Cyclooxygenase-2 Expression in Gingival Biopsies From Periodontal Patients Is Correlated With Connective Tissue Loss

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Background: The objective of this study is to compare cyclooxygenase-2 (COX-2) protein expression in gingival biopsies from patients with chronic periodontitis (CP), patients with gingivitis (GV), and individuals with no periodontal disease (control group) and to establish its relationship with clinical variables and connective tissue loss in the lamina propria.

Methods: A cross-sectional and analytic study was conducted in 108 gingival biopsies from 52 patients with CP, 39 with GV, and 17 controls. All biopsies were processed for conventional histopathologic study, immunohistochemical determination of COX-2 protein expression, and automatic quantification of connective tissue by image analysis.

Results: The protein expression of COX-2, mainly produced by plasma cells and monocytes, was significantly related to the presence of periodontal disease, bleeding index, intensity of inflammatory infiltrate, and loss of connective tissue in the lamina propria of gingival biopsies ($P<0.01$, Spearman test). COX-2 expression was also directly correlated with attachment loss ($P<0.05$, Spearman test).

Conclusions: COX-2 protein expression is higher in patients with GV and CP than in individuals without periodontal disease and is inversely correlated with the amount of connective tissue in the lamina propria as determined by image analysis. This finding suggests that COX-2 participates in mechanisms and pathway signaling related to the destruction of fibrillar support structures of the periodontium. *J Periodontol 2012;83:1538-1545.*

KEY WORDS

Biopsy; chronic periodontitis; cyclooxygenase-2; gingivitis; image processing, computer assisted; immunohistochemistry.

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Cyclooxygenase-2 (COX-2) expression has been reported in gingival tissues of patients with periodontal disease.[1-3] COX-2 is a marker of chronic inflammatory diseases, including chronic periodontitis (CP). It converts arachidonic acid into prostaglandins, such as PGE_2, a vasoactive eicosanoid produced by activated macrophages and fibroblasts in the gingival crevicular fluid of patients with CP, and is considered the main inflammatory mediator of alveolar bone destruction.[4,5]

There are two isoforms of cyclooxygenase. COX-1 is a housekeeping gene expressed constitutively in most tissues,[6] whereas COX-2 is an inducible enzyme believed to be responsible for prostaglandin synthesis at inflammation sites. COX-2 is an immediate, early-response gene that is highly inducible by mitogenic and inflammatory stimuli.[7] COX-2 protein expression is significantly upregulated ≤20-fold in inflamed periodontal tissues.[8]

Periodontitis has been associated with COX-2 polymorphisms rs20417 and rs689466 in Taiwanese[9] and Chinese[10] populations and with rs20417 in European populations, suggesting a general genetic risk of COX-2 variants in the pathogenesis of periodontitis.[11] COX-2 expression in gingival tissues has been correlated with the severity of periodontitis and gingival inflammation, indicating a major role for this protein in CP pathogenesis.[12] The effects of COX-2 on

doi: 10.1902/jop.2012.110561

Table 1.

Demographic Data and Periodontal Indices (N = 108)

Parameter	Value
Sex	
Male	59 (54.62%)
Female	49 (45.38%)
Age (mean ± SD years)	
CP	45.3 ± 11.4
GV	45.5 ± 18.6
Controls	38.6 ± 15.2
BI (mean ± SD)	
CP≠	66.6% ± 24.1%
GV≠	12.4% ± 9.5%
Controls	0.0% ± 0.0%
Arbes index (AL in patients with CP)	
3 to 5 mm	25 (48.1%)
6 to 15 mm	27 (51.9%)

The symbol ≠ indicates a significant paired comparison (P <0.001, Mann-Whitney U test).

gingival connective tissue have been indirectly investigated through the determination of collagenases or their inhibitors,[2] but no published study has associated the amount of connective tissue with the expression of this mediator in gingival inflammatory cells.

The objective of this study is to compare COX-2 protein expression in gingival biopsies from patients with gingivitis (GV), patients with CP, and non-periodontal patients and to establish its relationship with clinical variables and the loss of connective tissue in the lamina propria.

MATERIALS AND METHODS

A cross-sectional and analytic study was conducted in adults requiring tooth extraction for orthodontic indications, caries, tooth fracture, endodontic failure, or tooth mobility (>1 mm in vestibulo-lingual direction and cause of discomfort). They were grouped according to clinical diagnosis of GV, CP, or absence of periodontal disease (controls). GV was defined by any gingival bleeding (>0%) and clinical attachment loss (AL) <3 mm. In GV patients, biopsies were obtained at sites with bleeding and AL <3 mm. CP was classified by the percentage of sites with an AL ≥3 mm.[13] In CP patients, biopsies were obtained at sites with probing depth (PD) ≥6 mm with bleeding on probing (BOP) and evidence of bone loss on periapical x-rays. All patients were treated at the School of Dentistry, University of Granada, Granada, Spain, from September 2010 to June 2011. Immediately before the tooth extraction, the same clinician (FM) used a scalpel to take a single biopsy from the mesial or distal gingival papilla of each patient after anesthetization with 2% mepivacaine.

Inclusion criteria were the same for all three groups: 1) age >18 years; 2) no periodontal treatment in the previous year; 3) no antibiotics or anti-inflammatories in the previous 2 months; and 4) the presence of ≥6 teeth. An informed consent form was signed by all participants, and the study was conducted in accordance with the Helsinki Declaration and approved by the ethics/research committee of the University of Granada.

Clinical Variables

Data were gathered on the age and sex of patients. Examiners (FM and PG-M) were trained and calibrated before conducting periodontal examinations. Intra-examiner and inter-examiner reliability was assessed by using the κ statistic, which was 0.78 and 0.82, respectively, evidencing a high degree of consistency. Gingival inflammation was assessed by applying the gingival bleeding index (BI) of Ainamo and Bay,[14] and PD and AL were determined by using a periodontal probe¶ at six sites per tooth (mesio-vestibular, vestibular, disto-vestibular, mesio-lingual, mid-lingual, and disto-lingual).

Histopathologic Study

For the histologic morphology study, buffered 10% formaldehyde-fixed, paraffin-embedded human gingival biopsies were stained with hematoxylin and eosin and periodic acid–Schiff stain. The extent of inflammatory infiltrate was also assessed by examining the lamina propria in a single papilla section. The morphologic study was done by masked examiners (FO and MA) on 4-μm sections with light microscopy. Values were determined semiquantitatively on a four-point scale (0 = absence; 1 = mild [<10% of lamina propria involved]; 2 = moderate [10% to 25%]; 3 = severe [>25%]).

For the immunohistochemical analysis, paraffin-embedded gingival sections were dewaxed, hydrated, and heat treated in 1 mM EDTA buffer for antigenic unmasking in a pretreatment module# at 95°C for 20 minutes. Sections were incubated for 30 minutes at room temperature with anti-COX-2 (clone SP21) rabbit monoclonal antibody diluted 1:50 to identify cell expression and with anti-CD3 (clone E272), anti-CD20 (clone L26), anti-CD68 (clone KP-1), and anti-CD38 (clone 38C03) prediluted monoclonal antibodies to identify T-lymphocytes, B-lymphocytes, macrophages,

¶ PCPUNC-15 periodontal probe, Hu-Friedy, Chicago, IL.
# PT module, Thermo Fisher Scientific, Fremont, CA.

Table 2.

Inflammatory Infiltrate and Connective Tissue in Gingival Biopsies (N = 108)

	Control (n = 17)		GV (n = 39)		CP (n = 52)		Comparison (*P* value)	
Variable	n	%	n	%	n	%	Global*	Paired†
Inflammatory infiltrate							<0.001	C≠GV,CP
Absent	14	82.4	0	0.0	0	0.0		
Mild	1	5.9	17	43.6	19	40.4		
Moderate	1	5.9	12	30.8	15	31.9		
Severe	1	5.9	10	25.6	13	27.7		
Missing (n)	—		—		5			
Connective tissue (%)								
9 to <25	2	14.3	5	13.5	9	19.6		
25 to <50	0	0.0	16	43.2	15	32.6		
50 to <75	8	57.1	16	43.2	17	37.0		
75 to <90	4	28.6	0	0.0	5	10.9		
Missing (n)	3		2		6			
Mean ± SD	62.2 ± 19.5		43.6 ± 17.1		48.6 ± 21.1		0.010	C≠GV,CP

C= control.
* Kruskal-Wallis test.
† The symbol ≠ indicates a significant paired comparison (*P* <0.05, Mann-Whitney *U* test).

and plasma cells, respectively. Immunohistochemical staining was performed with an automatic immunostainer** using the polymer-peroxidase-based method, followed by development with diaminobenzidine. All reagents and antibodies were purchased from the same company.†† A millimeter scale in the eyepiece of a microscope‡‡ with a ×40 objective was used to count the number of positive cells per square millimeter.

Morphometric Study

For this study, the formalin-fixed paraffin-embedded samples were sectioned at 5-μm thickness and then stained with 1% picro-sirius red F3BA§§ for image analysis quantification. Staining was enhanced by keeping tissue sections in 70% alcohol as mordent for 3 to 5 days after deparaffination. Picro-sirius red stains connective fibers deep red and cell nuclei and cytoplasmic structures light red/bright yellow.[15]

Image analysis system and processing method. The system used consisted of a black and white charge coupled camera‖‖ fitted onto a microscope¶¶ with an adapter## and connected to a compatible personal computer. To automatically quantify connective tissue on histologic sections of gingival papilla histologic sections, several image processing algorithms were combined in a single image analysis application[16] using software.***

Image acquisition and preprocessing. Ten histologic images of lamina propria from gingival papilla were digitized in black and white at 8-bit intensity resolution (256 gray levels) at a global magnification of ×200. The analog images were acquired using an immunofluorescent 550 green optical filter.

Image processing and analysis. Automatic thresholding of the areas specifically stained with sirius red is done through application of the global thresholding method defined by Masseroli et al.[16] This yields a binary image that represents the different connective tissue areas in images of sirius red-stained gingival papilla sections acquired with excess light and preprocessed as described above. All extracted areas were automatically quantified in absolute (square micrometers) and relative (percentage) values.

A specific computer program††† was used for the statistical analyses (performed by MB). The tests used are reported in the table footnotes. *P* <0.05 was considered significant.

RESULTS

The study included 108 biopsies obtained from 52 patients with CP, 39 with GV, and 17 controls; Table

** Autostainer 480, Thermo Fisher Scientific.
†† Master Diagnóstica, Granada, Spain.
‡‡ BH2 microscope, Olympus Optical, Tokyo, Japan.
§§ Gurr, BDH Chemicals, Poole, UK.
‖‖ Sony, Tokyo, Japan.
¶¶ BH2 microscope, Olympus Optical.
MTV-3 adaptor, Olympus Optical.
*** Visilog v.6.1, Noesis, Saint Aubin, France.
††† SPSS for Windows v.15.0, IBM, Chicago, IL.

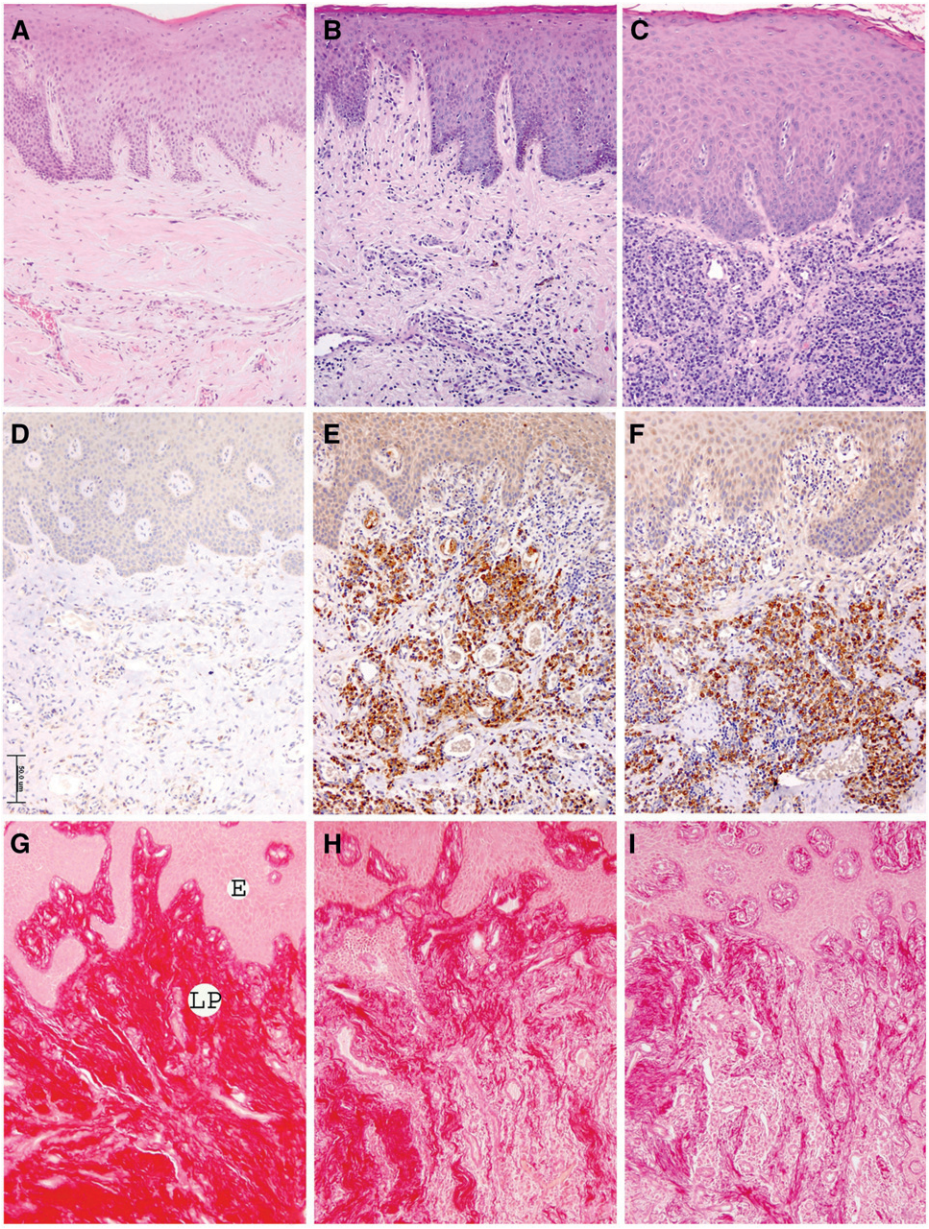

Figure 1.
Representative gingival biopsies from the three study groups. ***A)*** *Disease-free gingival biopsy from the control group. Note moderate/severe chronic inflammatory infiltrate in lamina propria in cases of GV* ***(B)*** *or CP* ***(C)*** *(hematoxylin and eosin stain, original magnification ×10). Immunohistochemical expression of COX-2 in chronic inflammatory infiltrate.* ***D)*** *Scant or absent COX-2 expression in the control group. Note increased immunostaining (brown deposit) in chronic infiltrate in gingival biopsies from patients with GV* ***(E)*** *or CP* ***(F)*** *(polymer-peroxidase-based method, original magnification ×10).* ***G)*** *Thick bundle of connective tissue (red) in lamina propria of gingival biopsy from healthy control. Note progressive connective tissue loss in GV* ***(H)*** *and CP* ***(I)****. E = epithelium; LP = lamina propria (sirius red stain, original magnification ×20).*

1 provides demographic data and periodontal indices. The BI significantly differed between CP and GV groups (P <0.001, Mann-Whitney U test). Patients with CP had a mean of 28.2 ± 16.3 sites, with AL ≥3 mm. Table 2 shows the intergroup comparisons of inflammatory infiltrate and connective tissue in the lamina propria. A similar amount of inflammatory infiltrate and loss of connective tissue was found in CP and GV biopsies. Moderate-to-severe chronic inflammatory infiltrate was observed in the lamina propria of patients with CP and GV, mainly composed of mature plasma cell (CD38 positive), macrophages (CD68 positive), and lymphocytes B (CD20 positive) and T (CD3 positive); this infiltrate was more prominent in the vicinity of the sulcular epithelium. The presence of neutrophils near the junctional epithelium was more evident in GV biopsies. Scattered inflammatory cells were observed in the lamina propria of the control group with no periodontal disease (Figs. 1A through 1C). Representative illustrations of the COX-2 immunohistochemical expression in each group are depicted in Figures 1D through 1F. COX-2 enzymatic protein was mainly expressed in plasma cells (moderate in 31.6% and severe in 37.8% of gingival biopsies) and monocytes (moderate in 40% and severe in 29.9% of biopsies), whereas COX-2 protein expression was weak in fibroblasts (weak in 66.3% of biopsies), epithelial cells (weak in 55%), and endothelial cells (weak in 54.1%). Intergroup comparisons of the immunohistochemical protein expression of COX-2 by inflammatory infiltrate cells are shown in Figure 2 and Table 3. A high percentage connective tissue in lamina propria was preserved in the control group (68.74% ± 11.04%) but was significantly lower in the GV (43.37% ± 16.88%) and CP (47.16% ± 20.93%) groups (P<0.0001, Kruskal-Wallis test). No significant difference was observed between the two periodontal groups (P = 0.356, Mann-Whitney U test). An inverse correlation was found between the

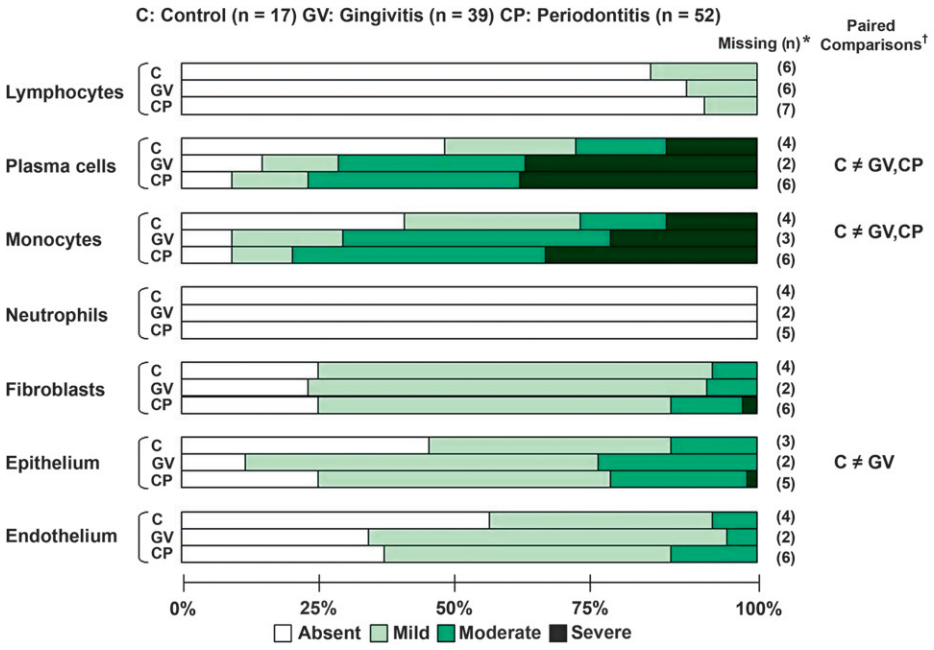

Figure 2.

COX-2. Percentage distribution of immunohistochemical expression in different inflammatory cells (N = 108). *******Excluding missing values.* †*When global comparison with Kruskal-Wallis test (data not shown) within each cell type was significant (P <0.05), paired comparisons between groups were performed with Mann-Whitney U test, using the symbol ≠ when significant (P <0.05).*

percentage connective tissue in lamina propria (also by image analysis) in CP biopsies and the percentage COX-2 immunohistochemical expression in plasma cells, monocytes, and total cells (r_s=–0.50, r_s=–0.50, and r_s = –0.43, respectively, Spearman rank correlation coefficient, P<0.05). Thick bundles of sirius red-stained collagen were observed up to the free border of papilla in gingiva samples from controls, whereas the gingiva from patients with GV and CP showed thin bundles interspersed with chronic inflammatory infiltrate or areas with no connective tissue (Figs. 1G through 1I). Table 4 shows the correlation between clinical data and immunohistochemical COX-2 expression in the patients GV and CP. Significant statistical associations were found among COX-2 protein expression in inflammatory cells, BI and AL values.

DISCUSSION

This cross-sectional and analytic study demonstrates higher COX-2 protein levels in the chronic inflammatory gingival infiltrate of patients with a clinical diagnosis of GV or CP than in the gingiva of healthy controls. These results are consistent with previous immunohistochemical findings of elevated amounts of COX-2 protein in inflamed gingiva[8] and with reports of elevated COX-2 mRNA and protein levels in gingival tissue from patients with CP.[12]

An important finding was the reduced connective tissue in the lamina propria from patients with GV and CP and its inverse correlation with COX-2 expression. This suggests that COX-2 protein indirectly participates in the destruction of periodontium support structures in humans and that pharmacological inhibition of COX-2 may have a beneficial effect by delaying the progression of CP.[17,18] Queiroz-Junior et al.[19] published evidence of the participation of COX-2 in early stages of periodontal disease in rats, reporting significantly reduced fiber attachment and alveolar bone losses and decreased leukocyte counts in gingival tissue after the systemic and local administration of non-selective and selective COX-2 inhibitors. Other experimental studies[20,21] demonstrated that the systemic administration of selective COX-2 inhibitors reduces alveolar bone loss and the secretion of collagenases and metalloproteinase-8 and decreases the secretion of proinflammatory cytokines interleukin-1β, interleukin-6, and PGE_2 by gingival fibroblasts.[22]

The initial pathogenesis of periodontitis is associated with tissue destruction produced by metalloproteinases,[23] which catalyze hydrolysis of collagen and proteoglycan; i.e., the major components of the extracellular matrix. The expression of these enzymes is regulated by the presence of PGs, which are derived from arachidonic acid via COX-2 catalysis. PGE_2 plays an important role in periodontitis by mediating inflammatory reactions in periodontal tissues and is partially responsible for the resorption of alveolar bone during its pathogenesis.[4,24] COX-2, which can be induced in bacteria-stimulated monocytes, is primarily responsible for PGE_2 production. The transcriptional control of COX-2 levels appears to act as a key regulator of tissue PGE_2 levels. PGE_2 has been found to predict AL[5] and has been associated with BOP,[11] suggesting that increased PGE_2 expression is associated with the progression of lesions. However, although PGE_2 levels rise during certain stages of disease progression, some downregulation must be necessary

Table 3.

Percentage COX-2 Immunohistochemical Expression in Inflammatory Cells (N = 108)

	Control (n = 17)		GV (n = 39)		CP (n = 52)		Comparison (*P* values)	
COX-2 expression (%)	n	(%)	n	(%)	n	(%)	Global*	Paired†
Inflammatory cells								
0	0	(0.0)	0	(0.0)	0	(0.0)		
1 to 10	12	(92.3)	11	(32.4)	15	(33.3)		
11 to 50	0	(0.0)	16	(47.1)	23	(51.1)		
51 to 100	1	(7.7)	7	(20.6)	7	(15.6)		
101 to 300	0	(0.0)	0	(0.0)	0	(0.0)		
Missing (n)	4		5		7			
Mean ± SD	8.2 ± 20.2		35.1 ± 29.8		34.5 ± 27.8		<0.001	C≠GV,CP
Plasma cells								
0	6	(54.5)	4	(11.8)	3	(6.7)		
1 to 0	4	(36.4)	6	(17.6)	8	(17.8)		
11 to 50	0	(0.0)	7	(20.6)	13	(28.9)		
51 to 100	0	(0.0)	7	(20.6)	5	(11.1)		
101 to 300	1	(9.1)	10	(29.4)	16	(35.6)		
Missing (n)	6		5		7			
Mean ± SD	21.6 ± 67.5		89.3 ± 90.0		88.7 ± 84.3		<0.001	C≠GV,CP
Monocytes								
0	5	(45.5)	2	(6.1)	3	(6.7)		
1 to 10	5	(45.5)	8	(24.2)	7	(15.6)		
11 to 50	0	(0.0)	7	(21.2)	14	(31.1)		
51 to 100	0	(0.0)	8	(24.2)	6	(13.3)		
101 to 300	1	(9.1)	8	(24.2)	15	(33.3)		
Missing (n)	6		6		7			
Mean ± SD	14.9 ± 44.8		75.0 ± 68.1		87.8 ± 83.6		<0.001	C≠GV,CP

C = Control
* Kruskal-Wallis test.
† The symbol ≠ indicates a significant paired comparison ($P < 0.05$, Mann-Whitney *U* test).

to prevent a continuing and ever-increasing loss of connective tissue.[25]

Some authors proposed the measurement of COX-2 levels as a useful biomarker of gingival inflammation.[12] Expression of COX-2 in the studied cells differed between the non-periodontal and periodontal patients but not between patients with CP and GV, indicating that COX-2 participates in the pathogenesis of both entities and cannot serve as an unequivocal biomarker of CP. It was reported that the mean concentration of COX-2 mRNA is ≈10-fold higher in healthy gingival tissue than in healthy non-gingival oral mucosas,[26] and it was suggested that COX-2 levels are higher in healthy gingiva than in other healthy oral tissues because of bacteria colonization or mechanical stress.[27] The increased COX-2 protein expression found in both patients with GV and CP in the present study (2.67 ± 2.06, 35.06 ± 29.75, and 36.26 ± 28.37 COX-2-positive cells/mm^2 in control, GV, and CP groups, respectively) may in part be explained by these factors and, especially, by the large chronic inflammatory infiltrate in the periodontal patients.

One method of quantifying the area occupied by connective tissue is by means of image analysis techniques. The image analysis-based application used in this study automatically and rapidly quantifies interstitial connective tissue in sirius red-stained sections. The image processing algorithms segment the connective tissue by automatic thresholding and morphologic filtering, producing robust, fully reproducible, accurate, objective and reliable quantifications; its use could improve the accuracy of clinico-pathologic analyses of gingival pathology in human biopsies.[16]

The authors were not able to adequately evaluate some cases with the immunohistochemical or image analysis techniques used because of the small size or inadequate depth of the biopsy. However, the percentage of missing values, which ranged from 4.1% to 17.6% for different variables, can be considered low and did not compromise the internal validity

Table 4.

Association (Spearman Rank Correlation) of COX-2 Expression and Connective Tissue (measured by image analysis) With Clinical Variables

	GV (n = 39)		CP (n = 52)					
	With BI		With BI		With Arbes Index		With AL	
COX-2 expression	n*	r_s	n	r_s	n	r_s	n	r_s
Lymphocytes	33	0.18	45	0.01	45	–0.04	45	0.11
Plasma cells	37	0.05	46	0.39†	46	–0.07	46	0.07
Monocytes	36	0.02	46	0.35†	46	–0.07	46	0.04
Fibroblasts	37	–0.23	46	–0.04	46	–0.23	46	–0.06
Epithelium	37	0.08	47	0.16	47	–0.08	47	0.10
Endothelium	37	–0.20	46	–0.01	46	–0.13	46	0.06
Global %	34	0.49†	45	0.50†	45	0.08	45	0.41†
Plasma cells (%)	34	0.38†	45	0.52†	45	0.07	45	0.40†
Monocytes (%)	33	0.43†	45	0.50†	45	0.05	45	0.40†
Connective tissue (%)	37	–0.17	46	–0.36†	46	0.01	46	–0.28

* The difference with n of each group corresponds to patients with missing values of COX-2 or connective tissue variables.
† $P < 0.05$.

of this study, and the degree of involvement of the sulcular epithelium could be evaluated in >90% of cases.

CONCLUSIONS

Our results demonstrate that COX-2 protein expression is higher in patients with GV and CP than in non-periodontal patients and is inversely correlated with the amount of connective tissue in the lamina propria, as determined by image analysis, and with the BI and AL. These findings suggest that COX-2 indirectly participates in mechanisms and pathway signaling related to the destruction of fibrillar support structures of the periodontium.

ACKNOWLEDGMENTS

This investigation was partially supported by Research Group CTS-138 and CTS-583 (Junta de Andalucia, Seville, Spain). The authors are grateful to María Dolores Rodríguez and Jorge A. Payá, laboratory technicians at the Department of Pathology of the University of Granada and Institute of Biopathology and Regenerative Medicine, for their valuable contribution to this study. The authors also thank Richard Davies for assistance with the English version. The authors report no conflicts of interest related to this study.

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Submitted September 21, 2011; accepted for publication January 20, 2012.