

# Elevation of collagen type I in fibroblast–keratinocyte cocultures emphasizes the decisive role of fibroblasts in the manifestation of the phenotype of cyclosporin A-induced gingival overgrowth

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**Background and Objective:** Collagen type I elevation in cyclosporin A-induced gingival overgrowth supports evidence that gingival fibroblasts play a decisive role in the manifestation of the phenotype. To analyze the role of gingival fibroblasts under more *in vivo*-like conditions, we evaluated the effect of cyclosporin A on collagen type I gene and protein expression in gingival overgrowth-derived gingival fibroblasts established as cocultures with gingival keratinocytes as well as in matched gingival fibroblast monolayers.

**Material and methods:** Monolayers and cocultures of primary gingival fibroblasts were treated with cyclosporin A for 6 and 72 h. The expression of collagen type I mRNA was analyzed by quantitative real time polymerase chain reaction, while expression and secretion of collagen type I protein was analyzed by indirect immunofluorescence and western blotting.

**Results:** Compared with controls, significant elevation of collagen type I mRNA was restricted to cocultures after 6 and 72 h of treatment with cyclosporin A. In keratinocytes, collagen type I remained undetectable. In monolayers and cocultures, indirect immunofluorescence showed a slightly higher level of collagen type I protein in gingival fibroblasts in response to stimulation with cyclosporin A. Semiquantitative detection of collagen type I by western blotting demonstrated a nonsignificant increase for cell extracts in monolayers and cocultures. For secreted collagen type I, western blot analysis of the supernatants revealed elevated protein levels in cultures stimulated with cyclosporin A. Compared with the corresponding monolayers, the stimulatory effect of cyclosporin A on protein secretion was significant only in coculture.

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**Conclusion:** Our results indicate that collagen type I is a target of cyclosporin A and that gingival fibroblasts are decisive for the manifestation of the gingival overgrowth-phenotype. Furthermore, the results suggest that cocultures of gingival overgrowth-derived gingival fibroblasts and gingival keratinocytes permit analysis of cyclosporin A-induced effects under more *in vivo*-like conditions.

Cyclosporin A is an immune suppressant, and gingival overgrowth is one of the reported side-effects of cyclosporin A medication, with a prevalence ranging from 2 to 70% (1). Despite the high incidence of gingival overgrowth, the mechanisms underlying the development of gingival overgrowth have not yet been completely elucidated. Moreover, this process seems to be multifactorial in nature, involving age and other demographic factors, such as drug variables, concomitant medication, periodontal variables and genetic factors (2). In cyclosporin A-induced gingival enlargement, quantitative modification of the extracellular connective tissue matrix occurs and collagen metabolism has been suggested as being the main target (3–6). The gingival connective tissue consists of a dense network of type I collagen fibril bundles, which provide firmness to the tissue and attach the gingiva to the tooth and alveolar bone (7). In a previous study we showed that the collagen type I status was increased in native tissue specimens derived from patients displaying cyclosporin A-induced gingival overgrowth (8). In this context, our results demonstrated that the high abundance of the collagen type I protein was caused by an increase in collagen type I synthesis. This was substantiated by an increase in the mRNA transcription level of collagen type I in the gingival connective tissue of patients suffering from cyclosporin A-induced gingival overgrowth (8). However, there is conflicting evidence concerning collagen type I as being a target of cyclosporin A. This conflict arises behind the background that evidence *in vivo*, irrespective of whether it is derived from rat animal models (3) or from patients' tissue (3), indicates the ambivalence of the results concerning the putative effects of cyclosporin A. In addition, several *in vitro* studies have revealed an increase (9), a decrease or unchanged levels of

fibrillar type I collagen (10). The reasons for this ambiguous evidence regarding collagen type I may be a result of the use of heterogeneous concentrations of cyclosporin A for cell culture treatment (3), or the use of fibroblasts derived either from normal or from gingival overgrowth-affected gingival connective tissue (3,4,11). A further important issue that should be noted is that current *in vitro* studies evaluating the effect of cyclosporin A are limited to monolayers of gingival fibroblasts. These simplified cell systems disregard the interactions between the mesenchymal connective tissue fibroblasts and the keratinocytes of the above-lying gingival epithelium. In analogy to skin, mesenchymal-epithelial interactions are also necessary for morphogenesis and physiological maintenance of the gingival epithelial phenotype (12,13). Thus, it cannot be excluded that fibroblast-keratinocyte interactions are also important for the manifestation of the gingival overgrowth phenotype in response to cyclosporin A.

Therefore, this study aimed to clarify the role of connective tissue fibroblasts and collagen type I as targets of cyclosporin A in the biological context of gingival overgrowth under more *in vivo*-like conditions. To achieve this aim, we established, for the first time, cocultures of gingival overgrowth-derived connective tissue fibroblasts and gingival keratinocytes and analyzed the expression and secretion of collagen type I in response to cyclosporin A in these cocultures and in matched gingival fibroblast monolayers.

## Material and methods

### Cell culture of human gingival fibroblasts and immortalized human gingival keratinocytes

Primary human gingival fibroblasts were derived from the gingival tissue

specimens of four cyclosporin A-treated renal transplant patients (29–61 years of age; three female and one male), who attended the Department of Operative Dentistry and Periodontology (University of Heidelberg, Germany) for periodontal therapy because of gingival overgrowth. For tissue harvest, informed consent was obtained from the patients according to the Helsinki Declaration, and the protocol was approved by the institution ethic committee. Specimens were taken from the interdental area, where the tissue revealed characteristic signs of gingival overgrowth. For each patient, primary cultures were established using the explant technique (12). Cultures were maintained in Dulbecco's Modified Eagle's Medium (PAA, Pasching, Austria), supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin).

Because of the limited life span of primary human keratinocytes, gingival keratinocytes immortalized with the human papilloma virus type 16 E6/E7 open reading frame (13) were used to generate the cocultures. Primary gingival fibroblasts were used between passages 5 and 13, and immortalized human gingival keratinocytes were used from passages 36 to 40.

Gingival fibroblasts in monolayer cultures were grown under the above-mentioned standard culture conditions. For the generation of cocultures, near-confluent cultures of gingival fibroblasts (i.e. 80% cell density) were trypsinized and then seeded onto the bottom of six-well multiwell plates with removable inserts (Falcon; Becton Dickinson GmbH, Heidelberg, Germany) at a cell density of  $1 \times 10^5$ . Immortalized human gingival keratinocytes were seeded at a cell density of  $2 \times 10^5$  on the inside of the insert's porous membrane, thereby facilitating spatially separated growth from

gingival fibroblasts. In addition, this culture device prevents cross-contamination between gingival fibroblasts and immortalized human gingival keratinocytes, and thus permits distinct RNA and protein extraction for each cell type.

### Cyclosporin A treatment

Cyclosporin A was kindly provided by Novartis Pharma AG (Novartis identifier number: 3142056, batch number 4CE7A; Nuernberg, Germany). A stock solution of 1 mg/mL was prepared by dissolving 1 mg of cyclosporin A in 45  $\mu$ L of ethanol and 5  $\mu$ L of Tween 20 as cosolvents. Taking into account the cyclosporin A concentrations used clinically, which range from 250 to 400 ng/mL, and the maximum dose of 1000 ng published in *in vitro* studies (3), we chose an intermediate concentration of cyclosporin A, of 750 ng/mL. Therefore, medium was subsequently added to give a final volume of 1.0 mL. For 6 and 72 h of treatment, cyclosporin A was generally added when the gingival fibroblasts were close to confluence (i.e. at 80%). Solvents were controlled by using a specific fluorescence polarization immunoassay method (Dimension X Pand, Dade Behring Inc., Deerfield, IL, USA; data not shown). The untreated controls were cultured without cyclosporin A but with the same concentration of both cosolvents for up to 72 h. Before stimulation, the viability of gingival fibroblasts and immortalized human gingival keratinocytes was ensured by using different concentrations of cosolvent (data not shown).

### RNA isolation

After the application of cyclosporin A, total RNA was isolated both from treated and from control cells (i.e. gingival fibroblasts and immortalized human gingival keratinocytes) by using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). To remove contamination with genomic DNA, RNA samples were treated with DNase I (Qiagen GmbH) during RNA purification. RNA concentration and quality was determined using an automated capillary-electrophoresis system (Experion; Bio-Rad Laboratories GmbH, Munich, Germany).

### Quantitative real-time polymerase chain reaction analysis

First-strand cDNA was synthesized from 1  $\mu$ g of total RNA in a reaction mixture containing random hexamer and oligo DT primer by performing the RevertAid™ reverse transcription protocol (#K1622; Fermentas GmbH, St Leon-Rot, Germany). The cDNA concentration was determined by fluorometry using a fluorescent dye (PicoGreen; Molecular Probes, Invitrogen GmbH, Karlsruhe, Germany) and adjusted to 5 ng/ $\mu$ L. Quantitative real-time polymerase chain reaction (PCR) analysis was performed using the iCycler Real-Time PCR Detection System (Bio-Rad Laboratories GmbH), according to the manufacturer's instructions. The standard temperature profile included initial denaturation for 3 min at 95°C, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 55–57°C (primer-dependent) for

30 s, and extension at 72°C for 40 s. Sequences of the primers used are listed in Table 1. Several commonly used reference genes (*GAPDH*, *YHWAZ*, *HMBS*, *HPRT1*, *ACTB*, *UBE2L3*) were tested to determine the most stably expressed genes in all samples by applying the geNorm applet (Primer-Design Ltd, Southampton Hants, UK). The two most stable reference genes (*HPRT1* and *UBE2L3*) were selected for further normalization of the expression data. The target gene expression level was calculated on the basis of a modification of the  $\Delta\Delta C_T$  equation, which allows differences in efficiency between the quantitative real-time PCR reactions to be measured using the GENEX® software (Bio-Rad Laboratories GmbH). In treated and control gingival overgrowth-derived gingival fibroblasts obtained from each patient, the relative mRNA expression of collagen type I and lysyloxidase was determined in monolayers and cocultures by three independent quantitative real-time PCR experiments, and calculation of the mean  $\pm$  standard deviation was performed. The relative expression levels were subjected to the Student's *t*-test; *p*-values of < 0.01 were considered statistically significant.

### Extraction of collagen type I protein from monolayer and coculture cell culture supernatants and lysed gingival fibroblasts, with and without cyclosporin A treatment

From cyclosporin A-treated (6 and 72 h) and nontreated cells, cell-culture supernatants and total cell extracts were collected to monitor the level of

Table 1. Primers used for quantitative real-time polymerase chain reaction

Gene accession number	Gene name	Primer sequence*	Product size (bp)
Hs.172928 NM_000088	Collagen 1, alpha 1 (COL1A1; E = 1.99)	Forward: 5'-CGGAGGAGAGTCAGGAAGG-3' Reverse: 5'-ACATCAAGACAAGAACGAGGTAG-3'	156
Hs.412707 NM_000194	Hypoxanthine phosphoribosyltransferase 1 (HPRT1; E = 1.98)	Forward: 5'-TGACACTGGCAAACAATGCA-3' Reverse: 5'-GGTCCTTTTACCAGCAAGCT-3'	94
Hs.108104 NM_003347	Ubiquitin-conjugated enzyme E2L3 (UBE2L3; E = 1.98)	Forward: 5'-ATTTGGGTCGCGTTCTT-3' Reverse: 5'-TGCCTTGACATTCTCGATGGT-3'	182
Hs.102267 NM_002317.3	Lysyloxidase (LOX)	RT <sup>2</sup> Primer-Set (Biomol, PPH01956A)	Not known

\*Sequences are as deposited in the National Center for Biotechnology Information database under the given accession number. E, efficiency of the reaction calculated from the slope of the standard curve ( $E = 10(-1/\text{slope})$ ).

collagen type I protein. First, supernatants were collected and concentrated using Centricon Plus-20 spin tubes (Millipore GmbH, Schwalbach, Germany). Then, fibroblasts were washed twice with phosphate-buffered saline, lysed in RIPA buffer (25 mM Tris, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) on ice for 2 min, followed by removal of DNA and cell debris by centrifugation through a shredder column at 14,000 *g* for 2 min at 4°C (Qiagen GmbH). The protein concentration in all samples was measured using the Experion™ automated electrophoresis system in combination with the Experion™ Pro260 analysis kit (Bio-Rad Laboratories GmbH) under denaturing conditions. The resulting supernatants and total cell extracts were stored at -80°C or used directly.

#### **Pepsin digestion of protein samples and western blotting**

All samples were adjusted to a total protein concentration of 15 µg and then digested for 2 h at 23°C by adding 0.1 vol% of a pepsin solution (1 mg/mL in 4 M urea, pH 3; 2500 units/mg; Sigma Aldrich GmbH, Munich, Germany) to digest noncollagenous proteins (14). This method results in cleavage of the telopeptides from the pro-form of the collagen type I molecule, thereby yielding the  $\alpha 1$  and  $\alpha 2$  chains (15). The reaction was stopped by the addition of an equal volume of pepstatin A (Sigma Aldrich GmbH). Subsequently, the proteins were precipitated using methanol/chloroform (16), and dried pellets were dissolved in an appropriate volume of NuPAGE lithium dodecyl sulphate sample buffer supplemented with NuPAGE reducing agent containing 500 mM dithiothreitol (Invitrogen GmbH). Samples were denatured for 10 min at 70°C and equal protein concentrations (15 µg) were loaded together with a protein standard (HiMark™ Pre-Stained High Molecular Weight Protein Standard, no. LC5699; Invitrogen GmbH) on a NuPAGE 12% Bis-Tris polyacrylamide gel (Invitrogen GmbH). After electrophoresis in NuPAGE 2-(N-morpholino) ethansul-

fonic acid sodium dodecyl sulfate running buffer (Invitrogen GmbH) at 200 V for 1 h under reducing conditions, proteins were blotted onto a poly(vinylidene) difluoride membrane (Invitrogen GmbH), according to the manufacturer's instructions. To ensure efficient and reproducible binding to the membrane, transfer proceeded under low-power conditions (30 V constant) for 70 min. Membranes were then blocked in blocking buffer (Invitrogen GmbH) at 4°C overnight. After blocking, membranes were rinsed and probed at 4°C for 1 h with rabbit polyclonal anti-collagen type I (1:1000, Catalog #T59103 R; Biorad International, Saco, ME, USA) in antibody dilution buffer (Invitrogen GmbH), followed by three washes with antibody wash solution for 5 min each (Invitrogen GmbH), and then incubated with an anti-rabbit alkaline phosphatase-conjugated secondary antibody (Western Breeze; Invitrogen GmbH) at 23°C for 1 h. Protein bands were visualized by chemiluminescence (alkaline phosphatase-activated CDP-Star substrate, Western Breeze; Invitrogen GmbH) and exposure to an autoradiography film (Hyperfilm ECL™; Amersham Biosciences, Piscataway, NJ, USA) for up to 1 min. The band density on representative gels was measured after scanning (Epson 1600Pro, 600 dots per inch; Epson Deutschland GmbH, Meerbusch, Germany) using image analysis software (IMAGEJ 1.37v; the public domain NIH image software developed by Wayne Rasband, Bethesda, MD, USA). The biologically relevant collagen type I  $\alpha 1$  and  $\alpha 2$  chains are shown in Figs 4 and 5, and the densitometric evaluation of the semiquantitative western blot analysis represents the mean ( $n = 3$ ,  $\pm$  standard deviation) of three individual western blot experiments using equal protein amounts and identical exposure times for all three western blots on the autoradiography films. The expression levels were subjected to the Student's *t*-test; *p*-values of  $< 0.01$  were considered statistically significant.

#### **Indirect immunofluorescence**

For indirect immunofluorescence, gingival fibroblasts in monolayers and

cocultures were grown on polyethylene coverslips (Thermanox™, 15 mm diameter; Nunc GmbH & Co. KG, Wiesbaden, Germany) to reach 80% confluence before treatment with cyclosporin A. In cocultures, immortalized human gingival keratinocytes were maintained in the inserts, as described previously. Coverslips and insert membranes were fixed in 80% methanol and in acetone (5 min each, -20°C). After overnight incubation with the primary rabbit polyclonal antibody for collagen type I (Catalog #T59103 R; Biorad International; dilution 1:50) at 4°C, samples were washed in phosphate-buffered saline (three washes, 5 min each) and then incubated with the secondary fluorochrome-conjugated antibody (Alexa Fluor® 488, anti-rabbit IgG, 1:100 dilution; MoBiTec GmbH, Goettingen, Germany) at 23°C for 1 h. For total nuclei staining, propidium iodide (10 µg/mL; Sigma-Aldrich GmbH, Munich, Germany) was added to the secondary antibody. After mounting in mounting medium (Vectashield, Weinheim, Germany), specimens were analyzed using a confocal laser-scanning microscope (Leica TCS/NTCLSM microscope; Leica Microsystems, Bensheim, Germany). To test the specificity of immunostaining, negative controls were run without primary antibodies. As expected, these specimens showed no staining (data not shown). Relative fluorescence intensity was scored by three experienced examiners (B.D., P.T. and T.S.) who were blinded to the status of the specimens, using a four-scale model (-, represented no immunoreactivity; + + +, represented the highest protein abundance). A summary of the scores is given in Table 2.

## **Results**

### **Cyclosporin A treatment increases collagen type I mRNA expression in gingival fibroblasts derived from gingival overgrowth in monolayers and cocultures**

To screen for cyclosporin A-sensitive genes, RNA of gingival fibroblasts from a monolayer of one patient (no.

Table 2. Results of relative mRNA expression level and protein status of collagen type I in monolayer and coculture of overgrowth-derived gingival fibroblasts in response to cyclosporin A and untreated controls

	Monolayer					Coculture						
	642	698	728	785	642	698	728	785	642	698	728	785
qRT-PCR (x-fold expression)	Control	1.0 ± 0.15	1.0 ± 0.25	1.0 ± 0.13	1.0 ± 0.1	1.0 ± 0.02	1.0 ± 0.3	1.0 ± 0.05	1.0 ± 0.02	1.0 ± 0.3	1.0 ± 0.05	1.0 ± 0.16
	6 h	1.57 ± 0.16	1.17 ± 0.06	1.66 ± 0.3	1.31 ± 0.1	2.01 ± 0.28	3.38 ± 0.4	1.85 ± 0.11	2.01 ± 0.28	3.38 ± 0.4	1.85 ± 0.11	1.93 ± 0.11
	72 h	1.02 ± 0.04	0.97 ± 0.14	1.62 ± 0.08	1.12 ± 0.15	2.1 ± 0.18	5.93 ± 0.2	2.84 ± 0.04	2.1 ± 0.18	5.93 ± 0.2	2.84 ± 0.04	2.95 ± 0.34
IIF <sup>a</sup>	Control			++				++			++	
	6 h			+++				+++			+++	
Cell extract, pixel values (% increase)	Control	103.6 ± 1.97	120.5 ± 1.81	156.3 ± 3.44	169.8 ± 1.95	130.0 ± 2.6	129.1 ± 1.96	111.9 ± 2.31	130.0 ± 2.6	129.1 ± 1.96	111.9 ± 2.31	159.8 ± 2.17
	6 h	108.8 ± 2.4	129.3 ± 1.42	163.5 ± 2.38	194.1 ± 3.61	139.7 ± 1.14	132.9 ± 1.13	134.1 ± 3.79	139.7 ± 1.14	132.9 ± 1.13	134.1 ± 3.79	146.6 ± 2.04
	72 h	107.8 ± 3.25	128.1 ± 2.6	168.2 ± 2.46	182.2 ± 2.2	140.0 ± 2.1	133.4 ± 1.71	138.2 ± 1.89	140.0 ± 2.1	133.4 ± 1.71	138.2 ± 1.89	167.4 ± 2.49
Supernatant, pixel values (% increase)	Control	135.4 ± 2.36	139.1 ± 2.33	106.8 ± 3.91	162.2 ± 2.82	75.7 ± 3.28	49.6 ± 1.53	56.5 ± 3.02	75.7 ± 3.28	49.6 ± 1.53	56.5 ± 3.02	96.2 ± 2.94
	6 h	145.2 ± 2.88	152.0 ± 3.66	123.5 ± 2.1	165.5 ± 4.0	145.8 ± 3.1	70.9 ± 1.64	92.0 ± 1.87	145.8 ± 3.1	70.9 ± 1.64	92.0 ± 1.87	149.7 ± 2.65
	72 h	157.3 ± 1.3	143.1 ± 2.64	120.9 ± 3.24	167.0 ± 3.54	144.4 ± 2.8	104.8 ± 2.5	96.5 ± 2.13	144.4 ± 2.8	104.8 ± 2.5	96.5 ± 2.13	185.5 ± 2.83
		(+21.9)	(+4)	(+14.1)	(+4.8)	(+68.7*)	(+55.2*)	(+40*)	(+68.7*)	(+55.2*)	(+40*)	(+89.3*)

<sup>a</sup>Relative expression of collagen type I in indirect immunofluorescence

(IIF) was scored on an arbitrary scale: + + +, strong; + +, intermediate; +, low; and -, no expression.

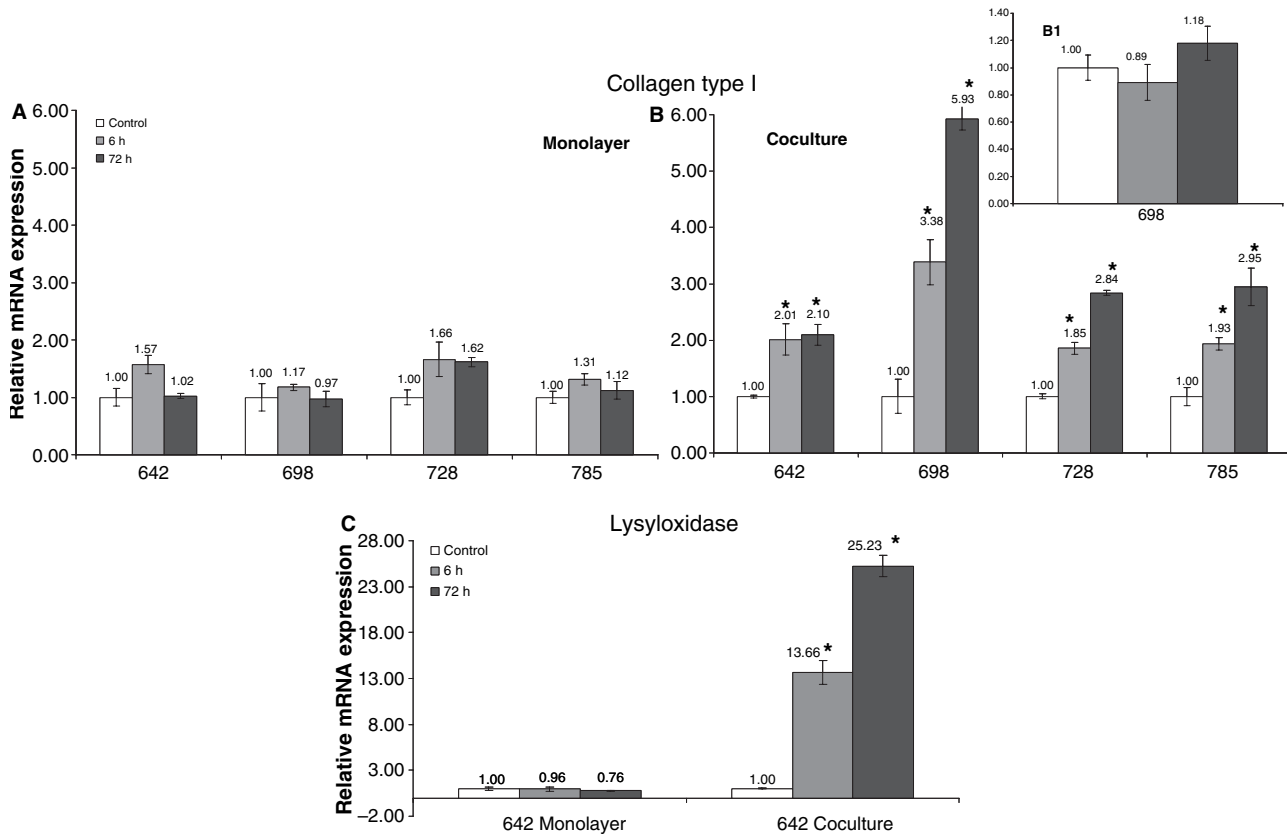
\*Significant x-fold expression values compared with the control are marked with an asterisk

(*n* = 3, mean ± SD, *p* > 0.01).

728) was subjected to an oligonucleotide-based whole human genome microarray (Affymetrix U133 Plus 2.0 Array; Affymetrix UK Ltd., High Wycombe, UK). The majority of genes displayed no significant difference in expression between the cyclosporin A-treated sample and the untreated control. Despite this, in addition to collagen type I, several extracellular matrix genes and genes involved in extracellular matrix synthesis showed an increase around or beyond the threshold level of 1.5-fold in the treated sample (data not shown). Although each of the genes showing these expression changes would be of potential interest as a candidate target of cyclosporin A, we focused on collagen type I, which is the major fibrillar collagen of the extracellular matrix in the gingival connective tissue. Compared with the untreated control, the microarray showed that the expression level of collagen type I was increased above the predefined cut-off value of 1.5 following treatment for 6 and 72 h with cyclosporin A.

To confirm the results obtained from the initial screening for cyclosporin A-sensitive genes by microarray analysis, relative mRNA expression of collagen type I was analyzed by quantitative real-time PCR for the whole panel of four patients (Fig. 1).

For all patients (nos 642, 698, 728 and 785), monolayers derived from gingival fibroblasts of gingival overgrowth displayed a slight, but nonsignificant, increase in relative mRNA expression levels of collagen type I, ranging from 1.17-fold (no. 698) to 1.66-fold (no. 728) after 6 h of stimulation with cyclosporin A (Fig. 1A, grey columns), when compared with the control (Fig. 1A, white columns). At 72 h (Fig. 1A, black columns), the relative mRNA expression level of collagen type I was very similar to that of the untreated control samples (Fig. 1a, white columns) in all patients. In marked contrast to monolayers, cocultures generally revealed significantly up-regulated relative mRNA expression levels of collagen type I at 6 h, in the range of 1.85-fold (patient no. 728) to 3.38-fold (patient no. 698) (Fig. 1B, grey columns) compared with



**Fig. 1.** Relative mRNA expression level of collagen type I $\alpha$ 1 and lysyloxidase in gingival overgrowth-derived gingival fibroblasts, as analyzed by the quantitative real-time polymerase chain reaction. Gingival fibroblasts were cultured in monolayers (A) and in cocultures with keratinocytes (B) and treated with 750 ng/mL of cyclosporin A for 6 and 72 h. In monolayers, the mRNA expression levels for all patients were slightly increased in all samples after 6 h, and had returned almost to the control level after 72 h of stimulation with cyclosporin A (A). The increase was more pronounced in cocultures, where all patients displayed significantly increased expression after 6 h and a further up-regulation of the collagen type I mRNA expression after 72 h compared with untreated controls. A control experiment with keratinocytes used in cocultures illustrated that the mRNA expression level of collagen type I $\alpha$ 1 was unaffected by stimulation with cyclosporin A and displayed only a basal expression level (inset B1, shown for patient no. 698). The relative mRNA expression level of lysyloxidase (shown for patient no. 642) demonstrated a significantly pronounced increase after 6 and 72 h of cyclosporin A treatment in the coculture system compared with the control, whereas the monolayers displayed expression at the control level (C). The data represent the mean of three independent quantitative real-time polymerase chain reaction experiments ( $n = 3$ , mean  $\pm$  standard deviation,  $p > 0.01$ ). The levels of expression were analyzed using the Student's *t*-test;  $p$ -values of  $< 0.01$  were considered statistically significant and are marked with an asterisk.

the untreated control (Fig. 1B, white columns). This significant up-regulation seen in cocultures was even more pronounced at 72 h, where the levels of increase ranged from 2.1-fold (patient no. 642) to 5.93-fold (patient no. 698) (Fig. 1B, black columns). This finding suggests that collagen type I appears to be a real target of cyclosporin A, and that the level of significant increase in relative collagen type I gene expression depends on whether the fibroblasts are growing in the absence (monolayers) or presence (cocultures) of keratinocytes, the latter facilitating intercellular interactions. The relative gene expres-

sion levels obtained from the transcription analysis are summarized in Table 2.

Intriguingly, the elevated gene expression found for collagen type I in the coculture system was restricted to the fibroblasts. This was indicated by the 0.18-fold increase of relative mRNA expression detected in the epithelial counterpart (immortalized human gingival keratinocytes) at 72 h, which is very similar to the level of the corresponding control (presented for patient no. 698, Fig. 1B, inset B1). This finding may be a hint that the fibroblasts occupy a crucial role in the accumula-

tion of extracellular collagen type I in the gingival connective tissue, which is associated with the manifestation of the gingival overgrowth phenotype.

#### Detection of collagen type I in gingival overgrowth-derived gingival fibroblasts by indirect immunofluorescence

Next, we were interested in whether the cyclosporin A-related elevation of collagen type I transcription, which was seen in gingival overgrowth-derived gingival fibroblasts in the coculture, was also found for the corresponding

protein. To address this question, we first detected collagen type I by indirect immunofluorescence. As shown for one patient (no. 728) in Fig. 2 (monolayer) and Fig. 3 (coculture), fibroblasts of both culture systems exhibited a slight elevation of collagen type I in response to cyclosporin A. This is shown by the increased intensity of green fluorescence when collagen type I in monolayer control fibroblasts (Fig. 2A) was compared with the presence of collagen type I 6 h (Fig. 2B) and 72 h (Fig. 2C) after treatment with cyclosporin A.

With respect to the increased intensity of the green fluorescence of collagen type I protein, this also applied to the coculture. Here, the weakest fluorescence of collagen type I was seen in the untreated control (Fig. 3A), while

an increased cyclosporin A-induced fluorescence was observed at 6 h (Fig. 3B) and at 72 h (Fig. 3C). This slight increase of the green fluorescence may mirror a small increase of the collagen type I protein in response to cyclosporin A. Although apparently affected by cyclosporin A, the collagen type I protein appeared to be only marginally elevated, and seemingly the stimulatory effect of cyclosporin A on collagen type I does not clearly discriminate gingival fibroblasts grown in monolayers from those established in cocultures. Concerning the coculture situation, collagen type I remained undetectable in the corresponding gingival keratinocytes at both 6- and 72-h time-points after treatment with cyclosporin A (Fig. 3B, insert B1, 6 h; Fig. 3C, insert C1, 72 h). The relative

expression of collagen type I in monolayers and cocultures, as detected by indirect immunofluorescence, is scored in Table 2.

#### Abundance of collagen type I protein in cell extracts and in cell culture supernatants of gingival overgrowth-derived gingival fibroblasts established in monolayers and cocultures

In the light of the results described so far for collagen type I protein, it appears rather vague whether collagen type I renders a true target of cyclosporin A or whether fibroblasts or fibroblast–keratinocyte interactions contribute to the elevated levels of collagen type I, as suggested by the oligonucleotide-based array and the quantitative real-time PCR experiments. This is mostly a result of the fact that indirect immunofluorescence revealed only slight cyclosporin A-related collagen type I elevation, regardless of the mode of culture of the fibroblasts. In this context, it appears noteworthy that the indirect immunofluorescence technique is limited in its validity concerning protein quantification. In addition, the collagen type I detected by indirect immunofluorescence only includes the intracellular or cell-bound fractions of the protein. Because collagen type I, as an extracellular matrix protein, can be secreted in the extracellular space (17–19), it cannot be ruled out that cyclosporin A-induced elevation of the protein becomes evident at the level of secreted collagen type I. Behind this background, we were motivated to employ a semiquantitative approach that allows detection of both the cellular/cell-bound collagen type I fraction and the secreted fraction. Thus, we performed semiquantitative western blot analysis on the cell extracts and corresponding supernatants of cyclosporin A-treated gingival overgrowth-derived gingival fibroblasts in monolayers and cocultures, and in matched controls.

To detect a biologically relevant part of the collagen type I protein, the collected fractions of the extracts and the supernatants were digested with pepsin; the bands depicted in Figs 4 and 5

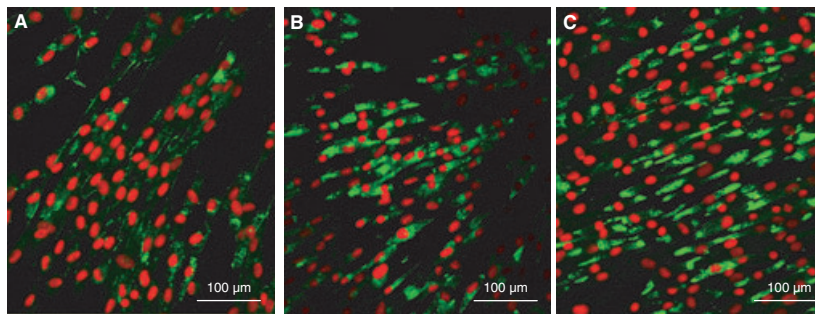


Fig. 2. Presence of collagen type I in monolayers, as detected using indirect immunofluorescence, in patient no. 728. The amount of collagen type I after 6 h (B) and 72 h (C) of stimulation with cyclosporin A was slightly higher than that of the untreated control (A). Green fluorescence staining indicates the presence of collagen type I. Red staining illustrates the propidium iodide nuclei counterstain. Bars represent 100 µm.

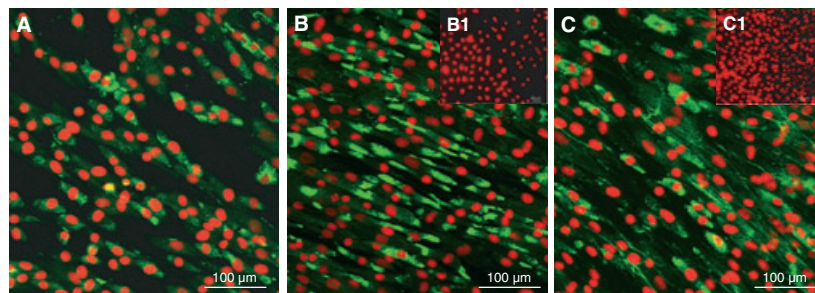


Fig. 3. Presence of collagen type I in cocultures, as detected by indirect immunofluorescence, in patient no. 728. The presence of protein after 6 h (b) and 72 h (c) of stimulation with cyclosporin A was seemingly enhanced compared with the untreated control (A). Green fluorescence staining indicates the presence of collagen type I. Inlays indicate that human gingival keratinocytes were devoid of the green collagen type I fluorescence, indicating lack of protein expression 6 h (b1) and 72 h (c1) after stimulation with cyclosporin A. The red staining illustrates the propidium iodide nuclei counterstain. Bars represent 100 µm.

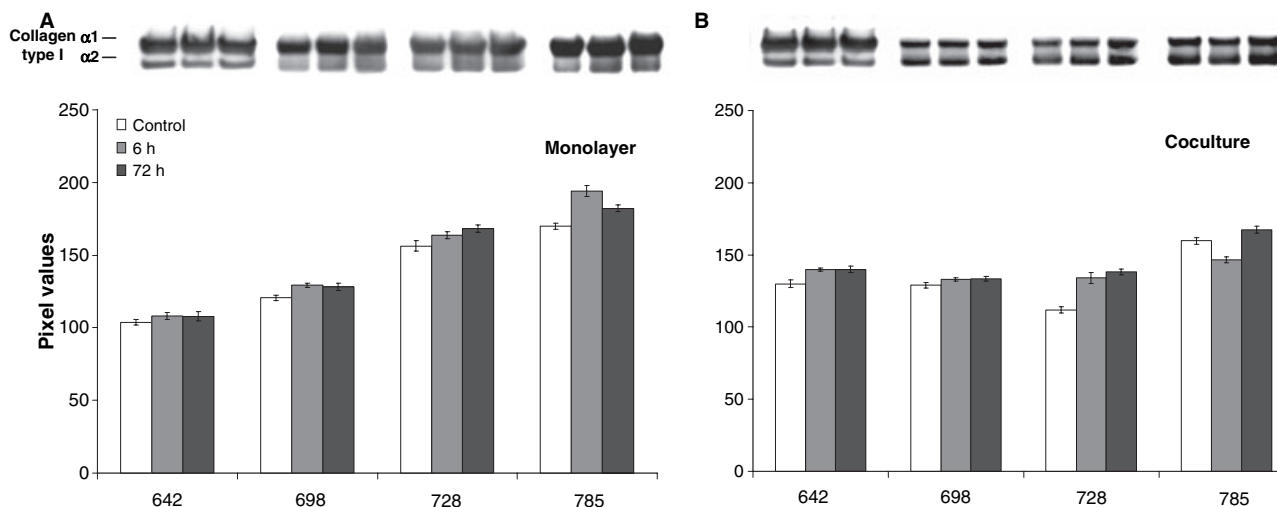


Fig. 4. Collagen type I expression by gingival overgrowth-derived gingival fibroblasts following cyclosporin A treatment, as analyzed by western blotting. Gingival fibroblasts were cultured in monolayers (A) and in cocultures with human gingival keratinocytes (B) and were treated with 750 ng/mL of cyclosporin A for 6 and 72 h. Proteins of the cell extracts were digested with pepsin and separated by electrophoresis on a 12% sodium dodecyl sulphate polyacrylamide gel followed by chemiluminescence immunodetection. Columns indicate the results from densitometric analysis of the collagen type I  $\alpha 1$  and  $\alpha 2$  chains obtained from western blot analysis. The data represent the mean of three independent western blot experiments for each patient ( $n = 3$ , mean  $\pm$  standard deviation). The banding pattern is shown of the collagen type I  $\alpha 1$  and  $\alpha 2$  chains derived from the pepsin digestion (15).

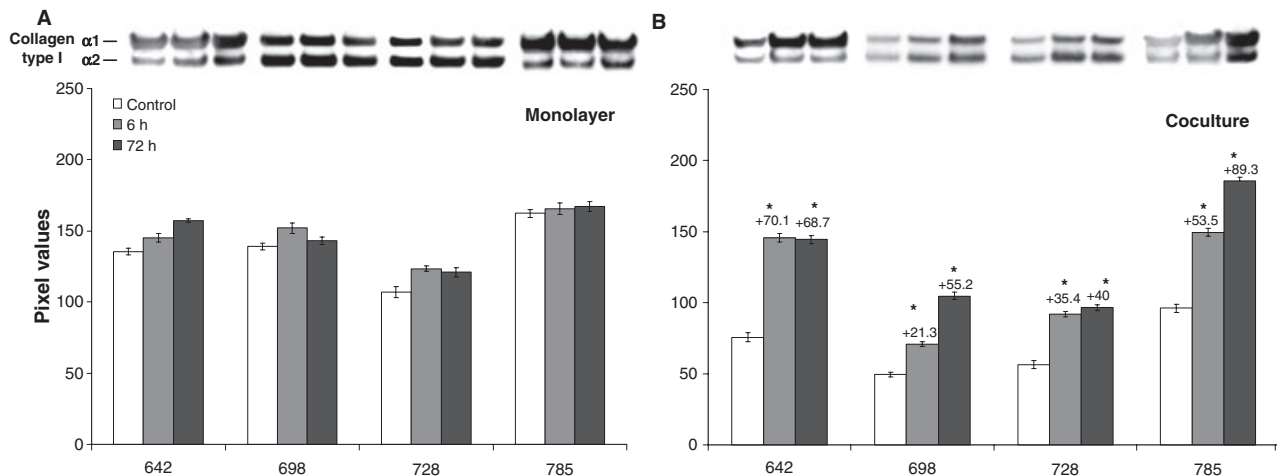


Fig. 5. Collagen type I expression of gingival overgrowth-derived gingival fibroblasts following treatment with cyclosporin A treatment, as analyzed by western blotting. Gingival fibroblasts were cultured in monolayers (A) and in cocultures (B) with human gingival keratinocytes and treated with 750 ng/mL of cyclosporin A for 6 and 72 h. Proteins of the cell culture supernatants were digested with pepsin and separated by electrophoresis on a 12% sodium dodecyl sulphate polyacrylamide gel followed by chemiluminescence immunodetection. Columns indicate the results from densitometric analysis of the collagen type I  $\alpha 1$  and  $\alpha 2$  chains obtained from the western blot analysis. The data represent the mean from three independent western blot experiments run for each patient ( $n = 3$ , mean  $\pm$  standard deviation,  $p > 0.01$ ). The banding pattern is shown of the collagen type I  $\alpha 1$  and  $\alpha 2$  chains derived from pepsin digestion (15) of the cell culture supernatants. The expression levels were analyzed by the Student's  $t$ -test;  $p$ -values of  $< 0.01$  were considered statistically significant and are marked with an asterisk. Column values indicate percentage increase compared with control.

show the collagen type I  $\alpha 1$  and  $\alpha 2$  chains (15). In this context, it should be noted that in addition to the cell extracts (Fig. 4), we have also included analysis of the corresponding super-

natants (Fig. 5) because they harbour the secreted proportion of collagen type I and, thus, mirror part of the extracellular status of this essential matrix molecule.

As shown in Fig. 4, western blot analysis revealed no obvious differences in the amount of collagen type I considering the intraspecific comparison of the untreated (white columns)



with the cyclosporin A-treated (grey columns, 6 h; black columns 72 h) situation for each of the four patients (nos 642, 698, 728, 785). This lack of obvious differences was irrespective of the culture mode of the gingival overgrowth-derived fibroblasts and therefore also applied to the monolayers (Fig. 4A) and the cocultures (Fig. 4B). This pattern of collagen type I found after assessment of the cell extracts suggests similar inpatient-related steady-state levels/amounts of the cell-bound form of this extracellular matrix molecule. While this inpatient-related similarity in protein content was also observed in the supernatants derived from gingival fibroblasts in monolayers (Fig. 5A), the coculture situation was characterized by a partly drastic, but basically significant, modulation of collagen type I in the supernatant (Fig. 5B).

This clear modulation was substantiated by the presence of significantly higher protein levels in the supernatants of each patient after 6 h of treatment with cyclosporin A, as indicated by comparison of the collagen type I status of the controls at 6 h (white columns) with the samples at 6 h (grey columns). Significant increases of collagen type I expression were as follows: 70.1% in patient no. 642 (Fig. 5B), 21.3% in patient no. 698 (Fig. 5B), 35.4% in patient no. 728 (Fig. 5B) and 53.5% in patient no. 785 (Fig. 5B). Compared with controls, the results at 6 h (grey columns) demonstrated that all patients exhibited increases of collagen type I that were similar to, or even higher than, those observed when gingival overgrowth-derived gingival fibroblasts in cocultures were treated with cyclosporin A for 72 h (black columns). While patient no. 642 produced a similar amount of collagen type I at 72 h (68.7%, black column; Fig. 5b), the other patients showed increased levels of collagen type I of 55.2% (patient no. 698, black column Fig. 5B), 40% (patient no. 728, black column; Fig. 5B) and 89.3% (patient no. 785, black column; Fig. 5B).

In view of the increased intensity of collagen type I green fluorescence in patient no. 728 (Fig. 2A–C, mono-

layer; and Fig. 3A–C, coculture), the corresponding western blot protein pattern of the cell extracts shown in Fig. 4A (monolayers) and Fig. 4B (cocultures) strongly suggests that the modulation exists, but is not significant. Although not shown by indirect immunofluorescence, the monolayer (Fig. 4A) and coculture cell extracts (Fig. 4B) of the other patients contributed to the suggestion of nonsignificant modulation by cyclosporin A. The data obtained from the supernatants indicate that regardless of the treatment duration, significant modulation of the level of collagen type I protein is restricted to the coculture situation (i.e. when gingival overgrowth-derived gingival fibroblasts were growing in the presence of gingival keratinocytes). This restriction to the coculture situation may indicate that the stimulatory effects of cyclosporin A on collagen type I were mediated through nonspecified fibroblast–keratinocyte interactions. A summary of the semiquantitative detection of collagen type I expression obtained from the cell extracts and their corresponding supernatants is given in Table 2.

## Discussion

Administration of the immunosuppressive drug cyclosporin A frequently leads to gingival overgrowth as an adverse effect. A feature of gingival overgrowth is the accumulation of extracellular matrix in gingival connective tissue, particularly collagenous components. Although the mechanisms underlying gingival overgrowth have still not been completely elucidated, the scientific evidence elaborated to date leads to the prevailing hypothesis that disruption of collagen homeostasis (i.e. the balance between synthesis and degradation in the gingival connective tissue) is a main causative factor (3).

Behind this background, our own studies carried out using native tissue specimens of patients suffering from gingival overgrowth, revealed elevated levels of collagen type I and the collagen type I-associated proteoglycan, decorin, in the connective tissue when

matched with healthy tissue (8). Because we were able to demonstrate that in addition to the proteins, collagen type I and decorin were also up-regulated at the transcriptional level, we could here contribute to the affirmation of the aspect that cyclosporin A affects collagen synthesis and its associated molecules, which has been postulated in the above-mentioned hypothesis. On the other hand, elevated mRNA and protein of the matrix metalloproteinases (MMPs) MMP-1 and MMP-10, and most strikingly for tissue inhibitor of MMP, described by our own group, support the other part of the hypothesis concerning the involvement of collagen degradation (20).

Although it appears likely from these *in vivo* studies that mesenchymal cells of the connective tissue and fibroblasts in particular may be involved in the expression of these gingival overgrowth features, they fail to prove direct evidence on designating gingival fibroblasts as the true source of origin of the molecules previously described. To elucidate whether collagen type I is a cyclosporin A target and whether fibroblasts derived from gingival overgrowth, as recipients of cyclosporin A, are the pivotal cell source for collagen type I up-regulation, we designed our cyclosporin A-treatment experiments in a dual manner. First, we established the gingival overgrowth-derived gingival fibroblasts in conventional monolayers, thereby paralleling the *in vitro* culture mode regularly described in contemporary literature. Second, to analyze the putative cyclosporin A-related effects under more *in vivo*-like conditions, we inaugurated cocultures that, similar to the native tissue situation, facilitate interactions between the gingival overgrowth-derived fibroblasts and gingival keratinocytes.

Intriguingly, all differences observed for collagen type I gene and protein expression in our study were detectable at the level of significance exclusively in the interactive coculture system. This applied for all patients in the study and included the quantitative transcription analysis reflecting the status of mRNA, and also the secreted protein,

which was detected by semiquantitative western blot. The fact that the differences in monolayers were marginal after cyclosporin A treatment may, on the one hand, point to a possible insufficiency of the monolayer in showing the effects of cyclosporin A. On the other hand, they suggest that although not explicitly analyzed in the present study, the fibroblasts may require interactions with their natural tissue counterpart (i.e. the gingival keratinocytes of the epithelium) in order to exhibit their competence in responding to cyclosporin A in a gingival overgrowth-like manner. Concerning the collagen type I protein, semiquantitative western blot analysis for the cell extracts revealed only small and nonsignificant differences between monolayers and cocultures. Although not detected on a quantifiable level, this finding was adumbrated by the indirect immunofluorescence studies. This considerable coincidence between the two methods of analyses indicates that the differences did not manifest themselves at the level of intracellular or cell membrane-bound collagen type I, but at the level of the secreted protein.

With respect to collagen type I gene or protein expression, the *in vitro* and *in vivo* studies available to date report ambiguous results following cyclosporin A treatment. While an increase in  $\alpha$ -procollagen chain and collagen secretion was noted in normal/healthy human gingival fibroblasts (12,21), other cell culture studies, where the gingival fibroblasts grew incorporated in a collagen cell culture lattice, reported an unaffected collagen type I status (22). Interestingly, in the latter study, control fibroblasts grown on plastic substrates exhibited inhibition of collagen synthesis. This discrepancy between a conventional culture mode and a mode seeking a more physiological environment for the fibroblasts may indicate that cyclosporin A did not reach the cells in the collagen gel because of the mechanical barrier function of the collagen lattice. A further explanation for the controversial findings on collagen type I in cyclosporin A-treated monolayers of normal/healthy gingival fibroblasts

may be the uncertainty of if the volunteers account to the group of patients which are cyclosporin A-sensitive at all in case of therapeutic administration. *In vivo* findings obtained from a rat model describe lower collagen type I transcription rates upon cyclosporin A administration. This decrease in collagen type I mRNA synthesis was seen at day 55, while a concomitant protein accumulation was noted in the subgingival connective tissue. These results indicate that although collagen type I transcription was decreased on day 55 when compared with the initial level, residual transcription could contribute to the collagen type I accumulation in the connective tissue over time. Moreover, the authors described lower levels of collagenase and a decline in gingival fibroblast phagocytosis of collagen type I (5). From this situation, observed in the rat model, it can be hypothesized that collagen type I elevation is a facultative, but not obligatory, criterion during manifestation of the gingival overgrowth phenotype. Thus, other key players in collagen homeostasis may be also targeted by cyclosporin A in the case of responding patients. Key players other than collagen type I itself, including its gene transcription, synthesized and cell-bound protein, and secreted protein, may include impairment in collagen phagocytosis, decrease in MMPs (3) and increase in TIMPs, the latter result reported by this study group (20). A further hallmark indispensable for the biosynthesis of proper collagen fibrils is a set of collagen-processing enzymes including lysyloxidase (LOX) (17). Interestingly, significantly increased levels of relative lysyloxidase gene transcription, resulting from cyclosporin A stimulation, were found in gingival overgrowth-derived gingival fibroblasts (13.67-fold at 6 h and 25.23-fold at 72 h in comparison with the control) again only in the coculture situation, whereas the results of the test monolayers remained similar to those of controls, as shown for patient no. 642 (Fig. 1C). This significant elevation, exclusively seen in the coculture situation, may lead to the assumption that the *LOX* gene is an as yet

unidentified target of cyclosporin A. Thus, for the general concept of cyclosporin A-induced gingival overgrowth, the multifactorial nature of this process described by Seymour (2) may also hold true at the molecular level, here addressing the molecular cornerstones that orchestrate collagen homeostasis.

Irrespective from being a facultative or an obligatory cyclosporin A-target, collagen type I expression should be restricted to the gingival overgrowth-derived gingival fibroblasts in the coculture situation, in order to emphasize the decisive role of the fibroblast located in the subgingival connective tissue for the manifestation of the gingival overgrowth phenotype. In fact, transcription analysis in treated cocultures indicated unchanged mRNA expression level at 72 h in gingival keratinocytes (Fig. 1B, insert B1, demonstrated for patient no. 698), the time-point where the most striking effects on collagen type I were seen in the cyclosporin A-treated cocultures. Because keratinocytes were also devoid of the protein (Fig. 3B, insert B1, 6 h; and Fig. 3C, insert C1, 72 h; shown for patient no. 728), it becomes evident that the epithelium contributes to the significant effects of cyclosporin A on collagen type I seen in the cocultures by so far unidentified fibroblast-directed synergistic mechanisms rather than by actively expressing collagen type I. At the transcriptional level, collagen type I elevation has been reported in a keratinocyte cell line subjected to cyclosporin A (23), but this may be a consequence of the monolayer situation. Herein, the epithelial cells are growing isolated from the influences of connective tissue-fibroblasts, representing part of their natural tissue environment.

Taken together, our studies on collagen type I gene and protein expression in monolayers and cocultures of gingival overgrowth-derived gingival fibroblasts indicate that collagen type I is a cyclosporin A-sensitive target, and that the cyclosporin A-related significant increase of collagen type I seen for the mRNA and the secreted protein implies fibroblast-keratinocyte interactions, provided by the cocultures.

Thus, cocultures allow for analysis of cyclosporin A-mediated effects under more *in vivo*-like conditions and further emphasize that gingival fibroblasts hold a decisive position in the onset and manifestation of the gingival overgrowth phenotype.

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