Direct Evidence for an Important Role of Sphingomyelinase in Ultraviolet-induced Activation of c-Jun N-Terminal Kinase*

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Sphingomyelinase (SMase) and its product ceramide have recently attracted a great deal of attention because of their possible role in the signal transduction pathway. However, the role of sphingomyelinase in UV-induced c-Jun N-terminal kinase (JNK) activation is still unclear. Thus, we investigated this issue directly using a genetic SMase-deficient (2–3% residual acid SMase activity) lymphoblast cell line, MS1418. The results showed that while UV irradiation markedly induces JNK activation in a normal human lymphoblast cell line, JY, it induces only weak JNK activation in MS1418 cells. This difference of JNK response to UV irradiation between these two cell lines was further observed in time course and dose-response studies. In contrast, 12-O-tetradecanoylphorbol-13-acetate-induced JNK activation could be observed in both JY and MS1418 cells. Furthermore, significant JNK activation can be observed in MS1418 cells by exposure of the cells to SMase or C2-ceramide, whereas phospholipase A2 or phospholipase C did not show significant induction of JNK activity, and C2-dihydroceramide and sphingosine induce only much weaker JNK activation in MS1418 cells than that by C2-ceramide. These data demonstrated that SMase plays an essential role in UV-induced JNK activation.

UV radiation can act as both a tumor initiator and as a tumor promoter (1, 2). Exposure of mammalian cells to UV light causes the activation of activator protein-1 (AP-1) and nuclear factor κB, which is known as the “UV response” and believed to be involved in the tumor-promotion effects of UV light (3–7). Previous studies indicated that exposure of cells to UV light rapidly activates Src-family tyrosine kinase, followed by activation of the Ha-Ras, the cytoplasmic serine-threonine kinase Raf-1 as well as c-Jun, an important component of AP-1 (8, 9). Since c-Src and Ha-Ras are involved in the UV-induced signal transduction pathway, the primary signal generated by UV must be initiated from upstream of Ras/Raf. Therefore, it was assumed that the signaling cascade leading to the activation of AP-1 by UV is generated from the plasma membrane (10). Very recently, we demonstrated that atypical PKC (aPKC) is required for UV-induced AP-1 activation by using both a mouse PKC-ζ antisense and a dominant negative mutant construct of Xenopus PKC-αI (6, 7). Although the upstream effector of aPKC in the UV signal transduction cascade is not clear, some lipids or their metabolites, such as phosphatidic acid and phosphatidylinositol-3,4,5-P3, are believed to be responsible for the activation of aPKC (11–14). Ceramide induces phosphorylation of aPKC in cells and activates the aPKC enzyme activity in vitro (15–17).

Ceramide is a sphingolipid that plays an important role in the regulation of cell growth and differentiation, cell-cell contact, and oncogenesis (17, 18). Increasing evidence also indicates important roles of ceramide as a second messenger (19, 20). A number of extracellular stimulations can result in activation of sphingomyelinase (SMase) that causes hydrolysis of sphingomyelin, a phospholipid largely confined to the outer leaflet of cellular membranes, and the generation of ceramide (22, 23). Among these inducers, UV irradiation leads to a rapid increase in ceramide above a basal level of 80 pmol/10⁶ cells (24). Based on this evidence and the results from different groups indicating that addition of exogenous ceramides to cells induces JNK activation (24), we proposed that UV-induced JNK activation is dependent on SMase. In the present study, we used genetic variants of cells to delineate the role of sphingomyelinase in the UV-induced JNK activation.

EXPERIMENTAL PROCEDURES

Reagents—C2-ceramide, C2-dihydroceramide (C2-Dhc), sphingosine, phospholipase A2 (PLA2), phospholipase C (PLC), and SMase were from BIOMOL; 12-O-tetradecanoylphorbol-13-acetate (TPA) was from Sigma; Eagle’s minimal essential medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), and RPMI 1640 were from Life Technologies, Inc.; fetal bovine serum (FBS) was from Life Technologies, Inc.; luciferase substrate was from Promega; and the SAPK/JNK assay kit was from New England Biolabs.

Cell Culture—EBV-transformed normal human lymphoblast cell lines, JY, or Niemann-Pick disease lymphoblast MS1418 (21), were a generous gift from Dr. Richard Kolesnick, Laboratory of Signal Transduction, Memorial Sloan-Kettering Cancer Center, New York. These two cell lines were maintained in the mixture of RPMI 1640 and DMEM (1:1, v/v) containing 15% FBS, 2 mM l-glutamine, and 25 mg of gentamicin/ml. Stable AP-1 luciferase reporter plasmid-transfected mouse epidermal JB6 P+ cells (25, 26) were cultured in Eagle’s minimal essential medium containing 5% fetal calf serum, 2 mM l-glutamine, and 25 μg of gentamicin/ml. All the cells were grown at 37 °C in a 5% CO2 atmosphere.

Assay for AP-1 Activity—Confluent monolayers of JB6 P+4 cells were trypsinized and 8 × 10⁶ viable cells suspended in 100 μl of 5% FBS MEM were added into each well of a 96-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO2. Twelve to twenty-four hours later, cells were starved by culturing cells in 0.1% FBS MEM for 12 h, and then exposed to UV, C2-ceramide, or SMase for AP-1

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induction for 24 h. Since the normal UVB lamp also generates a small amount of UVC light, the UVB irradiation was carried out in a UVB exposure chamber fitted with a Kodak Kodacel K6808® filter that eliminates all wavelengths below 290 nm. The cells were extracted with lysis buffer and luciferase activity was measured using a luminometer (Monolight 2010®). The results are expressed as the relative AP-1 activity.

**JNK Assay**—JNK assay was carried out as described by protocol of New England Biolabs. In brief, JB6 C141 cells or lymphoblasts JY or MS1418 cells were starved for 24 h in 0.1% FBS MEM or 0.5% FBS. After cultured at 37 °C overnight, the cells were starved by replacing the medium with 0.1% FBS MEM medium for 24 h. Then, the cells were or were not exposed to UVB (3 kJ/m² with filter), UVC (30 J/m²), B, different concentrations of C₂-ceramide or sphingosine; or C, SMase. After a 24-h culture, the AP-1 activity was measured by luciferase activity assay as described previously (25). The results are presented as relative AP-1 activity.

**FIG. 1. Induction of AP-1 activity by UV irradiation, SMase, or C₂-ceramide in JB6 cells.** Eight × 10⁴ JB6 AP-1 luciferase reported stable transfected P1–1 cells suspended in 5% FBS MEM medium were seeded into each well of 96-well plates. After cultured at 37 °C overnight, the cells were starved by replacing the medium with 0.1% FBS MEM medium for 24 h. Then, the cells were or were not exposed to A, UVB (3 kJ/m² with filter), UVC (30 J/m²); B, different concentrations of C₂-ceramide or sphingosine; or C, SMase. After a 24-h culture, the AP-1 activity was measured by luciferase activity assay as described previously (25). The results are presented as relative AP-1 activity.
FIG. 2. JNK activation by UV irradiation, SMase, or C2-ceramide in JB6 cells. JB6 Cl 41 were cultured in monolayers in 100-mm-diameter dishes until to 90% confluent. The cells were starved by changing the medium with 0.1% FBS MEM for 24–48 h. Then, the cells were exposed to A, UVB (3 kJ/m² with filter); UVC (30 J/m²) and cultured for 30 min. The cells were harvested, and JNK activity was measured as described under “Experimental Procedures.”

FIG. 3. UV irradiation induces JNK activation in JY cells. Normal lymphoblasts, JY, or SMase-deficient lymphoblasts MS1418 were starved for 24–48 h in 0.5% FBS mixture of RPMI 1640 and DMEM at 37 °C, 5% CO₂ atmosphere incubator. The cells were washed once with culture medium, then the cells (5 × 10⁶/sample) were or were not exposed to TPA (10 ng/ml), UVB (2 kJ/m² with filter) or UVC (30 J/m²) and cultured for 30 min. The cells were harvested, and JNK activity was measured as described under “Experimental Procedures.”

RESULTS

AP-1 Activation Induced by UV Irradiation, Sphingomyelinase, or Ceramide—Previous work indicated that UV irradiation induces both activation of transcription factor AP-1 and a rapid increase of ceramide (6, 7, 24). One report showed that exogenous ceramide induced the activation of JNK (24). Therefore, we proposed that SMase and its product are involved in UV-induced AP-1 activation. To test this hypothesis, we exposed P1 cells, a stable AP-1 luciferase transfectant of epidermal JB6 cells, to UV irradiation, SMase, or cell-permeable synthetic C2-ceramide for AP-1 induction. The results showed that, in addition to UV irradiation, both SMase and C2-ceramide induced the transactivation of AP-1 activity in a dose-dependent manner (Fig. 1), whereas sphingosine, a metabolite of ceramide, did not induce AP-1 activity (Fig. 1). We further measured the JNK activation of JB6 cells exposed to these agents. Significant JNK activations were observed in cells exposed to UV irradiation or stimulated by either SMase or C2-ceramide (Fig. 2).
exposed cells, and exogenous ceramide could induce activation of JNK activity by C2-Dhc or sphingosine. MS1418 cells were starved for 48 h in 0.5% FBS mixture of RPMI 1640 and washes once with culture medium, then the cells (5 x 10^6 sample) were exposed to A, SMase or different concentration of PLA2 or PLC; B, UVC (30 J/m^2), C2-ceramide (20 μM) or different concentration of C2-Dhc or sphingosine, and cultured for 30 min. The cells were harvested, and JNK activity was measured as described under “Experimental Procedures.”

UV Irradiation Induces JNK Activation in Normal JY Lymphoblasts—To examine the role of SMase in UV-induced JNK activation directly, we compared the activation of JNK by UV irradiation between EBV-transformed normal human lymphoblast cell line, JY, and EBV-transformed SMase-deficient (2–3% residual acid SMase activity) lymphoblast cell line, MS1418. The results are shown in Fig. 3. UV irradiation markedly induced JNK activation in normal human lymphoblast, JY, while only very weak JNK activation was found in MS1418, a SMase-deficient lymphoblast cell line. In contrast, similar levels of TPA-induced JNK activation were observed in both JY cells and MS1418 cells (Fig. 3). The results from time course and dose-response studies are consistent with these findings (Fig. 4). These results strongly suggest that SMase is involved in the UV-induced JNK activation pathway.

Rescued JNK Activation by SMase or Ceramide in SMase-deficient MS1418 Cells—To further demonstrate a role for SMase in JNK activation, we investigated the effect of SMase and C2-ceramide on JNK activation in MS1418 cells. Addition of exogenous SMase directly into the culture medium caused activation of JNK in both JY and MS1418 cells (Fig. 5). Furthermore, exposure of MS1418 cells to C2-ceramide also caused activation of JNK (Fig. 5). In contrast, PLA2 or PLC did not show significant induction of JNK activity, and C2-Dhc (an inactive form of ceramide) or sphingosine (a metabolic of ceramide) induces only very weak JNK activation in MS1418 cells (Fig. 6). These data are consistent with previous reports that C2-Dhc slightly induces JNK activation in U937 cells (Fig. 3 of Ref. 24). These experiments support the model that the defects of UV-induced JNK activation in MS1418 cells is due to its deficiency of SMase.

DISCUSSION

Growing evidence indicates the important role for SMase and its product ceramide in TNF-α- and interleukin-1-induced signal transduction. Verheij et al. (24) reported that UVC or x-ray irradiation lead to an increase of ceramide production in exposed cells, and exogenous ceramide could induce activation of both Erks and JNKs. However, it is not clear whether ceramide and SMase are required for UV-induced activation of a signal transduction pathway. In this study, we addressed this issue by using a genetic SMase-deficient lymphoblast. While UV irradiation induces a high level of JNK activity in normal human lymphoblasts, it induces little JNK activation in MS1418 cells, SMase-deficient human lymphoblasts. In contrast, TPA induces activation of JNK in both JY and MS1418 cells. Moreover, significant JNK activation is observed in MS1418 lymphoblasts by exposure of cells to exogenous SMase or C2-ceramide. In a JB6 mouse epidermal cell line, SMase and C2-ceramide also induce the activation of JNK and transcription factor AP-1. All this evidence demonstrated that SMase plays an essential role in UV-induced signal transduction.

Sphingomyelin is preferentially localized in the outer leaflet of the plasma membrane of most mammalian cells (19). It is comprised of a long chain sphingosine backbone, a fatty acid, and a phosphocholine head group (19). The activation of SMase results in the hydrolysis of sphingomyelin to yield ceramide and phosphocholine (27). It has long been known that a number of extracellular stimulators can lead to activation of SMase. These stimulators include UV or ionizing irradiation, heat shock, nerve growth factor, TNF-α, endotoxin, interferon-γ, interleukin-1, Fas, and CD28 (17, 22, 23). Some evidence revealed involvement of sphingolipids in signaling transduction pathways that are associated with the regulation of cell growth, differentiation, and apoptosis (17–21). Synthetic ceramide mimicked vitamin D3 in inducing monocytic differentiation in HL60 cells (22, 23). TNF-α induced apoptosis in a number of cellular models, including U937 monocytic cells, HL60 cells, and L929 fibrosarcoma cells. TNF-α also induced rapid sphingomyelin hydrolysis to ceramide. Further, synthetic ceramide analogs and sphingomyelinase mimicked the action of TNF-α in the initiation of apoptosis (19, 28, 29). All of these experiments involving the role of ceramide rely largely on intact cells and their exposure to permeable ceramides (18). Although it has been reported that ceramide activates the Erks and JNKs and that UV irradiation causes the increase of ceramide, it is still not clear whether SMase and its product, ceramide, play a role in UV-induced JNK activation and the transactivation of AP-1. In the present study, we found that UV irradiation, SMase, or C2-ceramide induces JNK activation and transactivation of AP-1 activity in the JB6 cell system. In the lymphoblast cell system, marked UV-induced JNK activation is observed in a normal human lymphoblast, JY, but only a little activation of JNK in MS1418, a SMase-deficient (2–3% residual acid SMase activity) lymphoblast cell line. Moreover, exposure of cells to SMase or C2-ceramide leads to significant JNK activation in either JY cells or MS1418 cells. These data suggest that the lack of response of MS1418 cells to UV irradiation in terms of JNK activation is due to its deficiency of SMase, but not the downstream location of SMase. Thus, we provide direct evidence that SMase and its products play an essential role in UV-induced JNK activation. The little activation of JNK activity in MS1418 cells induced by UV irradiation may be due to the response of cells through the 2–3% residual acid SMase activity.

The use of cell-permeable ceramide has shown that different serine/threonine protein kinase cascades, as well as protein phosphatases, are activated. A ceramide-activated protein kinase can phosphorylate Raf-1, which in turn activates the Erk2 (30). Ceramide might also act as an upstream activator of Ras (31). In stress-response kinase cascades, ceramide activates the JNKs possibly through stress-activated protein kinase/Erk kinase (4, 32). It has been suggested that ceramide induces the activation of a signal transduction pathway leading to activa-
tion of nuclear factor κ B by activation of PKC-ζ (16). However, the direct target of ceramide is not known at present. The direct target of ceramide should be activated by ceramide in vitro, and it should mediate most of the biological effects of ceramide in cells. Our previous studies demonstrated that aPKCs are required for UV-induced AP-1 activation (6, 7). Since UV responses are believed to be initiated from the plasma membrane (10), sphingomyelin hydrolysis by sphingomyelinase and stimulation of ceramide-activated protein kinase likely also occur within the plasma membrane (19). Taken together with the evidence that ceramide induces phosphorylation of PKC-ζ in cells and it activates the PKC-ζ in vitro (19), we speculate that the direct target of ceramide in UV-induced JNK and AP-1 activation may be aPKC.

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