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Gingival crevicular fluid and serum matrix metalloproteinase-8 and tissue inhibitor of matrix metalloproteinase-1 levels in renal transplant patients undergoing different immunosuppressive therapy

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Abstract

Aim: We investigated gingival crevicular fluid (GCF) and serum matrix metalloproteinase-8 (MMP-8) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) levels from renal transplant patients receiving cyclosporine-A (CsA) and having gingival overgrowth (GO), from patients receiving CsA therapy and having no GO and patients receiving tacrolimus therapy.

Material and Methods: GCF samples were collected from sites with GO (GO+) and without GO (GO −) in CsA patients having GO; and GO − sites in CsA patients having no GO; sites from tacrolimus, gingivitis and healthy subjects. GCF and serum MMP-8 and TIMP-1 levels were determined by a time-resolved immunofluorometric assay (IFMA) and enzyme-linked immunosorbent assay.

Results: GO+ sites in CsA patients having GO had elevated GCF MMP-8 levels compared with those of CsA patients having no GO, tacrolimus and healthy subjects ($p < 0.005$), but these levels were similar to those of gingivitis. The GCF MMP-8 level was higher in GO+ compared with GO − sites in CsA patients having GO ($p < 0.05$). GCF TIMP-1 levels were similar between groups. Tacrolimus patients had lower GCF MMP-8 levels than gingivitis ($p < 0.005$), but levels similar to the healthy group.

Conclusion: These results show that CsA and tacrolimus therapy has no significant effect on GCF MMP-8 levels, and gingival inflammation seems to be the main reason for their elevations.

Key words: gingival crevicular fluid; gingival overgrowth/pathogenesis; MMP-8, TIMP-1

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Conflict of interest and source of funding statement

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Cyclosporine-A (CsA) is a potent immunosuppressive drug that has been used successfully to prevent graft rejection in organ transplant patients as well as for the treatment of various systemic disorders (Kahan 1989, Hassell & Hefti 1991). Gingival overgrowth (GO) is one of the well-recognized side effects of CsA therapy (Rateitschak-Pluss et al. 1983). The pathogenesis of GO may be multifactorial in nature, involving poor dental hygiene, gingival inflammation, genetics and drug dosage (Pernu et al. 1992, Seymour et al. 1996, 2000). Tacrolimus or FK506 is a macrolide molecule that was introduced as an immunosuppressive drug for use to prevent organ rejection (Berloco et al. 2001). Although structurally different from CsA, it has a similar mechanism of action at the molecular level (Jacobson et al. 1998). It has more potent immunosuppressive properties compared with CsA and is known to be an effective alternative to CsA in primary and rescue therapy (Peters et al. 1993, Spencer et al. 1997, Jacobson et al. 1998). Tacrolimus has many unwanted side effects similar to CsA (Jacobson et al. 1998). However, the prevalence and severity of GO is less in patients taking tacrolimus compared with CsA (Ellis et al. 2004, Sekiguchi et al. 2007).

GO results from the alterations in the turnover of epithelial and connective tissue components as well as alterations in extracellular matrix (ECM) metabolism. Collagen metabolism is precisely regulated by a homeostatic balance between collagen synthesis and degradation (Kataoka et al. 2000). Several studies have shown that interstitial collagen is the main target of CsA, and an altered collagen metabolism is associated with this pathology (Bolzani et al. 2000, Kataoka et al. 2000, Hyland et al. 2003). Whether the accumulation of ECM is attributable to an increased synthesis of matrix components or changes in collagen degradation remains unclear (Bolzani et al. 2000, Kataoka et al. 2000, Hyland et al. 2003). Researchers have suggested that a reduced production of matrix metalloproteinases (MMPs) or an increased production of the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) levels by gingival fibroblasts in response to CsA may contribute to the ECM accumulation seen in CsA-induced GO (Duymelinck et al. 1998, Sugano et al. 1998, Thomason et al. 1998, Bolzani et al. 2000, Yamada et al. 2000, Dannewitz et al. 2006). MMPs can degrade almost all ECM and basement membrane components as well as process serpins, growth factors, pro- and anti-inflammatory cytokines and apoptotic signals to modulate immune responses (Hu et al. 2007, Page-McCaw et al. 2007). Thus, previously surrogate MMPs have been implicated in the mediation of tissue destruction in inflammatory diseases, but recently certain MMPs such as MMP-8 have been found to also exhibit defensive anti-inflammatory properties by regulating inflammatory cell activity and apoptosis (Hu et al. 2007, Page-McCaw et al. 2007).

The role of MMPs and TIMP-1 in CsA GO was investigated previously in the in vitro studies (Tipton et al. 1991, Duymelinck et al. 1998, Sugano et al. 1998, Thomason et al. 1998, Bolzani et al. 2000, Yamada et al. 2000, Dannewitz et al. 2006). The findings from these studies suggest that the effect of CsA on MMP production is unclear. There are a limited number of clinical studies investigating the level of MMP-8 and TIMP-1 in the gingival tissue or gingival crevicular fluid (GCF) samples from patients with CsA GO (Thomason et al. 1998, Atilla et al. 2001). Also, the levels of MMP-8 and TIMP-1 in patients under tacrolimus therapy have not been investigated so far. It can be hypothesized that altered GCF and serum levels of MMP-8 and TIMP-1, and the MMP-8/TIMP-1 ratio in GCF are related to the CsA-induced GO. Furthermore, especially serum MMP-8 and TIMP-1 levels may reflect systemic chronic inflammatory and immune responses (Soder et al. 2006, Sorsa et al. 2006, Tuomainen et al. 2007). Therefore, the aim of the present study was to investigate the presence of GCF and serum MMP-8 and TIMP-1 levels in renal transplant patients receiving CsA therapy and having GO, patients receiving CsA therapy and having no GO and patients receiving tacrolimus therapy.

Material and Methods

Study population

A total of 143 subjects (68 male and 75 female) were included in the present study. They were recruited from the Ege University School of Dentistry Department of Periodontology over a period of 1 year between 2004 and 2005. The purpose of the study, was completely explained to each subject before entering the study and informed consent in accordance with Helsinki declaration was obtained from each subject. Complete medical and dental histories were taken from all subjects. Renal transplant patients who have been followed by the Nephrology Department at the University of Ege, İzmir, were considered for this study. The immunosuppression protocols used for kidney transplantation were organized and monitored by the medical team as shown in Table 1. These patients had been taking either CsA or tacrolimus for more than 6 months and the dose of CsA was adjusted to maintain stable serum levels of 150–300 ng/ml. CsA and tacrolimus were used in combination with azathioprine and prednisolone or mycophenolate mofetil and prednisolone. They had not been taking any other drugs reported to cause drug-induced GO. Subjects with gingivitis and healthy ones had no serious systemic disease that could impair the immune response. All subjects had not been taking medications such as antibiotics, anti-inflammatory drugs or contraceptives that could affect their periodontal status for at least 3 months before the study. Pregnant females were excluded from the study.

Study groups

CsA patients having GO

Twenty-five renal transplant patients received CsA therapy and had GO (moderate to severe GO) (12 females and 13 males between the ages of 20 and 47, mean of 31.6 ± 7.9 years). CsA patients having GO had no clinical attachment loss > 2 mm, no sites with alveolar bone loss present in radiography (i.e., distance between the cemento-enamel junction and bone crest at $> 95\%$ of the proximal tooth sites $\leqslant 3$ mm) and had clinical signs of inflammation.

CsA patients having no GO

Thirty renal transplant patients received CsA therapy and had no CsA-induced

Table 1. Serum concentration (ng/ml) of CsA and tacrolimus according to post-transplant periods (mean ± SD)

	CsA patients having GO ($N = 25$)	CsA patients having no GO ($N = 30$)	Tacrolimus ($N = 21$)
6–12 months	164.78 ± 62.38	105.0 ± 44.86	7.24 ± 4.04
After 12 months	142.63 ± 57.43	130.61 ± 44.86	6.36 ± 3.18

CsA, cyclosporine-A; GO, gingival overgrowth.

GO [moderate to severe GO; hyperplastic index (HI): 2 or 3) (16 females and 14 males aged from 17 to 47 years, mean of 30.9 ± 9.1 years). CsA patients having no GO had no clinical attachment loss >2 mm, no sites with alveolar bone loss present in radiography and had clinical signs of inflammation.

Tacrolimus

Twenty-one renal transplant patients received tacrolimus therapy and had no GO (moderate to severe GO; HI: 2 or 3) (12 females and 9 males aged from 18 to 50 years, mean of 29.7 ± 9.5 years). Tacrolimus patients had no clinical attachment loss >2 mm, no sites with alveolar bone loss present in radiography and had clinical signs of inflammation.

Gingivitis

Twenty-seven patients had gingivitis and with no history of treatment with drugs known to cause drug-induced GO (9 females and 18 males aged from 14 to 57 years, mean of 28.3 ± 12.6 years). They had no clinical attachment loss >2 mm, no sites with alveolar bone loss present in radiography (i.e., distance between the cemento-enamel junction and bone crest at >95% of the proximal tooth sites ⩽3 mm) and had clinical signs of inflammation.

Healthy

Forty subjects had a clinically healthy periodontium and no clinical evidence of drug-induced GO (26 females and 14 males aged from 17 to 59 years, mean of 31.72 ± 12.1 years). They had at least 20 teeth and at least 90% of the measured sites exhibited probing depth <3 mm and CAL ⩽2 mm as well as no bleeding on probing (BOP) at examination and no alveolar bone loss present in radiography (i.e., distance between the cemento-enamel junction and bone crest at >95% of the proximal tooth sites ⩽3 mm).

Determination of periodontal status

Upon entering the study, all subjects received a full-mouth clinical periodontal examination including the presence of supragingival plaque, presence of BOP, as well as a radiographic examination. The HI (Pernu et al. 1992) was recorded in the studied groups. The degree of GO was classified into four categories on the basis of the criteria of Angelopoulos & Goaz (1972) modified by Pernu et al. (1992). Based on the examination, CsA-treated patients who had clinically significant (moderate or severe) GO (HI: 2 or 3) and did not have alveolar bone loss were selected for our study. Clinical measures were carried out by the same periodontist to minimize variability.

Collection of GCF Samples

GCF samples were collected from the following sites:

GO+ sites in CsA patients having GO; two sites with CsA-induced GO in CsA-treated patients,
GO − sites in CsA patients having GO; two sites without CsA-induced GO in CsA-treated patients,
GO − sites in CsA patients having no GO; two sites of patients receiving CsA therapy and exhibiting no CsA-induced GO,
GO − sites in tacrolimus patients; two sites of patients receiving tacrolimus therapy and exhibiting no GO,
Diseased sites in gingivitis patients; two inflamed sites from patients with gingivitis exhibiting BOP and the presence of supragingival plaque,
Healthy sites in periodontally healthy subjects; two clinically uninflamed sites with no BOP and no presence of supragingival plaque from periodontally healthy subjects.

Study sites were selected from mesial and distal aspects on the buccal located on the same papilla. Thus, 336 GCF samples were obtained from 143 subjects. Before GCF sampling, the supragingival plaque was removed from the interproximal surfaces with a sterile curette in the CsA- and tacrolimus-treated patients and gingivitis patients; these surfaces were dried gently by an air syringe and were isolated by cotton rolls. GCF was sampled with a filter paper. Paper strips were carefully inserted into the crevice until mild resistance was felt and left there for 30 s (Lamster et al. 1985). Care was taken to avoid mechanical injury. Strips contaminated with blood were discarded (Cimasoni 1983). The absorbed GCF volume of each strip was determined by Periotron 8000 (ProFlow Inc., Amityville, NY, USA) placed in sterile eppendorf vials and kept at −40°C until analysis. The readings from the Periotron 8000 were converted to an actual volume (μl) by reference to the standard curve.

GCF processing

The absorbed fluid was eluted from each strip into 75 μl 50 mM Tris-HCl, pH 7.8, containing 0.2 M NaCl and 1 mM $CaCl_2$ for 2 h at 22°C on the shaker as described previously (Emingil et al. 2006). The eluted GCF samples were frozen until enzyme-linked immunosorbent assay (ELISA) and immunofluorometric assay (IFMA).

Collection and processing of serum samples

Five milliliters of whole-blood samples for MMP-8, and TIMP analysis and for determination of CsA- and tacrolimus levels was collected in sterile tubes by a standard venipuncture method from the patients at the same time. The serum CsA and tacrolimus level that is given in Table 1 is the mean of the three measurements obtained within 6 months. The whole-blood samples collected for MMP-8 and TIMP analysis were processed to serum samples as described previously (Bergmann et al. 1989).

Analysis of TIMP-1 and MMP-8 in GCF and serum by ELISA and IFMA

TIMP-1 levels in the studied GCF groups were determined by ELISA assays (GE Healthcare, Amersham, Little Chalfont, UK) according to the manufacturer's instructions. GCF samples were assayed at dilutions (1:10) for TIMP-1 and (1:20) for MMP-8. The ELISA for TIMP-1 detects native,

complexed and fragmented species of TIMP-1. The detection limit for TIMP-1 is 1.25 ng/ml.

The levels of MMP-8 in GCF and serum (10 μl) were determined by a time-resolved IFMA as described by Hanemaaijer et al. (1997) and Mäntylä et al. (2003). Briefly, the monoclonal MMP-8-specific antibodies 8708 and 8706 (Medix Biochemica Oy Ab, Kauniainen, Finland) were used as a catching and tracer antibody, respectively. The tracer antibody, was labelled using europium-chelate. The assay buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/l diethylenetriamine-pentaacetic acid. Samples were diluted in assay buffer and incubated for 1 h, followed by incubation for 1 h with the tracer antibody. Enhancement solution was added, and after 5 min. fluorescence was measured using a 1234 Delfia Research Fluoremeter (Wallac, Turku, Finland). The specificity of the monoclonal antibodies against MMP-8 was the same as that of the polyclonal MMP-8 antibodies (Hanemaaijer et al. 1997, Mäntylä et al. 2003).

Total MMP-8 and TIMP-1 data were determined by averaging the sampling sites per subjects. The results for MMP-8 and TIMP-1 are converted as total MMP-8 and TIMP-1 (ng/sample) for MMP-8 and TIMP-1 in the GCF sample. Calculation of the concentration data for each enzyme was performed by dividing the amount of each mediator by the volume of the sample.

Statistical analysis

In the present study, the patient was used as the unit of observation. Statistical analysis was performed using non-parametrical techniques. The clinical measures, the total amount and the concentration of each mediator between sites with GO+ and GO − in CsA patients having GO were compared using paired Wilcoxon's signed-rank test (confidence interval of $p<0.05$). Comparisons of all study groups were performed using the Kruskal–Wallis test. It was also used for the comparison of GO − sites in CsA patients having GO with that of GO − sites in CsA patients having no GO, and sites in tacrolimus, gingivitis and in healthy subjects. Where there were significant differences, post-hoc two-group comparisons were assessed with Bonferroni-corrected Mann–Whitney *U*-tests, and *p*-values <0.005 or 0.0125 were considered to be statistically significant as appropriate. Spearman's rank correlation analysis was used to analyse the correlations between GCF MMP-8, TIMP-1 total amount and GCF MMP-8/TIMP-1 and clinical parameters, and $p<0.05$ was considered to be significant.

Results

Clinical findings

There was no difference in the mean age between the studied groups ($p = 0.3520$, Kruskal–Wallis test). The mean clinical data for the sampling areas are shown in Table 2.

Percentage of sites with BOP and supragingival plaque, and GCF volume

The percentages of sites with BOP and GCF volumes of GO+ sites were significantly higher compared with those of GO − sites in CsA patients having GO ($p = 0.0455$, $p = 0.0022$, respectively). On the other hand, the percentages of sites with supragingival plaque were similar between sites with GO+ and with GO − in CsA patients having GO ($p = 0.102$).

All patient groups had a significantly higher percentage of sites with BOP, supragingival plaque and GCF volume compared with the healthy group ($p<0.005$). GO+ sites in CsA patients having GO had a significantly higher percentage of sites with BOP, supragingival plaque and GCF volume compared with those of the CsA patients having no GO and tacrolimus patients ($p<0.005$), but similar values compared with those of gingivitis patients ($p>0.005$). CsA patients having no GO had a similar percentage of sites with BOP, supragingival plaque and GCF volume to the tacrolimus ($p>0.005$), whereas these values were significantly lower than those of the gingivitis patients ($p<0.005$). Tacrolimus patients also had a significantly lower percentage of sites with BOP, supragingival plaque and GCF volume compared with gingivitis patients ($p<0.005$).

The clinical periodontal parameters of GO − sites in CsA patients having GO were also compared with those of CsA patients having no GO and tacrolimus patients as well as gingivitis patients and healthy subjects. GO − sites in CsA patients having GO had a percentage of sites with BOP, supragingival plaque and GCF volume similar to those of GO − sites in CsA patients having no GO and in tacrolimus patients ($p>0.0125$). On the other hand, GO − sites in CsA patients having GO had a lower percentage of sites with BOP, supragingival plaque and GCF volume

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

	CsA patients having GO ($N = 25$)		CsA patients having no GO ($N = 30$)	Tacrolimus ($N = 21$)	Gingivitis ($N = 27$)	Healthy ($N = 40$)
	GO+ sites	GO − sites				
Percentage of sites with bleeding on probing	$100 \pm 0^{*,\S}$	$80.0 \pm 41^{\dagger}$	$70.8 \pm 33.5^{\P}$	$66.2 \pm 2.6^{\P}$	100 ± 0	$0^{\ddagger}$
Percentage of sites with plaque	$96 \pm 20^{\S}$	$82.5 \pm 37.3^{\dagger}$	$80.0 \pm 41^{\P}$	$72.6 \pm 35.3^{\P}$	100 ± 0	$0^{\ddagger}$
Hyperplastic index	2.72 ± 0.46	–	–	–	–	–
Gingival crevicular fluid (μl)	$0.40 \pm 0.1^{*,\S}$	$0.25 \pm 0.1^{\dagger}$	$0.19 \pm 0.1^{\P}$	$0.20 \pm 0.1^{\P}$	0.34 ± 0.1	$0.14 \pm 0.1^{\ddagger}$

*Significant difference from GO − sites in CsA patients having GO ($p<0.05$, Wilcoxon's signed rank test).
‡Significant difference from other groups (Mann–Whitney *U*-test, $p<0.005$).
§Significant difference from CsA patients having no GO and tacrolimus (Mann–Whitney *U*-test, $p<0.005$).
¶Significant difference from gingivitis (Mann–Whitney *U*-test, $p<0.005$).
†Significant difference from gingivitis and healthy groups (Mann–Whitney *U*-test, $p<0.0125$).
CsA, cyclosporine-A; GO, gingival overgrowth.

compared with those of inflamed sites in gingivitis patients ($p<0.0125$), but a higher percentage of sites with BOP, supragingival plaque and GCF volume than those of healthy sites from healthy subjects ($p<0.0125$).

Biochemical findings

The GCF MMP-8 levels of the studied groups are presented in Table 3.

GCF MMP-8 total amount

The GCF MMP-8 total amounts of GO+ sites were significantly elevated compared with GO− sites in CsA patients having GO ($p=0.0004$) (Table 3).

GO+ sites in CsA patients having GO had a significantly higher GCF MMP-8 total amount compared with those of the CsA patients having no GO, tacrolimus patients and healthy subjects ($p<0.005$). On the other hand, the GO+ sites in CsA patients having GO had a GCF MMP-8 total amount similar to those of gingivitis patients ($p>0.005$). CsA patients having no GO had a GCF MMP-8 total amount similar to those of tacrolimus patients ($p>0.005$). These patients had a significantly elevated GCF MMP-8 total amount compared with healthy subjects, but a lower GCF MMP-8 total amount than that of gingivitis patients ($p<0.005$). Tacrolimus patients also had a lower GCF MMP-8 total amount than that of gingivitis patients, but had a GCF MMP-8 total amount similar to healthy subjects. Gingivitis patients had a significantly higher GCF MMP-8 total amount compared with healthy subjects ($p<0.005$) (Table 2).

In CsA patients having GO, the GO− sites had a GCF MMP-8 total amount similar to those of sites in CsA patients having no GO ($p=0.2304$) and of sites in tacrolimus patients ($p=0.0169$). On the other hand, GO− sites in CsA patients having GO had a lower GCF MMP-8 total amount compared with those of sites in gingivitis patients ($p=0.0002$), but higher amounts than those of healthy sites from healthy subjects ($p=0.0007$).

GCF MMP-8 concentration

GCF MMP-8 concentration data of all groups are also presented in Table 3. The GCF MMP-8 concentrations of sites with GO+ were significantly elevated compared with sites with GO− in CsA patients having GO ($p=0.0008$) (Table 3).

GO+ sites in CsA patients having GO had a significantly higher GCF MMP-8 concentration compared with those of CsA patients having no GO, tacrolimus patients and healthy subjects ($p<0.005$). GO+ sites in CsA patients having GO had a GCF MMP-8 concentration similar to those of gingivitis patients ($p>0.005$). CsA patients having no GO had a GCF MMP-8 concentration similar to those of tacrolimus patients ($p>0.005$). CsA patients having no GO had a significantly lower GCF MMP-8 concentration than that of gingivitis patients ($p<0.005$) but similar GCF MMP-8 concentration compared with healthy subjects ($p>0.005$). Tacrolimus patients also had a lower GCF MMP-8 concentration than that of gingivitis patients ($p<0.005$), but had a GCF MMP-8 concentration similar to healthy subjects ($p>0.005$). Gingivitis patients had a significantly higher GCF MMP-8 concentration compared with healthy subjects ($p<0.005$).

GO− sites in CsA patients having GO had a lower GCF MMP-8 concentration compared with those of inflamed sites in gingivitis patients ($p=0.0015$). There was no significant difference in the GCF MMP-8 concentration between GO− sites in CsA patients having GO and GO− sites in CsA patients having no GO, sites in tacrolimus patients and healthy subjects ($p>0.0125$).

GCF TIMP-1 total amount and concentration

Both the GCF TIMP-1 total amount and the concentration of sites with GO+ were similar to those of sites with GO− in CsA patients having GO ($p=0.0581$, $p=0.067$, Wilcoxon's signed-rank test, respectively) (Table 2).

There was no significant difference in the GCF TIMP-1 total amount and concentration among GO+ sites in CsA patients having GO and other studied groups (Kruskal–Wallis test; $p=0.0982$ and $p=0.2031$, respectively) (Table 3).

The GCF TIMP-1 levels of GO− sites in CsA patients having GO were also compared with CsA patients having no GO, with sites in tacrolimus as well as gingivitis patients and healthy subjects. The Kruskal–Wallis test showed no significant differences between groups ($p=0.5937$).

GCF MMP-8/TIMP-1 ratio

GCF MMP-8/TIMP-1 ratios of GO+ sites were similar to those of GO− sites in CsA patients having GO ($p=0.421$, Wilcoxon's signed-rank test) (Table 3).

GO+ sites in CsA patients having GO had a significantly lower GCF MMP-8/TIMP-1 ratio compared with those of healthy subjects ($p=0.0030$),

Table 3. GCF MMP-8, TIMP-1 levels and MMP-8/TIMP-1 of the groups studied (mean ± SD)

	CsA patients having GO ($N=25$)		CsA patients having no GO ($N=30$)	Tacrolimus ($N=21$)	Gingivitis ($N=27$)	Healthy ($N=40$)
	GO+ sites	GO− sites				
MMP-8 (ng/sample)	10.06 ± 10.5*,‡	2.94 ± 3.2†	1.73 ± 1.6‡,§	1.06 ± 0.9§	9.80 ± 7.8‡,¶	0.82 ± 0.8
MMP-8 (ng/μl)	27.06 ± 25.9*,‡	9.74 ± 8.3†	11.47 ± 10.5§	5.18 ± 3.6§	29.91 ± 23.4‡,¶	7.89 ± 9.1
TIMP-1 (ng/sample)	0.14 ± 0.2	0.07 ± 0.1	0.04 ± 0.1	3.40 ± 15.4	0.05 ± 0.1	0.07 ± 0.1
TIMP-1 (ng/μl)	0.47 ± 0.8	0.23 ± 0.3	0.34 ± 0.4	12.18 ± 55.1	0.13 ± 0.2	0.52 ± 0.9
MMP-8/TIMP-1	140.3 ± 202.5 ‡	175.25 ± 564.1	195.69 ± 355.4	32.55 ± 33.9	223.8 ± 250.0 ‡,¶	192.78 ± 590.6

*Significant difference from GO− sites in CsA patients having GO ($p<0.05$, Wilcoxon's signed rank test).
‡Significant difference from healthy (Mann–Whitney *U*-test, $p<0.005$).
§Significant difference from GO+ sites in CsA patients having GO (Mann–Whitney *U*-test, $p<0.005$).
¶Significant difference from CsA patients having no GO and tacrolimus (Mann–Whitney *U*-test, $p<0.005$).
†Significant difference from gingivitis and healthy groups (Mann–Whitney *U*-test, $p<0.0125$).
CsA, cyclosporine-A; GO, gingival overgrowth; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase.

but similar with those of gingivitis patients ($p = 0.1495$). Gingivitis patients had a significantly elevated GCF MMP-8/TIMP-1 ratio compared with CsA patients having no GO, tacrolimus and healthy subjects ($p < 0.005$). GO − sites in CsA patients having GO and CsA patients having no GO had a GCF MMP-8/TIMP-1 ratio similar to those of healthy subjects.

Serum MMP-8 levels

The distributions of serum MMP-8 levels in all the studied groups can be seen in Fig. 1. CsA patients having GO had significantly higher serum MMP-8 levels compared with CsA patients having no GO and healthy subjects ($p < 0.005$). On the other hand, CsA patients having GO had serum MMP-8 levels similar to tacrolimus and gingivitis patients ($p > 0.005$). CsA patients having no GO had serum MMP-8 levels similar to tacrolimus, gingivitis and healthy subjects ($p > 0.005$). Tacrolimus patients had higher serum MMP-8 levels than that of healthy subjects ($p < 0.005$), but had serum MMP-8 levels similar to gingivitis patients ($p > 0.005$). Gingivitis patients had significantly higher serum MMP-8 levels compared with healthy subjects ($p < 0.005$).

Serum TIMP-1 levels

The distributions of serum TIMP-1 levels in all groups can be seen in Fig. 2. CsA patients having GO had serum TIMP-1 levels similar to CsA patients having no GO, tacrolimus and gingivitis patients ($p > 0.005$), but higher serum TIMP-1 levels than those of healthy subjects ($p < 0.005$). CsA patients having no GO had serum TIMP-1 levels similar to tacrolimus patients ($p > 0.005$), but had significantly elevated serum TIMP-1 levels compared with gingivitis patients and healthy subjects ($p < 0.005$). Tacrolimus patients had higher serum TIMP-1 levels than those of gingivitis patients and healthy subjects ($p < 0.005$). Gingivitis patients had serum TIMP-1 levels similar to healthy subjects ($p > 0.005$).

The correlations between GCF MMP-8, TIMP-1 total amount and clinical parameters are presented in Table 4. GCF MMP-8 total amount and GCF MMP-8/TIMP-1 were positively correlated with all the clinical periodontal parameters ($p < 0.05$). There was no correlation between GCF TIMP-1 total amount and clinical periodontal parameters ($p > 0.05$).

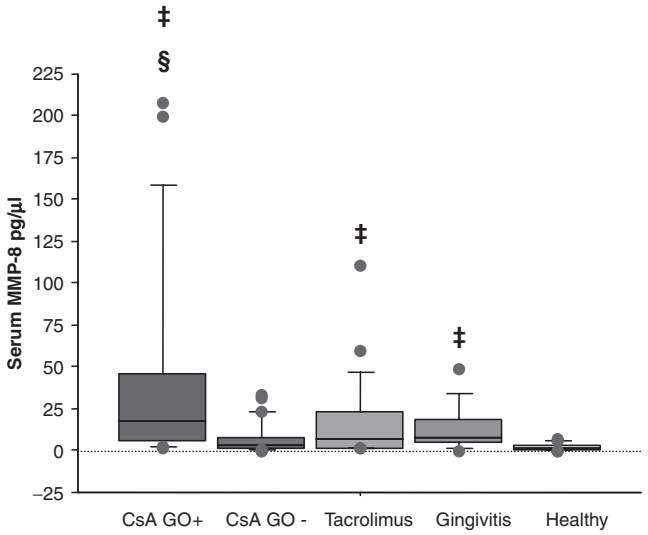

Fig. 1. Serum MMP-8 levels of the groups studied. Box plots show medians, 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers. Outside values are shown as closed circles. ‡Significant difference from the healthy group (Mann–Whitney *U*-test, $p < 0.005$). §Significant difference from CsA patients having no GO (CsA GO) (Mann–Whitney *U*-test, $p < 0.005$). CsA, cyclosporine-A; GO, gingival overgrowth; MMP-8, matrix metalloproteinase-8.

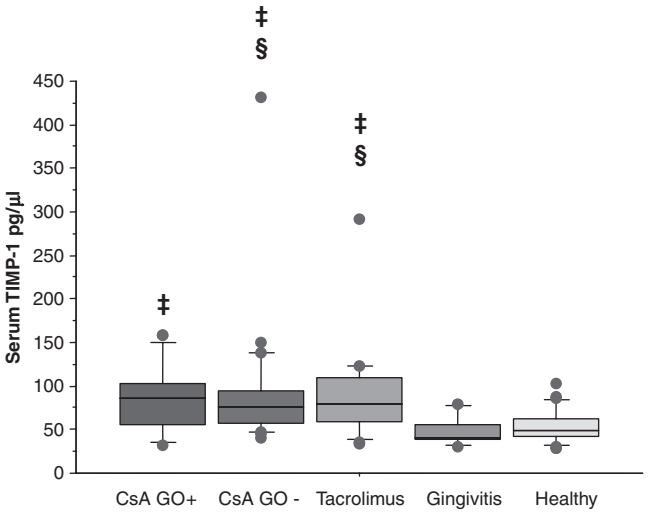

Fig. 2. Serum TIMP-1 levels of the groups studied. Box plots show medians, 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers. Outside values are shown as closed circles. ‡Significant difference from the healthy group (Mann–Whitney *U*-test, $p < 0.005$). §Significant difference from the gingivitis group (Mann–Whitney *U*-test, $p < 0.005$). TIMP-1, tissue inhibitor of matrix metalloproteinase-1.

There was also no correlation between GCF MMP-8 and TIMP-1 total amount ($R = 0.151$, $p > 0.05$).

Discussion

In the present study, we investigated the MMP-8 and TIMP-1 levels in GCF and serum samples of patients under different immunosuppressive therapy. We found significantly elevated GCF MMP-8 levels in GO+ sites of CsA patients having GO as the degree of clinical inflammation increased. On the other hand, tacrolimus patients had lower GCF MMP-8 levels compared with CsA patients having GO and gingivitis patients. GCF TIMP-1 levels did not differ between the groups studied. GO+ sites in CsA patients having GO had a significantly lower GCF MMP-8/TIMP-1 ratio compared with those of

Table 4. Correlations between GCF MMP-8, TIMP-1 total amount and GCF MMP-8/TIMP-1 and clinical parameters

Clinical parameters	MMP-8	TIMP-1	MMP-8/TIMP-1
Percentage of sites with BOP	0.692‡	0.255	0.486‡
Percentage of sites with plaque	0.631‡	0.188	0.564‡
GCF (μl)	0.659‡	0.214	0.424‡

‡$p < 0.05$.

BOP, bleeding on probing; GCF, gingival crevicular fluid; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase.

the healthy subjects who had normal tissue turnover.

Previous studies have pointed out the importance of the evaluation of CsA GO− sites in patients receiving CsA (Nares et al. 1996). With respect to this, to investigate the levels of proteolytic enzyme and its inhibitor on CsA patients having GO, MMP-8 and TIMP-1 levels were analysed both in GO+ and GO− sites in the CsA patients having GO as well as in CsA patients having no GO. Because MMP-8 in GCF increases along with the local degree of gingival inflammation (Chen et al. 2000, Tervahartiala et al. 2000, Kiili et al. 2002, Mäntylä et al. 2003, Pozo et al. 2005), both gingivitis patients and healthy subjects were included as a control group in the present study.

In the present study, GO+ sites in CsA patients having GO had a higher total amount of GCF MMP-8 as well as clinical degrees of inflammation compared with those of the GO− sites in CsA patients having GO and CsA patients having no GO. On the other hand, GO+ sites in CsA patients having GO had a GCF MMP-8 total amount similar to those of inflamed sites in gingivitis patients, whose gingival inflammation levels are also similar. Nevertheless, GCF MMP-8 total amounts were positively correlated with all clinical periodontal parameters. Overall, these findings might suggest that the increased GCF MMP-8 total amount is related to the increase in gingival inflammation; CsA therapy does not seem to have a significant effect on the GCF MMP-8 total amount. This may also be beneficial, because recently especially MMP-8 has been found in addition its surrogate tissue-destructive properties to have protective and defensive characteristics against inflammation due to its ability to process anti-inflammatory cytokines and chemokines (Owen et al. 2004, Gueders et al. 2005).

In general, the prominent changes in serum MMP-8 and TIMP-1 levels as well as MMP-8/TIMP-1 have been reported recently in systemically healthy patients with severe forms of periodontitis (Sorsa et al. 2006), or patients with diabetes or atherosclerosis patients with or without periodontitis (Safkan-Seppala et al. 2006, Soder et al. 2006, Tuomainen et al. 2007). Regarding serum of immunosuppressive patients, here, we found significant but modest changes in serum MMP-8, TIMP-1 levels and MMP-8/TIMP-1. Our patients did not suffer from severe forms of periodontitis or diabetes or atherosclerosis, and accordingly the changes in serum MMP-8 and TIMP-1 as well as in MMP-8/TIMP-1 were mainly moderate, but significant as presented.

It has been shown that expressions of several MMPs, such as MMP-1 and MMP-3, at both the mRNA and the protein level are reduced in CsA-induced overgrown gingival tissues (Sugano et al. 1998, Thomason et al. 1998, Bolzani et al. 2000, Kataoka et al. 2000, Yamada et al. 2000, Hyland et al. 2003). It was suggested that reduced collagenolytic activity in the tissues could account for the ECM accumulation that characterizes GO (Hyland et al. 2003). On the other hand, others have shown MMP-1 and MMP-10 mRNA expression to be unimpaired or increased, and cathepsins capable of activating latent collagenases were markedly suppressed after CsA exposure (Tüter et al. 2002, Yamaguchi et al. 2004, Dannewitz et al. 2006). We have previously shown that CsA did not have a significant effect on GCF MMP-8 and MMP-9 levels, and gingival inflammation was the main reason for the elevated enzyme levels (Atilla et al. 2001). The present findings are in agreement with and further extend the results of the previous investigation. Noteworthy, the main cellular source of GCF MMP-8 is degranulating triggered and recruited neutrophils (Kiili et al. 2002, Sorsa et al. 2004), which, different from resident gingival fibroblasts, epithelial and endothelial cells, release pre-synthetized and pre-packed MMP-8 without de novo expression (Tervahartiala et al. 2000, Sorsa et al. 2006).

Regarding GCF TIMP-1 levels, we found similar TIMP-1 levels in the groups studied. To the best of our knowledge, the present study has investigated TIMP-1 levels in CsA-treated patients' GCF for the first time. In vitro studies investigating the effects of CsA on TIMP-1 levels gave conflicting findings (Duymelinck et al. 1998, Yamada et al. 2000, Tüter et al. 2002, Hyland et al. 2003, Dannewitz et al. 2006). These different results could at least in part be explained by the heterogeneity of fibroblasts and macrophages in response to CsA, which eventually could play a role in the pathogenesis of CsA-induced GO (Tipton et al. 1991, Seymour et al. 1996). Accelerated matrix synthesis caused by decreased activity of MMPs and/or a quantitative imbalance between MMP and TIMP can result in drug-induced GO. A much lower MMP-8/TIMP-1 ratio was measured in GCF from GO+ sites in CsA patients having GO compared with healthy subjects. This suggests that it might prevent the turnover of collagen within gingival tissue and promote GO in CsA patients having GO. Recently, Sukkar et al. (2007) have reported that pro-inflammatory cytokines significantly up-regulate the levels of MMP-1 and TIMP-1 in cultured gingival fibroblasts from overgrown gingiva induced by CsA. Additionally, these researchers suggested that CsA alters the TIMP/MMP-1 ratio within the overgrown tissue induced by CsA. In the present study, an altered MMP-8/TIMP-1 ratio was found in GO+ sites in CsA patients having GO. Also, the similar GCF MMP-8/TIMP-1 ratio of GO− sites in CsA patients having GO, CsA patients having no GO to those of healthy subjects could reflect no tissue increase in these groups.

In a recent experimental study, Gagliano et al. (2005) investigated the cellular and molecular effects of tacrolimus in the collagen metabolism pathway. They demonstrated that collagen type I, TGF-β1 and TIMP-1 expressions did not change, while the MMP-1 and MMP-2 levels were elevated after tacrolimus treatment. They suggested that unlike CsA, tacrolimus may not induce GO. In the present study, the GCF MMP-8 and TIMP-1 levels were investigated for the first time in patients undergoing tacrolimus therapy, and

these patients had no clinically significant GO. Although the degree of gingival inflammation was higher than that in the healthy subjects, GCF MMP-8 levels of tacrolimus patients were similar to those of healthy subjects. The GCF MMP-8 total amount and clinical degrees of inflammation of tacrolimus patients were similar to those of GO − sites in CsA patients having GO and patients having no GO. Although statistically not significant, tacrolimus patients had the highest GCF TIMP-1 levels. Nevertheless, the similar GCF MMP-8/TIMP-1 ratios between tacrolimus patients and healthy subjects could suggest that there is no tissue increase in this group. On the other hand, we found that tacrolimus could slightly but significantly elevate the serum levels of both MMP-8 and TIMP-1. It seems that tacrolimus can in vivo up-regulate the systemic serum levels of MMP-8 and TIMP-1 and seemingly enhance the defensive process (Owen et al. 2004, Gueders et al. 2005, Sorsa et al. 2006). However, regarding other MMPs such as MMP-9, it has been demonstrated in vitro that tacrolimus (FK 506) can inhibit the induction of MMP-9 through the NF-κ pathway (Migita et al. 2006). Further in vivo studies at molecular levels are needed to clarify the role of these enzymes in patients undergoing tacrolimus therapy.

In conclusion, the results of this study indicate that CsA therapy does not have a significant effect on GCF MMP-8 and TIMP-1 levels; gingival inflammation seems to be the main reason for their elevations. The MMP-8/TIMP-1 ratio might be related to the CsA-induced GO. Tacrolimus therapy also does not have a significant effect on MMP-8 and TIMP-1 levels, which were investigated for the first time in GCF. However, tacrolimus may enhance the systemic serum MMP-8 and TIMP-1 levels and strengthen, at least partially, the systemic anti-inflammatory responses. Further studies are needed to elucidate the mechanism of GO in CsA-treated patients as well as why tacrolimus patients did not develop GO at molecular levels.

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Clinical Relevance

Scientific rationale for the study: Despite the current knowledge about the role of MMPs in CsA-induced GO, the pathogenesis of CsA GO is not yet clear. This study aimed to investigate the GCF and serum MMP-8 and TIMP-1 levels from renal transplant patients receiving CsA therapy and exhibiting CsA GO, from patients receiving CsA therapy and exhibiting no CsA GO and patients receiving tacrolimus therapy.

Principal findings: CsA patients having GO had higher GCF MMP-8 levels compared with CsA patients having no GO, tacrolimus and healthy subjects, but levels similar to gingivitis patients. On the other hand, tacrolimus patients had GCF MMP-8 levels similar to healthy subjects, but lower levels than gingivitis patients.

Practical implications: This study has shown that CsA and tacrolimus therapy do not have a significant effect on GCF MMP-8 levels; gingival inflammation seems to be the main reason for their elevations.