Interferon-γ inhibits the myofibroblastic phenotype of rat palatal fibroblasts induced by transforming growth factor-β1 in vitro

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Abstract Interferon-γ (IFN-γ), a multifunctional cytokine, has been noted as a potential therapeutic agent for various fibrotic disorders, including excessive scar tissue formation. We previously reported that transforming growth factor-β1 (TGF-β1) induced the myofibroblastic phenotype in palatal fibroblasts derived from palatal mucosa, and that such effects might have a close link to palatal scar formation. In the present study, we examined the effects of IFN-γ on TGF-β1-pretreated palatal fibroblasts for the purpose of clarifying the suppressive potency against myofibroblastic phenotype expression in vitro. IFN-γ significantly altered the spindle morphology of TGF-β1-pretreated palatal fibroblasts into the polygonal one that was similar to the non-treated palatal fibroblasts. This change was parallel with a decrease in the expression of α-smooth muscle actin protein, a marker for myofibroblast, as determined by immunoblot analysis. Northern blot analysis showed that IFN-γ inhibited proα2(I) collagen mRNA expression that was stimulated by TGF-β1 pretreatment for 24 h. Furthermore, IFN-γ decreased the cell contractility enhanced by TGF-β1 pretreatment for 24 h in a three-dimensional collagen gel culture system. These results suggest that IFN-γ may have negative effects with regard to controlling the myofibroblastic phenotype induced by TGF-β1 in palatal fibroblasts.

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Key words: Transforming growth factor-β; Interferon-γ; Myofibroblast; Scar; Wound healing; α-Smooth muscle actin

1. Introduction

A myofibroblast is a fibroblast subtype that possesses characteristics between those of fibroblasts and smooth muscle cells. Gabbiani et al. [1] have described that myofibroblasts exhibit several ultrastructural features of smooth muscle cells, including the presence of nuclear folds, abundant cytoplasmic microfilaments with dense bodies, complex cellular junctions, and microfilament bundles. Myofibroblasts appear in granulation tissue during the dermal wound healing process, and they are considered to function during wound contraction [2] as well as during the accumulation of extracellular matrix components including type I collagen [3]. α-Smooth muscle (α-SM) actin, an actin isoform of the vascular smooth muscle cell type, has been observed to be one of the most useful markers for myofibroblasts since Darby et al. described that a high proportion of fibroblasts expressed this protein during granulation tissue contraction [4]. Some reports illustrate that myofibroblasts persist under pathological circumstances, such as a hypertrophic scar [5]. We previously reported that palatal scar fibroblasts possessed more myofibroblastic characteristics, including higher collagen synthesis and α-SM actin protein expression, than normal palatal fibroblasts, and that among a number of growth factors, transforming growth factor-β1 (TGF-β1) induced the myofibroblastic phenotype in normal palatal fibroblasts [6,7]. Thus, it is conceivable that myofibroblasts play important roles in the palatal scar formation process.

On the other hand, the interferon family of peptides are highly pleiotropic cytokines that exert immunomodulatory and antiproliferative effects on many cell types both in vitro and in vivo [8]. Interferon-γ (IFN-γ) is a product of activated T lymphocytes, macrophages, and natural killer cells. In addition to its well-recognized roles in immune and inflammatory responses, IFN-γ may also influence the metabolism of connective tissue cells. IFN-γ regulates collagen accumulation by inhibiting the synthesis of type I and type III [9–11] and abrogating the stimulatory effects of TGF-β1 [12,13]. Furthermore, the antifibrotic activity of IFN-γ was confirmed in vivo [13,14].

Therefore, the aim of the present study was to examine whether IFN-γ can modulate the myofibroblastic phenotype of rat palatal fibroblasts which were induced by TGF-β1 in vitro.

2. Materials and methods

2.1. Growth factors

Porcine TGF-β1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA), and rat recombinant IFN-γ was purchased from Gibco BRL (Grand Island, NY, USA).

2.2. Cell culture

Palatal fibroblasts were obtained from the explants of the oral palatal mucosa of 12-week-old male Sprague-Dawley rats according to the procedure described in previous reports [6,7]. Cells were grown in Dulbecco's modified Eagle's medium (D-MEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS, CSL, Ltd., Australia) and 1% penicillin-streptomycin solution (Gibco BRL) under conditions of humidified 5% CO_2/95% air at 37°C. The medium was changed every 2–3 days, and the cells were passaged with trypsin-EDTA (Gibco BRL) when they became confluent. All experiments were done using early passage cells (passage 5–10) derived from at least three independent animals without freezing.

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2.3. Immunoblotting

Confluent palatal fibroblasts were pretreated with TGF-β1 (5 ng/ml) in D-MEM containing 10% FBS for 24 h. The cultures were washed after a 24 h exposure to TGF-β1 and then incubated with fresh medium containing IFN-γ (1000 U/ml) for an additional 48 h. Thereafter cells were trypsinized and counted with a hemocytometer and then homogenized in lysing buffer (0.4 M Tris-HCl, 1% SDS, 0.5% DTT, 1 mM PMSF, pH 6.8) and boiled for 3 min. The protein contents were determined using a protein quantitative assay kit (Stratagene, La Jolla, CA, USA). Aliquots of 10 μg protein from approximately 2×10^4 cells were denatured in reducing sample buffer, separated by 10% (w/v) SDS-PAGE, and transferred to PVDF membranes (Immobilon-P, Millipore Co., Bedford, MA, USA) in transblotting buffer (20 mM Tris, 150 mM glycine, 20% methanol, pH 8.0) for 16 h at 4°C. The membranes were blocked with 5% BSA, followed by incubation with α-SM actin-specific mouse monoclonal antibodies (1A4, ARP, Inc., Belmont, MA, USA) diluted at 1:1000 with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.2) containing 1% BSA and with goat anti-mouse Ig(G+A+M) conjugated with horseradish peroxidase that was diluted at 1:1000 (Cappel Research Products, Durham, NC, USA). The membranes were developed using the ECL system (Amersham International, plc., Buckinghamshire, UK) according to the manufacturer's protocol. The photographic images of the band were scanned with a color image scanner (GT-6000, Seiko-Epson, Nagano, Japan). The integrated densities of the band were quantitated by image analyzing computer software from NIH Image.

2.4. Northern blot analysis

Confluent palatal fibroblasts were pretreated with TGF-β1 (5 ng/ml) in D-MEM containing 10% FBS for 24 h. The culture was washed after a 24 h exposure to TGF-β1 and then incubated with fresh medium containing IFN-γ (1000 U/ml) for an additional 48 h. Thereafter the total cellular RNA was extracted by Isogen (Wako Co., Japan). Aliquots of total RNA (15 μg) were electrophoresed in 1% agarose-formaldehyde gel and blotted onto a nylon membrane (Zeta-probe, Bio-Rad, Richmond, CA, USA) with 10×SSC. Probes for proα2(I) collagen and matrix metalloproteinase 1 (MMP1) were labeled with [^{32}P]dCTP (ICN Biomedicals, Inc., Costa Mesa, CA, USA) using a Ready-To-Go DNA Labeling Kit (Pharmacia Inc., USA). Prehybridization and hybridization were performed with hybridization buffer (50% formamide, 0.12 M Na_2HPO_4, 0.25 M NaCl, 7% SDS, 1 mM EDTA, pH 7.2) at 43°C. The membrane was washed in each washing solution (2×SSC/0.1% SDS, 0.5×SSC/0.1% SDS, 0.1×SSC/0.1% SDS) for 15 min at room temperature and exposed to Kodak XRP-5 film with an intensifying screen at −80°C.

2.5. Collagen gel contraction assay

Confluent palatal fibroblasts were treated with 5 ng/ml of TGF-β1 in D-MEM containing 10% FBS for 24 h in the monolayer culture. These cells were detached from the culture plates by trypsinization, and the cell numbers were counted with a hemocytometer. Collagen solution was prepared from a mixture of seven volumes of bovine acid-soluble type I collagen (Cell matrix, 3 mg/ml, Nitta Gelatin Co., Osaka, Japan), two volumes of five times concentrated D-MEM, and one volume of pH adjusting buffer (0.05 M NaOH, 0.26 M $NaHCO_3$, 0.2 M HEPES) according to the manufacturer's instructions. Relevant amounts of the cell suspension and FBS were mixed into the collagen solution, and the final cell number and FBS concentration were adjusted to 2×10^5/ml and 10%, respectively, to make the total mixture. Twelve-well culture plates were precoated with 1% Bacto agar (Difco Lab., MI, USA) to make it easier to release the collagen gels from the wells at the beginning of the collagen gel culture. 1 ml of the total mixture was dispensed into each well of the precoated culture plates, and they were polymerized in an atmosphere of 5% CO_2/95% air at 37°C for 30 min. Immediately after the polymerization, 1 ml of D-MEM containing 10% FBS was added to each well; the gels were then detached from the wells by gently shaking the culture plates until they floated in the medium. Collagen gel cultures were maintained in the culture medium with or without IFN-γ (1000 U/ml) during the experimental period. The longest and the shortest diameters of each gel were measured at each time point, and the mean of the two measurements was used as a parameter of the contractility of the cells. The data represent the average diameter ± S.E. (mm) of triplicate gels.

3. Results

3.1. IFN-γ modulates morphological change by TGF-β1 in palatal fibroblasts

We have previously reported that confluent palatal fibroblasts, which exhibited a polygonal shape, became elongated with TGF-β1 treatment in accordance with the myofibroblastic phenotype expression [7]. Therefore, we first examined the effects of IFN-γ on the morphological change induced by TGF-β1 in palatal fibroblasts in order to determine if IFN-γ can change a phenotype of myofibroblasts. Palatal fibroblasts were pretreated with TGF-β1 for 24 h under monolayer culture conditions, and then cells were treated with or without IFN-γ for 48 h in the absence of TGF-β1. Confluent palatal fibroblasts exhibited a polygonal shape in the monolayer culture (Fig. 1a) and the palatal fibroblasts pretreated with TGF-β1 and without IFN-γ exhibited an elongated shape (Fig. 1b). However, the elongated shape of the palatal fibroblasts induced by the TGF-β1 pretreatment was changed to a polygonal shape by the treatment with IFN-γ (Fig. 1c).

3.2. Effects of IFN-γ on α-SM actin expression induced by TGF-β1

TGF-β1 stimulates the α-SM actin expression in a variety of cells [15,16]. We also demonstrated that TGF-β1 stimulated the α-SM actin expression in palatal fibroblasts [7]. Therefore, in order to evaluate the effects of IFN-γ on the TGF-β1-induced α-SM actin expression in palatal fibroblasts, we performed an immunoblot study using antibodies specific to α-SM actin. Palatal fibroblasts, which were pretreated with 5 ng/ml of TGF-β1 for 24 h, kept expressing an almost three times higher level of α-SM actin as compared with the non-treated palatal fibroblasts even in the culture after the removal of TGF-β1 for 48 h (Fig. 2A,B). When the palatal fibroblasts, on the other hand, were cultured with 1000 U/ml of IFN-γ for 48 h after TGF-β1 deprivation, the increment level of α-SM actin was suppressed by approximately half as much as that observed in the culture treated by only TGF-β1 (Fig. 2A,B).

3.3. Effects of IFN-γ on proα2(I) collagen and MMP1 mRNA expression in palatal fibroblasts

TGF-β regulates the transcription of a wide spectrum of matrix proteins including collagen and matrix-degrading pro-

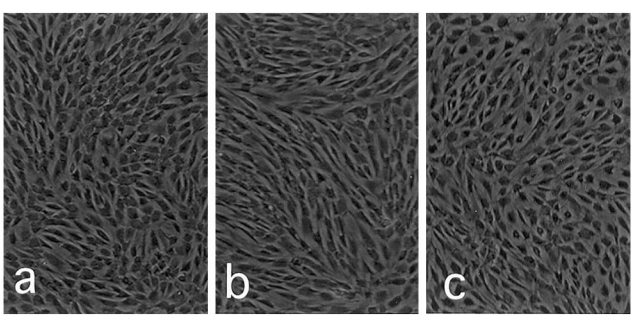

Fig. 1. Effects of IFN-γ on cell morphology of palatal fibroblasts pretreated with TGF-β1. Palatal fibroblasts were pretreated with TGF-β1 (5 ng/ml) in D-MEM containing 10% FBS for 24 h and then treated with (1000 U/ml; c) or without IFN-γ (b) for an additional 48 h. Non-treated palatal fibroblasts showed a polygonal shape (a). Phase contrast microscopy showed the morphological change by TGF-β1 and IFN-γ treatment (×28).

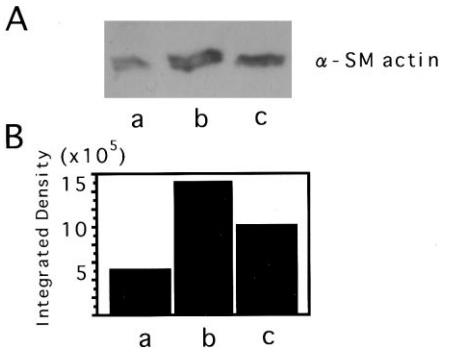

Fig. 2. α-SM actin expression was detected by immunoblot analysis. A: Palatal fibroblasts were pretreated with TGF-β1 in D-MEM containing 10% FBS for 24 h and then treated with (1000 U/ml, lane c) or without IFN-γ (lane b) for an additional 48 h. Cell lysates were prepared and subjected to 10% SDS-PAGE (10 μg protein/lane), followed by immunoblotting with anti-α-SM actin monoclonal antibody. IFN-γ decreased the expression of α-SM actin protein enhanced by the TGF-β1 pretreatment. Non-treated palatal fibroblasts are shown in lane a. B: The integrated density of the band was quantitated by scanning densitometry.

teinase. Therefore, we investigated the effects of IFN-γ on proα2(I) collagen and the MMP1 mRNA expression in palatal fibroblasts. When palatal fibroblasts were treated with (Fig. 3, lane b) or without (Fig. 3, lane a) TGF-β1 (5 ng/ml) for 24 h, the proα2(I) collagen mRNA expression was enhanced, and the MMP1 mRNA expression was inhibited by TGF-β1 treatment. Subsequent to the 24 h of TGF-β1 pretreatment, the palatal fibroblasts were cultured in the fresh medium with (Fig. 3, lane d) or without (Fig. 3, lane c) IFN-γ (1000 U/ml) for 48 h in the absence of TGF-β1, and a Northern blot analysis was then performed. IFN-γ treatment inhibited the proα2(I) collagen mRNA expression induced by the TGF-β1 pretreatment, but appeared to have no effects on the MMP1 mRNA expression in the palatal fibroblasts.

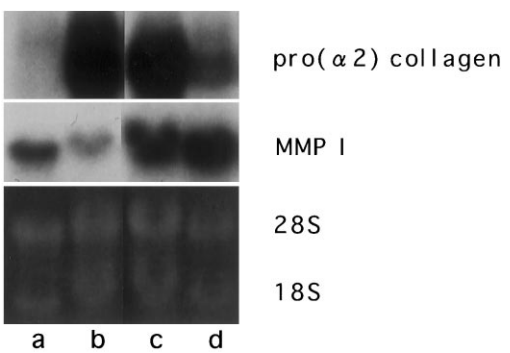

Fig. 3. Proα2(I) collagen and matrix metalloproteinase 1 (MMP1) mRNA expression in palatal fibroblasts analyzed by Northern blot. When the palatal fibroblasts were treated with (lane b) or without (lane a) TGF-β1 treatment for 24 h, proα2(I) collagen mRNA expression was enhanced, and MMP1 mRNA expression was inhibited by the TGF-β1 treatment. Subsequent to 24 h of the TGF-β1 pretreatment, palatal fibroblasts were cultured in fresh medium with (lane d) or without (lane c) 1000 U/ml IFN-γ for 48 h in the absence of TGF-β1. IFN-γ significantly inhibited proα2(I) collagen mRNA expression, whereas it did not change MMP1 mRNA expression in the palatal fibroblasts. Ethidium bromide stainings of the corresponding gels are shown in the lower panels.

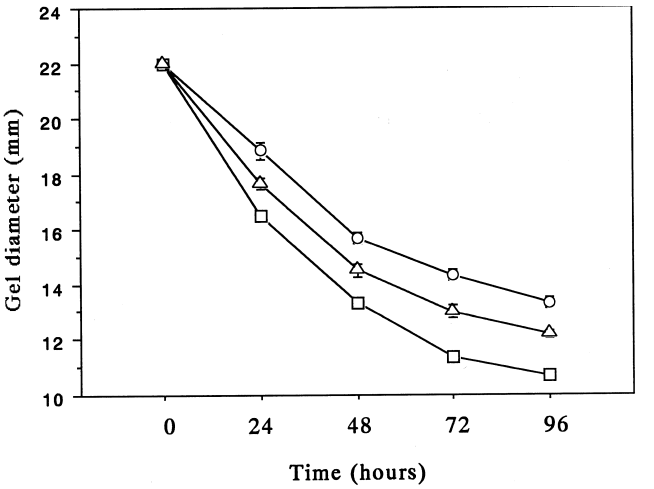

Fig. 4. Effects of IFN-γ on contractility of the myofibroblastic phenotype induced by TGF-β1. Monolayer palatal fibroblasts were treated with or without TGF-β1 (5 ng/ml) for 24 h, and then were transferred to the collagen gel culture system. The TGF-β1-pretreated palatal fibroblasts (□) showed a remarkably high contractility as compared to the non-treated palatal fibroblasts (○) through the 96 h culture period. When IFN-γ (1000 U/ml, △) was administered to the collagen gel culture, the contractility enhancement of the TGF-β1-pretreated palatal fibroblasts was partially blocked.

3.4. IFN-γ modulates contractility of myofibroblastic phenotype induced by TGF-β1

We previously demonstrated that palatal fibroblasts with TGF-β1 pretreatment for 24 h showed high contractility as compared with the non-treated palatal fibroblasts. In order to clarify the effects of IFN-γ on cell contractility, we investigated whether IFN-γ inhibited the contractility of palatal fibroblasts stimulated by the TGF-β1 pretreatment. Monolayer palatal fibroblasts were pretreated with or without TGF-β1 (5 ng/ml) for 24 h, after which they were transferred to a collagen gel culture system. Palatal fibroblasts with the TGF-β1 pretreatment (Fig. 4, □) showed a remarkably high contractility as compared with the palatal fibroblasts without the TGF-β1 pretreatment (Fig. 4, ○) through the 96 h culture period. When IFN-γ (1000 U/ml, Fig. 4, △) was administered to the collagen gel culture, the contractility enhancement of the palatal fibroblasts with the TGF-β1 pretreatment was partially blocked.

4. Discussion

The TGF-β family is a multifunctional regulator of cell growth, differentiation and extracellular matrix deposition including type I collagen [17]. Recently, it was reported that neutralization of TGF-β1 and TGF-β2 reduced scarring in rat cutaneous wounds [18]. Similar preventive effects of neutralizing antibodies or antagonists to TGF-β1 on other fibrotic diseases have been reported [19–22]. Thus, TGF-β1 is implicated in pathogenic fibrotic conditions and scarring of skin wounds. We previously reported that TGF-β1 induced α-SM actin expression and collagen synthesis in palatal fibroblasts and plays an important role in the expression of the myofibroblastic phenotype [6,7]. In order to easily evaluate whether IFN-γ can regulate myofibroblastic differentiation in this study, we preconditioned palatal fibroblast cultures by administering TGF-β1 to them for 24 h, because palatal fi-

broblasts hardly expressed the α-SM actin protein and proα2(I) collagen mRNA. By administering TGF-β1 to this culture for 24 h, the palatal fibroblasts were made to express the α-SM actin protein and proα2(I) collagen mRNA much more strongly than palatal fibroblasts without TGF-β1 treatment. Recently, with a combined immunohistochemical and in situ hybridization study, it has been shown that α-SM actin-expressing myofibroblasts are the main producer of collagen during pulmonary fibrosis [23], which agreed with our results.

We showed in the present study that IFN-γ decreased up to approximately 50% the α-SM actin expression induced by TGF-β1. Similarly, inhibition of the α-SM actin expression has also been reported in other cell types, such as rat dermal fibroblasts [24] and smooth muscle cells [25]. These results suggested that IFN-γ is a potential inhibitor of myofibroblastic differentiation. As for the mechanism by which IFN-γ downregulates α-SM actin expression, it was recently reported that NO-dependent increases in the cellular cGMP levels mediate the inhibitory effect of IFN-γ on α-SM actin expression in rat hepatic stellate cells [26].

Wound healing is an orderly process that involves inflammation, reepithelialization, matrix deposition, and tissue remodeling. MMPs that degrade various extracellular matrices are required to remove damaged tissue and the provisional matrix, and MMP1 in particular, seems to play a critical role in various stages of the wound healing process. Regulation of MMP1 expression is cell type-specific, as demonstrated by the fact that TGF-β repressed the basal and TNF-α induced MMP1 gene expression in dermal fibroblasts, whereas it activated its expression in epidermal keratinocytes [27]. Our result showed that TGF-β1 inhibited MMP1 gene expression in palatal fibroblasts, whereas IFN-γ had no effect on its gene expression. Our results are not consistent with previous reports that IFN-γ prevented MMP1 gene expression in human dermal fibroblasts [28], but we confirmed that IFN-γ decreased its gene expression in palatal fibroblasts without TGF-β1 pretreatment (data not shown). The TGF-β1 pretreatment may be responsible for the diverse effects of IFN-γ on MMP1 gene expression.

The three-dimensional collagen gel culture system has been developed to study wound contraction in vivo [29]. Contractility in collagen gel has been reported to be enhanced by serum or purified growth factors, such as TGF-β [30–32]. We have shown in the present study that IFN-γ inhibited the high contractility induced by TGF-β1 pretreatment in palatal fibroblasts. These results are consistent with previous reports [33,34]. The effects of IFN-γ were totally independent of any alteration of cell number or viability. The mechanism of inhibition might be related either to a decreased expression of cell adhesion molecules on the cell surface or to a decreased cell locomotion or cell contraction forces.

Taken together, we demonstrated that IFN-γ partially abrogated myofibroblastic phenotype expression, including α-SM actin, proα2(I) collagen mRNA expression and cell contractility, induced by TGF-β1 in vitro and might become a therapeutic agent to block scarring in oral palatal mucosa in the future.

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