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Elevated levels of cyclooxygenase 1 and 2 in human cyclosporine induced gingival overgrowth

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ABSTRACT

Objective: This study was carried out to immuno-localize and estimate the levels of cyclooxygenase 1 and 2 in human gingival tissue samples from healthy individuals, chronic periodontitis patients and patients with cyclosporine induced gingival overgrowth.
Methods: Group I consisted of individuals with healthy gingiva ($n=6$), Group II – cyclosporine induced gingival overgrowth ($n=9$) and Group III – chronic periodontitis patients ($n=6$). Gingival tissue samples were collected from subjects of all the three groups. COX-1, COX-2 levels were estimated in tissue homogenates by enzyme activity assay. Immuno-localization for COX-1 and COX-2 was also done in sections of gingival tissue.
Results: The study results demonstrated a significantly higher mean levels of COX-1 and 2 in drug induced gingival overgrowth samples ($p<0.05$). COX-1 and COX-2 was localized to epithelium and connective tissue in human gingival tissue sections from cyclosporine induced gingival overgrowth.
Conclusion: Cyclooxygenase enzymes appear to be potential mediators involved in the pathogenesis of cyclosporine induced gingival overgrowth.

1. Introduction

A number of drugs used for the management of epilepsy, hypertension and renal transplant rejection have a propensity to induce excessive growth of the gingiva termed drug induced gingival overgrowth [1]. The term overgrowth was coined by Hassel [2] which refers to the proportional increase in the cellular and connective tissue components in contrast to hyperplasia/hypertrophy of the gingiva. Histopathologically, this condition is characterized by 'test-tube' like elongation of rete pegs, inflammatory cell infiltration and fibrosis of the gingival corium [3]. In addition sulphated glycosaminoglycans [4], growth factors, binding proteins [5], cytokines and chemokines [6] have been shown to be up-regulated in gingival overgrowth. Inflammation has been proposed as a predisposing/aggravating factor for the development of gingival overgrowth in several studies [7,8]. Cyclooxygenase (prostaglandin-endoperoxide synthase) pathway represents a universal pathway of inflammation with widespread tissue distribution. Cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) are the two key isoforms which are involved in the synthesis of prostaglandins (PG). COX-1 is considered a constitutive enzyme which produces prostaglandins involved in homeostasis and COX-2 is an inducible form mediating production of PG's involved in the pathologic processes [9]. The renin–angiotensin system appears to play a role in the pathogenesis of renal fibrosis. It has been demonstrated that angiotensin II, the effector peptide of the renin–angiotensin system is a pro inflammatory molecule and is known to exert its effect through two receptor subtypes, AT 1 and AT 2 [10]. Elevated levels of angiotensin II and angiotensin receptor AT1 have been demonstrated in renal fibrosis [10]. Angiotensin II influences many aspects of cell metabolism, one such being, the action it has on induction of enzymes like phospholipases, cyclooxygenases and lipoxygenases [11]. Cyclosporine A has been shown to attenuate COX-2 intra-cellularly [12]. Chiang et al. [13], in their study have demonstrated the inhibition of expression of gingival COX-2 by cyclosporine A in edentulous ridges of Sprague–Dawley rats. COX-2 has been implicated in the production of prostaglandins which are vital factors that induce bone resorption as seen in chronic periodontitis [14]. The objective of this study was to immuno-localize and measure the COX-1 and COX-2 activity in human gingival tissue samples of

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individuals with healthy gingiva, patients with chronic periodontitis and cyclosporine induced gingival overgrowth (Cs A GO).

2. Materials and methods

A total of 21 patients attending the outpatient clinic of the Department of Periodontology at Faculty of Dental Sciences, Sri Ramachandra University, Chennai, were recruited for this study after obtaining written informed consent. The study was approved by the Institutional Ethics Committee of Sri Ramachandra University. The study was conducted from February 2013 to October 2013. The study comprised of three groups: Group I – individuals with healthy gingiva ($n = 6$), Group II – patients manifesting cyclosporine induced gingival overgrowth (Cs A GO) ($n = 9$) and Group III – patients with chronic periodontitis ($n = 6$). Control samples were obtained from systemically healthy individuals having clinically healthy gingiva as determined by the absence of clinical signs of inflammation, presence of probing pocket depth $\leq$3 mm, absence of bleeding on probing, no clinical attachment loss, no mobility or furcation involvement and no radiographic evidence of bone loss. Patients with Cs A GO were chosen from renal transplant patients consuming cyclosporine A for a minimum of 6 months, manifesting gingival overgrowth as per gingival overgrowth index (Angelopoulos and Goaz, 1972) [15], no attachment loss and no radiographic evidence of bone loss. Chronic periodontitis samples were obtained from systemically healthy individuals who were diagnosed to have generalized chronic periodontitis as per Armitage et al. [16]: presence of at least 10 natural teeth, attachment loss $\geq$1 mm in greater than 30% of the sites examined, abundant local factors, radiographic evidence of bone loss. The exclusion criteria for groups I and III are as follows: history of tobacco usage in any form, individuals who had consumed antibiotics in the past 6 months, individuals who had consumed anti-inflammatory medication in the past 1 week, pregnant and lactating women, presence of any other systemic disease and individuals who had undergone previous periodontal treatment. The exclusion criteria for group II was; history of tobacco usage in any form, individuals who had consumed anti-inflammatory medication for past 1 week, individuals who were on any medication other than cyclosporine A which can influence the overgrowth status, pregnant and lactating women, presence of any other systemic disease and individuals who had undergone previous periodontal treatment.

2.1. Sample collection

A total of 42 gingival tissue samples were obtained from the 21 study participants. Two gingival tissue samples were obtained from each of the study subject, by excision of gingiva using a sterile surgical Bard Parker blade under local anaesthesia (2% Xylocaine, 1: 200,000 adrenaline) and one sample was intended for the COX activity assay while the other was used for immunohistochemistry. The control tissue samples were obtained from individuals with healthy gingiva undergoing crown lengthening procedure/extraction for orthodontic purpose. In Cs A GO group, sample collection was done from sites with overgrowth severity of grade 2 (Angelopoulos and Goaz gingival overgrowth index) [15], pocket depth >3 mm with no attachment loss, no radiographic evidence of bone loss. Chronic periodontitis tissue samples were collected prior to non surgical periodontal therapy, from sites with probing depth of $\geq$5 mm, attachment loss $\geq$1 mm. The gingival tissue samples intended for COX enzyme activity assay (COX-1 and 2) were transferred to sterile Eppendorf tubes containing phosphate buffered saline (pH = 7.4) and stored at −20 °C till processing. The gingival tissue samples intended for immuno-histochemistry were fixed in 10% neutral buffered formalin for a minimum of 24 h before processing. Paraffin embedded sections of human skin were used as a positive control for COX-1 and tissue sections of human colon carcinoma were used as a positive control for COX-2. The tissue sections of human skin and human colon carcinoma were sourced from archival samples available with Department of General Pathology, Sri Ramachandra University.

2.2. Sample processing for COX enzyme activity assay

The pre-weighed tissue samples were thawed and ground in a mortar with a pestle in phosphate buffered saline (pH = 7.4). The supernatant obtained after homogenization was used for the study after centrifugation at 10,000 *g* for 10 min at 4° C. The COX enzyme activity in all the collected samples was measured using a COX activity assay kit[1] as per the manufacturer's instructions. In brief, the COX activity assay kit measures the peroxidase activity of the COX enzymes in a colorimetric manner by monitoring the appearance of N,N,N′,N′-tetramethyl-*p*-phenylenediamine at 590 nm. Iso-enzyme specific inhibitors were used to distinguish COX-2 activity from COX-1 activity. The assay was carried out in a 96 well titre plate with the standards and samples being run in triplicate. 40 μl of the sample, 110 μl of the assay buffer, and 10 μl of heme was added in each well. In alternate wells, 10 μl of DuP-697 (to eliminate all COX-2 activity) and 10 μl of SC-560 (to eliminate all COX-1 activity) were added and the plate was gently shaken to mix the components and incubated at 25 °C for 5 min. This was followed by addition of 20 μl of colorimetric substrate. The reaction was initiated by adding 20 μl of arachidonic acid solution to all the wells and incubating at 25 °C for 5 min. Next, the COX activity readings were obtained after subjecting the titre plate to 590 nm under a spectrophotometer. The wells in which DuP-697 (COX-2 inhibitor) were added demonstrated the COX-1 activity levels and those in which SC-560 (COX-1 inhibitor) were added demonstrated the COX-2 activity levels in the tissue homogenate samples of the three study groups.

2.3. Sample processing for immuno-histochemistry

The formalin fixed samples were embedded in paraffin and two 4 micron serial sections were taken on polylysine coated slides intended for immuno-histochemistry. The sections thus obtained were de-paraffinized and rehydrated in three changes each of xylene and serial dilutions of propanol. Antigen retrieval was done using citrate buffer (pH 6.2) according to manufacturer's protocol. Peroxide and protein blocking were performed following which overnight incubation with rabbit monoclonal antibody against human cyclooxygenase 1[2] (1:200 dilution) and rabbit monoclonal antibody against cyclooxygenase 22 (1:250) [17] was performed. The slides were washed in Tris-Buffer (pH-7.6) following which secondary antibody was added and incubated according to manufacturer's protocol. DAB (3,3-diamino benzidine) was used as the chromogen and Harris haematoxylin was used to counterstain the slides. The slides were mounted on coverslips using distrene dibutyl phthalate xylene and viewed under light microscope at 40× magnification. A positive staining was identified by a golden brown colour.

2.4. Data analysis

The mean and frequency of the continuous variables were calculated. Inter-group comparison of the mean levels of COX-1 and

[1] Cayman Chemical Company, Ann Arbor, MI, USA.
[2] Abcam Cambridge, MA, USA.

Table 1
Descriptive data of study population.

Variable	Control (Group I) $n=6$	Cs A GO (Group II) $n=9$	Chronic periodontitis (Group III) $n=6$
Age (years)			
Range	18–24	15–43	37–56
Mean ± SD	21 ± 1.88	35.2 ± 11.43	44.7 ± 6.25
Gender			
Male	4	5	5
Female	2	4	1
OHI (S) mean	0.114 ± 0.21	2.91 ± 0.81	4.19 ± 0.95

COX-2 in gingival tissues was performed using one way ANOVA (Bonferroni correction) and Post hoc Tukey's test. The difference in mean was considered statistically significant at $P<0.05$. Statistical analysis was performed using SPSS software version 15.

3. Results

The descriptive variables of the study population are presented in Table 1.

3.1. COX activity assay

The mean levels of COX-1 and COX-2 in the study groups are summarized in Table 2. The group II had the highest mean COX-1 levels followed by group I and group III and the difference was statistically significant (P value = 0.000) (Table 2). Similarly, the group II had the highest mean COX-2 levels followed by group III and group I and the difference was statistically significant (P value = 0.000). Intergroup comparison of the mean COX-1 and COX-2 levels was done by Post Hoc Tukey's test. With regard, to COX-1, a statistically significant difference was obtained when comparing healthy with chronic periodontitis group ($P=0.002$) and Cs A GO with chronic periodontitis group ($P=0.000$) (Table 3). With regard to COX-2, a statistically significant difference was obtained when comparing healthy with Cs A GO group ($P=0.002$), healthy with chronic periodontitis group ($P=0.000$) and Cs A GO with chronic periodontitis group ($P=0.002$).

3.2. Immuno-localization of COX-1 and COX-2

All the gingival tissue samples, demonstrated a positive staining for COX-1 and COX-2. The intensity of staining was found to vary in the gingival tissue sections of the three groups. In the healthy gingiva group, COX-1 staining was expressed predominantly in the basal layer of the epithelium with mild staining in the fibroblasts of the connective tissue stroma. In the chronic periodontitis group, COX-1 was observed to be present in inflammatory cells in the connective tissue with no staining observed in gingival epithelium and connective tissue. In the Cs A GO group, a higher staining intensity for COX-1 was observed in the basal and suprabasal layers of the epithelium, with intense staining in fibroblasts of the stroma (Fig. 1). With reference to COX-2 staining, the healthy gingival tissue sections revealed a mild staining in few cells of the basal layer of gingival epithelium and connective tissue whereas in chronic periodontitis group intense staining was observed in the basal and suprabasal layers of the epithelium, inflammatory cell infiltrate, fibroblasts in the stroma. The staining intensity for COX-2 was observed to be minimal in Cs A GO tissue sections with positive staining being observed only in the inflammatory cells of the stroma (Fig. 2). The tissue sections of human skin (positive control for COX-1) revealed a staining in the basal layer of epithelium. The human colon carcinoma sections (positive control for COX-2) revealed a staining in the lining epithelium and cells of the stroma. Negative control sections were prepared by incubating the human skin and human colon carcinoma sections with the Universal secondary antibody to rule out a non-specific staining. No non-specific staining was observed in any of the tissue sections tested (Fig. 3).

Table 2
Comparison of mean COX-1 and COX-2 levels between three groups by One Way ANOVA.

Groups	Mean ± SD (U/ml)	Std. error	95% CI		P value
			Lower	Upper	
COX-1					
Control	3.81 ± 0.80	0.32	2.969	4.656	
Cs A GO	10.02 ± 4.51	1.50	6.555	13.493	0.000**
Chronic periodontitis	2.54 ± 0.80	0.32	1.697	3.382	
COX-2					
Healthy gingiva	1.27 ± 1.39	0.57	1.191	2.732	
Cs A GO	6.92 ± 2.30	0.77	5.149	8.687	0.000**
Chronic periodontitis	5.29 ± 0.96	0.39	4.291	6.296	

** Statistically significant, one way ANOVA.

Table 3
Intergroup comparison of mean COX-1 and COX-2 levels by Post Hoc Tukey's test.

(I) Group	(J) Group	Mean difference (U/ml) (I–J)	Std. error	95% CI		P value
				Lower	Upper	
						COX − 1
Healthy gingiva	Cs A GO	1.27	1.58	−3.294	5.840	1.000
	Chronic periodontitis	−6.21	1.45	−10.380	−2.042	0.002††
Cs A GO	Chronic periodontitis	−7.48	1.45	−11.653	−3.315	0.000††
						COX − 2
Healthy gingiva	Cs A GO	−4.02	0.93	−6.716	−1.330	0.002††
	Chronic periodontitis	−5.64	0.85	−8.106	−3.190	0.000††
Cs A GO	Chronic periodontitis	−1.62	0.85	−4.083	0.833	0.002††

†† Statistically significant, post hoc Tukey's test.

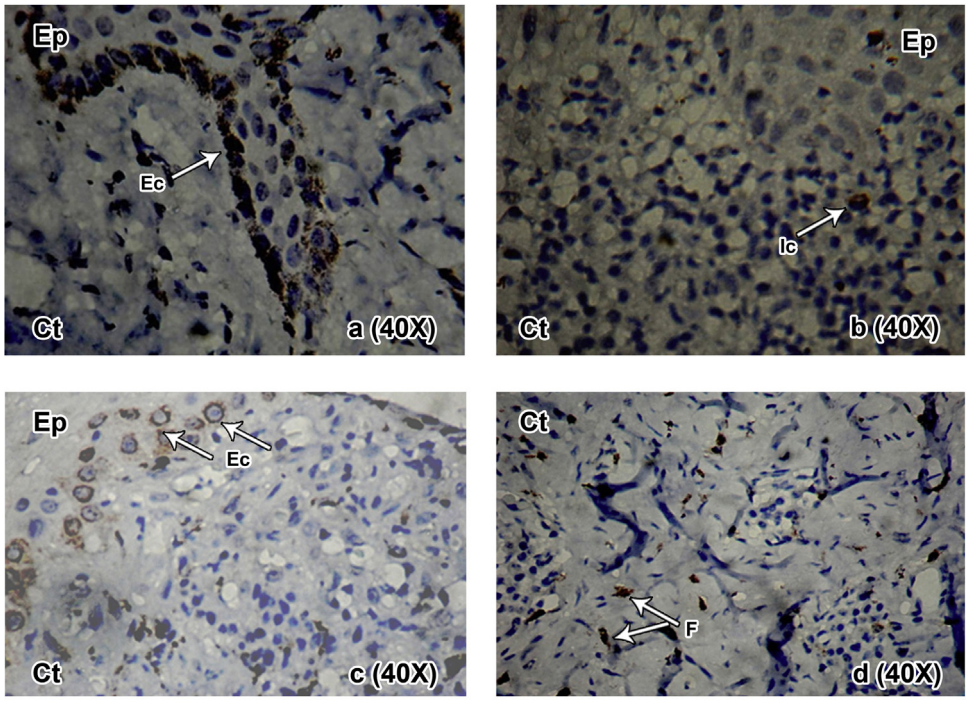

Fig. 1. Representative sections of human gingival tissue biopsies from the three groups stained with COX-1 antibody at magnification 40×. (a) Healthy gingival tissue section with positive staining of the cells (Ec) of the basal layer of the epithelium. (b) Gingival tissue section of chronic periodontitis patients with positive staining of the inflammatory cells (Ic) in the connective tissue stroma. (c) Cyclosporine induced gingival overgrowth tissue section with intense positive staining of the basal and suprabasal layers of the epithelium (Ec) and (d) fibroblasts (F) of the connective tissue.

4. Discussion

The objective of this study was to estimate the levels of and immuno-localize COX-1 and COX-2 in human gingival tissue samples of individuals with healthy gingiva, chronic periodontitis patients and patients with Cs A GO. In the present study, mean COX-1 levels in the control group was more than in the chronic periodontitis group. The interpretation of this result is that COX-1 is present constitutively in gingiva and decreases during inflammation. The mean COX-2 levels in the chronic periodontitis group were significantly higher than in the controls, thereby reinforcing its role as an enzyme induced during inflammation. Reddy et al. [18] found that the production of PGD_2 within the first 30 min of stimulation depends almost entirely on COX-1 while as the inflammation progress COX-2 becomes the major source of prostanoids. The involvement of COX-1 in the initial inflammatory response is substantiated by Langenbach et al. [19], in a model of gastric inflammation where there was a protective effect and enhanced survival rate upon indomethacin treatment in COX 1 deleted mice as compared to wild type. In our study, gingival samples were taken from periodontitis patients which is a chronic condition, this explains far more COX-2 and less COX-1 activity as compared to control. COX-1 and COX-2 levels were significantly elevated in Cs A GO group. An elevated COX-1 level in Cs A GO is a significant finding since many researchers have proved that constitutive COX-1 can be influenced by external stimuli. Feitoza et al. [20] subjected C57Bl/6 male mice to 60 min of unilateral renal pedicle occlusion with or without indomethacin, an universal COX inhibitor and concluded that blockade of COX-1 and 2 resulted in less profibrotic factors like TGF, Collagen-1, CTGF, IL-1, vimentin leading to impaired tissue fibrosis confirming the role of COX-1 and COX-2 in fibrosis. In our study, the gingival tissue samples of the Cs A GO group were

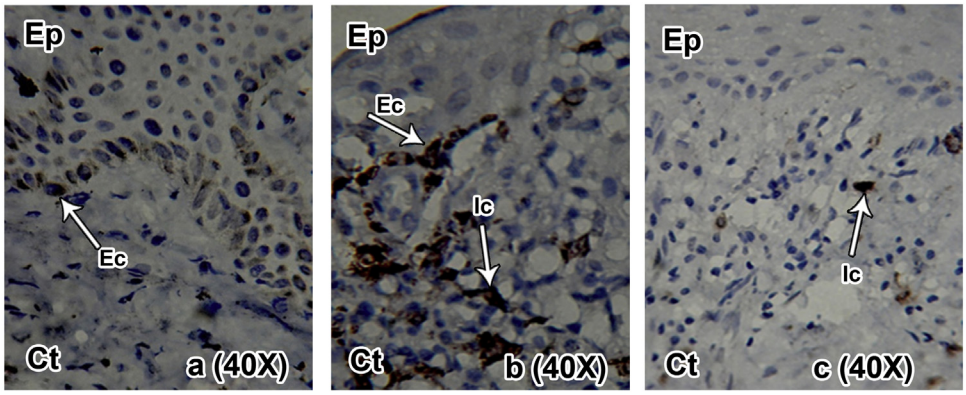

Fig. 2. Representative sections of human gingival tissue biopsies stained with COX-2 antibody. (a) Healthy gingival tissue section with positive staining of few basal cells of the epithelium (Ec). (b) Gingival tissue section of chronic periodontitis patients with intense positive staining of the basal cells of the epithelium (Ec) and inflammatory cells (Ic) in the connective tissue stroma. (c) Cyclosporine induced gingival overgrowth tissue section with positive staining of few inflammatory cells (Ic) in the connective tissue.

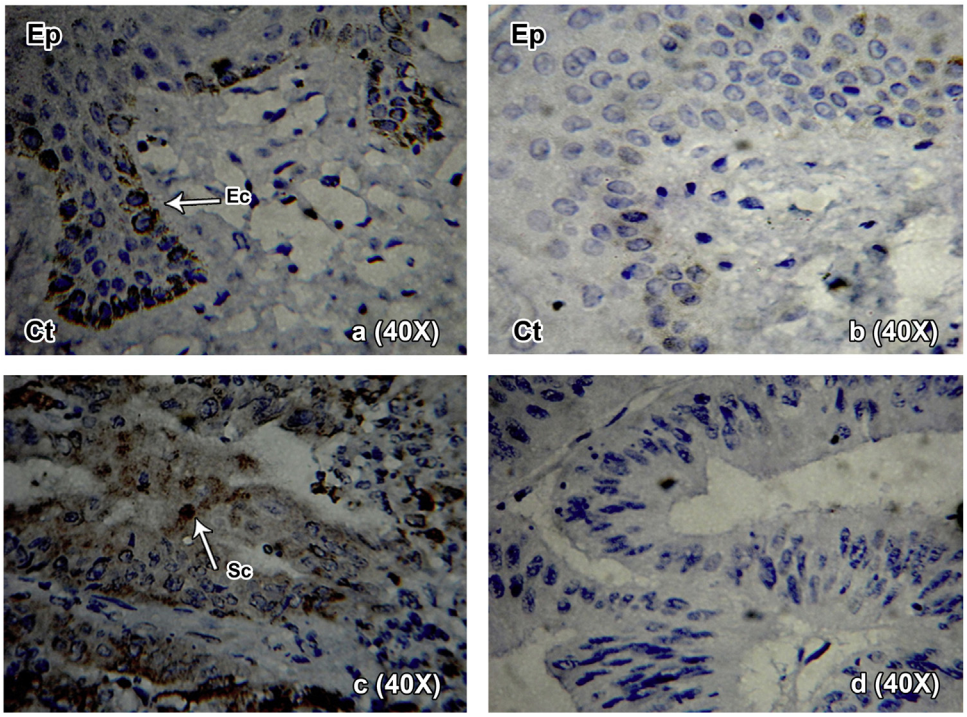

Fig. 3. Representative sections of human skin and colon carcinoma stained respectively with COX 1 and COX 2 antibody as positive control at magnification 40×. (a) Section of human skin showing positive staining with COX 1 in the basal layer of the epithelium (Ec) (b) section of human skin stained with secondary antibody only showing absence of staining (negative control). (c) Section of human colon carcinoma showing positive staining with COX 2 in the stromal cells (Sc). (d) Section of human colon carcinoma stained with secondary antibody showing absence of staining (negative control).

collected prior to non surgical therapy. Thereby, a higher level of COX-2 in the Cs A GO group is expected as compared to COX-1 to account for the inflammatory status of the gingiva. However, the findings of our study revealed a lower level of COX-2 (6.91 U/ml) as compared to COX-1 (10.02 U/ml). Since COX-2 is expected to be increased in inflammation, there are no other influences apart from cyclosporine, which can attenuate COX-2 and augment the levels of COX-1. In rat renal mesangial cells (RMC), CsA has been found to effectively inhibit PGE2 synthesis by pro inflammatory cytokines like interleukin-1 or tumour necrosis alpha (TNF alpha) (mediated by COX-2). On the contrary, the expression of constitutional cyclooxygenase isoform (COX-1), remained unaltered inspite of IL-1 or CsA [21]. Chiang et al. [13] have also demonstrated decreased expression of COX-2 in human gingival fibroblast and epithelial cell culture following cyclosporine A treatment. Similar suppression of COX-2 mRNA was evident when inducers like lipopolysaccharide or ionophore A23187 were used [21]. Höcherl et al. [22] has demonstrated inhibition of COX-2 gene expression by CsA through interference with the NFAT-phospholipase C pathway. Cyclosporine does not interfere with the expression of COX-1, since COX-1 is constitutively present in the nuclear and endoplasmic membrane and not dependent on NFAT like transcription factors. With regard to immuno-histochemistry findings, our study demonstrated the presence of COX-1 predominantly in epithelium and to a lesser extent in the corium in healthy gingival tissue. A previous study by Siegel et al. [23] demonstrated the presence of COX-1 in healthy gingiva by immunohistochemistry, the observations of which were similar to the findings of our study. In healthy rat gingival tissue sections, Nakatsuka et al. [24] have shown increased COX-1 in arteriolar endothelium and mast cells which may be the triggering step for the production of prostaglandin. With reference to COX-2 staining, the healthy gingival tissue sections revealed a mild staining in few cells of the basal layer of epithelium and connective tissue. COX-2 localization has been performed in gingival tissue sections of rats [25] and humans [26] in several previous studies and has been localized to the epithelium, inflammatory cell infiltrate, fibroblasts and endothelial cells. Additionally, in tissue sections from chronic periodontitis subjects, COX-2 was found to be intensely expressed in basal and suprabasal epithelial cells, inflammatory cells and stromal cells. In contrast, COX-1, was expressed only in few inflammatory cells of connective tissue in serial tissue sections. COX-2 being the inducible form of the enzyme, is up-regulated in the inflamed human gingival tissues [27] which explains the immunohistochemical observations in the chronic periodontitis group of our study.

To the best of our knowledge, this is the first study which has demonstrated the localization of COX-1 and COX-2 in human gingival tissue sections from cyclosporine A induced gingival overgrowth patients. A higher intensity of staining for COX-1 was observed in the epithelial, stromal cells of tissue sections of Cs A GO with minimal staining for COX-2 in the same tissue sections. Over-expression of COX-1 was observed in the epidermis of human scar tissue [28] which is also a fibrotic condition. The minimal staining observed in Cs A GO sections for COX-2 provides further evidence for the suppressive activity of cyclosporine A on COX-2 expression.

In conclusion, this study has demonstrated elevated levels of COX-1 and COX-2 in gingival tissues of cyclosporine induced gingival overgrowth patients as compared to chronic periodontitis and healthy gingival tissues. This study has also localized for the first time the COX-1 and COX-2 in cyclosporine induced gingival overgrowth.

Conflict of interest

The authors report no conflict of interest.

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