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Heterogeneous presence of myofibroblasts in hereditary gingival fibromatosis

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Abstract

Background/Aim: Hereditary gingival fibromatosis (HGF) fibroblasts are characterized by an increased production of collagen and transforming growth factor-β1 (TGF-β1), resulting in a fibrotic enlargement of the gingiva of affected patients. A common feature of interstitial fibrosis is the occurrence of myofibroblasts, which are regarded as the predominant cells in matrix synthesis. The goal of this article is to describe the presence of myofibroblasts in HGF in order to elucidate the mechanisms underlying HGF gingival overgrowth.

Materials and Methods: Fibroblast cell lines and gingival samples from patients of two distinct families affected by HGF and from normal gingiva (NG) were included in this study. To characterize the presence of myofibroblasts, the expression of specific myofibroblast marker smooth muscle isoform of α-actin (α-SMA) was examined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), Western blot, immunofluorescence, and flow cytometric analysis. Immunohistochemistry against the α-SMA antigen was performed in the gingival tissue samples.

Results: Our results demonstrated a significant increase in the expression of the myofibroblast marker α-SMA in cells from one HGF family (designed as HGF Family 2), which are also characterized by an elevated expression of type I collagen, TGF-β1 and connective tissue growth factor (CTGF). Additionally, α-SMA-positive cells were broadly detected in the gingival tissue samples from HGF Family 2 patients. In contrast, α-SMA expression by HGF Family 1 cells was quite similar to NG cells and no myofibroblasts were detected immunohistochemically, despite the higher levels of TGF-β1 and type I collagen in HGF Family 1 fibroblasts than in NG cells. The expression of CTGF, which has been considered a key molecule to promote the transdifferentiation of myofibroblasts via TGF-β1 activation, by HGF Family 1 cultures was significantly lower compared with HGF Family 2 and similar to NG control cells.

Conclusions: Our results suggest that the presence of myofibroblasts in HGF could be dependent on CTFG expression levels, and different biological mechanisms may account for the gingival overgrowth observed in HGF patients. This could be an underlying reason for the high variable clinical expressivity of disease.

Key words: connective tissue growth factor; gingival overgrowth; hereditary gingival fibromatosis; myofibroblast; smooth muscle isoform of α-actin; transforming growth factor-β1

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Myofibroblasts are cells related to fibroblasts and exhibit a hybrid phenotype between fibroblasts and smooth muscle cells (Gabbiani 1992). These cells are characterized by expression of the specific smooth muscle isoform of α-actin (α-SMA) and, when activated, synthesize high levels of extracellular matrix proteins, particularly collagen (Desmouliere et al. 2005). Several reports have shown the presence of cells with myofibroblastic features in specialized normal tissues and in a variety of pathological situations, such as Dupuytren's disease, cyclosporine-induced pancreatic fibrosis, and pulmonary, renal and hepatic fibrosis (Bisson et al. 2003; Desmouliere et al. 2003; Ahmed, et al. 2004). Furthermore, myofibroblasts are the main cellular type involved in extracellular matrix deposition during tissue repair. In spite of extensive studies, the exact mechanism of myofibroblast transdifferentiation is unknown. Evidences obtained from both human and

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animal models show that transforming growth factor-β1 (TGF-β1) stimulates myofibroblast transdifferentiation. This effect has been observed in several cell types and culture conditions, including human renal cell lines (Slattery et al. 2005), human pulmonary fibroblasts (Rehan et al. 2005), and rat cells (Grotendorst et al. 2004). However, recent studies suggested that TGF-β1 is probably not a direct mediator of the transformation of fibroblasts to myofibroblasts (Zhang et al. 2004; Grotendorst & Duncan 2005).

Hereditary gingival fibromatosis (HGF) is a rare oral disease characterized by a slow and progressive enlargement of both the maxilla and mandible gingiva (Bozzo et al. 1994). The enlarged gingiva is of normal colour, firm consistency, non-haemorragic, and asymptomatic (Bozzo et al. 2000). HGF occurs as an isolated finding or associated with other features such as hypertrichosis, mental retardation, and epilepsy (Ramon et al. 1967; Horning et al. 1985). HGF has an autosomal dominant mode of inheritance with variable penetrance and expressivity (Martelli-Junior et al. 2005). The most prominent pathologic manifestation of this disease is an excessive accumulation of extracellular matrix, predominantly type I collagen (Araujo et al. 2003). Many studies have shown increased transcriptional and translational levels of type I collagen in both tissue and fibroblast cultures derived from gingiva of HGF patients (Coletta et al. 1999a; Martelli-Junior et al. 2003). Furthermore, HGF fibroblasts produce excessive amounts of TGF-β1 (Coletta et al. 1999b; de Andrade et al. 2001; Martelli-Junior et al. 2003), which is, in association with an elevated synthesis of collagen, an intrinsic characteristic of myofibroblasts (Desmouliere et al. 2005). As HGF provides an excellent model for the study of connective tissue fibrosis, HGF cells produce abundant extracellular matrix and high levels of TGF-β1, and myofibroblasts represent a hallmark of interstitial fibrosis, the purpose of this study was to analyse the pattern of expression of α-SMA, as a marker of myofibroblast, in HGF compared with normal gingiva (NG).

Materials and Methods

Patients

The study included gingival samples from patients with NG and from members of two large families with HGF. Clinical, histological, and genetic data from both families were previously reported (Bozzo et al. 1994; Martelli-Junior et al. 2005) Only patients with fibrous gingival overgrowth without evidences of inflammation or a history of taking drugs associated with gingival overgrowth were selected from both families. From one family (Bozzo et al. 1994) five fibroblast cell lines from affected members with HGF (designed Family 1 for the purposes of this study) were established, and from the other (Martelli-Junior et al. 2005) were established two fibroblast cell lines from affected members (designed Family 2) were established. Five NG cell lines were used for comparative analysis. The study protocol was approved by the Ethical Committee in Research at the University of Campinas Dental School. All patients were informed about the study's purpose before they consented to participate.

Cell culture and gingival samples

Human gingival cultures were obtained using standard explant culture as described previously (Coletta et al. 1998). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS), 100 μg/ml penicillin, and 120 μg/ml kanamycin sulphate at 37°C in a 5% CO_2 air atmosphere. To assess the serum-starvation effect on α-SMA expression, cells were grown for 3 days in medium containing 0.1% FBS before the experiments. Each experiment was performed with cultures at a similar passage number (maximum difference of two passages), and cells were discarded after the 10th passage.

For immunohistochemistry, NG, and HGF samples were retrieved from the files of the University of Campinas Dental School, Department of Oral Pathology. New sections were cut from the paraffin blocks and stained with haematoxylin and eosin (H&E), or immunostained with anti-α-SMA (Dako Corp., Carpenteria, CA, USA).

Immunohistochemistry

α-SMA immunostaining was performed using 3-μm sections from 10% formalin-fixed, paraffin-embedded specimens. After dewaxing and hydration in graded alcohol solutions, the sections were treated with 3% H_2O_2, followed by incubation with 10 mM citric acid, pH 6.0, in a microwave for 24 min., divided into two cycles for antigen retrieval. After washing with phosphate-buffered saline (PBS), the sections were treated with 1% bovine serum albumin (BSA) in PBS for 1 h, and then incubated with monoclonal mouse anti-α-SMA diluted 1:200, followed by the ABC method (Dako Corp.). Reactions were developed by incubating the sections with 0.6 mg/ml 3,3"-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) containing 0.01% H_2O_2. Control reactions performed by the omission of the primary antibodies did not show any staining.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNAs were isolated from NG and HGF cells following the methods of Chomezynski and Sacchi (Chomczynski & Sacchi 1987) using the Trizol reagent (Invitrogen). The concentration and purity of RNA in each sample were determined by analysing spectrophotometric absorption at 260/280 nm. Following DNase I treatment in order to eliminate genomic DNA contamination, 2 μg of total RNA per sample was used to generate cDNA using the Superscript II RT enzyme (Invitrogen). The resulting cDNAs were subsequently amplified, analysed, and quantified as described previously (Martelli-Junior et al. 2003). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Primer sequences, PCR conditions, and the amplified lengths for α-SMA, type I collagen, TGF-β1, CTGF, and GAPDH are shown in Table 1.

Western blot analysis

Fibroblasts were washed with cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40%, 1% deoxycholic acid, 0.5% sodium dodecyl sulphate, 1 mM phenymethylsulphony fluoride, 1 mM *N*-ethylmaleimide, 1 mM dithiothreitol, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After centrifugation, protein concentrations were measured using a protein assay according to the manufacturer's instructions (Bio Rad protein assay, Bio Rad, Hercules, CA, USA). Fifty micrograms of total protein

Table 1. Primers used in the RT-PCR analysis

Gene	Sequence (5′ → 3′)	Annealing temperature (°C)	Number of cycles	Product length (pb)
α-SMA	Forward: GCT CAC GGA GGC ACC CCT GAA Reverse: CTG ATA GGA CAT TGT TAG CAT	61	40	589
Type I collagen	Forward: CTG GCA AAG AAG GCG GCA AA Reverse: CTC ACC ACG ATC ACC ACT CT	58	25	503
TGF-β1	Forward: AAG TGG ATC CAG GAG CCC AA Reverse: GCT GCA CTT GCA GGA GCG CAC	55	30	247
CTGF	Forward: AAC TAT GAT TAG AGC CAA CTG CCT G Reverse: TCA TGC CAT GTC TCC GTA CAT CTT C	55	35	477
GAPDH	Forward: GAA GGT GAA GGT CGG AGT C Reverse: GAA GAT GGT GAT GGG ATT TC	55	30	226

RT-PCR, reverse transcriptase-polymerase chain; α-SMA, smooth muscle isoform of α-actin; TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

per sample was resolved in a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and transferred onto nitrocellulose membranes (Invitrogen). The membranes were blocked for 2 h with 10% non-fat dry milk in PBS containing 0.1% Tween 20, rinsed in the same buffer, and incubated for 1 h with anti-α-SMA antibodies diluted 1:200 or with anti-β-actin (Sigma) diluted 1:40,000 in 5% milk in PBS. After washing, the membranes were developed using the Enhanced Chemiluminescent Western blot kit (Amersham Pharmacia Biotech, Arlington Heighs, IL, USA).

Immunofluorescence

Fibroblasts were plated at 5×10^3 cells per well in 8-well culture chamber slides (Lab Tek, Nunc, Naperville, IL, USA) and incubated at 37°C in humidified air containing 5% CO_2 for 24 h. Following incubation for 3 days in medium with 0.1% FBS, cells were washed in PBS and fixed in 70% ethanol for 15 min. To prevent non-specific binding, the cells were blocked with 3% BSA in PBS for 1 h. The cells were incubated for 1 h with mouse anti-α-SMA antibody diluted 1:100, followed by incubation with goat anti-mouse IgG conjugated with fluorescein (Vector Labs, Burlingame, CA, USA) at 1:250. Cells were mounted with Vectashield containing DAPI (Vector Labs), and then examined under a photomicroscope equipped with epifluorescence (DMR Microscope, Leica Microsystems, Nussloch, Germany). To generate fluorescent-labelled images, cells were excited at 480/40 nm with a 527/30 band pass filter. Cells untreated with primary antibodies were used as negative controls.

Flow cytometry

Single-cell suspensions were fixed with 70% ethanol, and stained for α-SMA as described above. Cells were washed, resuspended in PBS, and analysed on a FACScalibur flow cytometer equipped with an argon laser (Becton Dickinson, San José, CA, USA). A minimum of 10,000 events was collected on each sample, and only cells with forward and orthogonal light scatter characteristics similar to whole and intact fibroblasts were included in the analysis. Quantitative flow cytometric analysis was performed with the aid of CellQuest software (Becton Dickinson), measuring the percentage of positive cells and the mean of fluorescence intensity.

Statistical analysis

All data were presented as mean ± SD. One-way analysis of variance (ANOVA) with post hoc comparisons based on the Tukey test was performed to test group effects. The Spearman rank correlation test was assessed to verify the association between expression levels of type I collagen and TGF-β1. In our comparisons, $p < 0.05$ was considered to indicate statistical significance.

Results

α-SMA expression in NG and HGF tissue samples

Histological examination of gingival overgrowth tissues from both Families 1 and 2 stained with H&E revealed very similar findings. The gingival tissues comprised a well-structured epithelium with elongated and thin papillae inserted into deep fibrous connective tissue with collagen fibre bundles running in all directions (Figs 1b, c, e, f). Figures 1a and 1d show, on comparative analysis, the histological characteristics of one of the NG samples of this study. To determine the presence of myofibroblasts in the gingival specimens, we performed immunohistochemistry against α-SMA protein. This analysis revealed that all NG tissue samples ($n = 11$) were negative for α-SMA in the connective tissue, except in the smooth muscle of the blood vessel walls (internal positive control) (Fig. 1g and Table 2). One of the samples of HGF Family 1 ($n = 12$) showed a focal area with α-SMA-positive cells, whereas the other 11 samples were negative in the connective tissue (Fig. 1h and Table 2). On the other hand, all four samples from HGF Family 2 showed a broad expression of α-SMA-positive cells in the lesional connective tissue (Fig. 1i and Table 2). Interestingly, areas with moderate to intense inflammatory cell infiltrate showed a reduction in the number of positive cells.

α-SMA expression in NG and HGF cells

To determine whether cells derived from patients of HGF Family 2 show an altered proportion of myofibroblasts compared with HGF Family 1 and NG cells, a number of modalities were used that included RT-PCR, Western blot, immunofluorescence, and flow cytometric analysis. The semi-quantitative RT-PCR assay was used in this study for the measurement of steady-state levels of α-SMA mRNA in the cell lines derived from NG and HGF patients. In vitro, NG fibroblasts constitutively expressed low but detectable levels of α-SMA mRNA (Fig. 2). No differences were observed in the α-SMA expression among the fibroblast cell lines belonging to NG and HGF Family 1 groups,

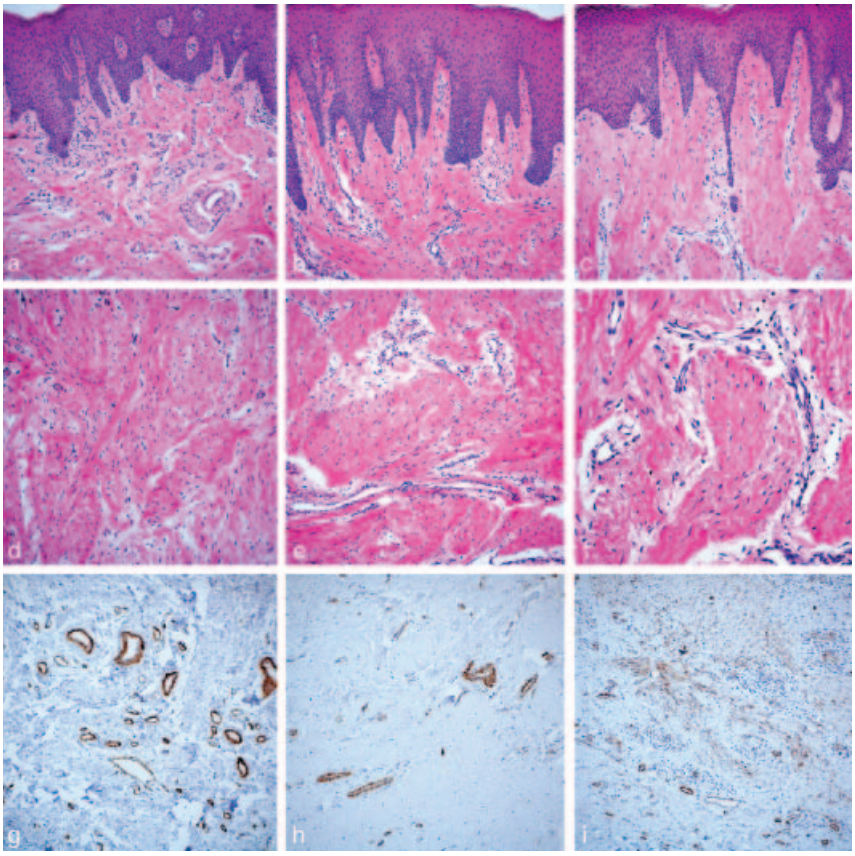


Fig. 1. Immunohistochemical detection of α-SMA in normal and HGF gingiva. Histological features and α-SMA immunohistochemical expression of a representative sample of NG (a, d, g), HGF Family 1 (b, e, h) and HGF Family 2 (c, f, i) of this study. Histological findings from both HGF families are very similar. The epithelium displays elongation of rate pegs, but the increased mass is primarily the result of a marked accumulate of dense fibrous connective tissue. α-SMA-positive cells were broadly observed in the lesional connective tissue of HGF Family 2 samples (i), whereas NG and HGF Family 1 samples were negative, except in the smooth muscle of the blood vessel walls (internal positive control). (a–c, original magnification × 100; d–i original magnification × 200)

Table 2. Immunohistochemical expression of α-SMA in normal gingiva and in tissue samples of patients from both HGF families

	Total (*n*)	α-SMA-negative samples	α-SMA-positive samples
NG	11	11	0
HGF Family 1	12	11	1 (8.33%)
HGF Family 2	4	0	4 (100%)

α-SMA, smooth muscle isoform of α-actin; HGF, hereditary gingival fibromatosis.

whereas HGF Family 2 fibroblasts clearly expressed higher levels of α-SMA mRNA in both 0.1% and 10% FBS concentrations (Fig. 2). To confirm these findings, Western blot was performed, revealing that HGF Family 2 cells produced higher amounts of α-SMA than normal and HGF Family 1 cells (Fig. 3). Fibroblasts cultured in 0.1% FBS, independent from the group, showed an increase in α-SMA expression and production compared with cells cultured in 10% serum concentration (Figs 2 and 3).

The distribution and proportion of α-SMA-positive cells in NG and HGF cultures were examined using immunofluorescence microscopy and flow cytometric analysis. Both NG and HGF Family 1 fibroblasts demonstrated vivid perinuclear staining for α-SMA, with the proportion of α-SMA-positive cells quite similar among them (Figs 4a and b). HGF Family 2 cells demonstrated cells with two distinct patterns of distribution: a perinuclear staining very similar to NG and HGF Family 1 cells, and another characterized by a brilliant striated staining throughout the cytoplasm that correlated with the pattern of stress fibres as reported previously (Figs 4c and d) (Gabbiani 2003). As expected, HGF Family 2 fibroblasts demonstrated a stronger fluorescence intensity and a higher number of positive cells for α-SMA compared with control (Fig. 5). The percentage of α-SMA-positive cells in the HGF Family 2 group ranged between 79% and 90% with a mean of 85.05% ± 6.81, whereas for the NG group, it ranged between 31% and 42%, with a mean of 37.08% ± 3.62, and for the HGF Family 1 group it ranged between 34% and 46%, with a mean of 42.41% ± 4.54.

HGF cells expressed high levels of TGF-β1 and type I collagen

To demonstrate that HGF fibroblasts have higher levels of TGF-β1 and type I collagen expression than NG fibroblasts, RT-PCR analysis was performed using total RNA isolated from fibroblast cultures established from NG, HGF Family 1, and HGF Family 2. Scanning densitometry of the PCR products, after normalization by the value from the GAPDH housekeeping gene, demonstrated statistically higher levels of type I collagen mRNA in fibroblast cultures from both HGF Family 1 and HGF Family 2 than in those from NG controls, in both 0.1% ($p<0.05$ for HGF Family 1 group, and $p<0.001$ for HGF Family 2 group) and 10% ($p<0.05$ for both HGF Family 1 and HGF Family 2) serum-containing medium (Fig. 6). HGF Family 2 cells showed higher levels of type I collagen in 0.1% serum-containing medium compared with HGF Family 1 fibroblasts ($p<0.05$). Type I collagen expression is significantly higher in fibroblasts growing in 0.1% serum-containing medium than in 10% serum-containing medium, independent of the cell line group ($p<0.01$; Fig. 6). The levels of TGF-β1 mRNA were also significantly higher in HGF cells from both Family 1 and Family 2 than in normal fibroblasts, but for the HGF Family 1 group, this was only when cells were grown in 0.1% FBS-DMEM (Fig. 7). TGF-β1 expression was significantly higher in the HGF Family 2 group, in both 0.1% and 10% serum-

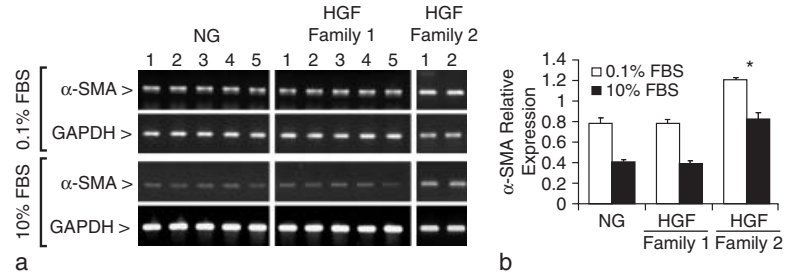


Fig. 2. Expression of α-SMA in gingival cells from NG and HGF. (a) Total RNA from cells cultured in the presence of 0.1% or 10% FBS was isolated, and cDNA synthesized by RT. After amplification using specific primers to α-SMA and GAPDH, the products were electrophoresed on a 1% agarose gel. (b) Densitometric analysis of the α-SMA bands demonstrated a significantly higher expression in HGF Family 2 fibroblasts compared to NG and HGF Family 1 groups. $^{*}p<0.05$

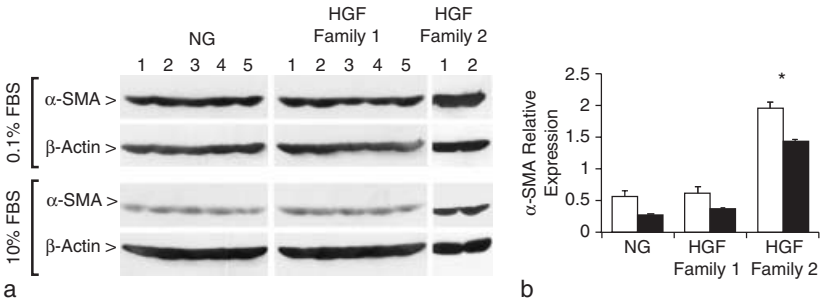


Fig. 3. Western blot analysis performed with total cellular proteins from NG and HGF cells. (a) Similar amount of protein of each sample was separated on SDS-PAGE, transferred to nitrocellulose membranes, and α-SMA production was analyzed utilizing anti-α-SMA antibodies. Blots were stripped and reprobed with anti-β-actin antibodies. (b) Comparison of production indicated that the amount of α-SMA produced by HGF Family 2 cells was significantly greater than NG and HGF Family 1 cells, in both concentrations of serum. $^{*}p<0.05$

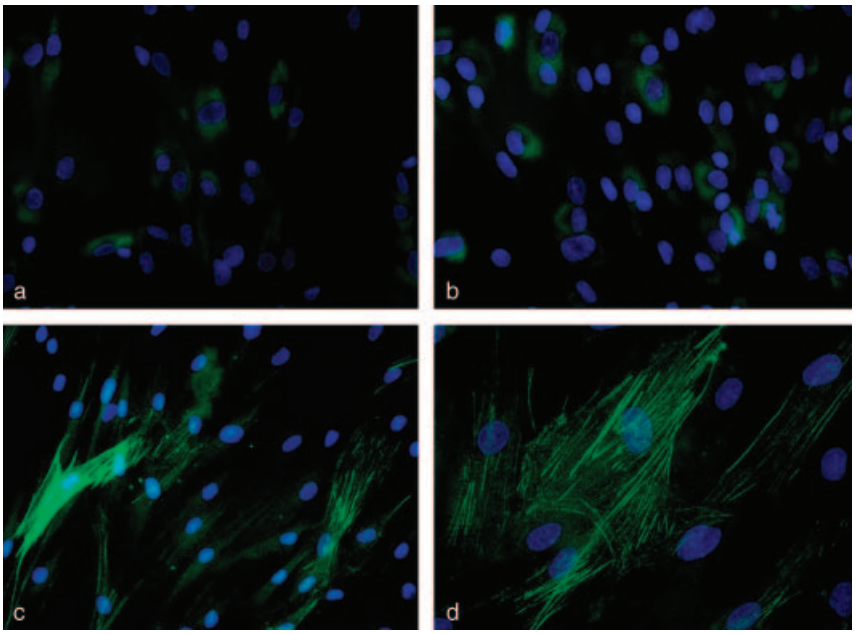


Fig. 4. Distribution of α-SMA in NG and HGF fibroblasts. Cells were stained with mouse anti-α-SMA primary antibodies and fluorescein anti-mouse IgG secondary antibodies, and analyzed in an immunofluorescence microscopy. DAPI was used for nuclear counterstain. Intense staining for α-SMA is observed in HGF Family 2. (a) represents NG cells, (b) HGF Family 1 cells, and (c–d) HGF family 2. (a–c, original magnification × 200; d, original magnification × 400)

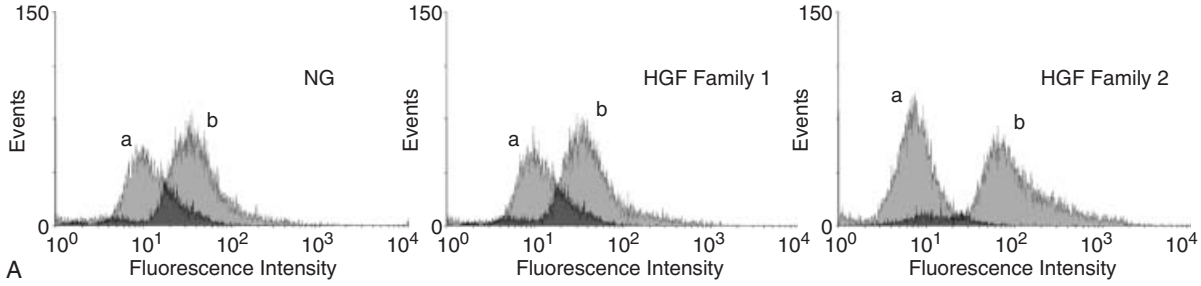
containing medium, than in the HGF Family 1 group ($p<0.01$).

CTGF expression is high in HGF Family 2 fibroblasts

As HGF Family 2 cultures demonstrate the presence of myofibroblasts and a higher expression of TGF-β1 than HGF Family 1, and TGF-β1 induces transdifferentiation of fibroblasts to myofibroblasts via stimulation of a CTGF-dependent pathway (Grotendorst et al. 2004; Zhang et al. 2004), we sought to determine whether CTGF expression levels could explain the difference in the presence of myofibroblasts between HGF Family 1 and HGF Family 2. RT-PCR analysis showed that CTGF expression is significantly higher in HGF Family 2 cell lines compared with control and HGF Family 1 cells in both 0.1% and 10% serum medium ($p<0.05$; Fig. 8). In addition, CTGF expression was quite similar between NG and HGF Family 1 groups. As expected, CTGF expression was higher in fibroblasts growing in 0.1% serum medium compared with 10% serum medium. Thus, the presence of myofibroblasts in HGF Family 2 may be due to the high CTGF expression levels.

Discussion

Although the underlying mechanisms of the gingival fibrosis that characterize HGF remain unclear, it has been proposed that gingival overgrowth develops through activation or selection of the resident tissue fibroblasts, phenotypically characterized by increased proliferation, low levels of extracellular matrix-degrading metalloproteinases (MMP-1 and MMP-2), and abnormally high collagen production (Coletta et al. 1998, 1999a, b). Furthermore, the autocrine stimulation by excessive amounts of TGF-β1 produced by HGF cells seems to contribute to these phenotypes (Coletta et al. 1999b; de Andrade et al. 2001; Martelli-Junior et al. 2003). A growing body of evidence has connected myofibroblasts with the pathogenesis of several fibrotic processes, as they are the main source of the increased extracellular matrix deposition (Gabbiani 2003; Desmouliere et al. 2004). The regulatory cytokine TGF-β1 has been traditionally considered an inducer of the myofibroblast phenotypes both in



B

	% α-SMA positive cells	Mean of fluorescence intensity
NG	37.08±3.62	127.92±8.51
HGF Family 1	42.41±4.54	125.39±9.48
HGF Family 2	85.05±6.81	213.27±12.73

Fig. 5. Flow cytometric analysis of NG and HGF fibroblasts with FITC-anti-α-SMA. Cells were plated, cultured for 3 days in 0.1% FBS medium, and analyzed by flow cytometry for α-SMA. Representative histograms with NG (left), HGF family 1 (middle) and HGF Family 2 (right) cells are depicted on panel (A). (a) control negative cells (FITC antibodies alone), and (b) FITC-anti-α-SMA stain. (B) Percentage of α-SMA positive cells and mean of fluorescence intensity for NG, HGF Family 1 and HGF family 2 cell groups. Note that the percentage of positive cells and the intensity of fluorescence are higher in HGF Family 2 group.

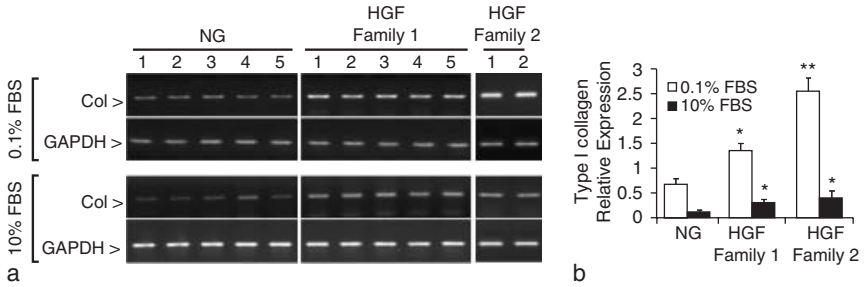


Fig. 6. Expression of type I collagen by NG and HGF cells. (a) RT-PCR analysis with specific primers for type I collagen and GAPDH. (b) Densitometric analysis demonstrated that type I collagen expression by HGF Family 1 and HGF Family 2 fibroblasts was significantly higher than NG fibroblasts, in both 0.1% and 10% serum medium. HGF Family 2 fibroblasts showed a significantly higher type I collagen expression compared to HGF Family 1 fibroblasts in 0.1% serum medium. Values represent the means ± SD from 2 independent experiments with all cell lines for each group. ($^{*}p<0.05$; $^{**}p<0.01$)

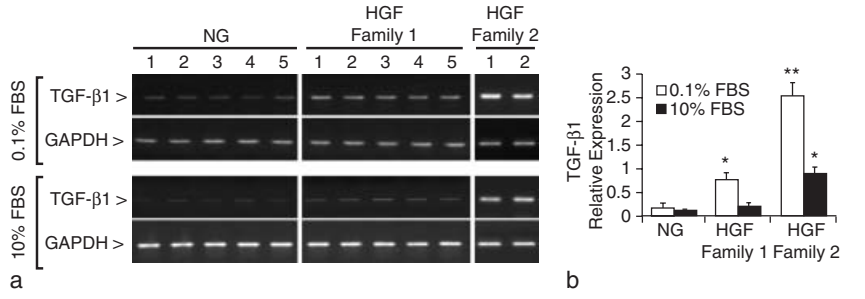


Fig. 7. Expression TGF-β1 by NG and HGF cells. (a) Representative RT-PCR analysis and (b) densitometric analysis. Expression of TGF-β1 was significantly higher in HGF Family 1 cells than control cells, but only when cells were cultured in 0.1% serum medium, whereas TGF-β1 expression by HGF Family 2 cells was significantly higher than control cells in both 0.1% and 10% serum medium. ($^{*}p<0.05$; $^{**}p<0.01$)

vivo and in vitro (Desmouliere et al. 1993, 1995). Because the HGF cells display their activated phenotype in the presence of elevated levels of TGF-β1, we were interested in determining whether myofibroblasts could play a role in the HGF gingival fibrosis. In this study, we have determined that there was an increased expression of α-SMA by HGF fibroblast cultures compared with NG controls in only one of the analysed families (HGF Family 2). Additionally, α-SMA expression was broadly presented in the lesional fibroblasts of all HGF Family 2 gingival tissues as revealed by immunohistochemical staining. There was no staining in the HGF family 1, with the exception of one focal area containing α-SMA-positive cells in one of the samples. Interestingly, α-SMA was detected in low levels in vitro in cultures from HGF Family 1 and NG fibroblasts but not in vivo, which is probably due to the stress fiber induction associated with cellular cultures. Furthermore, there was clearly a proportion of α-SMA-negative cells in all cell lines from either normal or HGF, suggesting heterogeneity of α-SMA expression in the fibroblast subpopulations. Such variations may be attributed to subsets of fibroblasts that exist in the gingiva and periodontum (Phipps et al. 1997). Given the fact that α-SMA is the specific marker of myofibroblasts, these results strongly suggest that myofibroblasts are involved in the pathogenesis of the gingival overgrowth of HGF Family 2, but not in HGF Family 1.

Several evidences have demonstrated that HGF is clinically, genetically, and biologically heterogeneous. Regarding the disease in the families studied here, we know from previous studies that

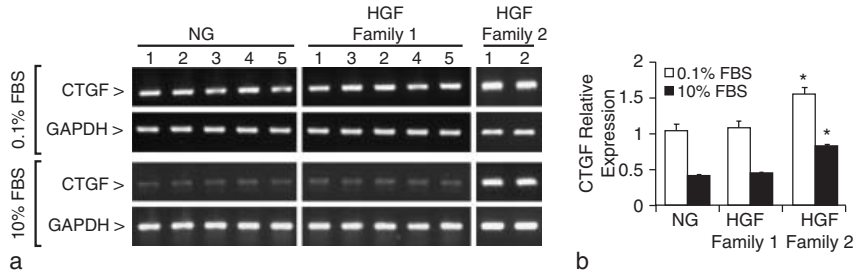


Fig. 8. RT-PCR analysis of CTGF mRNA in NG and HGF cells. (a) Representative RT-PCR results of expression of CTGF by NG, HGF Family 1 and HGF Family 2 cell lines. (b) Relative mRNA levels of CTGF normalized with GAPDH levels. The results showed that CTGF expression is significantly higher in HGF Family 2 cell lines compared with control and HGF Family 1 cells, which were quite similar, in both 0.1% and 10% serum medium. ($^*p < 0.05$)

there are clinical and genetic differences (Bozzo et al. 1994; Martelli-Junior et al. 2005). Although in both families the disease manifests as an isolated finding affecting all the gingival tissue, in Family 1 it is more severe and frequently associated with both aesthetic and functional problems, including diastemas, malpositioning of teeth, prolonged retention of primary dentition, delayed eruption, cross and open bites, prominent lips, and open lip posture (Bozzo et al. 2000). Moreover, recurrence is more frequent in patients from Family 1 than in patients from Family 2. While gene penetrance is incomplete and very low in HGF Family 2, as revealed by an offspring recurrence risk of 0.078 (7.8%) and a sibling recurrence risk of 0.085 (8.5%), in HGF Family 1 it is complete, with approximately half of the descendants being affected by HGF (Martelli-Junior et al. 2005). In addition, unaffected individuals in Family 2 transmit HGF in an autosomal dominant pattern to their offspring without themselves being clinically affected. In the present study, we add new heterogeneity for HGF, demonstrating that myofibroblasts are associated with HGF Family 2 gingival overgrowth tissues, but not with HGF Family 1. Taken together, these findings suggest that the HGF phenotype may be caused by distinct biological mechanisms.

The identification of the regulatory elements for myofibroblast transdifferentiation is of considerable importance for the molecular understanding of the mechanism of interstitial fibrosis, in general, as well as, in HGF. To date, it is suggested that myofibroblast transdifferentiation is critically dependent on TGF-β1. TGF-β1, a multifunctional cytokine with fibrogenic properties, has been implicated in the pathogenesis of fibrosis in several organs in both experimental and humans (Gressner et al. 2002; Chapman 2004). This is most clearly illustrated by the deliberate overexpression of TGF-β1 within the normal tissues, which results in fibrosis (Akagi et al. 1996). In addition, TGF-β1 stimulates extracellular matrix deposition by increasing the synthesis on the one hand while acting to inhibit their degradation on the other (Cotrim et al. 2002). Recent studies have indicated that CTGF plays a crucial role in mediating various profibrotic actions of TGF-β1. For example, CTGF has been reported to mediate TGF-β1-induced myofibroblast transdifferentiation, and TGF-β1-induced extracellular matrix synthesis and accumulation in both fibroblasts and renal epithelial cells (Yokoi et al. 2002; Zhang et al. 2004). Furthermore, a recent study demonstrated that CTGF binds to TGF-β1, resulting in stimulation of its activity (Abreu et al. 2002). In both HGF families, TGF-β1 levels are increased but CTGF expression levels are higher in fibroblasts from HGF Family 2 in both 0.1% and 10% serum concentrations, which correlated with the presence of myofibroblasts. Collectively, these findings suggest that elevation in TGF-β1 levels alone seems to be insufficient to induce myofibroblast transdifferentiation, and that elevated levels of CTGF are needed for this phenomenon. Interestingly, CTGF expression is higher in phenytoin-induced gingival overgrowth tissues, but not in cyclosporin- or nifedipine-induced gingival overgrowth (Uzel et al. 2001). Future studies will be necessary to determine the exact role of CTGF in the pathogenesis of different forms of HGF.

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Clinical Relevance

Scientific rationale for study: Information regarding the biological mechanism underlying the abnormal accumulation of excessive amounts of fibrous connective tissue in HGF is limited. *Principal findings*: We demonstrated that there likely is more than one biological mechanism resulting in the gingival overgrowth in HGF patients. Although fibroblasts isolated from two distinct families affected by HGF showed elevated expression of type I collagen and TGF-β1, only one of these families showed myofibroblasts.

Practical implications: HGF will be better understood when the clinical features are considered in conjunction with new genetic and cell biological data that are becoming available. These data will also have therapeutic relevance to treatment and recurrence of HGF gingival overgrowth.