Roles of Neutral Sphingomyelinase 1 on CD95-Mediated Apoptosis in Human Jurkat T Lymphocytes

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Abstract — CD95 receptor is a member of tumor necrosis factor receptor family that mediates apoptosis in many cell types when bound by CD95 ligand or cross-linked by agonistic anti-CD95 antibodies. To determine the role of neutral sphingomyelinase (nSMase) on CD95-mediated apoptosis, human Jurkat T lymphocytes were exposed to recombinant human CD95 ligand. Treatment with CD95 ligand induced cell death in a concentration and time-dependent manner. CD95-induced cell death was suppressed by inhibitors of SMase such as AY9944 or desipramine. Transfection with human nSMase1 siRNA plasmid into CD95 ligand-treated cells significantly prevented CD95-mediated cell death. CD95-mediated elevation of intracellular ceramide level detected by FACS analysis with anti-ceramide antibody was also decreased by nSMase1 siRNA. Knock-down of nSMase1 expression also blocked cytochrome c release into cytosol and caspase-3 cleavage in CD95-treated cells. Taken together, these results suggest that nSMase1 may play an important role in CD95-mediated apoptotic cell death in Jurkat T cells.

Keywords: CD95 ligand, nSMase1, Jurkat T cell, siRNA, Apoptosis

INTRODUCTION

CD95 (Fas or Apo-1) receptor is a member of tumor necrosis factor receptor family that mediated apoptosis in many cell types when bound by the CD95 ligand or cross-linking with agonistic anti-CD95 antibodies (Mitsumasa et al., 2004; Inna et al., 2005). Activation of CD95 receptor results in the formation of the death-inducing signaling complex and activation of initiator caspase-8, which then triggers a variety of regulated downstream events, which may finally result in apoptotic cell death (Martin et al., 1995; De Maria et al., 1998; Oliver et al., 2000). Many studies also showed that CD95-inducing cell death is related with intracellular ceramide generation (Obeid and Hannun, 1995; Cock et al., 1998; Norma et al., 2004).

Ceramide is one of various sphingolipid metabolites known to have biological activities, serving as a lipid-derived second messenger that modulates the induction of cell differentiation, cell cycle arrest, and/or cell death (Ram et al., 2004; Sawada et al., 2004). Intracellular ceramide accumulation results from multiple stimuli, such as tumor necrosis factor-α (TNF-α), irradiation, heat shock, CD95 cross linking, and other anticancer drugs (Nieves et al., 2000). These stimuli have been observed to initiate ceramide-mediated signaling cascade and the stimulation of caspase activity, which ultimately leads to DNA fragmentation and cell death (Chalfant et al., 2001; Kondo et al., 2002).

Generation of ceramide through sphingomyelinase (SMase) activation has been shown to play a role in cell death (Jarvis and Grant, 1998). SMase are enzymes that cleave the phosphodiester linkage of sphingomyelin into ceramide and phosphocholine, and they are implicated several signaling transduction and cell regulation (Norma et al., 2003). Activation of acidic SMase (aSMase) has been observed after treatment with anti-CD4 antibodies in vivo (Kirschnek et al., 2000), although the involvement of aSMase in apoptosis induced by TNFα or CD95 is somewhat controversial (Segui et al., 2000; Bezombes et al., 2001). Attention has also focused on the neutral SMase (nSMase) for its role of mediating a variety of cellular responses including differentiation, cell cycle regulation, and apoptosis through the generation of the ceramide (Hannun and Luberto, 2000). NSMase activation has been observed after ligation of CD95 and TNF receptor and UV ir-
radiation, and also upon heat stress and serum starvation (Hirofumi et al., 1999).

Based on similarity searches with bacterial SMases, two putative nSMases such as nSMase1 (Tomiuk et al., 1998) and nSMase2 (Hofmann et al., 2000) were identified. The recombinant nSMase1 has been found to efficiently hydrolyze phosphatidylcholine and lyso-platelet-activating factor in addition to SM and to be localized in the endoplasmic reticulum (Hirofumi et al., 1999; Tomiuk et al., 2000). In contrast nSMase1, nSMase2 specifically catalyzes the hydrolysis of sphingomyelin and is activated by phosphatidylserine, both of which are characteristics of the mammalian plasma membrane SMase (Norma et al., 2003). Recently nSMase3 gene has been cloned and expressed in MCF7 cells (Krut et al., 2006). However, the role of nSMases on CD95-mediated apoptosis in cells is still unclear. In these studies, the roles of nSMase1 on CD95-mediated apoptotic cell death in Jurkat T lymphocyte cells were studied using small interference RNA (siRNA)-mediated gene silencing and we suggest that nSMase1 may play an important role in CD95-mediated apoptotic cell death in Jurkat T cells.

MATERIALS AND METHODS

Cell culture

Human acute leukemic T-cell Jurkat, clone E6-1 cells were cultured in RPM 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2. For treatment, 5×105 cells were plated in 1 ml of culture medium and incubated for 24-48 h, as indicated.

Cell viability assay

Cells were plated onto 96-well plates and incubated at 37°C in a 5% CO2 atmosphere. After incubation for the designated time, cells were treated with 10 μl of CCK-8 solution (Dojindo, Kumamoto, Japan). After 210 min incubation, formazan generation was quantified by using a GENios Pro microplate reader (Tecan, Switzerland) at 450 nm. The percentages of cells surviving from each group relative to control, defined as 100% survival, were calculated.

Construction of siRNA plasmids and transfection

For design of small interference RNA oligonucleotides targeting human SMases, DNA sequence for the human nSMase1 (5’-AATCGACAGAAGGACATCTAC-3’) was selected using BLAST search. Double stranded siRNA oligonucleotides were synthesized (Genotech, Daejeon, Korea) and annealed at 15°C for about 12 h. Annealed oligonucleotides were ligated with pSilencer 2.0-U6 plasmid (Ambion, Austin, TX) digested with BamHI and HindIII. A nonspecific scrambled siRNA was used as a negative control. Constructed siRNA plasmids were transfected in cells using DMRIE-C transfection reagent as recommended by the manufacturer (Invitrogen). Briefly, cells suspended in serum and antibacterial reagent-free medium were incubated in opti-MEM medium along with 5 μg of plasmid DNA and 12.5 μl of DMRIE-C for 5 h at 37°C. Cells were maintained in RPMI medium containing 10% FBS for 24 h after transfection.

Subcellular fractionation

Cells were harvested at the end of treatment and washed with ice-cold PBS. Cells were resuspended in 0.5 ml of buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol) containing 0.25 M sucrose and a mixture of protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin, and 1 μg/ml chymostatin). To lyse the cells, the cell suspension was passed 10 times through a 26-gauge needle fitted to a syringe. Unbroken cells, large plasma membrane piece, and nuclei were removed by centrifuging the homogenates at 1,000×g at 4°C for 10 min. The supernatant was subjected to 10,000×g centrifugation at 4°C for 20 min. The pellet fraction (i.e., mitochondria) was first washed with the buffer A containing 0.25 M sucrose and then solubilized in 50 μl of 10 mM Tris-acetate buffer (pH 8.0) containing 0.5% NP-40, and 5 mM CaCl2. The supernatant was centrifuged at 100,000×g at 4°C for 1 h to generate cytosol.

Determination of intracellular ceramide

After treatment, cells were resuspended in 0.3 ml of PBS containing 6 μg of mouse anti-ceramide 15B4 antibody (Alexis, Grunberg, Germany) and stained for 40 min at room temperature. After incubation, cells were centrifuged at 1,000×g for 5 min and washed with ice-cold PBS. Cells were incubated in 1 ml of PBS containing 15 μg of Cy-3 conjugated anti-mouse antibody (Jackson Immuno-Research laboratories, West Grove, PA) and stained for 40 min in a dark condition at room temperature. Cells were centrifuged and washed again with ice-cold PBS once. Pellets were suspended in 1 ml of PBS, and cells (104 cells/well) were analyzed on a FACSCaliber (BD Biosciences, San Jose, CA). The intensity of Cy-3 fluorescence was measured with excitation at 550 nm and emission at 570 nm.
RT-PCR

Total RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD). RT-PCR was performed using an Access RT-PCR system purchased from Promega (Madison, WI) according to the manufacturer’s instructions. Human nSMase1 cDNA was amplified by PCR using a sense primer (5'-GGCTGCTGCTGCTGAA-3') and an antisense primer (5'-TAGAGCTGGGTTCTGCTGT-3') with 30 cycles by denaturation at 94°C for 20s, annealing at 58°C for 20s, and extension at 72°C for 40s (Zhou et al., 2004). Human β-actin cDNA was amplified using a sense primer (5'-CTACAAATGAGCTGCGTG-3') and an antisense primer (5'-TAGCTTCTTCCAGGGAGGA-3') with 30 cycles by denaturation at 94°C for 20s, annealing at 52°C for 20s, and extension at 72°C for 40s. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining.

Western blotting

Cells were solubilized with ice-cold lysis buffer containing 25 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Insoluble materials were removed by centrifugation at 10,000 × g for 10 min. Extracted proteins were fractionated by 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene difluoride membrane. For nonspecific binding, blocking was performed in Tris-buffered saline (TBS) containing 5% skim milk powder and 0.1% Tween-20 at 4°C overnight. The membranes were washed with three changes of TBS and incubated with antibodies against cytochrome c (1.5 μg/ml in TBS-T, Pharmingen, San Diego, CA), and caspase-3 (1:1,000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h at room temperature. After blotting with a primary antibody, the membranes were washed three times with TBS. The membranes were incubated for 90 min with TBS-T containing HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:5,000 dilution). The visualization of the blots was carried using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ). Protein content was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard.

Statistical analysis

Data are expressed as the mean ± S.D. and the statistical differences between means were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's Pairwise Multiple Comparison t-test. The difference was considered statistically significant at p < 0.05.

RESULTS

Effect of CD95 ligand on cell viability in human Jurkat T lymphocytes

To examine the effect of CD95 ligand on cell viability in Jurkat T cells, cells were treated with human recombinant CD95 ligand for 24 h, and cytotoxicity of CD95 ligand was determined using CCK-8. Treatment with CD95 ligand exerted a concentration-dependent inhibition of cellular proliferation in the cultured cells (Fig. 1A). At 8 ng/ml, cell death effect was significant and this concentration was selected for the further studies. As shown in Fig. 1B, treat-
Blockage of CD95 ligand-induced cell death by SMase inhibitors

To investigate the role of SMase on CD95 ligand-induced cell death, the effect of SMase inhibitors such as AY9944 and desipramine was determined (Yoshida et al., 1985) (Fig. 2). Cells were preincubated with AY9944 (10 μM) or desipramine (20 μM) for 30 min, and then were treated with 8 ng/ml CD95 ligand for 24 h. Treatment with inhibitors of SMases significantly recovered the decreased cell viability by CD95 ligand. The data suggest that SMases may be involved in the pathway of CD95 receptor-mediated cell death.

Transfection with nSMase1 siRNA results in recovery of cell death by CD95 ligand

To determine whether nSMase1 siRNA is able to suppress nSMase1 mRNA expression in T cells, cells were transfected with 5 μg of nSMase1 siRNA plasmids for 24 h. The scrambled siRNA with no human target mRNAs was used as a transfection control. Transfection with nSMase1 siRNA plasmid significantly reduced nSMase1 mRNA expression in cells although scrambled siRNA-transfected cells showed no difference in nSMase1 expression (Fig. 3A). The effect of nSMase1 knock-down on CD95-mediated cell death was investigated. After transfection, cells were incubated with CD95 ligand for additional 24 h. Transfection with nSMase1 siRNA showed a significant recovery of cell death by CD95 ligand (Fig. 3B). These results suggest that nSMase1 may play an important role in

Fig. 2. Effects of SMase inhibitors on CD95-mediated cell death in T cells. Cells were pre-treated with 10 μM AY9944 (A) or 20 μM desipramine (B) for 30 min, and then incubated with CD95 ligand (8 ng/ml) for 24 h. The cell viability was determined by CCK assay. Results are the mean ± S.D. of three separate experiments. *Significantly different from vehicle-treated cells (p < 0.05). #Significantly different from the CD95 ligand-treated cells (p < 0.05).

Fig. 3. Involvement of nSMase1 in CD95-mediated T cell death. (A) RT-PCR analyses. Cells were transfected with nSMase1-specific siRNA or with control scrambled siRNA for 24 h. Total RNA was isolated and the partial coding sequence of human nSMase1 was amplified with specific primers. Expression of β-actin mRNA was determined as a RNA control. (B) After transfection with nSMase1-specific siRNA or with control scrambled siRNA for 24 h, cells were incubated with CD95 ligand (8 ng/ml) for 24 h and the cell viability was determined by CCK assay. Results are the mean ± S.D. of three separate experiments. *Significantly different from vehicle-treated cells (p < 0.05). #Significantly different from the CD95 ligand-treated cells (p < 0.05).
CD95 receptor-mediated apoptotic cell death pathway.

Knockdown of nSMase1 expression suppresses intracellular ceramide generation

To address a potential involvement of nSMase1 in generating ceramide in T cells, we determined amount of intracellular ceramide levels using flow cytometry with anti-ceramide antibody. Intracellular ceramide levels were significantly elevated in CD95 ligand-treated cells and nSMase1 siRNA was effective at reducing intracellular ceramide levels although scrambled siRNA showed no difference (Fig. 4). The results revealed that nSMase1 may control intracellular ceramide generation in Jurkat cells.

Suppression of nSMase1 prevents apoptotic cell death signals

Based on the results described above, we asked whether knock-down of nSMase1 expression and decrease in ceramide production may suppress apoptotic cell death signaling because ceramide is well-known as an inducer of apoptotic cell death in various cells. Immunoblot analysis of cytochrome c protein showed that treatment with nSMase1 siRNA prevents translocation of cytochrome c from mitochondria to cytosol in CD95 ligand-exposed cells (Fig. 5A). In vehicle-treated control cells or scrambled siRNA-transfected cells, release of cytochrome c to cytosol was significantly increased by CD95 ligand. Because activation of caspase-3 may play a central role in the execution stage of apoptosis, we performed Western blot analyses with antibody against caspase-3 to measure cleavage of procaspase-3. Significant cleavage of procaspase-3 was showed in CD95 ligand-treated cells and these effects were completely blocked by nSMase1 siRNA (Fig. 5B).

**DISCUSSION**

Ceramide is known to play a key role in the regulation of diverse cellular functions, including cell growth, differentiation, stress response and apoptosis (Spiegel and Merrill, 1996; Hannun and Luberto, 2000). Because of the significant roles of ceramide in cells, the interest in the SMases that initiate the generation of intracellular ceramide rose to identify crucial enzymes that participate in the biochemical pathways leading to ceramide accumulation.

To date, at least five types of SMases have been reported, including the Zn^{2+}-dependent acidic SMase, Zn^{2+}-independent acidic SMase, the Mg^{2+}-dependent neutral SMase, Mg^{2+}-independent neutral SMase, and alkaline SMase (Clarke et al., 2006; Martin et al., 2007). Among these, acidic SMase and Mg^{2+}-dependent neutral SMase are postulated as candidate enzymes for regulating ceramide-mediated cellular processes (Horinouchi et al.,
Acidic SMase is present in lysosomes/endosomes of all cells where it degrades sphingomyelin of endocytosed membranes and acidic SMase activity is deficient in patients affected with types A and B Niemann-Pick disease (Brady et al., 1966; Schneider and Kennedy, 1967). A pivotal role of acidic SMase in CD95-triggered apoptosis has been studied using acidic SMase knockout mice and Niemann-Pick disease cell (Kirschne et al., 2000; Lin et al., 2000; Paris et al., 2001).

Three types of Mg$^{2+}$-dependent neutral SMases (nSMase 1, 2, and 3) have been identified so far (Tomiuk et al., 1998; Hofmann et al., 2000; Krut et al., 2006). Human nSMase1 gene was first cloned as Mg$^{2+}$-dependent nSMase gene pursuing similarity with gene sequences of bacterial SMases such as Bacillus cereus and Staphylococcus aureus (Tomiuk et al., 1998). The nSMase1 is known to express ubiquitously with the highest mRNA and protein amounts in kidney and localized to the endoplasmic reticulum (Sawai et al., 1999; Neuberger et al., 2000; Tomiuk et al., 2000). Previous report suggested that in vivo function of nSMase1 might be a lysophospholipase C with lysophosphatidylcholine and lyso-platelet activating factor as substrates (Sawai et al., 1999). Human nSMase2 gene encodes proteins of 655 amino acid residues with two putative transmembrane domains at the N terminus. The nSMase2 is mainly expressed in brain colocalizing with a Golgi marker in neuronal cells (Hofmann et al., 2000). Stable expression of nSMase2 gene in MCF7 cells causes an increase in the levels of ceramide and a decrease in the level of sphingomyelin (Marchesini et al., 2003). Mice deficient for nSMase2 develop a novel form of dwarfism and delayed puberty, suggesting that nSMase2 may play an important role in the control of developmental stages (Stoffel et al., 2007). Recently, a new mammalian nSMase gene named nSMase3 was cloned and the high level of nSMase3 expression in heart muscle raises the possibility of role of nSMase3 in the pathogenesis of cardiac disease (Krut et al., 2006).

Ligation of CD95 ligand is known to induce the production of ceramide that results in apoptosis in a variety of cell types (Ogasawara et al., 1993; Grassme et al., 2001; Miyaji et al., 2005). Several studies have implicated that CD95 ligation activates acidic SMase to hydrolyze sphingomyelin to produce ceramide (Tonnetti et al., 1999; Cremesti et al., 2001; Grassme et al., 2001). However, involvement of other SMases such as neutral SMases on CD95-mediated apoptosis should be addressed because ceramide production following CD95 ligation still occurred in mice deficient in the acidic SMase gene and CD95 ligand activates both acidic and neutral SMases (Tonnetti et al., 1999; Lin et al., 2000).

The present work was designed to elucidate whether nSMase1 plays an important role in apoptotic cell death by controlling ceramide levels in Jurkat T cells treated with CD95 ligand. We have shown that the SMase inhibitors such as AY9944 or desipramine are able to prevent cell death of T cell by CD95 treatment and down-regulation of nSMase1 using specific siRNA significantly recovered apoptotic cell death in CD95-treated cells. Gene knock-down of nSMase1 can also block generation of ceramide. The release of cytochrome c from mitochondria to cytosol by CD95 was suppressed when the cells were transfected with nSMase1 siRNA plasmid although scrambled siRNA showed no changes in cytochrome c release compared to the control cells. Procaspase-3 cleavage by CD95 was also blocked by nSMase1 knock-down. All of the data reported here indicate that human nSMase1 is involved in CD95-mediated apoptosis pathway. Several lines of evidence suggest that nSMase1 activity is regulated by changes in the glutathione level (Liu and Hannun, 1997; Liu et al., 1998a; Martin et al., 2007). Glutathione could be an inhibitor of nSMase activity and glutathione depletion is necessary for TNFα-stimulated nSMase activation (Liu et al., 1998a). Depletion of glutathione has also been observed in response to CD95 activation (Chiba et al., 1996; van den Dobbelsteen et al., 1996). Because CD95 ligand triggers a depletion of cellular glutathione, rapid formation of reactive oxygen species, and apoptosis induction (Watanabe et al., 2004; Reinehr et al., 2005), increased intracellular ceramide by activating nSMase1 through glutathione depletion and transient increases in glutathione disulfide may induce apoptosis.

There are conflicting data on the role of nSMase1 on apoptotic cell death. Stimulation of cells overexpressing nSMase1 with TNF or H$_2$O$_2$ did not elevate ceramide levels or induce apoptosis (Tomiuk et al., 1998). Tepper et al. (2001) showed that overexpression of human nSMase1gene in Jurkat cells increased in vitro nSMase activity. However, basal sphingolipid levels such as ceramide, sphingomyelin or glucosylceramide were not changed by nSMase1 expression. The nSMase1 was predominantly localized at the ER membrane, but not at the plasma membrane in HeLa cells. Interestingly, Jurkat cells expressing nSMase1 failed to undergo apoptosis after CD95 ligation. However, Tonetti et al. (1999) have showed that T cell receptor-mediated ceramide production in T cell hybridoma 3DO is inhibited by expressing antisense RNA complementary to nSMase1 cDNA. Recent study also showed that nSMase1 from zebrafish embryonic cell acts as a mediator of
stress-induced apoptosis (Yabu et al., 2008). These observations were also supported by our data that downregulation of human nSMase1 prevents CD95-mediated apoptotic response in Jurkat cells. The observed discrepancies may come from the fact that we and Tonetti group studied the effect of downregulation of nSMase1 using siRNA instead of overexpression. Suppression of nSMase1 expression in T cells by gene silencing may be able to modulate the action of other unknown factor(s) regulated by nSMase1 that can control intracellular ceramide level and apoptotic responses. Although the role of nSMase1 is still not clear, but the function of nSMase1 as a controller of cell signaling needs to be elucidated in more detail.

In conclusion, we demonstrate here that nSMase1 may play a pivotal role in CD95-mediated apoptosis in human Jurkat T cells. Stimulation of T cells with CD95 ligand induces nSMase1 activation which subsequently led to accumulation of ceramide, which in turn induces caspase-3 activation and apoptosis. Although the detailed mechanism needs to be further studied, our finding may be beneficial to understand the mechanism and physiological significance of SMases in apoptotic signal pathways.

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