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Expression of cell-surface heparan sulfate proteoglycans in human cyclosporin-induced gingival overgrowth

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Background and Objective: Cyclosporin A-induced gingival overgrowth comprises a variety of signaling pathways (including growth factors and proteoglycans) that are still not completely understood. In the present study, gingival overgrowth was investigated in transplant patients receiving cyclosporin A (cyclosporin A group) and compared with gingival tissues never exposed to the drug (control group) by analyzing the gene expression of the cell-surface heparan sulfate proteoglycans syndecan-2, syndecan-4 and betaglycan.

Material and Methods: mRNA analysis was carried out by reverse transcription–polymerase chain reaction amplification of pooled samples from nine patients of the cyclosporin A group and six control subjects. The groups were compared by the Student's *t*-test.

Results: The expression of heparan sulfate proteoglycans was increased in the cyclosporin A group (165% for syndecan-2, 308% for syndecan-4, and 42% for betaglycan) compared with the control group.

Conclusion: Our findings agree with the current concept of cyclosporin A-induced gingival overgrowth and provide new evidence that its noncollagenous extracellular matrix is overexpressed.

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Gingival overgrowth is a common side-effect associated with the systemic use of cyclosporin A, an immunosuppressant extensively used in transplant patients to prevent graft rejection as well as in the treatment of immunological diseases (1). Many questions regarding this overgrowth, such as the dose-dependent action of the drug on gingival cells and the extracellular matrix, are still not understood. In this multifactorial tissue response, plaque simultaneously induces inflammatory reactions that impair the interpretation of the tissue response as a whole, and only fragmented evidence of the pathogenesis is available. However, recent studies have revealed some peculiarities that partly clarify this gingival overgrowth and differentiate it from other drug-induced gingival overgrowth.

Although marked by some controversies, the pathogenesis of cyclosporin A-induced gingival overgrowth is apparently characterized by the accumulation of extracellular matrix, mainly because of the deficient degradation of collagen and other matrix components (2–4), as demonstrated by the analysis of their gene expression (5–7). The resulting accumulation of collagen is accompanied by an increase in the synthesis of noncollagenous components, and the over-expression of cytokines and growth factors corroborates with the most accepted

paradigm of matrix stimulation and growth. An increase in the levels of interleukin-6 (8,9) and interleukin-1β (10), the fibrogenic factors platelet-derived growth factor-B (11) and transforming growth factor-β (12,13), and the proliferative factors keratinocyte growth factor (14–16), scatter factor (16) and basic fibroblast growth factor (17), has been reported. However, analysis of gingival crevicular fluid did not show differences in transforming growth factor-β levels in subjects with cyclosporin A-induced gingival overgrowth compared with normal gingiva (18), but rather an increase in interleukin-15 (19).

Besides the fact that the increase in extracellular matrix is not well understood in cyclosporin A-induced gingival overgrowth, there is no evidence of the expression of heparan sulfate proteoglycans, such as syndecans and betaglycan, in this lesion. Only generic evaluations of proteoglycans and glycosaminoglycans have been published (20–23). In a previous study, we observed a higher expression of the proteoglycan perlecan, found in basement membranes (24). Perlecan belongs to the family of heparan sulfate proteoglycans that show a high affinity for growth factors, especially basic fibroblast growth factor. These proteoglycans retain the growth factor in the tissue and regulate its bioavailability, modulating important proliferative signaling pathways and the regulation of neoangiogenesis (25).

Like perlecan, the family of syndecans (syndecan-1, -2, -3 and -4) and betaglycan are important proteoglycans in cell–cell and cell–matrix interactions, and are involved in signaling pathways that stimulate growth and angiogenesis in several human tissues. These proteoglycans have never been studied in cyclosporin A-induced gingival overgrowth and are known to be associated with growth factors that seem to be increased in this gingival lesion (17). In the present study we analyzed the mRNA levels of syndecan-2, syndecan-4 and betaglycan in human gingival tissues in order to test the hypothesis of their higher expression in cyclosporin A-induced gingival overgrowth.

Material and methods

Series and clinical procedures

The protocol was approved by the Ethics Committee of the University of São Paulo. A total of 30 subjects were included in the study. The sample was divided into two groups: 15 transplant patients aged 20–64 years (median age 36 years) (cyclosporin A group, 11 kidney transplants, four liver transplants; eight men, seven women) undergoing cyclosporin A therapy for at least 6 mo (daily dose ranging from 2.5 to 5.0 mg/kg) and exhibiting gingival overgrowth; and 15 nondrug users (control; six men, nine women) aged 38–50 years (median age 40 years). Each analysis comprised its own sample size, as follows: all 30 subjects in qualitative mRNA expression analysis; and nine subjects of the cyclosporin A group and six control subjects in semiquantitative mRNA expression analysis. Patients receiving phenytoin, sodium valproate, nifedipine, diltiazem, verapamil and/or azalide antibiotics, which are known to interfere with gingival extracellular matrix composition (26,27), were excluded, as were clinical cases of mild gingival overgrowth.

During periodontal evaluation, the score of gingival overgrowth severity was recorded for the patients of the cyclosporin A group, based on an adaptation of the semiquantitative index developed by Aas (28) for patients receiving phenytoin, as follows: 0, no overgrowth; 1, blunting of the gingival margin (mild gingival overgrowth); 2, moderate gingival overgrowth (less than one-third of the crown length); and 3, severe gingival overgrowth (more than one-third of the crown length). Ten patients presented with moderate gingival overgrowth and five with severe gingival overgrowth. Gingival samples of the cyclosporin A group were collected during corrective surgery of the anatomical deformity, as included in the periodontal treatment plan of the patient, after informed consent had been obtained. Control samples were obtained from gingival tissue that had never been exposed to cyclosporin A treatment and with clinically controlled inflammation, as determined during routine preprosthetic surgical procedures (for example, resective surgery for crown lengthening).

Analysis of transcriptional gene expression

Total RNA was isolated from the 30 gingival samples using TRIzol™ Reagent (Gibco BRL Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol, as described previously (24). RNA quality was assessed by measuring the absorbance at 260 and 280 nm (GeneQuant pro RNA/DNA Calculator; Amersham Pharmacia Biotech, Uppsala, Sweden) and its integrity was determined by agarose-gel electrophoresis. cDNA was synthesized using a commercially available pre-amplification system (Superscript II kit; Gibco BRL Life Technologies), and amplified by the polymerase chain reaction (PCR), according to the manufacturer's instructions. Initially, primers for syndecans 1, 2, 4 and for betaglycan were tested in human tissues known to be their positive controls. However, only syndecan-2, syndecan-4 and betaglycan provided a regular reaction pattern and a reliable validation method for semiquantitative analyses. Therefore, amplifications were carried out with these selected primers whose sequences are listed in Table 1. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was used as an internal standard for nonregulated expression. A reaction mixture of 50 μL was amplified in a thermal cycler (PCR PTC-200 Peltier; MJ Research, Watertown, MA, USA) using the following program: 5 min at 94°C, 35 cycles of 1 min at 94°C (denaturing), 1 min at 58°C (annealing) and 1 min at 72°C (extension), and a final extension step at 72°C for 10 min. The PCR products were subjected to agarose-gel electrophoresis (1% gel) with ethidium bromide staining, and band densities were determined using an image analysis system (EagleEye II; Stratagene, La Jolla, CA, USA). The identity of each PCR product was confirmed by digestion with restriction enzymes (Gibco BRL Life Technologies).

Table 1. Primer sequences and expected product length

Gene	Primer sequences		Length
GAPDH	Sense:	5′-AACGGGAAGCTCACTGGCATG-3′	305 bp
	Antisense:	5′-TCCACCACCCTGTTGCTGTAG-3′	
Syndecan-1	Sense:	5′-GATGGCTCTGGGGATGACTC-3′	811 bp
	Antisense:	5′-TGTTTGGTGGGCTTCTGGTAG-3′	
Syndecan-2	Sense:	5′-GGGAGCTGATGAGGATGTAG-3′	394 bp
	Antisense:	5′-CACTGGATGGTTTGCGTTCT-3′	
Syndecan-4	Sense:	5′-CGAGAGACTGAGGTCATCGAC-3′	528 bp
	Antisense:	5′-GCGGTAGAACTCATTGGTGG-3′	
Betaglycan	Sense:	5′-CTGTTCACCCGACCTGAAAT-3′	502 bp
	Antisense:	5′-CGTCAGGAGGCACACACTTA-3′	

bp, base pairs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

In a semiquantitative PCR assay, nine samples of moderate gingival overgrowth from the cyclosporin A group, and six samples from the control group, were used. The linear range of exponential amplification of the genes was determined using a cDNA pool obtained from the control group. For each primer pair (Table 1), PCR was performed with 18, 21, 24, 27, 30, 35 and 40 amplification cycles. The products obtained in each amplification were visualized on a 1% agarose gel. After band densitometry (Eagle-Eye II; Stratagene), the linear range of amplification was determined for each gene, and the number of cycles whose product was in the center of this range was chosen. This standardization was then used for subsequent amplifications of cDNA pools obtained from the two groups (cyclosporin A and the control) and the amount of cDNA used was fixed at 1 μg in order to permit direct comparison between the amplified band densities. For this, the PCR products were analyzed by gel electrophoresis, documented and quantified by densitometry.

Statistical analysis

The semiquantitative PCR assays were repeated at least five times. For electrophoresis gel densitometry, a standard size area was defined for the bands. The background density of the gel was recorded in an identical area and subtracted from the calculations. The densities of the bands, corresponding to each primer studied, were divided by the density of the standard DNA ladder band with a similar size on the gel (relative density) and then divided by the relative density for glyceraldehyde-3-phosphate dehydrogenase calculated in the same pooled sample (normalized density). The results are reported as the mean (± standard error) of normalized densities of five PCR repetitions obtained for each pooled sample.

Once a normal distribution was confirmed by the Kolmogorov–Smirnov test, differences between the cyclosporin A and control groups were analyzed by the Student's *t*-test, and statistical significance was set at the 95% confidence level. Computation was performed using a software program (SPSS, Version 13.0; SPSS Inc., Chicago, IL, USA).

Results

The total RNA extracted from the gingiva samples of the two groups was free of protein, as demonstrated by the 260/280 nm absorbance ratios ranging from 1.6 to 1.8. Electrophoresis of total RNA on a 1% agarose gel showed the integrity of 28S and 18S ribosomal RNA, indicating that the RNA preparations were intact. The qualitative PCR products obtained with the primers for syndecan-1, syndecan-2, syndecan-4, betaglycan and glyceraldehyde-3-phosphate dehydrogenase were digested with *Nci*I, *Hin*dII, *Xba*I, *Afl*III and *Apa*I, respectively. The fragments found after digestion with the appropriate enzyme were of the expected size, confirming their identity (Fig. 1).

The semiquantitative PCR protocol was validated after analysis of curves of increasing amplification cycles obtained for glyceraldehyde-3-phosphate dehydrogenase, syndecan-1, syndecan-2, syndecan-4 and betaglycan. As mentioned previously, syndecan-1 was not included in this method because it provided an irregular reaction pattern. The linear range of amplification was determined for each gene, and the number of cycles whose product was in the center of this range was chosen. The number of cycles selected for the amplifications was 24 for glyceraldehyde-3-phosphate dehydrogenase and 35 for the heparan sulfate proteoglycans. Figure 2 shows the run of the semiquantitative PCR assay for all the genes studied. For the heparan sulfate proteoglycan primers, the relative density of each band was normalized against the relative density of the glyceraldehyde-3-phosphate dehydrogenase band in the same gel. mRNA expression was higher in the cyclosporin A group than in the control group for the three heparan sulfate proteoglycans analyzed, with the cyclosporin A/control ratio being 2.65 times higher for syndecan-2, 4.08 times higher for syndecan-4 and 1.42 times higher for betaglycan ($p < 0.0001$) (Table 2).

Discussion

The present study is a continuation of our line of research regarding the role of heparan sulfate proteoglycans in cyclosporin A-induced gingival overgrowth. In a previous study, involving the same series as in the present investigation, we observed a 54% higher mRNA expression of perlecan in the cyclosporin A group compared with subjects not exposed to the drug (24). In our search for data that corroborate our results, we did not find any other study in the literature evaluating heparan sulfate proteoglycan expression in cyclosporin A-induced gingival overgrowth, or even in normal human gingiva.

Using reverse transcription–PCR and northern blotting, Shimazu *et al.* (29) observed the qualitative expression of mRNA for syndecan-2, syndecan-4 and fibroblast growth factor receptor type 1 in fibroblast cultures of human periodontal ligament. Worapamorn *et al.* (30) also demon-

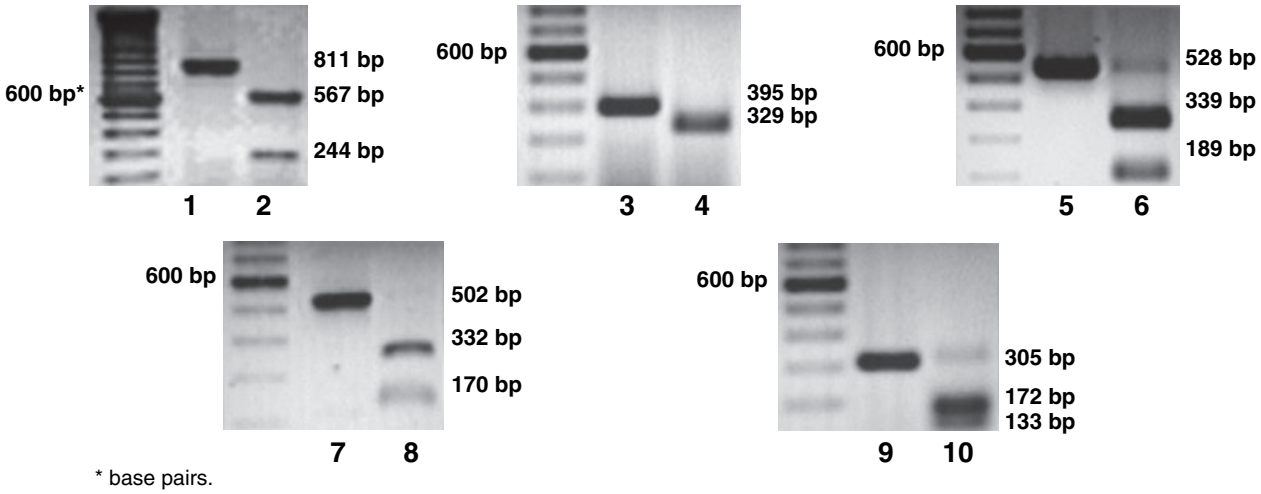

Fig. 1. Agarose-gel electrophoresis of polymerase chain reaction amplifications of one cyclosporin A sample. Lane 1, syndecan-1 (811-base pair product); lane 2, *Nci*I restriction products (567 and 244 base pairs); lane 3, syndecan-2 (395-base pair product); lane 4, *Hin*dII restriction products (329 and 66 base pairs); lane 5, syndecan-4 (528-base pair product); lane 6, *Xba*I restriction products (339 and 189 base pairs); lane 7, betaglycan (502-base pair product); lane 8, *Afl*III restriction products (332 and 170 base pairs); lane 9, glyceraldehyde-3-phosphate dehydrogenase (305-base pair product); and lane 10, *Apa*I restriction products (172 and 133 base pairs).

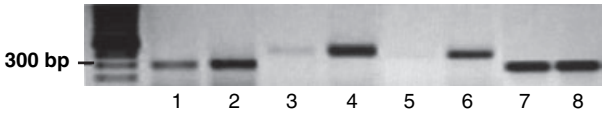

Fig. 2. Semiquantitative polymerase chain reaction using 1 μg of cDNA pools obtained from the two groups (cyclosporin A and control). Lane 1, syndecan-2, control; lane 2, syndecan-2, cyclosporin A; lane 3, syndecan-4, control; lane 4, syndecan-4, cyclosporin A; lane 5, betaglycan, control; lane 6, betaglycan, cyclosporin A; lane 7, glyceraldehyde-3-phosphate dehydrogenase, control; lane 8, glyceraldehyde-3-phosphate dehydrogenase, cyclosporin A.

Table 2. Comparative mean normalized density (MND)[a]

Gene	Cyclosporin A (MND)	Control (MND)	Transcriptional increase (cyclosporin A/control)	*p*-Value
Syndecan-2	0.220 ± 0.045	0.083 ± 0.008	265	< 0.0001
Syndecan-4	0.535 ± 0.152	0.131 ± 0.013	408	< 0.0001
Betaglycan	0.882 ± 0.098	0.619 ± 0.100	142	< 0.0001

[a]Means ± standard error obtained for the repetitions of polymerase chain reaction amplification for each pooled sample.

strated qualitatively the expression of syndecan-2, syndecan-4, glypican and betaglycan in rat periodontal cell lines. In the present study, syndecans and betaglycan were analyzed for the first time in human gingival tissues. Like perlecan, these heparan sulfate proteoglycans are important metabolic signaling molecules in several other human tissues. As they contain heparan sulfate in their structure, these proteoglycans present high affinity for basic fibroblast growth factor, regulating angiogenesis, among other phenomena (25,31). Yoshida *et al.* (17) indicated basic fibroblast growth factor as one of the growth factors that might be increased in cyclosporin A-induced gingival overgrowth, based on observations made in cultures of rat gingival fibroblasts stimulated with the drug. As for basic fibroblast growth factor, heparan sulfate proteoglycans function as receptors for acidic fibroblast growth factor, granulocyte–macrophage colony-stimulating factor and the cytokines interleukin-3 and interferon-γ (31), permitting that the extracellular matrix serves as a large reservoir of tissue growth stimulators.

The present study provides semiquantitative data on human gingival tissues comprising a higher transcription of mRNA for syndecan-2, syndecan-4 and betaglycan in the cyclosporin A group compared with the control group. The increased vascular component and the characteristics of the inflammatory infiltrate may explain, in part, the higher expression of these molecules observed in the cyclosporin A group gingival samples.

Syndecans modulate the phenotype, adhesion, proliferation, differentiation and migration of cells. These molecules function as growth factor and cytokine coreceptors, mediating the binding and modifying their actions (32). Besides regulating growth factor bioavailability, syndecans individually stimulate important mitogenic signaling pathways in tissues. Studies on angiogenesis have shown that basic fibroblast growth factor requires not only its receptor fibroblast growth factor receptor type 1, but also syndecan-4 to act during thrombin-mediated mitogenesis (33). Whitworth *et al.* (34) observed labeling of active

neoangiogenesis mediated by basic fibroblast growth factor in tissue adjacent to ovarian adenocarcinomas, with the participation of syndecan-3.

It is important to consider that the complexes formed between heparan sulfate proteoglycans and other factors remain active until they are released by enzymes such as plasmin, heparitinase and other matrix metalloproteinases (MMPs) (35). However, convergence exists in the literature, indicating reduced expression and activity of MMPs in cyclosporin A-induced gingival overgrowth (3,5,6). The hypothesis of a possible cyclosporin A-mediated down-regulation of the release of complexes formed between heparan sulfate proteoglycans and growth factors deserves special attention in future studies. This hypothesis offers the possibility that the increased expression of growth factors might be a compensatory response to the reduced activity of MMPs to make these factors available.

In addition to syndecans, we found a higher mRNA expression for betaglycan (transforming growth factor-β1 type III receptor) in the cyclosporin A group. Transforming growth factor-β, in turn, signals a variety of metabolic functions and its expression has been shown to be increased under the action of cyclosporin A (12). Transforming growth factor-β binds to the protein core of the proteoglycans through a protein kinase receptor. Whereas transforming growth factor-β is mainly controlled by the proteoglycans decorin and biglycan, betaglycan regulates transforming growth factor-β by presenting this factor to its signaling receptors, thus forming a ternary compound (36–38). If, on the one hand, betaglycan increases the binding capacity of transforming growth factor-β to its sites, on the other hand it competes for the same binding sites, depending on modulatory factors (39). Betaglycan is also able to eliminate differences between the transforming growth factor-β isoforms and binds to basic fibroblast growth factor through its heparan sulfate chains.

Heparan sulfate plays a key role in basic fibroblast growth factor binding to its receptor, by increasing their affinity (40). Although few modulatory mechanisms have been studied, Rapraeger *et al*. (41) reported that the binding blockade between heparan sulfate proteoglycans and basic fibroblast growth factor reduces binding of the growth factor to its receptor, inhibits its action in Swiss 3T3 fibroblasts and induces cell differentiation normally repressed by basic fibroblast growth factor.

In vivo biological phenomena involve signaling and feedback mechanisms in a dynamics that makes its investigation difficult. Cells are exposed to different cytokines and growth factors in a combined and sequential manner, and it is believed that each gene is induced within a characteristic period of time since exposure of the cell to the biological stimuli (32). In addition, the heparan sulfate proteoglycans studied are extensively modified during cellular expression. Protein alterations, and especially structural changes in the glycosaminoglycans chains, are common events. Most changes affect the number of chains, chain length and the sulfation pattern, and result in transformations of the type and function of the proteoglycan (42).

Therefore, our results provide data regarding higher mRNA levels for the heparan sulfate proteoglycans analyzed in moderate and severe cyclosporin A-induced gingival overgrowth. These findings should be interpreted as a partial event of matrix proliferation because translational parameters still need to be elucidated. However, therapeutic perspectives for this overgrowth should consider the local blockade of heparan sulfate proteoglycan expression or heparan sulfate bioavailability.

Our findings agree with the current concept of cyclosporin A-induced gingival overgrowth and provide new evidence that its collagen accumulation is accompanied by the expression of important noncollagenous matrix components. As these pathways become elucidated in greater detail, the pathogenesis of this type of gingival overgrowth will be better understood and its treatment will be improved.

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