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Opposite effects of TGF-β1 and IFN-γ on transdifferentiation of myofibroblast in human gingival cell cultures

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Abstract

Background/Aim: Previously, we have shown that myofibroblasts, the main cell type associated with interstitial fibrosis, may be implicated with the gingival overgrowth observed in hereditary gingival fibromatosis (HGF) patients. The goal of this study was to determine whether transforming growth factor-β1 (TGF-β1) stimulates myofibroblast generation in gingival fibroblast cultures. Moreover, we analysed how interferon-γ (IFN-γ) interferes in this process.

Material and Methods: Fibroblast cultures from normal gingiva and myofibroblast cells from HGF were included in this study. To determine the effects of TGF-β1 and IFN-γ stimulation in these cells, the expression of the 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 and immunofluorescence. Enzyme-linked immunosorbent assay (ELISA) for type I collagen was performed to measure the myofibroblast activity.

Results: Our results demonstrated that TGF-β1 promotes a dose- and time-dependent increase in the expression of α-SMA, whereas IFN-γ blocks it and markedly prevents the fibroblast–myofibroblast switch induced by TGF-β1 on normal gingiva cultures. IFN-γ altered HGF myofibroblasts metabolism with a decrease of both α-SMA and type I collagen expression. Additionally, IFN-γ treatment stimulated SMAD7 expression and inhibited connective tissue growth factor, which has been considered a key molecule to promote the transdifferentiation of myofibroblasts via TGF-β1 activation.

Conclusions: These findings demonstrate that TGF-β1 induces gingival fibroblast–myofibroblast transdifferentiation, whereas IFN-γ blocks this process. More importantly, this study suggests that IFN-γ may be clinically effective in attenuating excessive accumulation of extracellular matrix produced by myofibroblasts in HGF.

Key words: hereditary gingival fibromatosis; interferon-γ; myofibroblast; transforming growth factor-β1

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Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.

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Hereditary gingival fibromatosis (HGF) is an oral condition clinically manifested by a generalized and fibrotic enlargement of the gingiva (Coletta & Graner 2006). The disease results in both aesthetic and functional problems for affected individuals, such as primary dentition retention with delay in the eruption of the permanent teeth, difficulties in mastication and phonation,

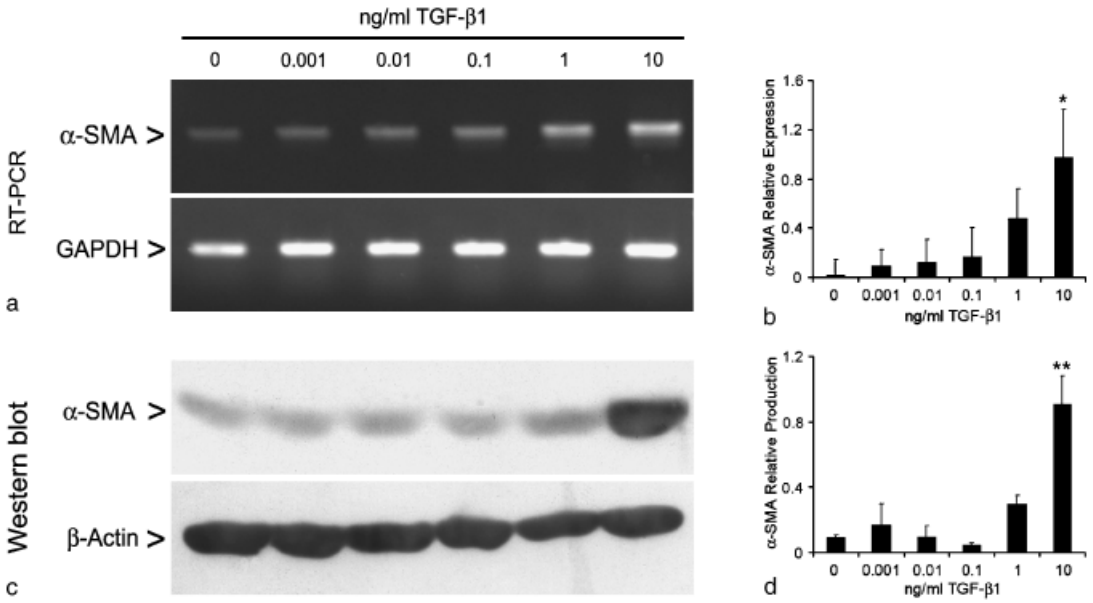

Fig. 1. Transforming growth factor-β1 (TGF-β1) induces transdifferentiation of normal gingival (NG) fibroblasts to myofibroblast. NG fibroblasts were cultured with different concentrations of TGF-β1 in culture medium containing 0.1% foetal bovine serum (FBS) for 3 days. Following treatment, cells were collected and subjected to either RNA purification and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (a) or total protein isolation and Western blot (c). Densitometric analysis of the α-actin (α-SMA) levels of RNA (b) and protein (d) demonstrated that cells grown with 10 ng/ml of TGF-β1 showed a significant increase in the expression and production of the myofibroblast marker α-SMA. $^{*}p<0.05$, $^{**}p<0.01$.

malpositioning of teeth and psychological problems for the patients and relatives (Martelli-Junior et al. 2005, Coletta & Graner 2006). The only treatment available is surgical resection of the overgrowth tissue, but recurrence is anticipated. We recently demonstrated that the presence of myofibroblasts is heterogeneous in HGF and may be associated with a pathogenesis of the gingival overgrowth observed in patients with this condition (Bitu et al. 2006). Myofibroblasts, characterized by the expression of the specific smooth muscle isoform of α-actin (α-SMA), contribute to the connective tissue fibrosis by increasing synthesis and decreasing degradation of extracellular matrix proteins (Desmouliere et al. 2005). However, relatively little is known about the underlying mechanisms that regulate myofibroblast emergence in HGF.

Several lines of evidence demonstrated an important role of cytokines in the regulation of the myofibroblast differentiation and activation. The regulatory cytokine transforming growth factor-β1 (TGF-β1) has been traditionally considered an inducer of the myofibroblast phenotype both in vivo and in vitro, but this differentiation process is not fully understood (Rehan et al. 2005, Slattery et al. 2005). Emerging experimental evidence suggests that TGF-β1-induced myofibroblast transdifferentiation is dependent of connective tissue growth factor (CTGF) stimulation (Zhang et al. 2004, Grotendorst & Duncan 2005).

Interferon-γ (IFN-γ), which is produced by inflammatory cells, shows opposite effects to TGF-β1, particularly on fibroblasts (Yamanaka et al. 2003, Ishida et al. 2004). This cytokine regulates extracellular matrix accumulation by inhibiting the synthesis of collagen and abrogating the stimulatory effects of TGF-β1 (Dooley et al. 2006, Ghosh et al. 2006). Furthermore, clinical studies demonstrated a therapeutic effect of IFN-γ injections into lesions of hypertrophic scars and Dupuytren's nodules (Pittet et al. 1994, Larrabee 1997). Whether IFN-γ might suppress myofibroblast transdifferentiation with subsequent effect on extracellular matrix accumulation in human gingival cell cultures is unknown. If so doing, IFN-γ might be an effective molecule for HGF therapy. To explore this hypothesis, in the present study we examined whether TGF-β1 and IFN-γ can modulate myofibroblast transdifferentiation of human normal gingival (NG) fibroblast cultures. Additionally, we have analysed whether IFN-γ could inhibit the myofibroblastic phenotype of human HGF cells.

Material and Methods

Cell culture and treatments

NG fibroblasts (designed as NG1 fibroblast cell line) and HGF myofibroblastic cell lines (designed as HGF1 and HGF2 myofibroblast cell lines) were described previously (Bitu et al. 2006). 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, 120 μg/ml kanamycin sulphate at 37°C in a 5% CO_2 air atmosphere. The study protocol was approved by the Ethical Committee in Research at the University of Campinas Dental School.

Lyophilized IFN-γ and TGF-β1 (R&D Systems, Minneapolis, MN, USA) were dissolved in culture medium, aliquoted and stored at -70°C. To assess the effect of these cytokines on myofibroblast transdifferentiation, cells were cultured in 0.1% FBS medium containing 0–10^6 U/ml of IFN-γ or 0–10 ng/ml of TGF-β1.

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

Total cellular RNA was isolated from NG1 and HGF cells using the Trizol reagent (Invitrogen, Carlsbad, CA,

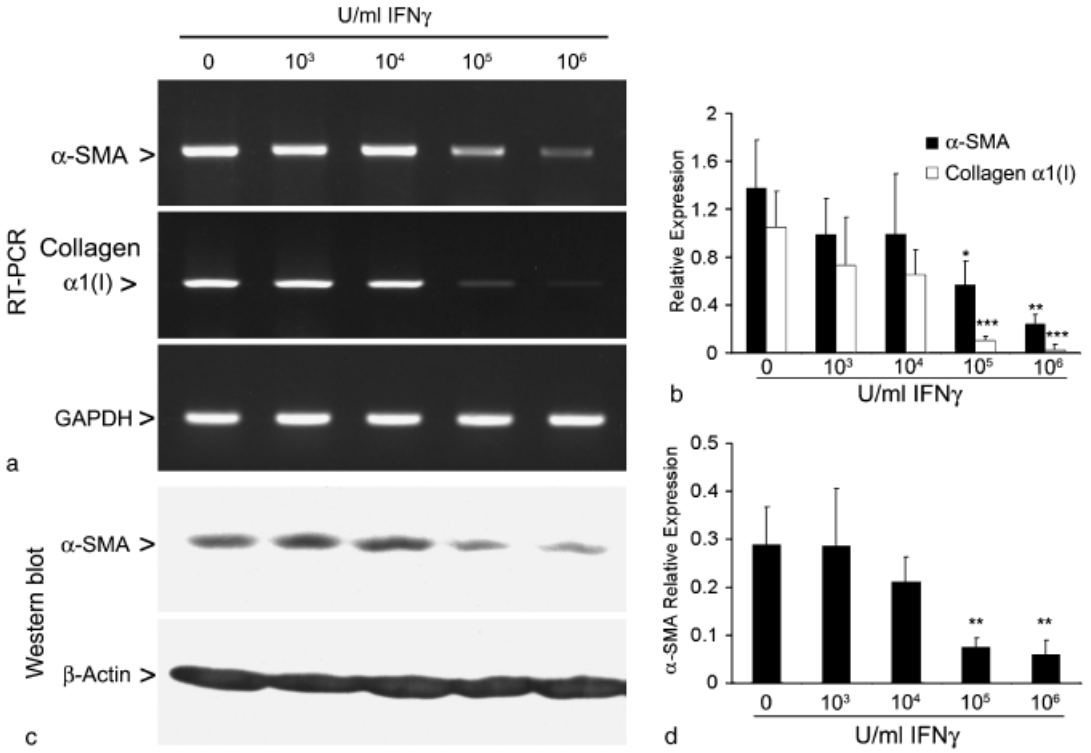

Fig. 2. Interferon-γ (IFN-γ) inhibits α-actin (α-SMA) expression and production by normal gingival (NG) fibroblasts. (a) After treatment for 3 days with increasing concentrations of IFN-γ, total RNA from fibroblasts was isolated and cDNA synthesized by reverse transcriptase (RT) and amplified with specific primers to α-SMA, type I collagen and glyceraldehyde-3-phosphate dehydrogenase. (b) Densitometric analysis of the α-SMA bands demonstrated that IFN-γ significantly inhibited α-SMA expression at concentrations of 10^5 and 10^6 U/ml. Similarly, type I collagen expression was dramatically decreased in NG fibroblasts under treatment with IFN-γ. (c) Western blot analysis performed with total cellular proteins from NG fibroblasts cultured with different concentrations of IFN-γ. (d) Comparison of production confirmed that the amount of α-SMA produced by NG1 cells is significantly inhibited by IFN-γ. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

USA). 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 in the TGF-β1 experiments and 3 μg per sample in the IFN-γ assays were used to generate cDNA using the Superscript II RT enzyme (Invitrogen, Carlsbad, CA, USA). 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. Linear amplification range for each gene was determined by preparing six similar reactions but amplified by different number of cycles (20, 25, 30, 35, 40 and 45 cycles). For all genes, the chosen cycle was in the exponential phase of the polymerase chain reaction (PCR) amplification. Primer sequences, PCR conditions and the amplified lengths have been described elsewhere (Bitu et al. 2006), with exception of SMAD6 and SMAD7. Primer pairs used to amplify SMAD6 were 5′-GCT ACC AAC TCC CTC ATC ACT-3′ and 5′-CGT CGG GGA GTT GAC GAA GAT-3′; and SMAD7 primers were 5′-CGT CCA CGG CTG CTG CAT AA-3′ and 5′-ATG CTG TGC CTT CCT CCG CT-3′.

Western blot analysis

α-SMA and β-actin detections by Western blot were performed after the methods of Bitu et al. (2006). Reactions were developed using the Enhanced Chemiluminescent Western blot kit (Amersham Pharmacia Biotech, Arlington Heighs, IL, USA).

Immunofluorescence

Ten thousand cells were plated in each well of a eight-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 with specific cytokines in medium with 0.1% FBS, cells were washed in phospahate-buffered saline (PBS) and fixed in 70% ethanol for 15 min. To prevent non-specific binding, the cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Cells were then 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, Burlingame, CA, USA), and examined under a photomicroscope equipped with epifluorescence (DMR Microscope, Leica Microsystems, 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.

Enzyme-linked immunosorbent assay (ELISA)

The production of type I collagen was determined by ELISA. To obtain condi-

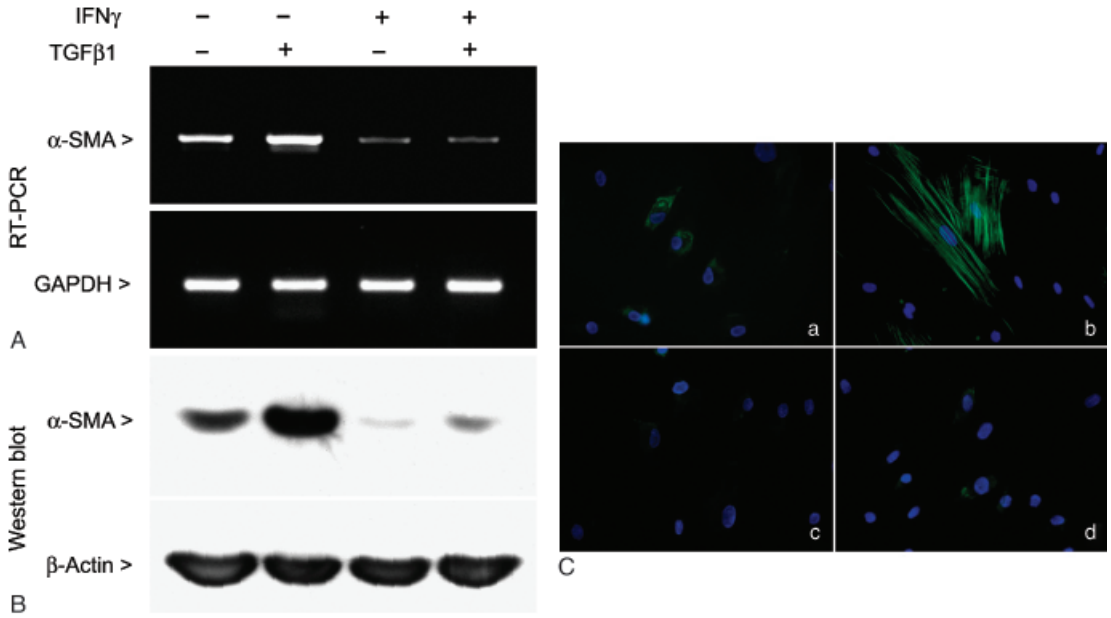

Fig. 3. Interferon-γ (IFN-γ) blocks fibroblast-myofibroblast transdifferentiation induced by transforming growth factor-β1 (TGF-β1). Normal gingival (NG) fibroblasts were cultured for 3 days with 10 ng/ml TGF-β1, 10^6 U/ml IFN-γ, or both, and examined for the inhibition of α-actin (α-SMA) expression and production. (a) reverse transcriptase-polymerase chain reaction (RT-PCR), and (b) Western blot analysis. IFN-γ strongly blocked the myofibroblast generation induced by TGF-β1, as manifested by the down-regulation of α-SMA. (c) Immunofluorescence microscopy revealed an intense staining for α-SMA in NG cells treated with TGF-β1, whereas IFN-γ blocked its effect. (a) Untreated cells, (b) cells treated with 10 ng/ml TGF-β1, (c) cells treated with 10^6 U/ml IFN-γ, and (d) cells treated with both TGF-β1 and IFN-γ (original magnification $\times$ 200).

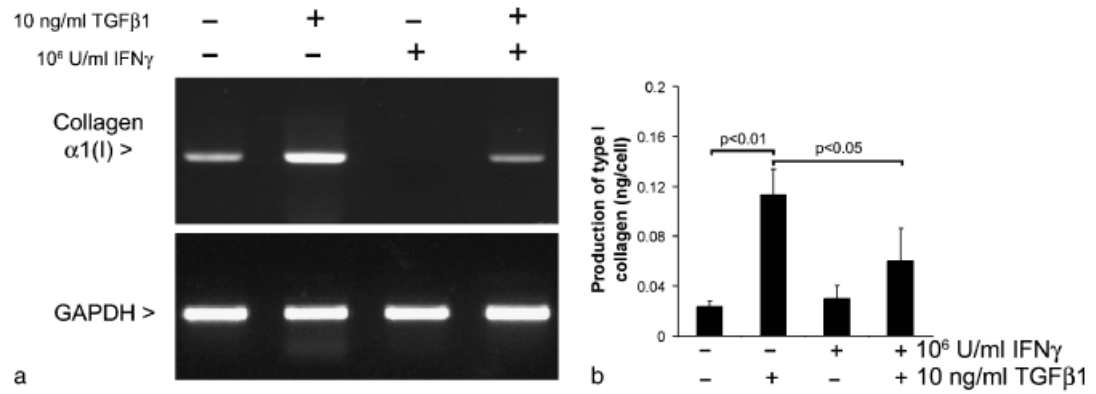

Fig. 4. Interferon-γ (IFN-γ) blocks significantly expression and production of type I collagen induced by transforming growth factor-β1 (TGF-β1). (a) reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed IFN-γ inhibits type I collagen expression and blocks TGF-β1 effects on it. (b) Production of type I collagen by monolayers of normal gingival (NG) cells treated with IFN-γ, TGF-β1 and both cytokines as determined by enzyme-linked immunosorbent assay. The values represent the mean of ng/cell of type I collagen production for triplicate wells $\pm$ SD. IFN-γ decreased significantly the amount of type I collagen production induced by TGF-β1 in NG cultures.

tioned cell culture medium and cells for this assay, NG1 and HGF cells were plated in 24-well culture plates at a density of 100 000 cells/well, in DMEM containing 10% FBS. After 16 h, the cells were rinsed with PBS and the medium replaced by 0.1% FBS-DMEM containing IFN-γ, TGF-β1 or both. After treatment, the culture medium was collected and the cells were harvested by scraping in 0.5 M acetic acid for 30 min. The medium and the cellular contents of each well were pooled, lyophilized and reconstituted in 300 μl of 0.5 M acetic acid. For the ELISA assays, microtitre plate wells were coated with the samples diluted in PBS (1:100) for 2 h at room temperature. The wells were then washed three times with 400 μl of 1% Tween 20 in PBS and non-specific binding sites were blocked with 3% BSA in PBS for 2 h. After washing, biotinylated anti-human type I collagen antibodies (Chemicon Int., Temecula, CA, USA), diluted 1:2000 in PBS, were added to the wells and incubated for 2 h. After another washing step, peroxidase-conjugated streptavidin (Vector Labs, Burlingame, CA, USA) diluted 1:500 in PBS was added and maintained for 1 h. The reaction was developed with 0.5 mg/ml of *o*-phenylenediamine (Sigma, St. Louis, MO, USA) in 0.5 M citric buffer pH 5.5 containing 0.01% H_2O_2 for 20 min. After terminating the reaction with 50 μl

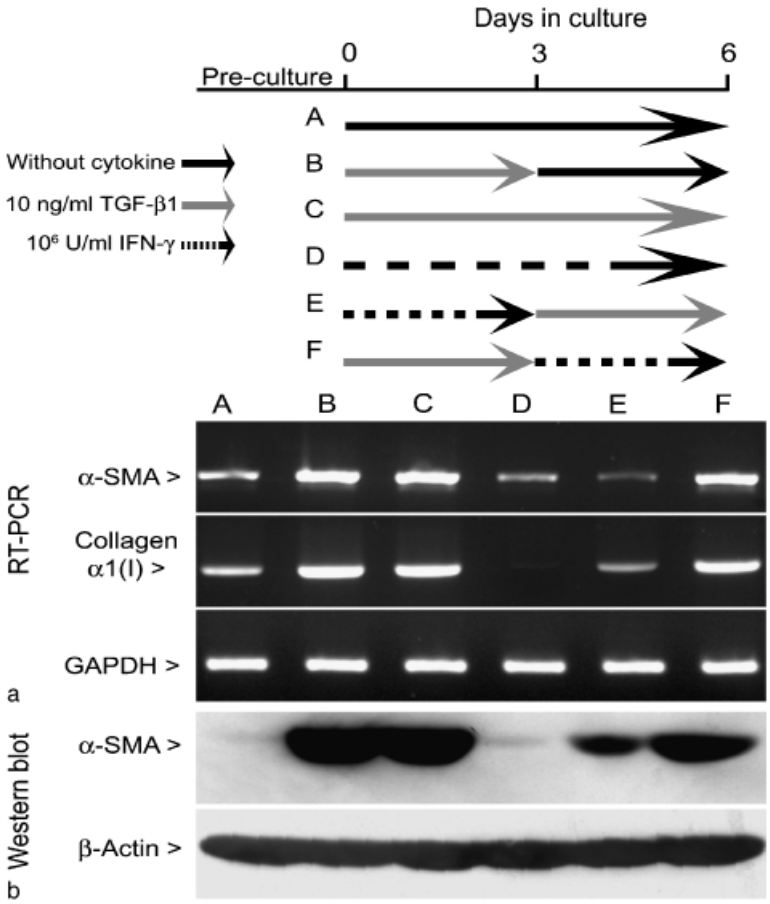

Fig. 5. Pre-treatment with interferon-γ (IFN-γ) prevents transdifferentiation of normal gingival (NG) fibroblasts to myofibroblasts induced by transforming growth factor-β1 (TGF-β1). Conditions of treatment with cytokines are depicted on the top. After 3 days of treatment, fresh medium containing specific cytokines was added. Following 6 days treatment, cells were collected and subjected to either reverse transcriptase-polymerase chain reaction (RT-PCR) (a) or Western blot analysis (b). Treatment with TGF-β1 for 6 days or for 3 days with further 3 days without the cytokine promoted similar phenotype. NG fibroblasts cultured in the presence of TGF-β1 after IFN-γ treatment showed a drastic reduction on α-actin (α-SMA) and type I collagen expression compared with those in the IFN-γ-untreated cells stimulated with TGF-β1.

of 2 N H_2SO_4, absorbance was read at 450 nm with λ correction at 570 nm. A standard curve was constructed using human placenta type I collagen purified protein (Chemicon) diluted in PBS, ranging from 0 to 800 ng/ml. The wells of replicate plates were treated in a like manner and used for cell counts. Cells were harvested using 0.2% trypsin and counted with a Coulter Counter (Beckman Coulter, Buckinghamshire, UK). The values were expressed as ng type I collagen/cell.

Statistical analysis

All experiments were performed at least twice. Data used in the statistical analysis are presented as mean ± SD of three experiments with the same cell line. Student's *t*-test (two-tailed) was used for statistical analysis, and in our comparisons, $p < 0.05$ pointed out to statistical significance.

Results

TGF-β1 induces expression of α-SMA in cultured NG fibroblasts

First we sought to verify whether TGF-β1 stimulates transdifferentiation of human NG1 fibroblasts to myofibroblasts using RT-PCR and Western blot. Total RNA was isolated from NG1 cells treated with 0–10 ng/ml of TGF-β1 for 3 days, and α-SMA mRNA and the constitutively expressed GAPDH mRNA (control for RT-PCR) were amplified using sequence-specific primer pairs (Fig. 1a). In vitro, NG1 fibroblasts constitutively expressed low but detectable levels of α-SMA. TGF-β1 increased α-SMA mRNA in a dose-dependent manner, with a maximal 10-fold increase over unstimulated cells at 10 ng/ml (Fig. 1b). Interestingly, Western blot analysis of cell lysates showed that TGF-β1 induced significantly α-SMA production at concentration of 10 ng/ml (Fig. 1c and d). Under phase contrast and fluorescence microscopy, NG1 cells cultured in 10 ng/ml of TGF-β1 showed profound morphological changes, with cells becoming elongated, flattened and with a vivid cytoplasmatic staining for α-SMA, which was not observed in cells cultured in TGF-β1 at concentration of 1 ng/ml or less (data not shown). Western blot analysis was used in the time–response study, revealing that TGF-β1 at 10 ng/ml stimulates α-SMA production after 1 day, with a marked induction after 3 days (data not shown). These results show that there is a threshold concentration of TGF-β1 that needs to be reached in order to achieve myofibroblast transdifferentiation.

IFN-γ simultaneously inhibits α-SMA and type I collagen expression by human-cultured NG fibroblasts

To verify whether IFN-γ could affect α-SMA expression and production by human gingival cells, we examined the expression of α-SMA in NG1 fibroblasts in the presence of increasing concentrations of IFN-γ. After being incubated with increasing concentrations of IFN-γ for 3 days, total RNA from NG1 cell line was extracted and subjected to RT-PCR analysis. As revealed in Fig. 2a, IFN-γ treatment resulted in an inhibition of α-SMA mRNA expression in a dose-dependent manner. IFN-γ at 10^5 and 10^6 U/ml inhibited significantly α-SMA mRNA expression (Fig. 2b). To confirm these findings Western blot analysis was performed revealing that NG1 cells treated with IFN-γ dramatically decreased the α-SMA production compared with untreated cells (Fig. 2c and d). When NG1 cells were cultured with 10^6 U/ml of IFN-γ for 24, 48 and 72 h, the levels of α-SMA mRNA began to decrease after 24 h treatment, reaching the lowest level after 3 days, whereas α-SMA protein levels were decreased later, after 3 days of treatment (data not shown). IFN-γ inhibition of α-SMA expression

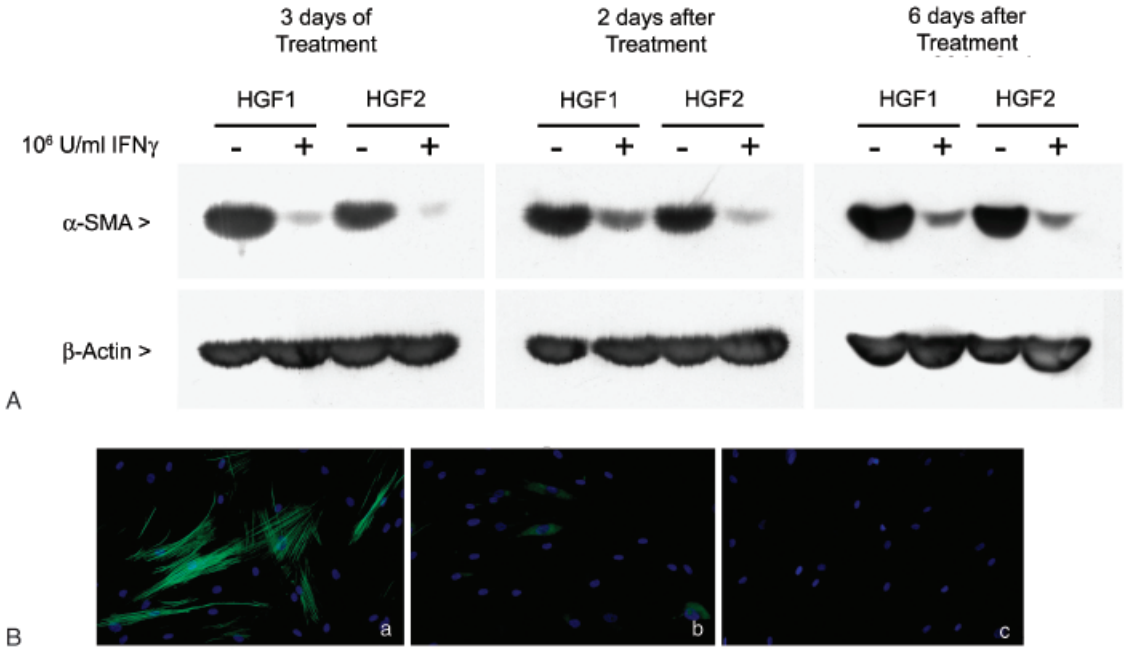

Fig. 6. Reversion of the hereditary gingival fibromatosis (HGF) myofibroblast phenotype with interferon-γ (IFN-γ) treatment. HGF cells were treated with 10^6 U/ml of IFN-γ for 3 days, and for additional 2 and 6 days without this cytokine. (a) Western blot analysis revealed that IFN-γ drastically inhibited α-actin (α-SMA) expression by HGF cells, and this effect was kept for at least 6 days. (b) Immunofluorescence analysis demonstrated that IFN-γ treatment of HGF cells for 3 days inhibit the α-SMA brilliant striated staining throughout the cytoplasm of the cells. (a) HGF1 myofibroblasts, (b) HGF1 myofibroblasts treated with 10^6 U/ml of IFN-γ, (c) negative control that represent cells untreated with primary antibodies but incubated with secondary antibodies.

in NG1 cells was accompanied by a decrease in type I collagen expression. Treatment of NG1 cells with IFN-γ for 3 days markedly inhibited type I collagen expression in a dose-dependent manner, from 10^5 U/ml (Fig. 2a and b).

IFN-γ blocks TGF-β1-induced myofibroblast transdifferentiation in human-cultured NG cells

To clarify whether IFN-γ interferes with the generation of myofibroblasts induced by TGF-β1, NG1 cells were cultured in the presence of IFN-γ plus TGF-β1. NG1 cells treated for 3 days with 10 ng/ml of TGF-β1 showed a higher α-SMA expression compared with the control cells (Fig. 3a and b). The concomitant presence of both IFN-γ and TGF-β1 in the culture medium blocked α-SMA mRNA expression and protein synthesis induced by TGF-β1 during fibroblast–myofibroblast transdifferentiation (Fig. 3a and b). Upon treatment with 10 ng/ml of TGF-β1 for 3 days, NG1 cells exhibited abundant bundles of α-SMA and had typical flattened myofibroblast morphology (Fig. 3c). IFN-γ at 10^6 U/ml completely prevented switching of NG1 cultures to myofibroblast induced by TGF-β1 (Fig. 3c). To demonstrate that the increased type I collagen activity of the myofibroblasts induced by TGF-β1 is also inhibited by IFN-γ, we performed RT-PCR and ELISA. Those results demonstrated that IFN-γ significantly decreased type I collagen expression and production induced by TGF-β1 in NG1 cultures (Fig. 4).

To further test the effect of IFN-γ, we examined whether this cytokine could reverse the effect of TGF-β1 on myofibroblast transdifferentiation. In NG1 cells cultured in the presence of TGF-β1 after IFN-γ treatment, both α-SMA and type I collagen were drastically reduced compared with those in the IFN-γ-untreated cells stimulated with TGF-β1 (Fig. 5, lane E compared with lane C). In a slighter way, IFN-γ inhibited myofibroblast transdifferentiation of NG1 cells pre-treated with TGF-β1 (Fig. 5, lane F compared with lane C). Interestingly, when NG1 cells were stimulated with 10 ng/ml of TGF-β1 for 3 days, followed for additional 3 days without cytokine, the intensity of α-SMA expression and production was quite similar as those in the NG1 cells stimulated throughout the entire culture period (6 days) with this cytokine (Fig. 5, lanes B and C).

IFN-γ inhibits α-SMA and type I collagen expression in human cultured HGF myofibroblastic cells

We previously demonstrated that myofibroblasts may be involved in the pathogenesis of the gingival overgrowth of HGF patients (Bitu et al. 2006). As IFN-γ blocked TGF-β1-induced myofibroblast transdifferentiation, we set out to determine whether IFN-γ could affect HGF myofibroblastic cells. To this end, we treated two HGF cell lines with 10^6 U/ml of IFN-γ for 3 days. As depicted in Fig. 6, production of α-SMA, the specific marker of myofibroblasts, was markedly inhibited by IFN-γ (Fig. 6a). After removal IFN-γ from the culture medium, α-SMA production began to increase, but still lower compared with control cell after 6 days (Fig. 6a). Additionally, immunofluorescence analysis demonstrated that IFN-γ strongly reversed the myofibroblastic phenotype of HGF cells as revealed by the absence of the brilliant-striated staining for α-SMA throughout the cytoplasm (Fig. 6b). Further, IFN-γ treatment significantly inhibited HGF myofibroblastic cell metabolism, as revealed by the statistically significant lower production of type I collagen in

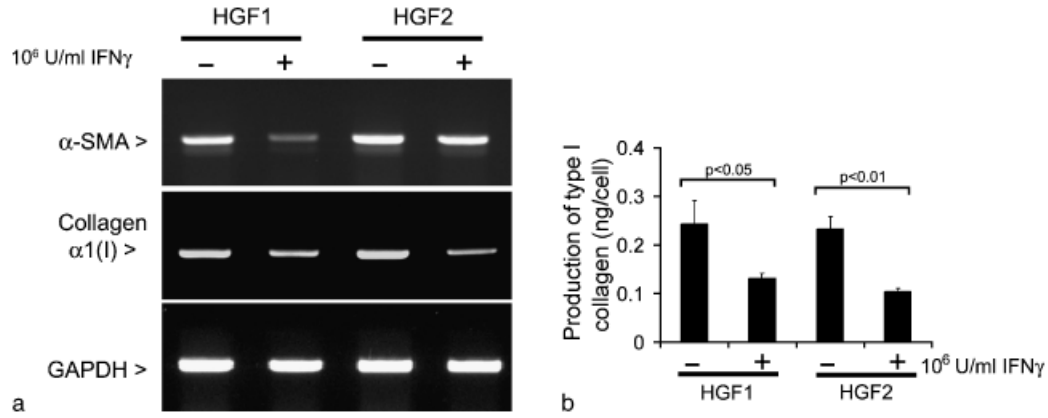

Fig. 7. Interferon-γ (IFN-γ) inhibits hereditary gingival fibromatosis (HGF) myofibroblasts activity as revealed by decreased type I collagen expression and production. (a) reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and (b) enzyme-linked immunosorbent assay for type I collagen.

HGF cells treated with 10^6 U/ml of IFN-γ compared with untreated cells (Fig. 7).

IFN-γ blocks myofibroblast transdifferentiation via inhibition of TGF-β1/SMAD pathway

Previous studies demonstrated that TGF-β1 has a central role in the transdifferentiation of fibroblasts to myofibroblasts, and that its activity is dependent of CTGF stimulation (Yokoi et al. 2001, Tanaka et al. 2003). To examine the effect of TGF-β1 on CTGF expression, NG1 fibroblast cells were incubated with TGF-β1 and CTGF mRNA expression levels determined by RT-PCR. As demonstrated in Fig. 8a, CTGF expression was markedly up-regulated by TGF-β1 from 0.01 to 10 ng/ml. Next, we treated NG1 fibroblasts with TGF-β1 at 10 ng/ml for 0, 12, 24, 48 and 72 h. CTGF expression was up-regulated after 12 h of exposure, whereas α-SMA expression increased from 48 h, with a more expressive up-regulation at 72 h after stimulation (Fig. 8b). Thus, the up-regulation of α-SMA expression by TGF-β1 is delayed compared with that of CTGF.

To determine whether IFN-γ influences TGF-β1 activity with consequent inhibition of CTGF expression, we performed RT-PCR analysis. We tested the hypothesis that IFN-γ inhibits the early step of TGF-β1 pathway, i.e. the activation of SMAD3. Possible mediators of this effect are SMAD6 and SMAD7, members of SMAD family that negatively interfere with TGF-β signalling (Faler et al. 2006). IFN-γ induced the expression of SMAD7, but not SMAD6, in both NG1 and HGF1 cells (Fig. 9). SMAD7 mRNA levels in both cell lines were increased in three- to fourfold over the basal levels after 2 h, and decreased progressively until 8 h, after which remained similar to basal levels. In contrast with TGF-β1 expression that was unaffected by IFN-γ, the expression of CTGF was markedly inhibited, suggesting that IFN-γ reduced TGF-β1 activity (Fig. 9). CTGF expression was down-regulated in both NG1 and HGF1 cells after 4 h of IFN-γ exposure (Fig. 9). Thus, down-regulation of CTGF expression by IFN-γ is delayed compared with that of SMAD7. Taken together, these data suggest that IFN-γ blocks myofibroblast transdifferentiation via stimulation of SMAD7, with consequent inhibition of TGF-β1-CTGF pathway.

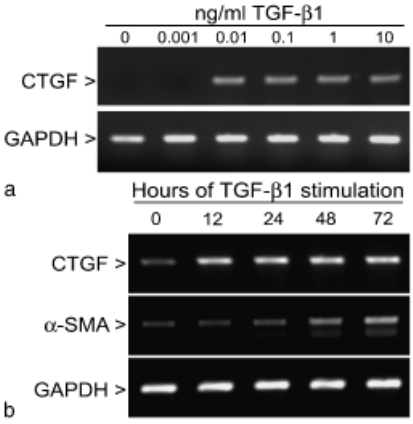

Fig. 8. Transforming growth factor-β1 (TGF-β1) induces connective tissue growth factor (CTGF) expression in a dose-dependent manner, and earlier compared with that of α-actin (α-SMA). (a) NG1 fibroblasts treated with increasing concentrations of TGF-β1 were collected, and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis using specific CTGF and glyceraldehyde-3-phosphate dehydrogenase primers. The results showed that TGF-β1 induced expression of CTGF from concentration of 0.01 ng/ml. (b) NG1 cells were incubated with 10 ng/ml TGF-β1, and harvested after 0, 12, 24, 48 and 72 h. CTGF expression was up-regulated by TGF-β1 stimulation after 12 h exposure and kept higher during all time-course. In contrast, α-SMA mRNA expression increased from 48 h, but showed a more significant up-regulation at 72 h.

Discussion

Although the underlying mechanisms that lead to the accumulation of abnormal amounts of gingival tissue in HGF are still unknown, we previously demonstrated that HGF cultures are characterized by increased production of TGF-β1 and collagen, and an elevated proportion of myofibroblast cells (Coletta et al. 1999, de Andrade et al. 2001, Bitu et al. 2006). Because myofibroblasts contribute to an increase of extracellular matrix deposition, they are identified as a key participant in abnormal remodelling and progressive fibrosis in a variety of pathological situations, such as Dupuytren's disease, cyclosporine-induced pancreatic fibrosis and different types of fibrosis in the lungs, kidneys and livers (Bisson et al. 2003, Desmouliere et al. 2003, Ahmed et al. 2004). Thus, identification of the regulatory elements of myofibroblast transdifferentiation is of considerable importance for the molecular understanding of the mechanism of interstitial fibrosis in general, as well as, in HGF. In this study, we explored whether TGF-β1, which is produced in elevate levels by HGF cells, could induce myofibroblast transdifferentiation in NG fibro-

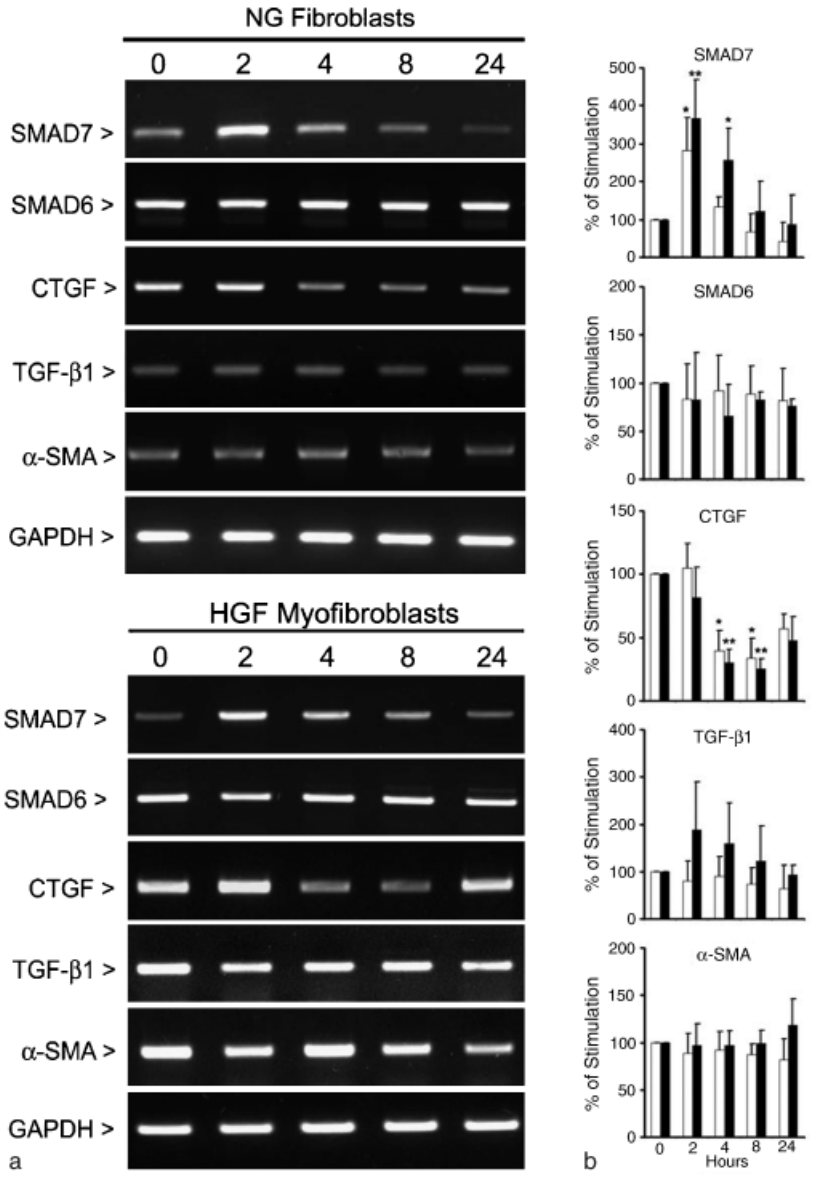

Fig. 9. Interferon-γ (IFN-γ) simultaneously up-regulates expression of SMAD7 and down-regulates connective tissue growth factor (CTGF) expression in both NG fibroblasts (□) and gingival fibromatosis (HGF) myofibroblasts (■). Cells were incubated with 10^6 U/ml of IFN-γ in culture medium containing 0.1% foetal bovine serum (FBS) for different periods and expression levels of SMAD7, SMAD6, CTGF, TGF-β1, α-actin (α-SMA) and glyceraldehyde-3-phosphate dehydrogenase were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Numbers on the top of the lanes represent hours of incubation with IFN-γ. (a) Representative RT-PCR analysis and (b) densitometric analysis. Expression of SMAD7 was significantly stimulated after 2 h of IFN-γ treatment, and decreased progressively until 8 h. In contrast, the expression of CTGF was markedly inhibited by IFN-γ after 4 h of treatment. The levels of SMAD6, TGF-β1, and α-SMA were not significantly affected by IFN-γ in this time-course. $^{*}p<0.01$, $^{**}p<0.05$.

blast cultures. Our results demonstrated that TGF-β1 leads to a dose- and time-dependent induction of gingival fibroblast transformation to myofibroblast in vitro. Importantly, we demonstrated that a minimum threshold concentration of TGF-β1 is needed to reach the complete transdifferentiation of NG1 cells. This was illustrated by the fact that NG1 fibroblasts treated with 1 ng/ml TGF-β1 showed a slight increase in the α-SMA mRNA and protein levels, but failed to drive a complete morphological transdifferentiation. Furthermore, the differentiated myofibroblasts could still retain their morphology, α-SMA expression and higher collagen production for at least 3 days in culture without TGF-β1. Recently, Smith et al. (2006) suggested that TGF-β1 induces transdifferentiation of gingival fibroblast to myofibroblasts via ρA-ROCK and c-Jun *N*-terminal protein kinase signalling pathways.

In parallel with the myofibroblast transdifferentiation induced by TGF-β1, CTGF expression was stimulated. Indeed, TGF-β1-induced CTGF expression occurred earlier than the α-SMA expression, leading to the hypothesis that CTGF may serve as a mediator of TGF-β1 stimulation of myofibroblast transdifferentiation. Previous studies demonstrated that CTGF is found in elevate levels in fibrotic diseases, including those of kidney, lung and liver (Kobayashi et al. 2005, Burns et al. 2006, Sanders et al. 2006), and its expression is strongly induced by TGF-β in vitro (Qi et al. 2006). Zhang et al. (2004) demonstrated that exogenous CTGF induces myofibroblast transdifferentiation in human renal cells, and that antisense oligonucleotides against CTGF abolished myofibroblast induction by TGF-β1. Such CTGF dependence in myofibroblast transdifferentiation by TGF-β1 has also been also demonstrated in epithelial–mesenchymal transdifferentiation (Yokoi et al. 2002). Moreover, Untergasser et al. (2005) demonstrated by using microarray technology that CTGF is a molecular target of TGF-β1 during transdifferentiation of prostate fibroblasts to myofibroblasts. More recently, we demonstrated that the presence of myofibroblasts in HGF is associated with the CTGF expression levels (Bitu et al. 2006). Myofibroblasts were identified in HGF tissues whose cells expressed high levels of both TGF-β1 and CTGF but not in tissues characterized for cells expressing elevated levels of TGF-β1 and low levels of CTGF. Collectively, these findings suggest that CTGF plays a crucial role in mediating TGF-β1 myofibroblast transdifferentiation.

To date, we know that neutralization of TGF-β1 synthesis or signalling with neutralizing antibodies, antisense technologies or antagonist drugs is effective in the treatment of animal models of fibrotic diseases (Gressner et al. 2002). However, whether this blockade of TGF-β1 interferes with myofibroblast generation and/or activity is still unclear. Drugs that can interfere with myofibroblast can be therapeutically useful in the control of the interstitial

fibrosis observed in HGF. The present study demonstrated that IFN-γ blocks the myofibroblast generation promoted by TGF-β1 and partially reverts myofibroblast phenotype of HGF myofibroblasts. However, HGF myofibroblast cell metabolism was significantly inhibited by IFN-γ, as revealed by the decreased synthesis of type I collagen. Our findings were similar to those reported by Hasegawa et al. (2003) using keloid-derived dermal fibroblasts and by Gu et al. (2004), who used human foetal lung fibroblasts. In both studies, IFN-γ inhibited TGF-β1-induced α-SMA and collagen production. Furthermore, IFN-γ has showed a potential antifibrotic agent by blocking myofibroblast activity in vivo. Clinical experience was, however, limited to a few clinical trials that demonstrated objective functional improvement in a small number of patients (Pittet et al. 1994, Larrabee 1997). For example, in a clinical trial with patients affected by Dupuytren's disease, injections of IFN-γ into nodular lesions revealed a clinical improvement, which was histologically associated with a significant reduction on myofibroblast activity (Pittet et al. 1994). Taken together, these data support that IFN-γ may be useful to prevent the gingival overgrowth in HGF patients, and clinical trials with locally delivery of this cytokine should be performed in those patients.

The results presented here also revealed that blockade of fibroblast–myofibroblast transdifferentiation induced by IFN-γ was likely mediated by antagonizing TGF-β1 signalling. We observed an increase in the amount of SMAD7 mRNA levels, and a reciprocal decrease in the amount of CTGF, suggesting that up-regulation of SMAD7 by IFN-γ prevents TGF-β1 activity. IFN-γ did not affect the expression levels of SMAD6, which is another anti-SMAD, and TGF-β1. Similar IFN-γ action was also observed in the HGF myofibroblastic cells. In CT cells, C2C12 myoblasts expressing constitutively elevated levels of TGF-β1, IFN-γ decreased TGF-β1 production and subsequently blocked the expression of vimentin and α-SMA (Foster et al. 2003). Induction of SMAD7 by IFN-γ interfering with TGF-β1 signalling has been reported by Ulloa et al. (1999) in U4A/Jak1 cells, a transfected cancer cell line. On the other hand, Gu et al. (2004) demonstrated that the action of IFN-γ in the myofibroblast transdifferentiation was neither dependent on alteration of SMAD7 expression, nor on interruption of SMAD2/3 phosphorylation and their nuclear translocation in human foetal lung fibroblasts. Furthermore, it was demonstrated that IFN-γ may block TGF-β1-induced type I collagen production, in part, by the antagonistic interaction of SMAD and Jak pathway at nuclear p300/CBP level (Ghosh et al. 2001). Further investigation should be done to elucidate whether IFN-γ effects on myofibroblast transdifferentiation occur through cell-specific mechanisms.

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Clinical Relevance

Scientific rationale for the study: We recently demonstrated that myofibroblasts, the main cellular type involved in extracellular matrix deposition in fibrotic diseases, may be associated with HGF aetiopathology. Information regarding the underlying mechanisms that regulate myofibroblast emergence in HGF is limited.

Principal findings: We demonstrate that while TGF-β1 induces, IFN-γ inhibits myofibroblast transdifferentiation and activity via stimulation of SMAD7 and interference in TGF-β1 activity.

Practical implications: IFN-γ might be an effective therapeutic agent for HGF therapy.