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ORIGINAL ARTICLE

Upregulation of lysyl oxidase expression in cyclosporin A-induced gingival overgrowth

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Background/purpose: Lysyl oxidase (LOX) is involved in the initial steps of converting soluble monomers of collagen and elastin into insoluble fibers in the extracellular matrix. LOX was found to be upregulated in some fibrotic diseases. However, little is known about the correlation between LOX and cyclosporin A (CsA)-induced gingival overgrowth. The aim of this study was to compare LOX expression in normal healthy gingival tissues and CsA-induced gingival overgrowth specimens.
Materials and methods: Fifteen CsA-induced gingival overgrowth specimens and five normal gingival tissues were examined by immunohistochemistry. Three oral submucous fibrosis specimens were used as positive controls. In addition, one section from each CsA-induced gingival overgrowth specimen was stained with hematoxylin and eosin to evaluate the magnitude of inflammation at the histologic level. Differences in LOX expression between tissues with low and high levels of inflammation were subsequently analyzed using Fisher's exact test.
Results: LOX staining in gingival tissue was stronger in the CsA-induced gingival overgrowth group than in the normal gingival group ($P<0.05$). LOX staining was detected in the epithelium, connective tissue, inflammatory infiltrates, and endothelium. The LOX signal was mainly expressed in inflammatory cells (100%), followed by endothelial cells (93.3%), fibroblasts (80%) and epithelial cells (60%). In addition, LOX expression was significantly higher in CsA-induced gingival overgrowth specimens with higher levels of inflammatory infiltrates ($P=0.017$).
Conclusion: LOX expression was significantly upregulated in CsA-induced gingival overgrowth specimens. In addition, the expression of LOX increased with the grade of inflammation in CsA-induced gingival overgrowth.

Introduction

Gingival overgrowth is a common side effect of the chronic use of the immunosuppressive drug, cyclosporin A (CsA). The incidence of CsA-induced gingival overgrowth varies from 8% to 85% among studies, depending on the criteria used.[1–3] CsA-induced gingival overgrowth is characterized by thickening of the gingival epithelium as well as a marked increase in the extracellular matrix (ECM)

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of the gingival connective tissue.[4] Etiologic factors causing and underlying gingival overgrowth have been reviewed, and it was determined that local, systemic and genetic factors may contribute to its development and progression.[5,6] Recently, our studies showed that upregulation of plasminogen activator inhibitor-1[7] and cystatin C[8] may contribute to ECM accumulation in CsA-induced gingival overgrowth. However, the exact mechanism whereby CsA induces gingival overgrowth remains largely obscure.

Lysyl oxidase (LOX) is a secreted, copper-dependent oxidase that deaminates the 3-amino group of lysines in collagen and elastin. The resulting aldehydes condense to form cross-linkages between collagen and elastin monomers.[9,10] LOX catalyzes the oxidative deamination of lysine residues in elastin and collagens as an initial step in their extracellular assembly into insoluble fibers.[11] This has the effect of converting soluble monomers of collagen and elastin into insoluble fibers in the ECM.[12] Upregulation of LOX expression and increased LOX activity have been seen in a variety of fibrotic diseases such as liver fibrosis,[9] scleroderma,[13] renal fibrosis,[14] and oral submucous fibrosis.[15,16]

Previously, LOX protein expression was detected in phenytoin-induced gingival overgrowth tissues.[17] The findings suggest that LOX may play an important role in the pathogenesis of CsA-induced gingival overgrowth. On the basis of these observations, the present work was undertaken to identify the *in situ* localization of LOX expression in normal gingival tissues and CsA-induced gingival overgrowth specimens.

Materials and methods

Tissue collection

Normal gingival tissue samples were obtained from five healthy individuals undergoing routine surgical crown lengthening with little, if any, evidence of inflammation and who were not receiving any systemic medication. Fifteen hyperplasic gingival biopsy specimens were obtained from 10 renal transplant patients receiving CsA therapy. These patients had been taking CsA for more than 1 year, and the dose had been adjusted to maintain stable serum levels of about 200 ng/mL. No sign of graft rejection was detected in these renal transplant patients. The samples were obtained during surgical removal of diseased gingival tissue as part of their routine clinical management, which also included intensive plaque control. Institutional Review Board permission at Chung Shan Medical University Hospital was obtained for the use of discarded human tissue.

Immunohistochemistry

The surgically removed gingival tissues were fixed with 10% buffered formalin overnight, and the specimens were then dehydrated in an ascending series of graded alcohol and embedded in paraffin. Five-micrometer sections were stained with the monoclonal anti-LOX antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 dilution) using a standard avidin-biotin-peroxidase complex method.[7,8] Diaminobenzidine (Zymed, South San Francisco, CA, USA) was then used as the substrate for localizing the antibody binding. Three biopsy specimens of oral submucous fibrosis were used as positive controls.[16] Negative controls included serial sections from which either the primary or secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany), and examined by light microscopy.

One section from each CsA-induced gingival overgrowth specimen was stained with hematoxylin and eosin to evaluate the magnitude of inflammation at the histologic level. Each specimen was graded at 200× magnification as low (<50% inflammatory cells per field) or high grade (>50% inflammatory cells per field). Grading of each specimen was based on the average inflammatory condition in three consecutive microscopic fields, beginning from the epithelial-connective tissue border and proceeding gradually deeper into the lamina propria.

After immunohistochemical processing for LOX expression, sections graded as low were represented by <50% of positively stained cells, while sections graded as high exhibited >50% positively stained cells on three sections per tissue at 400× magnification. The same fields were used to grade inflammation and LOX staining.

Statistical analysis

Three replicates of each experiment were performed for each test. All assays were repeated three times to ensure reproducibility. Fisher's exact test was used to test for differences in LOX between normal healthy gingival tissues and CsA-induced gingival overgrowth specimens.

Results

Fig. 1 shows gingival tissue obtained from the normal gingival group with faint LOX expression, which was almost totally limited to the epithelium and endothelium. In the CsA-induced gingival overgrowth group, intensive LOX expression was mainly observed in the cytoplasm of fibroblasts, epithelial cells and

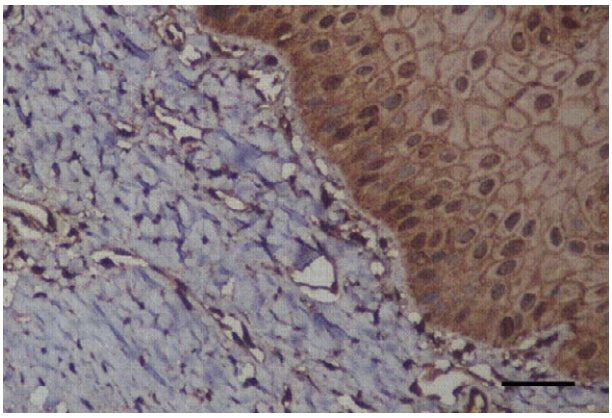

Fig. 1 Very faint immunoreactivity of lysyl oxidase was observed in normal human gingival tissues, and it was almost totally limited to the epithelium and endothelium (original magnification ×400). The bar represents 20 μm.

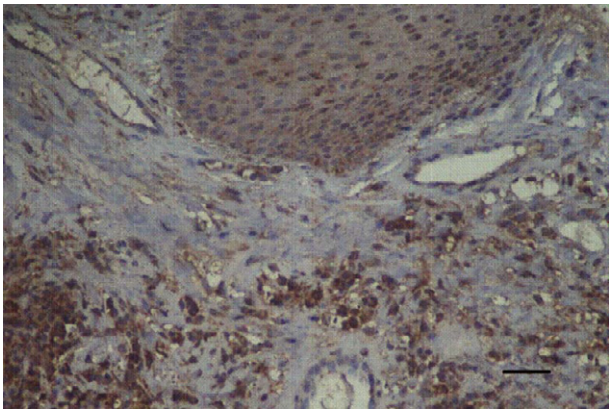

Fig. 2 Strong immunostaining for lysyl oxidase was noted in cyclosporin A-induced gingival overgrowth specimens. Lysyl oxidase was evident as an intense reddish-brown color in the cytoplasm of fibroblasts, epithelial cells, endothelial cells and inflammatory cells (original magnification ×200). The bar represents 20 μm.

inflammatory cells (Fig. 2). LOX staining of gingival tissue was stronger in the CsA-induced gingival overgrowth group than in the normal gingival group ($P<0.05$). The rank order of cells positively stained for LOX was found to be as follows: inflammatory cells (100%) > endothelial cells (93.3%) > fibroblasts (80%) > epithelial cells (60%).

LOX expression levels in CsA-induced gingival overgrowth specimens with either low or high levels of inflammation are given in Table 1. Differences in LOX expression between tissues with low and high levels of inflammation were subsequently analyzed using Fisher's exact test. Significantly greater LOX expression was noted in CsA-induced gingival overgrowth tissues with high levels of inflammation ($P=0.017$).

Table 1. Results of lysyl oxidase (LOX) expression and the grade of inflammation in cyclosporin A (CsA)-induced gingival overgrowth tissues

	Level of inflammation	
	High	Low
LOX expression*		
Low	1	4
High	8	2

*Significantly greater LOX expression was noted in CsA-induced gingival overgrowth tissues with high levels of inflammation compared with tissues with low levels of inflammatory cell infiltrates by Fisher's exact test ($P=0.017$).

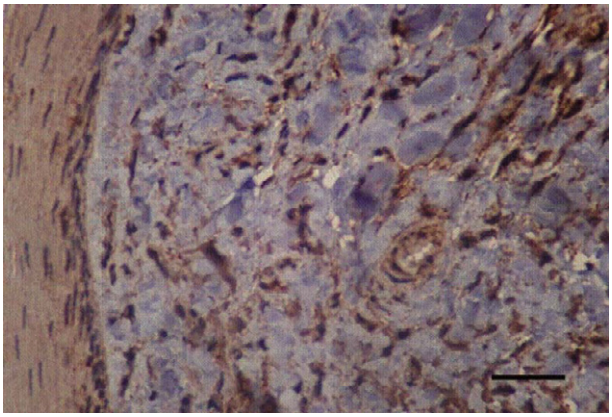

Fig. 3 Photomicrograph showing staining by a peroxidase-labeled streptavidin-biotin technique for lysyl oxidase in an oral submucosal fibrosis specimen which served as the positive control (original magnification ×400). The bar represents 20 μm.

Oral submucosal fibrosis specimens were used as positive controls. As shown in Fig. 3, LOX staining was detected in fibroblasts and endothelial cells.

Discussion

Besides the fact that the increase in the ECM is not well understood in CsA-induced gingival overgrowth, there is no evidence for expression of LOX in this type of lesion. In oral fibrotic disorders, the expression of LOX was found to be significantly upregulated in oral submucosal fibrosis[15,16] and phenytoin-induced gingival overgrowth tissues.[17] It is reasonable to speculate that LOX may be directly related to the pathogenesis of CsA-induced gingival overgrowth.

LOX, an extracellular enzyme, plays a key role in the post-translational modification of collagens and elastin, catalyzing inter- and intra-crosslinking reactions.[12] Because the crosslinked ECM is highly

resistant to degradative enzymes, it is thought that overexpression of LOX may cause severe fibrotic degeneration. Many reports have clearly demonstrated that LOX is consistently and dramatically upregulated in a variety of fibrotic diseases.[9,13–17] To the best of our knowledge, this is the first report of LOX expression being upregulated in CsA-induced gingival overgrowth specimens compared with normal gingival tissues. Strong immunostaining for LOX was detected in fibroblasts, epithelial cells, and inflammatory cells. LOX deposition is associated with CsA-induced gingival overgrowth, suggesting that it may play an important role in ECM turnover. From this phenomenon, we propose that CsA-induced gingival overgrowth may be due to the increased synthesis and deposition of ECM proteins, their altered degradation, or both.

Many studies have suggested that plaque-induced inflammation is associated with the onset or severity of drug-induced overgrowth,[18,19] and histologic findings have shown the presence of some level of inflammatory infiltrate in overgrown gingival tissues.[7,8] Here, the expression of LOX increased with the grade of inflammation in CsA-induced gingival overgrowth specimens. The LOX protein has consistently been found to be highly expressed in rat inflamed oral lesions *in vivo*, while normal non-inflamed periapical tissue contained no LOX-positive cells.[20] Our results suggest that CsA may predispose tissues to fibrosis via LOX overexpression in an inflammatory environment.

At present, no effective antifibrotic therapy is available that can be used for patients with CsA-induced gingival overgrowth. β-Aminopropionitrile is an irreversible inhibitor of LOX,[21] an extracellular enzyme that promotes crosslink formation in nascent fibrils of both collagen and elastin by conversion of lysine and hydroxylysine side chain residues into aldehydes. β-Aminopropionitrile was found to significantly decrease the collagen content in bleomycin-induced pulmonary fibrosis in rats.[22,23] Based on experimental evidence, anti-LOX activity may be suitable as an antifibrotic therapeutic target to prevent or delay CsA-induced gingival overgrowth.

As far as we know, this is the first attempt to evaluate the role of LOX expression in CsA-induced gingival overgrowth *in vivo*. We have demonstrated that LOX is elevated in CsA-induced gingival overgrowth compared with normal gingival tissues. LOX expression was significantly higher in CsA-induced gingival overgrowth specimens with higher levels of inflammatory infiltrates. CsA may predispose tissues to gingival overgrowth in inflammatory environments. More detailed *in vitro* and *in vivo* studies are needed to clarify the roles of LOX in CsA-induced gingival overgrowth in humans.

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