Enhancement of Fibroblast Collagenase (Matrix Metalloproteinase-1) Gene Expression by Ceramide Is Mediated by Extracellular Signal-regulated and Stress-activated Protein Kinase Pathways* (Received for publication, March 11, 1997, and in revised form, September 30, 1997)

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Inflammatory cytokines tumor necrosis factor-α and interleukin-1 trigger the ceramide signaling pathway, initiated by neutral sphingomyelinase-elicited hydrolysis of cell membrane phospholipid sphingomyelin to ceramide, a new lipid second messenger. Here, we show that triggering the ceramide pathway by sphingomyelinase or C2- and C6-ceramide enhances collagenase-1 (matrix metalloproteinase-1; MMP-1) gene expression by fibroblasts. C2-ceramide activates three distinct mitogen-activated protein kinases (MAPKs) in dermal fibroblasts, i.e. extracellular signal-regulated kinase 1/2 (ERK1/2), stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), and p38. Stimulation of MMP-1 promoter activity by C2-ceramide is dependent on the presence of a functional AP-1 cis-element and is entirely inhibited by overexpression of MAPK inhibitor, dual specificity phosphatase CL100 (MAPK phosphatase-1). Activation of MMP-1 promoter by C2-ceramide is also effectively inhibited by kinase-deficient forms of ERK1/2 kinase (MEK1/2) activator Raf-1, ERK1 and ERK2, SAPK/JNK activator SEK1, or SAPKβ. In addition, ceramide-dependent induction of MMP-1 expression is potentely prevented by PD 98059, a selective inhibitor of MEK1 activation, and by specific p38 inhibitor SB 203580. These results show that triggering the ceramide signaling pathway activates MMP-1 gene expression via three distinct MAPK pathways, i.e. ERK1/2, SAPK/JNK, and p38, and suggest that targeted modulation of the ceramide signaling pathway may offer a novel therapeutic approach for inhibiting collagenolytic activity, e.g. in inflammatory disorders.

Matrix metalloproteinases (MMPs)† are a family of zinc-dependent metalloendopeptidases collectively capable of degrading essentially all extracellular matrix components (1, 2). 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, e.g. rheumatoid arthritis, osteoarthritis, autoimmune blistering disorders of skin, dermal photoaging, and periodontitis (1, 2). In addition, degradation of basement membrane and extracellular matrix by MMPs is crucial for invasion and metastasis of tumor cells. To date, the MMP gene family consists of 16 members, which according to structure and substrate specificity can be divided into subgroups of collagensases, gelatinases, stromelysins, and membrane-type MMPs (2). Collagenase-1 (MMP-1) is the principal fibroblast-derived secreted 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. The expression of MMP-1 by fibroblasts is potently up-regulated by cytokines, growth factors, and tumor promoters (see Refs. 1–3).

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine, which potently inhibits accumulation of connective tissue components. TNF-α stimulates degradation of extracellular matrix by inducing the expression of MMP-1 and stromelysin-1 (MMP-3) by fibroblasts (4–6). In addition, TNF-α inhibits type I collagen gene expression by fibroblasts in culture (6–9) and in vivo (10), down-regulates elastin (11) and decorin (12) gene expression at the transcriptional level, and is able to abrogate the activation of type I collagen and elastin gene expression by transforming growth factor-β (9, 11). The effects of TNF-α on extracellular matrix formation partially overlap with those of interleukin-1 (IL-1), which also induces expression of MMP-1 and MMP-3 in fibroblasts (see Refs. 1–3 and 5). The cellular effects of TNF-α are mediated by two distinct cell surface receptors: TNF-R1 (TNF-R55) and TNF-R2 (TNF-R75), both of which are expressed by fibroblastic cells (see Ref. 13). We have recently shown that the effects of TNF-α on the expression of MMP-1, MMP-3, and type I collagen in dermal fibroblasts are primarily mediated by TNF-R55 (6). It has been shown that binding of TNF-α to TNF-R55 activates neutral sphingomyelinase, a cell membrane-associated phospholipase, which hydrolyzes cell membrane structural phospholipid sphingomyelin to
phosphocholine and ceramide, a novel lipid second messenger (see Refs. 14 and 15). The role of the ceramide pathway in TNF-α-induced apoptosis in various cells has been recently elucidated (15). In addition, it has been shown that ceramides activate the expression of cyclooxygenase, stimulate synthesis of prostaglandin E₂ (16), and enhance production of IL-6 by cultured fibroblasts (17), indicating a role for this signaling pathway in mediating the inflammatory effects of TNF-α on fibroblasts. However, the role of the ceramide pathway as a mediator of the effects of TNF-α and IL-1 on the synthesis and degradation of extracellular matrix is not known.

In this study, we show for the first time that triggering the ceramide pathway with neutral sphingomyelinase or cell-permeable ceramides in human skin fibroblasts results in marked stimulation of MMP-1 expression and that this effect is dependent on the presence of a functional AP-1 cis-element in the MMP-1 promoter region as well as on the activity of extracellular signal-regulated kinase 1/2 (ERK1/2), stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), and p38 mitogen-activated protein kinases (MAPKs). These results show that the effects of TNF-α and IL-1 on MMP-1 gene expression can be mimicked by activating the ceramide pathway in dermal fibroblasts, suggesting that targeted modulation of this pathway may offer a novel approach for therapeutic inhibition of matrix degradation, e.g. in inflammatory disorders.

**EXPERIMENTAL PROCEDURES**

**Materials**—Neutral sphingomyelinase (from Staphylococcus aureus) and cycloheximide were obtained from Sigma. C₆ and C₇-ceramide, C₄-dihydroceramide, and PD 98059 were obtained from Calbiochem. SB 203580 was provided by SmithKline Beecham (King of Prussia, PA). Human recombinant interleukin-1β was obtained from Boehringer Mannheim (Mannheim, Germany). Human TNF-R55-specific TNF-α (double mutant R32W/S86T) (18) were kindly provided by Dr. Walter Fiers (University of Gent, Belgium). TNF-R55-specific TNF-β (5 units/ml), and the incubations were continued for 24 h. Equal aliquots of the conditioned media, relative to cell number (27) were analyzed for the amount of MMP-1 produced. Western blotting was performed using a polyclonal rabbit antiserum against human MMP-1 (kindly provided by Dr. Henning Birkedal-Hansen, NIDR, National Institutes of Health, Bethesda, MD), in a 1:2000 dilution, and the enhanced chemiluminescence detection system (Amersham). The levels of immunoreactive MMP-1 were quantitated by densitometric scanning of the x-ray films.

**Transient CAT Transfections**—Construct pCLCAT3 (2 µg), which contains 3.8 kb of the 5'-flanking region of human MMP-1 gene linked to the CAT gene (29) (kindly provided by Dr. Steven Frisch (La Jolla Cancer Research Foundation, La Jolla, CA)), together with 10 µg of the following expression plasmids: RSV/AS-c-jun (30), a c-jun antisense expression construct (kindly provided by Dr. Alain Mauviel (Thomas Jefferson University, Philadelphia, PA)); SG5-β-catenin (31) for MAPK-phosphatase CL100 (MAPK phosphatase-1) (32) (kindly provided by Dr. Steven Keyse (Ninewells Hospital, Dundee, Scotland)); RSV-Raf-C4 (33) for kinase-deficient Raf-1 and SAPK/JNK-RR (34) for kinase-deficient SAPKβ (both kindly provided by Dr. Ulf Rapp (University of Würzburg, Germany)); pEBG-SEK1-R (35), specific for kinase-deficient SEK1 (kindly provided by Dr. John Kyriakis (Harvard University, Boston, MA)); and CEp4LK71RERK1 or CEp4LK52RERK2 (36), specific for kinase-deficient ERK1 and ERK2, respectively (kindly provided by Dr. Melanie Cobb, Southwestern Medical Center, Dallas, TX). Control cultures were co-transfected in parallel with the respective empty expression vectors.

Transfections were performed with the calcium phosphate/DNA co-precipitation method, followed by a 2-min glycerol shock, as described previously (20). The cultures were then maintained in DMEM and 1% CS for 16 h, C₇-ceramide was added, and the incubations were continued for 24 h. Cells were harvested and lysed by three cycles of freezing and thawing. As an index of promoter activity, CAT activity was measured from aliquots of cell extracts in reactions containing 0.25 μM Tris-HCl buffer (pH 8.0), 5 μg of n-butyryl-coenzyme A (Sigma), and 0.0625 μg/ml of chloramphenicol (Amersham), in a total volume of 125 µl. The butyrylated chloramphenol products were extracted with 300 µl of xylene after overnight incubation at 37 °C. The CAT activity was quantitated by scintillation counting after two back-extractions of the xylene phase with 100 µl of 0.25 μM Tris-HCl. The transfection efficiency was monitored by co-transfecting cells with 4 µg of RSV/β-galactosidase construct and correcting the CAT activities for β-galactosidase activity (28). CAT Activity Assays—For assay of ERK1/2 and SAPK/JNK activity, confluent cultures of human skin fibroblasts were incubated for 18 h in DMEM containing 0.5% FCS. Thereafter, ceramide was added, and the incubations were continued for different periods of time. Cells (2 × 10⁵/sample) were lysed in 400 µl of lysis buffer (PBS, pH 7.4; 1% Nonidet P-40; 0.5% sodium deoxycholate; 1 mM Na₃VO₄; 0.1% SDS; 1 mM EDTA; 1 mM EGTA; 20 mM NaF; 1 mM PMSF; and 1 µg/ml aprotinin, leupeptin, and pepstatin). For immunoprecipitation of ERK1/2, cell lysates were centrifuged (3000 × g for 15 min), and the supernatant was incubated with an antibody generated against ERK2 (p42 MAPK; Transduction Laboratories, Lexington, KY), coupled to protein A-Sepharose (Sigma). This antibody also cross-reacts with ERK1. Immunoprecipitates were washed three times in lysis buffer and three times in kinase assay buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol). The kinase reaction was carried out by adding to the immunoprecipitate 20 µl of kinase assay buffer, including 25 µM ATP, 2.5 µCi of [γ-³²P]ATP (Amersham), and 1 µg/ml myelin basic protein (Sigma) as substrate. The reaction was carried out for 15 min at 37 °C and stopped by adding 3 x Laemmli sample buffer. The samples were resolved on 12.5% SDS-polyacrylamide gel electrophoresis, and myelin basic protein phosphorylation was quantified with a phosphor imager (Bio-Rad).

To measure SAPK/JNK activity, the cells were lysed in 400 µl of lysis buffer as described above. The cell lysates were centrifuged (3000 × g for 15 min), and SAPK/JNK was immunoprecipitated by incubating the supernatant with an antibody generated against SAPK/JNK (Santa
Sphingomyelinase and Ceramides Enhance MMP-1 Expression in Dermal Fibroblasts—To elucidate the role of ceramide pathway in the regulation of fibroblast collagenase (collagenase-1, MMP-1) gene expression, we first activated this signaling pathway by treatment of human skin fibroblasts with S. aureus neutral sphingomyelinase for 24 h and assayed MMP-1 mRNA levels with Northern blot hybridizations. As shown in Fig. 1A, sphingomyelinase treatment of cells with 1 mU/ml resulted in a marked enhancement (7-fold) in MMP-1 mRNA expression, and an even more potent increase (14-fold) was noted with a concentration of 100 mU/ml after correction of the MMP-1 mRNA abundance for the level of GAPDH mRNA in the same samples (Fig. 1A).

Next, we triggered the ceramide pathway in dermal fibroblasts by treatment with cell-permeable ceramide analogs C6- and C2-ceramide. Exposure of cells to C6-ceramide resulted in a dose-dependent enhancement of MMP-1 mRNA abundance (6- and 19-fold) with concentrations of 10 and 100 μM, respectively (Fig. 1B). The mRNA abundance for TIMP-1 was slightly (25%) reduced with the highest C6-ceramide concentration when corrected for the levels of GAPDH mRNA (Fig. 1B). In a parallel experiment, fibroblasts were treated with different concentrations of C6-ceramide, which stimulated the expression of MMP-1 mRNA even more potently (22- and 385-fold) with concentrations of 10 and 100 μM). Treatment of cells with an inactive ceramide analog, C6-dihydroceramide (10 μM) had no effect on MMP-1, MMP-3, or TIMP-1 mRNAs (not shown). The viability of fibroblasts was only affected by 100 μM C6-ceramide, whereas lower concentrations of C6-ceramide as well as all concentrations of C2-ceramide or sphingomyelinase used did not affect the viability of cells as estimated by trypan blue exclusion (not shown).

Sphingomyelinase and Ceramide Augment Stimulation of MMP-1 Production by TNF-α and IL-1β—To examine the effect of sphingomyelinase and C2-ceramide on the production of MMP-1 by dermal fibroblasts, we assayed the amount of immunoreactive MMP-1 in the conditioned media of cells treated with sphingomyelinase (100 mU/ml) and C2-ceramide (10 μM) using Western blot analysis. As shown in Fig. 2A, both sphingomyelinase and C2-ceramide stimulated production of MMP-1 by fibroblasts by 5-fold.

We have recently shown that the effect of TNF-α on MMP-1 expression in dermal fibroblasts is primarily mediated via TNF-R55 (6). In this context, we examined whether the activation of the ceramide pathway by sphingomyelinase augments the maximal enhancement of MMP-1 production obtained with 20 ng/ml TNF-R55-specific TNF-α (6). In this experiment, TNF-R55-specific TNF-α enhanced production of immunoreactive MMP-1 into the culture media of dermal fibroblasts 45-fold,

![Fig. 1. Enhancement of MMP-1 mRNA levels in human skin fibroblasts by sphingomyelinase, C6-ceramide, and C2-ceramide.](image)

![Fig. 2. Sphingomyelinase augments induction of MMP-1 production by TNF-α and IL-1β.](image)
and simultaneous treatment with sphingomyelinase (100 mU/ml) potentiated this stimulation of MMP-1 production 1.8-fold, the final stimulation being 81-fold, as compared with the untreated control cultures (Fig. 2B).

The cellular signaling of IL-1 also involves activation of neutral sphingomyelinase and subsequent triggering of the ceramide pathway (14, 15). Therefore, we also examined the effect of sphingomyelinase on IL-1β-elicited induction of MMP-1 production. As expected, IL-1β (5 units/ml) enhanced (18-fold) the production of immunoreactive MMP-1 by dermal fibroblasts (Fig. 2C), and sphingomyelinase augmented this maximal IL-1β-elicited induction of MMP-1 production by 2.7-fold, the final enhancement being 49-fold over the control cells (Fig. 2C).

**C2-ceramide Activates the MMP-1 Promoter via AP-1**—Stimulation of MMP-1 gene transcription by various stimuli involves induction of dimeric AP-1 trans-activating factor complex (Jun plus Fos), which binds to the corresponding cis-element in the MMP-1 promoter (see Refs. 1–3). We therefore examined the effect of C2-ceramide on the expression of members of Jun and Fos families in dermal fibroblasts. Treatment of cells with C2-ceramide (100 μM) resulted in rapid stimulation of c-jun mRNA expression, first noted at 1 h of incubation with further increase up to 6 h (Fig. 3A). The levels of c-jun mRNA were enhanced by C2-ceramide, the peak induction noted at 2 and 6 h (Fig. 3A). Interestingly, the levels of c-jun, c-Jun, and c-Fos mRNAs were still enhanced after 24 h (Fig. 3A). In the same experiment, the expression of MMP-1 mRNA was first induced after a 6-h exposure to C2-ceramide (Fig. 3A). In parallel experiments, C6-ceramide (100 μM) and sphingomyelinase (100 mU/ml) also induced expression of MMP-1 mRNA with similar kinetics (not shown). As shown in Fig. 3B, enhancement of MMP-1 expression by C2-ceramide was abrogated by co-treatment of cells with cycloheximide (10 μg/ml). Similarly, activa-
tion of MMP-1 expression by sphingomyelinase was inhibited by cycloheximide (not shown). These observations show that ceramide-elicited activation of MMP-1 gene expression is dependent on synthesis of new regulatory proteins.

To examine whether ceramide treatment of fibroblasts results in transcriptional activation of MMP-1 promoter, we transiently transfected NIH-3T3 fibroblasts with an MMP-1 promoter/CAT construct p2278CLCAT, in which a 2.278-kb human MMP-1 promoter segment is linked to the CAT reporter gene. Treatment of transiently transfected NIH-3T3 cells with C2-ceramide (50 μM) potently (7.7-fold) enhanced the activity of this MMP-1 promoter construct (Fig. 3C). To elucidate the role of AP-1 in ceramide-elicited activation of the MMP-1 promoter, we transfected parallel cultures with an MMP-1 promoter/CAT construct containing the same promoter segment except with a mutation rendering the AP-1 binding site at −72 to −65 incapable of binding AP-1. As shown in Fig. 3C, loss of the functional AP-1 cis-element reduced the basal activity of p2278CLCAT by 95%. Interestingly, the C2-ceramide-elicited enhancement of the MMP-1 promoter construct lacking the AP-1 element was clearly lower (2.4-fold), as compared with the wild type MMP-1 promoter, the final promoter activity being 12% of the untreated wild type (Fig. 3C).

To elucidate the role of c-Jun in ceramide-mediated activation of the MMP-1 promoter, we transiently co-transfected NIH-3T3 fibroblasts with a 3.8-kb human MMP-1 promoter construct, pCLCAT3, together with a c-Jun antisense expression construct previously shown to inhibit both basal and TNF-α induced expression of c-Jun mRNA in NIH-3T3 cells (30). In this set of experiments, enhancement of MMP-1 promoter activity by C2-ceramide was clearly (by 53%) inhibited by expression of c-Jun antisense mRNA, indicating involvement of c-Jun in ceramide-dependent activation of MMP-1 gene transcription (Fig. 3D). Co-transfection of cells with the c-Jun antisense construct also reduced the basal MMP-1 promoter activity by 60%, indicating that c-Jun-containing AP-1 complexes play a role in maintaining MMP-1 gene transcription (Fig. 3D).

Ceramide-dependent Activation of the MMP-1 Promoter Is Mediated by ERK1/2 and SAPK/JNK Pathways—Induction of the expression of AP-1 components c-Fos and c-Jun by various stimuli, e.g. growth factors, cytokines, and tumor promoters, is mediated by activation of three distinct MAPKs, specifically ERK1/2, SAPK/JNK, and p38 (see Ref. 37). Of these, the ERK1/2 cascade (Raf-1/MEK1,2/ERK1,2) is activated by Ras and protein kinase Ca at the level of Raf-1 (38). In addition, Raf-1 is activated by ceramide-activated protein kinase (39) and directly by ceramide (40). The SAPK/JNK pathway (MEK kinase 1–3/SEK1/JNK) is also activated by Ras (see Ref. 37). In this context, we wanted to elucidate the role of the ERK1/2 and SAPK/JNK cascades in mediating the effects of ceramide on MMP-1 expression in fibroblasts. For this, we first examined the effects of C2-ceramide on ERK1/2 and SAPK/JNK activity in dermal fibroblasts. Treatment of cells with C2-ceramide (100 μM) resulted in a maximal increase (4-fold) in ERK1/2 activity after 1 h of incubation (Fig. 4A). In parallel cultures, incubation with C2-ceramide (100 μM) resulted in maximal stimulation (2-fold) of SAPK/JNK activity at time points of 1 and 2 h (Fig. 4A). These results show that treatment of dermal fibroblasts with ceramide activates both the ERK1/2 and SAPK/JNK pathways.

To examine the functional role of MAPKs in mediating the up-regulatory effect of ceramides on MMP-1 expression, we transiently co-transfected NIH-3T3 cells with a 3.8-kb human MMP-1 promoter construct, pCLCAT3, together with the expression vector for MAPK inhibitor, dual specificity phosphatase CL100 (MAPK phosphatase-1), which inactivates ERK1/2, SAPK/JNK, and p38 (31, 32, 41, 42). Interestingly, overexpression of CL100 entirely inhibited the activation (5.1-fold) of MMP-1 promoter by C2-ceramide and also potently (by 90%) suppressed the basal activity of MMP-1 promoter (Fig. 4B). These experiments show that the effect of ceramide on MMP-1 promoter activity is dependent on MAPK activity.

In the next set of experiments, we examined in detail the role of two distinct MAPK pathways, Raf-1/MEK1,2/ERK1,2 and MEK kinase 1–3/SEK1/JNK in the ceramide-elicited activation of MMP-1 gene transcription in fibroblasts. First, we co-transfected NIH-3T3 cells with the MMP-1 promoter construct pCLCAT3 in combination with the expression vector for kinase-deficient Raf-1 to block the ERK1/2 pathway at the level of Raf-1. In these experiments, C2-ceramide (50 μM) activated MMP-1 promoter 6.5-fold, and overexpression of the mutant Raf-1 potently (by 77%) inhibited this effect (Fig. 4C). Interestingly, overexpression of the kinase-deficient Raf-1 also suppressed the basal activity of the MMP-1 promoter by 46% (Fig. 4C). In a parallel experiment, co-transfection of pCLCAT3 construct together with kinase-deficient ERK1 and ERK2 expression vectors also inhibited (by 50%) the activation of MMP-1 promoter by C2-ceramide (Fig. 4D).

To elucidate the role of SAPK/JNK in the ceramide-elicited enhancement of MMP-1 promoter activity, NIH-3T3 cells were transiently co-transfected with the MMP-1 promoter construct pCLCAT3 in combination with the expression vector coding for the kinase-deficient form of SEK1, the activator of SAPK/JNKs, and subsequently treated with C2-ceramide. In these experiments, C2-ceramide (50 μM) up-regulated the MMP-1 promoter 7.6-fold (Fig. 4E), and overexpression of the kinase-deficient SEK1 inhibited this activation by 58% (Fig. 4E). In addition, overexpression of the mutant SEK1 slightly (by 27%) reduced the basal MMP-1 promoter activity (Fig. 4E). In parallel cultures, co-transfection of MMP-1 promoter/CAT construct together with the expression vector for kinase-deficient SAPKβ also inhibited (by 32%) the C2-ceramide-elicited activation of MMP-1 promoter (Fig. 4F).

Ceramide-dependent Activation of MMP-1 Expression Is Abrogated by MEK1 and p38 Inhibitors—To further elucidate the role of specific MAPK cascades in the ceramide-elicited induction of the expression of the endogenous MMP-1 gene, we utilized two specific chemical inhibitors to distinctly block the ERK1/2 or p38 MAPK pathways. First, we treated dermal fibroblasts with C2-ceramide in combination with PD 98059, a specific inhibitor of MEK1 activation, which prevents activation of ERK1 and ERK2 (43). As demonstrated in Fig. 5A, PD 98059 potently and dose-dependently, but not entirely, inhibited the induction of MMP-1 mRNA levels by C2-ceramide, corroborating the role of the ERK1/2 pathway in ceramide-dependent activation of MMP-1 expression.

As shown above in the co-transfection experiments, overexpression of kinase-deficient SEK1 potently inhibited ceramide-elicited stimulation of MMP-1 promoter activity (Fig. 4E). It has been shown that in addition to SAPK/JNK, SEK1 is also able to activate p38 MAPK (44). To examine whether the ceramide-dependent stimulation of MMP-1 expression involves activation of p38 MAPK, we first determined the level of activated p38 in ceramide-treated skin fibroblasts by Western blotting using a phosphospecific p38 antibody. C2-ceramide treatment clearly activated p38 after 1 h (8-fold), and elevated levels of the phosphorylated form of p38 MAPK were still detected after 6 h of incubation (Fig. 5B). To directly elucidate the role of activated p38 in the stimulation of the expression of the endogenous MMP-1 gene, we then treated dermal fibroblasts simultaneously with various concentrations of selective p38 inhibitor SB 203580 (45) and C2-ceramide (100 μM). As demon-
Stratified in Fig. 5C, SB 203580 (20 μM) partially (by 50%) inhibited the stimulatory effect of C2-ceramide on MMP-1 expression, indicating that p38 MAPK is also activated by ceramide and is involved in mediating the stimulatory effect of ceramide on endogenous MMP-1 gene expression.

**DISCUSSION**

In this study, we demonstrate for the first time that triggering the ceramide signaling pathway in human skin fibroblasts by neutral sphingomyelinase and cell-permeable ceramides induces the expression of fibroblast collagenase-1 (MMP-1), as determined at promoter, mRNA, and protein levels. In addition, sphingomyelinase and ceramides activate the expression of stromelysin-1 (MMP-3), a potent activator of latent MMP-1 (1, 2). Sphingomyelinase also augments the maximal enhancement of MMP-1 expression by TNF-R55-specific TNF-α and IL-1β, indicating that exogenous sphingomyelinase may further potentiate the signaling pathways triggered by these inflammatory cytokines. Based on these observations, it is possible that bacteria-derived sphingomyelinase may play a role in extracellular matrix degradation by stimulating MMP-1 expression, either alone or in combination with inflammatory cytokines. In this context, it should be noted that lipopolysaccharide, a constituent of the cell wall of Gram-negative bacteria, shows structural similarity to ceramides (46). However, although lipopolysaccharide induces expression of MMP-1 by monocytes, it has no effect on the expression of MMP-1 by fibroblasts (28), indicating that its biological effects are not identical with those of ceramides.
The transient transfection experiments show that C2-ceramide potently activates a 2.3-kb MMP-1 promoter/CAT construct and that activation of a similar MMP-1 promoter construct lacking a functional AP-1 binding element is clearly less potent. These results provide evidence that the maximal stimulation of MMP-1 transcription by ceramide is dependent on the binding of the AP-1 trans-acting factor complex to the corresponding cis-element in the MMP-1 promoter. This notion is also supported by results of co-transfection experiments, in which co-expression of c-jun antisense mRNA clearly, but not entirely, inhibited ceramide-dependent activation of MMP-1 promoter. However, since the low basal activity of the AP-1-deficient MMP-1 promoter construct was also somewhat up-regulated by ceramide, it is possible that the effect of ceramide on MMP-1 promoter also involves activation of other trans-acting factors. In addition to inducing expression of mRNAs for c-Jun and c-Fos, the components of the classical AP-1 dimer, C2-ceramide also induces rapid and transient expression of junB mRNA. Although JunB has been shown to counteract trans-activation of the AP-1-responsive promoter by c-Jun (26), we have recently demonstrated that JunB can also directly activate the MMP-1 promoter (47) and mediate its stimulation by tumor promoter okadaic acid (20). Therefore, we cannot exclude the possibility that JunB-containing AP-1 complexes may be involved in ceramide-elicited activation of the MMP-1 promoter in fibroblasts.

In the present study, triggering the ceramide pathway in dermal fibroblasts with exogenous ceramide activates both ERK1/2 and SAPK/JNK classes of MAPKs, and the ceramide-elicited induction of the endogenous MMP-1 gene is potently inhibited by PD 98059, a specific inhibitor of activation of MEK1, the MAPK kinase, which activates ERK1/2. This is in contrast to observations on U937 and bovine aortic endothelial cells in which ceramide treatment activates SAPK/JNK but has minimal effect on ERK1/2 activity (48). However, ceramide-treatment of promyelocytic HL-60 cells also activates the ERK1/2 class of MAPKs (49). In our transient transfection experiments, overexpression of MAPK inhibitor CL100, a dual specificity phosphatase, which inactivates ERK1/2, SAPK/JNKs, and p38 MAPK, entirely inhibited the activation of the MMP-1 promoter by C2-ceramide in fibroblasts. However, overexpression of kinase-deficient forms of Raf-1, ERK1 and ERK2, SEK1, or SAPKβ clearly, but not entirely, inhibited ceramide-dependent activation of the MMP-1 promoter in NIH-3T3 fibroblasts. Together, these results provide evidence that the effect of ceramides on MMP-1 gene expression is mediated via both ERK1/2 and SAPK/JNK pathways and that activation of both is required for maximal activation of the MMP-1 promoter by ceramide. It is likely that ERK1/2 and SAPK/JNK cascades are distinctly activated by ceramide at the level of MAPK kinase kinases, since Raf-1 does not activate the SAPK/JNK pathway and SEK1 does not activate ERK1/2 (44, 50). However, ceramide can also activate MEK1 and ERK1/2 independently of Raf-1 via protein kinase Cζ (51) and MEK kinase 1–3 of the SAPK/JNK pathway can also activate MEK1 and MEK2 (see Ref. 37), providing a putative Raf-independent pathway for activation of ERK1/2.

mRNA levels were quantitated by scanning densitometry, corrected for the levels of GAPDH mRNA, and shown relative to the level in untreated control cells (1.00) (bottom of panels A and C). MMP-1 mRNA levels were quantitated by scanning densitometry, corrected for the levels of GAPDH mRNA, and shown relative to the level in untreated control cells (1.00) (bottom of panels A and C). Human skin fibroblasts in DMEM supplemented with 0.5% FCS were incubated for 24 h without (white bars) or, with C2-ceramide (C2-cer (black bars), 100 µM) alone or in combination with PD 98059 (A), a specific inhibitor of MEK1 activation, or SB 203580 (C), a selective p38 MAPK inhibitor, in different concentrations, as indicated. Aliquots of total cellular RNA were analyzed by Northern blot hybridizations for the levels of MMP-1 and GAPDH mRNA (top of panels A and C). MMP-1 mRNA levels were quantitated by scanning densitometry, corrected for the levels of GAPDH mRNA, and shown relative to the level in untreated control cells (1.00) (bottom of panels A and C). Human skin fibroblasts in DMEM supplemented with 0.5% FCS were incubated with C2-ceramide (50 µM) for different periods of time, as indicated, and with TNF-α for 20 min. The levels of activated p38 MAPK (p38-P) were determined by Western blotting using a phosphospecific p38 MAPK antibody. The migration positions of the molecular weight markers (M, × 10− 5) are shown on the left.
As mentioned above, the role of the SAPK/JNK pathway in mediating the stimulatory effect of ceramide on MMP-1 expression is supported by the observation that overexpression of kinase-deficient SEK1 or SAPKα clearly, although not entirely, inhibited the up-regulatory effect of ceramide on the MMP-1 promoter. However, SEK1 may also activate p38 MAPK, which induces expression of c-Jun via phosphorylation and activation of ATF-2 (37, 44). Our observation that ceramide activates p38 MAPK and that the up-regulatory effect of C2-ceramide on endogenous MMP-1 expression is partially prevented by the selective p38 inhibitor SB 203580 also provides evidence for the role of p38 MAPK in the ceramide-mediated activation of MMP-1 expression in dermal fibroblasts. This is in contrast to endothelial cells, in which ceramide does not activate p38 (52), indicating cell specificity in the ceramide-dependent activation of MAPK pathways. p38 MAPK is also activated independently of the SAPK/JNK cascade by its specific MAPK kinases, MEK3 and MEK6, in response to environmental stress and TNF-α (53). It is therefore possible that in fibroblasts ceramides activate p38 independently of SEK1 via activation of MEK3 or MEK6 by TAK1 (transforming growth factor-β-activated protein kinase) (54). Nevertheless, the effect of C2-ceramide on the expression of the endogenous MMP-1 gene was not entirely inhibited by SB 203580, providing further evidence that the activity of three distinct MAPKs, i.e. ERK1/2, SAPK/JNK, and p38 is required for maximal activation of MMP-1 expression by ceramide.

In promyelocytic HL-60 cells and in human monocytic leukemia cell line U937, the induction of apoptosis by C2-ceramide is suppressed by sphingosine-1-phosphate, which inhibits activation of SAPK/JNK and activates ERK1/2 (48). In addition, the initiation of programmed cell death by ceramide in U937 and bovine aortic endothelial cells appears to be entirely mediated via the SAPK/JNK pathway and can be inhibited by overexpression of dominant negative c-Jun (55). In the present study, activation of the ceramide pathway in human skin fibroblasts by treatment with C2-ceramide (100 μM) somewhat affected the viability of cells. However, induction of MMP-1, as well as activation of c-Jun expression was also noted with the lower (10 μM) C2-ceramide concentration, with C2-ceramide, and with sphingomyelinase, all of which had no effect on the viability of these cells. Together, these observations indicate that ceramide-dependent stimulation of MMP-1 expression is not associated with altered cell viability and suggest that, in dermal fibroblasts, triggering the sphingomyelin pathway is not alone sufficient to induce programmed cell death.

In conclusion, the results of this study show that the activation of the ceramide-dependent signaling pathway either by endogenous ceramides generated by neutral sphingomyelinase or by exogenously added ceramide analogs potently activates the expression of collagenase-1 (MMP-1) and stromelysin-3 (MMP-3) in fibroblastic cells. We also show that in these cells ceramide treatment activates ERK1/2 and SAPK/JNK classes of MAPKs, both of which mediate the ceramide-elicted activation of MMP-1 gene expression. In addition, activity of p38 MAPK is required for maximal activation of MMP-1 gene expression by ceramides. However, our results indicate that inhibition of any one of these MAPK pathways alone is not sufficient to inhibit ceramide-dependent activation of MMP-1 expression. Based on the results of this study, it can be suggested that the ceramide signaling pathway plays an important role in mediating the effects of TNF-α and IL-1 on the expression of MMP-1 and also MMP-3. Inflammatory cell-derived TNF-α and IL-1 play an important role in the induction of MMP-1 expression in conditions characterized by increased collagenolytic activity, such as rheumatoid arthritis, osteoarthritis, autoimmune blistering disorders of skin, and periodontitis. It is likely that targeted inhibition of the ceramide pathway and three distinct MAPKs implicated in this study may be feasible for inhibiting induction of MMP-1 expression by TNF-α and IL-1, thus offering novel possibilities for therapeutic intervention of extracellular matrix degradation.
42. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J.,
and Davis, R. J. (1995) *J. Biol. Chem.* 270, 7420–7426
43. Dudley, D. T., Pang, L., Decker, S. T., Bridges, A. J., and Saltiel, A. R. (1995)
*Proc. Natl. Acad. Sci. U. S. A.* 92, 7686–7689
44. Lin, A., Minden, A., Martinetto, H., Clare, F.-X., Lange-Carter, C., Mercurio,
F., Johnson, G. L., and Karin, M. (1995) *Science* 268, 286–290
45. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F.,
Young, P. R., and Lee, J. C. (1995) *FEBS Lett.* 364, 229–233
46. Joseph, C. K., Wright, S. D., Bornmann, W. G., Randolph, J. T., Kumar, E. R.,
Bittman, R., Liu, J., and Kolesnick, R. N. (1994) *J. Biol. Chem.* 269,
17606–17610
47. Westermarck, J., Seth, A., and Kähäri, V.-M. (1997) *Oncogene* 14, 2651–2660
48. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind,
J. S., and Spiegel, S. (1996) *Nature* 381, 800–803
49. Raines, M. A., Kolesnick, R. N., and Golde, D. W. (1993) *J. Biol. Chem.* 268,
14572–14575
50. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and
Templeton, D. J. (1994) *Nature* 372, 796–800
51. Berra, É., Díaz-Meco, M. T., Lezane, J., Frutos, S., Municio, M. M., Sanchez,
P., Sanz, L., and Moscat, J. (1995) *EMBO J.* 14, 6157–6163
52. Modur, V., Zimmerman, G. A., Prescott, S. M., and McIntyre, T. M. (1996)
*J. Biol. Chem.* 271, 13094–13102
53. Moriguchi, T., Toyoshima, F., Gotoh, Y., Iwamatsu, A., Irie, K., Mori, E.,
Kuroyanagi, N., Hagiwara, M., Matsumoto, K., and Nishida, E. (1996)
*J. Biol. Chem.* 271, 26981–26988
54. Shirakabe, K., Yamaguchi, K., Shibuya, H., Irie, K., Matsuda, S., Moriguchi,
T., Gotoh, Y., Matsumoto, K., and Nishida, E. (1997) *J. Biol. Chem.* 272,
8141–8144
55. Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J.,
Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuks, Z., and
Kolesnick, R. (1996) *Nature* 380, 75–79
