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Extracellular glycosaminoglycan changes in healthy and overgrown gingiva fibroblasts after cyclosporin A and cytokine treatments

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BACKGROUND: It has been demonstrated that cyclosporin A (CyA) blocks the immune system, acts on cytoskeleton and stimulates the production of extracellular matrix (ECM) and transforming growth factor-β_1 (TGF-β_1). This cytokine, such as transforming growth factor-α (TGF-α), induces deposition of glycosaminoglycans (GAG), proteoglycans and collagen fibres in the ECM.
METHODS: In this work, we examined the effect induced by CyA, TGF-β_1 and TGF-α on cultures of healthy and overgrown human gingival fibroblasts in order to evaluate the glycosaminoglycan, cytoskeletal changes and the behaviour of fibroblasts after concanavalin A (Con A) treatment. Moreover, we examined gingival biopsies by Alcian blue histochemical staining and electron transmission microscopy.
RESULTS: Total and extracellular sulphated GAG in overgrown gingiva specimens and in derived fibroblast cultures treated with CyA and cytokines were significantly higher than controls. The action of cytokines was increased ($P \leq 0.01$) compared with CyA with a greater effect of TGF-α in comparison with TGF-β_1; the electron microscopy showed ECM accumulation. The agglutinations showed the heterogeneity of fibroblast populations.
CONCLUSIONS: Stimulation with Con A showed that the fibroblast population had cell surface heterogeneity, and could respond in a different way to both CyA and cytokine stimulus. Moreover, increased synthesis of GAG in overgrown gingiva compared with synthesis in normal fibroblasts before CyA treatment suggests a possible genetic origin of damage. As not all CyA-treated patients develop gingival overgrowth, a genetic predisposition may explain the different responses of gingival fibroblast populations.
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Keywords: cyclosporin A; gingival overgrowth; glycosaminoglycans; transforming growth factor-α; transforming growth factor-β_1

Introduction

Cyclosporin A (CyA) is a potent immunosuppressive drug that acts directly on T cells, but is not effective on humoral immunity (1). For these properties it is used by patients submitted to organ transplantation. Unluckily, in addition to this positive action, unwanted side-effects may sometimes arise such as nephrotoxicity, hepatoxicity, neurotoxicity, Kaposi's sarcoma and gingival overgrowth (2–6). Suggested causes of the human gingival overgrowth induced by CyA are altered immune response (7), decreased degradation of type I collagen (8), increased activity of interleukin-6 (9), increased volume of the extracellular matrix (ECM) for inhibition of enzymes' production and their degradation activity (10, 11), transforming growth factor (TGF) activation (12), increased bacterial lipopolysaccharide of dental plaque, which is considered a cofactor of gingival overgrowth (13, 14), and increased volume of ECM (15, 16).

Numerous authors have demonstrated that CyA acts on fibroblasts both *in vivo* and *in vitro* by stimulating the synthesis of total extracellular glycosaminoglycans (GAG), i.e. hyaluronic acid (HA) and sulphated GAG such as chondroitin 4- and 6-sulphate (CS), dermatan sulphate (DS) and heparan sulphate (HS) (9, 12, 17), without cytotoxicity (18–20) or cytoskeletal alteration (21).

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In contrast, other authors have shown that the total GAG and single classes, including HA, are not altered in CyA-induced gingival overgrowth (13, 22, 23), and that CyA increases lamin and β-tubulin fragmentation (24).

The action of immunosuppressive drugs in transplanted organ care is intended to prevent organ rejection as a result of immune response. The cells mainly involved are T-lymphocytes, macrophages and B-lymphocytes, which are activated by a complex series of events mediated by the lymphokines, acting directly on foreign cells. CyA blocks the immune system, preventing the production of cytokines, but stimulates the production of TGF-β_1 (25, 26). TGF-β_1 is a cytokine involved in the regulation of ECM production (27), HA binds the TGF-β_1 (28), and β-glycan competes with the TGF-β site-binding (29), so the ECM is involved in control cytokine activity: this suggests that the ECM could be involved in the mechanism of gingival overgrowth. Moreover, *in vitro*, the ECM synthesis and cell proliferation are stimulated even by other cytokines such as TGF-α (30, 31). In previous experiments using concanavalin A (Con A), we demonstrated that different GAG syntheses are related to the cell surface heterogeneity of the human adult fibroblast population (32). As the ECM is very important for cell functions and for preserving the functionality of adult human organs (33, 34), in this investigation we studied the effects of CyA, TGF-α and TGF-β_1 on components of extracellular GAG synthesized by human fibroblasts derived from healthy and overgrown gingiva, as well as on the cytoskeletal apparatus. The behaviour of fibroblasts after Con A treatment and histochemical analysis of the ECM of gingival biopsies by Alcian blue staining was also analysed.

Materials and methods

Tissue specimens of healthy and overgrown gingiva were obtained from the premolar area during oral surgery (Dental Clinic of the University of Ferrara) in renal transplant patients undergoing chronic CyA treatment (six females 40–55 years old) and healthy, non-inflamed periodontal tissue donors (five females 30–50 years old). Each donor had undergone dental prophylaxis and 3 weeks of intensive oral hygiene.

Light microscopy
For light microscopy studies, the samples of overgrown and normal gingiva were fixed in 10% neutral formalin and embedded in paraffin, sectioned in 5 μm slices, and stained with hematoxylin and eosin.

Ultrastructural microscopy
Gingival biopsies were fixed in 1% glutaraldehyde in sodium cacodylate buffer, pH 7.4. Subsequently they were post-fixed in cold 2% OsO_4. After dehydration in acetone, the specimens were embedded in Epon-Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by a Hitachi 800 transmission electron microscope (Hitachi, Tokyo, Japan).

Histochemical study
For histochemical analysis, in order to distinguish non-sulphated GAG such as HA, and sulphated GAG such as CS, DS and HS, we used staining with Alcian blue 8 GX (AB) (Fluka, Buchs, Switzerland) 1% in 0.3 M $MgCl_2$ (Hoechst 0.1% in 0.2 M phosphate buffer, pH 6, 37°C, 1 h). For enzymatic digestion, the sections were incubated with testicular hyaluronidase (Merck, Darmstadt, Germany; 1 mg/ml 0.1 M phosphate buffer, pH 7) for 6 h at 37°C. Control sections were incubated in buffer alone. The GAG was identified by critical electrolyte concentrations at which the polyanions change from binding Alcian to binding Mg^{++} (35). Alcian stained the polyanions with increasing selectivity as the $MgCl_2$ concentration of the staining solution was raised. At 0.3 M $MgCl_2$ the only macromolecules which were positively stained were the non-sulphated (HA) and sulphated GAG (CS, DS, KS, HS). The action of specific enzymes on the sections, followed by Alcian staining, allowed us to determine the distribution of sulphated and non-sulphated GAG. Digestion with testicular hyaluronidase, in particular, selectively removed HA (completely) and CS (partially), which might no longer be bound to proteoglycans. As a good relation between colour and GAG concentration has been demonstrated (36), we obtained GAG values by measuring the optical density of five random areas, connecting a Zeiss Axioplane Microscope (Carl Zeiss Jena, Germany) to a Kontron Electronic Scanner by Vidas Software. We performed two slides per sample; the values were expressed as relative optical density (arrangement: black = 0, white = 1), and were the mean ± SD of five determinations per slide.

Fibroblast cultures
Each biopsy was washed with Hank's balanced salt solution containing 50 μg/ml of gentamycin, and immediately minced with sterile scissors. Tissue fragments were transferred to 25 cm^2 Nunc flasks and after adherence, were supplemented with 5 ml 199 medium (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (Gibco), 100 IU/ml penicillin, 100 μg/ml streptomycin. Cultures were maintained in humidity-satured atmosphere (5% CO_2, 37°C) and routinely subcultured after use of 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in Ca^{++} and Mg^{++} free Hank's buffered saline for cell release. Experiments were performed with fibroblasts between the fifth and eighth passage in serum-free medium. Cells were seeded at the density of 2.5×10^5 cells/cm^2 Nunc flasks for GAG determination. Duplicate cultures were carried out for each experiment. Cultures were routinely monitored by phase-contrast Leitz inverted microscope (Ernst Leitz, Wetzlar, Germany) and fibroblast viability was measured by the cells' ability to exclude trypan blue (37).

CyA, TGF-α and TGF-β_1 treatments
The CyA stock solution was prepared by dissolving the drug in olive oil at a concentration of 50 mg/ml. The final concentration of drug in the medium was 100 μg/ml. This concentration was selected because it is in the range

of drug serum concentration that elicits a therapeutic effect on renal transplanted patients. The TGF-β_1 was added at 4 μg/ml, and TGF-α at 4 ng/ml concentration. Control cells received vehicle alone. Incubation was continued for 24 h in medium 199 serum-free cultures.

Immunofluorescence

The cells were washed with phosphate-buffered saline (PBS), containing 2 mM ethylglycol-bis-(B-aminoethyl-ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (Sigma Chemical Co., St Louis, MO, USA), and 2 mM $MgCl_2$, pH 7.3 (PBS), fixed in cold methanol for 5 min at room temperature, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min, incubated with swine serum (one of 10) (Sigma) for 30 min at room temperature, and then with primary antibodies overnight at −4°C. After incubation, the cells were rinsed in PBS and further incubated with fluoresceine isothiocyanate conjugate-goat antimouse IgG, cytokeratin A IgM low-molecular weight (MW), cytokeratin B IgG1 high MW, actin IgG1, α-actinin IgG1, vimentin IgG1, and β-tubulin IgG1, for 45 min at room temperature (Table 1). The slides were mounted in (30:70) PBS/glycerol solution and examined using a Leitz Ortoplan epifluorescence microscope. The cell fluorescences were quantified using a green- or red-specific canal of the ADOBE PHOTOSHOP 7.0 computer program (Adobe Systems, San Jose, CA, USA). The values are expressed as relative optical density, and are the mean ± SD of 10 independent determinations.

Newly synthesized GAG isolation and identification

Confluent cultures were incubated with 5 ml 199 medium without or with CyA, TGF-α or TGF-β_1 and labelled with 5 μCi/ml ^{3}H-glucosamine (Amersham Int., England, UK; s.a. 22 Ci/mmol). After 24 h intracellular and extracellular GAG were isolated and analysed. Cells and media were separately recovered, boiled for 5 min and digested at 37°C with 1 mg/ml of pre-digested (37°C, 30 min) pronase E from *Streptomyces griseus* (Sigma) for 3 days at 37°C in the presence of 1% toluene. Fresh pronase was added daily. Digested samples were precipitated with 10% trichloroacetic acid (TCA) and centrifuged. GAG were precipitated from supernatants by three volumes of 5% potassium acetate in absolute ethanol (4°C, 7 days), recovered by centrifugation (12 000 *g*, 20 min) and dissolved in 0.1 M Tris-HCl, pH 7.2. Aliquots of ^{3}H-labelled GAG were mixed with 10 ml Pico-Fluor 40 (Camberra Packard Int. S.A., Zurich, Switzerland) and counted in a Packard Tris-Carb 2425 liquid scintillation counter (Packard, Downers Grove, IL, USA). Individual ^{3}H-labelled GAG were separated with a dielthylaminoethyl (DEAE)-cellulose anion-exchange column (0.7 × 13 cm; DE 52, Whatman, England, UK) equilibrated with 10 mM Tris-HCl (pH 7.2). Following washing with 0.1 M NaCl in 10 mM buffer to remove glycopeptides, the column was eluted at room temperature with 100 ml 0.3 M NaCl (non-sulphated GAG) and 100 ml 0.8 M NaCl (sulphated GAG) in fractions of 2 ml at room temperature. 0.2 μl of each fraction were mixed with 2 ml of Aquasol-2 (NEN), and the samples were counted in a Packard Tri-carb 2425 liquid scintillator. Column fractions corresponding to each peak were pooled, dialyzed and lyophilized. The GAG were identified by elution profiles and enzymatic susceptibility to chondroitinase ABC and AC *Streptomyces* hyaluronidase according to Conrad et al. (38). Total GAG was determined by directly computing the amount of radioactivity over the range of column fractions eluted from 0.3 to 0.6 M NaCl. Data were expressed as cpm/mg protein. Proteins were determined according to Lowry et al. (39) on aliquots of medium and cell lysate.

Con A treatment

Cultured cells after 24 h of CyA treatment were washed with Ca^{2+} and Mg^{2+} free PBS then treated with 0.02% EDTA (30 min at 37°C) in PBS, and finally suspended in PBS at 1×10^6 cells/ml. 0.5 ml of cell suspension was mixed with 0.5 ml PBS containing Con A at 80 μg/ml in Petri dishes. After 30 min incubation at room temperature with gentle shaking, cell agglutination was observed with a microscope. The degree of agglutination was expressed in percentage of total cells present in Petri dishes.

Statistical analysis

The values are the mean ± SD of six duplicate experiments; inferential statistical analysis was performed using Students' *t*-test for paired and unpaired data. Differences were considered significant at 0.05 level.

Results

Control gingiva

At light microscopy, the control gingiva appeared to be composed of a weakly keratinized stratified squamous epithelium and a lamina of dense connective tissue.

Table 1 Monoclonal antibodies anti-intermediate filament proteins and antitubulin used cytoskeleton

Specificity	*IG classes*	*Clones*	*Dilution*	*Source*
Cytokeratin A (low-molecular weight)	IgM	35BH11	1/2000	Ortho Diagn. Sy
Cytokeratin B (high-molecular weight)	IgG1	34BE12	1/2000	Ortho Diagn. Sy
Actin	IgG1	A25	1/100	Dakopatts Sera Lab.
	IgG1	211*	1/100	
α-Actinin	IgG1	A15	1/100	Dakopatts/Sera Lab.
Vimentin	IgG1	V9	1/10	Dakopatts
	IgG1	39F4803*	1/100	Sigma
β-Tubulin	IgG1	128F4808*	1/100	Sigma

*Immunofluorescent determination.

Upon electron microscopic examination, the cytoplasm of basal and spinous layers showed evident desmosomes (Fig. 1a) and bundles of microfilaments which were irregularly arranged (Fig. 1b).

Overgrown gingiva

At light microscopy the epithelium of overgrown gingiva appeared to be composed of the same cell layers, with enlarged intercellular spaces. The lamina propria was constituted by dense connective tissue with abundant ground substance. The most significative ultrastructural feature of the overgrown gingiva confirms more abundant ground substance in extracellular epithelial space (1c) when compared with normal gingiva (Fig. 1a). The cytoplasm of fibroblasts showed a well-developed Golgi apparatus, evident rough endoplasmic reticulum and numerous mitochondria. The nucleus was rich in finely scattered chromatin. Cells of basal and spinous layers showed oriented bundles of microfilaments (Fig. 1d).

Fibroblast morphology

Cultured fibroblasts from normal and overgrown gingiva exhibited a continuous monolayer of fusiform cells. No morphological differences were observed between normal and overgrown fibroblasts. Addition of CyA, TGF-β_1 and TGF-α did not affect the morphology or the viability of the cells compared with controls.

The actin, α-actinin, vimentin and tubulin patterns were uniformly distributed in the cytoplasm in untreated and CyA-, TGF-β_1- and TGF-α-treated fibroblasts. The immunofluorescence of cytoskeletal components such as actin, α-actinin, vimentin and tubulin (expressed as relative optical density) in overgrown fibroblasts did not show significant differences after CyA, TGF-β_1 and TGF-α treatments compared with normal fibroblasts (Table 2).

The absence of cytokeratin indicates that the cultures were free of epidermal cell contamination.

Histochemical data

The ground substance of both normal and overgrown gingiva exhibited marked alcianophylic staining at 0.3 M $MgCl_2$ (Table 3). The alcianophylia at 0.3 M $MgCl_2$ was partially abolished by hyaluronidase treatment: this indicated sulphated GAG content. The levels of sulphated GAG were greater ($P < 0.01$) in overgrown gingiva when compared with normal gingiva.

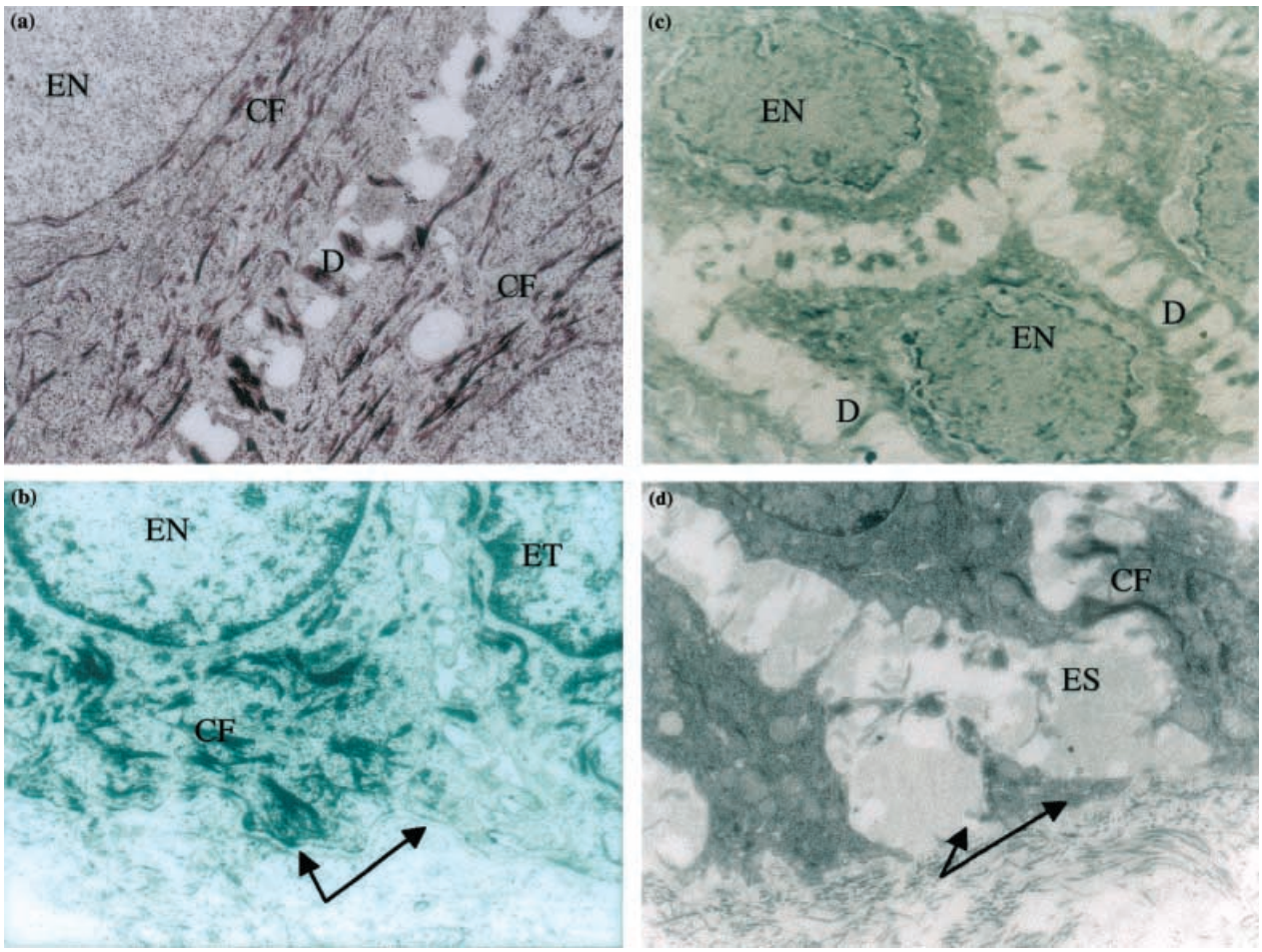

Figure 1 (a–d) Electron microscopic sections of normal and overgrown gingiva. Electron microscopy: normal (a, b) and overgrown gingiva (c, d). EN, euchromatic nucleus; D, desmosome; CF, cytoskeletal filaments; ES, extracellular electrondense substance; ET, etectrochromatin; arrows, basal membrane (uranyl acetate and lead citrate staining, magnification ×14 000 for a, b; ×9000 for c, d).

Table 2 Immunofluorescence of cytoskeletal components on control and overgrown human gingival fibroblast cultures treated with CyA, TGF-β_1 and TGF-α

	CyA	*TGF-β_1*	*TGF-α*
Actin			
Control	80.28 ± 16.06	73.29 ± 15.39	79.06 ± 14.67
Overgrown	76.11 ± 16.00	69.10 ± 16.58	67.23 ± 15.94
α-Actinin			
Control	88.92 ± 19.86	85.20 ± 13.61	83.00 ± 19.90
Overgrown	87.96 ± 17.08	89.50 ± 18.22	87.10 ± 14.00
Vimentin			
Control	92.66 ± 20.46	97.25 ± 21.44	95.33 ± 14.92
Overgrown	99.97 ± 23.93	96.32 ± 19.97	98.44 ± 22.64
Tubulin			
Control	54.90 ± 10.23	50.76 ± 9.88	51.92 ± 11.01
Overgrown	54.38 ± 12.96	52.81 ± 11.62	54.77 ± 10.97

The data (relative optical density) are the mean ± SD of six duplicate experiments. CyA, cyclosporin A; TGF, transforming growth factor.

Table 3 Histochemical GAG evaluation in normal and CyA-dependent overgrown gingival biopsies

	Alcian blue in $MgCl_2$ (0.3 M)	
	Before Jal	*After Jal*
Normal gingiva	115.6 ± 6.8	28.9 ± 2.3
Overgrown gingiva	120.2 ± 7.7	37.3 ± 3.1*

The values (relative optical density) are the means ± SD of five determinations per slide. Before Jal = before hyaluronidase treatment; after Jal = after hyaluronidase treatment; GAG, glycosaminoglycans; CyA, cyclosporin A.
*$P \leq 0.01$ when compared with normal gingiva.

As HA was sensitive and chondroitin sulphates partially sensitive to the enzyme, we can deduce that the higher amounts of sulphated GAG observed in overgrown gingiva were partly due to the CS themselves, and partly to other sulphated GAG such as DS, KS and HS.

Glycosaminoglycan analysis

The amount of total GAG (Table 4) was significantly increased in CyA, TGF-α, and TGF-β_1-treated cultures derived from normal gingiva when compared with controls. The amount of total GAG was much higher ($P \leq 0.01$) in CyA-, TGF-α- or TGF-β_1-treated fibroblast cultures derived from overgrown gingiva compared with controls. Then the effect of TGF-α and TGF-β_1 was significantly higher ($P \leq 0.01$) than that of CyA both in normal and overgrown cultures. The total GAG in control overgrown fibroblasts were significantly increased when compared with normal fibroblasts. The intracellular GAG variations were significantly increased in overgrown fibroblasts treated with CyA, TGF-α and TGF-β_1 with respect to controls, whereas the extracellular GAG variations increased ($P \leq 0.01$) in CyA-, TGF-α- and TGF-β_1-treated normal gingival fibroblasts with respect to controls. Overgrown cultures showed an identical trend, with greater effect ($P \leq 0.01$) compared with normal fibroblasts.

The intra-/extracellular GAG ratio was decreased ($P \leq 0.01$) in cultures treated with CyA, TGF-α and TGF-β_1 when compared with controls. Only the intra-/extracellular GAG ratio in overgrown cultures treated with TFG-α was significantly decreased compared with normal TGF-α-treated fibroblasts.

Table 5 shows non-sulphated and sulphated extracellular GAG variations in normal and overgrown fibroblasts treated with CyA, TGF-α and TGF-β_1. Non-sulphated GAG (HA) increases ($P \leq 0.01$) in TGF-α-treated normal cultures compared with controls. Overgrown fibroblasts showed a high non-sulphate synthesis in CyA, TGF-α and TGF-β_1 cultures. Extracellular sulphated GAG (CS, DS and HS) showed high levels in TGF-α and TGF-β_1 treated normal and overgrown fibroblasts compared with controls, whereas CyA showed an increase ($P \leq 0.01$) of extracellular sulphated GAG only in overgrown cultures compared with controls. The CyA, TGF-α and TGF-β_1 showed greater effect ($P \leq 0.01$) in overgrown cultures with respect to normal fibroblasts.

In the ECM, the non-sulphated/sulphated GAG ratio showed significant decreases in TGF-α and TGF-β_1 normal and overgrown cultures compared with controls, while CyA showed decreased non-sulphated/sulphated GAG ratio only in overgrown cultures.

Table 4 ^{3}H-glucosamine incorporation in normal and overgrown gingival fibroblasts after 24 h in vitro maintenance without serum

	Glycosaminoglycans (GAG)			
Fibroblasts	*Total GAG*	*Intracellular GAG*	*Extracellular GAG*	*Intracellular GAG/ Extracellular GAG*
Normal gingiva				
Control	148 246 ± 15 342	71 751 ± 9328 (48.4)	76 495 ± 10 709 (51.6)	0.937 ± 0.141
CyA	168 623 ± 18 793[a]	63 065 ± 9305 (37.4)	105 557 ± 12 902 (62.6)*	0.597 ± 0.09[a]
TGF-α	240 158 ± 25 843[ab]	91 260 ± 13 905 (38.0)[ab]	148 898 ± 20 613 (62.0)[ab]	0.613 ± 0.100[a]
TGF-β_1	193 908 ± 16 713[ab]	78 145 ± 9849 (40.3)	115 763 ± 13 258 (59.7)[a]	0.675 ± 0.09[a]
Overgrown gingiva				
Control	255 387 ± 25 345[c]	94 493 ± 13 300 (37.0)[c]	160 894 ± 24 134 (63.0)[c]	0.587 ± 0.08[c]
CyA	322 654 ± 24 478[ac]	110 992 ± 17 649 (34.4)*[c]	211 661 ± 23 032 (65.6)*[c]	0.524 ± 0.100[a]
TGF-α	416 280 ± 34 244[abc]	138 205 ± 22 113 (33.2)[abc]	278 075 ± 40 494 (66.8)[abc]	0.497 ± 0.09[ac]
TGF-β_1	345 240 ± 30 881[ac]	127 048 ± 20 057 (36.8)[ac]	218 191 ± 31 911 (63.2)[ac]	0.582 ± 0.111[a]

The values (cpm/mg protein) are the mean ± SD of six duplicate experiments.
[a]When compared with controls ($P \leq 0.01$); [b]when compared with CyA ($P \leq 0.01$); [c]when compared with normal gingiva ($P \leq 0.01$); *$P \leq 0.05$, when compared with controls. Values of percentage variations expressed in brackets. CyA, cyclosporin A; TGF, transforming growth factor.

Table 5 Extracellular glycosaminoglycans (GAG) expressed in terms of ^{3}H-glucosamine incorporation

	Extracellular glycosaminoglycans			
Fibroblasts	*Total extracellular GAG*	*Non-sulphated GAG*	*Sulphated GAG*	*Non-sulphated/ Sulphated*
Normal gingiva				
Control	76 495 ± 10 709	54 923 ± 8689 (71.8)	21 571 ± 4020 (28.2)	2.546 ± 0.306
CyA	105 557 ± 13 258	73 890 ± 9984 (70.0)	31 667 ± 4461 (30.0)	2.333 ± 0.355
TGF-α	148 898 ± 20 613[ab]	94 997 ± 14 449 (63.8)[ab]	53 901 ± 8546 (36.2)[a]	1.762 ± 0.264[a]
TGF-β_1	115 763 ± 13 902[a]	75 246 ± 9682 (65.0)	40 517 ± 5866 (35.0)[a]	1.857 ± 0.300[a]
Overgrown gingiva				
Control	160 894 ± 24 134[c]	107 799 ± 16 170 (67)[c]	53 095 ± 7433 (33)[c]	2.030 ± 0.212
CyA	211 661 ± 30 032[ac]	138 849 ± 19 439 (64.3)[ac]	75 563 ± 9877 (35.7)[ac]	1.837 ± 0.209[a]
TGF-α	278 075 ± 40 494[abc]	183 529 ± 25 695 (66.0)[abc]	94 545 ± 14 182 (34.0)[abc]	1.941 ± 0.330[a]
TGF-β_1	218 191 ± 31 911[ac]	141 387 ± 19 894 (64.8)[abc]	76 803 ± 10 852 (35.2)[abc]	1.841 ± 0.298[a]

Human normal and CyA–TGF-α–TGF-β_1-dependent overgrown gingival fibroblasts maintained *in vitro* for 24 h without serum. The values (cpm/cell proteins) are the mean ± SD of six duplicate experiments.
[a]When compared with controls ($P \leq 0.01$); [b]when compared with CyA ($P \leq 0.01$); [c]when compared with normal gingiva ($P \leq 0.01$).
Values of percentage variations expressed in brackets. CyA, cyclosporin A; TGF, transforming growth factor.

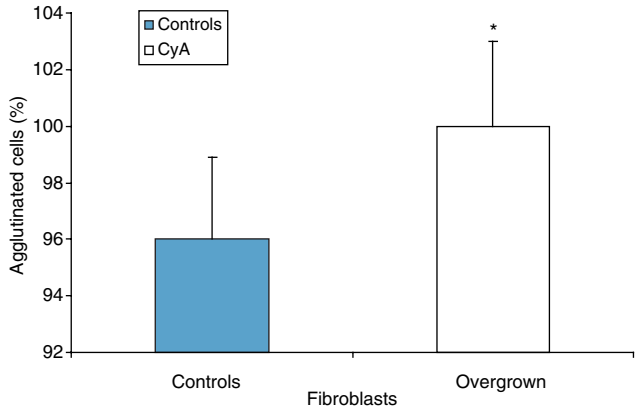

Figure 2 Effect of concanavalin A (Con A) (20 μg/ml) treatment on cell agglutination of healthy and cyclosporin A (CyA)-treated overgrown gingival fibroblasts. The values are the mean percentage ± SD of six duplicate experiments. *$P \leq 0.01$, when compared with controls.

Con A treatment

Normal and overgrown gingival fibroblasts treated with Con A showed variations in shape, with a less fusiform and more globular appearance, but no change in viability was detected. Agglutination testing 24 h after CyA treatment showed that agglutinability was greater in fibroblast cultures derived from overgrown gingiva with respect to normal gingiva (Fig. 2). The percentage of agglutinated cells in normal cultures was lower ($P \leq 0.01$) than that in overgrown gingival cultures.

Discussion

Our observations show that GAG increase in fibroblast cultures derived from overgrown gingiva with respect to healthy cultures; this increase is even more marked in fibroblast cultures derived from overgrown gingiva when they were further treated with CyA, TGF-α and TGF-β_1. The observations of fibroblast cultures derived from normal or overgrown gingiva do not show morphological differences in form and in the cytoskeletal apparatus, but some differences exist between the fibroblast populations obtained from normal and overgrown gingiva as far as GAG synthesis and Con A treatment are concerned. The cytokines are able to increase GAG secretion (27, 40), and the modifications in Con A agglutinability are well-known to depend on changes in the cell surface. The specific cell surface of gingival cells implies a different physiological role in gingival tissue for these cells, in agreement with Worapamorn et al. (41). As surface lectin ligands are involved in controlling GAG secretion by fibroblasts (42), the different agglutinability of human fibroblasts is related to GAG synthesis (32), and shows that these cells could respond differently to CyA stimulus. Control fibroblasts derived from overgrown gingiva show an increased synthesis of GAG compared with normal fibroblasts. These data suggest that the fibroblasts derived from overgrown gingiva have high synthesis activity, before culture maintenance. *In vitro* these differences of GAG synthesis increase especially after CyA, TGF-α and TGF-β_1 treatments with higher and more significative effect of cytokines than CyA. The diminished intra-/extracellular GAG ratio shows that CyA, TGF-α and TGF-β_1 increase GAG in the ECM, whereas the decrease of non-sulphated/sulphated GAG ratio shows that this increase was particularly evident for sulphated GAG. TGF-α stimulates the synthesis and secretion of HA; HA binds and inactivates TGF-β_1, and the increased extracellular sulphated GAG and diminished non-sulphated GAG/sulphated GAG ratio suggest that the relation between GAG and cytokine could be altered, with a diminished inactive effect on TGF-β_1. *In vivo* the control of fibroblast behaviour involves the action of hormones, growth factors and other cytokines, which are of course missing *in vitro*. Extrapolation of data obtained *in vitro* must consequently be considered with the appropriate limitations, when extended to the *in vivo* situation.

Moreover, the accumulation of GAG in the ECM increases trapped fluids in tissues due to the

accumulation of negative charges. This abnormal hydration of tissue could lead to gingival overgrowth, in contrast with Rocha et al. (23), who do not observe any GAG changes in CyA-treated gingival fibroblasts. As GAG is able to bind cytokines, to reduce their concentration in the ECM (28) and consequently the possibility of linkage with their receptor (29), changes in ECM composition could alter interactions between cytokines and the gingival fibroblast population. These speculations are in accordance with literature data, which demonstrate that the blockade of TGF-β_1 is related to mammary tumour cell viability and progression (43) and that their disregulation is related to oral carcinoma tumour progression (44).

The differences in agglutinability confirm that the gingival fibroblasts are heterogeneous (45, 46), and the real role of single populations on pathological development must be further studied; however, the fact that *in vitro* fibroblasts derived from overgrown gingiva have different GAG synthesis compared with normal fibroblasts suggests a possible genetic sensitivity according to Tipton et al. (47), who observed an inter-individual heterogeneity in collagenolytic response of gingival fibroblasts after CyA treatment.

As cytokines, such as TGF-β_1, are required for modulation of the intestinal epithelium, TGF-β_1 plays a central role in determining susceptibility to injury (48); therefore, in responsive subjects, the contribution of the gingiva epithelium should not be excluded and further studies are required to explain gingival overgrowth.

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