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Inhibition of induction of myofibroblasts by interferon γ in a human fibroblast cell line

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Abstract

Interferon γ (IFNγ) has been reported as a possible therapeutic agent for contractile diseases in clinical trials and in vitro studies. It is not yet clear, however, whether IFNγ simply inhibits myofibroblast generation or downregulates α smooth muscle actin (αSMA) production in myofibroblasts. In this study, we attempted to clarify how IFNγ acts in the generation of myofibroblasts, and the production of αSMA by myofibroblasts, using immunofluorescence staining, cell capture enzyme immunoassay (CC-EIA) and the reverse transcription polymerase chain reaction (RT-PCR) for αSMA.

We examined whether IFNγ could block the TGFβ1-promoted changes in myofibroblasts or the generation of myofibroblasts by TGFβ1. IFNγ strongly blocked the generation of myofibroblasts and moderately inhibited the production of αSMA in TGFβ1-promoted myofibrobasts. These findings indicate that IFNγ may be effective in the early stage of contractile diseases to prevent the progression of contractile lesions.

Keywords: In vitro assay; Myofibroblast; α Smooth muscle actin (αSMA); Interferon γ (IFNγ); Transforming growth factor β1 (TGFβ1); Dupuytren's contracture

1. Introduction

Dupuytren's contracture is a contractile disease in the fascia of the human palm. The disease involves the functional obstruction of finger movement by formation of nodules in the fascia of the palm. The only treatment is surgical resection of the nodule, but the nodule formation recurs frequently. Since myofibroblast-rich nodules occur in the lesion, and myofibroblasts are closely associated with the pathogenesis of Dupuytren's contracture, the majority of myofibroblasts appear to arise from fibroblasts by enhancing production of α smooth muscle actin (αSMA) [1]. Since the myofibroblast is a common cell in granulation tissue, and artificial overexpression of αSMA does not induce contracture of tissue [2,3], αSMA in the myofibroblast may not itself be directly associated with the induction of Dupuytren's contracture. In

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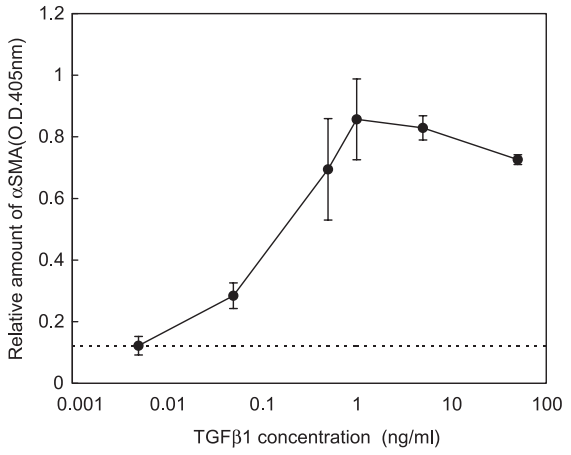

Fig. 1. Induction of αSMA production by TGFβ1 in fibroblasts. Fibroblasts at a density of 1×10^4 cells/well were cultured for 3 days in the presence of TGFβ1 at the designated concentrations. αSMA production was measured by CC-EIA.

contrast, Tomasek et al. [1,4] described a significant correlation between αSMA expression and the generation of contractile force in Dupuytren's nodules. At present, although it is not clear whether αSMA is directly associated with the contracture of tissue, αSMA is considered to be a useful marker of myofibroblasts in contractile diseases.

Pittet et al. [5] demonstrated in their clinical pilot study that injection of interferon γ (IFNγ) into lesions of hypertrophic scars and Dupuytren's nodules revealed a therapeutic effect in humans. They qualitatively demonstrated that IFNγ reduces αSMA production in cells from the lesions. Sanders et al. [6] reported that IFNα2b decreases contraction of Dupuytren's cells, and Tanaka et al. [7] quantitatively demonstrated that IFNγ reduces αSMA production in Dupuytren's cells and transforming growth factor β1 (TGFβ1)-treated cells, both in vitro. These studies indicated that IFNs could be promising drugs for the treatment of contractile diseases such as Dupuytren's contracture. It is not yet clear, however, whether IFNγ simply inhibits fibroblast transformation or downregulates αSMA production in myofibroblasts.

Tanaka et al. [7] demonstrated the downregulation of αSMA production by IFNγ in a TGFβ1-stimulated human fibroblast cell line. They also showed that such downregulation in TGFβ1-stimulated fibroblasts is similar to that in cultured Dupuytren's cells. In this study, we constructed a model for generating myofibroblasts using TGFβ1-stimulated human fibroblasts, and attempted to clarify whether IFNγ inhibits the generation of myofibroblasts or the downregulation of αSMA production in myofibroblasts.

2. Materials and methods

2.1. Cells

The human fibroblastic cell line, WI-38, was cultured in Earl's minimum essential medium (E-MEM, Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum. The cells were cultured in a 96-well microplate (Becton Dickinson, New Jersey, USA) at a density of 1×10^4 cells/ml at 37 °C for 24 h. In some experiments, TGFβ1 (R&D Systems, Minneapolis, MN, USA) was used for the induction of αSMA in fibroblasts. To induce αSMA in WI-38 cells, TGFβ1 solution at final concentrations of 0–5 ng/ml was added to 1×10^4 cells/well in a microplate, and the cells were cultured at 37 °C for 7 days under 5% CO_2 atmosphere with a medium change on the third day of culture. In some experiments, to demonstrate the downregulation of αSMA production, TGFβ1-treated WI-38 cells were cultured in the presence of 100 U/ml IFNγ (Roche Diagnostics,

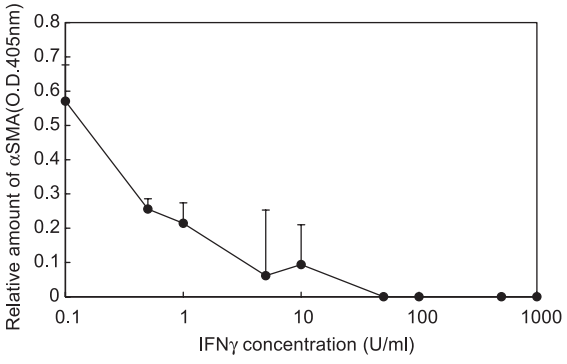

Fig. 2. Inhibitory effect of IFNγ on αSMA production induced by TGFβ1 stimulation. Cells were seeded at a density of 1×10^4 cells/well in a microplate and cultured for 3 days at various IFNγ concentrations and 5 ng/ml TGFβ1. The cells were examined for αSMA production by CC-EIA.

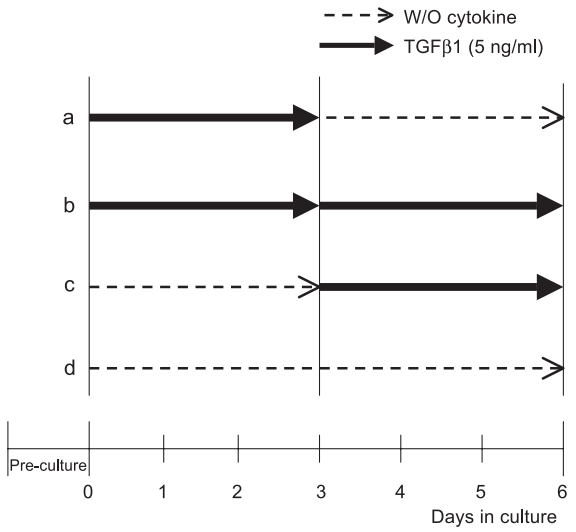

Fig. 3-1. Fibroblasts were treated with 5 ng/ml TGFβ1 for the first 3 days in culture, and then cultured without cytokines for the next 3 days of culture (a). Fibroblasts were stimulated with the same concentration of TGFβ1 throughout the entire culture (b). Fibroblasts were cultured without cytokines for the first 3 days, and then stimulated with the same concentration of TGFβ1 only in the next 3 days of culture (c). TGFβ1-unstimulated fibroblasts are presented (d).

Mannheim, Germany), and examined by the assays described below.

2.2. Antibodies, chemicals and cell capture enzyme immunoassay (CC-EIA) for αSMA

The cell capture enzyme immunoassay (CC-EIA) for αSMA [7] was applied. In brief, cultured cells were fixed in a 96-well microtiter plate with 4% formalin as described previously [8], and treated with 3% bovine serum albumin to block nonspecific immunoreactions. The fixed cells were treated with 32.5 μg/ml anti-αSMA mouse monoclonal antibody (Sigma, St. Louis, MO, USA) in 150 mM phosphate-buffered saline (PBS, pH 7.2) for 30 min at 37 °C and washed in PBS. The cells were reacted again with 50 μg/ml alkaline phosphatase (ALP)-labeled anti-mouse goat antibody (Cappel, Ohio, USA) in 150 mM PBS, for 30 min at 37 °C and washed in PBS. For colorization, a paranitrophosphate kit (MonotestALPot; Roche Diagnostics) was used with the addition of 4.8% levamisole to block endogenous ALP. The optical density of the colored solution was measured in a

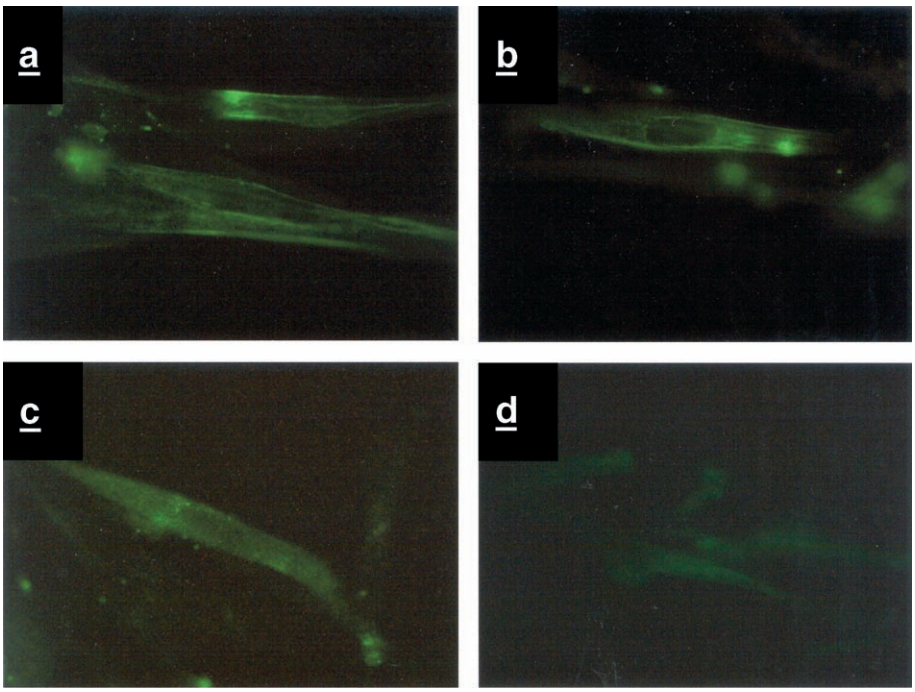

Fig. 3-2. Representative immunofluorescence micrographs of fibroblasts treated with TGFβ1. Fibroblasts were cultured in the manner as described in Fig. 3-1. The fluorescence signal intensity for αSMA in the fibroblasts was observed by immunofluorescence microscopy.

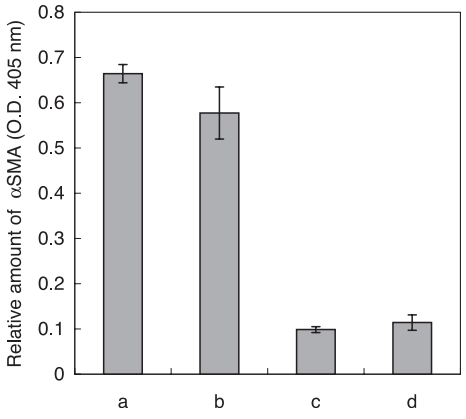

Fig. 3-3. Quantification of αSMA in TGFβ1-treated fibroblasts. Fibroblasts were cultured in the manner as described in Fig. 3-1, and examined for αSMA production by CC-EIA.

microplate reader (NJ-200, Japan InterMed, Tokyo, Japan) at 405 nm wavelength. The background O.D. value was subtracted from the direct O.D. value; the difference represented the amount of αSMA. This assay revealed a linear correlation between the number of cells which fully expressed αSMA and the O.D. value between 0.2 and 1.0 (data not shown), and was considered to be semiquantitative.

2.3. Immunofluorescence staining

WI-38 cells were cultured on a chamber glass slide with or without TGFβ1 for 3 days and air-dried for 1 h. The cells were fixed with 5% formalin in 150 mM PBS (pH 7.2) for 30 min and washed in 150 mM PBS (pH 7.2). The fixed cells were reacted with anti-αSMA mouse monoclonal antibody (Sigma) at 37 °C for 30 min in a moist chamber and washed in 150 mM PBS (pH 7.2). After the washing, the cells were reacted with the fluorescence isothiocyanate-labeled anti-mouse goat antibody (ZYMED Laboratories, CA, USA) at 37 °C for 30 min in a moist chamber and washed again in 150 mM PBS (pH 7.2). The cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Polymerase chain reaction (PCR)

The primer pair used in reverse transcription (RT)-PCR for αSMA was purchased from Stratagene (California, USA). The sequences of the primers were as follows: sense primer, 5′GCTCACGGAGGCACCCC-TGAA3′, antisense primer, 5′CTGATAGGACATTGT-TAGCAT3′. The expected size of the PCR product was 590 bp [9]. Prior to the gene amplification with Gene Amp PCR system 9700 (PE Applied Biosystems, California, USA), mRNAs were isolated using the Micro-FastTrack 2.0 kit (Invitrogen, California, USA) from 5×10^5 cells. The amplification reaction was performed using GeneAmp EZ r*Tth* RNA PCR Kit (PE Applied Biosystems), following the instruction manual attached to the kit. Temperature profiles were as follows: one cycle of denaturation for 60 s at 94 °C and annealing and reverse transcription for 30 min at 60 °C, followed by 35 cycles of denaturation for 30 s at 94 °C and annealing for 60 s at 65 °C, and a final extension for 10 min at 65 °C. The PCR products obtained were mixed with loading buffer and subjected to electrophoresis.

Chromosomal DNA was extracted from half the amount of the same fibroblasts and amplified with the primer pair for β-globin (Stratagene). The expected

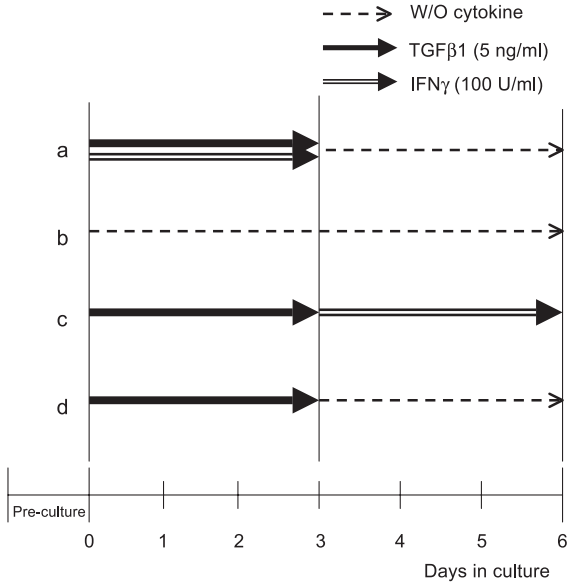

Fig. 4-1. Fibroblasts were cultured in the presence of 5 ng/ml TGFβ1 and 100 U/ml IFNγ for 3 days, and further incubated without the cytokines for 3 days (a). Fibroblasts were cultured without cytokines for 6 days (b). Fibroblasts were treated with 5 ng/ml TGFβ1 for 3 days, and then cultured in the presence (c) or absence (d) of 100 U/ml IFNγ for 3 days.

size of the PCR product was 109 bp. The amplification reaction was performed in a 50-μl volume with temperature profiles as follows: one cycle of denaturation for 60 s at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C and annealing for 60 s at 65 °C, and a final extension for 10 min at 65 °C with Gene Amp PCR system 9700 (PE Applied Biosystems).

The gel was stained using the SYBR Green I nucleic acid gel staining kit (FMC Bioproducts, Maine, USA) and observed through a transilluminator at a wavelength of 312 nm.

3. Results

3.1. Optimum concentrations of TGFβ1 and IFNγ

To determine the optimal concentrations of TGFβ1 for constructing a model of Dupuytren's cell, WI-38 cells were cultured at various concentrations of the cytokine. αSMA production was increased by the addition of TGFβ1 in a concentration-dependent manner (Fig. 1). The maximum induction was observed at TGFβ1 concentrations of 1–5 ng/ml.

To determine the optimal concentrations of IFNγ in the subsequent experiments for inhibition of αSMA production, WI-38 cells were treated with 5 ng/ml TGFβ1 and cultured in the presence of IFNγ. αSMA production was inhibited by IFNγ in a concentration-dependent manner (Fig. 2) and was completely blocked at concentrations equal to or greater than 50 U/ml.

From the above results, we determined the optimal transformation to be at 5 ng/ml of TGFβ1 and optimal inhibition to be 100 U/ml IFNγ for subsequent experiments.

3.2. Initiation of αSMA production

To determine when TGFβ1 initiates αSMA production in culture, TGFβ1 was added at different stages of culture. In our immunofluorescence micro-

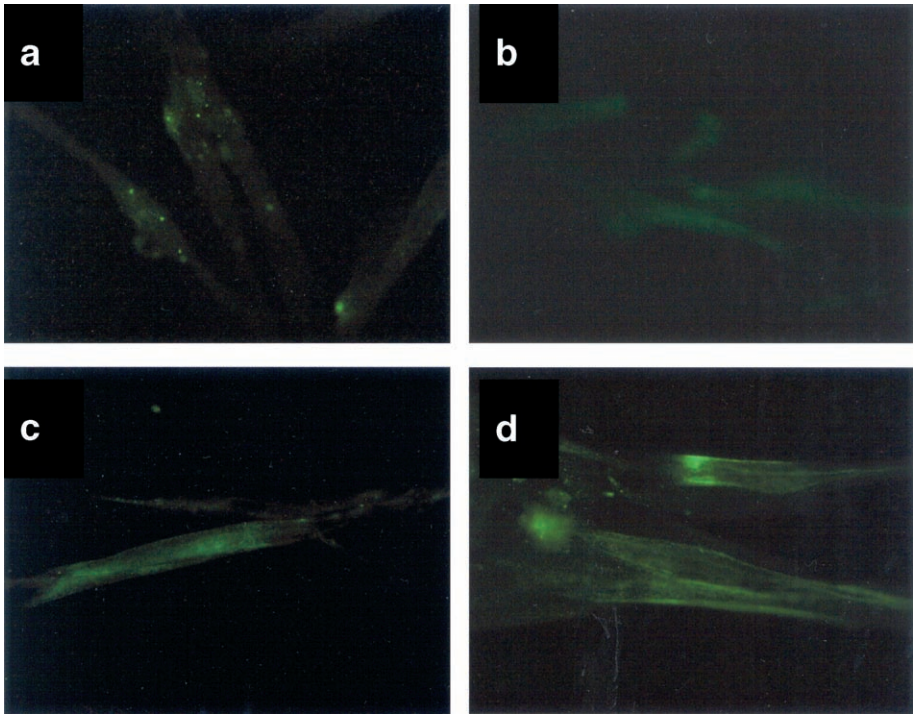

Fig. 4-2. Representative immunofluorescence micrographs of TGFβ1-stimulated fibroblasts inhibited by IFNγ. Fibroblasts were cultured in the manner as described in Fig. 4-1. The fluorescence signal intensity for αSMA in the fibroblasts was observed by immunofluorescence microscopy.

scopic study, when fibroblasts were stimulated with TGFβ1 in the first 3 days of culture, the intensity level of the fluorescence signal for αSMA in the cells (Figs. 3-1a and 3-2a) was almost the same as that in the fibroblasts stimulated throughout the entire culture period (Figs. 3-1b and 3-2b). In contrast, when fibroblasts were cultured in the presence of TGFβ1 in the next 3 days of culture, the fluorescence intensity for αSMA in the cells (Figs. 3-1c and 3-2c) was almost the same as that in the unstimulated fibroblasts (Figs. 3-1d and 3-2d). Similar results were obtained in the measurement of the relative amount of αSMA by CC-EIA (Fig. 3-3). Almost the same relative amount of αSMA was detected in the fibroblasts treated with TGFβ1 for the first 3 days of culture as that for the entire culture period. The TGFβ1 treatment in the next 3 days of culture did not stimulate αSMA production. Since these results suggest that αSMA production can be initiated in the early stages of the culture of fibroblasts, we used fibroblasts treated with TGFβ1 for the first 3 days in culture as a model of myofibroblast generation in the subsequent experiments.

3.3. IFNγ treatment of TGFβ1-stimulated fibroblasts

To clarify whether IFNγ interferes with generation of myofibroblasts in the first 3 days of culture, fibroblasts cultured in the presence of TGFβ1 and IFNγ were observed by immunofluorescence microscopy. The fluorescence signal intensity for αSMA in the fibroblasts treated with IFNγ during the TGFβ1 stimulation (Figs. 4-1a and 4-2a) was almost the same as that in the unstimulated fibroblasts (Figs. 4-1b and 4-2b). In fibroblasts cultured in the presence of IFNγ after TGFβ1 stimulation, the fluorescence signal intensity level for αSMA (Figs. 4-1c and 4-2c) was slightly reduced compared with that in the IFNγ-untreated fibroblasts stimulated with TGFβ1 (Figs. 4-1d and 4-2d). The signal pattern became homogenous in TGFβ1-promoted cells following treatment with IFNγ (Fig. 4-2c), compared with IFNγ-untreated preparations (Fig. 4-2d). In the CC-EIA assay, αSMA production in the fibroblasts treated with IFNγ during TGFβ1 stimulation (Fig. 4-3a) decreased to the same level as that in the unstimulated fibroblasts (Fig. 4-3b). When IFNγ was added to fibroblasts after TGFβ1 stimulation, αSMA production decreased to about 50% of that in the IFNγ-untreated fibroblasts

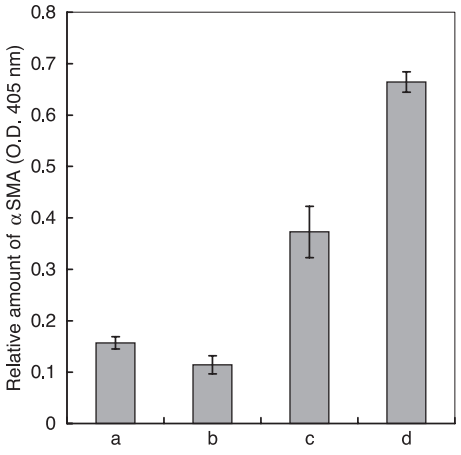

Fig. 4-3. Inhibition of αSMA production by IFNγ in TGFβ1-treated fibroblasts. Fibroblasts were cultured in the manner as described in Fig. 4-1, and examined for the inhibition of αSMA production by CC-EIA.

(Fig. 4-3c,d). These results indicate that IFNγ strongly blocks the myofibroblast generation as manifested by the initiation of αSMA production and moderately inhibits the αSMA production in the myofibroblast. These decreases were statistically analysed by Student's t analysis and appeared to be significant ($p < 0.002$).

3.4. Production of mRNA for αSMA

To clarify whether the blockage of αSMA production is due to the suppression of mRNA production, the level of mRNA expression for αSMA in the same number of cells was examined by RT-PCR. Assay control for RT-PCR kit was presented in Fig. 5A, lane 1. The expression was not detectable in TGFβ1-unstimulated fibroblasts of mRNA for αSMA, but was readily apparent in treated cells (Fig. 5A, lanes 2 and 3). When the TGFβ1-stimulated fibroblasts were cultured in the presence of IFNγ, the signal intensity decreased to almost undetectable levels (Fig. 5A, lane 4). To confirm the number of fibroblasts in this PCR experiment, the amount of amplicon of the chromosomal gene for β-globin was examined. All three samples of fibroblasts revealed bands with almost the same intensity (Fig. 5B). The densitometric analyses of the bands were 1.194 ± 0.066, 1.168 ± 0.065 and 1.264 ± 0.065 in fibroblasts, TGFβ1-promoted myofibroblasts and IFNγ-treated myofibroblasts stimulat-

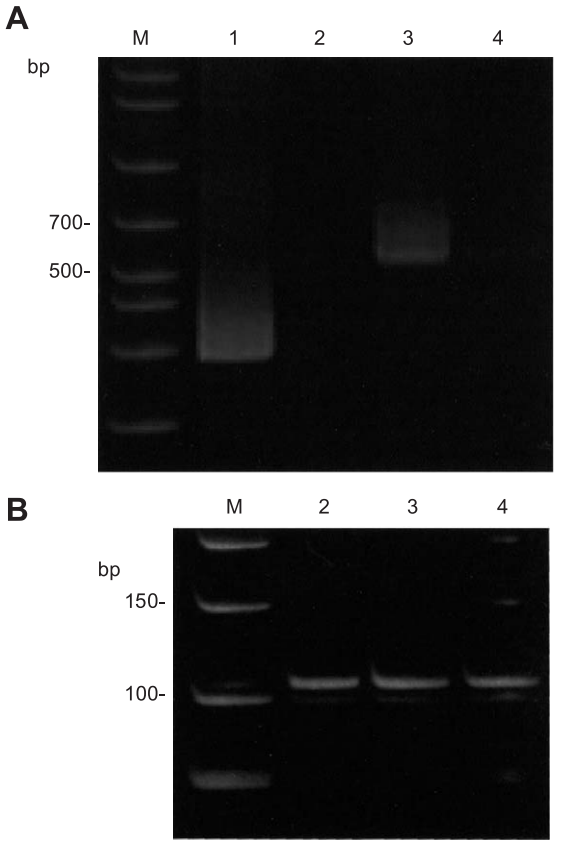

Fig. 5. RT-PCR products of αSMA mRNA in fibroblasts treated with TGFβ1 and/or IFNγ. A representative photograph of results of gel electrophoresis for RT-PCR analysis is shown (A). RNA was extracted from 5×10^5 fibroblasts, and used for RT-PCR. Assay control from *Escherichia coli* PAW RNA is shown in lane 1. Fibroblasts (lane 2) were stimulated with 5 ng/ml TGFβ1 in the absence (lane 3) or presence of 100 U/ml IFNγ (lane 4). Clear bands were observed at the expected position of the marker (lane M). Chromosomal DNA was extracted from the same number of cultured fibroblasts from the same culture, and examined for β-globin-specific sequence by PCR analysis (B).

ed with TGFβ1, respectively. Since no significant differences were observed by Student's *t* analysis ($p < 0.002$), the number of cells examined were almost identical.

4. Discussion

Baird et al. [10] demonstrated that the concentrations of interleukin-1α, interleukin-1β, TGFβ and basic fibroblast growth factor are high in Dupuytren's nodules. Since TGFβ1 stimulates αSMA expression in fibroblasts [11], and induces the formation of myofibroblast-rich Dupuytren's nodules [12], the cytokine is considered to be associated with the pathogenesis of Dupuytren's disease [13]. The myofibroblast is defined as an αSMA-rich fibroblast. Although the αSMA expression by myofibroblasts appears to be transient in granulation tissue, αSMA is a suitable marker of myofibroblasts in vitro [4,14]. In this study, we constructed a model for the generation of myofibroblasts from a TGFβ1-stimulated human fibroblast cell line by monitoring αSMA production, and found that TGFβ1 induces myofibroblasts in the early stages of culture. The fibroblasts that were stimulated with TGFβ1 only in the first 3 days of culture actively produced αSMA. However, those that were stimulated only in the next 3 days did not. In previous studies [4,6], an in vitro model of Dupuytren's cells was obtained from a diseased nodule. Our results indicate that the fibroblasts stimulated by TGFβ1 only in the first 3 days are initiated cells for myofibroblast formation, and are considered to be a model of Dupuytren's cells.

Using generating/generated myofibroblasts, we examined whether IFNγ could block the TGFβ1-promoted changes or the generation of myofibroblasts. Since the αSMA production was blocked by IFNγ in TGFβ1-stimulated fibroblasts, the cytokine could block the generation of myofibroblasts. When TGFβ1-stimulated fibroblasts were cultured further in the presence of IFNγ, the αSMA and its mRNA production decreased in the cells. These results are similar to that of IFNα2b as Sanders et al. [6] reported. Vaughan and Tomasek [15,16] reported that IFNγ blocks the TGFβ1-promoted changes of myofibroblasts including αSMA production and cellular fiber assembly in cultured Dupuytren's cells. In our study, the signal pattern was homogenous in TGFβ1-promoted cells cultured with IFNγ. The phenomenon may indicate that IFNγ also blocks the αSMA assembly as well as αSMA production.

The new findings of our study are that IFNγ strongly blocks the generation of myofibroblasts by TGFβ1, and that IFNγ moderately blocks αSMA production. These findings may suggest that IFNγ is effective in the early stage of Dupuytren's contracture in preventing the progression of this disease.

The above findings may also be important for a better understanding of the pathogenesis of Dupuytren's disease. In the very early stage of the disease, TGFβ1 is locally produced as an inflammatory cytokine. When a sufficient amount of IFNγ is produced in the local lesion following TGFβ1 production, the promotion of myofibroblasts may be suppressed [1]. As Tomasek et al. [1] suggested, a possible mechanism for the worsening of Dupuytren's disease might involve the local suppression of CD4+ T cells which secrete IFNγ, or the insufficient filtration of T cells due to a disturbance in microcirculation in Dupuytren's nodule. Our findings may support these hypothetical explanations.

A few clinical trials [5,17] of IFNγ therapy for contractile disease have been performed. Since our study has provided in vitro evidence for the effectiveness of IFNγ on myofibroblasts, further clinical trials of IFNγ treatment for contractile diseases should be performed.

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