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Immunosuppression and gingival overgrowth: gene and protein expression profiles of collagen turnover in FK506-treated human gingival fibroblasts

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Abstract

Aim: FK506 is an immunosuppressive agent that, unlike cyclosporin A (CsA), does not induce gingival overgrowth (GO). CsA-induced GO is caused by quantitative modifications of the extracellular matrix components, particularly collagen (COL). Up to now, clinical trials have only investigated FK506 in relation with GO, so we aimed at analysing the effect of FK506 on COL turnover using a molecular approach, to evaluate the expression of genes and proteins related to this process.

Materials and Methods: Human gingival fibroblasts were incubated with FK506 or its vehicle (VH) for 24, 48 and 72 h. COL type I (COL-I), matrix metalloproteinases (MMP)-1 and 2, tissue inhibitor of MMP (TIMP)-1 and transforming growth factor (TGF)-β1 mRNA were assayed by Reverse transcriptase polymerase chain reaction; COL-I protein levels were determined by dot blot, MMP-1 and MMP-2 activity by zymography.

Results: Fibroblast proliferation decreased 48 and 72 h after treatment. COL-I gene and protein expression, TGF-β1 and TIMP-1 mRNA levels were not significantly affected, whereas MMP-1 gene and protein expression and MMP-2 mRNA levels rose significantly in treated fibroblasts compared with VH.

Conclusions: These findings suggest that increased MMP-1 gene and protein expression may be important for regulating COL-I homeostasis in the gingival connective compartment of FK506-immunosuppressed subjects.

Key words: collagen turnover; FK506; gingival overgrowth

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FK506 is a potent immunosuppressive agent used as an alternative to cyclosporin A (CsA) to prevent graft rejection and to treat autoimmune diseases (Faulds et al. 1993). It has been used successfully to prevent renal, liver and cardiac allograft rejection, although it can cause side effects such as nephrotoxicity, neurotoxicity and glucose metabolism disorders. Unlike CsA, however, FK506 does not appear to induce gingival overgrowth (GO) (McKaig et al. 2002), a pathology that afflicts 25–80% of immunosuppressed subjects using CsA (Seymour & Jacobs 1992) and that can only be solved by repeated periodontal surgery. Moreover, in renal, liver and cardiac transplant recipients shifted from CsA to FK506, CsA-induced GO resolved promptly, within a few months (Bader et al. 1998, Busque et al. 1998, James et al. 2000).

CsA-induced GO is caused by the accumulation of extracellular matrix (ECM) components, particularly collagen (COL), in the gingival connective compartment. Experimental investigations indicate interstitial COL as the main target of CsA, and an altered COL degradation pathway seems to be responsible for this pathology (Bolzani et al. 2000, Kataoka et al. 2000, Hyland et al. 2003).

Collagen turnover is, in fact, very tightly regulated. Under physiological conditions, there is a dynamic balance between COL synthesis and degrada-

tion, finely tuned by matrix metalloproteinases (MMPs), a family of proteolytic enzymes responsible for the remodelling and degradation of ECM (Woessner 1991, Birkedal-Hansen 1993). Matrix metalloproteinases activity is closely controlled at the level of gene expression, activation and inhibition by MMP tissue inhibitors [tissue inhibitor of MMP (TIMP)]. If these regulatory mechanisms are deranged, an increase of COL may occur, leading to GO.

In this tightly regulated mechanism, transforming growth factor (TGF)-β1 is the major mediator influencing COL turnover in fibroblasts. Since in vitro COL synthesis by gingival fibroblasts may be affected by CsA, TGF-β1 might also be involved in the mechanisms of development of GO (James et al. 1998).

Up to now, clinical trials have only shown that FK506 treatment is not commonly associated with the development of GO. However, no detailed experimental study has investigated the cellular and molecular effects of FK506 on the ECM components of the gingival connective tissue and, in particular, on interstitial COL turnover.

Therefore, our study analysed, for the first time, the effect of FK506 on the COL turnover pathway, using a molecular approach, and evaluating the expression of genes and proteins related to this process.

Material and Methods

All reagents, if not otherwise specified, were purchased from Sigma, Milan, Italy.

Cell culture

Human gingival fibroblasts were obtained from the pre-molar area of the upper dental arch from four healthy volunteers (two males and two females, aged 20–27 years). All had clinically normal gums with no signs of inflammation, hyperplasia and no history of use of drugs associated with GO. Each volunteer gave informed consent.

Gingival biopsies were washed with sterile phosphate-buffered saline (PBS), plated in T-25 flasks (Greiner, Frickenhausen, Germany), incubated in Dublecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum and antibiotics (10 U/ml penicillin, 10 mg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2. When fibroblasts grew from the explant, they were trypsinized [0.1% trypsin-0.02% ethylenediamine-tetraacetic acid (EDTA)] for secondary cultures. Triplicate cultures were carried out for each treatment.

Treatment of gingival fibroblasts with FK506

When human gingival fibroblasts between the fourth and fifth passage grew to confluence in T-75 flasks (Greiner, Frickenhausen, Germany), the culture medium was replaced with serum-free DMEM containing FK506 (1 μM) dissolved in a vehicle (VH) (0.008% ethanol, 0.0016% Tween-20). We selected a dose of FK506 previously described for treating fibroblasts (Ginevri et al. 1998, Migita et al. 2000). This dose corresponds to the whole blood trough levels $\times 10^2$ in patients receiving FK506 immunosuppressive therapy. We used this single high dose because FK506 did not directly stimulate ECM expression in vitro in fibroblasts treated with serial doses of the drug (Ginevri et al. 1998). In addition, the treatment of mesenchymal cells with different concentrations of FK506 from 1 nM to 1 μM did not influence the expression of ECM components (Tang et al. 2002).

The cultures were then incubated at 37°C for 24, 48 and 72 h. Cultured fibroblasts of each sample incubated in the VH served as control. At the established intervals, the cell culture supernatants were collected and fibroblasts were washed in PBS, trypsinized and harvested by centrifugation (100 × *g*, 5 min.).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from fibroblasts using a modification of the guanidine isothiocyanate method (Extract-All, Eurobio, Les Ulis Cedex, France). After DNase I digestion, 1 μg of total RNA was reverse-transcribed in 20 μl final volume of reaction mix (Promega, Milan, Italy).

The following primers were used for RT-PCR: glyceroldehyde 3-phosphate dehydrogenase (GAPDH) 5′-ATTCCATGGCACCGTCAAGGCT, 3′-TCAGGTCCACCACTGACACGTT (571 bp); COL-I 5′-GGCGGCCAGGGCTCCGAC, 3′-AATTCCTGGTCT GGGGCACC (347 bp); MMP-1 5′-GGTGATGAAGCAGCCCAG, 3′-CAGTAGAATGGGAGAGTC (437 bp); MMP-2 5′-CCTCTCCACTGCCTTCGATACACC, 3′-AGCATCTATTCTTGGGCACCG, (162 bp); TIMP-1 5′-AGTCAACCAGACCACCTTATACCA, 3′-TTTCAGAGCCTTGGAGGAGCTGGT (386 bp); TGF-β1 5′-CAGAAATACAGCAACAATTCCTGG, 3′-TTGCAGTGTGTTATCCCTGCTGTC (186 bp).

Amplification reactions were conducted in a final volume of 25 μl containing

Table 1. Reverse transcriptase polymerase chain reaction amplification conditions

Gene	Protocol			Cycles no.
COL-I	Denaturation	94°C	1 min.	35
	Annealing	61°C	90 s	
	Elongation	72°C	2 min.	
MMP-1	Denaturation	94°C	30 s	32
	Annealing	53°C	1 min.	
	Elongation	72°C	1 min.	
	+72°C 10 min. to finalize extension			
MMP-2	Denaturation	94°C	1 min.	30
	Annealing	60°C	2 min.	
	Elongation	72°C	3 min.	
TIMP-1	Denaturation	94°C	1 min.	30
	Annealing	60°C	2 min.	
	Elongation	72°C	3 min.	
TGF-β1	Denaturation	94°C	1 min.	35
	Annealing	60°C	90 s	
	Elongation	72°C	2 min.	
GAPDH	denaturation	94°C	30 s	25
	Annealing	62°C	1 min.	
	Elongation	72°C	1 min.	
	+72°C 10 min to finalize extension			

COL-I, collagen type-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinases; TIMP, tissue inhibitor of MMP; TGF, transforming growth factor.

2.5 µl of cDNA, 200 µM of the four dNTPs, 100 pmol of each primer and 2.5 U of Taq DNA polymerase (Taq 2000, Stratagene, La Jolla, CA, USA). The RT-PCR protocols used are listed in Table 1. The RT-PCR products were resolved by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and quantified by densitometric analysis (Image Pro-Plus, Gleichen, Germany).

Dot blot

Media from VH- and FK506-treated fibroblasts were concentrated 20-fold with Centricon 10 columns (Amicon, Millipore, Milan, Italy). Protein content was determined by a standardized colorimetric assay (DC Protein Assay, Bio Rad, Segrate, Milan, Italy); 20 µg of total proteins per sample in a final volume of 200 µl of Tris buffer saline were spotted onto a nitrocellulose membrane in a Bio Dot SF apparatus (Bio Rad). Membranes were blocked for 1 h and incubated overnight at 4°C in monoclonal antibody to COL-I (1:1000 in Tris buffered saline/Tween 20 (TBST)) and then, after washing, in HRP-conjugated rabbit anti-mouse serum (1:40,000). Immunoreactive bands revealed by the Opti-4CN substrate (Amplified Opti-4CN, Bio Rad) were scanned densitometrically.

Sodium dodecyl sulphate (SDS)-zymography

Concentrated culture media were mixed 3:1 with sample buffer (containing 10% SDS). Four microgram total proteins per sample were run under non-reducing/non-denaturing conditions onto a 7.5% polyacrylamide gel [Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)] co-polymerized with 1 mg/ml of type I gelatin. The gels were run at 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min. each and incubated overnight in a substrate buffer at 37°C (Tris-HCl 50 mM, $CaCl_2$ 5 mM, NaN_3 0.02%, pH 7.5). The MMP gelatinolytic activity was detected after staining the gels with Coomassie brilliant blue R250, as clear bands on a blue background (Kleiner & Stetler-Stevenson 1994, Gagliano et al. 2002). To confirm the identity of MMP gelatinolytic activity, purified MMP-1 and MMP-2 (100 ng, Calbiochem, San Diego, CA, USA) were run as controls.

Statistical analysis

All the experiments were run in triplicate. Data generated from the three repetitions are expressed as mean standard error (SEM), and were analysed by one-way ANOVA, followed by Student's *t*-test. *p* values less than 0.05 were considered significant.

Results

Cell viability and proliferation

Phase contrast microscopy of VH- and FK506-treated fibroblasts did not indicate any real difference in their morphology. However, FK506 induced less proliferation at all intervals: at 24, 48 and 72 h after treatment these reductions amounted to, respectively, 10% p = ns, 16% $p < 0.05$, and 7% $p < 0.05$, compared with VH (Fig. 1).

Gene expression

Representative PCR amplifications of COL-I, MMP-1 and 2, TIMP-1 and TGF-β1 mRNA are shown in Fig. 2, and steady-state levels after densitometric analysis are presented in Fig. 3. COL-I mRNA tended to be up-regulated compared with VH, although without reaching statistical significance, in FK506-treated fibroblasts at 48 and 72 h (36% and 24%, respectively) (Fig. 3a). MMP-1 mRNA levels were substantially higher in FK506-treated fibroblasts at all intervals (106%, 137% and 125%, all $p < 0.05$, respectively, 24, 48 and 72 h after treatment), compared with VH (Fig. 3b).

The pattern was similar for MMP-2 gene expression, which rose by, respectively, 5% (ns), 36% ($p < 0.05$) and 49%

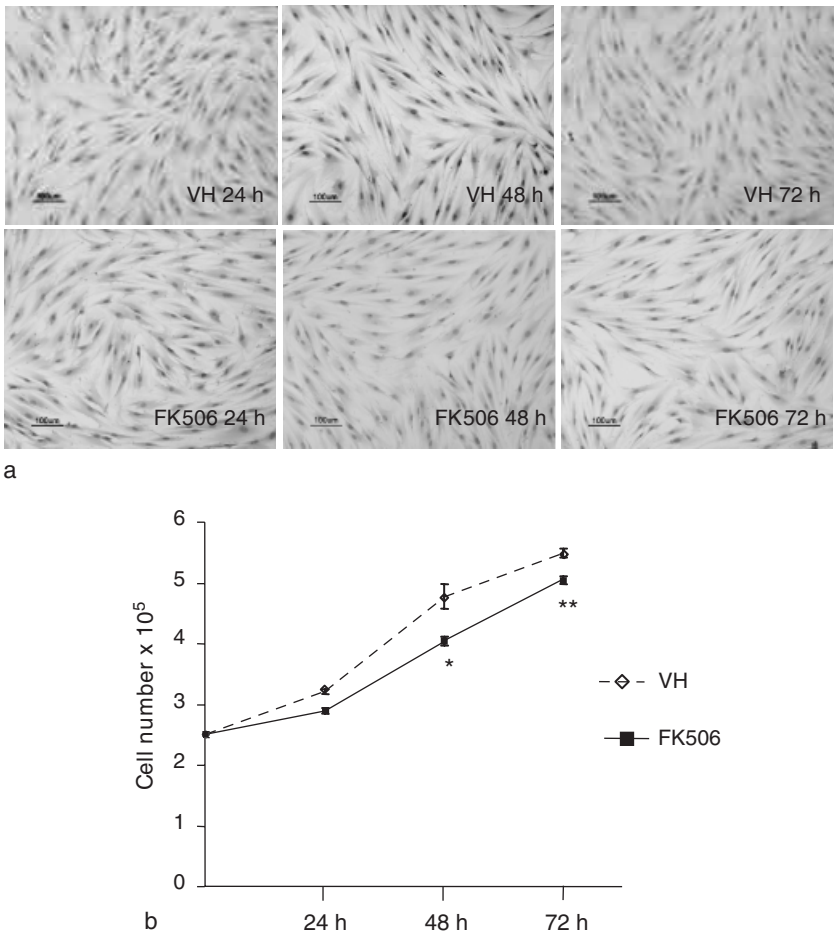

Fig. 1. (a) Microphotographs of cultured gingival fibroblasts (vehicle (VH); FK506-treated fibroblasts at different times). Haematoxylin–eosin stain. Scale bar: 100 µm. (b) Time-dependent effect of FK506 on cultured fibroblast proliferation. Cells were plated in T-75 flasks (250,000 cells/flask) and allowed to attach. Fresh medium containing FK506 1 µM was added, and cells were counted at the indicated times in the presence of 10% foetal bovine serum (FBS). Fibroblasts incubated with FK506 VH were used as controls. °$p < 0.05$ *versus* VH 48 h; °°$p < 0.05$ *versus* VH 72 h.

($p<0.05$) in FK506-treated fibroblasts 24, 48 and 72 h after treatment compared with VH (Fig. 3c).

TIMP-1 and TGF-β1 gene expression were slightly affected by FK506, but the quantitative modifications were not statistically significant (Fig. 3d, e).

COL protein

COL-I protein levels were assessed by dot blot. Densitometric scanning of immunoreactive bands showed that COL-I tended to decrease (by, respectively, 31%, 16% and 9%, after 24, 48 and 72 h, p = ns) in FK506 treated fibroblasts compared with VH (Fig. 4).

COL degradation

Zymographic analysis of MMP activity in conditioned human gingival fibroblast supernatants showed lytic bands at the molecular weights of 66 and 60/50 kDa. The bands at 66 kDa are consistent with proMMP-2 or gelatinase, while bands weighing 60/50 kDa corresponded to proMMP-1, as confirmed by purified MMP-1 and MMP-2 migration (Fig. 5a).

For MMP-1, SDS-zymography evidenced two bands at 60 and 55 kDa, corresponding, respectively, to the minor and the predominant zymogens, produced by fibroblasts, as previously reported (Stricklin et al. 1983). Densitometric analysis of lytic bands showed that FK506 steeply raised proMMP-1 levels (by, respectively, 122% ($p<0.05$), 70% (ns) and 30% ($p<0.05$) in treated fibroblasts, compared with VH after 24, 48 and 72 h). By contrast, gelatinase levels were not significantly modified by FK506 (Fig. 5b, c).

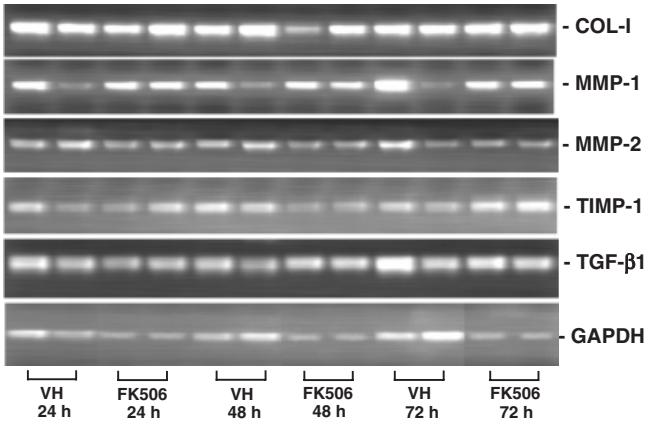

Fig. 2. Representative Reverse transcriptase polymerase chain reaction for collagen type-I (COL-I), matrix metalloproteinases (MMP)-1 and 2, transforming growth factor (TGF)-β1, tissue inhibitor of MMP (TIMP)-1 and GAPDH. Each lane represents one fibroblast strain. Each amplified cDNA was normalized on GAPDH gene expression.

Discussion

GO is one of the major side effects of CsA, causing both periodontal and aesthetic problems. Unlike FK506, the relationship between CsA and the development of GO is well established in humans and in animal experimental models (Hassell & Hefti 1991, Seymour & Jacobs 1992, Fu et al. 1995, Hyland et al. 2003). Gingival expansion induced by CsA mainly results in a disturbance in connective tissue homeostasis, interfering with COL metabolism in particular. By contrast, clinical trials indicate that patients immunosuppressed with FK506 did not develop GO and that

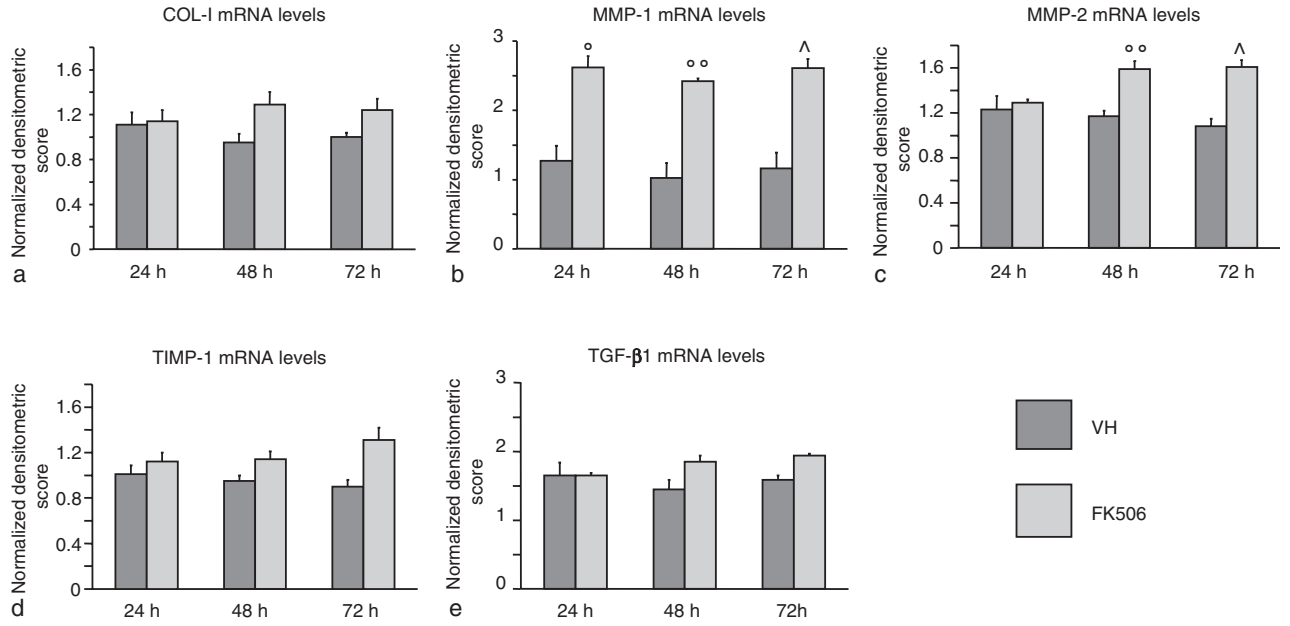

Fig. 3. Bar graph illustrating collagen type-I (COL-I) (a), matrix metalloproteinases (MMP)-1 (b), MMP-2 (c), tissue inhibitor of MMP (TIMP)-1 (d) and transforming growth factor (TGF)-β1 (e) gene expression in vehicle (VH)- and FK506-treated cultured human gingival fibroblasts at the times indicated. Changes in mRNA are expressed as normalized optical densities relative to GAPDH mRNA. Values are means ± SEM for four triplicate samples. $^{\circ}p<0.05$ *versus* VH after 24 h; $^{\circ\circ}p<0.05$ *versus* VH after 48 h; $^{\wedge}p<0.05$ *versus* VH after 72 h.

the pathology spontaneously resolved when CsA was discontinued or when the patient was switched to FK506.

As far as we know, this is the first detailed experimental study of the cellular and molecular effects of FK506 in the COL metabolism pathway. Using a single dose of 1 μM FK506, we investigated at the molecular level as to why FK506 does not trigger the mechanisms involved in CsA-induced GO pathogenesis and does not alter the balance between COL synthesis and degradation, inducing excessive COL-I deposition.

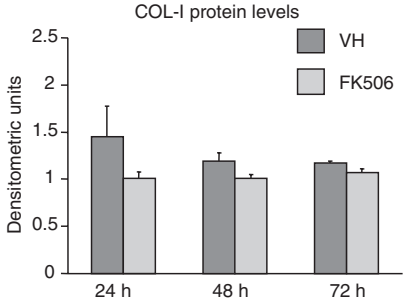

Fig. 4. Bar graph presenting collagen type-I (COL-I) protein levels assessed by dot blot analysis. Data are reported as densitometric units after scanning of the immunoreactive bands. Values are means ± SEM for four triplicate samples.

We found that FK506 had no effect on the fibroblasts' morphology. Interestingly, the drug induced less fibroblast proliferation at the three intervals tested (Fig. 1).

COL-I is the main COL species in all layers of gingival connective tissue. Its content is determined by a dynamic balance between synthesis and degradation and any derangement of this equilibrium may be important in the development of GO. Our data show that COL-I mRNA and protein are not significantly affected by FK506. However, we observed a tendency towards up-regulation of COL-I mRNA after 48 h in FK506-treated fibroblasts and, by contrast, COL-I protein content in conditioned media of drug-treated fibroblasts tended to be lower than in VH at all times. These findings suggest that FK506 does not increase COL-I deposition by gingival fibroblasts and are consistent with the clinical evidence that FK506, unlike CsA, does not induce GO. Moreover, this tendency to up-regulation of the COL-I gene and the opposite pattern of COL-I protein levels suggests that COL-I expression may be regulated at the post-translational level, probably because of regulation of the COL-I degradation pathway by MMPs.

MMPs with collagenase and gelatinase activities are secreted in the extracellular space as latent pro-enzymes and activated extracellularly by proteolytic cleavage within the matrix environment; their activity is regulated at multiple levels and under pathological conditions any mismatch could result in excessive ECM accumulation or degradation.

Interstitial collagenase or MMP-1 starts up the interstitial COL degradation pathway by cleaving its native triple helical region into 3/4- and 1/4-COL degradation fragments (Sakai & Gross 1967, Woessner 1991), the so-called gelatins. These cleavage products can be further degraded by other proteinases such as gelatinase or MMP-2, leading to complete digestion of fibrillary COL.

We found that FK506 strongly induced MMP-1 gene expression, and the pattern was similar for MMP-1 collagenolytic levels in fibroblasts after FK506, compared with VH. By contrast, MMP-2 mRNA levels increased 48 and 72 h after FK506 treatment, while gelatinase protein levels seemed unaffected by FK506.

In the same fibroblast samples we evaluated the effect of CsA on MMP levels, and found that CsA lowered MMP-1 in the supernatants of CsA-treated fibroblasts (Gagliano et al. 2004). This confirms that MMP-1 has a key role in the mechanisms of GO development. We can also speculate that the mechanism activated by FK506 differs from that of CsA toxicity.

The extracellular activity of MMPs is tightly regulated at various steps, including inhibition by TIMPs. TIMP-1 binds non-covalently to interstitial collagenase forming inactive 1:1 stoichiometric TIMP-1/MMP-1 complexes, thereby inhibiting the active form of MMP-1 (Woessner 1991, Brew et al. 2000).

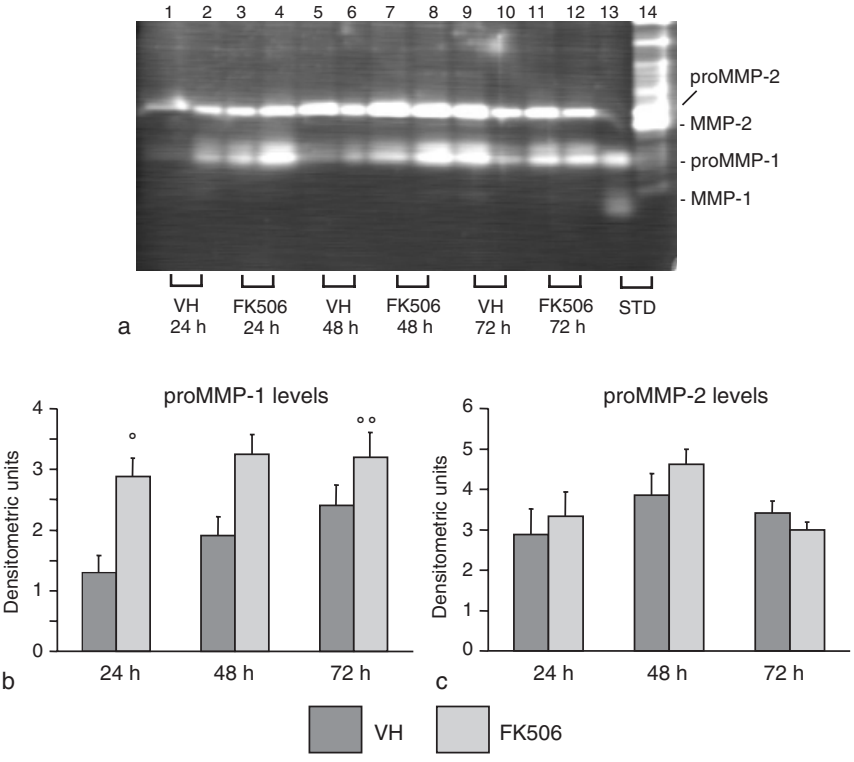

Fig. 5. (a) Representative gelatin zymogram of matrix metalloproteinases (MMPs) in serum-free conditioned human gingival fibroblast supernatants from VH- and FK506-treated fibroblasts at the indicated times. The lytic bands weighing 66 kDa and in the 60/50 kDa region correspond to proMMP-1 and proMMP-2, respectively, as confirmed by purified MMP-1 (lane 13) and MMP-2 (lane 14) electrophoretic patterns. Standard (STD): purified MMP-1 and MMP-2. (b, c) MMP amounts in fibroblast serum-free conditioned media after densitometric analysis of lytic bands following sodium dodecyl sulphate (SDS)-zymography. (b) proMMP-1 levels; (c) proMMP-2 levels. Data are mean ± SEM of four samples in triplicate. $^{\circ}p<0.05$ *versus* VH 24, $^{\circ\circ}p<0.05$ *versus* VH 24.

Recent studies investigating the role of TIMP-1 in CsA-induced GO have found that TIMP-1 expression is not strongly modified by the drug (Tuter et al. 2002, Hyland et al. 2003). In addition, FK506 does not significantly affect TIMP-1 expression, although we observed a slight – but not significant – increase in TIMP-1 mRNA levels in FK506-treated fibroblasts compared with VH at all three test times.

The constantly shifting COL-I balance is controlled by factors such as TGF-β1, a multifunctional cytokine involved in healing and fibrogenic processes, capable of directly activating gene expression for the synthesis of ECM components (Sporn & Roberts 1990, Border & Ruoslahti 1992). In mammals, three TGF-β isoforms have been identified, with high sequence homology and similar biological activities, TGF-β1 being the best characterized. TGF-β1 increases COL by activating COL transcription and synthesis and inhibiting MMP expression. Since it has powerful pro-fibrogenic activity in vitro and in vivo, it has been suggested that this cytokine may also be pivotal in the GO associated with CsA treatment. In fact, it has been reported that cultured fibroblasts treated with CsA produce high levels of TGF-β1 (James et al. 1998).

A recent immunohistochemical study found a stronger expression of TGF-β in patients treated with CsA than FK506 in renal graft biopsies one year after kidney transplantation (Mohamed et al. 2000) and in the liver (Mohamed et al. 2001). There is evidence that FK506 may have less fibrogenic influence on transplant glomeruli than CsA (Bicknell et al. 2000).

Our finding of an insignificant increase in TGF-β1 mRNA at all times after FK506 suggests that this cytokine does not play an important role in the molecular events triggered by FK506 in gingival fibroblasts.

Since MMP-1 is the interstitial collagenase responsible – and necessary – for starting interstitial COL breakdown, it is conceivable that FK506 does not trigger the development of GO because it induces MMP-1 gene and protein expression, thereby counteracting COL-I accumulation in the connective compartment.

We hope these results will help clarify the unresolved problem of drug-induced GO and therefore contribute to the development of new therapeutic approaches in chronic immunosuppressed subjects. Considered the interesting results obtained, in our laboratory we are testing the effect of FK506 on gingival fibroblasts COL turnover utilizing shorter times and a broader dose range of the drug.

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