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Original

Sphingosine 1-phosphate acts as a signal molecule in ceramide signal transduction of TNF-α-induced activator protein-1 in osteoblastic cell line MC3T3-E1 cells

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Abstract: We previously demonstrated that tumor necrosis factor (TNF)-α stimulated the production of activation protein (AP)-1, a transcriptional factor, in mouse osteoblastic MC3T3-E1 cells. Recent studies have shown the importance of ceramide and its metabolites as signal molecules for TNF-α-induced gene expression in several cell types. Therefore, our interest was to investigate whether sphingosine metabolites are involved in TNF-α-induced signaling in MC3T3-E1 cells. *DL-threo*-1-phenyl-2-hexadecanoyl-amino-3-pyrrolidino-1-propanol (PPPP), which causes accumulation of intracellular ceramide, stimulated the TNF-α-induced expression of the c-fos and c-jun genes. Gel shift assay clearly showed that PPPP increased the cytokine-induced specific binding of nuclear proteins to the 12-tetra-decanoyl phorbol 13-acetate-responsive element (TRE), a consensus sequence for AP-1. In addition, cell-permeable ceramide (*N*-acetylsphingosine, *N*-hexanoylsphingosine or *N*-octanoylsphingosine) stimulated expression of the c-fos and c-jun genes and nuclear protein binding to TRE. Interestingly, *DL-threo*-dihydrosphingosine (DHS), an inhibitor of sphingosine kinase, clearly blocked the ceramide analogue-induced stimulation. Sphingosine 1-phosphate (SPP) actually induced expression of these oncogenes and activated AP-1. Although TNF-α stimulated the AP-1-mediated expression of the monocyte chemoattractant JE/MCP-1, this stimulation was inhibited by DHS. SPP also stimulated JE/MCP-1 gene expression. The present study thus suggests that SPP acts as a signal molecule in ceramide-dependent signal transduction in TNF-α-induced AP-1 in osteoblastic MC3T3-E1 cells. (J. Oral Sci. 47, 43-51, 2005)

Keywords: sphingosine 1-phosphate; TNF-α; ceramide; osteoblast; AP-1; sphingomyelin.

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Introduction

Periodontal disease and rheumatoid arthritis are typical inflammatory bone diseases (1-6). It is well known that tumor necrosis factor (TNF)-α and interleukin (IL)-1 are powerful inflammatory cytokines that mediate bone-resorbing activity in several cell types, and thus both of these cytokines likely play a central role in the initiation and development of such bone diseases (2-12). At present, it is widely recognized that differentiation and activation of osteoclastic cells are stimulated via the supporting activity of osteoblastic cells (9-18). Many investigators (14-18) have therefore focused on the signaling mechanism(s)

of these cytokines in osteoblastic cells. With respect to TNF-α signaling in osteoblastic cells, we previously showed that TNF-α is a potent stimulator of activation protein (AP)-1, a transcriptional factor formed by the c-fos and c-jun gene products, in osteoblastic MC3T3-E1 cells (11,19,20).

The breakdown of sphingomyelin (SM) through the action of SMase results in the formation of ceramide, which is subsequently metabolized to sphingosine and sphingosine 1-phosphate (SPP) (21-25). It has been shown that ceramide or SPP plays a functional role as a second messenger in the cellular signal transduction of TNF-α, IL-1, platelet-derived growth factor, and vitamin D_3. As shown in several studies, these sphingosine metabolites are able to mimic the biological actions of several factors in osteoblasts (26-35). In osteoclasts, we showed that ceramide inhibits bone resorption by suppressing osteoclast activity (36). These studies prompted us to propose the possibility that ceramide and sphingosine metabolites may act as signaling molecules in both osteoblasts and osteoclasts.

Therefore, in the present study, we examined whether sphingosine metabolites play a functional role as signaling molecules in the TNF-α-induced expression of AP-1 in mouse osteoblastic MC3T3-E1 cells.

Materials and Methods

Reagents

Human recombinant TNF-α (specific activity of 2.0×10^6 units /mg of protein) was provided by the Suntory Institute for Biomedical Research (Osaka, Japan) and purified to homogeneity (> 98.9%, as determined by SDS-polyacrylamide gel electrophoresis analysis). *N*-acetylsphingosine (C_2-ceramide), *N*-hexnoylsphingosine (C_6-ceramide), *N*-octanoylsphingosine (C_8-ceramide), SPP, *DL-threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP), *DL-threo*-1-phenyl-2-decanoylamino-3-morpholion-1-propanol (PDMP), and *DL-threo*-dihydrosphingosine (DHS) were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). SMase and genisteun came from Sigma (St. Louis, MO). α-MEM came from Flow Lab (McLean, VA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). 5'-[α-^{32}P]-dCTP megaprimed DNA labeling system and [γ-^{32}P]-ATP were obtained from Amersham Biosciences (Piscataway, NJ). 1-(5-isoquinolinesulfonyl)-2-methypiperazine dihydrochloride (H-7) and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) were purchased from Seikagaku Corporation (Tokyo, Japan).

Cell Culture

Clonal osteoblastic MC3T3-E1 cells derived from C57BL/6 mouse calvaria were cultured in α-MEM with 10% FBS in 90-mm plastic dishes (3.0×10^5 cells per plate)at 37°C under 5% CO_2 in air until near confluence and were subcultured every 3 days, as previously described (11,19,20). Cells were then washed, incubated for 24 h in serum-free α-MEM, and treated for the indicated periods in FBS-free α-MEM with or without test samples at various concentrations.

Northern blotting analysis

Total cellular RNA was extracted using the guanidine isothiocyanate procedure (37). As previously described (11), RNA was subjected to 1% agarose electrophoresis, and blotted onto a nylon membrane (MSI Magnagraph, Westboro, MA). Membranes were subsequently baked, prehybridized, and then hybridized with cDNA probes that had been labeled with 5'-[α-^{32}P]-dCTP using the megaprimed DNA labeling system. After hybridization, membranes were washed, dried, and exposed to X-ray film (Eastman Kodak, Rochester, NY) at -70°C. β-Actin was used as an internal standard for quantification of total mRNA in each lane of the gel.

Preparation of Nuclear Extracts

Confluent monolayers in 15-cm-diameter dishes were treated with the test samples indicated in the figure legends, and nuclei were isolated as described above. Thereafter, nuclear extracts were prepared as described previously (19,20). Protein concentration was measured using Bradford method (38).

Gel Mobility Shift Assay

This assay was carried out as described previously (19,20). Binding reactions were performed for 20 min on ice with 5 μg of nuclear protein in 20 μl of binding buffer (2 mM HEPES[pH 7.9], 8 mM NaCl, 0.2 mM EDTA, 12%[v/v] glycerol, 5 mM DDT, 0.5 mM PMSF, 1 μg poly[dI-dC]) containing 20,000 c.p.m. of ^{32}P-labeled oligonucleotide in the presence or absence of nonlabeled oligonucleotide. Poly(dI-dC) and nuclear extract were first incubated at 4°C for 10 min before labeled oligodeoxynucleotide was added. Thirty-mer double-stranded oligonucleotides containing -TGACTCA- (Oncogene Science) of the AP-1 binding site were end-labeled using the oligonucleotide 5'-end labeling system-[γ-^{32}P]-ATP method. Reaction mixtures for binding were incubated for 15 min at room temperature after adding the labeled oligonucleotide. Unlabeled double-stranded oligonucleotide was used as a competitor. DNA-protein complexes were electrophoresed on native 6% polyacrylamide gels in 0.25x TBE buffer (22 mM Tris, 22

mM boric acid, and 0.5 mM EDTA, pH 8.0). Finally, the gels were vacuum-dried, and exposed to Kodak X-ray film at -70°C.

Results

Previous studies (11,19,20) showed that TNF-α is able to stimulate c-fos and c-jun gene expression at the transcriptional level and that the cytokine induces binding of nuclear protein to TRE, which is the consensus sequence for AP-1, in osteoblasts. Since recent studies have suggested that sphingosine metabolites act as signaling molecules in cellular signal transduction operated by several cytokines, our interest in this study was to explore whether sphingosine metabolites also act as signaling molecules in the TNF-α-induced expression of the c-fos and c-jun genes.

Involvement of ceramide in TNF-α-induced expression of c-fos and c-jun genes in MC3T3-E1 cells

Firstly, to examine whether endogenous ceramide is involved in TNF-α-induced expression of the c-fos and c-jun genes in MC3T3-E1 cells, we used the potent glucosylceramidase inhibitors PPPP and PDMP, which cause intracellular accumulation of endogenous ceramide (39,40). As shown in Fig. 1(A), TNF-α-induced expression of c-fos and c-jun was clearly increased in cells pretreated with either inhibitor. We also observed that the effect of PPPP was dose dependent [Fig. 1(B)]. These data suggest that PPPP pretreatment may increase cytokine-induced production of AP-1 in the cells; AP-1 is a transcription factor encoded by c-fos and c-jun (41-44). Using gel mobility shift assay, we investigated this point. As expected, PPPP pretreatment increased the cytokine-induced binding of nuclear proteins to TRE, a consensus sequence of AP-1, in the cells [Fig. 1(C)]. Since many studies have demonstrated that ceramide is generated from sphingomyelin via hydrolysis by SMase and that TNF-α-activated SMase causes accumulation of endogenous ceramide (21-24), we investigated whether exogenous SMase is able to induce expression of c-fos and c-jun. As shown in Fig. 2(A, B), we observed that SMase induced expression of both oncogenes as well as the binding of nuclear protein to TRE. These results strongly suggest that ceramide plays a functional role as a second messenger in TNF-α-induced AP-1 production in MC3T3-E1 cells.

Cell-permeable ceramide activated AP-1 via expression of c-fos and c-jun gene products in MC3T3-E1 cells

Several studies have suggested that ceramide acts as a second messenger in the response to TNF-α in several cell types. Since cell-permeable C_2-ceramide, C_6-ceramide, and

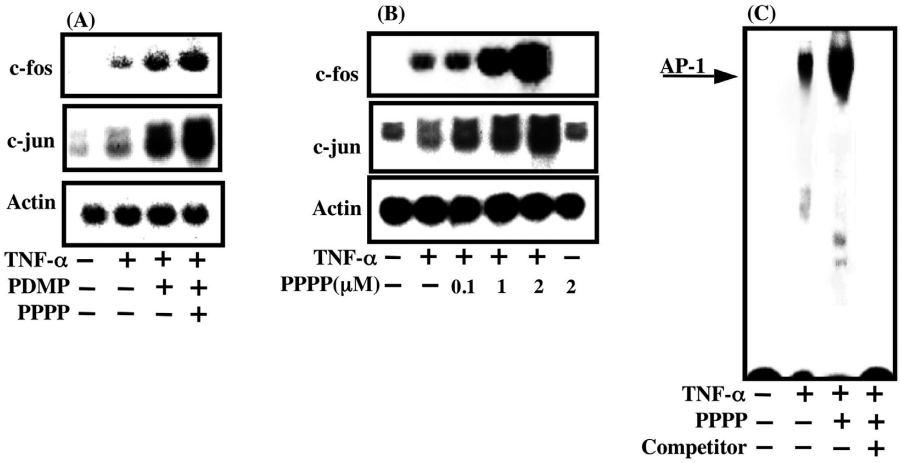

Fig. 1 Involvement of ceramide in TNF-α-induced expression of c-fos and c-jun genes and in TNF-α-activated AP-1of MC3T3-E1 cells.
(A) MC3T3-E1 cells were pre-treated with PDMP (2 μM) or PPPP (2 μM) for 3 h, followed by treatment with TNF-α (100 ng/ml). Total RNA was prepared at 0.5 h after initiation of treatment. Northern blot analysis was performed with mouse c-fos, c-jun, and β-actin cDNAs used as probes. (B) Confluent monolayers of MC3T3-E1 cells were pre-treated with PPPP at various doses. After 3 h, the cells were treated with TNF-α (100 ng/ml), and total RNA was prepared at 0.5 h after initiation of treatment. Northern blot analysis was performed with mouse c-fos, c-jun, and β-actin cDNAs used as probes. (C) MC3T3-E1 cells were treated with TNF-α at a dose of 100 ng/ml after pre-treatment with PPPP (2 μM), and nuclear proteins were prepared 3 h later. In the presence of nuclear proteins, gel mobility shift assays were performed with ^{32}P-labeled oligonucleotide containing the TRE sequence. The arrow indicates the position of DNA-protein complexes. Unlabeled oligonucleotide containing the AP-1 sequence was used as a competitor.

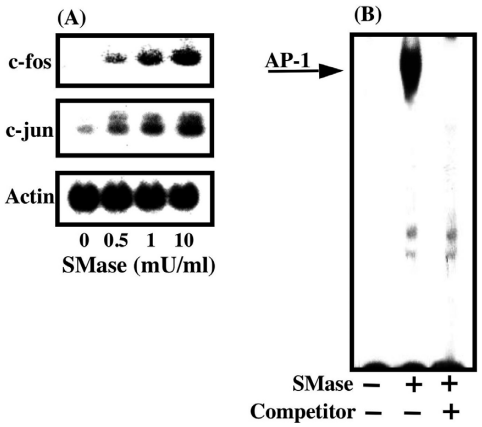

Fig. 2 Exogenous SMase is able to induce nuclear protein binding to TRE in MC3T3-E1 cells.
(A) MC3T3-E1 cells were treated with SMase at various doses [m units (mU)/ml]. Total RNA was prepared at 2 h after initiation of treatment. Northern blot analysis was performed with mouse c-fos, c-jun, and β-actin cDNAs used as probes. (B) MC3T3-E1 cells were treated with 1 mU of SMase, and nuclear proteins were prepared 3 h later. Gel mobility shift assay was performed with ^{32}P-labeled oligonucleotide containing the TRE sequence in the presence of nuclear proteins. The arrow indicates the position of DNA-protein complexes. Unlabeled oligonucleotide containing the AP-1 sequence was used as a competitor.

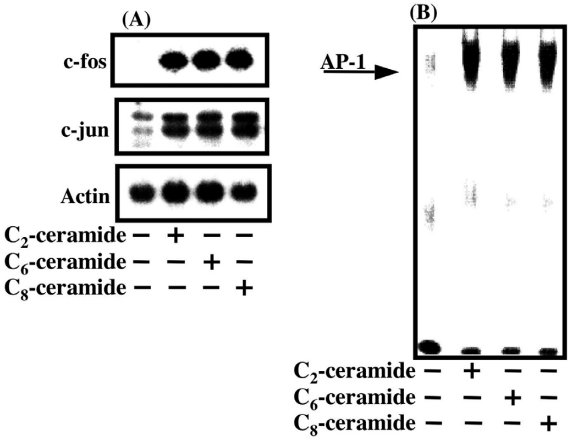

Fig. 3 Cell-permeable ceramide is able to induce AP-1 binding activity in MC3T3-E1 cells.
(A) MC3T3-E1 cells were treated with 1 μM C_2-ceramide, C_6-ceramide, or C_8-ceramide. Total RNA was prepared at 2 h after initiation of treatment. Northern blot analysis was performed with mouse c-fos, c-jun, and β-actin cDNAs used as probes. (B) MC3T3-E1 cells were treated with 1 μM C_2-ceramide, C_6-ceramide, or C_8-ceramide, and nuclear proteins were prepared 3 h later. Gel mobility shift assay was performed with ^{32}P-labeled oligonucleotide containing the TRE sequence in the presence of nuclear proteins. The arrow indicates the position of DNA-protein complexes.

C_8-ceramide are able to mimic biological activities mediated by endogenous ceramide (21-27), we used these cell-permeable ceramides to examine whether ceramide actually acts as a signaling molecule in TNF-α-induced AP-1 production in MC3T3-E1 cells. As shown in Fig. 3(A), C_2-ceramide, C_6-ceramide, and C_8-ceramide stimulated expression of both oncogenes. Stimulation by each ceramide was dose dependent (data not shown). These ceramides were also able to induce specific binding of nuclear proteins to TRE [Fig. 3(B)]. Taken together with the data shown in Figs. 1 and 2, these results suggest that TNF-α stimulates expression of c-fos and c-jun through ceramide action in MC3T3-E1 cells.

SPP mediated TNF-α-induced expression of c-fos and c-jun through ceramide signal pathway in MC3T3-E1 cells

Several studies (21-24) have shown that TNF-α exerts its biological activities via an SPP-dependent pathway. Therefore, in MC3T3-E1 cells, we addressed whether the cytokine-stimulated expression of c-fos and c-jun was SPP dependent by using DHS, a potent inhibitor of sphingosine kinase (21-27,31,35). As shown in Fig. 4(A), DHS pretreatment inhibited the TNF-α-induced expression of c-fos and c-jun in a dose-dependent manner. DHS also

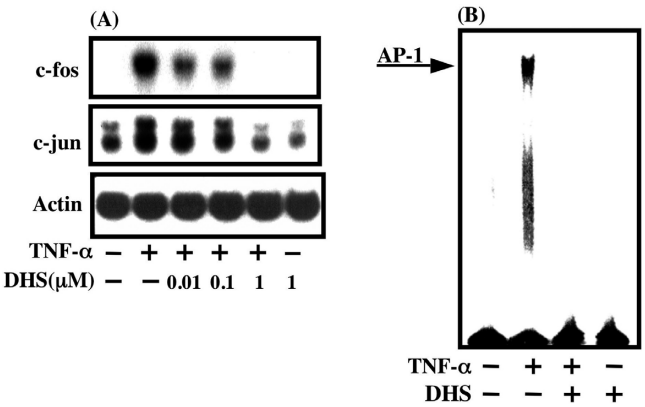

Fig. 4 SPP mediates TNF-α-induced AP-1 formation in MC3T3-E1 cells.
(A) MC3T3-E1 cells were incubated in the presence or absence of DHS at various concentrations. After 3 h, TNF-α at 100 ng/ml was added, and total RNA was prepared 0.5 h later. Northern blotting analysis was performed with mouse c-fos, c-jun, and β-actin cDNAs used as probes. (B) Cells were incubated in the presence or absence of DHS at 1 μM. After 3 h, TNF-α at 100 ng/ml was added, and nuclear proteins were prepared. Gel mobility shift assay was performed in the presence of nuclear proteins with ^{32}P-labeled oligonucleotide containing the TRE sequence. The arrow indicates the position of DNA-protein complexes.

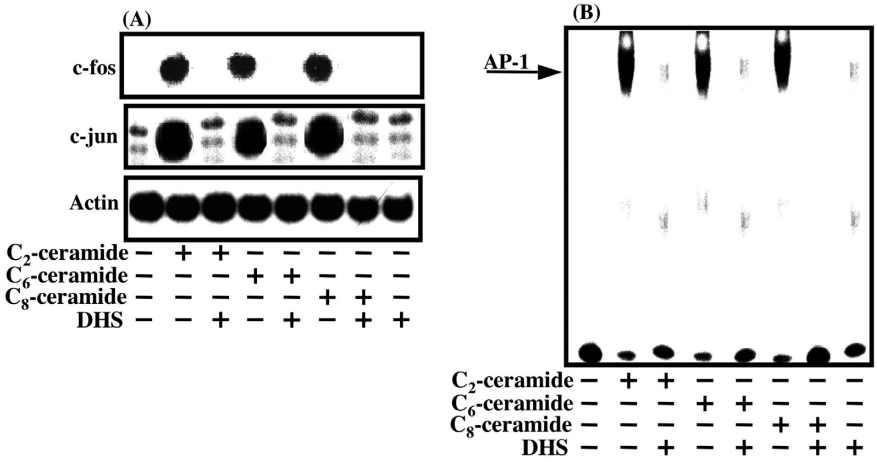

Fig. 5 SPP plays a functional role in ceramide-induced AP-1 formation in MC3T3-E1 cells.
(A) MC3T3-E1 cells were incubated in the presence or absence of DHS (1 μM). After 3 h, C_2-ceramide, C_6-ceramide, or C_8-ceramide at 1 μM was added, and total RNA was prepared 2 h later. Northern blotting analysis was performed with mouse c-fos, c-jun, and β-actin cDNAs used as probes. (B) Cells were incubated in the presence or absence of DHS at 1 μM. After 3 h, C_2-ceramide, C_6-ceramide, or C_8-ceramide at 1 μM was added, and nuclear proteins were prepared 3 h later. Gel mobility shift assay was performed with ^{32}P-labeled oligonucleotide containing the TRE sequence in the presence of nuclear proteins. The arrow indicates the position of DNA-protein complexes.

completely inhibited the cytokine-induced specific binding of nuclear proteins to TRE [Fig. 4(B)]. These data suggest that endogenous ceramide generated by TNF-α stimulation may induce expression of genes of both oncogenes via an SPP-dependent pathway. We also investigated whether DHS inhibited the cell-permeable ceramide-stimulated expression of c-fos and c-jun. As shown in Fig. 5(A), the inhibitor completely blocked the ceramide-induced expression of both oncogenes. Furthermore, DHS inhibited the ceramide-induced binding of nuclear proteins to TRE [Fig. 5(B)]. Taken together, these results suggest the involvement of SPP in the ceramide signal pathway operating in the TNF-α-induced expression of c-fos and c-jun in MC3T3-E1 cells.

SPP-induced expression of c-fos and c-jun genes and specific binding of nuclear protein to TRE in MC3T3-E1 cells

Our data suggested the involvement of SPP in the ceramide signal pathway in TNF-α-induced expression of c-fos and c-jun in MC3T3-E1 cells, and thus we were interested in examining whether SPP itself was able to stimulate expression of both oncogenes. As shown in Fig. 6(A), SPP potently stimulated the expression of these genes in the cells and increased the binding of nuclear proteins to TRE in the cells [Fig. 6(B)]. These results strongly suggest that SPP plays an important role as a signal molecule in the ceramide signal pathway in the TNF-α-induced production of AP-1 in MC3T3-E1 cells.

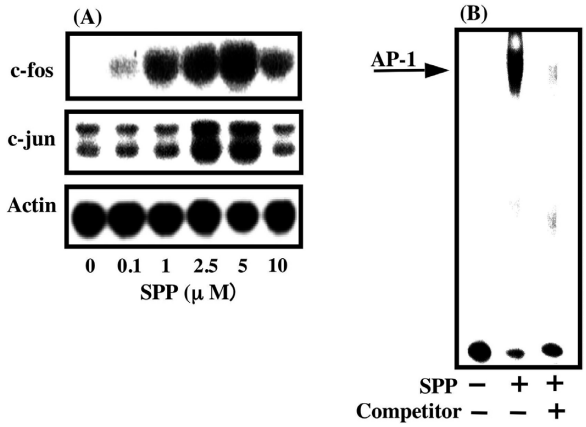

Fig. 6 SPP is able to stimulate expression of c-fos and c-jun in MC3T3-E1 cells.
(A) MC3T3-E1 cells were treated with SPP at various concentrations, and total RNA was prepared at 1.5 h after initiation of treatment. Northern blotting analysis was performed with mouse c-fos, c-jun, and _-actin cDNAs used as probes. (B) MC3T3-E1 cells were treated with SPP (5 μM), and nuclear proteins were prepared 3 h later. Gel mobility shift assay was performed with ^{32}P-labeled oligonucleotide containing the TRE sequence in the presence of nuclear proteins. The arrow indicates the position of DNA-protein complexes. Unlabeled oligonucleotide containing the AP-1 sequence was used as a competitor.

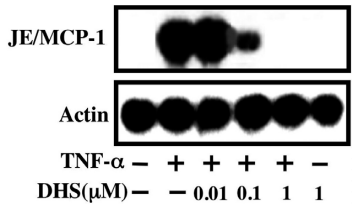

Fig. 7 SPP is involved in AP-1-mediated JE/MCP-1 gene expression in TNF-α-treated MC3T3-E1 cells.
MC3T3-E1 cells were incubated in the presence or absence of DHS at various concentrations. After 3 h, TNF-α at 100 ng/ml was added; and total RNA was prepared 1.5 h later. Northern blotting analysis was performed with mouse JE/MCP-1 and β-actin cDNAs used as probes.

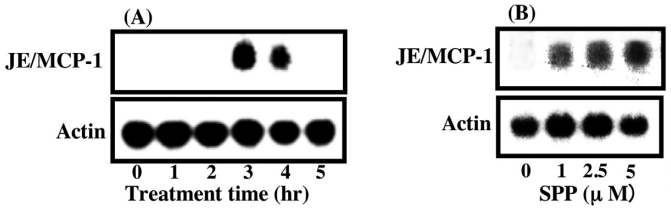

Fig. 8 SPP is able to stimulate JE/MCP-1 gene expression in MC3T3-E1 cells.
(A) MC3T3-E1 cells were treated with SPP at 5 μM, and total RNA was prepared at the indicated times after initiation of treatment. Northern blot analysis was performed with mouse JE/MCP-1 and β-actin cDNAs used as probes. (B) MC3T3-E1 cells were treated with SPP at various concentrations, and total RNA was prepared at 3 h after initiation of treatment. Northern blotting analysis was performed with mouse JE/MCP-1 and β-actin cDNAs used as probes.

Involvement of SPP in TNF-α-induced expression of monocyte chemoattractant JE/MCP-1 gene via AP-1 in MC3T3-E1 cells

It was of interest to determine whether SPP is involved in TNF-α-mediated physiological effects in osteoblastic cells. Indeed, it is well known that JE/MCP-1, a chemotaxis factor, plays functional roles in several inflammatory bone diseases (11,45,46). Moreover, we (11,19,20) previously demonstrated AP-1-mediated expression of the monocyte chemoattractant JE/MCP-1 in TNF-α-treated MC3T3-E1 cells. Based on these earlier findings, we explored the possibility that SPP actually acts as a signal molecule in the expression of the JE/MCP-1 gene in cytokine-treated cells. As shown in Fig. 7, DHS clearly inhibited the cytokine-stimulated expression of JE/MCP-1. In addition, SPP stimulated chemokine expression [Fig. 8 (A, B)]. These data thus indicate a functional role for SPP as a signal molecule in TNF-α-induced chemokine expression via AP-1 in MC3T3-E1 cells.

Discussion

Morphological and functional studies in c-fos transgenic and knock-out mice have strongly suggested that transcriptional factor AP-1 plays an important role in bone remodeling and metabolism (41-44). Many studies (11,19,20,47-51) have shown that osteogenic and osteolytic factors are able to stimulate expression of the c-fos and c-jun genes in ostoblastic cells. We have also previously shown that TNF-α is able to stimulate expression of c-fos and c-jun and to activate AP-1 in mouse osteoblastic MC3T3-E1 cells (11,19,20). Therefore, it was of interest to investigate which second messenger molecule is involved in TNF-α-induced expression of AP-1.

We first observed that PPPP and PDMP, which cause the accumulation of intracellular ceramide, potentiated the TNF-α-induced expression of c-fos and c-jun in MC3T3-E1 cells. These observations suggested that PPPP was able to increase the level of AP-1 binding to TRE. Gel mobility shift assay supported this suggestion. We observed that exogenous SMase actually induced expression of these genes and increased AP-1 activity. Because cell-permeable C_2-ceramide, C_6-ceramide, and C_8-ceramide also stimulated expression of both oncogenes and nuclear protein binding to TRE, it appears that ceramide may act as a second messenger in TNF-α-stimulated AP-1 binding to TRE in the cells.

Several studies (21-27,31,35) have demonstrated that SPP plays a functional role as a signal molecule in ceramide signaling in several types of cell, including osteoblasts. It is known that ceramide-induced apoptosis of human monocytic cells is blocked by endogenous SPP (21-25). We thus decided to examine whether SPP acts as a significant molecule in ceramide signal transduction associated with TNF-α-induced AP-1 production in MC3T3-E1 cells. We found that DHS, an inhibitor of sphingosine kinase, clearly inhibited both TNF-α- and ceramide analogue-induced AP-1 expression. These data suggest that TNF-α activates AP-1 through SPP-dependent ceramide signal transduction in MC3T3-E1 cells.

We then examined whether SPP is able to stimulate the expression of AP-1-responsive genes in MC3T3-E1 cells. It has been already shown that TNF-α-induced expression of JE/MCP-1 is AP-1 dependent (11,19,20), and thus we investigated the effects of DHS on TNF-α-induced expression of JE/MCP-1. We found that DHS completely inhibited this cytokine stimulation. Furthermore, we observed that SPP was able to stimulate the expression of JE/MCP-1. DHS also completely blocked C_2-ceramide-stimulated JE/MCP-1 gene expression (data not shown).

We previously found that DHS completely inhibited TNF-α-induced expression of the osteopontin gene, the expression of which requires AP-1 action (49,51), in MC3T3-E1 cells and that SPP was able to induce its expression (unpublished data). Interestingly, Kozawa et al. (31) also showed the involvement of an SPP signal pathway in TNF-α-stimulated expression of IL-6. Because numerous studies (7,10-14,19,20,31-33,45,46,48,49) have demonstrated the importance of IL-6, osteopontin, and JE/MCP-1 in bone resorption, it is highly possible that the SPP signal pathway contributes to TNF-α stimulation of osteoblasts to form osteoclasts. We are planning to examine the possible involvement of SPP in TNF-α-stimulated expression of receptor activator of nuclear factor-kappaB ligand (RANKL)/osteoclast differentiation factor (ODF) in MC3T3-E1 cells because recent studies (4,10,18) have demonstrated the importance of RANKL/ODF produced by osteoblasts in TNF-α-induced bone resorption.

Recent studies (21-24,27,31) have shown that intercellular SPP regulates signal molecules such as extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), focal adhesion kinase, and protein kinase C (PKC), to affect DNA synthesis, apoptosis, differentiation, and cell motility in several cell types. Our preliminary data showed that H-7, a potent inhibitor of serine/threonine kinases (particularly PKC), was able to suppress SPP-induced expression of c-fos and c-jun in MC3T3-E1 cells (unpublished data). On the other hand, H-89 and genistein, which are potent inhibitors of protein kinase A and tyrosine kinase, respectively, had no effect on c-fos and c-jun expression (unpublished data). We previously showed that H-7 also inhibits TNF-α-induced expression of c-fos and c-jun (11). Therefore, in subsequent experiments, we plan to determine the nature of the SPP-activated signal molecule functioning downstream of the ceramide signal pathway in MC3T3-E1 cells.

In conclusion, the present data suggest a novel pathway of TNF-α action in osteoblastic cells that functions in an SPP-dependent fashion.

Acknowledgments

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References

1. Mercado FB, Marshall RI, Bartold PM (2003) Inter-relationships between rheumatoid arthritis and periodontal disease. A review. J Clin Periodontol 30, 761-772
2. Krane, SM, Goldring MB, Goldring SR (1988) Cytokines. In Cell and molecular biology of vertebrate hard tissues. (Ciba Found Symp. 136), Evered D, Harnett S eds, John Wiley & Sons, Chichester, 239-256
3. Buchan G, Barrett K, Turner M, Chantry D, Maini RN, Feldmann M (1988) Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 α. Clin Exp Immunol 73, 449-455
4. Romas E, Gillespie MT, Martin TJ (2002) Involvement of receptor activator of NFκB ligand and tumor necrosis factor-α in bone destruction in rheumatoid arthritis. Bone 30, 340-346
5. Graves DT, Cochran D (2003) The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol 74, 391-401
6. Assuma R, Oates T, Cochran D, Amar S, Graves DT (1998) IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. J Immunol 160, 403-409
7. Mundy GR (1993) Cytokines and growth factors in the regulation of bone remodeling. J Bone Miner Res 8 (Suppl 2), S505-S510
8. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR (1986) Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factors. Nature 319, 516-518
9. Tashjian AH Jr, Voelkel EF, Lazzaro M, Goad D, Bosma T, Levine L (1987) Tumor necrosis factor-α (cachectin) stimulates bone resorption in mouse calvaria via a prostaglandin-mediated mechanism. Endocrinology 120, 2029-2036
10. Takahashi N, Udagawa N, Suda T (1999) A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. Biochem Biophys Res Commun 256, 449-455
11. Hanazawa S, Takeshita A, Amano S, Semba T, Nirazuka T, Katoh H, Kitano S (1993) Tumor necrosis factor-α induces expression of monocyte chemoattractant JE via *fos* and *jun* genes in clonal osteoblastic MC3T3-E1 cells. J Biol Chem 268, 9526-9532
12. Manolagas SC (1998) The role of IL-6 type cytokines and their receptors in bone. Ann NY Acad Sci 840, 194-204
13. Yoshitake H, Rittling SR, Denhardt DT, Noda M (1999) Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. Proc Natl Acad Sci USA 96, 8156-8160, 10944
14. Webb SJ, McPherson JR, Pahan K, Koka S (2002) Regulation of TNF-α-induced IL-6 production in

MG-63 human osteoblast-like cells. J Dent Res 81, 17-22
15. Yao GQ, Sun BH, Insogna KL, Weir EC (2000) Nuclear factor-κB p50 is required for tumor necrosis factor-α-induced colony-stimulating factor-1 gene expression in osteoblasts. Endocrinology 141, 2914-2922
16. Kaji K, Katogi R, Azuma Y, Naito A, Inoue JI, Kudo A (2001) Tumor necrosis factor α induced osteoclastogenesis requires tumor necrosis factor receptor-associated factor 6. J Bone Miner Res 16, 1593-1599
17. Kumar S, Votta BJ, Rieman DJ, Badger AM, Gowen M, Lee JC (2001) IL-1- and TNF-induced bone resorption is mediated by p38 mitogen activated protein kinase. J Cell Physiol 187, 294-303
18. Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, Sakai H (2000) Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-κB ligand: modulation of the expression by osteotropic factors and cytokines. Biochem Biophys Res Commun 275, 768-775
19. Hanazawa S, Takeshita A, Kitano S (1994) Retinoic acid suppression of *c-fos* gene inhibits expression of tumor necrosis factor-α-induced monocyte chemoattractant JE/MCP-1 in clonal osteoblastic MC3T3-E1 cells. J Biol Chem 269, 21379-21384
20. Takeshita A, Yasuda H, Ishida M, Ochiai K (2002) $1\alpha25(OH)_2D_3$ interferes with retinoic acid-induced inhibition of *c-fos* gene expression for AP-1 formation in osteoblastic cells. J Oral Sci 44, 27-34
21. Spiegel S, Milstien S (2000) Sphingosine-1-phosphate: signaling inside and out. FEBS Lett 476, 55-57
22. Pyne S, Pyne NJ (2000) Sphingosine 1-phosphate signalling in mammalian cells. Biochem J 349, 385-402
23. Maceyka M, Payne SG, Milstien S, Spiegel S (2002) Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. Biochim Biophys Acta 1585, 193-201
24. Olivera A, Kohama T, Edsall L, Nava V, Cuvillier O, Poulton S, Spiegel S (1999) Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. J Cell Biol 147, 545-558
25. Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind S, Spiegel S (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature 381, 800-803
26. Rani CS, Wang F, Fuior E, Berger A, Wu J, Sturgill TW, Beitner-Johnson D, LeRoith D, Varticovski L, Spiegel S (1997) Divergence in signal transduction pathways of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors. Involvement of sphingosine 1-phosphate in PDGF but not EGF signaling. J Biol Chem 272, 10777-10783
27. Kozawa O, Niwa M, Matsuno H, Tokuda H, Miwa M, Ito H, Kato K, Uematsu T (1999) Sphingosine 1-phosphate induces heat shock protein 27 via p38 mitogen-activated protein kinase activation in osteoblasts. J Bone Miner Res 14, 1761-1767
28. Chae HJ, Chae SW, Kang JS, Bang BG, Cho SB, Park RK, So HS, Kim YK, Kim HM, Kim HR (2000) Dexamethasone suppresses tumor necrosis factor-α-induced apoptosis in osteoblasts: possible role for ceramide. Endocrinology 141, 2904-2913
29. Carpio LC, Shiau H, Dziak R (2000) Changes in sphingolipid levels induced by epidermal growth factor in osteoblastic cells. Effects of these metabolites on cytosolic calcium levels. Prostaglandins Leukot Essent Fatty Acids 62, 225-232
30. Carpio LC, Stephan E, Kamer A, Dziak R (1999) Sphingolipids stimulate cell growth via MAP kinase activation in osteoblastic cells. Prostaglandins Leukot Essent Fatty Acids 61, 267-273
31. Kozawa O, Suzuki A, Kaida T, Tokuda H, Uematsu T (1997) Tumor necrosis factor-α-autoregulates interleukin-6 synthesis via activation of protein kinase C. Function of sphingosine 1-phosphate and phosphatidylcholine-specific phospholipase C. J Biol Chem 272, 25099-25104
32. Shinoda J, Kozawa O, Tokuda H, Uematsu T (1999) Effect of ceramide on interleukin-6 synthesis in osteoblast-like cells. Cell Signal 11, 435-441
33. Luegmayr E, Varga F, Glantschnig H, Fratzl-Zelman N, Rumpler M, Ellinger A, Klaushofer K (1998) 1, 25-Dihydroxy vitamin D_3 and tri-iodothyronine stimulate the expression of a protein immunologically related to osteocalcin. J Histochem Cytochem 46, 477-486
34. Kitajima I, Soejima Y, Takasaki I, Beppu H, Tokioka T, Maruyama I (1996) Ceramide-induced nuclear translocation of NF-κB is a potential mediator of the apoptotic response to TNF-α in murine clonal osteoblasts. Bone 19, 263-270
35. Takeshita A, Watanabe A, Takada Y, Hanazawa S (2000) Selective stimulation by ceramide of the expression of the α isoform of retinoic acid and

retinoid X receptors in osteoblastic cells. A role of sphingosine 1-phosphate-mediated AP-1 in the ligand-dependent transcriptional activity of these receptors. J Biol Chem 275, 32220-32226
36. Takeda H, Ozaki K, Yasuda H, Ishida M, Kitano S, Hanazawa S (1998) Sphingomyelinase and ceramide inhibit formation of F-actin ring in and bone resorption by rabbit mature osteoclasts. FEBS Lett 422, 255-258
37. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning. A Laboratory Manual, Cold Spring Habor Laboratory, Cold Spring Habor, New York, 196
38. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254
39. Nicholson KM, Quinn DM, Kellett GL, Warr JR (1999) Preferential killing of multidrug-resistant KB cells by inhibitors of glucosylceramide synthase. Br J Cancer 81, 423-430
40. di Bartolomeo S, Spinedi A (2001) Differential chemosensitizing effect of two glucosylceramide synthase inhibitors in hepatoma cells. Biochem Biophys Res Commun 288, 269-274
41. Jochum W, Passegue E, Wagner EF (2001) AP-1 in mouse development and tumorigenesis. Oncogene 20, 2401-2412
42. Wang ZQ, Liang J, Schellander K, Wagner EF, Grigoriadis AE (1995) *c-fos*-induced osteosarcoma formation in transgenic mice: cooperativity with *c-jun* and the role of endogenous *c-fos*. Cancer Res 55, 6244-6251
43. Grigoriadis AE, Schellander K, Wang ZQ, Wagner EF (1993) Osteoblasts are target cells for transformation in *c-fos* transgenic mice. J Cell Biol 122, 685-701
44. Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, Wagner EF (1994) *c-Fos*: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. Science 266, 443-448
45. Graves DT, Jiang Y, Valente AJ (1999) The expression of monocyte chemoattractant protain-1 and other chemokines by osteoblasts. Front Biosci 4, D571-D580
46. McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM (2002) Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties *in vivo*. Blood 100, 1160-1167
47. Gurlek A, Kumar R (2001) Regulation of osteoblast growth by interactions between transforming growth factor-β and 1 α,25-dihydroxyvitamin D_3. Crit Rev Eukaryot Gene Expr 11, 299-317
48. Takeshita A, Chen Y, Watanabe A, Kitano S, Hanazawa S (1995) TGF-β induces expression of monocyte chemoattractant JE/monocyte chemoattractant protein 1 via transcriptional factor AP-1 induced by protein kinase in osteoblastic cells. J Immunol 155, 419-426
49. Takeshita A, Imai K, Kato S, Kitano S, Hanazawa S (1998) 1 α,25-dehydroxyvitamin D_3 synergism toward transforming growth factor-β 1-induced AP-1 transcriptional activity in mouse osteoblastic cells via its nuclear receptor. J Biol Chem 273, 14738-14744
50. Chen Y, Takeshita A, Ozaki K, Kitano S, Hanazawa S (1996) Transcriptional regulation by transforming growth factor β of the expression of retinoic acid and retinoid X receptor genes in osteoblastic cells is mediated through AP-1. J Biol Chem 271, 31602-31606
51. Chang PL, Prince CW (1991) 1 α, 25-dehydroxyvitamin D_3 stimulates synthesis and secretion of nonphosphorylated osteopontin (secreted phosphoprotein1) in mouse JB6 epidermal cells. Cancer Res 51, 2144-2150