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Cell line dependent involvement of ceramide in ultraviolet light-induced apoptosis

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Abstract

Ultraviolet light (UV) activates an acid sphingomyelinase (ASMase) pathway, which hydrolyzes sphingomyeline to ceramide. Ceramide has been found to be a second messenger, which activates the c-jun N-terminal kinase (JNK) that is required for apoptotic cell death. However, the role of ceramide in UV-induced JNK activation and apoptosis remains controversial. In this study, we examined the correlation among ceramide production, JNK activation and cell apoptosis after UV-irradiation in three cell lines: 293 (kidney), Jurkat (lymphocytes) and MCF-7 (breast) were used in this study. The ceramide production was analyzed using the diacylglycerol kinase assay method. The JNK activation was measured by Western blot analysis using an antibody specifically recognizing phosphorylated JNK. Cell apoptosis was determined by morphological change or flow cytometry. Our data show that UV-irradiation induces ceramide production in both 293 and Jurkat cells. Inhibition of ceramide production by desipramine (25–50 μM) reduced UV-induced JNK activation in both 293 and Jurkat cells; and protects 293 cells from UV-induced apoptosis. However, inhibition of ceramide production does not prevent Jurkat cells from UV-induced apoptosis. In addition, our data demonstrates that UV-irradiation induces JNK activation and apoptosis of MCF-7 cells without production of detectable amounts of ceramide after UV-irradiation. These results suggest that UV-induced JNK activation and apoptosis can be mediated through a ceramide dependent or an independent pathway. (Mol Cell Biochem **219**: 21–27, 2001)

Key words: ultraviolet light, ceramide, sphingomyelinase, apoptosis

Abbreviations: UVC – ultraviolet light, 254 nm; ASMase – acid sphingomyelinase; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Ultraviolet light (UV) is an important environmental factor, which has wide ranging effects in every day life. Overexposure to UV light can lead to skin inflammation, premature aging and ultimately cancer. UV-induced apoptosis eliminates UV-damaged cells and thus reduces the likelihood that DNA damaged cells will develop into cancers. Apoptosis is a complex biological process. Recent extensive investigations have elucidated some key components that are involved in UV-induced apoptosis pathways. UV-induced apoptosis consists of two pathways: a nuclear DNA damage dependent pathway and a membrane dependent pathway. The DNA-damage caused by UV-irradiation activates p53, which induces its own gene expression and activates downstream genes that induce apoptosis [1]. UV-irradiation also activates membrane death receptors, such as Fas-receptor and TNFα-receptor. UV-irradiation induces aggregation of Fas-receptors, which activates Fas-FADD-caspase-8 axis for the production of active caspases. Overexpression of a dominant negative FADD (DN-FADD) blocked UV-induced apoptosis of BJAB cells [2, 3]. The aggregation of Fas-receptor or TNFα-receptor also activates an acid sphingomyelinase (ASMase) pathway, which is an ubiquitous signaling system that links specific

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cell surface receptors and environmental stresses to cellular responses [4–7]. ASMase is a lysosomal enzyme (pH optimum 4.5–5.0), which hydrolyzes sphingomyelin to ceramide and serves as a second messenger to induce apoptosis [8, 9]. It was suggested that ceramide mediates the UV-induced activation of the N-terminal c-jun kinase (JNK), which is required for transcriptional activation of pro-apoptotic genes. Inhibition of JNK activation by overexpressing a dominant negative JNK (DN-JNK) blocked UV-induced apoptosis of 293 cells [10]. The UV-induced JNK activation is blocked in ASMase-deficient cell lines. [11]. However, this result contradicted the finding that the fibroblasts from Niemann-Pick A patients deficient in ASMase did not show altered activation of JNK in response to either TNFα or UVC [12]. Therefore, the actual role of ASMase and ceramide in UV-induced JNK activation and apoptosis remains unclear.

In this manuscript, we provide evidences that UV-induced JNK activation and apoptotic signaling are mediated either by a ceramide dependent or ceramide independent pathways. Our results suggest that requirement of ceramide for UV-induced JNK activation and apoptosis is cell type dependent. Based on our results, a model for UV-induced JNK activation and apoptosis is proposed.

Materials and methods

Cell lines and culture conditions

293 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. Jurkat and MCF-7 cells were cultured in RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. All of the cell culture reagents were obtained from Life Technologies (Gaithersburg, MD, USA).

UV-irradiation

Cells were irradiated with UVC generated from a 15 W UVC light source (UVP Inc., Upland, CA, USA). The intensity of UVC was measured using an UV-meter (UVP Inc., Upland, CA, USA). For low dose (below 100 J/m^2) of UV-irradiation, the intensity of UVC was set at 15 W/cm^2. For high dose (above 100 J/m^2) of UV-irradiation, the intensity of UVC was set at 30 W/m^2. Adherent cells (70–80% confluent on 60 mm plate) were irradiated with 200 ml culture media. Suspension cells (10–20 × 10^6) were pelleted, resuspended in 0.5 mL media and plated on a 60 mm plate for irradiation. After UV-irradiation, 2 mL media was added to each 60 mm plate.

Western blot analysis

Cells were harvested and lysed with NP-40 lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris-HCl, 0.1% SDS, 1× Complete™ protease inhibitor cocktail). After centrifugation at 14,000 rpm for 10 min, supernatants were collected, and the protein concentration was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The protein samples were resolved by SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were immunoblotted with antibodies against JNK or phosphorylated JNK (New England Biolab Inc., Beverly, MA, USA). The proteins of interest were then probed with correlated HRP-conjugate-antibodies and analyzed by the SuperSignal Chemiluminescent detection system (Pierce, Rockford, IL, USA) according to the manufacturer's protocol (Pierce, Rockford, IL, USA).

Ceramide extraction and analysis

Ceramide was quantified by diacylglycerol kinase assay following the procedure as previously described [13] with modifications. The cultured cells (20 × 10^6) after UV irradiation (100 J/m^2) were pelleted by centrifugation (300 × g for 10 min), and washed twice with ice-cold phosphate buffer saline (PBS). The cells were lysed with 1 mL of $CHCl_3$:MeOH (1:2) and the cell lysates were vortexed for 30 min. Then, 1 mL of $CHCl_3$ was added to the lysates. The mixtures were vortexed for another 30 min. Finally, 1 mL of H_2O was added to the mixtures and vortexing was continued for an additional 30 min. The resultant mixtures were centrifuged at 12,000 rpm for 10 min to separate the aqueous and organic layer. The organic phases, which contained the purified lipid, were isolated, dried and subjected to mild alkaline hydrolysis (0.1 N methanolic KOH for 1 h at 37°C) to remove glycerophospholipids. The samples were extracted again following the same procedure as mentioned above and the organic phases were isolated and dried under a vacuum. Ceramide in each sample was resuspended in 100 μL of reaction mixture containing 150 μg of Cardiolipin, 280 μM diethylenetriamine-pentaacetic acid, 51 μM octyl-β-D-glucopyranoside, 50 mm NaCl, 51 mM imidazole, 1 mM EDTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.7% glycerol, 70 μM β-mercaptoethanol, 1 mM ATP, 10 μCi of (γ-^{32}P) ATP (3000 Ci/mmole) and 35 μg/mL *E. coli* diacylglycerol kinase at pH 6.5. After 30 min at room temperature, the reaction was stopped by extraction of lipids with 1 mL of $CHCl_3$:MeOH:1 N HCl (100:100:1), 170 μL of buffered saline solution (BSS) (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose and 10 mM HEPES (pH 7.2) and 30 μl of 100 mM EDTA. The organic phases were recovered and dried under a vacuum. Ceramide-1-^{32}P-phosphate was resolved by thin layer chromatography

(TLC) on silica gel 60 plates (Sigma Corp. St. Louis, MO, USA) using solvent system of chloroform:methanol:acetic acid (65:15:5) and was detected by autoradiography. The spots containing ceramide-1-^{32}P-phosphate were excised from the TLC plate and the incorporated ^{32}P was quantified by liquid scintillation counting.

Desipramine treatment

Desipramine was added to the medium from a 1 mM stock solution in dimethyl formamide to achieve a final concentration of 25 µM. Treatment was carried out for 2 h before the cells were subjected to UV-irradiation.

Study of cell morphology after UV-irradiation

293 and MCF-7 cells were plated at a density of 5×10^4 cells per plate. Both control and desipramine pretreated 293 and MCF-7 cells were irradiated with 0 or 15 J/m^2 of UVC. At 48 h of post-irradiation, cell morphology was examined using Olympus CK2 microscope (Olympus America Inc., Melville, NY, USA) and cell images were captured using Kodak DC120 megapixel digital camera. The images were resolved using MDS120 microscopy documentation software (Kodak Scientific, New Haven, CT, USA).

Estimation of percentage of apoptotic cells using Flow Cytometry

Percentage of apoptotic cells was estimated by measuring sub-G1 DNA content using Flow Cytometry. Both control and desipramine pretreated 293, Jurkat and MCF-7 cells were irradiated with 0 or 15 J/m^2 of UVC. At 48 h post-irradiation, the cells were rinsed with PBS, fixed in 70% Ethanol in PBS, rehydrated in PBS, treated with RNase A (20 µg/ml) and stained with propidium iodide (50 µg/ml in PBS) at room temperature for 30 min. The stained cells were analyzed on an EPICS C flow cytometer in the Flow Cytometry Core of University of Michigan, USA.

Results

Inhibition of ASMase activity protects 293 cells from UV-induced apoptosis

Since the activation of ASMase is suggested to mediate the UV-induced apoptosis, we first determined if inhibition of ASMase activity protects cells from UV-induced apoptosis. 293, Jurkat and MCF-7 cells were treated or untreated with 25 µM desipramine, which is an ASMase inhibitor [14] and then UV-irradiated. At 48 h post-irradiation, the apoptosis of cells were analyzed using flow cytometry. Upon UV-irradiation, untreated 293 cells showed a 2.4-fold increase in apoptosis. Whereas, desipramine-treated 293 cells showed only 0.7 fold increase in apoptosis (Fig. 1, panel A). UV-irradiation also induced apoptosis of untreated Jurkat and MCF-7 cells at the same levels as 293 cells, which is 1.95- and 2.65-fold respectively (Fig. 1, panel A). However, desipramine treatment of Jurkat and MCF-7 cells did not significantly alter the fold increase of apoptosis of these cells after UV-irradiation (Fig. 1, panel A). To confirm the flow cytometry results, we also analyzed the morphology of the UV-irradiated adherent cells. Our data show that desipramine protects against UV-induced apoptosis of 293 cells, even thought it is slightly toxic to the cells (Fig. 1, panel B). Desipramine has no effect on MCF-7 cells, which were UV-irradiated or non-irradiated (Fig. 1, panel C). These data agree with the flow cytometry data shown in the same figure. These results suggest that activation of ASMase is only required in certain cell types for apoptotic signaling.

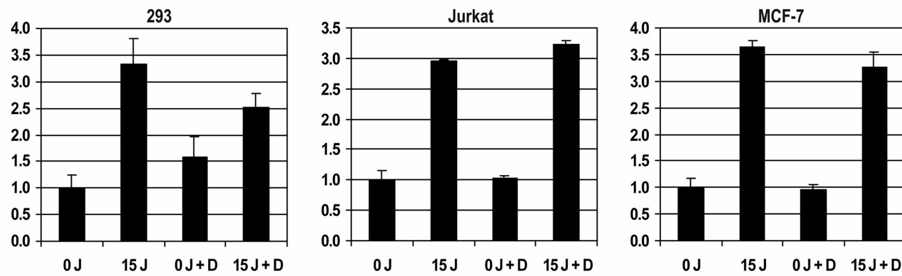
UV-irradiation resulted in induction of ceramide in 293 and Jurkat cells but not in MCF-7 cells

To confirm that the desipramine treatment inhibits ASMase activity for generating ceramide, we analyzed the level of ceramide in desipramine treated and untreated cells, which were irradiated with 0 or 100 J/m^2 of UVC. Our data shows that in both 293 and Jurkat cells, intracellular ceramide levels were increased after UV-irradiation (Fig. 2, panels A and B, lane 2 vs. 1). Ceramide quantification revealed 34 ± 8 and 44 ± 10% increase of ceramide in UV-irradiated 293 and Jurkat cells over non-irradiated cells (Fig. 2, panel D). After desipramine treatment, ceramide production was reduced by 4 ± 4% in 293 cells and 15 ± 6% in Jurkat cells (Fig. 2, panels A and B, lane 3; panel D). UV-irradiation did not induce the production of ceramide in these cells after desipramine treatment (Fig. 2, panels A and B, lanes 4 vs. 3). Ceramide was not detected in MCF-7 cells even after UV-irradiation (Fig. 2, panel C). Our results suggest that ASMase activity is required for UV-induced ceramide production. However, increased ceramide production is not always required for UV-induced apoptosis.

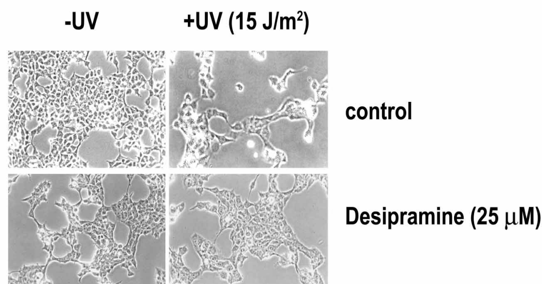
The UV-induced JNK activation is partially mediated by ceramide in 293 cells

Although a coordinated regulation via the sphingomyelin pathway and JNK activation has been suggested, the effect

A) Percentages of apoptotic cells after UV irradiation



B) Morphology of 293 cells



C) Morphology of MCF 7 cells

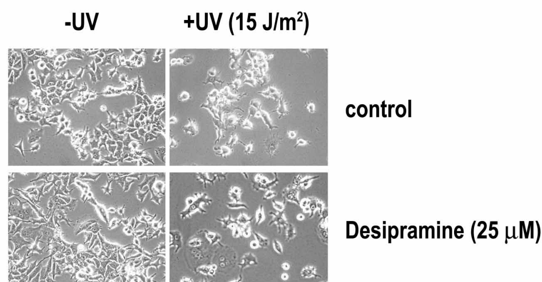


Fig. 1. Desipramine prevents apoptosis of 293 cells but not Jurkat and MCF-7 cells. In panel A, 293, Jurkat and MCF-7 cells were treated with desipramine or left untreated for 2 h. Both desipramine treated or non-treated cells were irradiated with 0 or 15 J/m^2 of UVC as indicated. At 48 h post-irradiation, the cells were rinsed with PBS, fixed in 70% Ethanol in PBS, rehydrated in PBS, treated with RNase A (20 μg/ml) and stained with propidium iodide (50 μg/ml in PBS) at room temperature for 30 min. The stained cells were analyzed on an EPICS C flow cytometer in the Flow Cytometry Core of University of Michigan, USA. In panels B and C, 293 and MCF-7 cells were either untreated or treated with desipramine and UV-irradiated in the same way as described above. At 48 h post-irradiation, the cell morphology was examined using Olympus CK2 microscope and cell images were captured using Kodak DC120 megapixel digital camera. The images were resolved using MDS120 microscopy documentation software (Kodak Scientific, New Haven, CT, USA).

of ceramide on JNK activation is still controversial [11, 15–17]. To elucidate the relationship between ceramide generation and JNK activation upon UV-irradiation, we studied the effect of ceramide induction on JNK activation in 293, Jurkat and MCF-7 cells. Our data shows that ceramide induction (Fig. 2, panels A and B, lane 2; panel D) is correlated with JNK activation in UV-irradiated 293 and Jurkat cells (Fig. 3, panels A and B, lane 2; panel D). The treatment of desipramine prior to UV treatment, which inhibited ceramide production (Fig. 2, panels A and B, lanes 3 and 4; panel D), partially inhibited JNK activation caused by UV-irradiation in 293 and Jurkat cells (Fig. 3, panels A and B, lanes 4 and 6 vs. 2; panel D). The lowered effectiveness of desipramine on the UV-induced JNK activation in Jurkat cells may be due to the fact that Jurkat expresses higher level Daxx (Fig. 4, lane 2), which also mediates Fas- and UV-induced JNK activation [18]. In contrast to 293 and Jurkat cells, ceramide production was undetectable in MCF-7 cells (Fig. 2, panel C) and UV-induced JNK activation was not affected by desipramine (Fig. 3, panel C, lanes 4 and 6 vs. 2). Our results suggest that ceramide partially mediates UV-induced JNK activation in certain cells.

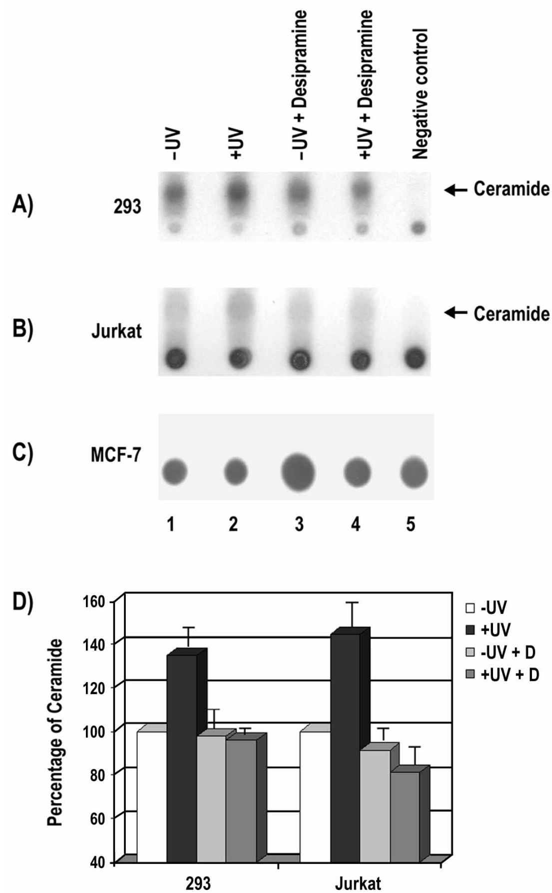


Fig. 2. Desipramine inhibits UV-induced ceramide production. 293 (panel A), Jurkat (panel B) and MCF-7 (panel C) cells were left untreated (lanes l and 2) or treated (lanes 3 and 4) with desipramine (25 μM) for 2 h. The cells were irradiated with 0 (lanes 1 and 3) and 100 J/m^2 (lanes 2 and 4) of UVC. At 30 min post-irradiation, ceramide was extracted following the procedure as described in 'Materials and methods'. A reaction without ceramide extract was performed as negative control (lane 5). The incorporated ^{32}P in ceramide-1-^{32}P-phosphate was quantified by liquid scintillation counting (panel D).

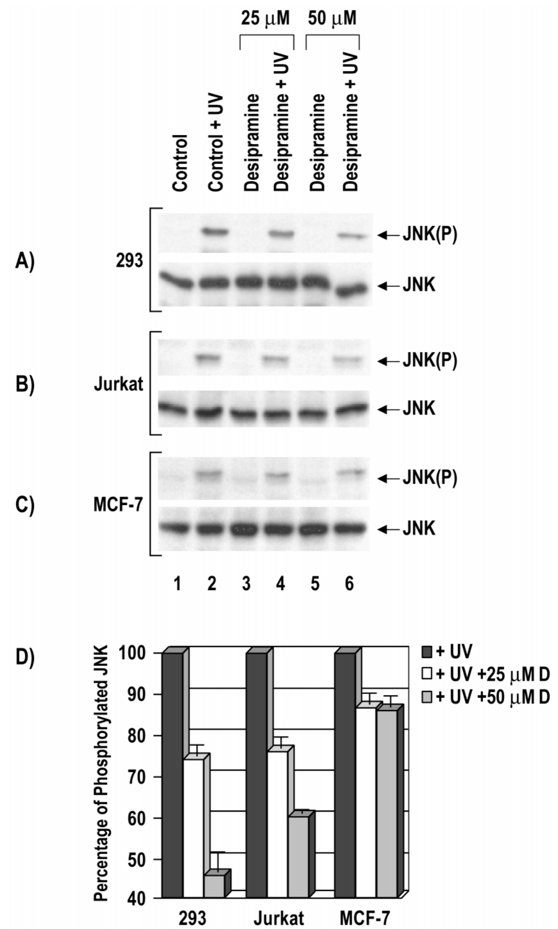


Fig. 3. JNK activation affected by ceramide synthesis inhibitor – desipramine. Jurkat and 293 cells were treated with 25 and 50 μM desipramine for 2 h and then irradiated with 0 J/m^2 (lanes 1 and 3, panels A and B) or 100 J/m^2 (lanes 2 and 4, panels A and B). After irradiation, cells were cultured in the media containing desipramine for another 30 min at 37°C. Cells were lysed and UV-induced JNK phosphorylation and total JNK were analyzed using SAPK/JNK Assay Kit. Densitometric scanning showed that desipramine treatment reduces the UV-induced phosphorylation of JNK in 293 and Jurkat cells (panel D).

Discussion

ASMase hydrolyzes sphingomyelin to ceramide, which plays a role in cell cycle arrest, cell differentiation and apoptosis [8, 19, 20]. However, the roles of ceramide in UV-induced JNK activation and apoptosis remains contradictory [11, 12]. In this manuscript, we provide evidence that UV-induced JNK activation and apoptosis is mediated either by a ceramide dependent or a ceramide independent pathway. In 293 and Jurkat cells, intracellular levels of ceramide is increased after UV-irradiation. This increased level of ceramide correlates with JNK activation in both cell lines. These results suggest that the ceramide is involved in UV-induced JNK activation. Furthermore, inhibition of ASMase activity by treating cells with desipramine significantly reduces JNK activation in 293 cells and Jurkat cells after 100 J/m^2 of UV-irradiation. The decreased inhibition of JNK activation in Jurkat cells may be due to the higher level of expression of Daxx in the cells. Daxx has been shown to mediate Fas JNK activation and apoptosis [18] and our recent study suggest that UV-induced ASMase activation is mediated by Fas-receptor [21]. The inhibition of ceramide induction and sequentially JNK activation in 293 cells reduces apoptosis of the cells. This result suggests that the combined effect of ceramide induction and JNK activation play a role in mediating UV-induced apoptosis in 293 cells. In contrast, Jurkat cells undergo apoptosis

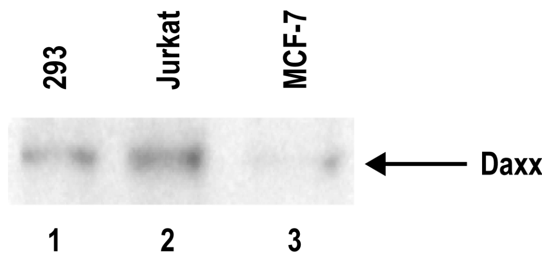


Fig. 4. Western blot analysis of Daxx. The expression of Daxx in 293 (lane 1), Jurkat (lane 2) and MCF-7 cells was measured by Western blot analysis using polyclonal antibodies against Daxx. Equal amount of proteins (40 μg) were subjected to the SDS-PAGE. Densitometric scanning showed the ratio for the expression of Daxx in these cell lines is 293:Jurkat:MCF-7 = 1.0:1.9:0.5 (with a deviation of ±0.3).

even when ceramide induction is blocked upon UV-irradiation. This result indicates that induction of ceramide is not required for UV-induced apoptosis in Jurkat cells. The high dosage (100 J/m^2) of UV used in the experiments was needed to visualize the ceramide induction and JNK. However, we believe, only an small increase of ceramide production and JNK activation is needed to induce apoptosis of cells. Therefore, with a lower dosage of irradiation (such as 15 J/m^2, Fig. 1), the cells are going through apoptosis although we cannot detect an increase of ceramide production and JNK activation at this dosage.

The pathways involved in UV-induced apoptosis in MCF-7 cell bears no resemblance with those involved in either 293 or Jurkat cells. The MCF-7 cells have undetectable amount of intracellular ceramide even after UV-irradiation. However upon UV-irradiation, MCF-7 cells show induction of JNK activation and finally undergo apoptosis. Furthermore, treatment of the cells with desipramine does not inhibit UV-induced JNK activation and apoptosis. These results suggest that induction of ceramide is not always required for UV-induced JNK activation and apoptosis. Based on these results, a model for UV-induced JNK activation and apoptosis has been proposed (Fig. 5). UV-irradiation induces aggregation of the cell surface receptor, which recruit intracellular molecules, such as Daxx and FADD. Depending on the cell type, these molecules lead to the activation of JNK via ceramide dependent and independent pathways. The activation of JNK then results in the activation of multiple transcriptional factors that activate pro-apoptotic genes and induce apoptosis.

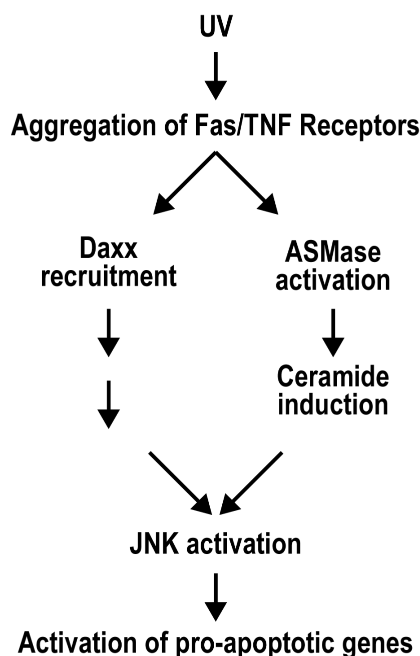


Fig. 5. Model for UV-induced JNK activation and apoptosis. UV-irradiation induces the JNK activation and apoptosis in ceramide dependent and independent pathways.

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