GER symptoms but a normal pH score, 27 patients (44%) had nonerosive GER disease, and 11 patients (18%) had endoscopic evidence of erosive esophagitis.

The COX-2 levels correlated with the pH score (r = 0.25, P < .05). None of the other genes analyzed correlated significantly with acid exposure parameters (Table 2). When multivariate linear regression was used to formulate a model including both COX-2 and Bcl-2, COX-2 expression levels were significantly associated with the pH score (r = 0.26, P = 0.05), percentage of supine GER (r = 0.26,

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Table 1. Clinical Characteristics of the Study Population*

<table>
<thead>
<tr>
<th>Clinical Characteristic</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Age, mean (range), y</td>
<td>49 (23-76)</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>32/29</td>
</tr>
<tr>
<td>Percentage of positive 24-hour pH scores</td>
<td>38 (62)</td>
</tr>
<tr>
<td>Patients using nonsteroidal inflammatory drugs</td>
<td>9 (15)</td>
</tr>
<tr>
<td>Patients with histologic-confirmed esophagitis</td>
<td>21 (34)</td>
</tr>
<tr>
<td>Endoscopic esophagitis</td>
<td>11 (18)</td>
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*Data are given as the number (percentage) of patients unless otherwise indicated. Sixty-one patients were included in the study.

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In an attempt to evaluate the importance of inflammation on gene expression, particularly COX-2 expression, we evaluated expression levels of each gene in association with either endoscopic or histologic evidence of esophagitis. Changes in COX-2 expression levels were not associated with either measure of inflammation (Figure 1). There was also no significant difference in COX-2 expression in patients who did or did not use NSAIDs (Figure 2).

COMMENT

These findings suggest for the first time that expression levels of COX-2 in esophageal squamous mucosa can be modulated by intraluminal esophageal acid exposure. This finding has important clinical implications, including the current applicability of COX inhibitors for chemoprevention of esophageal adenocarcinoma, the possibility of adding additional factors (gene expression measurement) to the traditional end points of symptom control and mucosal healing in patients with GER disease, and finally that chemoprevention may be possible in the prevention of Barrett esophagus prior to its development.

The genes of interest were chosen because of their role in cellular functions important in metaplasia and neoplasia. They can be broadly grouped as anti-apoptosis (Bcl-2, survivin, COX-2), genome maintenance (TS, telomerase, and CDX2), cell/adhesion signaling (SPARC), and cellular proliferation (COX-2, TSPAN). The Bcl-2 gene inhibits apoptosis and has been found to be strongly expressed in Barrett tissue. Survivin belongs to the inhibitor of apoptosis protein family and has been found to be elevated in different

| Table 2. Correlation Between 24-Hour pH Score and Gene Expression |
|------------------------|-----------------|-----------------|
| Gene       | Pearson Correlation Coefficient | P Value |
| COX-1      | -0.17            | .19             |
| TS         | -0.071           | .59             |
| hTERT      | 0.16             | .23             |
| Bcl-2      | -0.055           | .67             |
| Survivin   | 0.11             | .47             |
| SPARC      | -0.048           | .72             |
| TSPAN      | -0.033           | .81             |
| CDX2       | 0.10             | .45             |

Abbreviations: CDX2, caudal-type homeobox transcription factor 2; COX-1, cyclooxygenase 1; hTERT, human telomerase reverse transcriptase; SPARC, secreted protein acidic and rich in cysteine; TS, thymidylate synthase; TSPAN, tetraspan.

| Table 3. Correlation Between COX-2 Gene Expression and Acid Exposure Parameters* |
|------------------------|-----------------|-----------------|
| Variable         | Partial Pearson r | P Value |
| pH score         | 0.26             | .05             |
| Time pH<4, %     | 0.24             | .07             |
| Time of upright GER, % | 0.18         | .18             |
| Time of supine GER, % | 0.26         | .04             |
| No. of GER episodes | 0.28             | .03             |
| No. of episodes lasting >5 min | 0.25         | .06             |
| Longest GER episode | 0.18             | .16             |

Abbreviations: COX-2, cyclooxygenase 2; GER, gastroesophageal reflux. *Sixty-one patients were included in the study. Boldfaced values indicate statistical significance.

P = .04), and total number of GER episodes (r = 0.28, P = .04, Table 3).

In an attempt to evaluate the importance of inflammation on gene expression, particularly COX-2 expression, we evaluated expression levels of each gene in association with either endoscopic or histologic evidence of esophagitis. Changes in COX-2 expression levels were not associated with either measure of inflammation (Figure 1). There was also no significant difference in COX-2 expression in patients who did or did not use NSAIDs (Figure 2).

Figure 1. A, Cyclooxygenase 2 (COX-2) gene expression and endoscopic esophagitis (n=61, P=.16). B, Cyclooxygenase 2 gene expression and histologic esophagitis (n=61, P=.10). The thick black line indicates the mean.

Figure 2. Cyclooxygenase 2 (COX-2) gene expression and nonsteroidal anti-inflammatory drug use (n=61, P=.10). NSAID indicates nonsteroidal anti-inflammatory drug; the thick black line, the mean.
tumor tissues. Cyclooxygenase 2 also inhibits apoptosis, but also has many other functions including important roles in angiogenesis and cellular proliferation. Thymidylate synthase represents the rate-limiting step in de novo synthesis of dTTP and is essential for DNA repair and replication. Telomerase maintains the ends of chromosomes, which naturally shorten during the cellular aging process. Telomerase activity is associated with cellular immortality. CDX2 is a homeobox gene that encodes for nuclear transcription factors and has been found to have increased expression in colorectal cancer. SPARC, or secreted protein acidic and rich in cysteine, is a bone matrix protein overexpressed in several cancers, including esophageal cancer. TSPAN is expressed in dendritic cells and plays a role in cellular proliferation.

Although COX-2 has been reported to be increased in the esophagus in response to duodenal reflux in rats, to our knowledge up-regulation of COX-2 in response to acid exposure has not been previously reported in the esophageal squamous mucosa of human subjects. Shirvani et al evaluated COX-2 protein expression (via immunohistochemistry) in esophageal biopsy specimens of Barrett epithelium, dysplasia, and adenocarcinoma. Faint expression was seen in the basal layers of esophageal squamous epithelium. Quantitative analysis revealed a 2.2-fold increase over baseline squamous tissue in nondysplastic Barrett esophagus, 4-fold in dysplastic Barrett esophagus, and 6-fold in adenocarcinoma. They went on to study the effects of acid and bile salts in 24-hour ex vivo organ culture of Barrett epithelium via immunoblotting and showed that COX-2 expression was greatest when exposed to a mixture of bile acids.

Two forms of the COX gene have been identified; both encode an enzyme important in the conversion of arachidonic acid to prostaglandin. COX-1 is a 22-kilobase (kb) gene located on chromosome 9, is constitutively expressed, and provides prostaglandin precursors under basal conditions. COX-2 is an 8-kb gene located on chromosome 1, normally expressed in brain, kidney, ovary, and uterus. COX-1 acts primarily as a housekeeping enzyme, while COX-2 expression is generally induced by inflammation, injury, and mitogens. Enhanced COX-2 expression is associated with many processes important in tumorigenesis, including apoptosis, cell adhesion, invasion and metastasis, and angiogenesis. Overexpression of the COX-2 gene has been shown to be present in both Barrett esophagus and adenocarcinoma and experimental evidence has shown that cyclooxygenase blockade may have clinical relevance.

To our knowledge only 1 other in vivo study of gene expression as it relates to acid exposure has been published. Menges et al reported DNA microarray analysis (1176 genes) in 5 patients with Barrett esophagus who were receiving and not receiving (on-and-off) proton-pump inhibitor therapy. Expression profiles were similar in proximal and distal biopsy specimens of the Barrett segment (91% concordance), but different in the same patient who was receiving and not receiving proton-pump inhibitor therapy (68% concordance). The authors concluded that the gene expression profiles varied depending on “different reflux situations.”

Our findings are significant for several reasons: first, the increased COX-2 expression occurred before any histologic changes were apparent. It is unknown why 10% to 20% of the patients with GERD develop Barrett esophagus, or how to identify these patients before metaplasia occurs. It is possible that genetic markers may predict which patients are at highest risk. Second, chemoprevention strategies using selective COX-2 gene inhibitors may help prevent progression of patients through the metaplasia-dysplasia-adenocarcinoma sequence and are under way. These data suggest that chemoprevention strategies may be better applied much earlier in the neoplastic process. However, several important questions are generated from this study, including which particular components of GER are responsible for changes in COX-2 expression and whether treatment of GER, such as fundoplication, can alter COX-2 gene levels. Further studies are ongoing to investigate these important questions.

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REFERENCES

have the potential for further investigations and might help explicate some elements of pathogenesis, to me it is still unclear that we can be certain what the implications or meaning of this finding are.

Dr Peters: Thank you. First, regarding the issue of clinical relevance of our findings, I would bring to your attention to the fact that there is a large, highly funded, multicenter, national trial evaluating COX-2 inhibitors in the prevention of cancer in patients with dysplastic Barrett esophagus. That trial got under way about 6 or 8 months ago. I believe it has enrolled about half of its cohort and is focused at the end of the spectrum of the metaplasia-dysplasia-carcinoma process. That is, it is taking patients with dysplasia and trying to prevent cancer. I suspect that that trial is going to fail because it has loaded the gun, if you will, with patients who are so far along the process it will be difficult to change the natural history. Our data suggest that maybe we ought to back that up a little bit and start thinking about clinical trials of COX-2 inhibitors in patients with nondysplastic Barrett esophagus or, perhaps even more interesting, in patients who do not yet have Barrett esophagus but have significant GER disease. I would venture to guess that there are a fair number of people in the audience who take a COX-2 inhibitor every day for reasons that are unrelated to GER. I suspect one day it will also be important in the pathophysiology of this process.

Second, you asked, what do we really know about the development of metaplasia vs. the development of neoplasia? That is a fundamental and insightful question. There is a lot more that has been studied regarding the neoplastic genetic events than the metaplastic genetic events. We really do not know much about what causes metaplasia. In this era of stem cell development, it almost certainly involves stimulation of stem cells to differentiate in a different way. We wanted to focus our investigations specifically on the early process to try to understand what is going on way before cancer develops, so we were looking at the events before Barrett esophagus.

Vic Velanovich, MD, Detroit, Mich: Although there are only a few patients taking NSAIDs, did you notice any difference with those patients who were taking NSAIDs? Did they have less esophagitis? Were they less likely to have Barrett esophagus? How was that associated with the gene expression?

The second question is related to the p-53 gene. You previously showed that this was important in the development of Barrett adenocarcinoma, and I would like to know your thoughts on this.

Dr Peters: We found no relationship with NSAID use, which may be a matter of numbers. These relationships do not jump out at you. It takes fairly large numbers of patients to find significant correlations. It may be clinically significant, but it is not dramatic. The lack of expression change with NSAID use was probably a matter of the few people who were using NSAIDs. We did not look at p-53 specifically because it is typically a later genetic event. It is reasonably well known that that does not occur in squamous mucosa; nor does it occur in most patients with quiescent, nondysplastic Barrett mucosa, and, as I mentioned earlier, we are more interested in some of the earlier events that occur than what we know is happening downstream in the dysplastic cancer sequence.

Raymond J. Joehl, MD, Hines, Ill: Are you beginning to stratify these patients and select patients who may be prone to these changes, specifically, have you looked at symptom duration, duration of proton-pump inhibitor therapy? Does proton-pump inhibitor therapy alter gene expression? What about the presence or absence of a hiatal hernia, the size of the hiatal hernia? And, lastly, looking at patients who have normal vs abnormal motility, and were any of these patients later found to have achalasia?
Dr Peters: To answer your last question, no, they were not. All of the other issues are, of course, important ones, the most intriguing of which is what happens with gene expression in the lower esophagus in patients with and without fundoplication or proton-pump inhibitor therapy. We are in the process of doing that right now, and hopefully in the next year or so we will have that answer. The other components of GER, the presence of hiatal hernia, the physiology of the reflux, the physiology of the patient, are all perhaps important, but I think we are going to need to get bigger numbers before we can answer those questions.

Elizabeth Peralta, MD, Springfield, Ill: I would suggest an alternative view. Isn’t it true that induced COX-2 is a physiologic reparative response and, therefore, you are picking up the earliest sign of reflux that is an asymptomatic state with no esophagitis but actually a lot of repair going on? Do you think that this might have some implications for where COX-2 inhibitors might reduce repair?

Dr Peters: It is interesting that cyclooxygenase was correlated with acid exposure and the other ones were not. This, of course, raises the question of whether inflammation and/or wound healing is responsible rather than the neoplastic process. As we learn more about the biology of neoplasia and the biology of wound healing, it is clear that there is a great deal of overlap between the two. They are not mutually exclusive processes. As a matter of fact, they may be matters of degree of the same process. So I do not think it really matters a whole lot. If we can inhibit this enzyme and change the natural history of this disease, it does not really matter which process or what stage of it is going on.

IN OTHER AMA JOURNALS

ARCHIVES OF INTERNAL MEDICINE

Genetic Counseling and Testing in Families With Hereditary Nonpolyposis Colorectal Cancer

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Background: Genetic testing to refine cancer risk is available. However, little is known about factors affecting the uptake of testing for the most common hereditary colon cancer, hereditary nonpolyposis colorectal cancer. This study investigated attitudes, intentions, and uptake of genetic testing within newly identified families with hereditary nonpolyposis colorectal cancer. Methods: Cohort study conducted at the National Institutes of Health between April 15, 1996, and November 20, 1999. Data were collected through questionnaires before semistructured education sessions, individual counseling sessions, and the offer of genetic testing. Results: Of the 111 eligible first-degree relatives, 51% chose to participate in education and individual counseling sessions. Participation was associated with greater numbers of first-degree relatives with cancer; no association was found between participation and personal history of cancer. Before education and individual counseling sessions, 64% of participants had heard little about genetic testing for cancers; however, most (97%) stated intentions to pursue testing. Fifty-one percent identified learning about their children’s risks as the most important reason to consider testing. Thirty-nine percent identified the potential effect on their health insurance as the most important reason to not undergo testing. Of the 111 eligible first-degree relatives, 51% chose to undergo genetic testing. Participants’ intentions to pursue genetic testing were significantly affected by concerns regarding the ability to handle the emotional aspects of testing and the psychosocial effect on family members. Conclusions: Genetic counseling and testing offers the potential to focus cancer screening resources in individuals truly at increased risk, thereby reducing mortality and morbidity. Fears of discrimination and concerns about psychological and psychosocial issues may present barriers to the use of current cancer prevention strategies, including genetic counseling and testing. (2003;163:573-582)

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Accepted for Publication: December 1, 2004.
Correspondence: Joel T. Allison, MS, Baylor Health Care System, 3500 Gaston Ave, Dallas, TX 75246 (joela @baylorhealth.edu).

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Correction
Misspelled Surname. In the article by Hamoui et al titled “Increased Acid Exposure in Patients With Gastroesophageal Reflux Disease Influences Cyclooxygenase-2 Gene Expression in the Squamous Epithelium of the Lower Esophagus,” published in the July 2004 issue of the ARCHIVES (2004;139:712-717), the surname of the sixth coauthor was misspelled in the byline on page 712. It should have read as follows: Daniel Vallböhmer, MD.