

ORIGINAL ARTICLE

Tacrolimus abrogates TGF- β 1-induced type I collagen production in normal human fibroblasts through suppressing p38MAPK signalling pathway: implications on treatment of chronic atopic dermatitis lesions

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Abstract

Background Atopic dermatitis (AD) is a commonly encountered inflammatory skin disease. Although acute lesions of acute AD are characterized by intense inflammation, the hallmarks of chronic AD lesions include lichenified fibrosis and thickening of the upper dermis. The increased expression of transforming growth factor beta 1 (TGF- β 1), a well-known fibrogenic cytokine, is observed in chronic AD lesions. Tacrolimus (FK506) ointment has been reported to be effective for treating AD as well as some TGF- β 1-induced fibrotic diseases.

Objectives To evaluate the effect of tacrolimus on TGF- β 1-stimulated cultured normal human dermal fibroblasts and explore the potential signalling pathways involved.

Methods Fibroblasts cultured from healthy adult human foreskins were treated with TGF- β 1 with or without tacrolimus. The impact on cell viability and proliferation were assessed by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay and BrdU incorporation assay respectively. Reverse transcription-polymerase chain reaction (RT-PCR), quantitative real-time PCR, enzyme-linked immunosorbent assay (ELISA) and western blotting were performed to evaluate the relevant expressions of mRNA or proteins in fibroblasts.

Results Our results revealed that the increased expressions of transforming growth factor- β receptor I (TGF- β RI) and TGF- β RII in TGF- β 1-treated fibroblasts were suppressed by tacrolimus treatment. In addition, tacrolimus significantly inhibited fibroblast proliferation enhanced by TGF- β 1. TGF- β 1 increased type I collagen production, and this enhancing effect was suppressed by tacrolimus. The down-regulation of MMP-1 and up-regulation of TIMP-1 induced by TGF- β 1 were reversed by tacrolimus. The increase in phosphorylated p38 mitogen-activated protein kinase (p38MAPK) expression stimulated by TGF- β 1 was down-regulated by tacrolimus. Moreover, the fibroblasts treated with p38MAPK inhibitor significantly reduced type I collagen expression induced by TGF- β 1.

Conclusions The present results demonstrated that tacrolimus significantly inhibited physiological functions of fibroblasts enhanced by TGF- β 1 *in vitro*. Clinically, we propose that topical tacrolimus may not only reduce AD recurrence but also ameliorate dermal fibrosis often seen in chronic AD lesions.

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Conflicts of interest

None declared.

Introduction

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease with increasing prevalence in both paediatric and adult populations.^{1–3} Acute phases of AD lesions are characterized by erythematous papules, excoriations, serous exudation and extreme pruritus. On the other hand, chronic AD is character-

ized by thickened skin, accentuated skin markings (i.e. lichenification) and fibrotic papules. Increased deposits of type I collagen were considered the major event responsible for the development of lichenification.^{4–6}

Transforming growth factor β (TGF- β) is a multifunctional cytokine that plays an important role in regulating cell growth,

differentiation and biosynthesis of extracellular connective tissue.^{7,8} TGF- β superfamily members transduce their signal from the membrane to the nucleus through transmembrane type I and type II receptors and their downstream effectors Smad proteins.^{8,9} In addition to Smad-related signalling, the mitogen-activated protein kinase (MAPK) pathway was shown to be an important Smad-independent TGF- β activated cascade.¹⁰ In mammals, three isoforms of TGF- β , namely TGF- β 1, TGF- β 2 and TGF- β 3 have been identified, with TGF- β 1 being the most well studied. TGF- β 1 is an important growth factor for promoting fibrosis.^{9,11} Results from Toda *et al.*⁵ demonstrated that the TGF- β 1 expression was markedly enhanced in chronic AD lesions. Therefore, it is likely that increased expression of TGF- β 1 in chronic AD lesions played an important role on the formation of skin thickening and lichenification.

Matrix metalloproteinase's (MMPs) are a family of zinc- and calcium-dependent endopeptidase that degrades the various extracellular matrix proteins.¹² In contrast, tissue inhibitors of MMPs (TIMPs) are secreted proteins that block the activity of MMPs with a one-to-one stoichiometry.^{12,13} Appropriate regulation of MMPs and their inhibitors TIMPs during the tissue repair processes is important for tissue remodelling. The aberrant expression of MMPs and TIMPs has been shown to be associated with several inflammatory disease activities, including AD,^{14,4} and this dysregulation of collagen degradation probably contributed to development of lichenification in chronic AD lesions.

Tacrolimus (FK506) ointment, a topical calcineurin inhibitor, has been used to treat AD with sustained efficacy and no significant side-effects.^{15–17} In addition to treating AD, tacrolimus has also been reported to be effective for treating certain fibrotic skin disorders such as scleroderma.^{18–20} Several studies have shown that topical tacrolimus significantly improved the skin thickening and lichenification as reflected by reduced dermal thickness in chronic AD lesions.^{21–23} These results suggested that in addition to immune suppressive effect, tacrolimus may also modulate the physiological functions of dermal fibroblasts. Tacrolimus is best known to exhibit its pharmacological effects on inflammatory cell including T cells, macrophages, eosinophils and neutrophils.^{24,25} In contrast, little remains known regarding the effects of tacrolimus on fibroblasts.

The current managements for AD focussed on alleviation of inflammatory response during acute phase. The need for treating lichenified lesions in AD was often ignored. In this study, we set out to investigate if tacrolimus suppresses TGF- β 1-stimulated fibrotic responses in normal human fibroblasts.

Materials and methods

Cell culture and treatments

The fibroblasts used in our study were cultured from the healthy adult human foreskins. Cell culture for fibroblasts was

performed as described elsewhere.²⁶ Briefly, the adipose tissues were removed by a sterilized scissor, and the remaining specimens including epidermis and dermis were sectioned into 5 mm by 5 mm blocks and laid onto 100 mm² Petri dishes. The dishes were maintained semi-opened into laminar flow for at least 40 min to adhere the blocks in the culture surface. Once the blocks were fixed in the Petri dish surface, 10 mL of Dulbecco's minimal essential medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (Gibco), penicillin (100 U/mL) and streptomycin (100 μ g/mL) (Gibco), was poured into the dishes. Then, the culture dishes were maintained in a humidified incubator and the culture medium was changed every 2 days. The fibroblasts gradually crawled out the minced blocks in 7–10 days. The third or fourth passage of the cultured fibroblast was used in the following experiments. In our study, the fibroblasts were cultured in the serum-free medium for 24 h for synchronization. Then, the cells were re-fed with medium containing 10% fetal bovine serum (Gibco) for the following experiments. Tacrolimus (Calbiochem; San Diego, CA, USA.) used in this study was dissolved in dimethyl sulphoxide (DMSO) and then was diluted with DMEM solution containing 10% fetal bovine serum (the final concentration of DMSO in the culture medium was less than 0.1%). The cultured fibroblasts were treated with TGF- β 1 5 ng/mL (R&D Systems, Minneapolis, MN, USA), with or without tacrolimus at doses of 100 and 1000 nmol/L. The rationale for tacrolimus concentrations used in our study was that fibroblasts treated with tacrolimus 1000 nmol/L demonstrated no stimulatory effect on the expressions of collagen and ECM components.^{27,28} Moreover, this dose closely corresponded to the whole blood trough levels $\times$ 100 in patients receiving tacrolimus immunosuppressive therapy.²⁹ Therefore, in this study, we use 1000 nmol/L and a lower tacrolimus 100 nmol/L as the treated concentrations for the following experiments.

Cell viability assay

A colorimetric method for determining the activity of living cells was based on mitochondrial function and was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (CellTiter 96[®] non-radioactive cell proliferation assay kit, Promega, Madison, WI, USA), as described previously.³⁰ Fibroblasts were seeded on 96-well plates with a density of 3×10^3 cells/well and incubated overnight. After treatments with tacrolimus 100 and 1000 nmol/L for 1, 3 and 5 days, 20 μ L of CellTiter reagent containing tetrazolium compound and electron coupling reagents were added into each well to be catalyzed by mitochondrial dehydrogenase enzymes in metabolically active cells. The plates were incubated for 4 h and the colorimetric absorbance was recorded at 570 nm by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

BrdU assay

The proliferation of fibroblasts was assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay using a commercially available BrdU-enzyme-linked immunosorbent assay (ELISA) cell proliferation assay kit (Roche, Basel, Switzerland). Briefly, fibroblasts were seeded on 96-well plates at a density of 1×10^4 cells/well and incubated overnight. Then, the fibroblasts were treated and incubated for 1, 3 and 5 days. The treated cells were fixed and stained according to the manufacturer's instructions. The optical density was measured at 450 nm with an ELISA plate reader (Model 450; Bio-Rad, Hercules, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from treated fibroblasts using the Trizol method (Gibco) and processed as recommended by the manufacturer. Briefly, single-stranded cDNA was reverse transcribed from total cellular RNA using the BcaBEST RNA PCR kit (Takara Shuzo, Kyoto, Japan). The primers used for TGF- β receptor I (TGF- β RI), TGF- β receptor II (TGF- β RII), MMP-1, TIMP-1, type I collagen, GAPDH were summarized in Table 1. The PCR products were electrophoresed through 1.8% agarose gels, stained with ethidium bromide and further visualized by UV illumination. The visualized gels were recorded and analysed on a digital imaging system (Alpha Imager 2000, Alpha Innotech, San Leandro, CA, USA). Levels of gene expression were expressed as the integrated density value of PCR products normalized to that of the GAPDH in the same sample.

Real-time quantitative polymerase chain reaction (real-time PCR)

The total RNA of the treated fibroblasts was extracted using TRI Reagent (Invitrogen, Carlsbad, CA, USA). Then, the extracted RNA was treated with TURBO DNase (Applied Biosystems) for

25 min at 37°C. Two micrograms of TURBO DNase-treated RNA were reverse transcribed using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The cDNA samples were then diluted with nuclease-free water to a final concentration equivalent to 20 ng/ μ L RNA input, and 6 μ L of these dilutions were then subjected to 40 cycles of quantitative Real-time PCR with the SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN, USA) was performed using ABI Prism 7500 Sequence Detection System (Applied Biosystems). Primers for type I collagen (sense: 5'-GCCATCAAA GTCTTCTGCAACAT-3'; antisense: 5'-ACCAGACATGCCTCTT GTCCTT-3') were tested alongside the normalizing gene β -actin (sense: 5'-TCTGTGTGGATTGGTGGCTCT-3'; antisense: 5'-GACTC ATCGTACTCCTGCTTGCT-3'). The fluorescent signals were collected during extension phase, Ct values of the sample were calculated, and the transcript levels were analysed by $2^{-\Delta\Delta C_t}$ method.

Enzyme-linked immunoassay for MMP-1 and TIMP-1 production

Concentrations of secreted MMP-1 and TIMP-1 were determined by enzyme-linked immunoassay (R&D Systems, Minneapolis, MN, USA) in culture supernatants of fibroblasts. The measurement of MMP-1 and TIMP-1 were carried out according to the manufacturer's instructions. The minimum detectable dose of MMP-1 and TIMP-1 were 0.021 and 0.08 ng/mL respectively.

Western blotting analysis

Total cellular proteins were extracted from treated fibroblasts with lysis buffer (1.5% SDS, 0.0625 mol/L Tris-HCl and 1 mmol/L Na_3VO_4 , pH 6.8) containing protease inhibitor cocktail (Roche, Mannheim, Germany). For Western blot analysis, 40 μ g of extracted total cellular proteins were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Subsequently, they were electroblotted onto a nitrocellulose membrane for 1 h using a Mini Trans-Blot Transfer Cell (Bio-Rad) at 90 V. After blocking and washing, the nitrocellulose membrane was incubated overnight at 4 °C with the following primary antibodies: pro-collagen IA1 (Santa Cruz Biotechnology), total ERK1/2 (Cell Signaling, Beverly, MA, USA), phosphorylated ERK1/2 (pERK1/2) (Cell Signaling), total JNK (Cell Signaling), phosphorylated JNK (pJNK) (Cell Signaling), total p38MAPK (Cell Signaling), phosphorylated p38MAPK (pp38MAPK) (Cell Signaling) and GAPDH (BD Biosciences, San Jose, CA, USA). Then, membranes were incubated with HRP-labelled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The immunoblots were developed using the ECL Western detection reagent (Santa Cruz Biotechnology). The results were analysed by digital imaging system (Alpha Imager 2000, Alpha Innotech Corp., San Leandro, CA, USA). For

Table 1 Primer sequence used for RT-PCR

	Primer sequence	Size (bp)
TGF- β RI	F: 5'CGGCGTTACAGTGTCTTCT3' R: 5'TGTCTTTATTGTCTGCTGCTAT3'	483
TGF- β RII	F: 5'CGTGAAGAACGACCTAACCT3' R: 5'AGTTCCACCTGCCAC3'	104
MMP-1	F: 5' GATCCAAGCCATATATGGACG 3' R: 5' GCAGTTGAACCAGCTATTAGC 3'	634
TIMP-1	F: 5' AAGATGCTGCTGTTTACGCGGG 3' R: 5' GTCCTCAGGGCACTGCAGGAT 3'	607
Type I collagen	F: 5' GTGAACCTGGCAAACAAGGT 3' R: 5' CTGAGACCAAGAGAAGCCAC 3'	413
GAPDH	F: 5' TCCTGTGGCATCCACGAAACT 3' R: 5' GAAGCATTTCGGTGGACGAT 3'	319

MAPKs blocking test, 20 $\mu\text{mol/L}$ PD98059 (ERK1/2 inhibitor) (Sigma, St. Louis, MO, USA), 20 $\mu\text{mol/L}$ SP600125 (JNK inhibitor) (Sigma) and 20 $\mu\text{mol/L}$ SB203580 (p38MAPK inhibitor) (Sigma) were added for 1 h, followed by addition of TGF- β 1. Then, the pro-collagen IA1 protein levels were determined.

Statistical analysis

SPSS system for Windows version 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were expressed as mean $\pm$ standard deviation (mean $\pm$ SD). The Student's *t*-test was used for statistical evaluation between control and experimental groups in the study. A *P*-value of <0.05 is considered statistically significant.

Results

Tacrolimus reduced the expressions of TGF- β 1-induced TGF- β RI, TGF- β RII mRNA and proteins in fibroblasts

It has been well recognized that among the cytokines affecting tissue fibrosis, TGF- β 1 is a key mediator of fibrotic reactions. The pro-fibrotic signalling cascade is activated by binding of TGF- β 1 to its type I and type II receptors. In our study, exogenous TGF- β 1 was introduced to simulate the condition *in vivo* (i.e. high TGF- β 1 environment in dermis of chronic AD lesions), and RT-PCR and Western blotting were performed to examine the expressions of TGF- β RI and TGF- β RII in fibroblasts. The results showed that TGF- β 1 significantly stimulated TGF- β RI and TGF- β RII mRNA (Fig. 1a), and protein (Fig. 1b) expressions in fibroblasts. In addition, tacrolimus 100 and 1000 nmol/L treatments significantly suppressed the TGF- β 1-enhanced TGF- β RI and TGF- β RII mRNA (Fig. 1a), and protein (Fig. 1b) expressions in fibroblasts.

Tacrolimus treatment alone showed no significant effect on cell viability but significantly suppressed the TGF- β 1-induced proliferation in fibroblasts

The results shown in Fig. 2 A revealed that tacrolimus at 100 and 1000 nmol/L exhibited no significant effect on fibroblast viability directly. Treatment with TGF- β 1 significantly increased the proliferation of fibroblasts on day 3 and day 5. However, addition of tacrolimus 100 and 1000 nmol/L significantly suppressed the enhancing effect of TGF- β 1 on cell proliferation (Fig. 2b).

Tacrolimus decreased the expression of TGF- β 1-induced type I collagen mRNA and protein in fibroblasts

The expression of type I collagen mRNA in fibroblasts was determined by RT-PCR. The results shown in Fig. 3a demonstrated that type I collagen mRNA expression was markedly increased by TGF- β 1 stimulation. However, TGF- β 1 induced type I collagen mRNA expressions in fibroblasts were markedly decreased

by tacrolimus 100 and 1000 nmol/L treatment. To further validate this phenomena, we used a more sensitive real-time quantitative PCR assay to detect the expression of type I collagen mRNA. As shown in Fig. 3b, the results from real-time PCR were in line with those from RT-PCR. Moreover, Western blotting showed that type I collagen protein expressions in fibroblasts correlated with the mRNA levels (Fig. 3c), demonstrating that tacrolimus decreased the TGF- β 1-induced type I collagen expression.

The decreased expression of MMP-1 and increased expression of TIMP-1 treated by TGF- β 1 were reversed by tacrolimus treatment

The deposition and degradation of type I collagen, the most abundant ECM component, were modulated by the expressions of MMPs and their tissue inhibitors (TIMPs). In this study, the expressions of MMP-1 and TIMP-1 mRNA were examined by RT-PCR. Our results showed that decreased MMP-1 mRNA and increased TIMP-1 mRNA expressions were observed in fibroblasts treated with TGF- β 1 (Fig. 4a). Furthermore, additional tacrolimus treatment significantly enhanced MMP-1 mRNA expression inhibited by TGF- β 1, and decreased TIMP-1 mRNA expression increased by TGF- β 1 (Fig. 4a). To further quantify the effects of TGF- β 1 with or without tacrolimus treatments on MMP-1 and TIMP-1 expressions, the ELISA assays specific for human MMP-1 and TIMP-1 were performed using relevant culture supernatants. The supernatants were collected at 48 h after treatments. The results shown in Fig. 4b demonstrated that the values of MMP-1 in control group, TGF- β 1 treated group, TGF- β 1 + tacrolimus 100 nmol/L group and TGF- β 1 + tacrolimus 1000 nmol/L group were 40.5 ± 8.7 ng/mL, 26.4 ± 6.4 ng/mL, 42.1 ± 10.1 ng/mL and 38.7 ± 3.7 ng/mL respectively. The concentrations of TIMP-1 in control group, TGF- β 1 treated group, TGF- β 1 + tacrolimus 100 nmol/L group and TGF- β 1 + tacrolimus 1000 nmol/L group were 107.8 ± 18.4 ng/mL, 220.1 ± 23.4 ng/mL, 124.3 ± 20.9 ng/mL and 89.4 ± 12.4 ng/mL respectively. In line with the results of mRNA expressions, the decreased protein levels of MMP-1 and increased protein levels of TIMP-1 stimulated by TGF- β 1 were significantly reversed by addition of tacrolimus.

Tacrolimus abrogated the TGF- β 1-induced type I collagen synthesis via downregulation of phosphorylated p38MAPK expression in fibroblasts

In addition to TGF- β /Smad signalling pathways, MAPKs is also reported to be involved in TGF- β 1-mediated pathway.³¹ As demonstrated in Fig. 5a, TGF- β 1 treatment enhanced pERK1/2, pJNK and pp38MAPK expressions in fibroblasts. However, only pp38MAPK, but not pERK1/2 and pJNK expressions induced by TGF- β 1, was markedly suppressed by tacrolimus (Fig. 5a). TGF- β 1 remains known to induce type I collagen expression in dermal fibroblasts.³² Moreover, MAPK

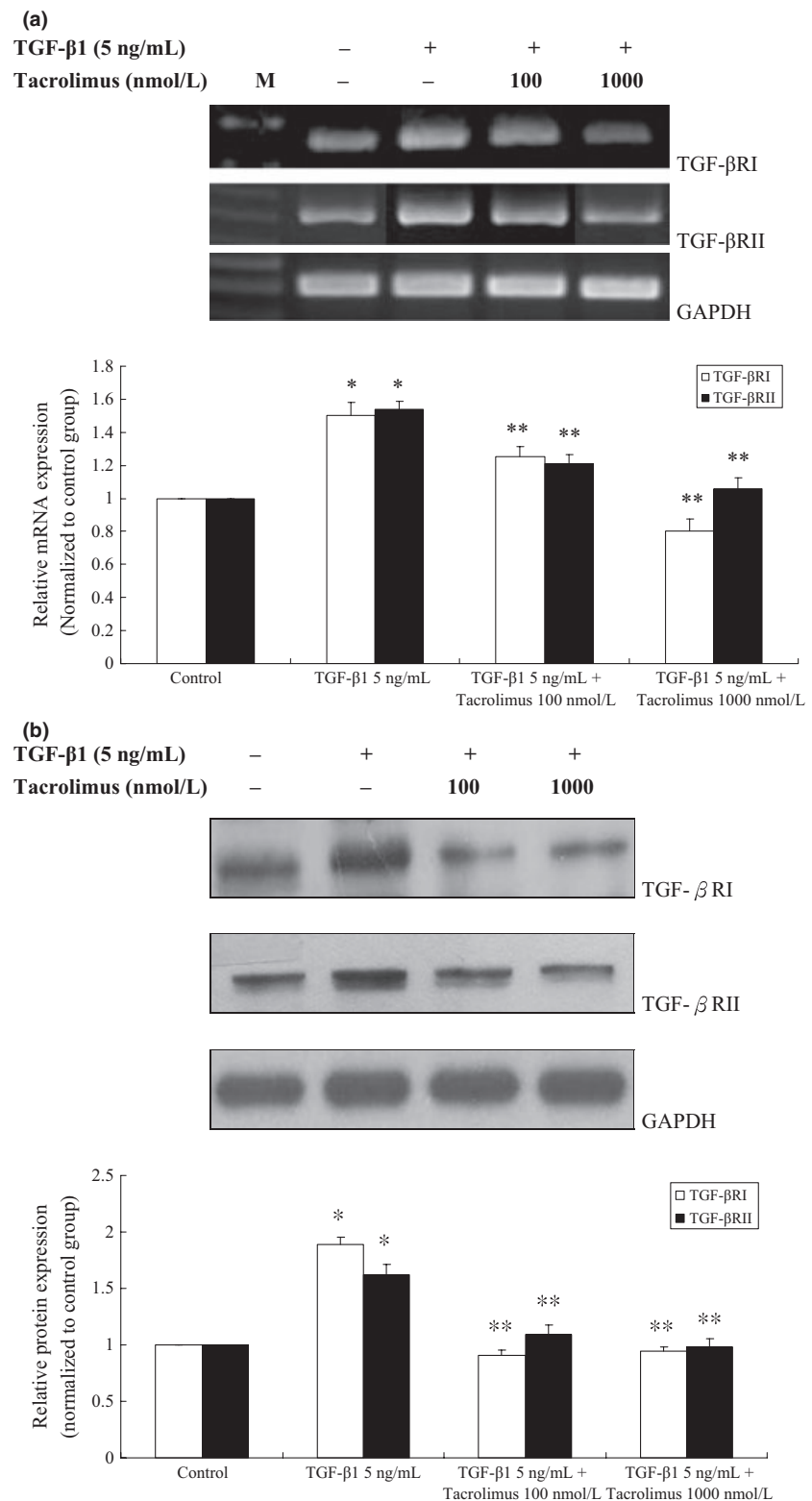


Figure 1 Tacrolimus reduced the expressions of TGF- β RI and TGF- β RII enhanced by TGF- β 1 in fibroblasts. (a) Cultured fibroblasts were treated with TGF- β 1 5 ng/mL with or without addition of tacrolimus 100 and 1000 nmol/L for 24 h. Then, the mRNA was extracted and subjected to RT-PCR for determination of TGF- β RI and TGF- β RII gene expressions. (b) 48 h after the same treatments as shown in (a), the total cellular protein lysates were prepared and subjected to Western blotting analysis with antibody against TGF- β RI, TGF- β RII or GAPDH. The bars indicated mean $\pm$ SD. Experiments were repeated twice with similar results. Densitometric analysis of (a) and (b) were derived from the ratios of TGF- β RI and TGF- β RII to GAPDH expressions of different treatment groups normalized to the control group respectively. The 'M' shown in graph (a) stood for 'Marker'. * P < 0.05 as compared with control group; ** P < 0.05 as compared with TGF- β 1-stimulated group.

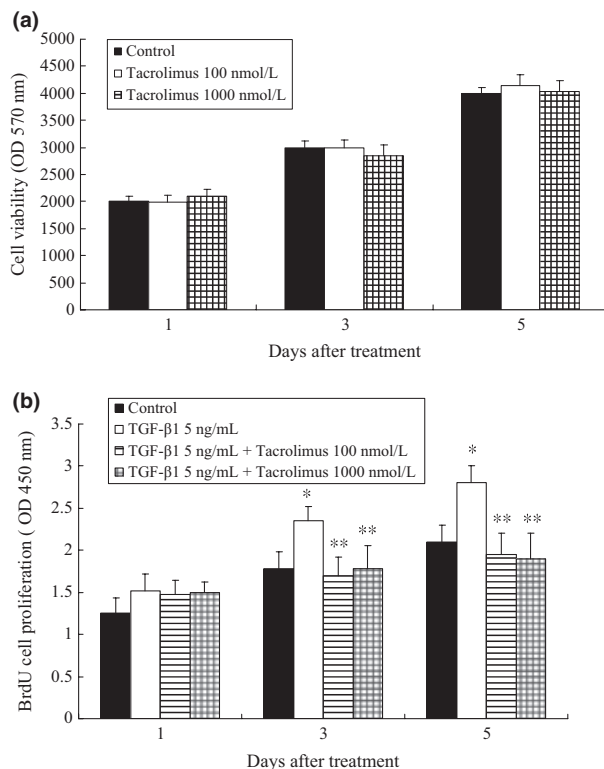


Figure 2 Tacrolimus did not affect the viability of fibroblasts but significantly reduced the proliferation increased by TGF-β1. (a) The viability of cultured fibroblasts treated with tacrolimus 0, 100 and 1000 nmol/L for 1, 3 and 5 days was assessed by MTT assay. (b) Cultured fibroblasts were treated with TGF-β1 5 ng/mL with or without tacrolimus 100 and 1000 nmol/L and then incubated at indicated time points for determination of cell proliferation. The proliferation of fibroblasts at 1, 3 and 5 days after treatments was determined using a 5-bromo-2'-deoxyuridine-enzyme-linked immunosorbent cell proliferation assay. The data shown here (mean ± SD) were from one representative experiment performed in triplicate, repeated three times with similar results. * $P < 0.05$ as compared with control group; ** $P < 0.05$ as compared with TGF-β1-treated group.

pathways activated by TGF-β1 play a role in TGF-β-stimulated type I collagen expression in certain cells.^{33–35} To clarify how tacrolimus reduced TGF-β1-induced type I collagen synthesis in fibroblast in the context of MAPK pathways, specific pharmacologic inhibitors of ERK1/2, JNK and p38MAPK were used. The results shown in Fig. 5b demonstrated that the inhibitors of ERK1/2 and JNK did not affect the enhancing effect of TGF-β1 on type I collagen expression. However, p38MAPK inhibitor SB203580 significantly decreased the collagen I α1 expression induced by TGF-β1. These results supported the notion that inhibition of p38MAPK pathway by tacrolimus is involved in reducing the increased type I collagen expression stimulated by TGF-β1.

Discussion

Atopic dermatitis (AD) is a chronic relapsing skin disease characterized by inflammation and lichenification. Similar to tissue remodelling seen in asthmatic airways, chronic lichenified skin lesions in human AD have often undergone tissue remodelling due to chronic inflammation.³⁶ Lichenified skin lesions characterized by dermal fibrosis is the hallmark of chronic AD.³⁷ Several studies have documented elevation of TGF-β1 expression in chronic AD skin lesions.^{5,38} TGF-β1 is well recognized as the major inducer for tissue fibrosis due to its stimulatory effect on ECM production and inhibitory effect on matrix proteins degradation.³⁹ Tacrolimus is known for its immune suppressive effects and has been used extensively in patients with organ transplant. In addition to immune suppression, tacrolimus has also been associated with less renal interstitial fibrosis as compared with other immune suppressant.^{40,41} These evidences suggested that tacrolimus exhibits an anti-fibrotic effect. Therefore, this study was designed to explore the effects of tacrolimus on TGF-β1-stimulated cultured normal human fibroblasts in terms of proliferation and collagen production, the two hallmarks of skin lichenification.

A large body of evidences supported the role of excess TGF-β1 during the pathogenesis of tissue fibrosis.^{42–44} TGF-β exerts its function by binding to TGF-βRI and TGF-βRII. Moreover, TGF-β1 regulates TGF-βRI and TGF-βRII expressions in an autocrine positive-feedback fashion.^{45–47} In agreement with previous studies demonstrating that TGF-β1 stimulation enhanced the expression of TGF-β receptors in certain types of cells including human lung fibroblasts,^{48,49} our results also showed an increased expression of TGF-β receptor I and II in cultured fibroblasts after TGF-β1 treatment. Addition of tacrolimus markedly inhibited the TGF-β1 enhanced TGF-βRI and TGF-βRII expressions in cultured fibroblasts. Therefore, tacrolimus treatment was able to abrogate the autocrine positive loop between binding of TGF-β1 with its cognate receptors via suppression of TGF-β receptor expressions.

As an important fibrogenic mediator, TGF-β1 is reported to induce proliferation in dermal fibroblasts via binding to its cognate receptors, followed by activating the downstream Smad pathway.⁵⁰ Our results demonstrated that tacrolimus concentration at 100 and 1000 nmol/L did not influence the fibroblast viability up to 5 days. However, 100 and 1000 nmol/L tacrolimus significantly suppressed the enhancing effect of TGF-β1 on fibroblast proliferation. These results indicated that tacrolimus reduced fibroblast proliferation only in presence of excessive TGF-β1 and the decreased expressions of TGF-βRI and TGF-βRII by tacrolimus treatment may contribute to this phenomenon.

Excess deposition of ECMs, especially collagens, in various tissues is the key characteristic of fibrosis. Therefore, both decreased degradation and increased formation of ECMs may

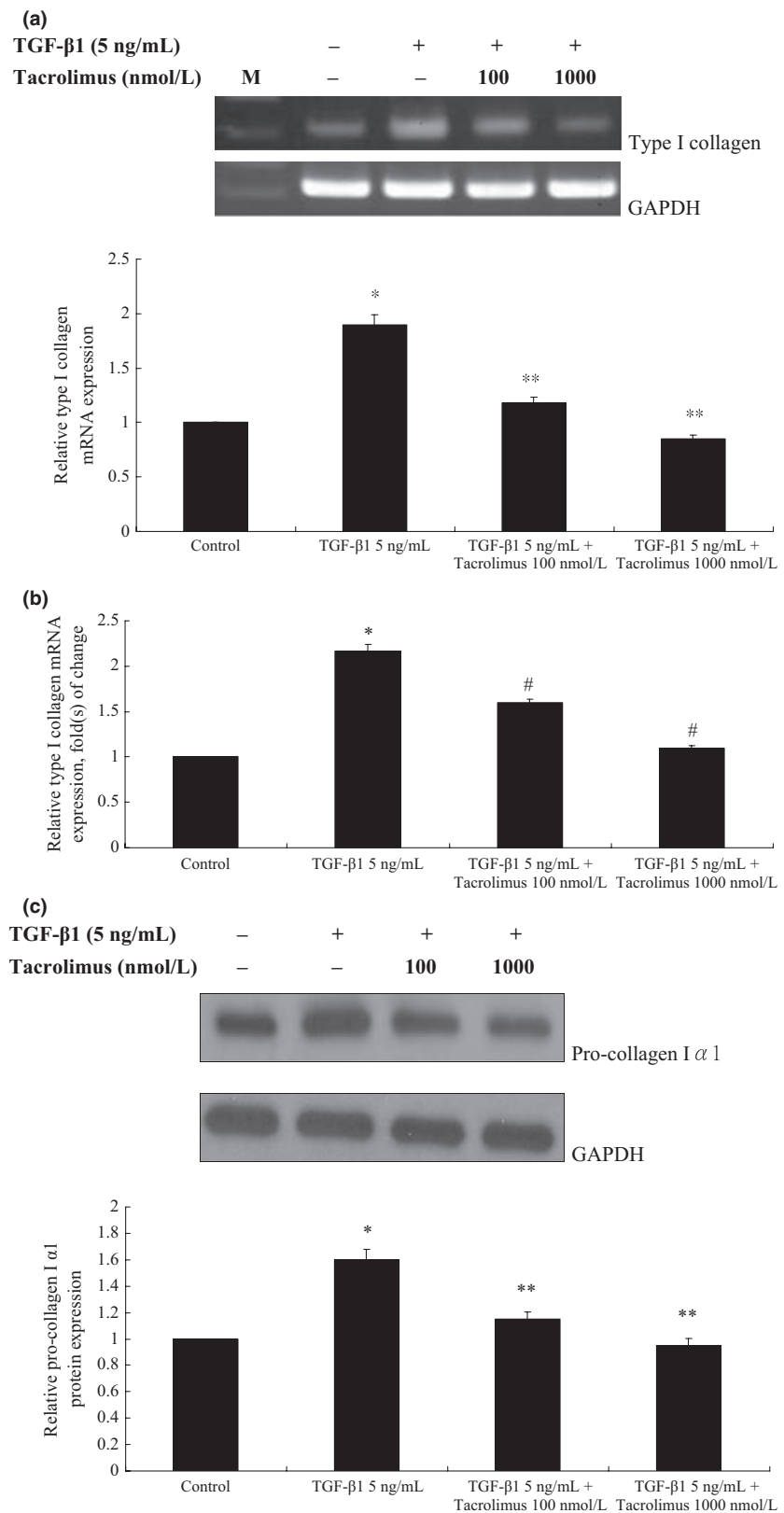


Figure 3 Tacrolimus significantly inhibited the increased type I collagen synthesis by TGF- β 1. Cultured fibroblasts were treated with TGF- β 1 5 ng/mL with or without addition of tacrolimus 100 and 1000 nmol/L for 24 h (for mRNA detection) or 48 h (for protein detection). Total mRNA and cellular protein lysates were extracted and subjected to RT-PCR/quantitative real-time PCR (a/b) or Western blotting analysis (c) respectively. The bars indicated mean $\pm$ SD. Experiments were repeated trice with similar results. The graphs shown here were the mean densitometric data showing the ratios of type I collagen mRNA or pro-collagen I α 1 proteins to GAPDH expressions of different treatment groups normalized to the control group respectively. * P < 0.05 as compared with control group; ** P < 0.05 as compared with TGF- β 1-stimulated group. The 'M' shown in graph (a) stood for 'Marker'.

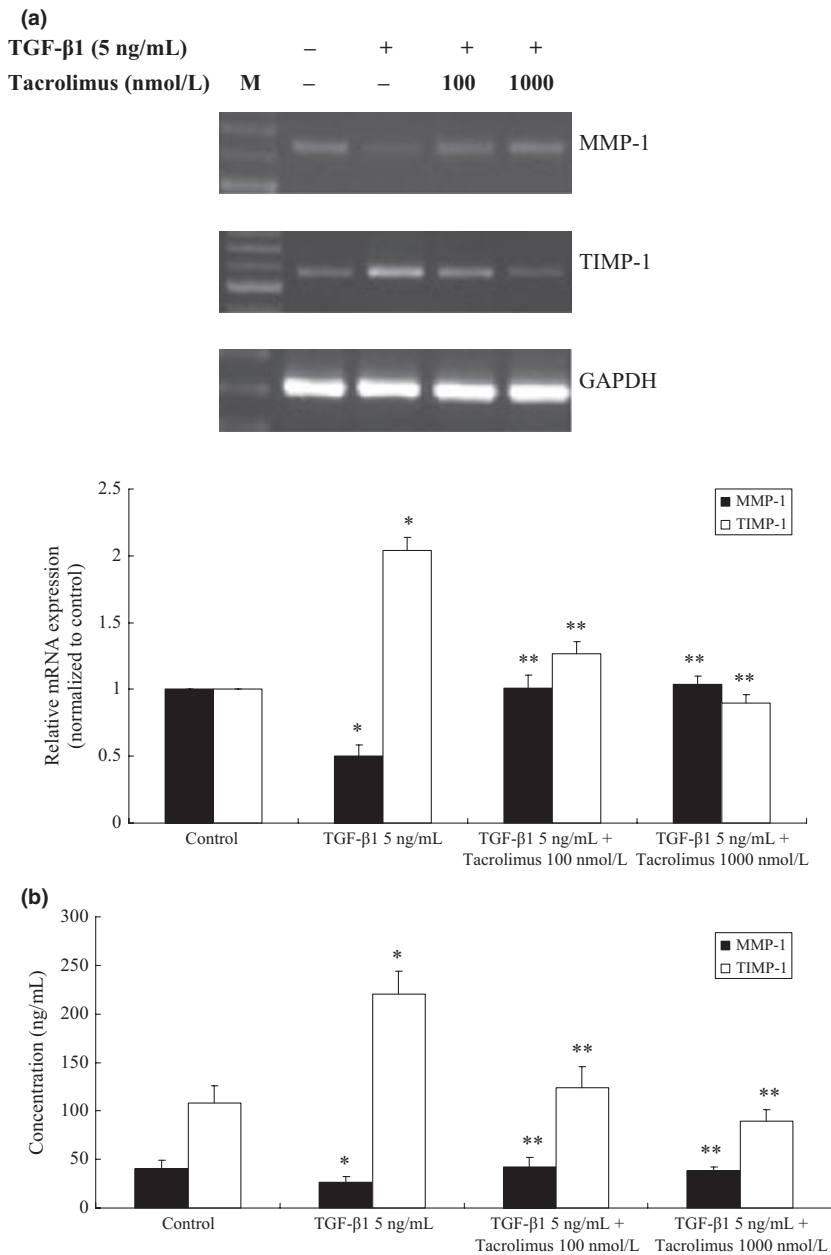
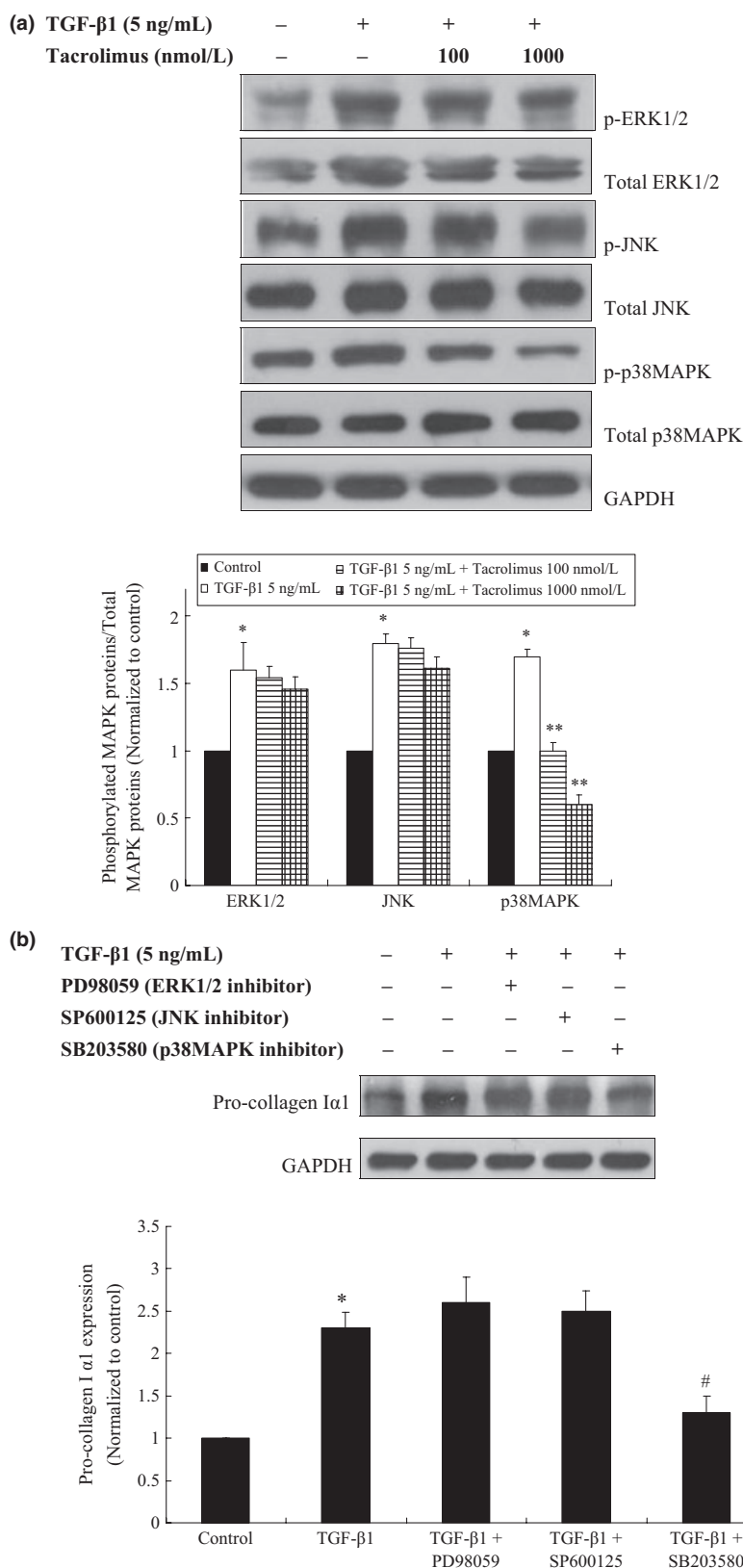


Figure 4 The decreased MMP-1 and increased TIMP-1 mRNA expressions induced by TGF- β 1 were markedly reversed by tacrolimus. Fibroblasts were cultured and treated with TGF- β 1 5 ng/mL followed by addition of tacrolimus 0, 100 and 1000 nmol/L. (a) 24 h after treatment, the total mRNA of treated fibroblasts was isolated and the levels of MMP-1 and TIMP-1 mRNA were determined by RT-PCR. (b) MMP-1 and TIMP-1 protein concentrations in supernatants after 48 h of treatment were evaluated by ELISA. The data shown here (mean $\pm$ SD) were repeated three times with similar results. Densitometric analysis of (a) was derived from the ratios of MMP-1 and TIMP-1 to GAPDH expressions of different treatment groups normalized to the control group respectively. * $P < 0.05$ as compared with control group; ** $P < 0.05$ as compared with TGF- β 1-treated group. The 'M' shown in graph (a) stood for 'Marker'.

lead to tissue fibrosis. The imbalance between MMPs and their specific inhibitors TIMPs has been implicated in pathogenesis of many fibrotic diseases.^{51–53} More specifically, several studies have demonstrated that decreased MMP-1 expression and increased TIMP-1 expression resulted in ECMs accumulation and facilitate the development of tissue fibrosis.^{52,54,55} It is well established that TGF- β 1 is a major inducer of tissue fibrosis at various sites throughout the body.⁴² Previously, TGF- β 1 was reported to up-regulate production of type I collagen and TIMP-1 expression, while down-regulating MMP-1 gene tran-

scription.^{56–59} Consistent with these previous results, our present findings showed that TGF- β 1 down-regulated MMP-1 mRNA and up-regulated TIMP-1 mRNA expressions in fibroblasts. Tacrolimus treatment significantly increased MMP-1 and decreased TIMP-1 activities after TGF- β 1 stimulation. The ratio of increased TIMP-1 and decreased MMP-1 is often observed in fibrotic diseases including scleroderma and keloids.⁵³ Moreover, this study demonstrated that the TGF- β 1-induced type I collagen synthesis was abrogated by tacrolimus. Therefore, it was reasonable to propose that tacrolimus may reduce the dermal

Figure 5 Tacrolimus abrogated TGF- β 1-induced type I collagen expression in fibroblasts via inhibition of phosphorylated p38MAPK signalling. (a) Culture fibroblasts were stimulated with TGF- β 1 5 ng/mL followed by addition of tacrolimus 0, 100 and 1000 nmol/L and incubated for 30 min. The levels of phosphorylated ERK1/2, phosphorylated JNK and phosphorylated p38MAPK were determined by Western blot using phosphospecific antibodies. The levels of ERK1/2, JNK and p38MAPK were determined by Western blot using ERK1/2, JNK and p38MAPK specific antibodies. The expressions of each phosphorylated MAPK proteins and each total MAPK proteins were first normalized to GAPDH and then the ratios of phosphorylated MAPK proteins to their cognate total MAPK proteins of different treatment groups were normalized to the control group. (b) The cultured fibroblasts were pre-treated with PD98059 (ERK1/2 inhibitor), SP600125 (JNK inhibitor) and SB203580 (p38MAPK inhibitor) for 60 min and then stimulated with TGF- β 1 5 ng/mL. Forty-eight h after treatment, the proteins of treated cells were extracted and subjected to Western blotting analysis for detection of type I collagen expression using pro-collagen I α 1 specific antibody. The results were expressed as mean $\pm$ SD. The immunoblots shown in (a) and (b) were representative of two independent experiments with similar data. * P < 0.05 as compared with control group; ** P < 0.05 as compared with TGF- β 1-treated group.



fibrosis induced by TGF- β 1 through up-regulation of MMP-1 and down-regulation TIMP-1.

Asano *et al.*⁶⁰ reported that tacrolimus showed an inhibitory effect on TGF- β 1-induced type I collagen production in normal fibroblasts. In line with these results, we also found that the TGF- β 1-induced type I collagen in normal fibroblasts was significantly suppressed by tacrolimus. To clarify whether the effect of tacrolimus on the TGF- β 1-induced type I collagen expression was caused by inhibition of TGF- β 1 signalling or simple reduction of type I collagen basal expressions, the influence of various concentrations of tacrolimus on type I collagen synthesis was performed in our study. The results revealed that tacrolimus treatment alone did not significantly affect the production of type I collagen (Fig. S1, supplementary data). These results indicated that the suppressive effect of tacrolimus on TGF- β 1-induced type collagen expressions was dependent on TGF- β 1 signalling pathway. Subsequently, the molecular signalling involved was investigated. MAPKs have been reported to play important roles in signal transduction downstream of TGF- β 1.^{61,62} MAPKs comprise at least three protein kinases including extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) in mammalian cells. Sato *et al.*³² demonstrated that activation of p38MAPK is critical for type I collagen production by TGF- β 1 in dermal fibroblasts. These results suggested that p38MAPK pathway played an important role on TGF- β 1-induced type I collagen production in fibroblasts. As demonstrated in our results, the increased level of phosphorylated p38MAPK by TGF- β 1 treatment was significantly abrogated by tacrolimus in fibroblasts. In addition, we found that the enhanced expression of type I collagen induced by TGF- β 1 was significantly reduced by pre-treatment with p38MAPK inhibitor. These results indicated that the inhibitory effect of tacrolimus on phosphorylated p38MAPK expression induced by TGF- β 1 contributed to decreased collagen production in dermal fibroblasts.

Current treatments for AD focused on acute lesions and emphasized on suppression of inflammation.⁶³ Little attention is paid to the management of dermal fibrosis in chronic AD lesions. Although lichenification, resulting from dermal fibrosis in chronic AD, is not life threatening, it can have significant negative impact on life quality and attract unwanted attention if lesions are located in exposed areas. Our current results provided evidences suggesting that tacrolimus may ameliorate dermal fibrosis in chronic AD via inhibiting fibrotic responses in TGF- β 1-stimulated dermal fibroblasts. Recently, proactive tacrolimus ointment application has been proposed to effectively prevent, delay and reduce the occurrence of AD exacerbation.^{64,65} We propose that proactive therapy using tacrolimus may not only reduce disease recurrence but also improve dermal fibrosis frequently observed in chronic AD. Further clinical validation of this hypothesis is warranted.

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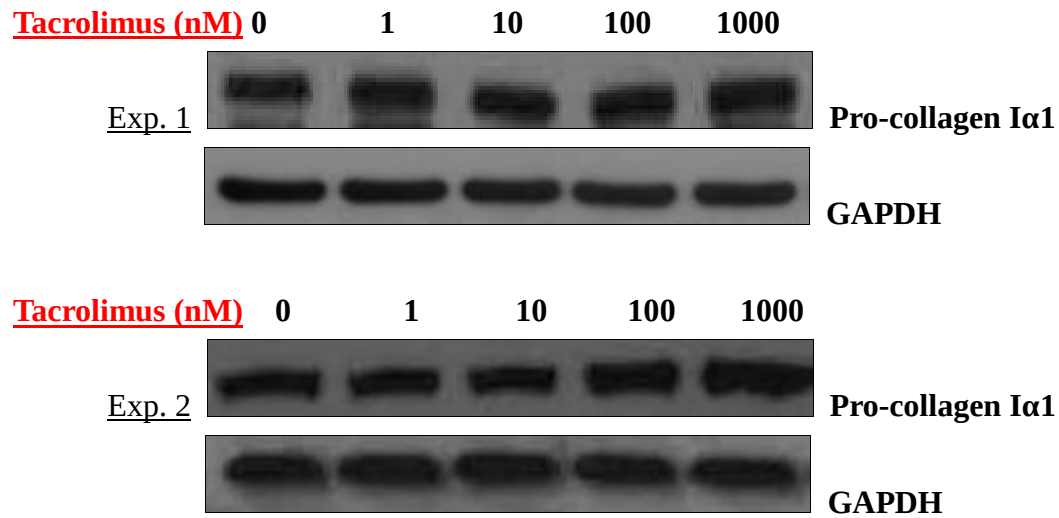
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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cultured fibroblasts were treated with tacrolimus 0, 1, 10, 100 and 1000 nmol/L and incubated for 48 h. Total cellular protein lysates were extracted and subjected to Western blotting analysis. GAPDH expression was served as internal control. The results showed that various concentrations of tacrolimus treatment did not significantly affect type I collagen expression in normal human fibroblasts. The plots shown here were from two different experiments with similar results.

Supplementary data



Legend for figure:

Cultured fibroblasts were treated with tacrolimus 0, 1, 10, 100, and 1000 nM and incubated for 48 hrs. Total cellular protein lysates were extracted and subjected to Western blotting analysis. GAPDH expression was served as internal control. The results showed that various concentrations of tacrolimus treatment did not significantly affect type I collagen expression in normal human fibroblasts. The plots shown here were from two different experiments with similar results.