The overexpression of cyclo-oxygenase-2 in chronic periodontitis

Background. The objective of this prospective cross-sectional study was to determine if cyclo-oxygenase-2, or COX-2, is overexpressed in the inflamed gingival tissue of patients diagnosed as having moderate-to-severe chronic periodontitis, or CP.

Methods. The authors evaluated clinical measures, crevicular fluid and gingival biopsy specimens from patients with moderate or severe CP (n = 16) and from healthy volunteers (n = 8). Patients were diagnosed as having CP based on clinical attachment loss, or CAL, of at least 5 millimeters at two sites in each quadrant and on evidence of alveolar bone loss as assessed from standard periapical or bitewing radiographs. Healthy patients exhibited no sites with CAL of more than 2 mm and no evidence of alveolar bone loss. The authors used standard techniques to perform biochemical measures.

Results. Levels of interleukin 1 beta, or IL-1β, in crevicular fluid were more than doubled in the CP group (P < .05). The amounts of COX-2 mRNA and protein also were elevated in gingival tissues from subjects with CP compared with those from healthy subjects. To gain further mechanistic insights, the authors conducted in vitro studies. The results showed that lipopolysaccharide and tumor necrosis factor alpha, or TNF-α, induced COX-2 in macrophages, while IL-1β and TNF-α induced COX-2 in oral epithelial cells.

Conclusions. Taken together, these results suggest that levels of COX-2 in gingivae reflect clinical measures of periodontitis and gingival inflammation.

Clinical Implications. The discovery of increased levels of COX-2 in inflamed gingival tissue suggests that COX-2 represents a pharmacological target for the prevention or treatment of CP.

Two isoforms of cyclo-oxygenase, or COX, catalyze the synthesis of PGs from arachidonic acid. COX-1 is a housekeeping gene that is expressed constitutively in most tissues. COX-2 is an immediate, early-response gene that is highly inducible by mitogenic and inflammatory cytokines. COX-2 is particularly upregulated in inflamed tissues as part of the normal inflammatory response to injury. The overexpression of COX-2 is associated with various chronic inflammatory diseases, including chronic periodontitis. The results of this study confirm the importance of measuring levels of COX-2 as a useful biomarker of gingival inflammation.
inflammatory stimuli. Elevated levels of COX-2 (enzyme) are detected in a variety of inflammatory conditions, including arthritis and inflammatory bowel disease. Researchers have used immunohistochemistry to demonstrate increased levels of COX-2 protein in inflamed gingiva. To further evaluate the potential significance of COX-2 in CP, we performed a prospective, cross-sectional study that compared clinical measures of CP severity with histologic and biochemical markers of gingival inflammation in patients with CP and in healthy volunteers.

**SUBJECTS, MATERIALS AND METHODS**

**Clinical examination.** Of the 24 systemically healthy adults enrolled in this study, 16 had moderate-to-severe CP and eight volunteers did not have periodontitis. A single examiner (S.E.) performed all clinical measurements. The diagnosis of moderate or severe periodontitis required clinical attachment loss, or CAL, of at least 5 mm at two sites in each quadrant, with radiographic evidence of moderate or severe alveolar bone loss. We determined bone loss by visually interpreting standard dental radiographs. We used a full-mouth series of radiographs to evaluate all cases of periodontitis.

For healthy subjects, we used bite-wing radiographs. Bite-wing radiographs with distances of less than 2 mm from the alveolar crest to the cementoenamel junction, or CEJ, were considered to be evidence of no bone loss. We considered patients to be healthy if they had no sites with CAL of greater than 2 mm, and exhibited no evidence of alveolar bone loss. In addition, we required all patients with CP to have at least one site that exhibited clinical evidence of gingival inflammation for sampling. A gingival index, or GI, was used to assess inflamed sites, and scoring was done on a scale from 0 (absence of inflammation) to 3 (severe inflammation), according to established criteria.

The examiner measured probing depth, or PD, with a manual probe. PD was defined as the distance in millimeters from the coronal-most margin of the free gingiva to the most apical penetration of the probe. We defined CAL as the distance from the CEJ to the most apical penetration of the probe.

None of the patients in our study had a history of recent periodontal treatment or long-term use of NSAIDs that, in the clinical judgment of the examiner, could have affected the outcome of the study. We recorded subjects’ smoking status. Exclusion criteria included the following: conditions that can affect infectious or inflammatory parameters such as rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, active tuberculosis, chronic osteomyelitis, HIV infection and nephrotic syndrome; active bacterial infectious diseases such as pneumonia or pyelonephritis within the previous two weeks; use of an antibiotic or selective COX-2 inhibitor within the previous six months; current use of heparin or coumadin; pregnancy; diabetes mellitus; and any condition necessitating antibiotic premedication for dental appointments.

The institutional review boards of Columbia University, New York, and the Weill Medical College of Cornell University, New York, approved the study, and all subjects gave their informed consent.

**GCF analysis.** The examiner collected a GCF specimen from the mesiobuccal and mesiopalatal sites adjacent to the biopsy site, as previously described. Teeth were isolated, supragingival plaque was gently removed and samples of GCF were collected on precut methylcellulose filter paper strips for 30 seconds. We snap froze the strips, stored them at -70°C for three months and then analyzed them. The GCF samples were then eluted and assayed in duplicate for interleukin 1 beta, or IL-1β, with the use of a commercially available enzyme-linked immunosorbant assay (Immunotech, Marseille, France). We report the results as the total amount of IL-1β in picograms per 30-second sample.

**Gingival biopsy.** The examiner collected biopsy specimens from sites with the maximum GI score for each patient. For example, if the maximum GI score for a patient was 0, then the biopsy specimen was from a 0 site; if the maximum GI score was 1, then a biopsy specimen was taken from a 1 site. All biopsy specimens from patients with periodontitis had a GI score of 2 or 3; all specimens from healthy control subjects had a GI score of 0 or 1.

Once the biopsy site was determined, the examiner administered local anesthetic and removed interdental tissue, as previously described. Two 3- to 4-mm pieces of tissue were obtained and fixed in 10 percent neutral-buffered formalin for histopathologic examination or snap frozen and stored at -70°C until analyzed. We used biopsy specimens from 18 subjects for quantitative reverse transcriptase–polymerase chain
reaction, or RT-PCR. Tissue from six (38 percent) of the 16 subjects with CP was used exclusively for immunoblot analysis. Several of the biopsy specimens were large enough for us to perform both COX-2 mRNA and immunoblot analyses.

**Reagents and cell lines.** Tumor necrosis factor alpha, or TNF-α, IL-1β and endotoxin (lipopolysaccharide, or LPS) (from Escherichia coli 026:B6) were purchased from Sigma Chemical Co. (St. Louis). We obtained the cell culture media from Life Technologies (Grand Island, N.Y.). The murine macrophagelike cell line RAW 264.7 and 1483 oral squamous carcinoma cell lines were maintained as described in previous studies.21,22

**Western blotting.** We conducted immunoblot analysis for COX-2 as described in previous studies23 using COX-2 polyclonal antiserum PG-27 (Oxford Biomedical Research, Oxford, Mich.) combined with enhanced chemiluminescent detection (ECL, Amersham, Piscataway, N.J.).

**Quantitative PCR for COX-2 in gingival tissue.** We isolated total RNA from biopsy tissue using RNeasy Mini Kits (Qiagen, Santa Clarita, Calif.). One microgram of total RNA was reverse transcribed with the use of the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer’s protocol. We carried out quantitative RT-PCR as previously described,23 using a competitor template as an internal reference and COX-2 specific primers (Sigma/Genosys, The Woodlands, Texas).

**Statistical analysis.** We used the Student t test to make comparisons between groups. A difference between groups of P < .05 was considered significant.

**RESULTS**

**Levels of IL-1β and COX-2.** Compared with healthy control subjects, patients with CP had elevated mean probing depths and GIs (Table). Moreover, using histologic evaluation, we confirmed the presence of inflammation in tissue biopsy specimens from patients with CP. Consistent with these findings, patients with CP had IL-1β levels in GCF that were more than double those seen in healthy control subjects (Table). Of the 16 patients with CP, 10 were nonsmokers, five were current smokers and one was a former smoker. Among the eight healthy control subjects, six were nonsmokers, one was a current smoker and one was a former smoker. IL-1β values were not significantly different between smokers and nonsmokers (data not shown). We measured IL-1β rather than PGE2 levels as an independent measure of inflammation, because levels of IL-1β and PGE2 in GCF are known to be highly correlated (r = .81, P < .001) in patients with CP.24

We used quantitative RT-PCR to determine the amount of COX-2 mRNA in gingival samples.

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**TABLE**

**CLINICAL MEASURES, DEMOGRAPHICS AND LEVELS OF IL-1β* IN GCF†.**

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>HEALTHY CONTROL SUBJECTS (n = 8)</th>
<th>PATIENTS WITH CHRONIC PERIODONTITIS (n = 16)</th>
<th>P VALUE‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SEM Age (years)</td>
<td>28.8 ± 0.6</td>
<td>44.9 ± 3.4</td>
<td>.0036</td>
</tr>
<tr>
<td>Mean ± SEM Probing Depth at Biopsy Site (millimeters)</td>
<td>2.13 ± 0.12</td>
<td>5.8 ± 0.57</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>Mean ± SEM Gingival Index at Biopsy Site</td>
<td>0.75 ± 0.16</td>
<td>2.29 ± 0.11</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Female Subjects (percentage)</td>
<td>37</td>
<td>43</td>
<td>–</td>
</tr>
<tr>
<td>Mean ± SEM IL-1β in GCF (picograms)¶</td>
<td>47.6 ± 17.2</td>
<td>120.6 ± 20.3</td>
<td>&lt; .05</td>
</tr>
</tbody>
</table>

* IL-1β: Interleukin-1 beta.  
† GCF: Gingival crevicular fluid.  
‡ Comparison between groups (Student t test).  
§ SEM: Standard error of the mean.  
¶ Total amount in picograms per 30-second sample.
This method relies on the coamplification in the same tube of known amounts of competitor DNA as an internal control, along with COX-2 cDNA obtained after reverse transcription from total-tissue RNA. The competitor and target use the same PCR primers, but yield amplicons with a different size (Figure 1A), allowing their separation on a gel at the end of the reaction. COX-2 mRNA was readily detectable in all tissue samples. Levels were increased by about 250 percent in the gingivae of patients with CP (Figure 1B).

Immunoblot analysis was performed on gingivae from seven subjects with CP and three healthy subjects. All of the biopsy specimens from patients with CP exhibited COX-2 protein. Moreover, patients with CP had higher levels of COX-2 protein than did those with healthy gingivae. Figure 2 shows a representative immunoblot.

**DISCUSSION**

The results of this cross-sectional study clearly demonstrate that the inflamed gingivae of patients with clinically defined CP exhibit increased levels of COX-2 mRNA and protein compared with the gingivae of healthy volunteers. These findings are consistent with previous reports, in which elevated amounts of COX-2 protein were detected immunohistochemically in inflamed gingivae.15,16

It is important to note that previous studies did not report clinical and biochemical measurements of periodontal disease severity. Thus, this report is the first to associate professionally accepted clinical measurements of disease severity with elevated amounts of COX-2 mRNA and protein in affected gingival tissue from patients with CP. We did not measure bacterial load or characterize the oral pathogens present. It is plausible that different species of periodontal pathogens will stimulate COX-2 expression to varying degrees.

A potential limitation of our study is that the patients with CP were somewhat older than the healthy volunteers (Table). Several recent studies have shown a relationship between aging and the expression of COX-2.25-27 It is intriguing to speculate, therefore, that proinflammatory stimuli could lead to greater induction of COX-2 in older subjects. Future studies will need to control for age to determine the relative importance of this factor as a determinant of COX-2 expression in CP.

There is ample evidence that COX-2 can be a rate-limiting enzyme in the PG biosynthetic pathway. As discussed above, PGE$_2$ is considered to be an important mediator of the bone and tissue destruction observed in active periodontitis.2,3 A number of nonselective COX
inhibitors have shown promise clinically for the treatment of CP. However, Kelm and colleagues believed that the risk of gastrointestinal side effects from long-term systemic administration of NSAIDs outweighed the benefit of using these agents to treat CP. Topical NSAID formulations were developed in an effort to avoid the gastrointestinal side effects associated with systemic use of nonselective NSAIDs.

Three separate randomized, double-blind, placebo-controlled phase II clinical studies evaluated the safety and efficacy of an oral rinse formulation of the potent COX-1/COX-2 inhibitor, ketorolac tromethamine. A surprising oral mucosal finding was observed in patients with CP and gingivitis in these studies. Specifically, after 12 months of treatment, the researchers observed a statistically significant dose-response relationship (P < .05) in the incidence of reported mouth ulcerations compared with the incidence when placebo rinse was used. Except for these infrequent oral soft-tissue findings, ketorolac tromethamine rinse was generally well-tolerated.

Selective COX-2 inhibitors are widely used to treat arthritis and acute postsurgical dental pain. Fewer serious gastrointestinal side effects were found with COX-2 inhibitors than with traditional NSAIDs. Likewise, it is reasonable to postulate that topically applied selective COX-2 inhibitors might not be associated with oral mucosal ulcerations. Based on the results of our current study, it will be important to determine whether selective COX-2 inhibitors administered systemically or topically are useful in the treatment of patients with CP.

Another interesting question raised by our findings is with regard to the factors regulating the expression of COX-2 in healthy versus inflamed gingivae. The mean concentration of COX-2 mRNA in healthy gingival tissue (28 femtograms/microgram of total RNA) was approximately 10-fold higher than the level (2.4 fg/µg of total RNA) we previously observed in healthy nongingival oral mucosa. It is possible that levels of COX-2 are higher in healthy gingivae than in other healthy oral tissues because of bacterial colonization or mechanical stress. It would be worthwhile to determine whether other genes involved in CP, such as matrix metalloproteinases, also are expressed at higher levels in healthy gingivae than in other oral tissues.

The continued expression of COX-2 (and possibly other genes) over time or its increase with a patient’s age could contribute to a subclinical inflammatory state leading to progressive attachment loss. This would be consistent with the known relationship between attachment loss and age, suggesting that topical anti-inflammatory
agents could play a valuable role in routine oral hygiene.

We observed a further increase in levels of COX-2 in inflamed gingivae. Bacterial toxins are key drivers of the inflammatory signal transduction cascade observed in gingivitis and periodontitis. The periodontal pathogen Porphyromonas gingivalis has been demonstrated to stimulate the expression of COX-2 and production of PGE2 in experimental models of inflammation. The potential importance of bacterial endotoxin is suggested by our in vitro findings. It is important to note that LPS induced COX-2 in macrophages.

Endotoxin also is known to stimulate the production of IL-1β and TNF-α by macrophages. It is significant, therefore, that IL-1β was a potent inducer of COX-2 in epithelial cells but not in macrophages. By contrast, COX-2 was induced by TNF-α in both macrophages and oral epithelial cell lines. Taken together, these results suggest that endotoxin initiates an inflammatory cascade whereby COX-2 is induced by both autocrine and paracrine mechanisms (Figure 4). Experiments using gingival fibroblasts suggest a role for specific tyrosine kinases in the regulation of COX-2 by cytokines in this cell type. Additional studies are needed to compare the effects of LPS produced by different periodontal pathogens and to define the signal transduction pathway by which COX-2 is induced in CP.

CONCLUSION

Both COX-2 mRNA and protein levels are significantly elevated in inflamed gingival tissue from patients with CP compared with levels in uninflamed tissue from healthy subjects. We defined the tissue from patients with CP as inflamed using clinical, biochemical and histologic criteria. These results confirm the importance of measuring levels of COX-2 as a useful biomarker of gingival inflammation. Our study also raises the
possibility that COX-2 represents a valid pharmacological target for the treatment, prevention or both of periodontitis.

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10. Kim HJ, Kim KW, Yu BP, Chung HY. The effect of age on cyclooxygenase-2 gene expression: NF-kB activation and IκB degrada-


13. Williams RC, Jeffcoat MK, Howell TH, et al. Altering the progression of human alveolar bone loss with the non-steroidal anti-inflammato-


20. Engebretson SP. “ajdannen@med.cornell.edu”. Address reprint requests to Dr. Dannenberg.


24. Cavanaugh PF Jr., Meredith MP, Buchanan W, Doyle MJ, Reddy MS, Jeffcoat MK.Coordinate production of PGE2 and IL-1β in the ginv-


27. Williams RC, Jeffcoat MK, Howell TH, et al. Altering the progression of human alveolar bone loss with the non-steroidal anti-

28. Williams RC, Jeffcoat MK, Howell TH, et al. Altering the progression of human alveolar bone loss with the non-steroidal anti-inflammato-

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