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Regulation of keratinocyte growth factor and scatter factor in cyclosporin-induced gingival overgrowth

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BACKGROUND: Epithelial proliferation is a histological characteristic of drug-induced gingival overgrowth. Keratinocyte growth factor (KGF) and scatter factor (SF) are fibroblast-derived growth factors with potent mitogenic and motogenic effects on epithelial cells, and, therefore, could be involved in the pathogenesis of gingival overgrowth. The aims of this study were to investigate: (i) the effects of cyclosporin on KGF and SF expression by gingival fibroblasts; and (ii) the expression levels of KGF and SF mRNA in normal and overgrown gingival tissue.
METHODS: The KGF and SF protein production was determined by enzyme-linked immunosorbent assay. Relative levels of KGF and SF mRNA expression were determined using semi-quantitative reverse transcriptase polymerase chain reaction. Expression levels in biopsies of normal and overgrown gum were also determined.
RESULTS: In overgrown fibroblasts, 500 ng/ml cyclosporin significantly inhibited KGF and SF mRNA and protein while 2000 ng/ml cyclosporin induced a stimulatory effect. In normal cells cyclosporin significantly increased both KGF and SF. KGF and SF mRNA was detected in both normal and overgrown tissues with a tendency towards increased expression levels in overgrown tissue.
CONCLUSION: These results suggest that KGF and SF may have an important role in cyclosporin-induced gingival overgrowth.
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Keywords: cyclosporin; fibroblast phenotype; gingival overgrowth; keratinocyte growth factor; scatter factor

Introduction

Cyclosporin is a potent immunosuppressant drug widely used in the treatment of immune-related conditions, but primarily for the prevention of organ transplant rejection. Cyclosporin therapy is associated with a number of major side-effects including nephrotoxicity, hepatotoxicity and, in approximately 30% of individuals, the development of gingival overgrowth (1). The pathogenesis of cyclosporin-induced gingival overgrowth remains unclear despite numerous clinical and scientific investigations. Most histological studies of overgrown tissue indicate that an accumulation of extracellular matrix (ECM) within the gingival connective tissue is a central feature of the overgrown tissue (2–4). However, subsequent studies investigating the effects of cyclosporin on both matrix synthesis and degradative activity by gingival fibroblasts have produced conflicting results, with no clear evidence of a drug-induced increase in matrix deposition as the central cause for this condition (5–10).

Histological studies have also reported changes in the epithelial component of cyclosporin-induced overgrown gingiva (11, 12), which is characterized by epithelial enlargement and elongated rete pegs, which penetrate deep into the underlying connective tissue. Ayanoglou and Lesty (13) suggested that the resulting increase in epithelial thickness was a consequence of cell hypertrophy in the keratinized epithelia and cell hyperplasia in the junctional epithelium. Increased epithelial mitotic activity, illustrated by upregulated Ki67 expression, has also been reported in cyclosporin-induced gingival overgrowth (14). Conversely, most studies investigating the effects of cyclosporin on epithelial cell cultures *in vitro* have reported an antiproliferative effect in these cells (15–18). Taken together these findings suggest that the increase in epithelial thickness seen in gingival overgrowth may not result from a direct effect of cyclosporin on epithelial cells, but may be an indirect result of drug interactions with other cells in the gingival tissues.

The phenotype of an epithelium is determined initially by developmental interactions with its adjacent connective tissue and it is now apparent that such tissue interactions persist and are required for both the maintenance and repair of epithelia in adult life (19, 20). Thus the epithelial changes seen in gingival overgrowth may reflect differences in the signals

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emanating from the subjacent connective tissue. The nature of the signalling molecules, which mediate interactions between an epithelium and its supporting connective tissue have been the subject of intense investigation. The expression patterns of two factors, keratinocyte growth factor (KGF) and hepatocyte growth factor/scatter factor (SF) in mesenchymal cells, in close vicinity to epithelial structures, combined with their distinctive target cell specificity, suggests that both these molecules may play a role in regulating epithelial phenotype (21, 22). Gingival fibroblasts are capable of synthesizing an array of biological mediators including KGF and SF. Two recent studies have investigated the potential role of KGF in the pathogenesis of gingival overgrowth, reporting increased expression of both KGF and its receptor (KGF-R) in overgrown compared with normal gingival tissue (23, 24). However, no studies have reported on SF expression in overgrown tissue. Cyclosporin has previously been shown to regulate expression of other cytokines and growth factors, including TGF-β1, PDGF, IL-1, IL-2 and IL-6, by both fibroblasts and inflammatory cells (25–27). A similar drug-induced increase in KGF and SF expression by gingival fibroblasts provides a potential biological mechanism underlying the epithelial changes seen in gingival overgrowth. The aims of this study were therefore to investigate: (i) the effects of cyclosporin on KGF and SF production by normal and overgrown gingival fibroblasts, and (ii) expression levels of KGF and SF mRNA in normal and overgrown gingival tissue.

Materials and methods

Reagents

All cell culture consumables were purchased from Invitrogen Ltd (Paisley, UK). Cyclosporin, donated by Novartis (Bern, Switzerland), was initially dissolved in ethanol and serially diluted to the required concentrations. Appropriate carrier controls were performed and the ethanol vehicle was found to have no effect on cell behaviour.

Cell culture

The three strains of fibroblasts derived from normal gingiva (NG-5, NG-10, NG-11), and the three strains derived from cyclosporin-induced gingival overgrowth (COG-8, COG-9, COG-10) used during the study were developed by explant culture from human tissue biopsies. In addition, biopsies of clinically healthy (N1, N2, N3) and cyclosporin-induced gingival overgrowth (OG1, OG2, OG3) were used to determine mRNA expression at the tissue level. All donors were male and aged between 20–25 years – fibroblast populations used in the study were not derived from the same tissue samples employed for mRNA expression studies. Ethical approval for the study was gained from the Research Ethics Committee, Queen's University, Belfast, and informed consent was given by all subjects. Fibroblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and incubated in a humidified atmosphere of 5% CO_2 at 37°C. Cells between the third and sixth transfer were used in the study. A keratinocyte cell strain derived from the gingival tissue of a 28-year-old female served as a negative control for KGF and SF mRNA expression.

Reverse transcriptase polymerase chain reaction

The effect of cyclosporin on mRNA expression was determined in one normal (NG-10) and one overgrown (COG-9) fibroblast strain. Fibroblasts were grown to confluence in duplicate 12 well plates and cultured in serum-free medium supplemented with 0.2% bovine serum albumin (BSA) and containing cyclosporin at 0, 100, 500 and 2000 ng/ml concentrations, reflecting both trough blood levels and the increased concentrations of drug found in gingival tissues. After 24 h in culture, total RNA was extracted from cultured cells using TRIZOL (Invitrogen Ltd) according to manufacturer's instructions. Concentration and purity of the isolated RNA samples was determined by spectrophotometry followed by electrophoretic separation on a 1% denaturing gel. For studies on gene expression in gingival tissue, total mRNA was extracted from 100 mg of tissue biopsies using the same method.

cDNA was synthesized from 1 μg of extracted total RNA using the oligo dT primers and the Superscript™ First Strand Synthesis System (Invitrogen Ltd). Newly synthesized cDNAs were then amplified using Platinum *Taq* DNA polymerase (Invitrogen Ltd), and 40 pmols of primers. Polymerase chain reaction (PCR) primers used for amplification of KGF, SF and β-actin cDNAs are shown in Table 1. Amplification was carried out for 27 cycles (cell cultures) or 32 cycles (tissues); 50 s denaturation at 94°C, 25 s annealing at 64°C, and 45 s elongation at 72°C. All PCR amplifications of cDNA samples were carried out within the exponential range of each product. An appropriate PCR-amplified negative blank (i.e. containing no cDNA), positive kit control (Invitrogen Ltd), negative RT^- control (without Superscript II RT), negative keratinocyte control (K^-) and a low molecular weight mass marker (Invitrogen Ltd) were used during all gel electrophoresis runs.

To confirm the presence or absence of KGF/SF transcripts in tissue specimens, a nested PCR was carried out on all tissue first round products using

Table 1 Primer sequences used for amplification of KGF, SF and β-actin cDNA. Also shown are the primers for nested (n) PCR amplification of KGF and SF

Primer	*Sequence 5′→3′*
KGF1	CATCCAAGCCATATATGGACGTTCC
KGF2	TCTGGAGAGTCAAAATTCTCTTCGT
KGFn3	GAAATCAGGACAGTGGCAGTTGG
KGFn3	CCATAGGAAGAAAGTGGGCTGTT
SF1	AGTCAACCAGACCACCTTATACCA
SF2	TTTCAGAGCCTTGGAGGAGCTGGTC
SFn3	CCATAGGAAGAAAGTGGGCTGTT
SFn4	AGCATCATCATCTGGATTTCGG
β-actin 1	ATCTGGCACCACACCTTCTACAATG
β-actin 2	GCTTCTCCTTAATGTCACGCACGA

20 pmols of the appropriate nested-primers (Table 1). Amplification was carried out for 25 cycles; 10 s denaturation at 94°C, 10 s annealing at 67°C, and 30 s elongation at 72°C. All PCR products were identified by size following electrophoresis on a 2% EtBr-stained agarose gel, and further confirmed by direct DNA sequencing. The specificity of each product was confirmed by direct cycle sequence analysis using the ABI PRISM Terminator Cycle Sequence Reaction Kit (Perkin Elmer, Bucks, UK) for preparation of reaction products, followed by electrophoresis on an ABI fluorescent automated DNA sequencer.

Analysis of mRNA transcripts

Net intensities of KGF, SF and β-actin bands were measured using Kodak 1D Image analysis software (Eastman Kodak Company, Rochester, NY, USA). Relative levels of KGF and SF mRNA in each sample were calculated to produce KGF and SF expression indices values (KEI and SEI, respectively) as previously described (23).

Enzyme-linked immunosorbent assay

Three normal (NG-5, NG-10, NG-11) and three overgrown (COG-8, COG-9, COG-10) gingival fibroblast strains were grown to confluence in triplicate on six-well plates in DMEM containing 10% FCS. Cells were then washed in Hanks balanced salt solution (HBSS) and cultured in serum-free medium supplemented with 0.2% BSA for 48 h in the presence of cyclosporin, over the concentration range 0, 100, 500 and 2000 ng/ml. At the end of the incubation period, culture supernatants were collected and assayed for KGF and SF by enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK) according to manufacturer's instructions. Protein production was expressed as pg/ml/10^5 cells.

Statistical analysis

The effects of cyclosporin on KGF and SF protein and mRNA expression were analysed by one-way analysis of variance (ANOVA). Comparison of KGF and SF production by normal and overgrown-derived fibroblast strains under control conditions, was performed by the Mann–Whitney *U*-test analysis. The level of statistical significance in all cases was set at $P < 0.05$.

Results

Protein production by gingival fibroblasts

Comparison of KGF protein production by normal and overgrown gingival fibroblasts under control conditions showed that although an overall 1.6-fold increase in mean KGF protein was observed in overgrown cultures compared with normal (73.7 ± 29.3 vs. 50.3 ± 7.5 pg/ml/10^5 cells), this difference was not statistically significant ($P = 0.63$; Fig. 1a,b). The mean level of SF protein produced by overgrown strains was also greater than in normal cultures (815.9 ± 140.4 vs. 505.8 ± 81.4 pg/ml/10^5 cells) – again this difference did not reach statistical significance (Fig. 2a,b).

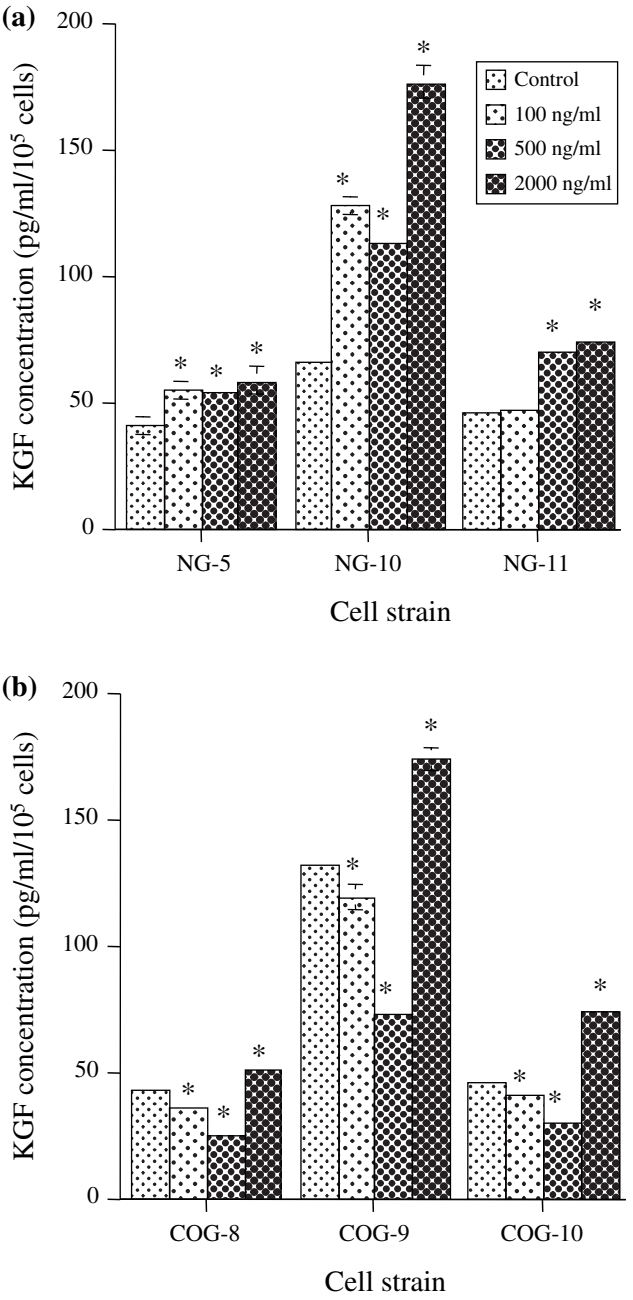

Figure 1 Keratinocyte growth factor (KGF) production (pg/ml/10^5 cells) by (a) normal and (b) overgrown fibroblasts in response to increasing concentrations of cyclosporin (mean ± SEM). *Indicates significant difference from controls ($P < 0.05$).

The effects of cyclosporin on KGF protein production are shown in Fig. 1a,b. At concentrations of 500 ng/ml and 2000 ng/ml, cyclosporin significantly stimulated KGF output by all three normal fibroblast strains. At the lowest drug concentration used (100 ng/ml), this stimulation of KGF production was seen in 2/3 strains (NG-5 and NG-10), with no significant effect noted in the third strain. The effect of cyclosporin on KGF production by overgrown fibroblasts was also concentration-dependent. As with normal fibroblasts, at a concentration of 2000 ng/ml, cyclosporin significantly

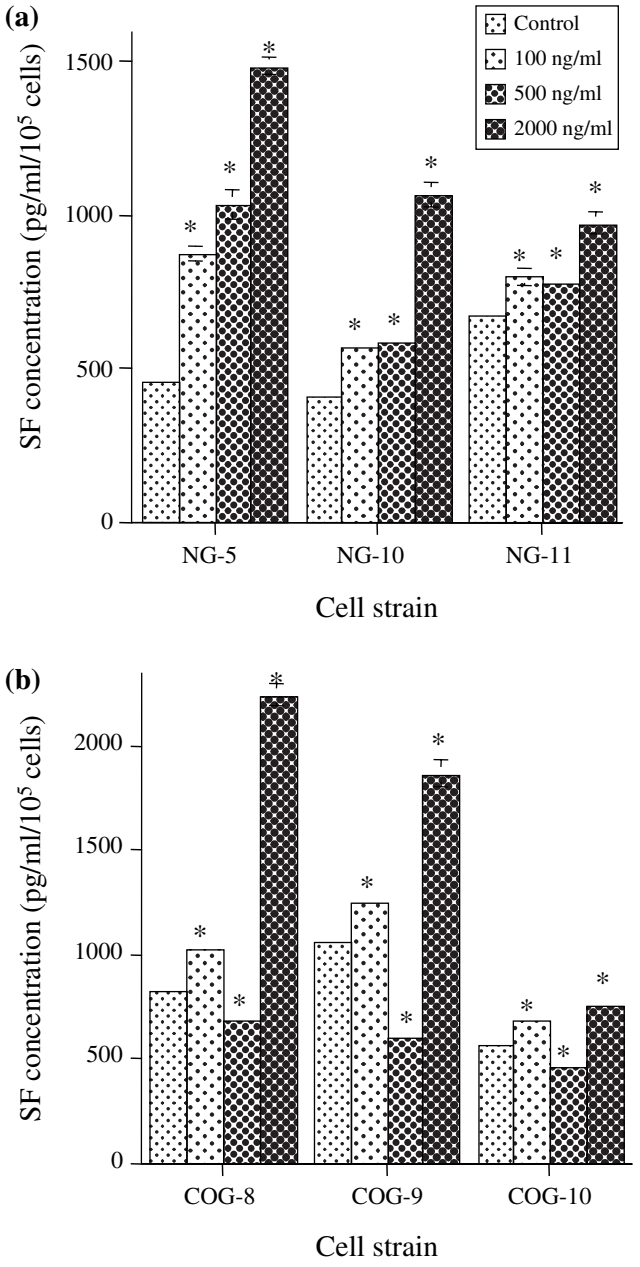

Figure 2 Scatter factor (SF) production (pg/ml/10^5 cells) by (a) normal and (b) overgrown fibroblasts in response to increasing concentrations of cyclosporin (mean ± SEM). *Indicates significant difference from controls ($P < 0.05$).

stimulated KGF production by overgrown fibroblasts over control cultures ($P < 0.05$). In contrast, addition of 100 ng/ml cyclosporin resulted in a small but still significant inhibition of KGF protein expression ($P < 0.05$). Cyclosporin at 500 ng/ml induced a marked reduction of KGF production in all three overgrown strains (Fig. 1b).

The effect of cyclosporin on SF protein production in cultures by normal fibroblasts mirrored the results for KGF, namely a significant stimulation of SF output by all drug concentrations in all three fibroblast strains ($P < 0.05$). Again the greatest level of stimulation occurred at the highest drug concentration (Fig. 2a). The effects of cyclosporin on overgrown fibroblasts were again dependent on drug concentration (Fig. 2b). The highest concentration of cyclosporin (2000 ng/ml) significantly stimulated SF production, while at a concentration of 500 ng/ml, SF output was significantly inhibited ($P < 0.05$). Addition of 100 ng/ml of cyclosporin resulted in a small but significant stimulation of SF protein production by all overgrown strains ($P < 0.05$).

mRNA expression by gingival fibroblasts

Under control conditions, levels of KGF and SF mRNA were significantly greater in overgrown compared with normal cell cultures ($P < 0.05$). All concentrations of cyclosporin significantly increased mRNA expression levels of both KGF and SF in normal fibroblasts (Fig. 3). This effect was particularly marked in cultures treated with 2000 ng/ml cyclosporin (Table 2). In overgrown cultures cyclosporin, at concentrations of 100 and 2000 ng/ml, also stimulated KGF and SF mRNA expression over control levels, with the greatest effect again seen at the highest drug concentration (Fig. 3; Table 2). In sharp contrast, 500 ng/ml of cyclosporin significantly reduced SF mRNA expression in overgrown cultures, with a lesser inhibitory effect on KGF mRNA (Fig. 3; Table 2).

KGF and SF mRNA expression in gingival tissues

Following first round amplification of tissue cDNAs, KGF bands were clearly observed in one normal (N3) and one overgrown (OG3) tissue sample (Fig. 4a). Bands were also present in a second normal (N2) and second overgrown (OG1) tissue sample, albeit at a much lower but still quantifiable level of expression (Table 3). In contrast, SF expression was observed in two of the overgrown samples following first round amplification, but were absent in all healthy tissue samples examined (Table 3). Nested-PCR confirmed the presence of KGF and SF transcripts in all negative first round products from both healthy and overgrown tissue samples (Fig. 4b).

Discussion

An increase in epithelial thickness along with deep penetrating rete pegs are characteristic histological features of cyclosporin-induced gingival overgrowth. Cell hypertrophy and increased mitotic activity have been suggested as possible mechanisms underlying these changes. Interestingly Ayanoglou and Lesty (13) reported that epithelial changes mainly occurred in areas in contact with enlarged and modified regions of connective tissue, suggesting that epithelial changes may be secondary to drug-induced alterations in the underlying lamina propria. Cross-recombination studies (19, 20) have shown that connective tissue continues to influence and modify epithelial growth and differentiation throughout adulthood. Two factors thought to play central roles in mediating such epithelial-mesenchymal interactions are KGF and SF, both of which exert

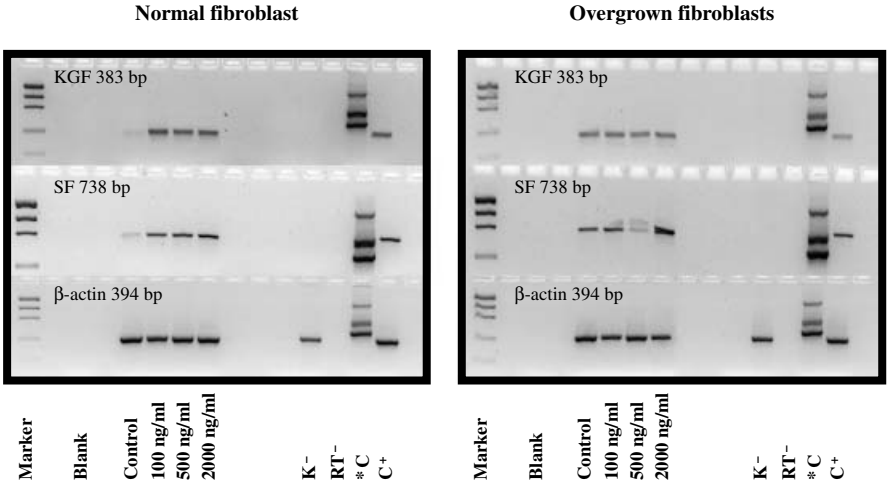

Figure 3 Reverse transcriptase polymerase chain reaction analysis (RT-PCR) for Keratinocyte growth factor (KGF), scatter factor (SF) and β-actin products from cultured normal and overgrown fibroblasts following incubation with 0, 100, 500 and 2000 ng/ml cyclosporin for 24 h. PCR-amplified negative blanks, negative keratinocyte controls (K^-), negative RT^- controls (without RT enzyme), positive kit controls (*C), cDNA controls (C^+) and molecular weight markers are shown.

Table 2 Effect of cyclosporin on the relative levels of KGF and SF mRNA in NG-10 and COG-9 gingival fibroblasts. Values are expressed as mean expression indices (KEI and SEI, ±SD)

	Control	*100 (ng/ml)*	*500 (ng/ml)*	*2000 (ng/ml)*
NG-10 KGF	5.1 (±1.0)	41.4 (±5.2)*	43.2 (±4.7)*	152.0 (±2.0)*
COG-9 KGF	47.7 (±0.64)**	50.7 (±0.60)*	39.0 (±0.50)*	58.0 (±1.0)*
NG-10 SF	13.3 (±2.1)	30.9 (±4.2)*	37.2 (±4.5)*	112.2 (±1.1)*
COG-9 SF	20.6 (±0.53)**	47.2 (±0.60)*	9.6 (±0.55)*	75.6 (±1.9)*

*Statistically different from controls, $P < 0.05$.
**Significantly greater than normal fibroblasts ($P < 0.05$).

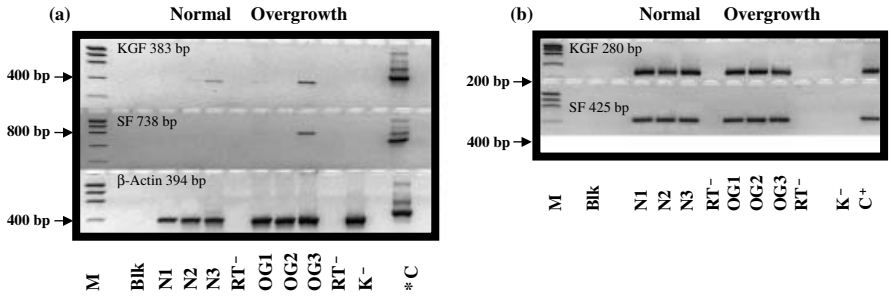

Figure 4 Representative gels of reverse transcriptase polymerase chain reaction analysis for; (a) KGF, SF and β-actin (first round products) from three normal (N) and three overgrown (OG) gingival biopsies, and (b) detection of KGF and SF mRNA using a second round nested-PCR. Molecular weight markers (M), PCR-amplified negative blanks (Blk), negative RT^- controls (without RT enzyme), negative keratinocyte controls (K^-), and positive kit controls (*C) are shown.

strong mitogenic and motogenic effects on epithelial cells. We therefore investigated a possible role for KGF and SF in mediating the epithelial changes that accompany gingival overgrowth.

Our studies on the levels of KGF and SF mRNA expression in normal and overgrown gingival tissue were unable to detect any major differences in KGF expression levels between the two tissue types, albeit in a small number of samples – quantifiable levels of expression were detected in two out of three biopsies of both normal and overgrown tissue after first round amplification. In contrast Das et al. (23) recently reported significantly higher levels of KGF mRNA and protein expression in overgrown compared with normal gingival tissue. Interestingly these authors were unable to detect KGF mRNA in any of six healthy biopsies. This is somewhat surprising as KGF expression has previously been demonstrated in healthy gingival connective tissue by *in situ* hybridization (28). The reasons for these differences in outcome between studies may simply reflect the different methodologies used, with subsequent variability in the levels of transcript detection.

Table 3 Relative levels of KGF and SF mRNA transcripts (KEI and SEI respectively) in normal (N) and overgrown (OG) gingival tissues

Tissue sample	*Individual KEI values ± SD*		*Combined average KEI ± SEM*	*Individual SEI values ± SD*		*Combined average SEI ± SEM*
Normal	N1	0 ± 0	3.52 ± 1.44 (0–8.6)	N1	0 ± 0	0 ± 0 (0)
	N2	3 ± 1.1		N2	0 ± 0	
	N3	7.55 ± 1		N3	0 ± 0	
Overgrown	OG1	2 ± 0.1	5.83 ± 3.08 (0–16.1)	OG1	0 ± 0	9.18* ± 4.31 (0–23.5)
	OG2	0 ± 0		OG2	5.1 ± 0.2	
	OG3	15.5 ± 0.6		OG3	22.5 ± 1.05	

*Statistically significant $P < 0.05$.
KEI and SEI range in parentheses.

Expression of SF in normal and overgrown gingival tissue has not been previously reported. Here we were unable to detect SF in any biopsies of healthy gingival tissue after first round PCR. SF mRNA was detectable in two of three overgrown biopsies, suggesting an upregulation in expression in gingival overgrowth.

Two possible explanations, not mutually exclusive, may account for any increased KGF and SF expression in gingival overgrowth. First the stimulatory effects of cyclosporin on the expression levels of both factors by normal gingival fibroblasts *in vitro* shown in this study support the concept of a direct, drug-induced effect. That the greatest level of stimulation was seen at the highest drug concentration (2000 ng/ml) may be of clinical relevance as cyclosporin has been reported to be concentrated in the gingival environment (29). However, increased expression of KGF and SF in overgrown tissue may simply reflect associated inflammation in the tissues. Ohshima et al. (30) recently reported increased levels of SF in gingival crevicular fluid retrieved from periodontitis compared with healthy periodontal sites. Overgrown gingival tissues are often inflamed because of associated difficulties with plaque control and thus contain a range of inflammatory cytokines such as TNF-α and IL-1β, both of which are known to stimulate KGF and SF expression by fibroblasts (31–34).

The concentration-dependent differences in effect of cyclosporin on normal compared with overgrown fibroblasts were surprising. Whereas cyclosporin increased KGF and SF expression by normal cells at all concentrations studied, this stimulatory effect was only seen in overgrown cultures treated with the highest drug concentration. At 100 ng/ml cyclosporin stimulated SF expression at both mRNA and protein levels, but inhibited KGF protein production in all three overgrown strains. This difference in effect is noteworthy given that KGF and SF share many biological effects on epithelial cells and previous studies have shown the two factors to be similarly regulated. The most striking findings, however, occurred in cultures treated with 500 ng/ml of drug. At this concentration cyclosporin markedly inhibited expression of both KGF and SF. The inhibition was seen at both the mRNA and protein levels, and the effect only occurred in overgrown cultures. The mechanisms underlying this concentration-dependent effect of cyclosporin are not clear. We and others have investigated the effects of cyclosporin on many parameters of gingival fibroblast function, including cell growth, production of ECM molecules, expression of proteases and their inhibitors and secretion of growth factors and cytokines (5–10). In all of these studies the concentration of added cyclosporin has been shown to dictate the extent of drug effect on the fibroblast response, but directly opposing effects by different concentrations of the drug have not been previously reported. Numerous studies have investigated the possible link between serum drug concentration and the development of gingival overgrowth. Although no clear relationship has been established, a number of studies suggest that drug concentration is an important factor in the clinical presentation of overgrowth. For example Hefti et al. (35) reported that those patients with trough blood levels greater than 400 ng/ml had an increased risk of developing overgrowth, while McGaw et al. (29) found significant correlations between salivary concentrations of cyclosporin and severity of overgrowth. Data presented in this study would support a role for drug concentration as a factor regulating epithelial changes in overgrown tissue.

The finding that the concentration-dependent inhibitory effect of cyclosporin was seen in all overgrown cultures but none of the normal cell populations was also striking. Phenotypic differences between these two cell types, in terms of growth potential and synthetic activity, have been reported (5, 9, 25), but never with such a clear distinction in response to the drug. This finding does lend support to the concept of a drug-induced selection and expansion of specific fibroblast subpopulations during the development of gingival overgrowth, resulting in a change in the overall phenotype of the resident cell population in overgrown tissue.

In conclusion, data presented in this study suggest the changes in epithelial histomorphology characterizing gingival overgrowth may be mediated by a drug-induced increase in KGF and SF expression by underlying gingival fibroblasts. The degree of this effect was dependent both on the drug concentration and the source of responding cells.

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