Ceramide accelerates ultraviolet-induced MMP-1 expression through JAK1/STAT-1 pathway in cultured human dermal fibroblasts

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Abstract **Ultraviolet (UV) irradiation accelerates formation of ceramide through hydrolysis of sphingomyelin and de novo synthesis. Here, we investigated the effects of ceramide on UV-induced matrix metalloproteinase-1 (MMP-1) expression in human dermal fibroblasts. Our results showed that acidic-sphingomyelinase (aSMase) and MMP-1 mRNA expression were increased by UV irradiation. Treatment of D609 (aSMase inhibitor) decreased the level of basal and UV-induced MMP-1 expression. On the other hand, basal and UV-induced MMP-1 expression was increased through induction of intracellular ceramide by D-MAPP, a ceramidase inhibitor. Our results also showed that MMP-1 protein expression was dose-dependently increased by C_2-ceramide or SMase treatment. The activation of ceramide pathway by C_2-ceramide enhanced phosphorylation of signal transducer and activators of transcription-1 (STAT-1), whereas ceramide-induced MMP-1 expression was potently prevented by piceatannol; Janus kinase (JAK1) inhibtor; and WHI-P131, a specific inhibitor of JAK3; but not by AG490, JAK 2 inhbitor, in human dermal fibroblasts. We also found that UV induced the phosphorylation of STAT-1, and UV-induced MMP-1 expression was significantly decreased by JAK1 inhibitor, piceatannol. Overall, we demonstrate that induction of intracellular ceramide by UV may activate MMP-1 gene expression via JAK1/STAT-1 pathway. Therefore, we suggest that targeted modulation of the ceramide signaling pathway may offer a novel therapeutic approach for inhibiting MMP-1 expression, which is a causing gene of skin aging.—Kim, S., Y. Kim, Y. Lee, and J. H. Chung. Ceramide accelerates ultraviolet-induced MMP-1 expression through JAK1/STAT-1 pathway in cultured human dermal fibroblasts. *J. Lipid Res.* 2008. 49: 2571–2581.**

Supplementary key words aSMase • UV • JAK3

UV (UV) irradiation is a major cause of epidermal inflammation, immunosuppression, altered epidermal permeability barrier function, premature aging, dyspigmentation, and the development of nonmelanoma and melanoma skin cancers (1–4). In addition, UV significantly increases the amount of intracellular ceramide, which is a major component of skin lipid and a lipid second messenger (5, 6). In addition, UV-damaged skin was more resistant to damage than normal skin, indicating improvement of the barrier function through induction of the amount of all stratum corneum lipids (7). These effects may be related to an increase in the stratum corneum ceramides (8). Activation of SMase by UV may be rapidly generated ceramide from hydrolysis of sphingomyelin at the plasma membrane (9).

Ceramide has a number of important physiological functions that regulate cellular homeostasis, such as cell proliferation, differentiation, and apoptosis (10–13). As an intermediate in sphingomyelin biosynthesis, ceramide plays a key role in the metabolism of molecules that comprise membranes (14, 15). In addition, membrane ceramides contribute to membrane structure by influencing membrane lipid ordering by affecting membrane porosity and by altering the permeability of cell membranes (15, 16). Ceramide activates downstream targets including ceramide activated protein kinase, stress-activated protein kinase/c-Jun N-terminal kinase (JNK), protein kinase C, ceramide activated phosphatase, cathepsin D, phospholipase A2, phospholipase D, and nuclear factor-κB cells, depending on tissue type (9, 17–20). Previously, triggering the ceramide pathway in dermal fibroblasts with exogenous ceramide

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Abbreviations: aSMase, acidic sphingomyelinase; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MOI, multiplicity of infection; MMP-1, matrix metalloproteinase-1; MTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxan; STAT-1, signal transducer and activators of transcription-1; UV, ultraviolet.

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was reported to activate both ERK1/2 and SAPK/JNK, and the ceramide-elicited induction of the endogenous MMP-1 gene was potently inhibited by PD98059, a MEK1 specific inhibitor (20). Also, Rodriguez-Lafrasse et al. and Mazière et al. (21, 22) suggested that ceramide enhances STAT-1 DNA binding activity, indicating that physio-pathological stimuli that generate endogenous ceramide also stimulate the JAK/STAT pathway. v-Src constitutively activates, which phosphorylates and activates STAT-3, thereby amplifying this signaling pathway (23).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent metalloendopeptidases that are collectively capable of degrading essentially all extracellular matrix components (24, 25). MMPs play an important role in tissue remodeling during fetal development, angiogenesis, and tissue repair, and they are also responsible for excessive breakdown of connective tissue in inflammatory disorders, such as rheumatoid arthritis, osteoarthritis, autoimmune blistering disorders of skin, dermal photoaging, and periodontitis (24, 25). In addition, degradation of basement membrane and extracellular matrix by MMPs is crucial for invasion and metastasis of tumor cells (24, 25). In particular, MMP-1 is the principal neutral proteinase capable of degrading native fibrillar collagens of types I, II, III, and V, and it apparently plays an important role in the remodeling of collagenous connective tissues in various physiological and pathological situations (24–26).

In this study, we show for the first time that the level of intracellular ceramide can regulate basal and UV-induced MMP-1 expression in human dermal fibroblasts. In particular, ceramide up-regulates MMP-1 expression through activation of JAK1/STAT-1 pathway in human dermal fibroblasts. Therefore, we suggest that targeted modulation of this pathway may offer a novel approach for therapeutic inhibition of matrix degradation.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), antibiotics, and TRIzol reagent were purchased from Life Technologies, (Rockville, MD). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Rabbit polyclonal anti-phospho-STAT-1 and total-STAT-1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-MMP-1 was from Oncogene (San Diego, CA). SMase (from *Staphylococcus aureus*), C_2-, C_6-, C_8-, C_{16}-ceramide, UO126, SP600125, piceatannol, AG490, and WHI-P131 were purchased from Calbiochem (San Diego, CA). D-MAPP and D609 were purchased from Biomol (Plymouth Meeting, PA).

Cell cultures

Primary human dermal fibroblasts were cultured from foreskins of healthy donors aged 20–30 years. The skin was minced and incubated with collagenase (1 mg/ml in DMEM) for 1–2 h at 37°C. Collagenase was then removed by washing with DMEM. The isolated cells were allowed to attach on plastic plates and cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicilline, and 100 μg/ml streptomycin. After six and eight passages the fibroblasts were used for experiments.

Chemical treatment

For experiments, the cells were maintained in serum-free media for 24 h. Thereafter, C_2-ceramide or SMase were added at the indicated concentration and the cells were further incubated for 72 h in serum-free media. In the experiments involving MEK inibitor, UO126, JNK inhibitor, SP600125, JAK1 inhibitor, piceastannol, JAK2 inhibitor, AG490, and JAK3 inhibitor, WHI-P131, respectively, each inhibitor was added to the cultures 30 min prior to the addition of UV irradiation or C_2-ceramide.

UV irradiation

Philips TL 20W/12 RS fluorescent sun lamps with an emission spectrum between 275 and 380 nm (peak, 310–315 nm) were used as a UV source, and a Kodacel filter (TA401/407; Kodak, Rochester, NY) was used to block UVC, which has wavelengths of <290 nm. UV strength was measured using a Waldmann UV meter (model 585100) (27). After serum starvation for 24 h, the cells were pretreated with D609 or D-MAPP for 30 min prior to UV treatment and then further culture for 72 in serum-free media.

MTT assay

To estimate the viability of cells after incubation with C_2-ceramide for 72 h, cells were washed with PBS. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to quantify living, metabolically active cells. Mitochondrial dehydrogenases metabolize MTT to a purple formazan dye, which is measured photometrically at 570 nm (28). Cell morphology was assessed by phase contrast microscophy (DP50, Olympus, Tokyo, Japan).

RT-PCR

Total RNA was extracted from cells using the TRIzol reagents as recommended by manufacturer. Isolated RNA samples were then used for RT-PCR. Samples of 1 μg total RNA were reverse transcribed into cDNA in 20 μl reaction mixtures using a first-strand cDNA synthesis kit for RT-PCR according to the manufacturer's protocol (MBI Fermentas, Lithuania). Semi-quantitative PCR was performed with 1 μl of the first-strand cDNA product using the primers for human genes listed in **Table 1** (29, 30). The PCR amplifications were carried out in cycle numbers correspondent to logarithmic amplification phase. Reaction products were electrophoresed in 2% agarose gels and visualized with ethidium bromide. The signal strengths were quantified using a densitometric program (Bio 1D; Vilber Lourmat, Marne La Vallec, France).

Western blotting

Cells were lysed with lysis buffer [50 mM Tris-HCl, (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT] containing 1% Triton X-100. Insoluble debris was removed by centrifugation at 12,000 rpm for 15 min, and protein content was determined using Bradford reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were resolved on gradient 10% SDS-PAGE gels and then electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon P; Amersham, Buckinghamshire, UK). Membranes were subsequently blocked with 5% skim milk in TBS/T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.05% Tween 20) and incubated with the indicated antibodies against MMP-1 and STAT-1. Blotting proteins were visualized by enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK). The signal strengths were quantified using a densitometric program (Bio 1D; Vilber Lourmat, Marne La Vallec, France).

TABLE 1. Primer sequences and RT-PCR conditions

Gene name	Primer sequences	PCR conditions	Size
aSMase			
Forward	CAG GGT TCC TGG CTG GGC AGC A	23 cycles of 94°C/40sec,	687 bp
Reverse	GGT CCT GGA CCA TGA GAC CTA C	65°C/40 sec, 72°C/40sec	
MMP-1			
Forward	ATT CTA CTG ATA TCG GGG CTT TGA	28 cycles of 94°C/40sec,	409 bp
Reverse	ATG TCC TTG GGG TAT CCG TGT AG	60°C/60 sec, 72°C/60sec	
36B4			
Forward	TGG GCT CCA AGC AGA TGC	21 cycles of 94°C/60sec,	413 bp
Reverse	GGC TTC GCT GGC TCC CAC	60°C/40 sec, 72°C/60sec	

Zymography

To assess the gelatinolytic activities of MMP-2 in culture media, equal amounts of supernatant were subjected to gelatin zymography using zymogram gels containing 10% gelatin, according to manufacturer's protocol (NOVEX, San Diego, CA). After electrophoresis, gels were renatured by incubating in renaturing buffer (50 mM Tris–HCl, pH 7.4, 2% Triton X-100) for 30 min at room temperature. Gels were then incubated in developing buffer (50 mM Tris, pH 8.0, 2.5 mM $CaCl_2$, 0.02% NaN_3) for 24 h at 37°C. Proteolytic bands were visualized by staining gels with 0.5% Coomassie brilliant blue solution.

Adenoviral gene transfer

Adenoviral human STAT-1 cDNA was obtained from Vector Biolabs (Philadelphia, PA). Briefly, for infection, recombinant adenovirus was diluted in DMEM containing 10% FBS, and added to the cells at 37°C for 24 h. After this, the media were replaced with serum-free media for 24 h and then treated with 10 μM C_2-ceramide for 72 h in serum-free media. The expression of each construct was confirmed by Western blotting.

Acidic SMase siRNA transfection

We purchased aSMase siRNA from Bioneer (Daejeon, Korea). The siRNA sequences were: human aSMase (sense: AAA GUC UUA UUC ACU GCU CUC; anti-sense: GAG AGC AGU GAA UAA GAC UUU). Silencer® Negative Control #1 siRNA (Ambion, Cambrige, UK) was used as scrambled control siRNA. Effectene (Qiagen, Valencia, CA) was used for transfections with siRNA (50 pM or as noted), following protocols provided by the manufacturer. Fresh serum-free media were added 8 h after the 48 h transfection.

Statistical analysis

Statistical significance was determined using the Student's *t*-test. Results are presented as means ± SEM. All quoted *P* values are two-tailed and differences are considered significant when $P < 0.05$.

RESULTS

ASMase and MMP-1 mRNA expression are enhanced by UV irradiation in human dermal fibroblasts

Acute cellular stress, including reactive oxygen species, UV irradiation, or inflammatory cytokines, is associated with increased cellular ceramide levels and aSMase activity (9, 17). We investigated the aSMase and MMP-1 mRNA expression induced by UV irradiation in cultured human dermal fibroblasts. The cells were exposed to various doses of UV irradiation and then further cultured for 8 h in serum-free media. Our results showed that aSMase and MMP-1 mRNA expression were dose-dependently increased by UV irradiation (**Fig. 1A**). The levels of aSMase and MMP-1 mRNA expression were increased significantly by 682% and 710% of control level by 100 mJ/cm^2 UV irradiation, respectively. The primer sequences of aSMase and MMP-1 were given in Table 1. The cell viabilities were not affected by UV irradiation as estimated by MTT assays (Fig. 1B).

On the other hand, we investigated the effect of aSMase siRNA on basal level of MMP-1 expression. After aSMase siRNA transfection for 48 h, cells were incubated for 24 h in serum-free media and then added with fresh serum free media for 8 h. We demonstrated that basal level of aSMase mRNA was decreased by aSMase siRNA transfection using the RT-PCR (Fig. 1C). In addition, MMP-1 protein expression was decreased by 50% of control level (Fig. 1C). Our results suggest that the induction of aSMase by UV irradiation may increase the intracellular ceramide level and then increase the level of MMP-1 expression in human dermal fibroblasts.

Basal and UV-induced MMP-1 expression is inhibited by D609, an aSMase inhibitor, in human dermal fibroblasts

To inhibit aSMase activity, we treated the cells with aSMase inhibitor, D609, for 8 h (mRNA) and 72 h (protein), respectively, at the indicated concentration, and then further cultured for 72 h in serum-free media. Basal MMP-1 mRNA (**Fig. 2A**) and protein expressions (Fig. 2B) were dose-dependently decreased by D609 treatment in whole cell lysates and culture media, respectively. The levels of MMP-1 mRNA and protein were decreased to 29.6 ± 9.2% and 2.8 ± 0.7% of control level, respectively, by 80 μM D609 treatment.

In the next study, we examined the effect of D609 on UV-induced MMP-1 mRNA and protein expression. The cells were pretreated with D609 (40 and 80 μM, respectively) for 30 min prior to 100 MJ/cm^2 UV irradiation and then further cultured for 8 h (mRNA) and 72 h (protein), respectively, in serum-free media. UV-induced MMP-1 mRNA (Fig. 2C) and protein (Fig. 2D) expressions were significantly decreased by D609 treatment in whole cell lysates and culture media, respectively. UV-induced MMP-1 mRNA and protein expressions were significantly increased by 526.1 ± 131.7% and 404.7 ± 69.5% of control level, respectively, by 100 mJ/cm^2 UV irradiation. However, UV-induced MMP-1 mRNA and protein expres-

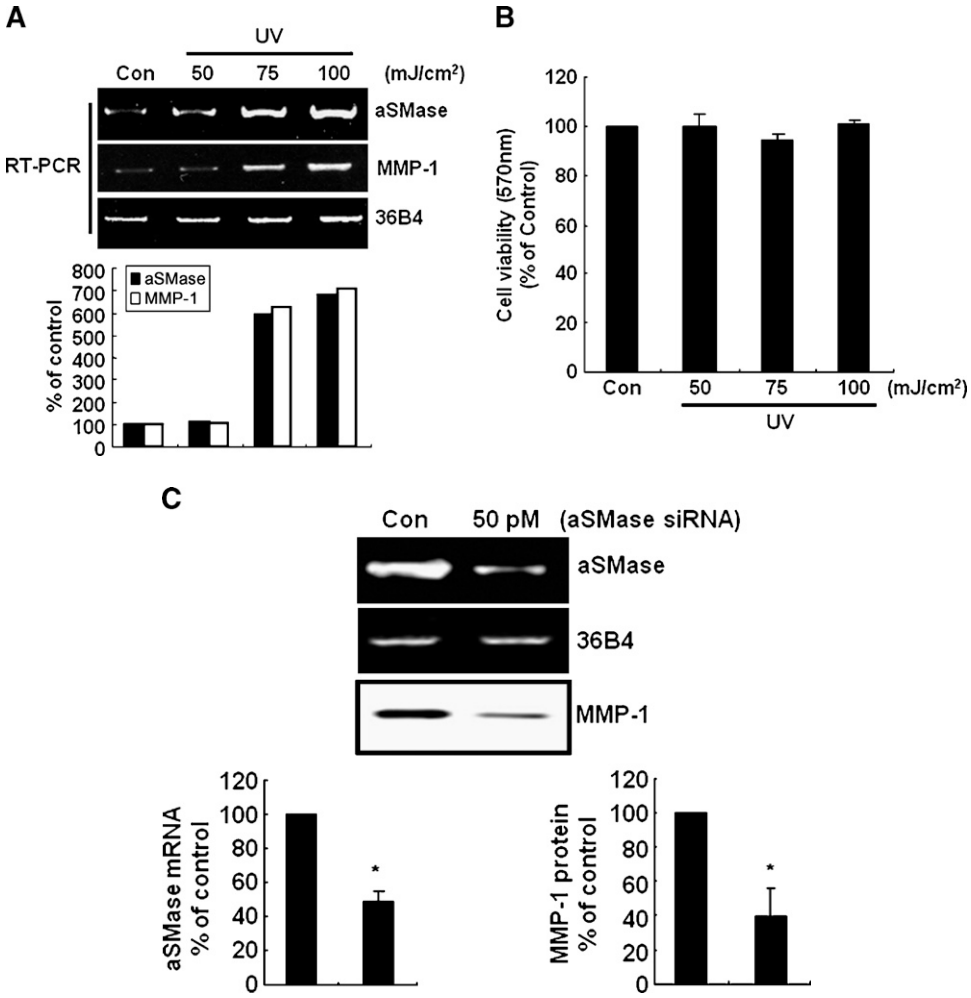

Fig. 1. The levels of acidic sphingomyelinase (aSMase) and matrix metalloproteinase-1 (MMP-1) mRNA expression are increased by ultraviolet (UV) irradiation in cultured human dermal fibroblasts. A: After serum-starvation for 24 h, the cells were exposed to various doses of UV irradiation and then further incubated for 8 h. B: After serum-starvation for 24 h, the cells were exposed to various doses of UV irradiation and then further incubated for 72 h. The cell viabilities were measured by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxan (MTT) assays. C: Cells were transfected with aSMase siRNA for 48 h and then further incubated for 8 h in serum-free media. The levels of aSMase and MMP-1 mRNA expression were analyzed by RT-PCR. The level of MMP-1 protein expression was analyzed by Western blotting. The results were representative of three independent experiments. Values shown are means ± SEM. * $P < 0.05$ vs. Con. Con, control.

sions were decreased significantly by 122.7 ± 62% and 95.7 ± 14% of control level, respectively, by 80 μM D609 treatment. Thus, aSMase activity plays an important role on UV-induced MMP-1 expression, and the inhibition of aSMase activity suppresses basal and UV-induced MMP-1 expressions through blockage of ceramide synthesis in cultured human dermal fibroblasts.

Basal and UV-induced MMP-1 expression is increased by D-MAPP, a ceramidase inhibitor, in cultured human dermal fibroblasts

To induce intracellular ceramide levels, we treated human dermal fibroblasts with ceramidase inhibitor, D-MAPP for 8 h (mRNA) and 72 h (protein), respectively, at the indicated concentration. Our results showed that basal levels of MMP-1 mRNA (**Fig. 3A**) and protein (Fig. 3B) were dose-dependently increased by D-MAPP treatment in whole cell lysates and culture media, respectively. The levels of MMP-1 mRNA were increased by 244.7 ± 12.7% and 262.1 ± 34.6% of control level by 5 and 10 μM D-MAPP treatment, respectively. MMP-1 protein expression was also increased by 4846.7 ± 1055.5% and 17457 ± 5786% of control level by 5 and 10 μM D-MAPP treatment, respectively.

Next, we investigated the effect of D-MAPP on UV-induced MMP-1 expression. The cells were pretreated with 10 μM D-MAPP for 30 min prior to 100 mJ/cm^2 UV irradiation and then further cultured for 8 h (mRNA) and 72 h (protein) in serum-free media. UV-induced MMP-1 mRNA (Fig. 3C) and protein (Fig. 3D) were significantly increased by 10 μM D-MAPP in whole cell lysates and culture media, respectively. UV-induced MMP-1 mRNA and

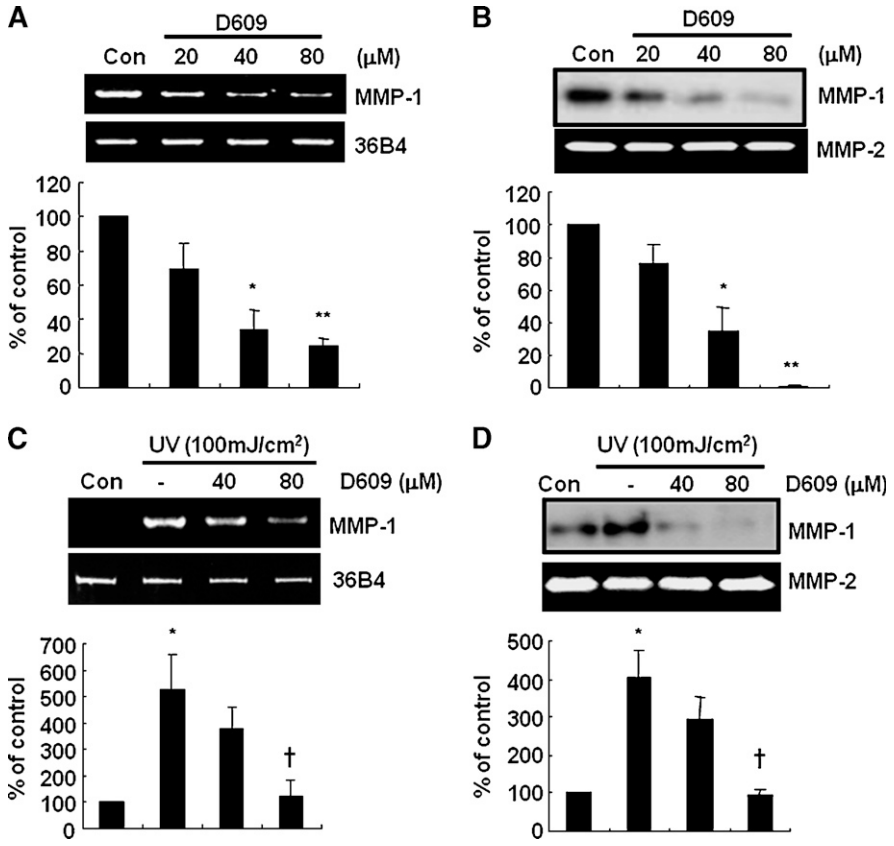

Fig. 2. Basal and UV-induced MMP-1 expression are inhibited by D609, an aSMase inhibitor, in human dermal fibroblasts. A, B: After serum-starvation for 24 h, the cells were pretreated with D609 at the indicated concentrations and then further cultured at 37°C for 8 h (mRNA) and 72 h (protein), respectively. C, D: The cells were pretreated with D609 at the indicated concentrations prior to 100 mJ/cm^2 UV treatment and then further incubated at 37°C for 8 h (mRNA) and 72 h (protein), respectively. MMP-1 mRNA (cell lysates) and protein (culture media) levels were analyzed by RT-PCR (A, C) and Western blotting (B, D), respectively. MMP-2 expression in culture media was analyzed by zymography. The results were representative of three independent experiments. Values shown are means ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. Con. † $P < 0.05$ vs. UV-treated cells. Con, control.

protein expressions were increased significantly by 3234 ± 1303.6% and 5363 ± 1440.6% of control level, respectively, by 100 mJ/cm^2 UV irradiation. In addition, these effects were more increased by 6450 ± 780.8% and 12427 ± 2830.1% of control level, respectively, by 10 μM D-MAPP treatment. Therefore, our results suggest that the ceramidase inhibitor, D-MAPP, suppresses the conversion ceramide into sphingosine and thus may enhance intracellular ceramide levels. We demonstrated that basal and UV-induced MMP-1 expression is related with intracellular ceramide levels in human dermal fibroblasts.

MMP-1 expression is increased by ceramide or SMase in human dermal fibroblasts

We investigated the effects of ceramide on the expression of MMP-1 protein. After 24 h serum starvation, cells were treated with 10 μM C_2-, C_6-, C_8-, and C_{16}-ceramide, respectively, for 72 h in serum-free media. We demonstrated that all types of ceramide significantly increased MMP-1 expression (**Fig. 4A**). Those ceramides showed decreasing abilities in MMP-1 induction with increasing chain length of ceramide. Because C_2-ceramide has the strongest effect on MMP-1 induction, we treated cells with C_2-ceramide for further experiments.

To investigate the dose response of ceramide on cell viabilities, we treated the cells with C_2-ceramide for 72 h at the indicated concentration in serum-free media. As shown in Fig. 4B, cell viabilities were greatly decreased to 24.3 ± 5.8% and 22.2 ± 5.2% of control level upon treatment with 20 μM and 50 μM C_2-ceramide, respectively. Based on phase contrast microscopy, the number of surviving cells was also significantly decreased by 20 μM and 50 μM C_2-ceramide (Fig. 4C).

In culture media, MMP-1 protein expression was dose-dependently increased by C_2-ceramide (Fig. 4D). The level of MMP-1 protein was increased significantly by 2318 ± 386.8%, 12583 ± 1177.4% and 16691 ± 144.9% of control level with 1 μM, 5 μM, and 10 μM of C_2-ceramide, respectively.

Next, we treated the cells with SMase to trigger the ceramide pathway through enzyme hydrolysis of sphingomyelin for 72 h in serum-free media. MMP-1 protein expression

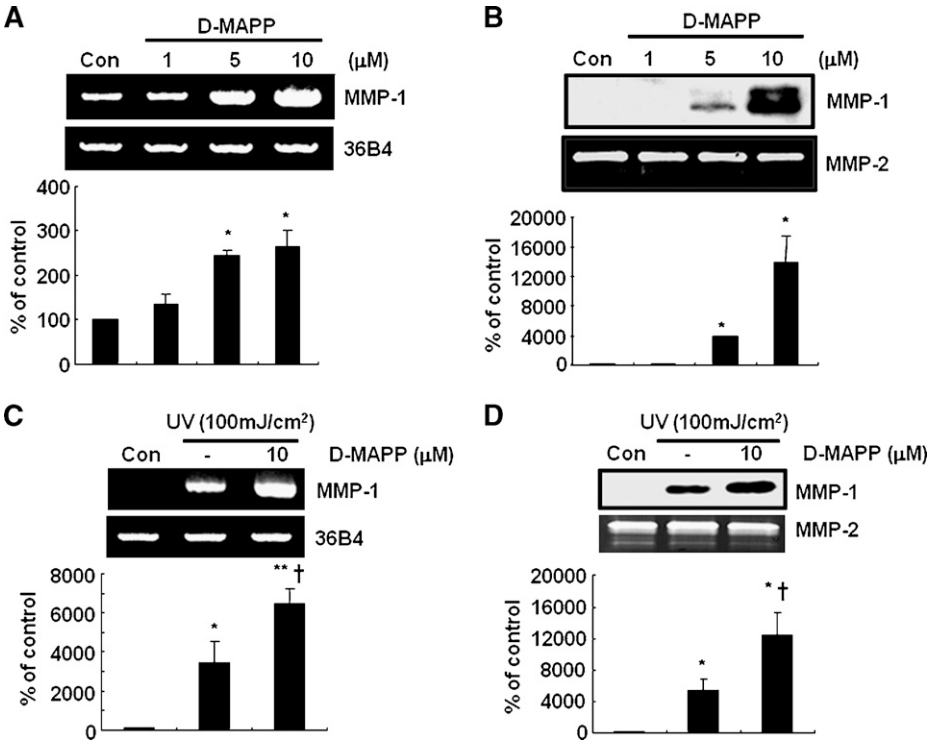

Fig. 3. Basal and UV-induced MMP-1 expression are increased by D-MAPP, a ceramidase inhibitor, in cultured human dermal fibroblasts. A, B: After serum-starvation for 24 h, the cells were pretreated with D-MAPP at the indicated concentrations and then further incubated at 37°C for 8 h (mRNA) and 72 h (protein), respectively. C, D: The cells were pretreated with 10 μM D-MAPP prior to 100 mJ/cm^2 UV treatment and then further incubated at 37°C for 8 h (mRNA) and 72 h (protein), respectively. MMP-1 mRNA (cell lysates) and protein (culture media) levels were analyzed by RT-PCR (A, C) and Western blotting (B, D), respectively. MMP-2 expression in culture media was analyzed by zymography. The results were representative of three independent experiments. Values shown are means ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. Con. † $P < 0.05$ vs. UV-treated cells. Con, control.

was dose-dependently increased by SMase treatment (Fig. 4E). The level of MMP-1 protein expression was increased significantly by 331 ± 28%, 486.5 ± 83.5%, 630.5 ± 1.5%, and 1011.5 ± 230.5% of control level with 1, 10, 50, and 100 mU of SMase, respectively. Therefore, the enhancement of intracellular ceramide level by C_2-ceramide or SMase treatment up-regulates MMP-1 expression in cultured human dermal fibroblasts.

Phosphorylation of STAT-1 was increased by C_2-ceramide and ceramide-induced MMP-1 expression is prevented by JAK1 inhibitor, piceatannol, or JAK3 inhibitor, WHI P131, but not by JAK2 inhibitor, AG490

To investigate the regulation of ceramide-induced MMP-1 expression, we treated cells with 10 μM C_2-ceramide for the indicated time in serum-free media. Ceramide-induced STAT-1 phosphorylation showed a maximal increase at 15 min (**Fig. 5A**). Furthermore, we investigated the involvement of JAKs/STAT-1 pathway in ceramide-induced MMP-1 expression. We pretreated with JAK1 inhibitor, piceatannol, JAK2 inhibitor, AG490, and JAK3 inhibitor, WHI-P131, respectively, for 30 min at the indicated concentration prior to C_2-ceramide treatment and then further incubated the cells for 72 h. Ceramide-induced MMP-1 expression was dose-dependently inhibited by JAK1 inhibitor, piceatannol (Fig. 5B) or JAK3 inhibitor, WHI-P131 (Fig. 5D), respectively, in the culture media of human dermal fibroblasts. However, JAK2 inhbitor, AG490, did not affect the MMP-1 induction by ceramide (Fig. 5C). Therefore, these results demonstrate that ceramide triggers the induction of MMP-1 through JAK1 and JAK3/STAT-1 pathways in cultured human dermal fibroblasts.

To confirm the regulation of MMP-1 expression via JAK/STAT-1 pathway, we infected the cells with adenoviral STAT-1 gene [10, 25, 50 multiplicity of infection (MOI), respectively] for 24 h and then treated with 10 μM C_2-ceramide for 72 h in serum-free media. Our results showed that ceramide-induced MMP-1 expression was increased by STAT-1 overexpression (25 and 50 MOI, respectively) in culture media (Fig. 5E). Based on these results, we suggest that increasing of intracellular ceramide level up-regulates MMP-1 expression through JAK1 and JAK3/STAT-1 pathway.

UV-induced MMP-1 expression is suppressed by JAK1 inhibitor, piceatannol, but not by JAK3 inhibtor, WHI-P131 in human dermal fibroblasts

Next, we investigated the effects of JAK inhibitors on UV-induced MMP-1 expression in cultured human dermal fibroblasts. We pretreated cells with JAK1 inhibitor,

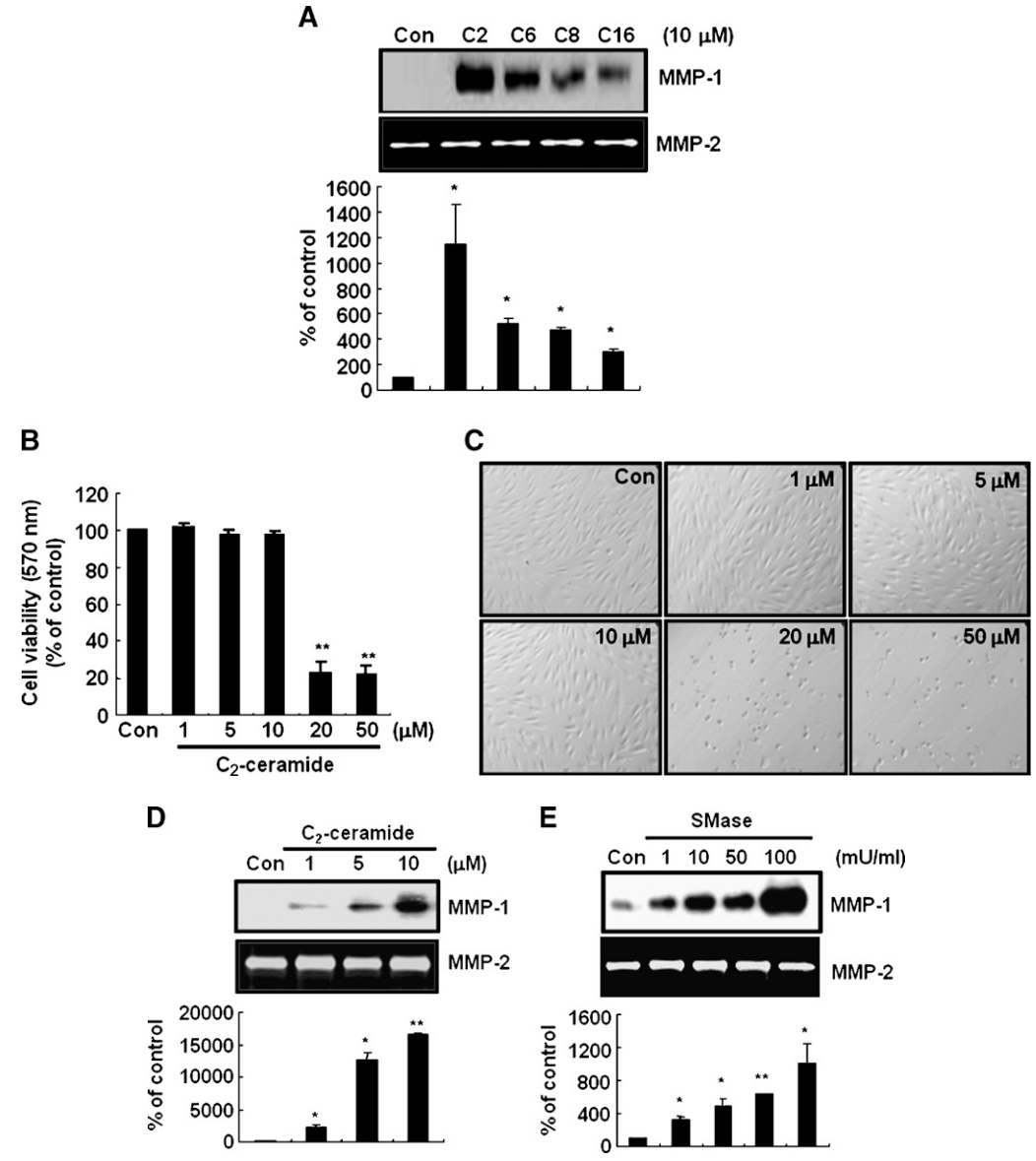

Fig. 4. MMP-1 expression is dose-dependently increased by ceramide and SMase in cultured human dermal fibroblasts. A: After serum starvation for 24 h, the cells were treated with 10 μM C_2-, C_6-, C_8-, and C_{16}-ceramide, respectively, for 72 h. After serum-starvation for 24 h, the cells were treated with C_2-ceramide (B–D) or SMase (E) at the indicated concentrations for 72 h. B: Cell viabilities were measured by MTT assay. C: Cell morphology was assessed by phase contrast microscopy (× 400). D, E: MMP-1 and MMP-2 expressions in culture media were determined by Western blotting and zymography, respectively. The results were representative of three independent experiments. Values shown are means ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. Con. Con; control.

piceatannol, and JAK3 inhibitor, WHI-P131, respectively, for 30 min prior to UV irradiation and then further incubated for 72 h in serum-free media. UV-induced MMP-1 expression was dose-dependently decreased by JAK1 inhibitor, piceatannol (**Fig. 6A**), whereas JAK3 inhibitor, WHI-P131, did not affect UV-induced MMP-1 expression (Fig. 6B). Therefore, our results suggest that JAK1/STAT-1 pathway plays an important role on UV-induced MMP-1 expression in cultured human dermal fibroblasts.

To verify the regulatory mechanism of UV-induced MMP-1 expression, we pretreated cells with MEK inhibitor, UO126, JNK inhibitor, SP600125, and JAK1 inhibitor, piceatannol, respectively, for 30 min prior to UV irradiation. As shown in Fig. 6C, the phosphorylation of extracellular signal-regulated kinase (ERK), JNK, and STAT-1 were significantly increased after 10 min following UV treatment. Interestingly, MEK inhibitor and JNK inhibitor also suppressed the phosphorylation of STAT-1. JAK1 inhibitor, piceatannol, significantly inhibited UV-induced STAT-1 phosphorylation, whereas it did not affect the UV-induced phosphorylation of ERK and JNK (Fig. 6C). Therefore, our results indicate that STAT-1 may be a down-

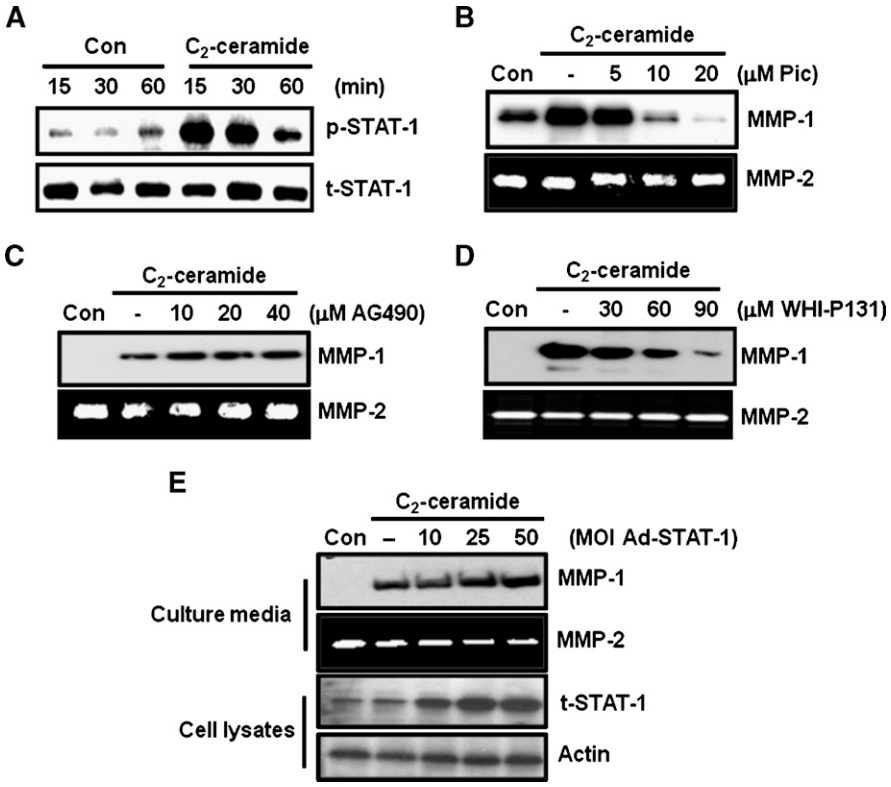

Fig. 5. Phosphorylation of STAT-1 is increased by C_2-ceramide and ceramide-induced MMP-1 expression is prevented by JAK1 inhibitor, piceatannol, and JAK3 inhibitor, WHI P131 in cultured human dermal fibroblasts. A: After serum-starvation for 24 h, cells were treated with 10 μM C_2-ceramide for indicated times. Using the whole cell lysates, phosphorylation of STAT-1 was measured by Western blotting with anti-phospho- and total-STAT-1 antibodies. B–D: After serum-starvation for 24 h, the cells were pretreated for 30 min JAK1 inhibitor, piceatannol (B), JAK2 inhibitor, AG490 (C), and JAK3 inhibitor, WHI-P131 (D) by the indicated concentration prior to 10 μM C_2-ceramide treatment and then further incubated at 37°C for 72 h. E: After infection of Ad-STAT-1 gene [10, 25, and 50 multiplicity of infection (MOI), respectively] for 24 h, the cells were treated with 10 μM C_2-ceramide and then further cultured for 72 h in serum-free media. MMP-1 and MMP-2 expressions in culture media were measured by Western blotting and zymography, respectively. The results were representative of three independent experiments. Con, control.

stream target of ERK and JNK pathways in cultured human dermal fibroblasts.

DISCUSSION

Ceramide is a major component of skin lipid, along with cholesterol and fatty acid, and plays a critical role in structuring and maintaining the water permeability barrier function of the skin (31). Activation of SMase occurs in response to a variety of stimuli including cytokines, G protein-coupled receptors and cellular stress (UV irradiation) (9, 11, 32); ceramide can then be generated rapidly by SMase-catalyzed hydrolysis of sphingomyelin at the plasma membrane (33). Also, ceramide has been implicated in apoptosis, cellular senescence, growth arrest, and differentiation (9, 15, 32, 34). Consistent with these reports, our results also show that the aSMase mRNA level is dose-dependently increased by UV irradiation. Therefore, our results suggest that the induction of aSMase mRNA expression may stimulate the synthesis of intracellular ceramide in human dermal fibroblasts.

Ceramide production by UV irradiation is mediated by activation of acid SMase and neutral SMase (35). The UV-induced apoptotic signaling pathway is known to be required for activation of aSMase (34). aSMase activity is also known to be involved in the apoptotic pathway initiated by TNF-α (36) and Fas ligand (37). In addition, aSMase deficient cells, Niemann-Pick lymphocytes, and thymocytes derived from aSMase- knockout mice do not undergo apoptosis in response to UV radiation (8). Consistent with these reports, our results showed that basal and UV-induced MMP-1 mRNA and protein expressions are significantly decreased by the aSMase inhibitor, D609, in cultured human dermal fibroblasts. Therefore, we suggest that inhibition of ceramide synthesis by aSMase inhibitor (D609) suppresses UV-induced MMP-1 expression in human dermal fibroblasts.

Recently, it has been reported that D609 significantly decreased the generation of ceramide in human immortalized keratinocytes, HaCaT cells (38), and that D609

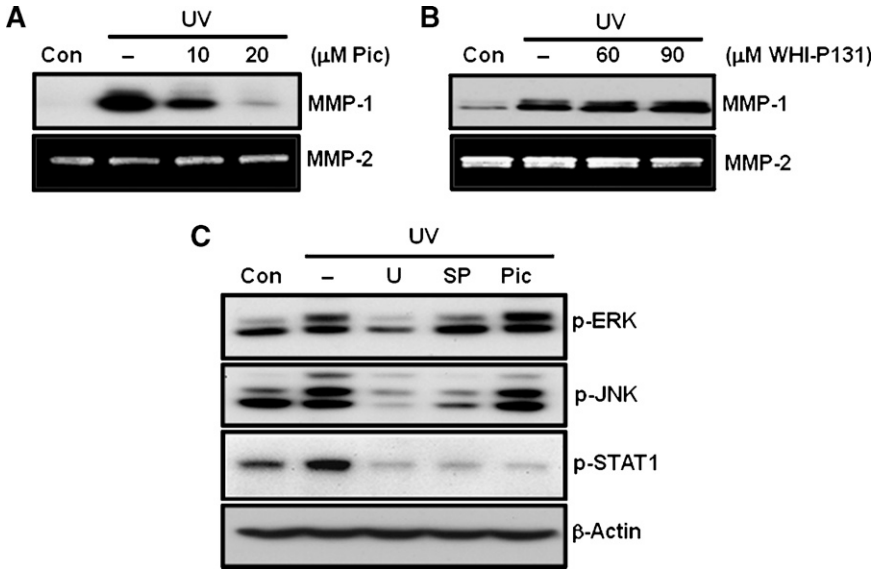

Fig. 6. UV-induced MMP-1 expression is inhibited by JAK1 inhibitor, piceatannol, and extracellular signal-regulated kinase (ERK) and JNK inhibitors inhibited UV-induced phosphorylation of STAT-1 in cultured human dermal fibroblasts. A, B: After serum-starvation for 24 h, the cells were pretreated with piceatannol or WHI-P131 at the indicated concentration for 30 min prior to 100 mJ/cm^2 UV treatment and then further incubated at 37°C for 72 h, respectively. The levels of MMP-1 and MMP-2 protein (culture media) were analyzed by Western blotting and zymography, respectively. C: After serum-starvation for 24 h, the cells were pretreated with 10 μM UO126, 10 μM SP600125, and 10 μM Pic, respectively, for 30 min prior to 100 mJ/cm^2 UV treatment, and then further incubated at 37°C for 10 min. The phosphorylation of JNK, STAT-1, ERK, and β-Actin (cell lysates) were dectected by Western blotting respectively. The results were representative of three independent experiments. Pic; piceatannol.

prevented UV- or platelet-activating factor-induced ceramide accumulation in Hela cells and rat lung, respectively (39, 40). Based on these evidences, we suggested that the down-regulation of UV-induced MMP-1 expression by D609 may result from the decreased level of intracellular ceramide in cultured human dermal fibroblasts.

The ceramide levels of stratum corneum were significantly increased by UV irradiation (41). UV-induced cer-

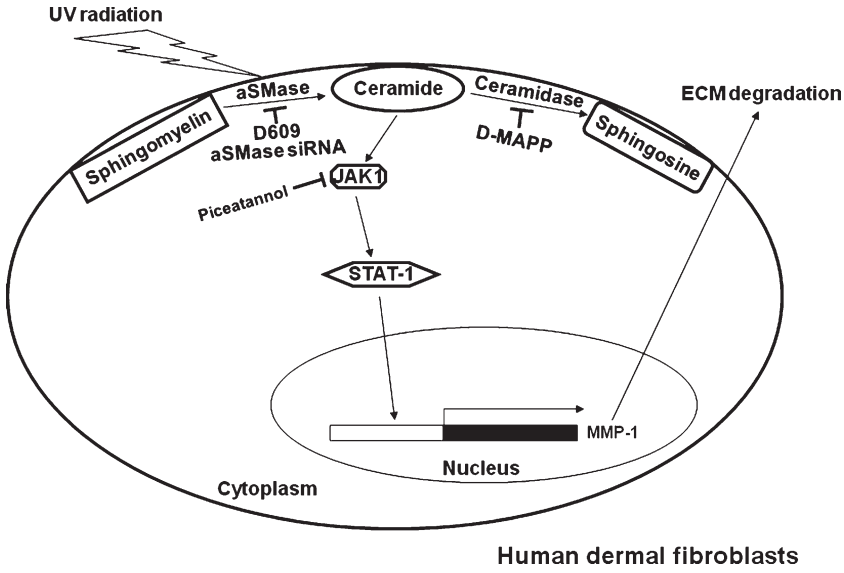

Fig. 7. A schematic model of our results. Extracellular stress such as UV radiation causes accumulation of intracellular ceramide through activation of aSMase (32). On the other hand, D-MAPP, ceramidase inhibitor, inhibits the conversion ceramide into sphingosine and thus augments the level of intracellular ceramide. In addition, ceramide activates JAK1 and JAK3/STAT-1 pathways, while UV activates only JAK1/STAT-1 pathways in cultured human dermal fibroblast. Both UV- and ceramide-induced MMP-1 expression was suppressed by corresponding JAK inhibitors. Therefore, our results suggest that induction of intracellular ceramide by UV may activate MMP-1 gene expression via JAK1/STAT-1 pathway in cultured human dermal fibroblasts.

amide accumulation was mediated through activation of neutral and acidic SMases (21). Induction of endogenous ceramide using inhibitors of sphingolipid metabolism, such as D-MAPP, DL-PDMP, and imipramine, maximized ionizing radiation-induced apoptotic cell death. In particular, D-MAPP, ceramidase inhibitor, inhibits the conversion ceramide into sphingosine (22). In accordance with these reports, our results showed that basal and UV-induced MMP-1 expression were significantly increased by D-MAPP. It is evidence that endogenous induction of ceramide levels also triggered MMP-1 mRNA and protein expression. Therefore, our results suggest that endogenous induction of ceramide levels also triggered MMP-1 mRNA and protein expression. In addition, UV-induced ceramide accumulation causes MMP-1 induction in photodamaged skin.

Interestingly, our results showed that C_2-ceramide or UV treatment activated phosphorylation of STAT-1 in cultured human dermal fibroblasts. Also, ceramide-induced MMP-1 expression was significantly decreased by JAK1 inhibitor, piceatannol, or JAK3 inhibitor, WHI-P131, but not by JAK2 inhibitor, AG490. Whereas ceramide-induced MMP-1 expression was mediated through JAK1, JAK3/STAT-1 pathway, we demonstrated that UV-induced MMP-1 expression was regulated only by JAK1/STAT-1 pathway. Based on these results, we demonstrate that UV up-regulates MMP-1 expression through JAK1/STAT-1 pathway, and JAK1/STAT-1 pathway may be a downstream of ERK or JNK pathways in cultured human dermal fibroblasts.

In conclusion, as shown in **Fig. 7**, we hypothesized that the enhancement of intracellular ceramide by activation of aSMase or inhibition of ceramidase mediates UV-induced MMP-1 expression. In particular, the treatment of exogenous ceramide triggers JAK1/STAT-1 signaling pathway and phosphorylation of STAT-1 induces the level of MMP-1 expression in human dermal fibroblasts. Both UV- and ceramide-induced MMP-1 is commonly suppressed by JAK1 inhibitor, piceatannol. Therefore, we demonstrate that JAK1/STAT-1 pathway plays a pivotal role on UV- and ceramide-induced MMP-1 expression in human dermal fibroblasts.

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