Immunohistochemical analysis of epidermal growth factor receptor in cyclosporin A-induced gingival overgrowth

Nurcan Buduneli, Özgül Sağol, Gül Atilla, Soner Duman and Palle Holmstrup

Department of Periodontology, Faculty of Dentistry, and Department of Nephrology, Faculty of Medicine, Ege University, and Department of Pathology, Faculty of Medicine, Dokuz Eylül University, İzmir, Turkey, and Department of Periodontology, School of Dentistry, Faculty of Health Sciences, Copenhagen University, Copenhagen, Denmark

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Cyclosporin A (CsA)-induced gingival overgrowth represents a tissue of fibrosis and epidermal growth factor (EGF) has been shown to induce extracellular matrix synthesis by fibroblasts. The purpose of this study was to evaluate the expression of EGF-receptor (EGF-r) in frozen sections of CsA-induced overgrown gingival tissue using immunohistochemical and semiquantitative techniques. Gingival biopsies were obtained from 12 renal transplant patients receiving CsA as well as 9 systemically and periodontally healthy individuals. Immunohistochemical staining procedures were carried out in frozen sections of gingival tissue and the expression of EGF-r was compared between the two study groups. The expression of EGF-r was more pronounced in the oral gingival epithelium of CsA-induced overgrown gingiva as compared to those of the clinically healthy gingival specimens. The reactivity in the inflammatory infiltrate and connective tissue cells of both of the study groups was similar. In conclusion, the results of the present study may suggest that CsA affects EGF-r metabolism in gingival keratinocytes resulting in an increased number of cell surface receptors, which may eventually play a role in the pathogenesis of gingival tissue alterations. □ *Cyclosporin A; epidermal growth factor receptor; gingival overgrowth/pathogenesis; immunohistochemistry*

Nurcan Buduneli, Department of Periodontology, Faculty of Dentistry, Ege University, 35100 Bornova, İzmir, Turkey. Fax: +90 232 388 03 25, e-mail: nurcan@bornova.ege.edu.tr

Cyclosporin A (CsA) has been widely used for its ability to act as a safe, low-toxicity immunosuppressant with a potential application in prevention of graft rejection and management of a wide range of systemic disorders (1). However, CsA is also associated with various adverse effects; the most notable one in terms of dental medicine is gingival overgrowth (2). As yet, the mechanisms through which CsA elicits a connective tissue reaction in the gingiva remain unclear. Recently, growth factors have received attention in relation to the pathogenesis of drug-induced gingival overgrowth. Cellular homeostasis in tissues is likely to be the result of a balance between complex interactions of antagonistic molecules, in which cytokines and growth factors play a major role (2).

Epidermal growth factor (EGF) is a polypeptide that is synthesized by a range of normal cell types, and human EGF consists of 53 amino acids with a molecular weight of 6045 Da (3). EGF displays many kinds of biological effects, including stimulation of proliferation and differentiation of both epithelium- and mesenchyme-derived tissues, stimulation of DNA, mRNA, protein, and hyaluronic acid synthesis (3–5). Furthermore, EGF can stimulate cell migration and proteinase production and accelerate epithelial regeneration and wound healing (3, 6). EGF stimulates proliferation and collagen and hyaluronan synthesis of fibroblasts (7). These properties have led to the idea that EGF plays an important role in tissue remodeling during development and in wound repair throughout life (8). It is likely that the responses of different cell types to EGF vary, and it has different functions in different tissues. EGF needs to bind to a specific cell surface receptor at the target cell to exert its action. The EGF receptor (EGF-r) is a single polypeptide chain (175 kDa) that spans the plasma membrane (9, 10). The response of target cells to EGF is regulated at several different levels, including modulation of receptor number and availability. Increased levels of certain growth factors and/or their receptors are likely in conditions with increased tissue volume, such as CsA-induced gingival overgrowth. Even healthy gingiva is in a continuous state of repair because of a constant environmental insult from microbial dental plaque (11). In view of these facts, it may be hypothesized that an increased expression of EGF-r may be relevant to the pathogenesis of CsA-induced gingival overgrowth. Therefore, the present study was conducted to evaluate the expression of EGF-r in frozen sections of CsA-induced overgrown gingiva, using immunohistochemical and semiquantitative techniques.

Materials and methods

Tissues

After informed consent a total of 21 biopsy specimens of interproximal gingival tissue were obtained from 21

Table 1. Clinical characteristics of the study groups

Group	Mean age ± standard deviation (years)	PPD (mm)	PI	PBI	HI
CsA-treated patients	36 ± 5.7	4–6	0	0	≥2
Healthy controls	17 ± 2.9	≤2	0	0	0

CsA = cyclosporin A; PPD = probing pocket depth; PBI = papilla bleeding index; HI = hyperplastic index.

individuals. The CsA group consisted of 12 gingival samples from 12 renal transplant patients (8 male, 4 female; 18 to 43 years old) receiving CsA for more than 1 year (ranging from 1 to 5 years) with a dose adjusted to maintain stable serum levels of 80 to 300 ng/mL. The patients had been taking no other drugs associated with gingival overgrowth. They had been followed up by the Department of Nephrology, Faculty of Medicine, Ege University, İzmir, and owing to the presence of moderate to advanced CsA-induced gingival overgrowth they were referred to the Department of Periodontology, Faculty of Dentistry. None of them had periodontitis, but chronic gingivitis was evident in all of the patients. Two months after completion of the initial phase of periodontal treatment in terms of patient instruction in oral hygiene and scaling, they were scheduled for periodontal surgery. Before the surgical interventions the patients' motivation was reinforced regularly once a week. The clinical periodontal indices comprising probing pocket depth (PPD), plaque index (PI) (12), papilla bleeding index (PBI) (13), and hyperplastic index (HI) (14) were recorded at the time of surgery. Gingival samples were obtained during the periodontal surgical interventions. The biopsy specimens were frozen immediately after removal and kept at −40°C for a maximum of 3 months until the day of immunohistochemical analysis.

The control group consisted of nine systemically and periodontally healthy individuals (3 male, 6 female; 15 to 24 years old). Biopsy specimens were taken after local anesthesia for tooth extractions for orthodontic reasons. The individuals had good oral hygiene, and there were no signs of inflammation in the gingiva (no plaque, no bleeding, probing pocket depth ≤2 mm).

Immunohistochemical analysis

Cryostat sections (7 μm thick) of all specimens were cut in the buccolingual plane, and three consecutive sections for each gingival specimen were mounted on one poly-L-lysine-coated slide. The sections were air-dried and fixed in cold acetone at 4°C for 10 min. They were then rinsed with tris buffered saline (TBS) (0.05M, pH 7.2–7.6) and incubated with the primary antibody (monoclonal mouse anti-human (IgG2b) EGF-r, code M0886, DAKO, Glostrup, Denmark) for 30 min in a humidity chamber at room temperature. The optimal working titer of the monoclonal antibody was determined to be 1:10 by checkerboard titrations. After being rinsed with TBS, the sections were incubated with alkaline phosphatase labeled polymer (Envision System, Code K1396, DAKO) for 30 min. The sections were rinsed again with TBS, and the substrate-chromogen solution (Fast red) was applied for 30 min. The slides were then rinsed with distilled water, and counterstaining was performed with Mayer hematoxylin. Finally, the slides were mounted and coverslipped with an aqueous-based mounting medium. Remaining frozen tissues were then fixed in 10% formalin, routinely processed, and embedded in paraffin. One section (5 μm thick) from each paraffin block was stained with hematoxylin and eosin (H & E) and used for orientation and routine histopathological evaluation. Semiquantitative analysis of EGF-r was performed on three consecutive sections mounted on the same slide for each gingival specimen. The staining pattern with EGF-r antibody was evaluated separately in the oral gingival epithelium, connective tissue, and inflammatory infiltrate in each section as previously defined (15). A range between 0 and 3 was used to establish the extent of antibody-positive cells present in the connective tissue fibroblasts and the inflammatory cells, as modified from the study by Gamonal et al. (16). In brief, 0 = no detectable cells; 1 = less than 25%; 2 = between 25% and 50%; and 3 = more than 50%. The entire connective tissue between the sulcular epithelium and the oral gingival epithelium was evaluated. The extent of positively stained cells present in the oral gingival epithelium was assessed as follows: 0 = no detectable cells; 1 = lower one third part of the epithelial thickness, limited to the stratum basale; 2 = lower two thirds of the epithelial thickness; 3 = more than two thirds of the epithelial thickness. The intraexaminer reliability of microscopic evaluations was tested by having the same investigator (Ö. Sağol) twice evaluate five randomly selected tissue specimens. Control reactions were carried out in two different ways: 1) conjugate control: substitution of the primary antibody with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS); 2) isotype control: substitution of the primary antibody with a mouse antibody of irrelevant specificity but with the same immunoglobulin isotype (mouse IgG2b, negative control, Code X0944 DAKO).

Statistical analysis

Mann–Whitney U nonparametric test with Bonferroni correction was used to examine the differences in EGF-r

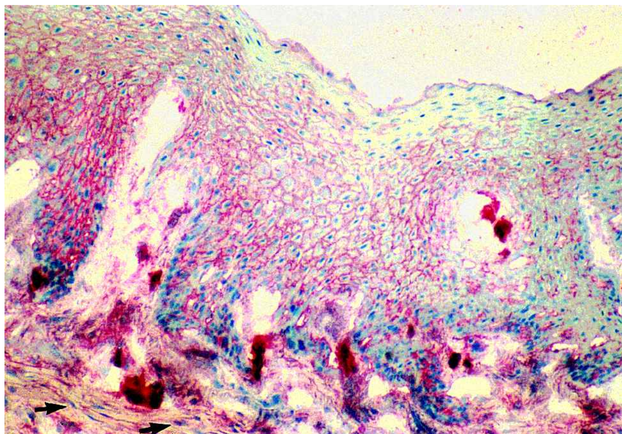

Fig. 1. The pronounced thickening due to acanthosis of the oral epithelium in the cyclosporin A group. Staining was observed in nearly all layers of epithelium. In addition, fibroblasts were stained (arrows). (Magnification, ×40.)

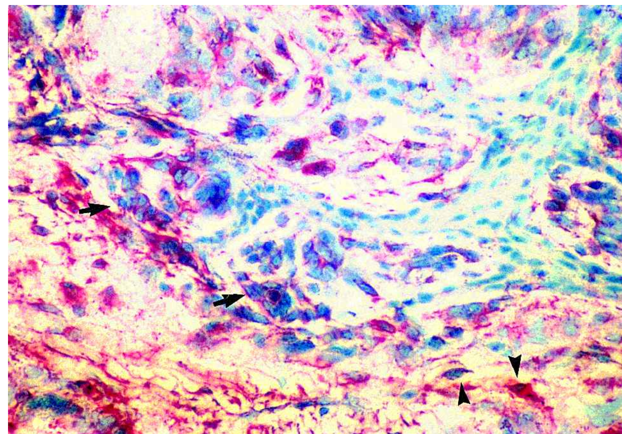

Fig. 3. Epidermal growth factor receptor expression in the inflammatory cells (arrows) and in the fibroblasts (arrowheads). (Magnification, ×200.)

expression between the two study groups. $P < 0.0167$ was the level of significance.

Results

Histopathology

The clinical characteristics of the study groups and gingival tissue specimens are outlined in Table 1. In the clinically healthy control group regular, well-developed rete pegs were observed in the epithelium. A few specimens showed mild inflammatory infiltration in the connective tissue of predominantly perivascular location and elongations of rete pegs in association with subclinical inflammation. In the CsA-induced gingival overgrowth group, thickening of epithelium was noted. High vascularization and focal accumulations of infiltrating inflammatory cells were also observed. The inflammatory infiltrate was rich mainly in plasma cells, and fewer lymphocytes were observed. Acanthosis and parakeratinization were frequent findings in the CsA-induced overgrown group (Fig. 1).

Immunohistochemical staining reactions

Overall, the EGF-r staining bound most strongly to the basal and parabasal layers of oral gingival epithelium and gave a typical cell-surface-associated staining (Fig. 1). Receptor expression diminished gradually towards the surface of the epithelium, and in some specimens in the control group EGF-r staining was limited to the basal layer, and the superficial layers appeared almost completely negative (Fig. 2). Positively stained fibroblasts and

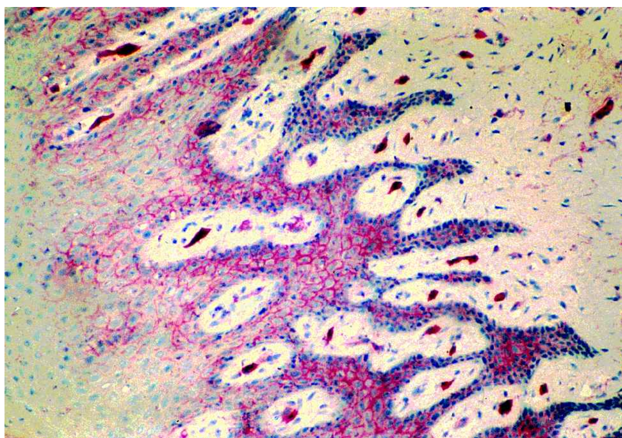

Fig. 2. The positive staining reaction with epidermal growth factor receptor antibody limited to the basal layer and suprabasal regions of the epithelium in a control specimen. (Magnification, ×100.)

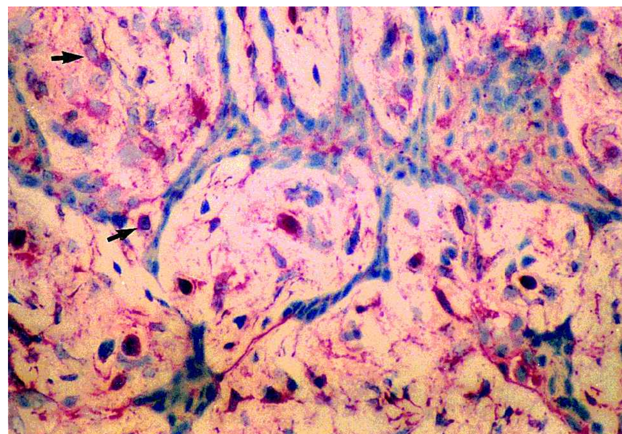

Fig. 4. The positive staining with epidermal growth factor receptor antibody in the inflammatory cells in a specimen from the cyclosporin A-treated patient group (arrows). (Magnification, ×200.)

Table 2. The percentages of scores showing the degree of positively stained keratinocytes in the oral gingival epithelium*

	Cyclosporin A	Control
0	—	—
1	—	66.7
2	58.3	22.2
3	41.7	11.1

* All values are expressed as percentages.

Table 3. The percentages of scores showing the degree of positively stained fibroblasts and inflammatory cells in the connective tissue*

	Connective tissue		Inflammatory infiltrate	
	CsA	Control	CsA	Control
0	—	—	16.7	44.4
1	25	22.2	66.7	44.4
2	25	33.3	8.3	11.1
3	50	44.4	8.3	—

CsA = cyclosporin A.
* All values are expressed as percentages.

Table 4. The median and range of scores showing the degree of positively stained cells

Group	Epithelium	Connective tissue	Inflammatory infiltrate
CsA-treated patients	2 (2–3)*	2.5 (1–3)	1 (0–3)
Healthy controls	1 (1–3)	2 (1–3)	1 (0–2)

CsA = cyclosporin A.
* Significant difference from the clinically healthy control group ($P < 0.0167$).

inflammatory cells were detected in the connective tissue of both of study groups (Figs. 3 and 4). The percentages of scores showing the degree of positively stained keratinocytes in the oral gingival epithelium are presented in Table 2, and those showing the degree of positively stained fibroblasts and inflammatory cells in connective tissue are presented in Table 3. The median and minimum-maximum scores established in various evaluation sites in the two study groups are shown in Table 4. When the two study groups were compared, a significant difference was found in EGF-r expression in the oral gingival epithelium ($P < 0.0167$). The reactivity in the connective tissue and inflammatory infiltrate was similar in the two groups.

Discussion

To date, the effects of EGF on various cell populations including gingival fibroblasts have been studied in cell culture (5, 17–19). Furthermore, immunohistochemical investigations of EGF and/or EGF-r on sections of inflamed and noninflamed human gingival tissue samples (20, 21) and gingival biopsy specimens from adult periodontitis and juvenile periodontitis patients have been published (22). However, to our knowledge, this is the first report describing the expression of EGF-r in CsA-induced overgrown gingival tissue. The expression of EGF itself was out of the scope of the present study, and the possible alterations in EGF-r expression due to CsA usage was looked for.

In an initial in vitro study Modeer et al. (18) reported that fibroblasts from the responder patients (with diphenylhydantoin (PHT)-induced gingival overgrowth) showed around 30% increase in the number of EGF-r, whereas in nonresponders (not showing any PHT-induced gingival overgrowth) there was a decrease. In another study, Modeer & Andersson (19) reported that PHT treatment of normal gingival fibroblasts derived from healthy children resulted in a significant increase in the number of cell surface EGF-r. Furthermore, Soory & Kasasa (23) have speculated that EGF isolated in inflammatory exudate could contribute to matrix synthesis, which may be relevant to PHT-induced gingival overgrowth. Likewise, the present study showed a significant increase in the expression of EGF-r in the keratinocytes of CsA-induced overgrown gingiva. Taken together, the findings of the previous reports and those of the present study suggest that common pathogenic mechanisms may act in PHT- and CsA-induced gingival overgrowth. Boltchi et al. (2) have stated that drugs known to induce gingival overgrowth as an adverse effect exacerbate the normal tissue turnover and wound healing signals.

It has been proposed that EGF plays a significant role in the development and maintenance of oral tissue integrity. The binding of EGF to the cell surface receptor is considered to be the first step in a chain of reactions that culminate in mitosis (24). Whitcomb et al. (25) have investigated the immunohistochemical mapping of EGF-r in normal human gingiva, buccal mucosa, the lip, the floor of the mouth, and the palate. Consistent with our findings, they reported that in all oral sites, the immunohistochemical reactivity was most pronounced in the basal layer, progressively diminishing in intensity towards the surface. This finding may indicate that synthesis occurs in basal cells and is maintained only briefly on the plasma membrane as the cells stratify and differentiate. It has previously been shown that the expression of EGF-r present on rapidly proliferating cells increases in the inflamed human junctional and pocket epithelium (20, 26). The present finding of more pronounced expression of EGF-r in the epithelium may be closely related to rapid proliferation of gingival keratinocytes. This may well be a component of gingival tissue alterations observed in CsA-induced gingival overgrowth. It is likely that the exposure of periodontal epithelial cells to EGF in connection with pocket formation could lead to their proliferation. The chronic inflammation in the dentogingival junction could

be considered a chronic wound healing (22). Thus, inflammation appears to trigger EGF-r expression (21). Varga et al. (27) showed that pre-existing plaque-associated gingival inflammation correlates with CsA-induced gingival overgrowth. Inflammation may eventually lead to tissue changes via various mediators secreted by activated inflammatory cells. Hence, CsA may have growth-promoting actions via EGF. In inflamed gingiva, a tissue normally expressing a high rate of remodeling, the effects of CsA could act synergistically with endogenous signals, resulting in excessive repair or overgrowth, and inflammatory mediators may act as cofactors for inducing gingival overgrowth (28).

In conclusion, from the results of the present study it may be suggested that CsA may affect EGF-r metabolism in gingival keratinocytes, resulting in an increased number of cell surface receptors, whereas fibroblasts and inflammatory cells do not seem to be affected significantly. It is quite clear that growth factors, drugs, and inflammatory mediators act in concert within the systems and bring about a response, which is reflected as gingival overgrowth (29). However, whether increased EGF-r expression of the epithelium in CsA-induced overgrown gingiva is causal or consequential remains to be elucidated.

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