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Gingival tissue proteoglycan and chondroitin-4-sulphate levels in cyclosporin A-induced gingival overgrowth and the effects of initial periodontal treatment

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Abstract

Objectives: Cyclosporin A (CsA) is a potent immunosuppressive drug used in organ transplant patients to prevent graft rejection. CsA-induced gingival overgrowth is one of the side effects of this drug and its pathogenesis is still unclear. The present study was planned to comparatively analyse total proteoglycan (PG) and chondroitin-4-sulphate (C4S) levels in CsA-induced overgrown gingival tissue samples obtained before and after initial periodontal treatment and to compare these findings with the situation in healthy gingiva.

Material and Methods: Gingival tissue samples were obtained from nine patients with CsA-induced gingival overgrowth before and 4 weeks after initial periodontal treatment including oral hygiene instruction and scaling and also from 10 healthy control subjects. Total PG and C4S levels were determined by biochemical techniques. PG levels were analysed using modified Bitter and Muir method. C4S assay was carried out using chondroitin sulphate lyase AC and chondroitin-6 sulphate sulphohydrolase enzymes. The results were tested statistically using non-parametric tests.

Results: All clinical measurements in the CsA-induced gingival overgrowth group demonstrated significant reductions 4 weeks after initial periodontal treatment ($p < 0.05$). There was no significant difference between the levels of baseline total PG in CsA-induced gingival overgrowth and healthy control groups ($p > 0.05$). The gingival tissue levels of PG in CsA-induced gingival overgrowth group decreased significantly 4 weeks after treatment ($p = 0.043$). Gingival tissue C4S levels in the overgrowth group were significantly higher than the healthy control group at baseline ($p = 0.000$). C4S levels of the overgrowth group were significantly reduced after treatment ($p = 0.033$), but these levels were still significantly higher than the healthy control group ($p = 0.000$).

Conclusion: The observed prominent increase in gingival tissue C4S levels may be interpreted as a sign of an increase in C4S synthesis in CsA-induced gingival overgrowth. Furthermore, remission of clinical inflammation by means of initial periodontal treatment had a positive effect on tissue levels of these extracellular matrix molecules.

Key words: chondroitin-4-sulphate/gingival overgrowth; cyclosporin A; gingiva; proteoglycan/gingival overgrowth

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Cyclosporin A (CsA) is an immunosuppressive drug used in organ transplant patients to prevent graft rejection (Kahan 1989). Cyclosporin therapy is associated with a number of side effects including nephrotoxicity, hepatotoxicity and gingival overgrowth. The reported incidence of CsA-induced gingival overgrowth ranges from 8% to 70%

and is often associated with poor dental hygiene and gingival inflammation (Rostock et al. 1983, Pernu et al. 1992).

The extracellular matrix (ECM) of gingival connective tissue is composed principally of collagens and non-collagenous proteins (Embery et al. 2000). Proteoglycans (PGs) and glycosaminoglycans (GAGs) are integral components of the non-collagenous part of ECM and constitute a major group of non-collagenous molecules. Prominent functions of PGs are maintenance of tissue integrity, tissue hydration, collagen fibre formation and providing resistance against pressure (Bartold et al. 1983, Bartold 1987, Uitto & Larjava 1991). GAGs which are the most important carbohydrate components of PGs exist in covalently bound form rather than free state (Bartold 1987). Particularly, four species of GAGs are present in gingiva; dermatan sulphate, chondroitin sulphate, heparan sulphate and hyaluronic acid (Sakamoto et al. 1978, Bartold 1987). Chondroitin-4-sulphate (C4S) is a major GAG component in gingival connective tissue, and the distribution of C4S is widespread throughout the gingival connective tissue (Bartold et al. 1982, 1984, Shibutani et al. 1989, Beighton et al. 1992).

The exact mechanism of pathogenesis of CsA-induced gingival overgrowth is not clear, although it has been suggested that resident fibroblasts are producing more collagen and other ECM molecules than normal (Rees 1998). The side effects of CsA remain to be of significant clinical interest and therefore, their mechanisms deserve further comprehensive investigations. Only relatively few studies have investigated the effects of CsA on human gingival PG and GAG composition (Zebrowski et al. 1994, Mariani et al. 1996, Newell & Irwin 1997, Rocha et al. 2000, Martins et al. 2003). However, conflicting data have been presented regarding the alteration of GAG content in CsA-induced gingival overgrowth lesions in terms of increases or no significant changes in GAG content. Mariani et al. (1996) have reported that CsA-induced overgrown gingival tissue contained higher levels of GAGs than the control. Furthermore, increased levels of these compounds have been found by CsA induction in gingival fibroblast cultures (Zebrowski et al. 1994, Newell & Irwin 1997). However, Rocha et al. (2000) indicated that there were no significant differences in the total and relative amounts of GAGs in CsA-induced overgrown gingiva compared with the healthy gingiva. In a more recent study, no significant differences were found between healthy tissue and CsA-induced overgrown gingiva with regards to the molecular size distribution of hyluronic acid and sulphated GAGs (Martins et al. 2003). Obviously, the nature of CsA-related alterations in gingival PG and GAG composition remains unclear.

Our previous study revealed significantly lower PG and C4S levels in inflamed gingiva obtained from the periodontitis groups when compared with the healthy control group (Vardar et al. 2004). It is well documented that CsA-induced gingival overgrowth is often clinically associated with gingival inflammation (Hassell & Hefti 1991, Seymour et al. 1996, Kantarci et al. 1999). The severity of drug-induced gingival overgrowth increases in the presence of poor plaque control and gingival inflammation (Thomason et al. 1993). The pathogenesis of CsA-induced gingival overgrowth has been reported to be multifactorial, with one of the main factors being drug effects and the effects of plaque-related inflammation (Seymour et al. 1996). Therefore, elimination of the plaque-related inflammation by means of periodontal treatment may help better clarifying the sole effects of CsA on the PG and C4S content of CsA-induced overgrown gingiva. Therefore, the aims of the present study were twofold; (1) to determine the composition of PG and C4S in CsA-induced gingival overgrowth and (2) to investigate whether gingival inflammation affects these non-collagenous macromolecules in this entity by evaluating total gingival PG and C4S levels also after initial periodontal treatment.

Material and Methods

The study was approved by the Ethics Committee of Ege University, Izmir, and written informed consent was obtained from each subject before their enrollment in the study. A total of 19 subjects (12 male, seven female) were included in this study, of whom nine were renal transplant patients exhibiting CsA-induced gingival overgrowth (33.6 ± 10.5 years of age) and 10 were subjects with clinically healthy periodontium without any sign of gingival overgrowth (34.4 ± 12.6 years of age). Renal transplant patients followed by the Department of Nephrology, Ege University, Izmir have been taking CsA for more than 6 months at a dose adjusted to maintain stable serum levels of 80 to 300 ng/ml. None of these patients had a history of any other drug related with gingival overgrowth. The control group comprised systemically and periodontally healthy individuals and this group was part of a recently published study (Vardar et al. 2004). The clinical periodontal parameters including probing pocket depth (PPD), plaque index (PI) (Sillness & Löe 1964), papilla bleeding index (PBI) (Saxer & Mühleman 1975) and hyperplastic index (HI) (Pernu et al. 1992) were recorded at the sampling sites at baseline. The degree of gingival overgrowth (HI) was classified in four categories based on the criteria of Angelopoulos & Goaz (1972) modified by Pernu et al. (1992). CsA-treated patients who had moderate or severe gingival overgrowth and did not have alveolar bone loss were selected for our study. Williams probe was used for clinical measurements and all measurements were performed by the same investigator (S. V.).

In CsA-treated patients, inter-dental sites in single rooted teeth with a HI score of 2 or 3 were selected as sampling sites, and tissue samples were obtained as described previously (Vardar et al. 2004) before the initial periodontal treatment. In the healthy control group, gingival tissue specimens were taken during tooth extractions for orthodontic reasons or crown lengthening procedures. One tissue sample from each subject was obtained which was then minced and divided into two parts. Half of the tissue was put into ethanol buffer for PG analysis while the rest was put into dry propylene tubes for C4S analysis. All samples were stored at -40°C until the day of laboratory analysis.

The patients received initial periodontal treatment in the sampling sites consisting instruction on daily plaque control and scaling procedures under antibiotic coverage. Periodontal therapy on other teeth was also provided as required. Clinical periodontal measurements as well as gingival tissue sampling were repeated on the same sampling sites during periodontal surgery 4 weeks after the initial periodontal treatment.

Extraction and analysis of PGs and C4S were performed by biochemical

techniques as previously described (Vardar et al. 2004). The PG content in the sample was expressed as mg PG/100 mg tissue and C4S as μmol/100 mg tissue.

Statistical analysis

The results of this study were tested statistically using non-parametric tests. Baseline and post-therapy values in the CsA-induced gingival overgrowth group were compared by Wilcoxon signed ranks test. Inter-group comparisons were done by Mann–Whitney *U* test. Gender and smoking status differences between the groups were tested by chi-squared test. The relations between the clinical parameters and non-collagenous matrix molecules in gingiva were tested by Spearman correlation analysis. $p<0.05$ was chosen as the level of significance.

Results

There was no statistically significant difference between the two study groups in terms of demographic characteristics ($p>0.05$) (Table 1). Baseline PPD, PI, PBI values of the CsA-induced gingival overgrowth group were significantly higher than those of the healthy control group ($p<0.05$). Four weeks after the initial periodontal treatment, all clinical measurements in the CsA-induced gingival overgrowth group showed statistically significant reductions ($p<0.05$), but they were still statistically higher than the healthy control group (Table 2).

No significant difference was found for baseline total PG level between CsA-induced gingival overgrowth and healthy control groups ($p>0.05$). Four weeks after treatment, the gingival tissue level of PG in CsA-induced gingival overgrowth group significantly decreased ($p=0.043$) and this level was similar to healthy control group ($p>0.05$) (Table 3).

Gingival tissue level of C4S in the CsA-induced gingival overgrowth group was significantly higher than the healthy control group at baseline ($p=0.000$). C4S levels of overgrown gingiva were significantly reduced after initial periodontal treatment ($p=0.033$), but these levels were still significantly higher than that of the healthy control group ($p=0.000$) (Table 3).

None of the study groups exhibited significant correlations between any of the clinical periodontal parameters and the total PG or C4S levels in the gingival tissue samples (data not shown).

Table 1. Demographic characteristics of the study groups

	CsA-GO ($N=9$)	H ($N=10$)
Age (mean ± SD)	33.6 ± 10.5	34.4 ± 12.6
Female/male	3/6	4/6
Smoker	0	3

CsA-GO, Cyclosporin A-induced gingival overgrowth; H, Healthy.

Table 2. Clinical periodontal parameters of the study groups (mean ± SD)

	CsA-GO ($N=9$)		H ($N=10$)
	Baseline	4 weeks	Baseline
PPD (mm)	5.2 ± 1.1*	3.2 ± 0.8*†	1.51 ± 0.3
PBI	2.7 ± 0.9*	0.9 ± 0.7*†	0.04 ± 0.09
PI	1.69 ± 0.6*	0.6 ± 0.4*†	0.12 ± 0.1
HI	2.77 ± 0.4	1.50 ± 0.5†	0

CsA-GO, Cyclosporin A-induced gingival overgrowth; H, Healthy; PPD, probing pocket depth; PBI, papilla bleeding index; PI, plaque index; HI, hyperplastic index.
*Significant difference from the healthy control group ($p<0.05$).
†Significantly lower than the baseline values within the same group ($p<0.05$).

Table 3. Gingival tissue levels of total PG (mg/100 mg tissue) and C4S (μmol/100 mg tissue) in the study groups (mean ± SD)

	CsA-GO ($N=9$)		H ($N=10$)
	Baseline	4 weeks	Baseline
PG	2.14 ± 0.5	1.57 ± 0.6†	1.9 ± 0.5
C4S	4.35 ± 0.9*	3.4 ± 0.5*†	2.04 ± 0.4

CsA-GO, Cyclosporin A-induced gingival overgrowth; H, Healthy; PG, proteoglycan; C4S, chondroitin-4-sulphate.
*Significant difference from the healthy control group ($p<0.05$).
†Significantly lower than the baseline values within the same group ($p<0.05$).

Discussion

Almost all of the studies investigating the non-collagenous matrix composition of drug-induced overgrown gingiva have been carried out in gingival tissue samples obtained after completion of initial periodontal therapy in an attempt to minimize the influence of gingival inflammation which is likely to affect the PGs and GAGs (Dahllöf et al. 1984, 1986, Rocha et al. 2000, Martins et al. 2003). To our knowledge, this is the first communication evaluating gingival tissue levels of total PG and C4S in CsA-induced overgrown gingiva obtained from the same sites both before and 4 weeks after the initial phase of periodontal therapy. This methodology enabled us to investigate the role of inflammation on gingival tissue levels of total PG and C4S. Furthermore, the effect of initial phase of periodontal therapy on these molecules could be determined. In addition, the age of the subjects in the CsA-induced gingival overgrowth and the healthy control groups were matched in the present study considering the effect of age on the PG and GAG content of gingiva (Sakamoto et al. 1978).

The first studies investigating the role of PGs and GAGs in drug-induced gingival overgrowth have been performed in phenytoin-induced overgrown gingiva (Ballard & Butler 1974, Kantor & Hassell 1983, Dahllöf et al. 1984, 1986). It is quite clear that the amounts of GAGs increase in gingival tissue affected by phenytoin. However, there is no similar consensus about CsA-induced overgrown gingiva. Conflicting results have been reported on the composition of non-collagenous matrix in overgrown gingiva of CsA-treated patients. Some studies suggested an increase in the synthesis of PGs and GAGs in CsA-induced overgrown gingiva (Wondimu et al. 1995, Mariani et al. 1996, Newell & Irwin 1997). More specifically, Mariani et al. (1996) have found that CsA-induced overgrown gingiva contained increased amounts of

both sulphated and non-sulphated GAGs. These researchers proposed that most of the dimensional changes are because of increased production of amorphous ground substance. On the other hand, a marked increase in the expression of perlecan, a typical basement membrane PG, has been documented in this entity (Gnoatto et al. 2003). CsA has been reported to stimulate fibroblast proliferation (Bartold 1989, Barber et al. 1992) and GAG production (Newell & Irwin 1997). In the present study, we found a slight increase in total PG level of overgrown gingiva, while significant increase in the level of C4S was observed. These findings may suggest that it is more likely that the production of C4S increases in CsA-induced gingival overgrowth. In case of a decrease in collagenolytic activity, the amount of PGs would have been increased significantly. However, this was not the case in the present study. The results of the present study may provide additional support in favour of an increase in GAG synthesis in CsA-induced gingival overgrowth.

CsA-induced gingival overgrowth may arise from a decrease in degradation of the ECM components of gingival connective tissue as well as from an increase in synthesis, or it may include the combination of both of these mechanisms. However, we previously showed that CsA therapy did not have a significant effect on GCF MMP-8 and MMP-9 levels, although lower GCF PMN-elastase levels have been detected (Atilla et al. 2001). A number of studies have investigated the direct effects of cyclosporine on ECM synthesis by fibroblasts. It has been assumed that CsA alters gingival fibroblast activity through effects on various cytokines and growth factors (Williamson et al. 1994, Nares et al. 1996). CsA has an inhibitory effect on MMP-1 and MMP-3 expression in overgrown gingiva (Thomason et al. 1998, Bolzani et al. 2000, Hyland et al. 2003). Furthermore, CsA inhibits collagenase gene expression in healthy gingival tissue fibroblasts (Sugano et al. 1998). Studies have demonstrated that CsA inhibited collagenase activity of fibroblasts derived from a healthy individual with non-inflamed gingiva (Fan & Scott 1989, Tipton & Dabbous 1989, Tipton et al. 1990). Most fibroblast strains showed significantly reduced collagenolytic activity, but some subpopulations were unaffected and some showed enhanced activity, (Tipton et al. 1991). The heterogeneity of the collagenolytic response and ECM synthesis of different gingival fibroblast strains to CsA treatment may partly explain the variable gingival response noted in patients taking this drug (Seymour et al. 1996). It has been suggested from above-mentioned studies that CsA-induced inhibition of collagenolytic activity and the fibroblast heterogeneity may underlie the mechanisms of gingival overgrowth. Our investigation into the tissue contents of C4S has been confirmed by this opinion.

An increased amount of GAGs in the ground substance leads to a greater amount of bound water and to a higher volume and an increased osmotic pressure in the ECM. This would produce waterlogged connective tissue and would account for gingival turgor which has all the characteristics of local gingival oedema (Mariani et al. 1996). Zebrowski et al. (1994) suggested that the increased tissue colloid osmotic pressure and resultant tissue oedema accompanying the accumulation of non-sulphated GAGs in gingival tissue is an important part of the overgrowth mechanisms. The present finding of significantly increased C4S levels is in line with the previous reports claiming the role of the change in GAG composition leading to water imbalance and eventual increase in tissue volume.

CsA-induced gingival overgrowth is often clinically associated with gingival inflammation (Hassell & Hefti 1991, Seymour et al. 1996, Kantarci et al. 1999). The severity of drug-induced gingival overgrowth increases in the presence of poor plaque control and gingival inflammation (Thomason et al. 1993). However, it remains unclear whether plaque is a contributory factor or a consequence of the gingival changes. Any alteration in gingival contour will compromise mechanical plaque removal and the gingival inflammation will further contribute to the gingival changes. Presumably, CsA alone is not directly influencing the fibroblasts' cellular metabolism, but rather acts through indirect networks via locally released inflammatory mediators or bacterial products (James et al. 1995). Inflammatory mediators have been proposed to act as co-factors for inducing drug-induced gingival overgrowth. In the present study, total PG and C4S levels in gingiva were evaluated before and after initial periodontal treatment in order to investigate the effects of gingival inflammation on gingival tissue levels of these molecules in CsA-induced gingival overgrowth. The present finding of reductions in tissue levels of these molecules along with the improvement in clinical periodontal indices may support the contributory role of inflammation in CsA-induced gingival overgrowth.

In in vitro studies, CsA has been shown to negate the inhibitory effect of lipopolysaccharide (LPS) and maintain the ability of the drug to stimulate fibroblast proliferation in the presence of LPS at a concentration that would normally inhibit fibroblast proliferation (Bartold 1989, James et al. 1995). *Escherichia coli* LPS coupled with CsA caused a synergistic effect in healthy fibroblasts in terms of PG synthesis (Barber et al. 1992). The reversal of LPS inhibition of gingival fibroblast proliferation by CsA may explain, in part, why gingival overgrowth is most prominent in areas of heavy dental plaque accumulation (Bartold 1989). In our previous study (Vardar et al. 2004), gingival tissue C4S levels were found to be decreased in aggressive and chronic periodontitis patients when compared with the healthy controls. On the contrary, the present study revealed increased levels of C4S in the presence of inflammation in CsA-induced overgrown gingiva that points out the role of CsA in converting the effect of LPS on tissue levels of this molecule.

As a conclusion, the observed prominent increase in gingival tissue C4S levels can be interpreted as a sign of an increase in GAG synthesis in CsA-induced gingival overgrowth. Furthermore, remission of clinical inflammation by means of initial periodontal treatment had a positive effect on tissue levels of PG and C4S. However, further studies comparatively evaluating markers of both synthesis and degradation of ECM components in the same samples may better clarify the exact mechanism of deviation from homeostasis in CsA-induced gingival overgrowth.

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