Immunoblotting analysis showed that the forced down-regulation of miR-205 using the specific miRNA inhibitor in normal dermal fibroblasts resulted in the increase of LRP-1 protein (Fig. 2a). miR-205 mimic decreased LRP-1 protein in cultured DFSP cells (Fig. 2b), indicating miR-205 regulates LRP-1. And miR-205 inhibitor significantly increased the luciferase activity of LRP-1 in normal dermal fibroblasts (Fig. 2c), indicating that LRP-1 is the direct target of miR-205. In addition, LRP-1 siRNA significantly decreased cell growth in a time-dependent manner in DFSP cells (Fig. 2d). Immunohistochemical staining showed LRP-1 expression was not seen in normal fibroblasts, but was detected strongly in DFSP in vivo (Fig. 2e, g). LRP-1 expression in DF was only slightly (Fig. 2f). Taken together, LRP-1 overexpression by the decreased miR-205 may promote the proliferation of DFSP cells.

LRP-1 regulates ERK phosphorylation in DFSP

We studied underlying mechanism in the miR-205/LRP-1-controlled cell proliferation in DFSP. LRP-1 is reported to sustain phosphorylation state of ERK [8]. As described above, DFSP cells overexpress phosphorylated ERK both in vitro and vivo [13, 20]. LRP-1 knockdown by the siRNA decreased ERK phosphorylation in DFSP cells while total ERK level was not affected (Fig. 3a). The decreased ratio

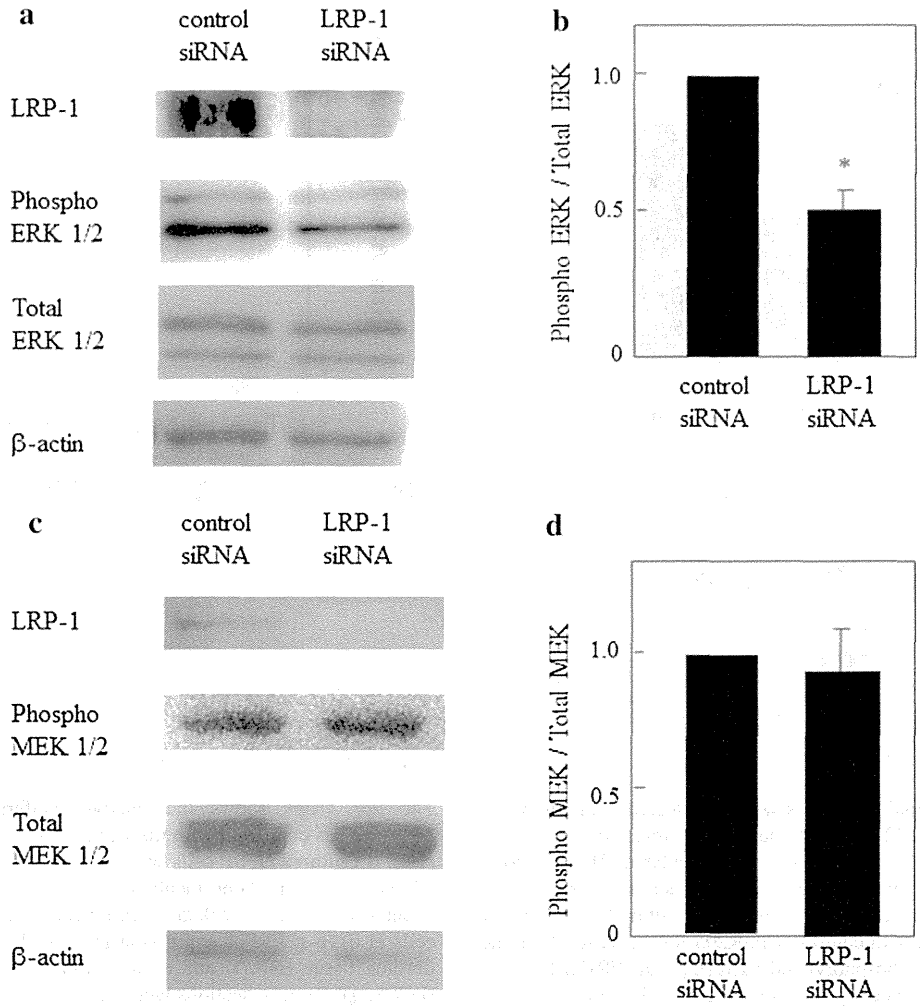

Fig. 3 **a** Cultured DFSP tumor cells were transfected with control or LRP-1 siRNA. After 96 h, the expression of LRP-1, phosphorylated ERK1/2, total ERK1/2 and β-actin levels was analyzed by immunoblotting. The results of one experiment representative of three independent experiments are shown. **b** Relative levels of phosphorylated ERK1/2 protein in DFSP cells treated with control or LRP-1 siRNA were quantified by scanning densitometry and corrected for the levels of total ERK1/2 in the same samples. $*P < 0.05$ as compared with the values in control cells (1.0, $n = 3$). **c** Cultured DFSP tumor cells were transfected with control or LRP-1 siRNA. After 96 h, the expression of LRP-1, phosphorylated MEK1/2, total MEK1/2 and β-actin levels was analyzed by immunoblotting. The results of one experiment representative of three independent experiments are shown. **d** Relative levels of phosphorylated MEK1/2 protein in DFSP cells treated with control or LRP-1 siRNA were quantified by scanning densitometry and corrected for the levels of total MEK1/2 in the same samples ($n = 3$). The value in the cells treated with the control was set at 1

of phosphorylated ERK/total ERK by the siRNA was statistically significant (Fig. 3b). miR-205 inhibitor in normal dermal fibroblasts increased ERK phosphorylation (Fig. 2a), while miR-205 mimic down-regulated the phosphorylation in cultured DFSP cells (Fig. 2b), indicating miR-205/LRP-1 regulates phosphorylation state of ERK. On the other hand, LRP-1 knockdown did not significantly affect the phosphorylation state of MEK, the upstream module of ERK in the MAP kinase cascade (Fig. 3c, d). These results suggested that LRP-1 overexpression caused by miR-205 down-regulation directly contributes to the increased ERK phosphorylation without affecting MEK phosphorylation in DFSP.

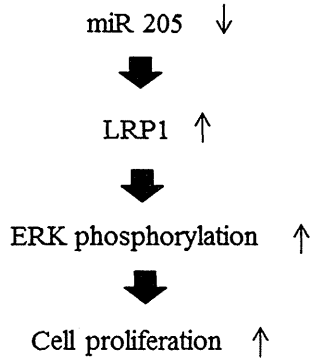

Fig. 4 A hypothetical model of the role of miR-205 and LRP-1 in the oncogenesis of DFSP

Discussion

In this study, we identified several overexpressed or suppressed miRNAs specifically in DFSP compared with normal skin and DF by miRNA PCR array. We focused on miR-205, that is known to be decreased in glioblastoma, head and neck squamous cell carcinoma, prostate cancer and breast cancer [5, 7, 14, 32]. To our knowledge, our study is the first to investigate miR-205 expression in skin diseases.

In addition, we found the new miRNA–target interactions: down-regulated miR-205 led to the overexpression of LRP-1 in normal dermal fibroblasts. Recent reports indicated that BCL2, ESRRG or ZEB2 as the targets of miR-205 in prostate cancer, endometrial carcinoma or esophageal squamous cell carcinoma, respectively [34, 35]. Although it is still unclear how the expression levels of miR-205 was reduced in DFSP, miRNA can be regulated by various mechanisms including methylation and cytokine stimulation in general. Further studies are needed to clarify the mechanism.

The forced down-regulation of miR-205 activated cell proliferation, whereas knockdown of LRP-1 inhibited cellular growth. Our results indicated these changes may involve the ERK phosphorylation. ERK is one of the most potent mitogenic pathways in various cell types [2]. LRP-1 is known to activate ERK phosphorylation [8]. ERK phosphorylation is usually regulated by MEK, but sometimes by other factors without altering MEK phosphorylation [19]. For example, several molecules including growth factor receptor-bound 2 (GRB2) or son of sevenless (SOS) are reported to alter ERK phosphorylation directly [18]. Although the exact mechanism is still unclear, our results suggested that miR-205-LRP-1 pathway may induce abnormal cell proliferation in DFSP by regulating the phosphorylation of ERK directly (Fig. 4).

Currently, the treatments of the tumor other than surgical excision are not established. Investigations of the regulatory mechanisms of oncogenesis by miR-205 and LRP-1 may lead to a new therapeutic approach for DFSP.

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Conflict of interest The authors have no financial conflict of interest.

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Effects of the immunosuppressant rapamycin on the expression of human α2(I) collagen and matrix metalloproteinase 1 genes in scleroderma dermal fibroblasts

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ABSTRACT

Background: Rapamycin has been shown to exert an anti-fibrotic effect on skin fibrosis in a certain subset of patients with systemic sclerosis (SSc) and in bleomycin-treated animal models.
Objectives: To investigate the mechanism responsible for the anti-fibrotic effect of rapamycin especially by focusing on human α2(I) collagen (COL1A2) and matrix metalloproteinase 1 (MMP1) genes in normal and systemic sclerosis (SSc) dermal fibroblasts.
Methods: The expression levels of type I procollagen and MMP1 proteins were analyzed by immunoblotting and the mRNA levels of COL1A2 and MMP1 genes were evaluated by quantitative real-time RT-PCR. The activities of COL1A2 and MMP1 promoters were determined by reporter analysis.
Results: Rapamycin significantly decreased the levels of type I procollagen protein and COL1A2 mRNA, while significantly increasing the levels of MMP1 protein and mRNA in normal dermal fibroblasts. Similar effects of rapamycin were also observed in SSc dermal fibroblasts. Importantly, the inhibitory and stimulatory effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes, respectively, were significantly greater in SSc dermal fibroblasts than in normal dermal fibroblasts. In SSc dermal fibroblasts, rapamycin affected the expression of COL1A2 gene at the post-transcriptional level. In contrast, rapamycin altered the expression of MMP1 gene at the transcriptional level through the JNK/c-Jun signaling pathway in those cells.
Conclusion: Rapamycin has a potential to directly regulate the deposition of type I collagen in extracellular matrix through inhibiting type I collagen synthesis and promoting its degradation by MMP1, suggesting that this drug is useful for the treatment of SSc.

1. Introduction

Systemic sclerosis (SSc) is a multisystem autoimmune disorder characterized by initial vascular injuries and resultant fibrosis of the skin and certain internal organs. Although the pathogenesis of SSc still remains unknown, there has been a wealth of evidence suggesting that a number of humoral and cellular immune abnormal reactions, including B cells, T cells, and macrophages [1,2], in early SSc extensively damage and activate microvascular endothelial cells and fibroblasts in the dermis, eventually leading to the promotion of the excessive extracellular matrix (ECM) deposition [3–9]. Consistent with the notion that immune abnormality is a primary event in the pathogenesis of SSc, several studies have demonstrated that various immunosuppressive agents, such as cyclosporine A [10,11], tacrolimus [11], cyclophosphamide [9], methotrexate [12,13], and mycophenolate mofetil [14,15], are useful against the vascular and/or fibrotic lesions in this disorder.

Rapamycin, isolated from *Streptomyces hygroscopicus*, is macrolide antibiotic, which has also been known as a potent immunosuppressive drug with the property of calcineurin inhibitor as well as

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cyclosporine A and tacrolimus. Similarly to tacrolimus, rapamycin exerts a variety of effects on various cell types via binding to immunophilin FK506-binding protein (FKBP12). Following the binding, the complex inhibits mammalian target of rapamycin (mTOR), which regulates cell growth and proliferation as well as metabolic homeostasis [16,17]. In T cells, for instance, the complex inhibits cell growth and proliferation by impairing the response to growth-promoting cytokines including interleukin-2 and subsequently interfering with the downstream signaling pathways required for G1 progression [18,19]. Indeed, rapamycin has been widely used for the prevention of graft rejection in kidney transplant recipients due to its relatively lower nephrotoxicity than other calcineurin inhibitors [20–22]. Importantly, several experimental studies have shown that rapamycin also exerts an anti-fibrotic effect on liver and pulmonary fibrosis in animal models [23,24], supporting the conventional idea that the suppression of inflammation prior to fibrosis is efficacious for the treatment of fibrotic diseases. Alternatively, these experimental data may be attributable to the direct anti-fibrotic effect of rapamycin on dermal fibroblasts.

Previous clinical trials [9,12,13] demonstrated that a certain subset of immunosuppressants is useful for the treatment of skin fibrosis in SSc. Given that SSc fibroblasts keep producing excessive amount of ECM even in the in vitro culture system, where these cells are free from inflammatory attacks, these immunosuppressants appear to exert their anti-fibrotic effect via directly affecting homeostasis of the ECM, which is maintained by a balance between the production and the degradation of ECM proteins. Supporting this idea, we have previously shown that tacrolimus suppresses the pro-fibrotic property of SSc fibroblasts through decreasing the stability of α2(I) collagen (COL1A2) mRNA [25]. Based on these backgrounds with our recent data that rapamycin suppresses bleomycin-induced skin fibrosis in animal models [26], in this study we investigated if rapamycin affects the expression of COL1A2 and matrix metalloproteinase 1 (MMP1) genes using normal and SSc dermal fibroblasts. We also discussed the potential mechanism by which rapamycin exerts its anti-fibrotic effect in human diseases and animal models.

2. Materials and methods

2.1. Reagents

Rapamycin and SP600125 were purchased from Calbiochem (La Jolla, CA, USA). Actinomycin D and antibody for β-actin were purchased from Sigma (St. Louis, MO, USA). Antibody for type I collagen was purchased from Southern Biotechnology (Birmingham, AL, USA). Luciferase assay kit and β-galactosidase enzyme assay kit were purchased from Promega (Madison, WC, USA). Antibody for MMP1 was obtained from Chemicon (San Francisco, CA, USA). FuGENE 6 was obtained from Roche Diagnostics (Indianapolis, IN, USA).

2.2. Cell cultures

After getting institutional approval and informed consent, dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of seven patients with diffuse cutaneous SSc (dcSSc) and <2 years of skin thickening and from the corresponding areas of 5 healthy donors. Primary explant cultures were established and cells were maintained as described previously [27].

2.3. Immunoblotting

Subconfluent quiescent normal dermal fibroblasts were treated with rapamycin at the indicated concentrations or an equal amount of vehicle (dimethyl sulfoxide, DMSO). Then, cells were cultured for an additional 72 h. Subconfluent quiescent SSc dermal fibroblasts were treated in parallel with normal dermal fibroblasts. Cell lysates were prepared and subjected to immunoblotting as described previously [28].

2.4. RNA preparation and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using an RNeasy Protect Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instruction. First strand-cDNA synthesis was performed with oligo (dT) primer using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT-PCR was performed by PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Primers for COL1A2, MMP1, COL3A1, COL5A1, fibronectin, tenascin C, tissue inhibitor of metalloproteinase 1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were purchased from Applied Biosystems.

2.5. Plasmid construction

The full-length clone of the human MMP1 promoter containing a fragment of promoter DNA linked to the luciferase (MMP1/Lux construct) reporter was kindly provided by Dr. Brinckerhoff [29]. A −772 COL1A2/Lux construct consisting of a COL1A2 gene fragment (+58 to −772 bp relative to the transcription start site) linked to the luciferase reporter gene was generated as previously described [30]. Plasmids used in transient transfection assays were purified twice on CsCl gradients. At least two different plasmid preparations were used for each experiment.

2.6. Transient transfection

Transfection of reporter constructs and the determination of promoter activity were performed as described previously [28].

2.7. Analysis of mRNA stabilities

To investigate the stability of COL1A2 and MMP1 mRNAs, subconfluent quiescent SSc dermal fibroblasts were pretreated for 1 h with actinomycin D (1.0 μg/ml), and treated with rapamycin (1.0 nM) or an equal amount of vehicle (DMSO), for the indicated times prior to RNA extraction. The mRNA levels of COL1A2, MMP1, and GAPDH genes were analyzed by quantitative real-time RT-PCR.

2.8. Statistical analysis

Statistical analyses were carried out with the Mann–Whitney *U* test for comparison of means. *P* values less than 0.05 were considered significant.

3. Results

3.1. Cell viability of normal and SSc dermal fibroblasts treated with rapamycin

Before starting a series of substantial experiments, we investigated toxic effect of rapamycin on dermal fibroblasts. To this end, cell viability of normal and SSc dermal fibroblasts treated with indicated concentration of rapamycin was determined by trypan blue exclusion test. As shown in Table 1, in both of normal and SSc dermal fibroblasts, rapamycin failed to significantly affect the cell mortality at all of the concentrations tested up to 72 h.

Table 1
Cell viability of normal and SSc dermal fibroblasts treated with rapamycin.

	24 h				
Rapamycin	0 nM	0.01 nM	0.1 nM	1.0 nM	10 nM
Normal fibroblasts	96.5 ± 2.7	96.6 ± 2.5	96.7 ± 3.1	96.6 ± 2.1	96.9 ± 1.1
SSc fibroblasts	97.6 ± 2.8	97.7 ± 1.2	97.6 ± 2.5	96.1 ± 2.7	97.2 ± 1.0
	48 h				
Rapamycin	0 nM	0.01 nM	0.1 nM	1.0 nM	10 nM
Normal fibroblasts	98.3 ± 1.2	97.3 ± 1.2	98.0 ± 1.0	97.3 ± 1.2	97.0 ± 1.0
SSc fibroblasts	98.0 ± 1.0	97.7 ± 1.5	97.3 ± 1.5	97.3 ± 1.5	97.0 ± 1.7
	72 h				
Rapamycin	0 nM	0.01 nM	0.1 nM	1.0 nM	10 nM
Normal fibroblasts	98.3 ± 0.6	97.7 ± 1.2	97.7 ± 1.2	96.3 ± 1.2	96.3 ± 1.2
SSc fibroblasts	96.7 ± 2.1	97.3 ± 1.5	97.3 ± 2.1	96.3 ± 2.5	96.6 ± 2.1

SSc, systemic sclerosis. The values represent mean ± standard deviation.

3.2. Effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins in normal dermal fibroblasts

To determine the effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins in normal dermal fibroblasts, cells were treated with various concentrations of rapamycin or the same amount of vehicle (DMSO) for 72 h. Each sample of cell lysate was analyzed by immunoblotting. As shown in Fig. 1A, the expression levels of type I procollagen proteins were reduced in a dose-dependent manner by the treatment with rapamycin. The inhibitory effect was statistically significant over the concentration of 1.0 nM. As shown in Fig. 1B, in contrast, the expression levels of MMP1 proteins were increased by the treatment with rapamycin. The stimulatory effect was statistically significant at the concentration of 0.1 nM and 1.0 nM.

3.3. Effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes in normal dermal fibroblasts

We next investigated the effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes in normal dermal fibroblasts. Under the same conditions described above, the mRNA levels of COL1A2 and MMP1 genes were determined by quantitative real-time RT-PCR. As shown in Fig. 1C, the mRNA levels of COL1A2 gene were decreased by the treatment with rapamycin in a dose-dependent manner, and the inhibitory effect was statistically significant over a concentration of 0.1 nM. In contrast, the mRNA levels of MMP1 gene were increased by the treatment with rapamycin in a dose-dependent manner and the stimulatory effect was statistically significant over a concentration of 0.1 nM (Fig. 1D). Taken together, these results suggest that rapamycin coordinately decreases the accumulation of type I collagen proteins in ECM through the suppression of type I collagen production and the acceleration of its degradation by MMP1.

3.4. Effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins in SSc dermal fibroblasts

We further investigated if rapamycin affects the expression levels of type I procollagen and MMP1 proteins in SSc dermal fibroblasts. As shown in Fig. 2A, the expression levels of type I procollagen proteins were reduced in a dose-dependent manner by the treatment with rapamycin. The inhibitory effect on type I procollagen proteins was statistically significant over a concentration of 0.1 nM. In contrast, as shown in Fig. 2B, the expression levels of MMP1 proteins were increased in a dose-dependent manner by the treatment with rapamycin. The stimulatory effect on MMP1 proteins was statistically significant over a concentration of 1.0 nM. Importantly, the stimulatory effect of rapamycin on MMP1 proteins was much greater in SSc dermal fibroblasts than in normal dermal fibroblasts at a concentration of 10 nM (mean ± SD [fold increase]: 2.80 ± 1.29 vs. 1.22 ± 0.22, $P < 0.05$).

3.5. Effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes in SSc dermal fibroblasts

We also determined the effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes in SSc dermal fibroblasts by quantitative real-time RT-PCR. As shown in Fig. 2C, the mRNA levels of COL1A2 gene were decreased by the treatment with rapamycin in a dose-dependent manner, and the inhibitory effect was statistically significant over a concentration of 0.01 nM. In contrast, as shown in Fig. 2D, the mRNA levels of MMP1 gene were increased by the treatment with rapamycin in a dose-dependent manner, and the stimulatory effect was statistically significant over a concentration of 1.0 nM.

3.6. Rapamycin exerts greater inhibitory and stimulatory effects on the mRNA expression of COL1A2 and MMP1 genes, respectively, in SSc dermal fibroblasts than in normal dermal fibroblasts

To further assess if rapamycin exerts differential effects on the expression levels of COL1A2 and MMP1 genes between normal and SSc dermal fibroblasts, we focused on the magnitude of its inhibitory and stimulatory effects on the mRNA levels of COL1A2 and MMP1 genes, respectively, at the highest concentration (10 nM). As shown in Table 2, the basal expression levels of COL1A2 gene was significantly increased in SSc dermal fibroblasts compared with normal dermal fibroblasts (2.59 ± 1.44 arbitrary unit [AU] vs. 1.00 ± 0.18 AU, $P < 0.01$). After the treatment of rapamycin, the mRNA levels of COL1A2 gene in SSc dermal fibroblasts were decreased to the levels comparable to those in normal dermal fibroblasts (0.79 ± 0.33 AU vs. 0.71 ± 0.09 AU). Importantly, the magnitude of its inhibitory effect on the mRNA levels of COL1A2 gene was much greater in SSc dermal fibroblasts than in normal dermal fibroblasts (fold increase: 0.36 ± 0.18 vs. 0.71 ± 0.09, $P < 0.05$). On the other hand, the mRNA basal levels of MMP1 gene were comparable between SSc and normal dermal fibroblasts (0.86 ± 0.43 AU vs. 1.00 ± 0.25 AU). After the treatment of rapamycin, there was a trend toward the elevation of MMP1 mRNA levels in SSc dermal fibroblasts compared with normal dermal fibroblasts, but it did not reach a statistical difference (2.51 ± 1.11 AU vs. 144 ± 0.27 AU, $P = 0.073$). Similarly to COL1A2 gene, the magnitude of its stimulatory effect on the mRNA levels of MMP1 gene was much greater in SSc

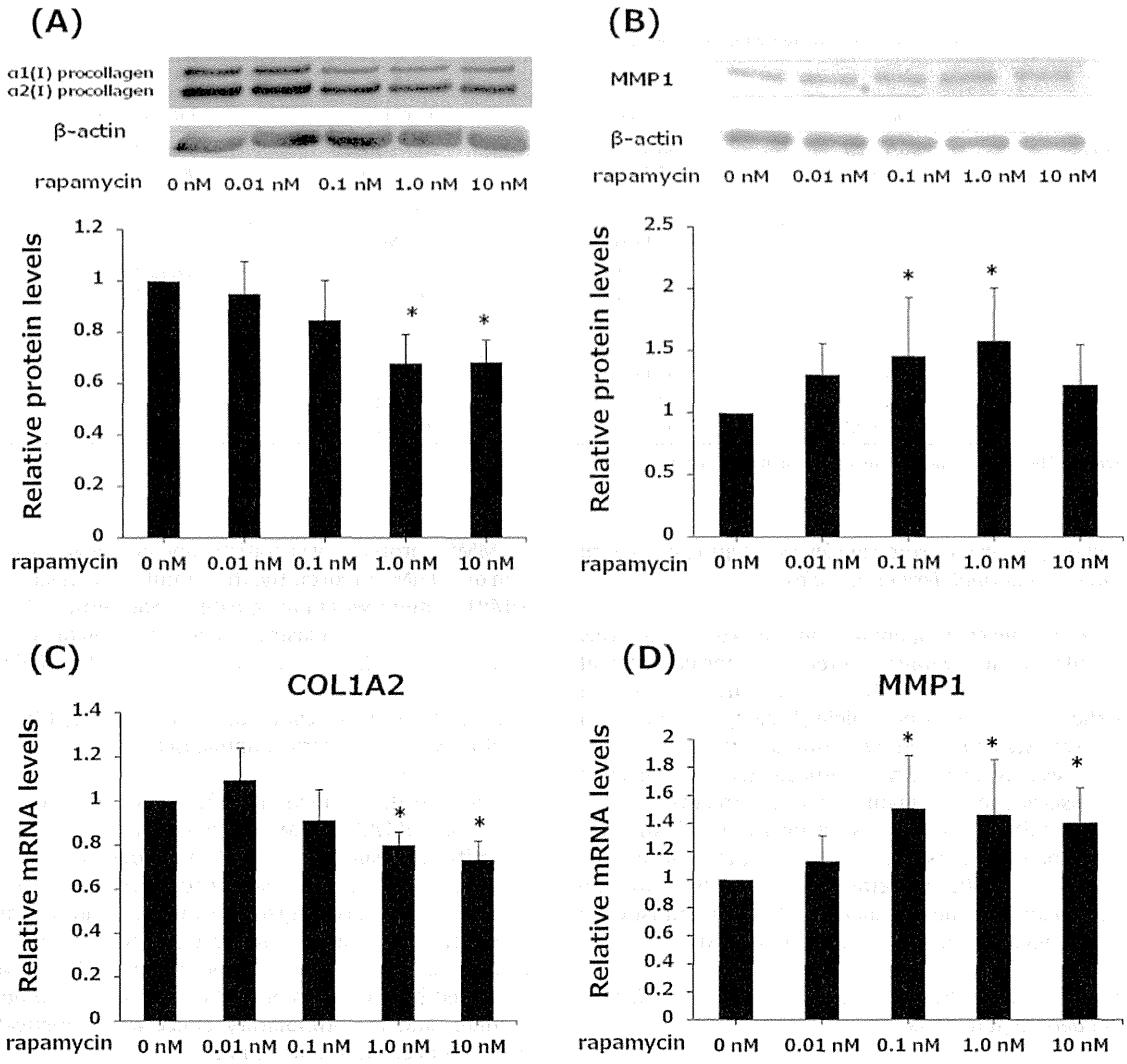

Fig. 1. Effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins, and the mRNA levels of COL1A2 and MMP1 genes in normal dermal fibroblasts. (A) Subconfluent normal dermal fibroblasts were treated with various concentrations of rapamycin or the same amount of vehicle (DMSO) and incubated for 72 h. Each sample of cell lysate was analyzed by immunoblotting using anti-type I collagen antibody. The levels of β-actin proteins are shown as a loading control (top). Shown is a representative experiment of four independent experiments. The levels quantified by scanning densitometry are shown relative to the levels in untreated normal fibroblasts (bottom). Data are expressed as the mean and SD of four independent experiments. $^*P < 0.05$, as compared with the value in untreated normal dermal fibroblasts. (B) Subconfluent fibroblasts were treated with various concentrations of rapamycin and incubated for 72 h. Each sample of cell lysate was analyzed by immunoblotting using anti-MMP1 antibody. The levels of β-actin proteins are shown as a loading control (top). Shown is a representative experiment of four independent experiments. The levels quantified by scanning densitometry are shown relative to the levels in untreated SSc dermal fibroblasts (bottom). Data are expressed as the mean and SD of four independent experiments. $^*P < 0.05$, as compared with the value in untreated normal dermal fibroblasts. (C) Subconfluent normal dermal fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The mRNA levels of COL1A2 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of COL1A2 corrected for the mRNA levels of GAPDH gene in the same samples are shown relative to those in untreated normal dermal fibroblasts. Data are expressed as the mean and SD of five independent experiments. $^*P < 0.05$, as compared with the value in untreated normal dermal fibroblasts. (D) Subconfluent normal dermal fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The mRNA for MMP1 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of MMP1 corrected for the levels of GAPDH gene in the same samples are shown relative to those in untreated normal dermal fibroblasts. Data are expressed as the mean and SD of four independent experiments. $^*P < 0.05$, as compared with the value in untreated normal dermal fibroblasts.

dermal fibroblasts than in normal dermal fibroblasts (fold increase: 3.06 ± 1.04 vs. 1.46 ± 0.14, $P < 0.01$). Of note, there was a trend toward a negative correlation between basal levels and fold increase values in the mRNA levels of COL1A2 gene, but it did not reach a statistical significance probably due to the small number of samples ($r = -0.72$, $P = 0.088$). Collectively, these results indicate that rapamycin exerts greater effects on the mRNA levels of COL1A2 and MMP1 genes in SSc dermal fibroblasts with prominent fibrotic phenotype.

3.7. Rapamycin decreases the expression of COL1A2 gene at the post-transcriptional level in SSc dermal fibroblasts

To further determine the mechanism responsible for the inhibitory effect of rapamycin on the expression of COL1A2 gene, we examined if rapamycin affects the expression levels of COL1A2 mRNA at the transcriptional level or at the post-transcriptional level. To this end, we investigated if rapamycin affects the stability of COL1A2 mRNA in SSc dermal fibroblasts using a transcriptional inhibitor, actinomycin D. One hour after the addition of actinomycin D, cells were treated with 1.0 nM rapamycin or the same amount of vehicle (DMSO) for the indicated period. The levels of remaining COL1A2 mRNA were determined by quantitative real-time RT-PCR. As shown in Fig. 3A, the stability of COL1A2 mRNA was significantly decreased in SSc dermal fibroblasts treated with 1.0 nM rapamycin compared with SSc dermal fibroblasts treated with DMSO at 24 h, indicating that rapamycin suppresses the expression of COL1A2 gene at the post-transcriptional level. To further examine if rapamycin affects the

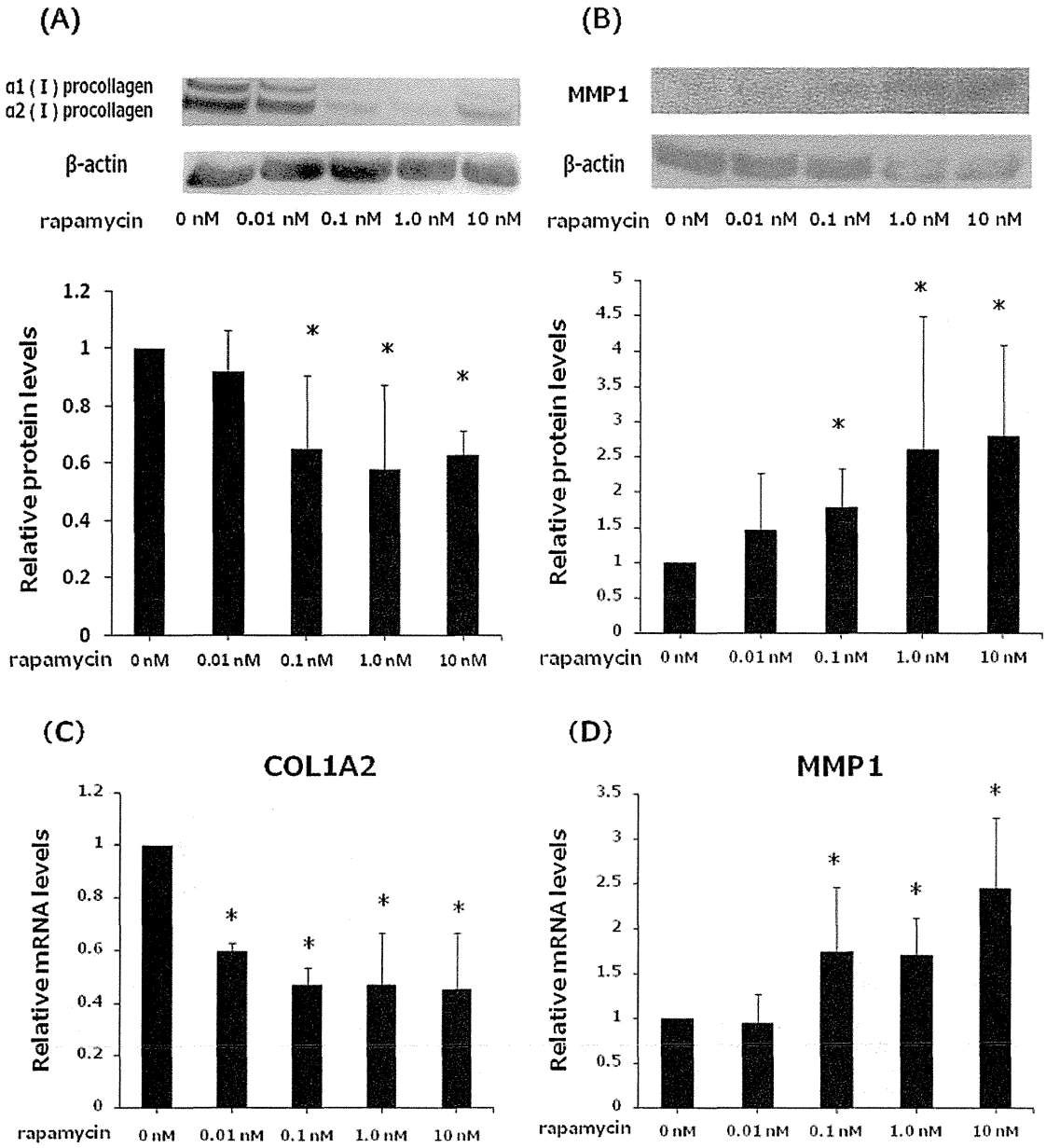

Fig. 2. Effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins, and the mRNA levels of COL1A2 and MMP1 genes in SSc dermal fibroblasts. Subconfluent SSc dermal fibroblasts were treated with various concentrations of rapamycin or the same amount of vehicle (DMSO) and incubated for 72 h. (A) Each sample of cell lysate was analyzed by immunoblotting using anti-type I collagen antibody. The levels of β-actin proteins are shown as a loading control (top). Shown is a representative experiment of six independent experiments. The levels quantified by scanning densitometry are shown relative to the levels in untreated SSc dermal fibroblasts (bottom). Data are expressed as the mean and SD of five or six independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (B) Each sample of cell lysate was analyzed by immunoblotting using anti-MMP1 antibody. The levels of β-actin proteins are shown as a loading control (top). Shown is a representative experiment of four independent experiments. The levels quantified by scanning densitometry are shown relative to the levels in untreated SSc dermal fibroblasts (bottom). Data are expressed as the mean and SD of five independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (C) Subconfluent SSc dermal fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The mRNA levels of COL1A2 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of COL1A2 corrected for the mRNA levels of GAPDH gene in the same samples are shown relative to those in SSc dermal fibroblasts treated with DMSO. Data are expressed as the mean and SD of four independent experiments. (D) Subconfluent SSc dermal fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The mRNA levels of MMP1 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of MMP1 gene corrected for the levels of GAPDH gene in the same samples are shown relative to those in SSc dermal fibroblasts treated with DMSO. Data are expressed as the mean and SD of four or five independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts.

transcriptional activity of COL1A2 gene, we performed transient transfection assay using the −772 COL1A2/Lux construct. As shown in Fig. 3B, the treatment with rapamycin for 48 h did not affect the promoter activity of COL1A2 gene in SSc dermal fibroblasts. These results indicate that rapamycin suppresses the expression of COL1A2 gene at the post-transcriptional level, but not at the transcriptional level.

3.8. Rapamycin increases the expression of MMP1 gene at the transcriptional level in SSc dermal fibroblasts

We also carried out the same analyses to investigate the mechanism responsible for the stimulatory effect of rapamycin on the expression of MMP1 gene. As shown in Fig. 4A, the treatment with rapamycin did not affect the stability of mRNA in SSc dermal

Table 2
Comparison of the inhibitory effect of rapamycin on mRNA levels of COL1A2 gene and the stimulatory effect of rapamycin on the mRNA levels of MMP1 gene between normal and SSc dermal fibroblasts.

	COL1A2 mRNA			MMP1 mRNA		
	Basal	Rapamycin	Fold increase	Basal	Rapamycin	Fold increase
Normal No. 1	0.88	0.65	0.74	0.70	1.12	1.59
Normal No. 2	0.94	0.58	0.61	0.90	1.31	1.46
Normal No. 3	0.91	0.75	0.82	1.13	1.71	1.52
Normal No. 4	1.26	0.83	0.66	1.27	1.60	1.26
Mean ± SD	1.00 ± 0.18	0.70 ± 0.11	0.71 ± 0.09	1.00 ± 0.25	1.44 ± 0.27	1.46 ± 0.14
SSc No. 1	1.31	0.54	0.41	0.39	0.81	2.07
SSc No. 2	5.26	0.89	0.17	0.62	2.42	3.90
SSc No. 3	1.59	0.65	0.41	0.64	3.00	4.69
SSc No. 4	3.21	1.01	0.33	0.73	2.45	3.36
SSc No. 5	3.35	0.70	0.21	0.83	2.54	3.06
SSc No. 6	1.98	1.36	0.72	1.08	1.85	1.71
SSc No. 7	1.40	0.36	0.26	1.70	4.48	2.63
Mean ± SD	2.59 ± 1.44**	0.79 ± 0.33	0.36 ± 0.18*	0.86 ± 0.43	2.51 ± 1.11	3.06 ± 1.04**

* $P < 0.05$ vs. normal dermal fibroblasts.
** $P < 0.01$ vs. normal dermal fibroblasts.

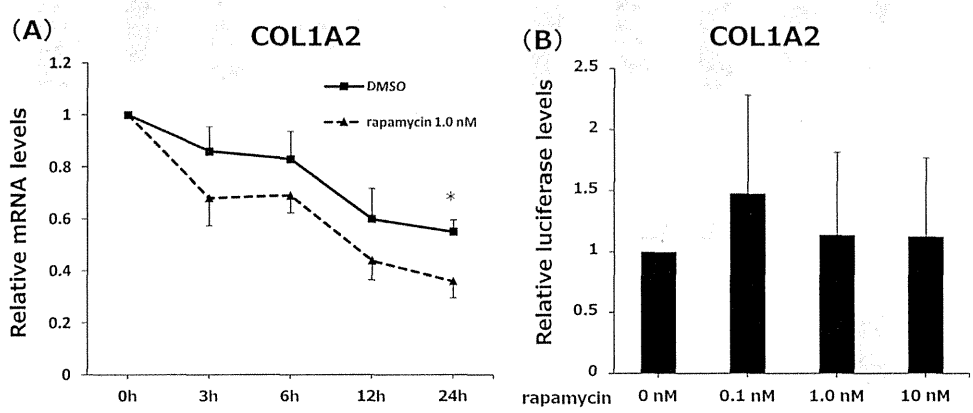

Fig. 3. Rapamycin decreases the expression of COL1A2 gene at the post-transcriptional level in SSc dermal fibroblasts. (A) Subconfluent SSc dermal fibroblasts were pretreated for 1 h with actinomycin D (1 μg/ml) and treated with 1.0 nM rapamycin or vehicle for the indicated period prior to RNA extraction. The levels of remaining COL1A2 mRNA were analyzed by quantitative real-time RT-PCR. The stability of COL1A2 mRNA corrected for the levels of GAPDH gene in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of four independent experiments. *$P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (B) SSc dermal fibroblasts were transiently transfected with the COL1A2/Lux construct. After incubation for 24 h, subconfluent fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The promoter activities of COL1A2 and β-galactosidase genes were analyzed by luciferase assay. The promoter activity of COL1A2 corrected for the levels of β-galactosidase in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of five independent experiments.

fibroblasts, suggesting that rapamycin increases the expression of MMP1 gene at the transcriptional level. Consistently, as shown in Fig. 4B, the promoter activity of MMP1 gene was significantly increased after the exposure to rapamycin in a dose-dependent manner. Since a previous study showed that rapamycin increases the expression of MMP1 gene through the activation of JNK/c-Jun signaling in human lung fibroblasts [31], we next determined whether rapamycin increases the expression of MMP1 gene through the activation of JNK/c-Jun signaling in SSc dermal fibroblasts. As shown in Fig. 4C, SP600125, a specific JNK inhibitor, almost completely inhibited the stimulatory effect of rapamycin on the mRNA levels of MMP1 gene in SSc dermal fibroblasts. These results indicate that rapamycin increases the expression of MMP1 gene through the activation of JNK/c-Jun signaling in SSc dermal fibroblasts as well as normal lung fibroblasts.

4. Discussion

This study was undertaken to clarify the effect of rapamycin on the expression levels of human COL1A2 and MMP-1 genes in normal and SSc dermal fibroblasts. A series of cell-based experimental data demonstrated that this reagent exerts anti-fibrotic effects on normal and SSc dermal fibroblasts through the suppression of COL1A2 gene expression and the up-regulated expression of MMP1 gene. Most importantly, the inhibitory effect of rapamycin on COL1A2 gene and the stimulatory effect of rapamycin on MMP1 gene were much greater in SSc dermal fibroblasts than in normal dermal fibroblasts. In addition to its immunosuppressive property, the present data provide another molecular basis for our previous finding that rapamycin inhibits the bleomycin-induced skin fibrosis in animal models [26]. These results indicate that this reagent possesses a therapeutic potential against skin fibrosis in SSc.

There are several different mechanisms regulating eukaryotic mRNA decay. A major mRNA decay pathway is initiated by shortening of poly(A) tail, followed by decapping of the 5′-end and subsequent 5′–3′ exonucleolytic degradation of the mRNA [32]. A number of studies have shown that the stabilization of type I collagen mRNA is deeply involved in fibrotic process due to excessive production of type I collagen proteins. The mechanism of mRNA stabilization has been well-studied in the mRNA of COL1A1 gene in human lung and dermal fibroblasts. Krupsky et al. [33]

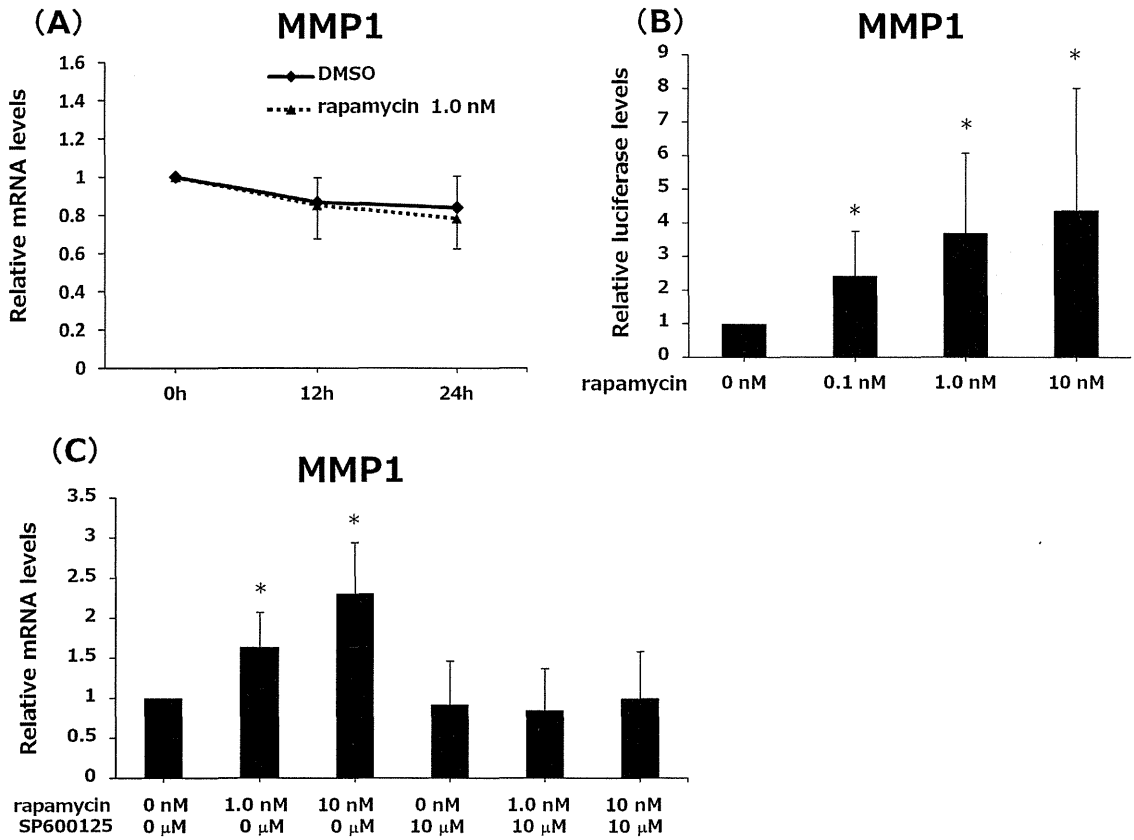

Fig. 4. Rapamycin increases the expression of MMP1 gene at the transcriptional level in SSc dermal fibroblasts. (A) Subconfluent SSc dermal fibroblasts were pretreated for 1 h with actinomycin D (1 μg/ml) and treated with 1.0 nM rapamycin or vehicle for the indicated period prior to RNA extraction. The levels of remaining MMP1 mRNA were analyzed by quantitative real-time RT-PCR. The stability of MMP1 mRNA corrected for the levels of GAPDH gene in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of four independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (B) SSc dermal fibroblasts were transiently transfected with the MMP1/Lux construct. After incubation for 24 h, subconfluent fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The promoter activities of MMP1 and β-galactosidase genes were analyzed by luciferase assay. The promoter activity of MMP1 corrected for the levels of β-galactosidase in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of six independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (C) Subconfluent SSc dermal fibroblasts were pretreated for 1 h with the indicated concentration of rapamycin and incubated in the presence or absence of SP600125 (10 μM) for an additional 48 h. The mRNA levels of MMP1 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of MMP1 gene corrected for the levels of GAPDH gene in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of five independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts.

demonstrated that the stability of COL1A1 mRNA is reduced by amino acid deprivation in human lung fibroblasts. Similarly, Varga et al. [34] disclosed that retinoic acid and prostaglandin E_2, which down-regulate the uptake of the transported amino acid, reduces the steady state mRNA levels of COL1A1 gene in human dermal fibroblasts. Importantly, another study revealed that rapamycin provokes a cellular stress response similar to amino acid decay [35]. Given that mTOR may act as a nutritional sensor, the inhibition of mTOR by rapamycin may reduce the stability of type I collagen mRNA through the similar signals caused by amino acid deprivation [31]. Although a stabilizing mechanism of human COL1A2 mRNA has not been well-studied, rapamycin may decrease the COL1A2 mRNA stability in SSc dermal fibroblasts through the mechanism similar to that regulating the stabilization of COL1A1 mRNA. These observations suggest that the treatment with rapamycin reduces the stability of COL1A2 mRNA through one or some of these pathways; however, further studies are required.

The post-transcriptional regulation of MMP1 gene expression still remains elusive, but a mechanism regulating the stability of MMP1 mRNA is the induction of rapid degradation through the binding of AU-rich element binding proteins to AU-rich sequences in 3′-untranslated region [36–38]. On the other hand, the transcriptional regulation of MMP1 gene has been well studied in various cell types [39]. Importantly, rapamycin increases the promoter activity of MMP1 gene through the activation of JNK/c-Jun/AP-1 signaling axis in human lung fibroblasts [31]. Consistently, in the present study, rapamycin increased the expression levels of MMP1 gene at the transcriptional level in SSc dermal fibroblasts. Furthermore, the specific JNK inhibitor, SP600125, almost completely prevented the stimulatory effect of rapamycin on the mRNA levels of MMP1 gene in SSc dermal fibroblasts. These results indicate that, similarly to lung fibroblasts, rapamycin increases the promoter activity of MMP1 gene through the activation of JNK/c-Jun cascade in SSc dermal fibroblasts.

Given that SSc dermal fibroblasts are characterized by the constitutive activation resulting in the excessive production of type I collagen proteins, the present observation that rapamycin reverses the mRNA levels of COL1A2 gene to normal levels by decreasing the mRNA stability strongly supports the potential of rapamycin to improve skin fibrosis in SSc. On the other hand, experimental data regarding the expression levels of MMP1 gene in SSc dermal fibroblasts are still controversial. Kuroda and Shinkai [40] reported that the mRNA levels of MMP1 gene in SSc dermal fibroblasts are altered in relation to disease duration. According to the report, although the mRNA levels of MMP1 gene in SSc dermal fibroblasts from patients with disease duration of <1 year are significantly higher than those in normal dermal fibroblasts, SSc dermal fibroblasts from patients with disease duration of 2–4 years

show low mRNA levels of MMP1 gene compared with normal dermal fibroblasts. Furthermore, the mRNA levels of MMP1 gene in SSc dermal fibroblasts from patients with disease duration of more than 6 years were comparable to those in normal dermal fibroblasts [40]. However, we could not reproduce the association of MMP1 mRNA levels with disease duration in SSc dermal fibroblasts in the present study as well as in a previous study [41]. In any case, one of the most important observations in this study was that rapamycin uniformly increased the mRNA levels of MMP1 gene in SSc dermal firboblasts irrespective of their basal expression levels. Collectively, rapamycin may achieve the reversal of skin sclerosis in early diffuse SSc by restoring altered homeostasis of the ECM as well as aberrant activation of immune system. These results support the previous clinical findings that rapamycin is efficacious for skin fibrosis equally to methotrexate in a certain subset of SSc patients [42].

Another important observation in this study was that rapamycin altered the expression levels of COL1A2 and MMP1 genes to a greater extent in SSc dermal fibroblasts than in normal dermal fibroblasts. This finding is plausible if mTOR is activated more greatly in SSc dermal fibroblasts than in normal dermal fibroblasts. Supporting this hypothesis, Pannu and Trojanowska reported that mTOR expression is elevated in SSc dermal fibroblasts in vitro [43]. Given that TGF-β stimulation activates mTOR in fibroblasts and that SSc dermal fibroblasts are constitutively activated by autocrine TGF-β stimulation [27,28,44,45], mTOR may be activated as a result of autocrine TGF-β stimulation in SSc dermal fibroblasts. Taken together with the evidence that rapamycin suppressed mRNA levels of COL3A1 and COL5A1 genes, while not those of fibronectin, tenascin C and tissue inhibitor of metalloproteinase 1 genes in SSc dermal fibroblasts (supplementary Fig. 1), it is likely that the activation of mTOR is largely involved in the regulation of fibrillar collagen (types I, III and V) and MMP1 and rapamycin reverses the altered expression of these genes in SSc dermal fibroblasts.

Although corticosteroid is likely to induce a couple of severe complications in SSc, including scleroderma renal crisis especially at the dose of >15 mg/day [46], it is widely used to suppress the inflammatory reactions prior to resultant fibrosis and to improve the skin fibrosis itself in early diffuse SSc [47]. This situation largely depends on the lack of established anti-fibrotic and anti-inflammatory treatments against this disorder. Since rapamycin has a relatively low-nephrotoxicity compared with other calcineurin inhibitors, such as cyclosporine A and tacrolimus [20–22], the combination therapy of rapamycin with corticosteroid may be potentially useful to decrease the dose of corticosteroid and to reduce the adverse events due to conventional calcineurin inhibitors, such as nephrotoxicity. Importantly, in case of renal transplantation, a suitable trough level of rapamycin to optimize efficacy and minimize adverse effects is thought to be between 5 and 15 ng/ml (5.5–16.5 nM) [48], where rapamycin achieves a maximal anti-fibrotic effect on SSc dermal fibroblasts in the present study. Therefore, rapamycin may be a safe and useful drug for the treatment of early diffuse SSc.

In summary, we elucidated that rapamycin has a potential to directly regulate the deposition of type I collagen in ECM through inhibiting type I collagen synthesis and promoting its degradation by MMP1. To the best of our knowledge, this is the first report demonstrating one of the potential mechanisms by which rapamycin exerts its anti-fibrotic effect in human diseases and animal models. Large clinical trials are required to assess the efficacy of rapamycin in patients with early diffuse SSc in the future.Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2014.02.002.

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ORIGINAL ARTICLE

miR-424 levels in hair shaft are increased in psoriatic patients

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ABSTRACT

Objective diagnostic markers have not been in clinical use for psoriasis. In this study, we investigated the levels of miR-424 in hair roots and hair shafts in psoriatic patients, and evaluated the possibility that miR-424 can be a biomarker of the disease. A single hair root and five pieces of hair shafts (~5 cm in length) were obtained from the non-lesional occiput of each individual of 26 psoriatic patients. Control hair samples were collected from nine normal subjects. Samples from 10 atopic dermatitis patients were also included as the disease control. miR-424 levels were determined by quantitative real-time polymerase chain reaction. Hair shaft miR-424 levels were significantly upregulated only in patients with psoriasis compared with normal controls and those with atopic dermatitis. By receiver–operator curve analysis of hair shaft miR-424 to distinguish psoriatic patients from normal subjects, the area under the curve was 0.77. However, relative miR-424 levels were not correlated with disease activity markers including disease duration, body surface area and Psoriasis Area and Severity Index. Hair root miR-424 was not useful for evaluating both diagnosis and severity of the disease. Our results indicated hair shaft miR-424 levels may be useful as a diagnostic marker of psoriasis.

Key words: hair, miRNA, psoriasis.

INTRODUCTION

Psoriasis is one of the chronic skin diseases. In Japan, the number of psoriatic patients gradually increased recently, because of lifestyle changes such as diet. Characteristic skin lesions are papules and erythemas with silver-white scales. Nail deformity or joint involvement are sometimes seen.

To date, the causes of the disease have not been completely clarified. However, recent studies indicate that inflammation, abnormal proliferation of epidermal keratinocytes and immune dysregulation may induce the skin lesion.[1–7] Especially, cytokines including tumor necrosis factor (TNF-α), interleukin (IL)-17 and IL-23 may play central roles in the pathogenesis.

We have focused on the possibility that miRNA are also involved in the keratinocyte proliferation in psoriasis epidermis. miRNA are non-coding short ribonucleic acid molecules, which are constituted of an average of 21–22 base pair nucleotides.[8] By binding to the 3′-untranslated region of target mRNA, the translation of the mRNA is inhibited, resulting in the decreased expression of the target. They may have diverse effects on cellular processes including cell development or proliferation, and are thought to be involved in the pathogenesis of various human diseases.

We previously identified miR-424 as one of the miRNA specifically downregulated in psoriatic skin using polymerase chain reaction (PCR) array analysis. Our study suggested that decreased miR-424 expression and subsequently increased mitogen-activated protein kinase kinase 1 (MEK1) or cyclin E1, predicted target genes of miR-424, may contribute to the increased keratinocyte proliferation of psoriatic skin.[9] Furthermore, although the association of miR-424 with IL-17 or IL-23 has not been described, Zhao *et al.*[10] reported that miR-424 can regulate TNF-α production, suggesting that miR-424 may contribute to the pathogenesis via several pathways. On the other hand, there has been much research indicating the usefulness of circulating miRNA as the disease markers.[11] However, we could not find statistically significant difference in serum miR-424 levels between normal and psoriatic patients. Thus, serum miR-424 levels are not available as the disease marker.

We have recently confirmed the presence of miRNA in hair roots and shafts, and showed that hair miRNA levels as well as serum miRNA levels can be novel and independent biomarkers. In the present study, we evaluated the usefulness of hair miRNA for the diagnosis of psoriasis.

METHODS

Clinical assessment and patient material

A single hair root and five pieces of hair shafts (~5 cm in length) were obtained from the non-lesional occiput of each

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individual of 26 psoriatic patients (age range, 22–84 years) by gently plucking or cutting hairs as described previously.[12] Control hair samples were collected from nine normal subjects. Samples from 10 atopic dermatitis patients were also included as the disease controls. All hair samples were stored at −80°C prior to use. Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study according to the Declaration of Helsinki. Clinical data reported in this study were obtained at the time of hair sampling. Patients were evaluated for the disease duration between the onset of disease and the first visit to the hospital, Psoriasis Area Severity Index (PASI) score, body surface area (BSA) of involved skin, the presence of arthritis and nail change.

RNA extraction from a hair root

After being washed in water and ethanol, a single hair root was incubated with Isogen (Nippon Gene, Toyama, Japan) overnight at room temperature. After chloroform was added, samples were mixed well by vortexing, and were incubated for 5 min at 4°C. The aqueous and organic phase were separated by centrifuge. RNA was precipitated from the aqueous phase by adding isopropanol and ethanol.[13]

RNA extraction from hair shafts

Hair shafts washed in water and ethanol were then dissolved by incubation in extraction buffer supplemented with enzyme solution and lysis solution of Isohair (Nippon Gene) for 2 h at 55°C. Then, RNA was purified using Isogen as described above.

Quantitative real-time PCR

For cDNA synthesis from total RNA including small RNA derived from hair roots and hair shafts, we used mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit (Takara Bio, Shiga, Japan).[12] The cDNA and primers were mixed with SYBR Premix for quantitative real-time PCR with Takara Thermal Cycler Dice TP800. The U6 primer pair and universal 3′-primer were included in the kit. The sequence of the 5′-primer of hsa-miR-424 was designed based on miRBase (http://www.mirbase.org). DNA amplification was performed by 40–50 cycles of denaturation for 5 s at 95°C and annealing for 20 s at 60°C. The levels of miR-424 were normalized by U6 levels in the same samples.

Statistical analysis

Differences between two groups were compared using Mann–Whitney's *U*-test. Correlations were assessed by Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

RESULT

First, we tried to confirm the presence of miR-424 in the hair shafts. The level of miR-424 was determined by quantitative real-time PCR using a primer set specific for miR-424 and miRNA obtained from hair shafts of a normal subject (Fig. 1).

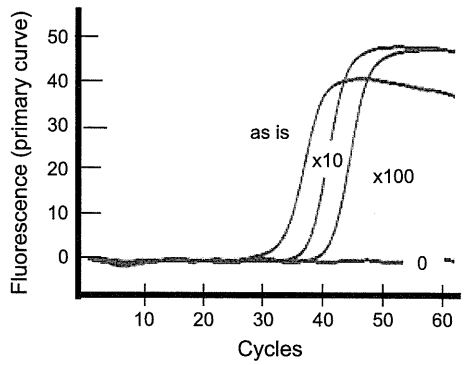

Figure 1. miR-424 is present in the hair shaft. Serial dilution of cDNA (as is, 10-fold, 100-fold dilutions and water) synthesized from hair shaft miRNA was used as the template for real-time polymerase chain reaction. Amplification curves of gene-specific transcripts are presented to illustrate the process of exponential increase of fluorescence.

As a result, the amplification of miR-424 was observed by the PCR. Also, Ct values were increased by the serial dilution of the miRNA. Therefore, miR-424 seemed to be quantifiable in hair shafts using our method.

The hair shaft miR-424 levels in the normal control subjects and in psoriatic patients are shown in Figure 2. miR-424 levels of atopic dermatitis patients were also included as the disease controls. miR-424 in psoriatic patients was significantly increased compared to that in normal subjects ($P = 0.0174$) and to that in patients with atopic dermatitis ($P = 0.0216$). In receiver–operator curve (ROC) analysis for hair shaft miR-424 to distinguish psoriatic patients from normal subjects, the area under the curve (AUC) was 0.77 (95% confidence interval [CI], 0.58–0.96) (Fig. 3): a ROC of more than 0.7 suggests that the level of miR-424 in hair shafts is useful for diagnosis of psoriatic patients.

We then investigated whether miR-424 in hair shaft can be also utilized as a biomarker for evaluating the disease activity of psoriasis. The correlations between the expression of miR-

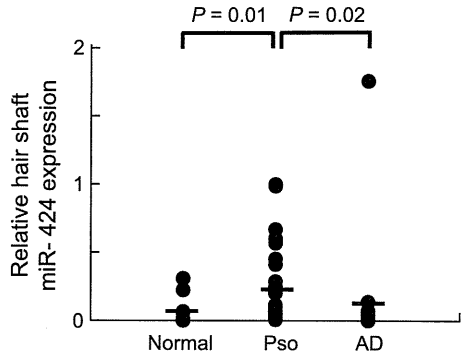

Figure 2. Hair shaft concentrations of miR-424 in patients with psoriasis and in normal subjects are shown on the ordinate. The maximum value in psoriatic patients was set at 1. Horizontal bars show means. *P*-values were determined by Mann–Whitney *U*-test. AD, atopic dermatitis; Pso, psoriasis.

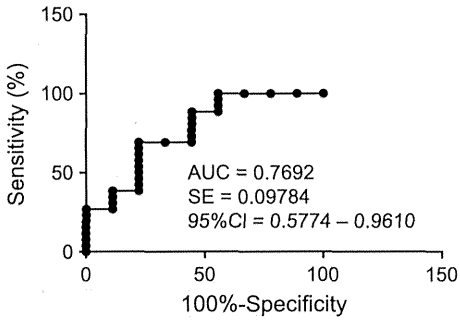

Figure 3. Receiver–operator curve for hair shaft miR-424 to distinguish psoriatic patients from normal subjects. AUC, area under the curve; CI, confidence interval; SE, standard error.

424 and disease duration (between symptom onset and the first visit to the hospital), BSA or PASI score (Fig. 4a-c) were evaluated, respectively. There was no significant correlation between hair miR-424 levels with each of these factors ($r = -0.0419$, $P = 0.8388$; $r = -0.1453$, $P = 0.4787$; and $r = -0.1901$, $P = 0.3523$, respectively). Also, no correlation was found between the miR-424 levels and nail change ($r = -0.0023$, $P = 0.9926$) or arthritis ($r = -0.0304$, $P = 0.8829$). Taken together, miR-424 in hair shafts could not indicate severity of the disease.

Next, we also determined the levels of hair root miR-424 in psoriatic patients: miR-424 was also found in hair roots of normal subjects (Fig. S1). As shown in Figure S2, there was no significant difference in the hair root miR-424 expression between psoriatic patients and normal subjects ($P = 0.2931$) as well as between psoriatic patients and atopic dermatitis patients ($P = 0.2203$). In ROC analysis for hair shaft miR-424 to distinguish psoriatic patients from normal subjects, the AUC was 0.64 (95% CI, 0.40–0.87) (Fig. S3). Therefore, the level of miR-424 in hair root is not effective for the diagnosis of psoriasis.

There were no significant correlations of hair root miR-424 levels and disease activity markers including disease duration, PASI or BSA ($r = -0.1296$, $P = 0.5858$; $r = -0.0609$, $P = 0.7983$; and $r = -0.0242$, $P = 0.9192$, respectively) (Fig. S4). In addition, we could not find significant association between miR-424 levels and nail change ($r = 0.3202$, $P = 0.2643$) or arthritis ($r = -0.1790$, $P = 0.4501$).

DISCUSSION

Psoriasis Area and Severity Index score and BSA have become recently available to evaluate the activity of psoriasis. However, an objective diagnostic marker of the disease is not in clinical use. Psoriasis is sometimes difficult to be clinically distinguished from eczemas (seborrheic dermatitis or atopic dermatitis), lymphomas, drug eruptions or syphilis, and histopathological examination by skin biopsy may be needed to confirm the diagnosis. Thus, non-invasive disease markers for the diagnosis of psoriasis will be beneficial for patients. As a candidate of such serum markers, we have previously determined the concentration of miR-424 in sera of psoriatic

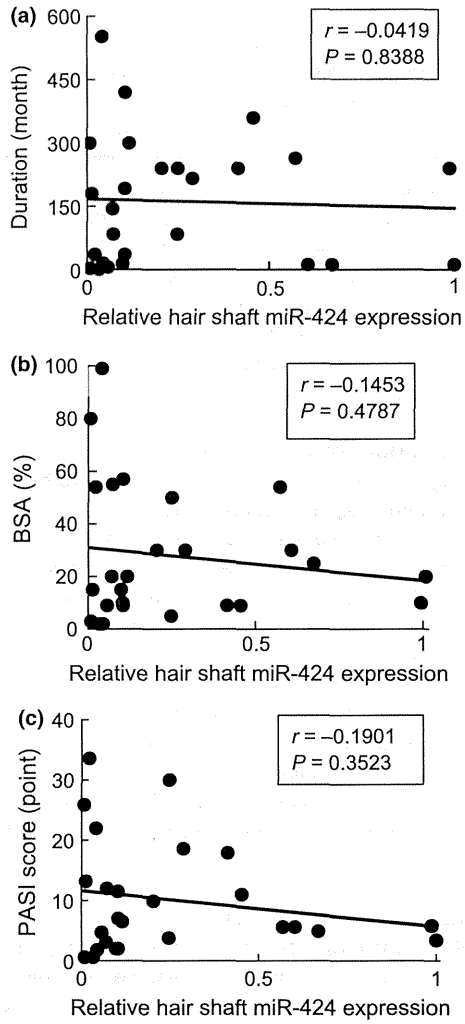

Figure 4. Correlation of hair shaft miR-424 levels with duration between the disease onset and the first visit to the hospital (a), body surface area (BSA) of involved skin (b) or Psoriasis Area Severity Index (PASI) score (c) in patients with psoriasis. The *X*-axis corresponds to the relative miR-424 levels shown in Figure 2. Correlations were assessed by Pearson's correlation coefficient.

patients, but they are not significantly different from those in normal subjects.[9]

Recently, we have reported that miRNA levels in hair shafts and hair roots can be disease markers which are independent of serum levels.[12] Given that hair samples are easier to obtain than blood samples, hair miRNA may have potential to be useful disease markers. We then demonstrated that serum miR-19a levels of psoriatic patients are not significantly altered in comparison to those of normal subjects,[14] while the expression of miR-19a in hair roots of psoriasis is significantly elevated than that in normal subjects.[13] In the present study, the concentration of miR-424 in hair shafts of psoriasis showed significant increase compared to that of normal subjects. According to the ROC analysis, hair shaft miR-424 levels are effective as a

diagnostic marker. This study is the first to demonstrate the clinical significance of hair shaft miRNA in psoriasis.

Because cells of hair roots including hair papilla or cortex have nuclear components, miRNA in the hair roots are thought to originate from these cells. On the other hand, because nuclear components are lost by apoptosis in hair shafts, miRNA in the hair shafts may be supplied by other surrounding tissues as well as the remnant of previously living cells:[15] miRNA levels in hair shafts may reflect the integration of the expression of adjacent tissue miRNA or circulating miRNA in association with hair growth. We previously described that miR-424 levels in psoriatic skin were decreased.[9] Although we have showed that hair miRNA levels are usually independent of serum levels or skin tissue levels as described above, the reason why miR-424 levels of psoriatic patients are elevated in hair shafts of non-lesional skin is not addressed in this study. For example, miR-424 levels may be increased in other tissues than skin (e.g. circulating lymphocytes or monocytes), which may contribute to the increased hair shaft miR-424. Further studies are needed to clarify the mechanism of miR-424 elevation in psoriatic hair shafts. In addition, investigation of the role of miR-424 in keratinocyte proliferation and TNF-α–IL-23/IL-17 axis dysregulation may clarify the pathogenesis of this disease.

Hair root miR-19a levels were correlated with disease duration, suggesting that the miR-19a levels can reflect the severity of symptoms in psoriasis.[13] However, the concentration of hair shaft miR-424 did not correlate with activity of the disease. Because this is the pilot study using a small number of patients' samples, larger studies are needed in the future to find other hair shaft miRNA which are more useful for the evaluation of disease activity of psoriasis. In addition, hair root miR-424 was not useful for evaluating either diagnosis or severity of the disease. Another limitation of this study is that we used only one hair root plucked to purify miRNA for each individual. The level of hair root miRNA may be variable among different stages of the hair cycle (anagen, catagen or telogen), although we have previously showed the reproducibility of miRNA levels in hair roots using samples obtained from different parts of the head in the same individual.[12] We used five hair shafts to average the difference between samples, whereas it was difficult to pluck multiple hair roots from each patient because of pain. Our results should be confirmed by the future study using an increased number of hair root samples obtained from each patient.

CONFLICT OF INTEREST: None.

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SUPPORTING INFORMATION

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

Figure S1. miR-424 is present in hair root. Serial dilution of cDNA (as is, 10-fold, 100-fold dilutions and water) synthesized from hair root miRNA was used as the template for real-time PCR. Amplification curves of gene-specific transcripts are presented to illustrate the process of exponential increase of fluorescence.

Figure S2. The hair root concentrations of miR-424 in patients with psoriasis and in normal subjects are shown on the ordinate. The maximum value in psoriatic patients was set at 1. Horizontal bars show means. *P* values were determined by Mann–Whitney test. Pso, psoriasis; AD, atopic dermatitis.

Figure S3. Receiver operating characteristic (ROC) curve for hair root miR-424 to distinguish psoriatic patients from normal subjects. AUC; areas under curves, SE; standard error, CI; confidence interval.

Figure S4. Correlation of hair root miR-424 levels with duration between the disease onset and the first visit to the hospital (a), body surface area (BSA) of involved skin (b) or Psoriasis Area Severity Index (PASI) score (c) in patients with psoriasis. The X axis corresponds to the relative miR-424 levels shown in Figure S2. Correlations were assessed by Pearson's correlation coefficient.

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CONCISE COMMUNICATION

Case of disseminated cutaneous *Mycobacterium chelonae* infection mimicking cutaneous vasculitis

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ABSTRACT

Mycobacterium chelonae **is a non-tuberculous, rapidly growing mycobacteria and is widely distributed in the natural environment. In the immunocompetent status, localized cutaneous infections such as cellulitis and subcutaneous abscesses commonly occur after traumatic injury. However, disseminated cutaneous infections occur on a background of immunosuppression. Cutaneous *M. chelonae* infection presents with a variety of skin eruptions. We report a case of disseminated *M. chelonae* infection mimicking cutaneous vasculitis. The patient was treated with long-term oral corticosteroids and injected etanercept for the treatment of rheumatoid arthritis and asthma. Because the skin eruptions were preceded by asthma and rheumatoid arthritis and the pathological findings showed fibrinoid necrosis around the vascular of dermis, cutaneous vasculitis was first suspected. The culture from the pus revealed the bacterium which grew within 5 days on Ogawa's culture medium suggesting a rapidly growing mycobacteria. This bacterium was identified as *M. chelonae* by the DNA–DNA hybridization method. We chose 800 mg/day clarithromycin and 500 mg/day levofloxacin as a result of the drug-sensitivity test. After 6 months of the treatment, infection symptoms disappeared. Rapidly growing mycobacteria should be considered in the differential diagnosis of infections in patients under immunosuppression caused by diseases or drugs such as corticosteroids and biologic agents. Repeated bacterial examinations are important and required for the diagnosis of rapidly growing mycobacteria.**

Key words: biologic agent, clarithromycin, dissemination, immunosuppression, rapidly growing mycobacteria.

INTRODUCTION

Mycobacterium chelonae is a non-tuberculous, rapidly growing mycobacteria (RGM) and is widely distributed in the natural environment such as soil, natural water and dust. It is classified into group IV by the Runyon classification of non-tuberculous mycobacteria.[1] The incidence of RGM infections has been increasing recently.[2] *M. chelonae* infection usually involves skin, soft tissues, bones and joints. The optimum growth temperature of *M. chelonae* is 28–30°C. It sometimes causes osteomyelitis, keratitis, otitis media and pulmonary disease. In the immunocompetent status, localized cutaneous infections such as cellulitis and subcutaneous abscesses commonly occur after traumatic injury including tattoos.[3] However, disseminated cutaneous infections occur on a background of immunosuppression caused by drugs (e.g. anti-tumor necrosis factor [TNF] therapy, corticosteroids and methotrexate) or diseases (e.g. AIDS, leukemia and internal malignancy).[4] In the disseminated type, indurated erythema, numerous nodules, abscesses and ulcers are often present. *M. chelonae* infections are also reported as iatrogenic infections following cosmetic surgery, liposuction, catheter insertions for dialysis or laser eye surgery.[5]

CASE REPORT

A 56-year-old Japanese woman with 18-year history of rheumatoid arthritis and 8-year history of asthma presented erythema and subcutaneous nodules on the lower legs. She was treated with long-term oral prednisolone (22.5 mg/day) and betamethasone phosphate (1.5 mg/day) and injected etanercept to control rheumatoid arthritis and asthma after failing to tolerate other therapies. She had no past history of traumatic injury. The skin eruptions gradually spread to her thighs in 1 month. She sometimes had fever. Physical examination showed disseminated infiltrated erythema with small pustules on the surface, subcutaneous nodules with tenderness and edema on her legs. (Fig. 1) When incisional drainage was performed in the pus-discharging subcutaneous nodules, white viscous pus was found. The skin biopsy lesion on her right lower leg was ulcerated.

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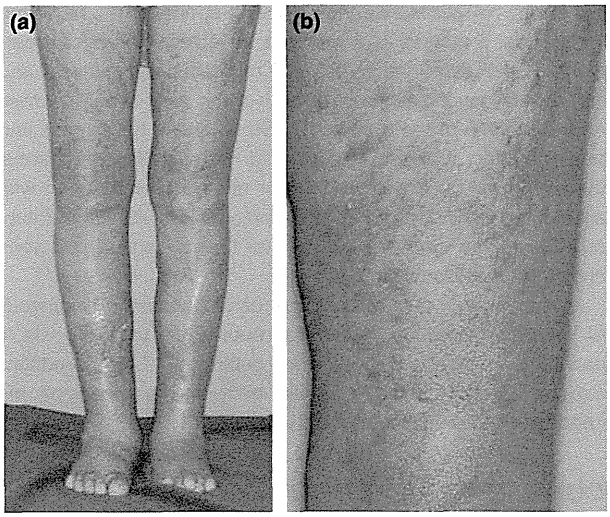

Figure 1. (a,b) Patient's limbs were covered with disseminated infiltrated erythema with small pustules on the surface, subcutaneous nodules and edema. The skin biopsy lesion on her right lower leg was ulcerated.

Laboratory blood examination including liver and renal functions, antinuclear antibody, antineutrophil cytoplasmic antibody, antiphospholipid antibody, QuantiFERON-TB Gold (QIAGEN, Hilden, Germany) and β-D-glucan were within normal range except for elevated C-reactive protein (12.48 mg/dL) and white blood cell count (28.7 × 10^3/mL, neutrophil 90%). Computed tomography (CT), magnetic resonance imaging (MRI) and ultrasonography showed no thrombus formation or vascular stenosis suggesting vasculitis. A skin biopsy specimen taken from the patient's thigh showed clusters of inflammatory cells composed of neutrophils and lymphocytes with abscess formation in the dermis and subcutaneous tissue. Granulomatous lesions with epithelioid histiocytes and Langerhans giant cells were seen in the dermis. Fibrinoid necrosis and red blood cell extravasation were also found around dermal vessels (Fig. 2). Periodic acid-Schiff and Grocott stain were negative. Because the skin eruptions were preceded by asthma and rheumatoid arthritis and the pathological findings showed fibrinoid necrosis around dermal vessels, cutaneous vasculitis related to rheumatoid arthritis or allergic granulomatous angiitis was first suspected. Immunofluorescence staining was not evident in vascular walls.

We performed bacterial cultures from the pus or skin tissues several times. Finally, the culture from the pus revealed the bacterium. The colonies were subcultured on Ogawa's culture medium, which grew within 5 days (35°C), suggesting an RGM. The color of colonies was moist milky white (Fig. 3a). Polymerase chain reaction analyses detected no tuberculosis or *Mycobacterium avium* complex. This bacterium was identified as *M. chelonae* by the DNA–DNA hybridization method. Drug-sensitivity tests revealed the resistance to streptomycin, kanamycin and most standard antituberculosis agents, including rifampin, rifabutin and ethambutol. The organism was

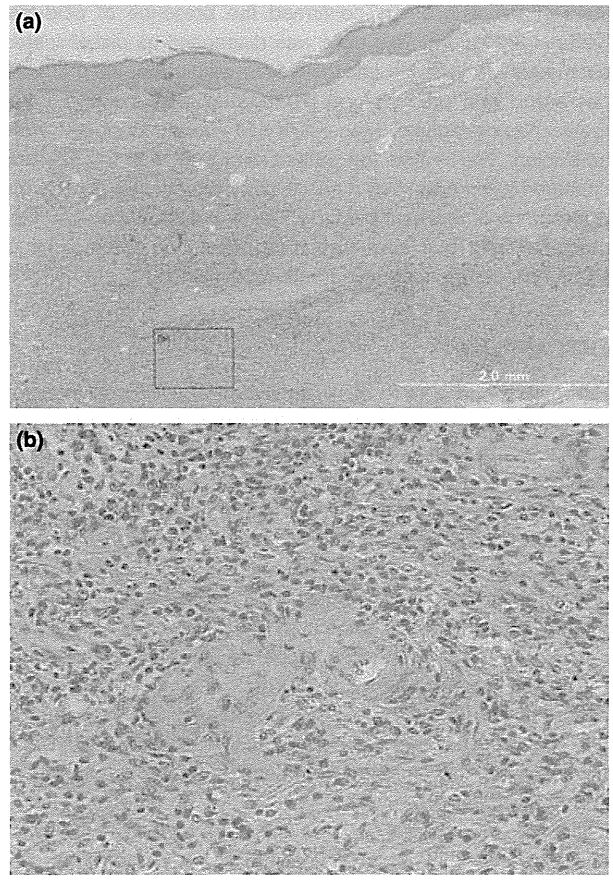

Figure 2. (a) Clusters of inflammatory cells mixed with neutrophils and lymphocytes in the dermis and subcutaneous tissue (hematoxylin–eosin [HE], original magnification ×40). (b) Fibrinoid necrosis and red blood cell extravasation were also found around the dermal vessels (HE, ×200).

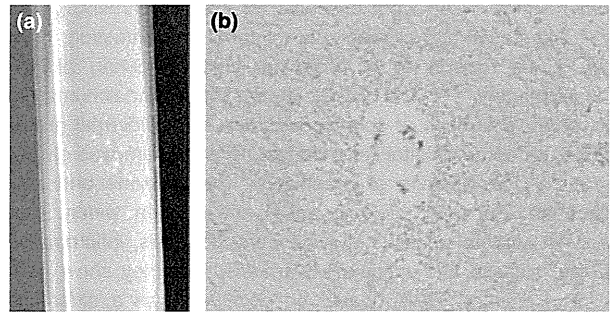

Figure 3. (a) Colonies on Ogawa's culture medium were moist milky white. (b) Ziehl–Neelsen stain of the skin tissue showed the presence of red rod-shaped microorganisms surrounded by inflammatory cells (original magnification ×200).

susceptible to clarithromycin, levofloxacin and ethionamide, and the minimum inhibitory concentration of clarithromycin was lowest at 1 mg/mL among these drugs. Ziehl–Neelsen

stain of the skin tissue confirmed the presence of acid-fast bacterium (Fig. 3b). This case was diagnosed as disseminated cutaneous *M. chelonae* infection. After admission, we gradually decreased oral prednisolone (10 mg/day) and stopped betamethasone phosphate. Etanercept had already stopped before admission. We treated the patient with both 800 mg/day oral clarithromycin and 500 mg/day levofloxacin to avoid the appearance of resistant bacteria. The erythema turned into pigmentation and pus discharge disappeared after 6 months of treatment, but the fibrous indurations remained.

DISCUSSION

Here, we report a case of disseminated *M. chelonae* infection mimicking cutaneous vasculitis. Cutaneous *M. chelonae* infection presents with a variety of skin eruptions which include abscesses, erythema, nodules, ulcers, cellulitis and "sporotrichoid" lesions. Therefore, previous reports show that cutaneous *M. chelonae* infection mimics suture granuloma after skin surgery, panniculitis, lupus erythematosus eruptions or soft tissue tumor.[6–8] The most frequent areas of skin involvement, the extremities, are also common to *M. chelonae* infection and cutaneous vasculitis. Before repeating bacterial cultures, we suspected eosinophilic granulomatosis with polyangiitis according to the history of asthma and pathological examination. We consider that the pathological findings of vasculitis in the biopsy specimen came from secondary change caused by *M. chelonae* infection.

Immunosuppression plays an important role in the incidence and progression of *M. chelonae* infection. Our case had risk factors such as long-term systemic corticosteroids and a TNF inhibitor, etanercept. Etanercept acts as a decoy receptor that binds to TNF.[9] TNF is a cytokine produced mainly by activated macrophages and is involved in cell survival, apoptosis, activation of pro-inflammatory mediators and the innate immune response in the defense mechanism against pathogens.[10,11] Roach *et al.*[12] said that TNF regulated the early induction of chemokines which were essential for cell recruitment, granuloma formation and clearance of mycobacterial infection. There are some reports of *M. chelonae* infection during biologic agent therapy.[13–15] The biologic agents include adalimumab for rheumatoid arthritis or spondyloarthritis, and epidermal growth factor receptor inhibitor for a cancer. One of them was given a combined application of prednisone, methotrexate and adalimumab. Recently, biologic agents have been widely used against various diseases (e.g. cancers, psoriasis, inflammatory bowel disease and rheumatoid arthritis) and they show good clinical efficacy. RGM should be considered as a differential diagnosis of infections in patients under biologic agents.

It is essential to examine drug sensitivities *in vitro* to determine treatment strategies. RGM such as *M. chelonae* are resistant to general antituberculosis drugs. However, they are widely recognized to be sensitive to tobramycin, amikacin, imipenem and clarithromycin.[4] Clarithromycin has been reported to be most effective against *M. chelonae*. In a clinical trial, clarithromycin alone is adequate for treatment of cutaneous *M.* chelonae infection,[16] although several recent cases of clarithromycin resistance have been reported.[17,18] Multidrug therapy for 6 months is supported as a recommended treatment. In our case, we chose 800 mg/day clarithromycin and 500 mg/day levofloxacin as a result of the drug-sensitivity test. After 6 months of the treatment, erythema with tenderness and pus discharge disappeared. Ota *et al.*[19] suggested that thermal therapy is effective and safe when used in combination with antibiotics, considering that the optimum growth temperature of *M. chelonae* is 28–30°C. Thermal therapy should be good choice for localized lesions.

In summary, we report a case of disseminated cutaneous *M. chelonae* infection mimicking cutaneous vasculitis. In a patient with immunosuppression, cutaneous RGM infections can be disseminated. Repeated bacterial examinations are important and required for the diagnosis of RGM.

CONFLICT OF INTEREST: None declared.

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