Tissue Inhibitor of Metalloproteinase-2 Suppresses Collagen Synthesis in Cultured Keloid Fibroblasts

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Background: Keloids are defined as a kind of dermal fibroproliferative disorder resulting from the accumulation of collagen. In the remodeling of extracellular matrix, the balance between matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) is as critical as the proper production of extracellular matrix. We investigate the role of TIMPs and MMPs in the pathogenesis of keloids and examine the therapeutic potential of TIMP-2.
Methods: The expression of TIMPs and MMPs in most inflamed parts of cultured keloid fibroblasts (KFs) and peripheral normal skin fibroblasts (PNFs) in the same individuals and the reactivity of KFs to cyclic mechanical stretch were analyzed by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay (n = 7). To evaluate the effect of treating KFs with TIMP-2, collagen synthesis was investigated by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay, and microscopic analysis was used to examine the treatment effects of TIMP-2 on ex vivo cultures of keloid tissue (n = 6).
Results: TIMP-2 was downregulated in cultured KFs compared with PNFs in the same individuals, and the reduction in TIMP-2 was exacerbated by cyclic mechanical stretch. Administration of TIMP-2 (200 or 300 ng/mL) significantly suppressed expression of Col1A2 and Col3A1 mRNA and collagen type I protein in KFs. TIMP-2 also significantly reduced the skin dermal and collagen bundle thickness in ex vivo cultures of keloid tissue.
Conclusion: These results indicated that downregulation of TIMP-2 in KFs is a crucial event in the pathogenesis of keloids, and the TIMP-2 would be a promising candidate for the treatment of keloids. *(Plast Reconstr Surg Glob Open 2015;3:e520; doi: 10.1097/GOX.0000000000000503; Published online 22 September 2015.)*

DOI: 10.1097/GOX.0000000000000503

Keloids are defined as a kind of dermal fibroproliferative disorder resulting from the accumulation of collagen. The progression and development of keloids, which are characterized by extending beyond the original skin injury, is thought to relate closely to abnormal wound healing, genetic factors, and a local microenvironment influenced by cytokines, growth factors, and mechanical forces.[1–5] Histologically, keloids are characterized by excessive

> **Disclosure:** *The authors have no financial interest to declare in relation to the content of this article. The Article Processing Charge was paid for by the authors.*

accumulation of thick, hyalinized collagen bundles within the reticular layer of the dermis and are the products of an excessive synthesis and an imbalance between the deposition and degradation of extracellular matrix (ECM).[1,6]

Fibroblasts are the primary type of mechanoresponsive cells and are highly heterogeneous.[7] Fibroblasts play pivotal roles in both tissue remodeling and wound healing, and it is their abnormal behavior that results in keloid formation. During ECM remodeling (eg, during wound healing), the balance between matrix metalloproteinases (MMPs) and their inhibitor, the tissue inhibitors of metalloproteinases (TIMPs), is as critical as the proper production of ECM,[8] which is regulated by several cytokines and growth factors including transforming growth factor β-1 (TGFβ-1).[9] TIMPs and MMPs are tightly regulated during normal wound healing, and their imbalance has been implicated in pathologic fibrosing diseases of the skin, including hypertrophic scars and keloids, and scleroderma.[10]

Discovered in 1989,[11] TIMP-2 is a unique member of the TIMP protein family, in that, it has multiple effects on cell growth, apoptosis, and differentiation in addition to its activity inhibiting MMPs including membrane type 1 (MT1)-MMP.[12,13] In addition, TIMP-2 plays a dual role in the regulation of MMP-2 activation: at low concentrations, it bridges the interaction between adjacent TIMP-2–free MT1-MMP and proMMP-2, leading to activation of proMMP-2, whereas at higher concentrations, it completely inhibits this reaction.[14,15] Recently, we have reported that collagen production and the expression levels of 2 collagen types (COL1A2 and COL3A1) are significantly increased in TIMP-2 knockdown keloid fibroblasts (KFs).[16]

From the evidence currently available, the level of TIMP-2 within keloids is uncertain. It has been reported, for example, that the relative levels of TIMP-2 mRNA expression are significantly higher in both hypertrophic scars and KFs than in normotrophic scars.[6,17,18] Other studies, however, have shown that the relative levels of TIMP-2 mRNA expression are significantly lower in keloids than in normal skin.[19] Moreover, as far as we know, no study has focused on the changes of TIMP-2 expression between KFs and normal skin in the same individuals. In addition, the influence of KFs on TIMP-2 expression stimulated by mechanical stretch remains unclear.[20–22] To determine the role of TIMP-2 in keloids, we investigated the expression profiles of TIMP-2 in most inflamed parts of cultured KFs and peripheral normal skin fibroblasts (PNFs) in the same individuals and explored the effect of cyclic mechanical stretch on TIMP-2 expression in KFs. Finally, we examined the

therapeutic potential of TIMP-2 using in vitro and ex vivo cultures of keloid tissue.

MATERIALS AND METHODS

Cell Origin and Culture

All keloid samples showed the typical behavior with invasion into the boundary of the original lesion caused by minor injury, which had been present for more than 3 years. In this study, discarded keloid samples and discarded adjacent strained healthy skin (so called "dog ear") from the sternum of 13 patients (7 males and 6 females, age 22–69 years) were collected.[23] "Healthy" skin present at the site that is more than 5 mm of clinical keloid margin was used.[24] Informed consent was obtained from each patient in accordance with the Declaration of Helsinki. Primary KF and PNF cultures were established as previously described.[16,25] Cells at passage 2 were used in the experiments of comparison between KFs and PNFs and cyclic mechanical stretch. Cells at passages 1–3 were used in the experiments with TIMP-2 treatments. The culture medium was Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Gibco-BRL), 1% antibiotic–antimycotic (Wako, Tokyo, Japan), and 50 μg/mL ascorbic acid 2-phosphate (Wako).[16,26]

Application of Mechanical Stretch

An STB-140 stretching instrument (Strex Ltd., Osaka, Japan) was used to apply cyclic mechanical stretch to KFs and PNFs. Stretching was performed as described previously.[21] In brief, to generate the stretch group, KFs were allowed to attach for 48 hours to the bottom of silicon chambers in 2 mL of culture medium. After the medium was changed, continuous uniaxial sinusoidal stretch (20%, 1 cycle/100 s) was applied at 37°C, 5% CO_2 for 72 hours. In the control group, the culture medium was changed, and the silicon chambers were incubated for 72 hours as described above but without stretching.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using RNeasy Mini Kits (Qiagen, Valencia, Calif.) according to the manufacturer's recommendations, after which cDNA was synthesized using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, South San Francisco, Calif.). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using an ABI Prism 7500 System (Applied Biosystems, Foster City, Calif.) with RT2 SYBR Green/ROX PCR master mix (SA Biosciences, Frederick, Md.) as described previously.[16] The PCR protocol entailed 10 seconds of de-

naturation at 95°C followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds. For each primer set, the optimal dilution was determined, and melting curves were used to determine the amplification specificity. glyceraldehyde 3-phosphate dehydrogenase (GAPHD) served as the internal control. The primer pairs used in this study are shown in Table 1. Relative changes in the levels of genes of interest were determined using the ΔΔCt method with correction for different amplification efficiencies.

Enzyme-Linked Immunosorbent Assay

To analyze the TIMP-2 secretion from KFs and PNFs, supernatant levels of TIMP-2 were assayed using a commercially available enzyme-linked immunosorbent assay (ELISA, DTM200, R&D Systems, Minneapolis, Minn.). KFs and PNFs were seeded into silicon chambers and grown to a density of 1.5×10^5 cells per chamber. Then the culture medium was changed, and the silicon chambers were incubated for 72 hours. To evaluate the effect of the treatment with TIMP-2, procollagen type I C-terminal peptide (PIP; MK101, TaKaRa, Shiga, Japan), TIMP-1, and MMP-1 (DTM100 and DMP100, R&D Systems) were assayed using commercially available ELISA. KFs were seeded into 6-well dishes and grown to a density of 1.5×10^5 cells per well. The medium was then changed to serum-free DMEM containing 50 μg/mL L-ascorbic acid with or without 100, 200, or 300 ng/mL active human TIMP-2 (ab82117, Abcam, Cambridge, Mass.). After 48 hours, the conditioned media were used in subsequent assays.

MTS Assay

To assess cell viability at 48 hours after TIMP-2 treatment, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) tests were performed using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, Wis.) according to the manufacturer's instructions. KFs were seeded into 96-well plates at a density of 1×10^4 cells per well and cultured in 100 μL DMEM supplemented with 10% fetal bovine serum. After starving in serum-free DMEM over-

night, KFs were incubated for 48 hours in the medium with or without 100, 200, or 300 ng/mL active human TIMP-2. Thereafter, 20 μL of MTS reagent was added and incubated for 3 hours, after which the Optical density $(OD)_{490}$ was recorded using a microtiter plate reader (Dynatech Laboratories Inc., Chantilly, Va.). Each assay was performed in quadruplicate.

Ex Vivo Culture of Keloid Tissues and Histopathology

Keloid tissues were cultured ex vivo as described previously.[16,27,28] Briefly, samples of keloid tissue were cut into 10×10 mm sections, after which control phosphate-buffered saline (PBS) or 200 ng/mL active TIMP-2 was injected intradermally in a 200 μL volume. The keloid tissue specimens were then incubated for 7 days before being fixed in 4% paraformaldehyde and stained with hematoxylin and eosin using standard protocols.

Measurement of the Thickness of Skin Dermis and Collagen Bundles

Skin dermal thickness and collagen bundle thickness were measured in skin sections stained with hematoxylin and eosin. The thickness of the dermis and individual collagen bundles was measured using Lumina vision software (Mitani, Fukui, Japan) as described previously.[27,28]

Immunohistochemistry

To detect expression of α smooth muscle actin (α SMA), immunohistochemical staining was performed using a Histofine SAB-PO(R) kit (Nichirei Biosciences Inc., Tokyo, Japan) in accordance with the manufacturer's procedure. The skin sections were immersed in 0.3% hydrogen peroxide for 15 minutes to inhibit endogenous peroxidase activity. After blocking with 10% goat normal serum, the sections were incubated with 1:200 rabbit polyclonal anti-human αSMA antibody (ab5694, Abcam) and preimmune rabbit immunoglobulin G for 1 hour at 37°C. After washing with PBS, the sections were incubated with biotin-conjugated anti-rabbit immunoglobulin G for 15 minutes at room temperature.

Table 1. Sequences of the Primers Used for Quantitative Real-Time Polymerase Chain Reaction

Primer	Forward	Reverse
GAPDH	5′-TGCACCACCAACTGCTTAG-3′	5′-GTTCAGCTCAGGGATGACC-3′
TIMP-1	5′-ACTTCCACAGGTCCCACAAC-3′	5′-GCATTCCTCACAGCCAACAG-3′
TIMP-2	5′-GAAGCATTTGACCCAGAGTG-3′	5′-CCTTTCAGACCGAACCTACT-3′
MMP-1	5′-GGCTGAAAGTGACTGGGAAACC-3′	5′-TGCTCTTGGCAAATCTGGCGTG-3′
MMP-2	5′-GACATACATCTTTGCTGGAGAC-3′	5′-TTCAGGTAATAGGCACCCTT-3′
MT1-MMP	5′-CCTTGGACTGTCAGGAATGAGG-3′	5′-TTCTCCGTGTCCATCCACTGGT-3′
Col1A2	5′-GGTGGTGGTTATGACTTTGG-3′	5′-GTTCTTGGCTGGGATGTTTT-3′
Col3A1	5′-GCTCTGCTTCATCCCACTATTA-3′	5′-TGCGAGTCCTCCTACTGCTAC-3′
αSMA	5′-CCG ACC GAA TGC AGA AGG A-3′	5′-ACA GAG TAT TTG CGC TCC GAA-3′

TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase.

After washing with PBS, they were incubated with peroxidase-conjugated streptavidin for 10 minutes at 37°C. The immunoreactions were visualized using a histofine DAB substrate kit (brown; Nichirei Biosciences Inc.) and counterstained with hematoxylin.

Statistical Analysis

All data are presented as mean ± standard deviations. Statistical analysis was performed using Microsoft Excel 2011 (Microsoft, Tokyo, Japan) and SPSS statistical software (SPSS, Chicago, Ill.). Comparisons between 2 groups were made using the Wilcoxon t test. To evaluate the effect of TIMP-2, comparisons of more than 2 groups were made using Friedman's χ^2 test, followed by application of the Wilcoxon t test with Bonferroni correction for comparison with the control group. Values of $P < 0.05$ were considered significant.

RESULTS

Downregulation of TIMP-2 and MMP-1 Expression in KFs and Decreased Production of TIMP-2 in KFs

To analyze the difference in TIMP and MMP expression between KFs and PNFs, RT-PCR was conducted to assess expression of TIMP-2, TIMP-1, MMP-2, MT1-MMP, and MMP-1 mRNA. Levels of TIMP-2 and MMP-1 mRNA were significantly lower in KFs than in PNFs ($P < 0.03$), whereas those of TIMP-1, MMP-2, and MT1-MMP mRNA did not significantly differ between KFs and PNFs (Fig. 1). To confirm the downregulation of TIMP-2 expression, we also analyzed TIMP-2 production in KFs and PNFs using an ELISA. TIMP-2 protein levels were
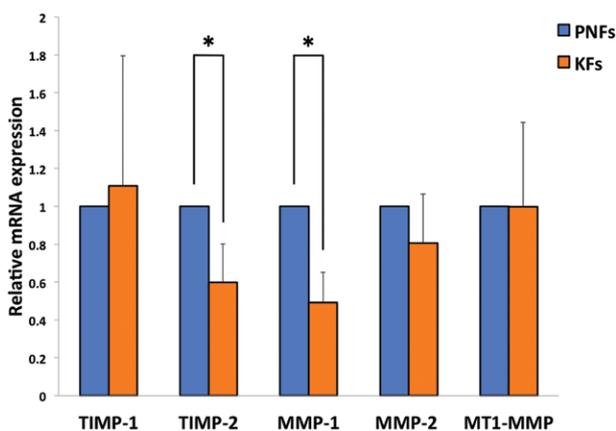

Fig. 2. Tissue inhibitor of metalloproteinase-2 (TIMP-2) protein in culture supernatants. Expression levels of TIMP-2 protein in culture supernatants conditioned by keloid fibroblasts (KFs) and peripheral normal skin fibroblasts (PNFs) were measured using an enzyme-linked immunosorbent assay (ELISA, n = 7). *$P < 0.03$.

significantly lower in the cultured supernatants of KFs (41.3 ± 13.3 ng/mL) than in the culture supernatants of PNFs (54.3 ± 12.5 ng/mL; $P < 0.03$; Fig. 2).

Downregulation of TIMP-2 and Upregulation of MMP-1 by Cyclic Mechanical Stretch in KFs

To further understand how TIMP-2 expression was changed by cyclic mechanical stretch in KFs, the effects of stretching on expression of TIMPs and MMPs were examined. After 72 hours of cyclic mechanical stretch, expression of TIMP-2 mRNA was significantly

Fig. 1. Expression of tissue inhibitor of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) in keloid fibroblasts (KFs) and peripheral normal skin fibroblasts (PNFs). Expression levels of TIMP-2, TIMP-1, MMP-2, MT1-MMP, and MMP-1 mRNA were determined using quantitative real-time polymerase chain reaction (qRT-PCR, n = 7). The normalized level in the control group (PNFs) was assigned a value of 1, after which levels in the keloid group (KFs) are expressed relative to control. *$P < 0.03$.
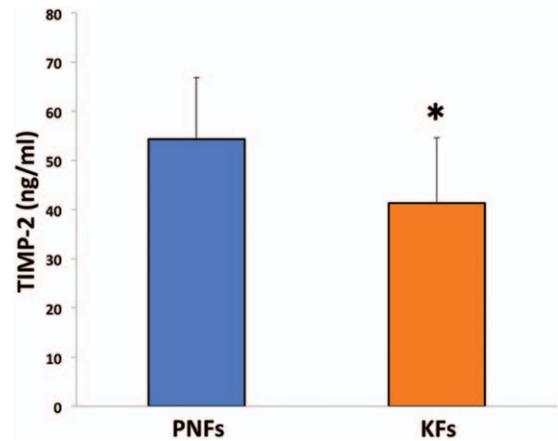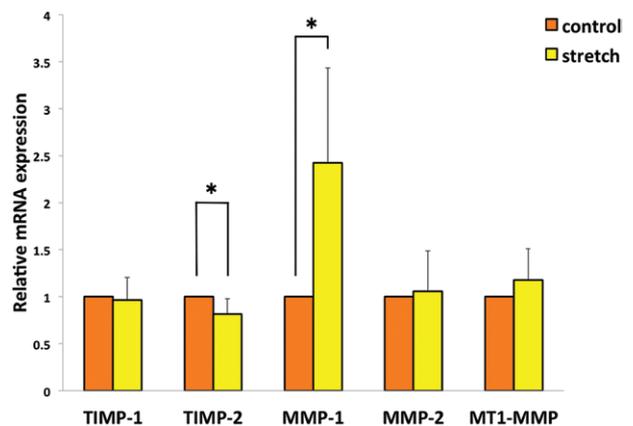

Fig. 3. The effect of cyclical uniaxial mechanical stretch on expression of tissue inhibitor of metalloproteinase-2 (TIMP-2), TIMP-1, matrix metalloproteinase-2 (MMP-2), MT1-MMP, and MMP-1 mRNA in keloid fibroblasts (KFs, n = 7). KFs were allowed to attach for 48 hours to the bottom of silicon chambers in 2 mL of culture medium. After the medium was changed, continuous uniaxial sinusoidal stretch (20%, 1 cycle/100 s) was applied at 37°C, 5% CO_2 for 72 hours. In the control group, the culture medium was changed, and the silicon chambers were incubated for 72 hours as described above but without stretching. *$P < 0.03$.
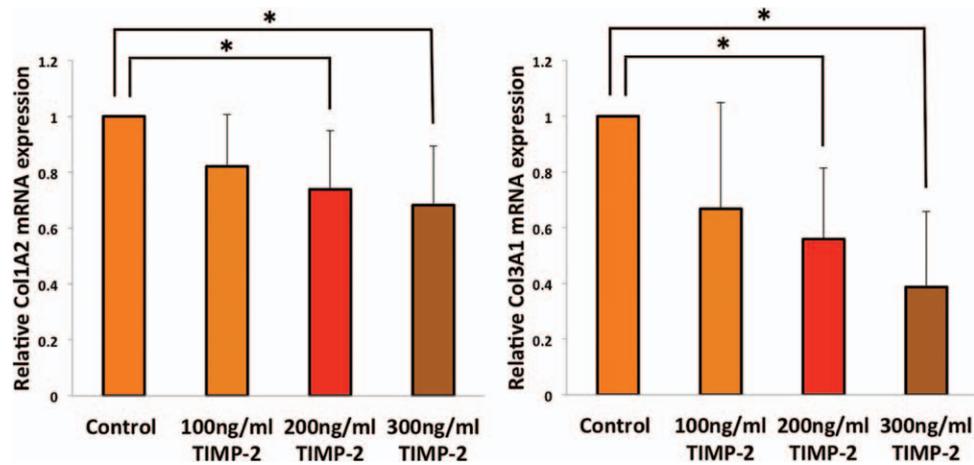
Fig. 4. The effects of tissue inhibitor of metalloproteinase-2 (TIMP-2) on collagen expression. Keloid fibroblasts (KFs) were exposed to the indicated concentration of TIMP-2 for 48 hours. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of Col1A2 and Col3A1 mRNA expression (n = 6). *$P < 0.05$.

reduced ($P < 0.03$), whereas expression of MMP-1 mRNA was significantly increased ($P < 0.03$). In contrast, levels of TIMP-1, MMP-2, and MT1-MMP mRNA expression were not affected significantly (Fig. 3). Thus, production of TIMP-2 was downregulated in KFs, and the reduction in TIMP-2 was exacerbated by cyclic mechanical stretch. And recently, we have reported that TIMP-2 knockdown leads to increase in collagen production and expression of Col1A2 and Col3A1.[16] Therefore, we also examine the therapeutic potential of recombinant TIMP-2 using in vitro and ex vivo cultures of keloid tissue.

Downregulation of Col1A2 and Col3A1 after Treatment of KFs with TIMP-2

Serum TIMP-2 levels are tightly regulated,[29] as low concentration of TIMP-2 (100 ng/mL) greatly increase
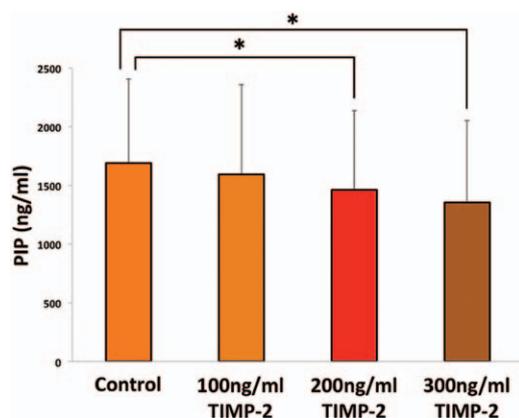

Fig. 5. The effects of tissue inhibitor of metalloproteinase-2 (TIMP-2) on collagen production. Levels of collagen type I protein (procollagen type I C-peptide; PIP) in culture supernatants conditioned by keloid fibroblasts (KFs) were measured using an enzyme-linked immunosorbent assay (ELISA, n=6). *$P < 0.05$.

MMP-2 activity in human dermal fibroblasts, whereas high concentrations of TIMP-2 ($\geq$ 400 ng/mL) inhibit the gelatinase activity of MMP-2.[12,30] Moreover, the concentrations of TIMP-2 in serum and tissues are in the 10–220 ng/mL range.[29,31–33] Therefore, the effects of 100, 200, and 300 ng/mL recombinant active human TIMP-2 on expression of Col1A2 and Col3A1 mRNA were examined. Expression of both Col1A2 and Col3A1 mRNA significantly reduced in KFs exposed to 200 or 300 ng/mL TIMP-2 ($P < 0.05$), whereas 100 ng/mL TIMP-2 had no significant effect on Col1A2 or Col3A1 expression (Fig. 4).

Decreased Production of Procollagen Type I C-Peptide in KFs Treated with TIMP-2

PIP levels reflect the total production of type I collagen. As a result of a specific ELISA, PIP levels were significantly lower in culture supernatants conditioned by KFs treated with 200 or 300 ng/mL TIMP-2 (1461.8 ± 674.1 and 1354.3 ± 696.3 ng/mL, respectively) than in supernatants from untreated control group (1690.2 ± 717.4 ng/mL; $P < 0.05$). In contrast, PIP levels in the supernatants from cells treated with 100 ng/mL TIMP-2 did not differ from that measured in the control group (1593.7 ± 764.8 ng/mL; Fig. 5).

No Significant Effect of TIMP-2 on Cell Viability

The effects of TIMP-2 on cell growth and viability remain controversial.[34] To analyze whether suppression of collagen is depend on cell viability, MTS assays were performed to examine the viability of KFs treated with or without TIMP-2. TIMP-2 (100, 200, and 300 ng/mL) had no significant effect on cell viability (Fig. 6). This means the fact that the observed TIMP-2–induced reduction in PIP production did not reflect a decrease in cell viability after treatment.
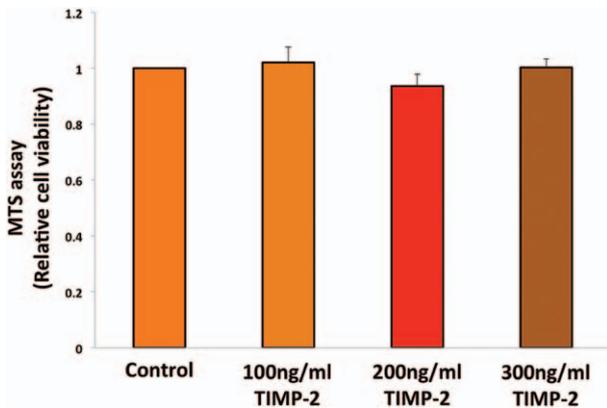
Fig. 6. The effects of tissue inhibitor of metalloproteinase-2 (TIMP-2) on cell viability. MTS assays of keloid fibroblast (KF) viability after exposure for 48 hours to the indicated concentration of TIMP-2 (n = 3).

TIMP-2–Induced Downregulation of αSMA in KFs

Because αSMA is the main activation marker of fibroblasts[35] and the primary indicator of keloid formation induced by TGFβ-1,[36,37] the effects of 200 ng/mL TIMP-2 on expression of αSMA were investigated. The concentration condition was chosen because it significantly reduced collagen expression and production, and moreover, the concentrations of TIMP-2 in serum and tissues are within 220 ng/mL.[28,31–33] Although levels of αSMA mRNA were significantly higher in KFs than in PNFs ($P < 0.03$), treatment of KFs with 200 ng/mL TIMP-2 significantly reduced expression of αSMA mRNA ($P < 0.03$) when compared with control (Fig. 7).

Upregulation of MMP-1/TIMP-1 Ratios in KFs Treated with TIMP-2

Because MMP-1/TIMP-1 ratios reflect the imbalance between collagen deposition and degradation,[38] the effects of TIMP-2 on expression of MMP-1/TIMP-1 ratios were also investigated. Although MMP-1/TIMP-1 ratios detected by mRNA in KFs were significantly downregulated compared with PNFs ($P < 0.05$), MMP-1/TIMP-1 ratios were significantly increased in KFs treated with 200 ng/mL TIMP-2 compared with that in untreated control KFs ($P < 0.03$; Fig. 8). We also analyzed MMP-1/TIMP-1 ratios in protein levels using ELISA. MMP-1/TIMP-1 ratios in protein levels were also significantly higher in culture supernatants conditioned by KFs treated with 200 ng/mL (11.5±2.8) TIMP-2 than in untreated control group (8.4±1.4; $P < 0.03$; Fig. 9). These results indicate that TIMP-2 treatment relatively leads to collagen degradation compared with control.

Therapeutic Effects of TIMP-2 in Ex Vivo Cultures of Keloid Tissues

Finally, to assess the potential efficacy of TIMP-2 in the treatment of keloid, we analyzed the thickness of the dermis and individual collagen bundles in the dermal layer using ex vivo cultures of keloid tissue. TIMP-2–treated specimens showed significant reductions (4.83±1.20 mm) in dermal thickness compared with control (6.52±1.92 mm; $P < 0.03$; Fig. 10). Treatment of TIMP-2 also results in significantly decreased thickness of individual collagen bundles (5.42±1.27 arbitrary units) compared with control (7.47±1.16 arbitrary units; $P < 0.03$; Fig. 11). These results support the idea that TIMP-2 may be a useful therapeutic target for the treatment of keloids. To examine the expression of αSMA protein in cells produced by ex vivo cultures of keloid tissue, immunohistochemical analyses for αSMA were performed. The number of αSMA positive cells was decreased in TIMP-2–treated specimens (Fig. 12). These results revealed that the decrease of skin thickness would be caused by the inactivation of dermal fibroblasts.
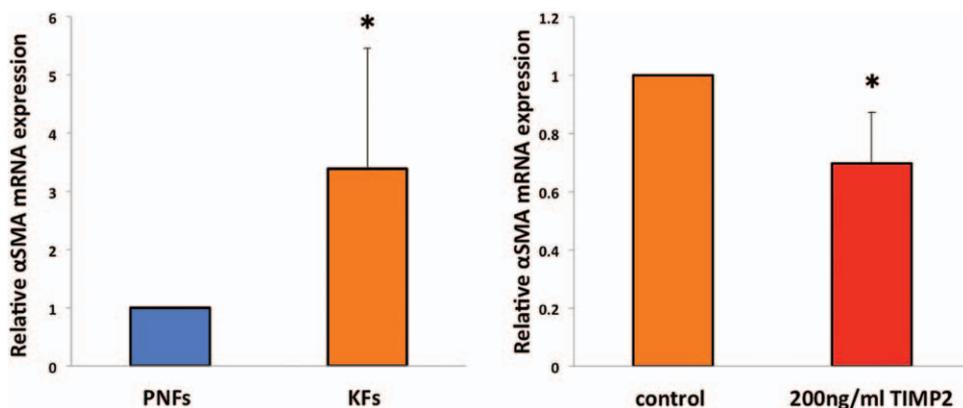

Fig. 7. Expression of αSMA mRNA. Expression of αSMA mRNA in keloid fibroblasts (KFs) and peripheral normal skin fibroblasts (PNFs, n=7) and expression of αSMA mRNA in KFs treated with 200 ng/mL tissue inhibitor of metalloproteinase-2 (TIMP-2, n = 6) were quantified using quantitative real-time polymerase chain reaction (qRT-PCR). *$P < 0.03$.
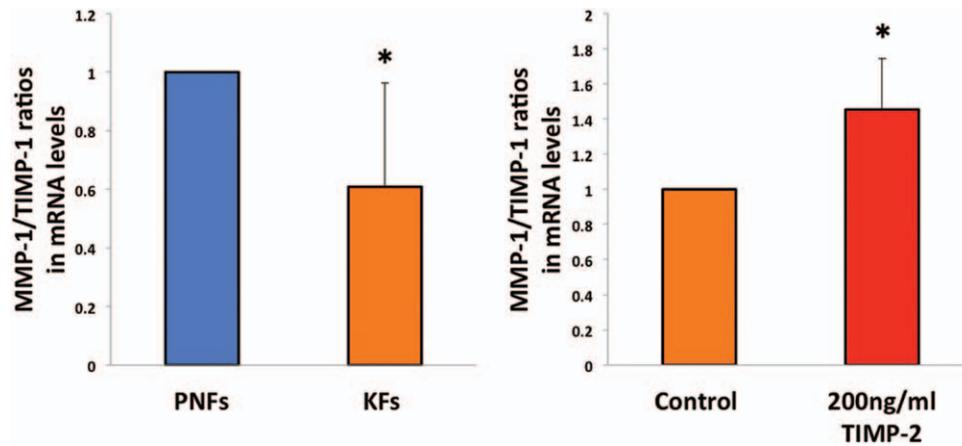
Fig. 8. Expression of matrix metalloproteinase-1 (MMP-1)/tissue inhibitor of metalloproteinase-1 (TIMP-1) ratios. Expression of MMP-1/TIMP-1 ratios in keloid fibroblasts (KFs) and peripheral normal skin fibroblasts (PNFs, *P < 0.05; n = 7) and expression of MMP-1/TIMP-1 ratios in KFs treated with 200 ng/mL TIMP-2 (*P < 0.03; n = 6) were analyzed by using quantitative real-time polymerase chain reaction (qRT-PCR).

DISCUSSION

Although earlier studies have suggested that TIMP-2 effectively inhibits post-myocardial infarction remodeling[39] and suppresses angiogenesis in metastatic cancer,[14,40] the effects of TIMP-2 on keloids had not been previously reported. We recently reported that TIMP-2 knockdown leads to increase in collagen production and expression of Col1A2 and Col3A1.[16] In this study, we found that production of TIMP-2 was significantly downregulated in KFs, when compared with PNFs, and that collagen synthesis (PIP levels) and expression of type I and III collagen were all suppressed by treatment with TIMP-2. Lastly, we confirmed that the thickness of

Fig. 9. Matrix metalloproteinase-1 (MMP-1)/tissue inhibitor of metalloproteinase-1 (TIMP-1) ratios in culture supernatants. Protein levels of MMP-1/TIMP-1 ratios in culture supernatants conditioned by keloid fibroblasts (KFs) treated with 200 ng/mL TIMP-2 were analyzed by using enzyme-linked immunosorbent assay (ELISA, n = 6). *P < 0.03.

the dermis and collagen bundles was reduced by TIMP-2 treatment in ex vivo cultures of keloid tissue. Thus, TIMP-2 seems to play a key role in the progression or development of keloids.

Our results also showed that whereas expression of TIMP-2 is significantly downregulated in KFs, expression of TIMP-1 is largely unaffected. Some reports have shown that expression of both TIMP-1 and TIMP-2 is significantly higher in keloids than in normotrophic scars.[6,17,18] In those studies, however, the normal control tissue used was not from the same individuals as the keloid tissue. To avoid the effects of individual differences, we analyzed KFs and PNFs from the same patients. This may be one reason for the discrepancy between our results and those reported previously. On the other hand, the MMP-1/TIMP-1 ratios observed in this study were consistent with earlier reports,[18] as was the observed imbalance in collagen deposition in KFs. It is commonly known that expression of TGFβ-1, which is secreted by many types of cells including macrophages,[36,41] is increased in keloid region.[24,37] Some reports suggested that TIMP-2 and MMP-1/TIMP-1 ratios were downregulated by TGFβ-1.[42–45] There was no discrepancy between our results and the effects of TGFβ-1.

Because mechanical force is one of the important factors contributing to the progression of keloids,[4,6,23] we also analyzed the expression of TIMPs and MMPs after cyclic mechanical stretch. Suppression of TIMP-2 mRNA expression subjected to stretching further suggests that downregulation of TIMP-2 contributes to the progression of keloids because of a relative increase of MT1-MMP activity. Although MMP-1 expression was weaker in KFs than in PNFs, it was significantly upregulated by
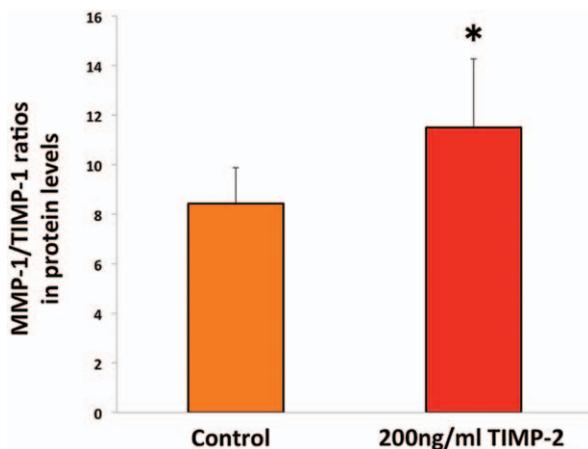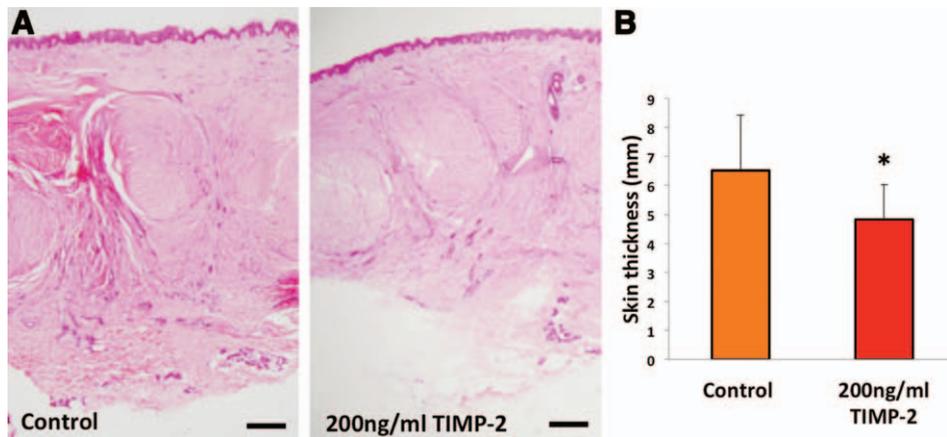
Fig. 10. The thickness of the dermis treated with tissue inhibitor of metalloproteinase-2 (TIMP-2). Keloid tissues were intradermally injected with control phosphate-buffered saline (PBS) or 200 ng/mL TIMP-2. The thickness of the dermis in the dermal layer was then assessed 1 week after injection. (A) Hematoxylin and eosin staining of ex vivo culture of keloid tissues. Photomicrographs were taken using an Olympus SZX12 stereo microscope (Olympus, Melville, N.Y.) and a DP70 digital camera (Olympus) for skin dermal thickness. Scale bars in black = 500 μm. (B) Quantitative analysis of dermal thickness. For each section, the thickness of the dermis was measured from the epidermal–dermal junction to the dermal–fat junction in 5 random fields using Lumina vision software. Data represent 6 independent experiments using skin explants from 6 different donors. *$P < 0.03$.

cyclic mechanical stretch, which is consistent with our earlier report claiming that MMP-1 expression was upregulated by stretching with enhanced cell migration.[21]

As for the progression of keloids, fibroblasts-derived MT1-MMP and active MMP-2 are thought to play crucial roles in keloid invading normal skin and tumor invasion.[6] Then, excessive synthesis and deposition of collagen contribute to the development of keloids on the invasion part with prolonged and excessive presence of TGFβ-1.[24,37]

Excessive synthesis of collagen was induced by the active fibroblasts, and excessive deposition of collagen was also induced by imbalance between TIMP and MMP, especially imbalance of MMP-1/TIMP-1.[9,10,38] Levels of Col1A2 and Col3A1 mRNA and PIP were all significantly downregulated in the KFs treated with TIMP-2. Likewise, expression of αSMA was also significantly downregulated. Moreover, the MMP-1/TIMP-1 ratios, which are the indicators of the antifibrotic effects of treatments,[46,47] were significantly increased by TIMP-2 treatment. Therefore,
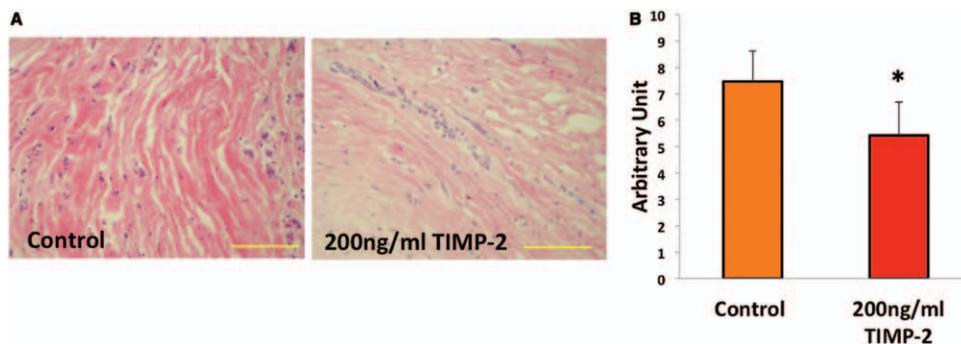

Fig. 11. Collagen bundles thickness treated with tissue inhibitor of metalloproteinase-2 (TIMP-2). (A) Hematoxylin and eosin staining of ex vivo culture of keloid tissues. Photomicrographs were taken using an Olympus AX70 microscope and a DP72 digital camera for collagen bundle thickness. Scale bars in yellow = 100 μm. (B) Quantitative analysis of collagen bundles thickness. The thickness of individual collagen bundles at 100 sites/field were measured in 5 random fields in each sample using Lumina vision software. Data represent 6 independent experiments using skin explants from 6 different donors. Data are shown in arbitrary units. *$P < 0.03$.
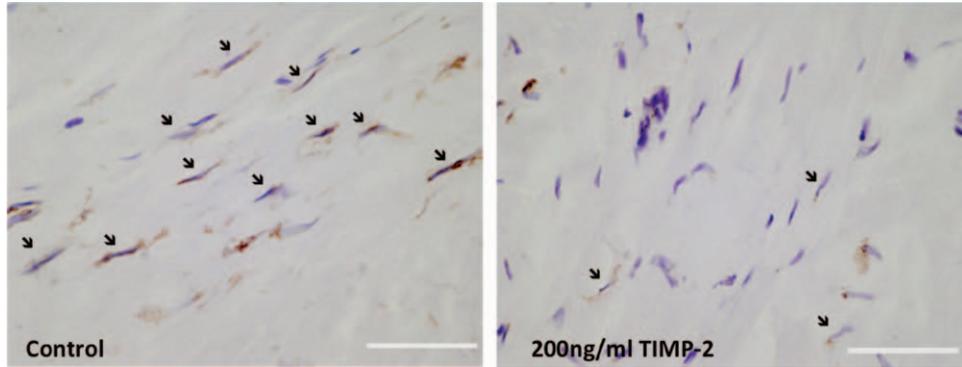
Fig. 12. Immunohistochemical staining of αSMA. Immunohistochemical staining for αSMA comparing sections of ex vivo culture of keloid tissues treated with tissue inhibitor of metalloproteinase-2 (TIMP-2) or control. αSMA-positive cells are shown in brown. Arrows represents the positive αSMA staining of cells. Scale bars in white = 50 μm.

these results strongly suggest that TIMP-2 may be useful for the treatment of keloids. We also recently reported that treatment with TIMP-1 siRNA is effective against keloids.[16] Therefore, a combination therapy involving knockdown of TIMP-1 together with overexpression of TIMP-2 may be more effective therapy for keloids.

It has been reported that MT1-MMP, which is inhibited by TIMP-2 or MMP inhibitors (GM-6001), enables localized cleavage of the TGFβ-1 latency-associated peptide to liberate active TGFβ-1.[48,49] As bioavailability of TGFβ-1 was crucial as pathogenesis of keloids, downregulation of TIMP-2 would be an essential change of keloid progression and development as a result of relative increase of MT1-MMP activity (Fig. 13).[48] Our results of immunohistochemical analyses for αSMA revealed that the decrease of collagen synthesis and skin thick-
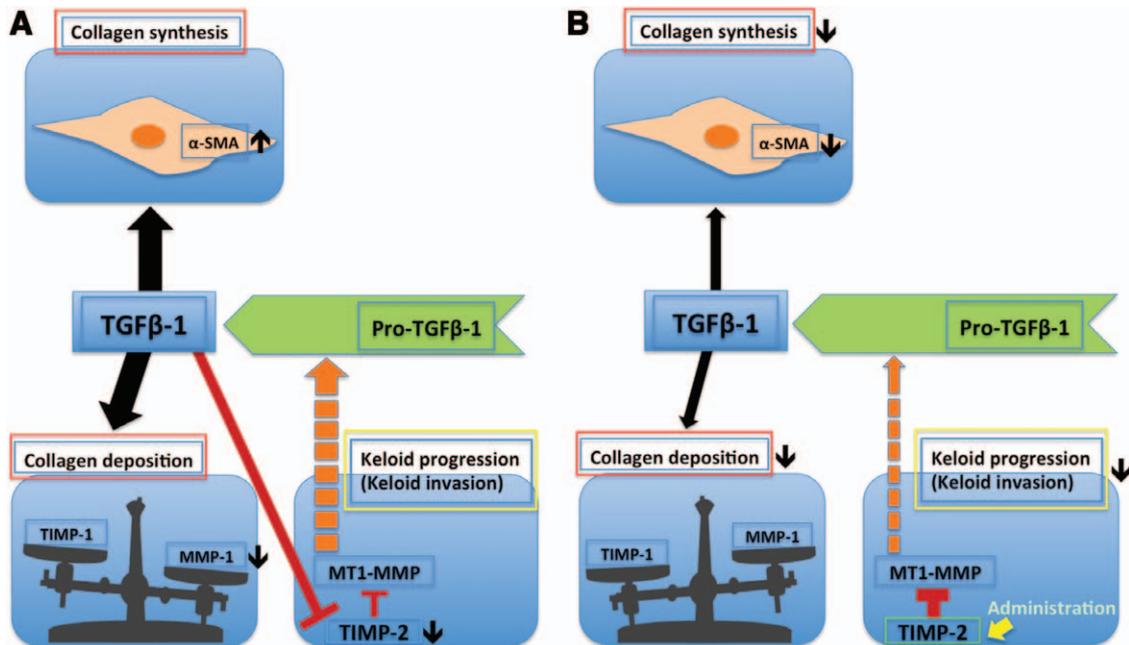

Fig. 13. Scheme for keloid pathogenesis and possible mechanism of treatment with tissue inhibitor of metalloproteinase-2 (TIMP-2). (A) Downregulation of TIMP-2 leads to the progression of keloids because of relative increase of MT1-MMP activity. MT1-MMP–regulated bioavailability of TGFβ-1 and the increase of active TGFβ-1 lead to collagen synthesis of fibroblasts and collagen deposition in keloid development. (B) Administration of TIMP-2 decreases progression of keloids by inhibiting MT1-MMP. Because activation of pro-TGFβ-1 was inhibited as a result of relative decrease of MT1-MMP activity with TIMP-2 treatment, collagen synthesis was decreased by inactivation of fibroblasts, and the imbalance of MMP-1/TIMP-1 ratios in collagen deposition was improved in keloid development.

ness would be caused by the inactivation of dermal fibroblasts. On the other hand, the imbalance of MMP-1/TIMP-1 ratios in collagen deposition was improved by TIMP-2 treatment. Therefore, we speculated that treatments with TIMP-2 decreased the progression of keloids, collagen synthesis, and collagen deposition as a result of inhibition of MT1-MMP activity. However, further studies will be needed to define the precise mechanism by which TIMP-2 acts on keloids.

In summary, our findings suggest that downregulation of TIMP-2 in KFs is a crucial change contributing to the progression and development of keloids, and that replenishing TIMP-2 may be a potentially effective approach to the reduction of the thick dermis and collagen bundles seen in keloid. Thus, TIMP-2 would be a promising candidate for the treatment of keloids.

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ACKNOWLEDGMENTS

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