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Original Research Article

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.

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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.

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