Quantitative, Tissue-Specific Analysis of Cyclooxygenase Gene Expression in the Pathogenesis of Barrett’s Adenocarcinoma

Hidekazu Kuramochi, M.D., Daniel Vallböhmer, M.D., Kazumi Uchida, M.D., Sylke Schneider, M.D., Nabid Hamoui, M.D., Daisuke Shimizu, M.D., Parakrama T. Chandrasoma, M.D, Tom R. DeMeester, Kathleen D. Danenberg, B.Sc, Peter V. Danenberg, Ph.D., Jeffrey H. Peters, M.D.

Cyclooxygenase (Cox-2) is implicated in the pathogenesis of many cancers including esophageal adenocarcinoma (EAC), whereas the role of the isoform Cox-1 in carcinogenesis is not well understood. To further elucidate the role of these factors in the development of EAC, we measured the gene expressions (mRNA levels) of Cox-2 and Cox-1 by real-time quantitative polymerase chain reaction (QRT-PCR) in tissues from normal esophagus with and without erosive gastroesophageal reflux disease (GERD), Barrett’s esophagus (BE), dysplasia, adenocarcinoma, and in healthy gastric antrum. All tissues were purified by laser capture microdissection from endoscopic or surgical resection specimens. Median Cox-2 gene expression did not differ significantly among the esophageal control groups but was elevated 5-fold in BE, 8-fold in dysplasia and 16-fold in EAC compared to normal esophageal controls with no erosive GERD. Erosive GERD tissue had slightly higher median Cox-2 expression but Cox-2 expression in normal antrum was much higher than that in a normal esophagus, close to that of dysplasia. In contrast to that of Cox-2, Cox-1 expression was significantly decreased in all neoplastic tissues compared to normal controls. Cox-1 and Cox-2 expression varied over a wide range in the neoplastic tissues but over a relatively narrow range in the esophageal normal tissues. The occurrence of substantial alterations in Cox-1 and Cox-2 expression at the BE stage indicates that these are early events in the development of EAC. These results confirm the important role of Cox-2 amplification in the pathogenesis of esophageal adenocarcinoma, but the unexpected down-regulation of Cox-1 raises questions about its role in carcinogenesis. (J GASTROINTEST SURG 2004;8:1007–1017) © 2004 The Society for Surgery of the Alimentary Tract

KEY WORDS: Cox-1, Cox-2, gene expression, Barrett’s esophagus, esophageal adenocarcinoma

Barrett’s esophagus (BE), a condition in which the normal squamous epithelium of the distal esophagus is replaced with metaplastic specialized intestinal-type epithelium as a sequel of chronic gastroesophageal reflux disease,1,2 is known to be the first stage of a multistep progression from metaplasia to dysplasia to adenocarcinoma,1,3 The ability to isolate tissue representing each of these stages by endoscopy or after surgery has made it possible to study and identify many molecular events associated with the pathogenesis of esophageal adenocarcinoma. Changes at the genomic level, such as p53 and p16 mutation, gene methylation and aneuploidy, as well as abnormal expression of growth factors, cell adhesion molecules, and cell signaling factors have been reported.4,5 These changes result in de-regulation of key cellular processes, including proliferation, apoptosis, and cellular differentiation.6,7
One of the most studied molecular events linked to gastrointestinal carcinogenesis is the up regulation of cyclooxygenase-2 (Cox-2). Cox-2 and the isoform Cox-1 are rate-limiting enzymes in the conversion of arachidonic acid to prostaglandins. Epidemiologic studies have shown that the long-term use of non-steroidal anti-inflammatory drugs, which inhibit cyclooxygenases, are associated with a reduced risk of developing cancer, especially digestive cancers.8–10 These findings have focused much interest on the role of cyclooxygenases in the pathophysiology of cancer and in the use of cox inhibitors as chemopreventive and chemotherapeutic agents.10 Data from most studies suggest that Cox-1 is constitutively expressed, whereas Cox-2 is inducible by various factors and is itself a transcriptional regulator of a number of genes involved in carcinogenesis.11

A number of previous studies have addressed the role of Cox-2 in the pathogenesis of esophageal adenocarcinoma.12–20 These studies have all reported increased Cox-2 levels at various stage of progression to EAC, but are quantitatively inconsistent as to the frequencies and extents of Cox-2 overexpression in the various tissue types. For example, reported detection frequencies of Cox-2 immunoreactivity in BE have ranged from 0% to 81%.16 Such discrepancies may have arisen in part from the use of semi-quantitative immunohistochemistry (IHC) methodology, which has standardization issues related to using different antibodies, staining and scoring protocols,12 and from isolating RNA without separation of tumor tissue from surrounding normal tissue.15,16 None of the previous studies concurrently analyzed Cox-1 expression.

The aim of the present study was to establish accurate ranges of Cox-1 and Cox-2 gene expressions in clinical tissue specimens representing metaplasia-dysplasia-EAC sequence in order to better characterize the role of these genes in each of the stages of progression to EAC. We utilized two recent technological advances—laser-capture microdissection (LCM)21 and quantitative real-time polymerase chain reaction (QRT-PCR)22 to overcome some of the previous methodologic limitations and to improve the accuracy of gene expression measurements in specific tissue types.

MATERIAL AND METHODS

Definition of Erosive Esophagitis Using Endoscopy

We used the Skinner-Belsey classification to grade the different steps of mucosal injury when performing endoscopy. The term erosive esophagitis was used when the patients had grade II (linear ulcerations) or grade III (cobblestone esophagitis) mucosal changes.

Definition of Histologic Injury/Reflux Esophagitis of the Squamous Epithelium

We defined reflux esophagitis in the squamous epithelium-lined mucosa by the presence of intraepithelial eosinophils and maturation abnormalities including basal cell hyperplasia and papillary elongation.

Definition of a Positive 24h pH-monitoring

Twenty-four-hour pH monitoring was performed by positioning a glass pH electrode (Mui Scientific, Toronto, Ontario, Canada) 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, TX), and pH was continually monitored for 24 hours. The patients’ diets were limited to foods having a pH in the range of 5 to 7. The stored data were transferred to a computer and analyzed with the use of a standard software package (Multigram, Gastrosoft, Irving, TX) according to our standard protocol. The following parameters were measured: total percent time in which the pH was less than 4, percent time the pH was less than 4 when the subject was upright, percent time the pH was less than 4 when the subject was supine, total number of reflux episodes longer than 5 minutes, time of the longest reflux episode, and a composite score based on these parameters (DeMeester score). The study was defined positive when the DeMeester score was greater than 14.72.

Tissue Samples for Real-Time PCR

Esophageal tissue samples (n = 91) were obtained from endoscopy or surgical specimens from 47 patients with Barrett’s metaplasia, dysplasia (high- or low-grade) or cancer and were immediately snap-frozen in liquid nitrogen. Through histologic evaluation, the samples were separated into 3 different groups:

1) 37 samples containing specialized intestinal metaplasia on biopsy from 22 patients (BE group)
2) 17 samples containing specialized intestinal metaplasia and either low- or high-grade dysplasia from 11 patients (dysplasia group)
3) 37 samples with confirmed adenocarcinoma of the esophagus from 28 patients (carcinoma group). Because we planned to use laser-capture microdissection (LCM) to separate tissue types, 12 patients were classified into separate
groups depending on whether their tissue samples showed metaplasia next to dysplasia or adenocarcinoma. A single specimen was analyzed from 31 patients and multiple specimens were analyzed from 16 patients. In those cases when multiple samples from one patient were analyzed, the average value was taken for the statistical analysis.

For normal tissue controls, 48 specimens of the squamous epithelium of the esophagus were taken 3 cm above the gastroesophageal junction. These tissues were taken from a total of 48 different patients and the following criteria (see definitions above) were used to classify them:

1) samples of patients with upper gastrointestinal symptoms but normal endoscopy, no histologic injury, and normal 24 hour esophageal pH monitoring (pH− group)
2) samples of patients with upper gastrointestinal symptoms and abnormal 24 hour esophageal pH monitoring but normal endoscopy and no histologic injury (pH+, non esophagitis group)
3) samples of patients with upper gastrointestinal symptoms, endoscopic evidence of erosive esophagitis, evidence of histological injury and abnormal 24 hour esophageal pH monitoring (pH+, erosive esophagitis group).

In these three control groups, one sample was analyzed for each patient and patients were classified in just one group. None of the patients of the control groups had evidence for BE, dysplasia, or carcinoma. Additionally, from 11 patients of the pH− group, a biopsy from the antrum was taken as another normal tissue control. All patients included in this study had undergone no prior foregut-operation and no prior neoadjuvant therapy, if they were cancer patients.

Tissue samples were obtained from a total of 95 patients. There were 34 women and 61 men in this group, with a median age of 56 years (21–86).

Approval for this study was obtained from the Institutional Review Board of the University of Southern California Keck School of Medicine and written informed consent was obtained from participating patients.

Microdissection

For microdissection, frozen samples were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek U.S.A., Inc., Torrance, CA) and cut into serial sections with a thickness of 20 µm. Sections were mounted on uncoated glass slides and stored at −80°C. For histologic diagnosis, three representative sections, consisting of the beginning, middle, and end of sectioning, were stained with hematoxylin and eosin (H&E) by the standard method.

Before microdissection, sections were air-dried, fixed in 70% ethanol for 3 minutes and washed in H2O for 2 min. Afterwards, they were stained with nuclear fast red (NFR, American MasterTech Scientific, Inc., Lodi, CA) for 10 seconds and again washed in H2O for 30 seconds. Samples were then dehydrated in stepwise manner with 70% ethanol, 95% ethanol and 100% ethanol for 30 seconds each, followed by incubation in xylene for 5 minutes and complete air-drying. All H&E stained sections were evaluated by a pathologist. Normal esophageal samples or normal gastric samples were dissected from the slides using a scalpel, if the histology of the samples was homogeneous and contained more than 90% tissue of interest. All other sections were selectively isolated by LCM (P.A.L.M. Microsystem, Leica, Wetzlar, Germany) according to the standard procedure. The dissected flakes of tissue were transferred to a reaction tube containing 400 µl of RNA lysis buffer.

RNA Isolation and cDNA Synthesis

RNA isolation from OCT-embedded samples was done according to a proprietary procedure of Response Genetics, Inc. (Los Angeles, CA; United States patent number 6,248,535). Afterwards, cDNA was prepared as previously described.23

Real-Time PCR Quantification of mRNA Expression

Quantification of Cox-1 and Cox-2 and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence detection System (TaqMan) Perkin-Elmer (PE) Applied Biosystems, Foster City, CA, USA), as described.26 The PCR reaction mixture consisted of 1200 nmol/of each primer, 200 nmol/probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nmol/each dATP, dCTP, dGTP, dTTP, 3.5 mM MgCl2 and 1x Taqman Buffer A containing a reference dye, to a final volume of 20 µl (all reagents from PE Applied Biosystems, Foster City, CA, USA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15s and 60°C for 1 min. The primers and probes used are listed in Table 1.

TaqMan measurements yield Ct values that are inversely proportional to the amount of cDNA in the tube; i.e., a higher Ct value means that more PCR cycles are required to reach a certain level of detection. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the genes of interest (Cox-1
Table 1. Primers and probes

<table>
<thead>
<tr>
<th>GenBank accession: NM_000962</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer cox-1</td>
</tr>
<tr>
<td>Sequences: 5'-CGCTGCTCTGGAGTCTTGGTTC-3'</td>
</tr>
<tr>
<td>Reverse primer: cox-1</td>
</tr>
<tr>
<td>Sequence: 5'-GGACTGGGTTGATAAGGTTTGGGA-3'</td>
</tr>
<tr>
<td>TaqMan probe: cox-1</td>
</tr>
<tr>
<td>Sequence: 6FAM 5'-CGAGAGATCGTCATGCGGC TGG-3' TAMRA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GenBank accession: NM_000963</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer cox-2</td>
</tr>
<tr>
<td>Sequence: 5'-GCTAAACATGATGTTTGATTCCATC-3'</td>
</tr>
<tr>
<td>Reverse primer: cox-2</td>
</tr>
<tr>
<td>Sequence: 5'-GCTGGCCCTCGCTTATGA-3'</td>
</tr>
<tr>
<td>TaqMan probe: cox-2</td>
</tr>
<tr>
<td>Sequence: 6FAM 5'-TGCCCAAGACTTCACGCACT-3' TAMRA</td>
</tr>
<tr>
<td>Gen BAM 5'-ACCACCCAGGGGCCGAGC-3' TAMRA</td>
</tr>
</tbody>
</table>

and Cox-2 in this case) and an internal reference gene (β-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

Statistical Analysis

Cox-1 and Cox-2 mRNA expression levels in samples with Barrett’s metaplasia, dysplasia, and carcinoma were compared to the pH−, pH+, non-esophagitis and pH+, erosive esophagitis groups and the antrum of the pH− group by using the Mann-Whitney U test to identify significant differences in the expressions of those groups. Also, gene expression levels among the control groups were compared to each other. Because of the larger number of tests undertaken, the Benjamini and Hochberg multiple comparison correction was performed afterward. Statistical significance was set at the 0.05 level for the corrected P-value.

RESULTS

The median values and ranges of the gene expressions of Cox-1 and Cox-2 determined by QRT-PCR (Taqman) analysis of all of the different tissue types comprising 139 tissue samples from 95 patients are listed in Table 2 and shown in Fig. 1. In Fig. 2, the results using a “patient-group-classification” instead of a “tissue-group-classification” for the Barrett’s, dysplasia and EAC group are shown. Thereby, a patient was classified in only one of those three groups, and Barrett’s and dysplasia samples of cancer patients for the analysis were excluded.

Cox-2 mRNA expression was lowest in the pH− group (median 0.11). Median Cox-2 gene expression levels were 0.12 in pH+, non esophagitis, 0.21 in pH+, erosive esophagitis, 1.01 in BE, 1.11 in dysplasia, and highest in the EAC group with 1.77. The increased Cox-2 mRNA gene expression in the BE, dysplasia, and carcinoma groups was significant compared to the pH− and pH+, non esophagitis group. The pH+, erosive esophagitis group, had a non-significantly decreased median Cox-2 expression compared to the BE and dysplasia group (p = 0.09), whereas compared to the carcinoma group a significant difference was detected. Within the control groups, there was no significant difference detectable, but a trend toward higher Cox-2 gene expression was present in the pH+, erosive esophagitis group. This group contained a number of high Cox-2 expression values not seen in either the pH− or pH+, non esophagitis group.

Cox-1 mRNA expression was highest in the pH+, non esophagitis group (median 4.39). Median gene expression levels of Cox-1 were 3.38 in the pH− group, 2.66 in the pH+ group, erosive esophagitis, 1.05 in the carcinoma, 0.82 in the BE group, and lowest in the dysplasia group with a value of 0.81. The apparent down-regulation of Cox-1 expression in the BE, dysplasia and EAC groups of the Cox-1 gene expression levels was significant compared to the three control groups, as listed in Table 3. There was no significant difference in the Cox-1 expression within the control groups.

Median Cox-2 gene expression in antrum tissues (median = 1.27) was significantly higher than in the squamous epithelium, similar to those of the BE, dysplasia and EAC groups. However, median Cox-1 expression in the antrum tissues (median = 3.58) was more similar to that of the control tissue and was significantly higher than in BE, dysplasia, and EAC tissues.

There was no statistically significant difference in median Cox-2 or Cox-1 gene expressions between BE samples of patients with only intestinal metaplasia compared to BE tissues and tissues from dysplasia or cancer patients, and also dysplasia samples of patients with just dysplasia compared to dysplastic tissue of cancer patients.

Discussion

The study reports quantitative analysis of Cox-1 and Cox-2 gene expressions in tissues of the metaplasia-dysplasia-carcinoma sequence in the esophagus. We made use of two recent technological developments, laser capture microdissection (LCM) and real-time quantitative PCR, in order to maximize the tissue-specific accuracy and reliability of the gene expression data. LCM makes it possible to effectively separate different types of tissues found in heterogeneous clinical specimens, thus providing greater
Table 2. *cox-1* and *cox-2* mRNA expression levels in the different tissue groups

<table>
<thead>
<tr>
<th>Tissue group</th>
<th>No. of samples</th>
<th>mRNA expression (median) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>cox-1</em> × 100/<em>β</em>-actin</td>
</tr>
<tr>
<td>Squamous, pH−</td>
<td>17</td>
<td>3.38 (0.6–7.1)</td>
</tr>
<tr>
<td>Squamous, pH+, NERD</td>
<td>17</td>
<td>4.39 (0.01–8.71)</td>
</tr>
<tr>
<td>Squamous, pH+, esophagitis</td>
<td>14</td>
<td>2.66 (0.01–13.48)</td>
</tr>
<tr>
<td>Barrett’s</td>
<td>22</td>
<td>0.82 (0.19–2.22)</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>11</td>
<td>0.81 (0.35–2.09)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>28</td>
<td>1.05 (0.07–4.15)</td>
</tr>
<tr>
<td>Gastric antrum (squamous, pH−)</td>
<td>11</td>
<td>3.58 (1.35–16.92)</td>
</tr>
</tbody>
</table>

NERD = nonerosive reflux disease.

assurance that any data generated are authentically representative of particular tissue types. Real-time QRT-PCR permits a more accurate and reproducible quantification of gene expression (relative mRNA level) and generates a range of numerical values, thus allowing more precise delineation of the difference in gene expressions between tissues.

Our data show progressively increased *Cox-2* expression at each successive stage of esophageal carcinogenesis. Consistent with most previous studies, we found that *Cox-2* is significantly elevated in BE compared to normal tissue, demonstrating up-regulation of *Cox-2* to be an early event in carcinogenesis. The study of Zimmerman et al., who failed to detect any *Cox-2* immunoreactivity in BE mucosa, is a lone exception. However, although qualitatively similar, our results and those reported in previous studies differ in some quantitative aspects. We found a 16-fold difference in median *Cox-2* mRNA expression between normal esophagus and EAC, but the greatest increase occurred in the transformation of normal tissue to BE (5-fold) with only a further 3-fold greater increase thereafter. Morris et al., using IHC to measure *Cox-2* protein, found the majority of the increase in *Cox-2* (5-fold) to occur in the progression of BE to high grade dysplasia. The IHC data of Cheong et al showed no difference between *Cox-2* expressions in BE and in EAC, but *Cox-2* in high grade dysplasia was higher than that of EAC. It is not yet clear whether the source of the quantitative
discrepancies among these studies is methodological or whether, in fact, gene expression levels do not always correspond to protein levels because of post-translational processing. However, the fact that the various studies using IHC reported widely different values for over-expression frequencies of Cox-2 in BE suggests considerable inherent variability in the IHC methodology.

**Fig. 1 (B).** Relative Cox-2 mRNA expression in the different tissue groups.

**Fig. 2 (A).** Relative Cox-1 mRNA in the different patient groups.
Only one of the previous studies that used IHC methodology reported detectable Cox-2 protein in normal esophagus. That we were able to detect and quantitatively measure Cox-1 and Cox-2 gene expression in all specimens illustrates the greater sensitivity of RT-PCR methodology. Consistent with the low Cox-2 protein levels suggested by the negative IHC results, we found uniformly low Cox-2 gene expressions in normal tissues with a relatively narrow range of expression values.

In addition to normal esophagus as a baseline gene expression control, we also included tissues from patients with GERD as controls for possible gene expression changes caused by acid reflux. Erosive

**Table 3.** Comparing the gene expression levels of different tissue groups with use of the Mann-Whitney U test and the corrected \( P \) value using the Benjamini and Hochberg correction

<table>
<thead>
<tr>
<th>Tissue group</th>
<th>Cox-1 ( P ) value</th>
<th>Cox-2 ( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous, pH–</td>
<td>0.21 (0.22)*</td>
<td>0.78 (0.78)</td>
</tr>
<tr>
<td>Squamous, pH–</td>
<td>0.46 (0.46)</td>
<td>0.12 (0.16)</td>
</tr>
<tr>
<td>Squamous, pH–</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
</tr>
<tr>
<td>Squamous, pH–</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
</tr>
<tr>
<td>Squamous, pH–</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
</tr>
<tr>
<td>Squamous, NERD</td>
<td>0.13 (0.14)</td>
<td>0.06 (0.09)</td>
</tr>
<tr>
<td>Squamous, pH+</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
</tr>
<tr>
<td>Squamous, NERD</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
</tr>
<tr>
<td>Squamous, pH+</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
</tr>
<tr>
<td>Squamous, NERD</td>
<td>0.003 (0.004)</td>
<td>0.06 (0.09)</td>
</tr>
<tr>
<td>Squamous, NERD</td>
<td>0.009 (0.01)</td>
<td>0.06 (0.09)</td>
</tr>
<tr>
<td>Squamous, NERD</td>
<td>0.01 (0.01)</td>
<td>0.002 (0.004)</td>
</tr>
<tr>
<td>Gastric antrum (squamous, pH–)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>0.74 (0.79)</td>
</tr>
<tr>
<td>Gastric antrum (squamous, pH–)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>0.74 (0.79)</td>
</tr>
<tr>
<td>Gastric antrum (squamous, pH–)</td>
<td>&lt;0.0001 (&lt;0.002)</td>
<td>0.31 (0.38)</td>
</tr>
</tbody>
</table>

NERD = nonerosive reflux disease.

*\( P \) value in parentheses indicates a collective value after using the Benjamini and Hochberg correction for multiple comparisons.
GERD, likely the immediate precursor condition to the development of Barrett’s esophagus, is characterized by endoscopically visible injury to the distal esophagus. All patients included in the pH+, erosive esophagitis group had an abnormal 24 hour esophageal pH monitoring and both endoscopic evidence of erosive esophagitis and histologic evidence of tissue injury. It is interesting to note that, although pH+, non esophagitis samples had the same median Cox-2 as normal epithelium, the pH+, esophagitis group had greater median Cox-2 expression and also contained a number of high Cox-2 expression values not seen in either of the two other control groups, but close to some values in the BE group. Because Cox-2 is known to be a mediator of inflammation, the elevation of Cox-2 seen in erosive esophagitis specimens may be up-regulation linked to inflammation associated with gastroesophageal reflux. In fact we have recently reported a relationship between Cox-2 expression and 24-hour pH parameters. Whether this represents a transitory up-regulation secondary to inflammation or a permanent up-regulation that sets the stage for the subsequent transformation of normal tissue to intestinal metaplasia is unknown. Investigations of Cox-2 expressions following treatment by either drugs or antireflux surgery may provide insight into the mechanisms at play.

We also isolated tissue from the antrum with the idea of possibly using it as a normal control tissue for columnar epithelium below the gastroesophageal junction. However, although Cox-1 expression in the antrum was similar to that of normal esophagus (Fig. 2), Cox-2 expression was very high, about 10-fold greater than normal esophagus and exceeding that of BE and dysplasia (Fig. 1). The antrum may represent an example of normal tissue with permanently up-regulated Cox-2, perhaps as a consequence of the extreme pH conditions in the stomach.

Although the up-regulation of Cox-2 expression at each step of the BE-dysplasia-EAC sequence is generally consistent with previously reported results, the approximately 4-fold down-regulation of Cox-1 expression that we observed in all neoplastic tissues was unexpected and, to our knowledge, has only one precedent in the literature: a study by Wiese et al, in which it was reported that COX-1 protein levels were significantly reduced in colorectal tumors compared to matched normal tissues. Most previous studies have reported that Cox-1 expression does not change during carcinogenesis, thus giving rise to the often-expressed belief that Cox-1 expression is constitutive and, in contrast to that of Cox-2, non-inducible. Although down-regulation of a gene during tumorigenesis is a prima facie indication of tumor suppressor activity, studies showing that both Cox-1 as well as Cox-2-deficient mice have reduced intestinal and skin papillomas suggest that Cox-1 also plays a positive role in tumorigenesis (at least in mice), albeit possibly by a somewhat different mechanism than Cox-2. If indeed Cox-1 does promote tumorigenesis, it is difficult to see how tumorigenic progression would thereby benefit from decreased Cox-1 expression. Thus, at this point, we can only speculate as to the significance of Cox-1 down-regulation in esophageal carcinogenesis. Possibly, because the decline of Cox-1 expression occurs almost entirely at the transition from normal tissue to BE, reduced Cox-1 expression specifically facilitates only the differentiation of esophageal epithelium to intestinal metaplasia. Among other possibilities are a) that the down-regulation of Cox-1 is not functional for tumorigenesis but Cox-1 is being co-regulated with some other gene that is the real tumor suppressor; b) the effect of Cox-1 in tumorigenesis is different in humans than in mice and it really does act as a tumor suppressor. In erosive GERD (pH+, erosive esophagitis group) tissues, the expression of Cox-1 tended in the same direction (downward) as in the BE tissues, supporting the idea that this tissue represents in some respects an intermediate stage of conversion to metaplasia.

One of our longer-term goals is to determine if it is possible to obtain unique gene expression profiles characteristic of each different stage of EAC carcinogenesis in order to test the feasibility of the concept of “molecular pathology,” i.e., histological characterization based on quantitative measurement of gene expression values, because current tests, including endoscopy and histopathologic examination, do not allow accurate diagnosis of each stage of the development of Barrett’s-associated adenocarcinoma. Because of the considerable overlap among the gene expression ranges for each tissue type, none of the intermediate stages of progression can be unequivocally identified based on just Cox-1 or Cox-2 expressions. However, as shown in Fig. 1, B the range of Cox-2 expression in normal esophagus is relatively narrow with a definite upper limit. Thus, finding high Cox-2 expression in the esophagus is likely to indicate the presence of some abnormal tissue that would call for further examination. Although the Cox-1 expression ranges in normal tissue do not appear to have a definite lower limit, most of the values in normal tissue are relatively high and thus a low Cox-1 expression might also indicate an abnormal condition, especially in conjunction with a high Cox-2 value. Additionally the ratio of Cox-2 and Cox-1 could therefore be used in the future as a more effective method to highlight/distinguish the different stages of disease, as shown in Fig. 3.
The determination of accurate ranges of Cox-1 and Cox-2 gene expressions in EAC and the other tissues will allow a more precise estimate of these gene expressions as risk factors for progression, recurrence, and survival. High Cox-2 expression is known to be an unfavorable prognostic factor in a number of cancers, including esophageal cancer.\textsuperscript{14,30–32} We have previously shown in lung cancer patients that if a data base of accurate quantitative values for gene expressions is generated and matched to clinical outcome data, it is possible to determine specific cut-off points of gene expression values that separate low- and high-risk groups.\textsuperscript{33} (In this group of lung cancer patients, those with Cox-2 gene expression $> 0.6$ had a 31% 5-year survival compared to 62% for those with lower Cox-2 gene expression).\textsuperscript{34} When clinical data for the set of patients in the present study mature and become available, it should be possible eventually to answer a number of questions, such as: 1) Are the patients with erosive GERD who have high Cox-2 more likely to progress to BE? 2) Are the patients with BE who have high Cox-2 values more likely to progress to dysplasia and cancer? 3) Can we quantitatively delineate a range of Cox-2 expression values that signify worse survival of EAC patients?

Drs. Kuramochi, Vallboehrmer, and Uchida contributed equally to this work.

REFERENCES

Discussion

Dr. G. Sarosi (Dallas, TX): That was a very interesting paper, and you are to be applauded for making a strong effort to get just the tissue you want by laser microdissection, because that has always been a problem with these tissue-based studies—you get a mix of tissue. I wanted to ask you a couple of questions about your methodology, and then I am going to push you to speculate a little bit about what the inverse relationship of Cox-1 and Cox-2 means.

How thick were your sections? And the reason that I ask is if you use very thin sections, you tend to just get the cells that you see; if you use very thick sections, you might conceivably get things that are underneath the sort of cells that you see.

The second question I wanted to ask is, in my experience with patient specimens, one of the great challenges is that individual variations between patients are sometimes as large as the individual variations between groups, and do you have any patients where you actually have the progression of Barrett’s, about whom you can actually make comments about the progression of Cox-1 and Cox-2 in an individual patient?

And finally, could you speculate about what this means for chemo prevention? We sort of constantly think of Cox-1 as a steady state and Cox-2 as sort of the important gene, and do you think that this may matter for chemo prevention? People advocate aspirin alone or specific Cox-2 inhibitors, and do you think that that is going to matter?

Dr. Vallbohmer: To the first question, the thickness of our sections was 20 micrometers. We made
some experiments and tested lower and higher thicknesses, and that was the best thickness we could take and get really enough tissue. As you know, when you are performing laser capture microdissection, it takes sometimes about one hour to just work on one sample of one patient, so you have to play with that to better it a little bit.

To the second question, the study is now in progress. We included so far over 200 patients in the study at different stages of disease, and we have patients right now in follow-up whose stage of disease might change.

And about chemo prevention, it is very suggestive. I think Cox-2 inhibitors are the right way for chemo prevention, but why is Cox-1 downregulated? There is only one paper in the literature that describes the same effect, looking at colorectal cancer, that also describes a Cox-1 downregulation, and they didn’t find the right answer. We have three suggestions why it could be.

Cox-1 could be downregulated to facilitate the difference or the development from squamous to columnar epithelium, but it could also be a tumor-suppressor gene itself or related to other tumor-suppressor genes. We are not sure. Even the Cox-2 upregulation with inflammation; is that just transient because of the reflux and it goes back after, let’s say, a Nissen? Or is it already the first step of the beginning of the development of Barrett’s metaplasia? We will look at that.

Dr. J. Svanvik (Linkoping, Sweden): Thank you for a nice presentation. Is there a risk that you could capture some inflammatory cells despite this microdissection, and that could influence the results, too?

Dr. Vallböhmer: When you laser capture, as you saw on the video clip, you can really be very sure that you just get the tissue you are looking for. You sit with a pathologist and look at an H&E slide, then mark the area that you would like to get, go to the machine, and ensure that you just get the area that you would like to analyze.

Dr. J. Fischer (Cincinnati, OH): Let me ask you a question. You are doing a preventative study with the Cox-2, and you have the Cox-1/Cox-2 system, but there have been a series of other abnormalities observed in Barrett’s esophagus at the molecular level. Where do you think that the Cox-1/Cox-2 system fits in? For example, with p53 or p27 abnormalities that have been explored in the area of Barrett’s, if you are right and this is “the big deal,” then you will see an effect, and if this is just one of a number of other molecular changes that you see in a disordered epithelium, manipulating the Cox-1/Cox-2 system won’t have any effect. So, do you have any thoughts about that?

Dr. Vallböhmer: First of all, I think the cyclooxygenase system has a very important role in the development of Barrett’s and finally of cancer. So I think Cox inhibitors will have an important role in that field. But you are right, there are other pathways that are involved in that development, like, for example, the apoptotic pathways. There are members of the apoptotic pathway that are important and perhaps there is another key or another way to block that system too. So it could be that not just Cox-1 or Cox-2 will be involved through chemotherapy—perhaps other pathways or parameters too.

Dr. R. Wong (Washington, D.C.): I have two questions. The first question is, when you did your microdissection, I think it is becoming more recognized that the lamina propria versus the epithelium may have a major role in terms of the Cox-2 stimulation, and the question is, did you actually try to microdissect the epithelium and compare that to the lamina propria, because there are many stromal cells in the lamina propria that may also produce Cox-2?

Dr. Vallböhmer: We didn’t do that comparison directly.

Dr. Wong: So you had a combination of both epithelial and lamina propria cells?

Dr. Vallböhmer: We did.

Dr. Wong: The other question was, if you take sections from a similar biopsy and compare the difference between one section versus another microdissection, how much variation do you have between these sections?

Dr. Vallböhmer: Sometimes there were variations. We took multiple biopsies from one section of a patient and analyzed if the gene expression is different. They were sometimes mainly in Barrett’s tissue variations, and we are looking now if that perhaps has something to do with the height; you know, when you take it a higher or a lower level, the gene expression is sometimes different. We have to collect more of these samples or take multiple biopsies to answer this question.

Dr. C. Pellegrini (Seattle, WA): Do you have any data on patients from whom you obtained the tissue as to whether the patients were treated with proton pump inhibitors, and if so, for how long? There is some data that has suggested in the past that pulses of acid have a different chance of stimulating Cox-2 expression, at least in isolated cells, as opposed to constant acid perfusion.

Dr. Vallböhmer: When patients come to us we perform a pH study and endoscopy. They have to stop taking proton inhibitors two weeks before. Now you can ask if there will still be an influence of the PPIs. So we have data for each patient if they took PPIs before our studies, and we are right now analyzing if this has an influence at the gene expression level. We don’t know right now, but we hopefully will have an answer.