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Actinomycin D enhances TRAIL-induced caspase-dependent and -independent apoptosis in SH-SY5Y neuroblastoma cells

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has attracted great attention as a promising anti-cancer reagent. Recombinant soluble TRAIL (rsTRAIL) derivatives induce apoptosis in various cancer cells, but not in most normal cells. However, a number of cancerous cell types are resistant to TRAIL cytotoxicity, limiting its application in cancer therapy. In the present study, we report that actinomycin D (Act D) pretreatment increases apoptosis in human neuroblastoma SH-SY5Y cells treated with rsTRAIL. Both caspase-9 and caspase-7, but not caspase-3, were activated during the apoptosis process. z-VAD-fmk, a pan-caspase inhibitor, only partially suppressed apoptosis of the cells, suggesting that the Act D-enhanced apoptosis of SH-SY5Y occurred via caspase-dependent and -independent manners. In cells pretreated with Act D, we found decreased mitochondrial transmembrane potential, high levels of reactive oxygen species (ROS), and up-regulated apoptotic-inducing factor (AIF). Cell death was blocked in cells stably transfected with AIF-siRNA plasmid. Taken together, these data indicate that Act D sensitizes SH-SY5Y cells to rsTRAIL-induced apoptosis via caspase activation, impairment of the mitochondrial membrane, release of ROS, and up-regulation of AIF expression. This study provides a novel strategy for the therapy of malignant neuroblastoma resistant to rsTRAIL cytotoxicity.

Keywords: Neuroblastoma cells; Apoptosis; Actinomycin D; Tumor necrosis factor-related apoptosis-inducing ligand; Caspases

1. Introduction

Neuroblastoma is the most common malignant sympathetic nervous system tumor. Unfortunately, it is resistant to many known chemotherapeutic drugs, possibly through its association with the multidrug-resistant P-glycoprotein. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) specifically induces apoptosis in various cancer cells, but not in most normal cells. There are, however, a number of cancers resistant to TRAIL-induced cytotoxicity, especially neural system carcinoma cells.

Caspases play a critical role in the process of apoptosis, and evidence is accumulating that different caspase cascades depend on the apoptotic stimulus. One explanation for the TRAIL-resistance seen in neuroblastoma (NB) cells is the lack of caspase-8 and caspase-10 expression (Eggert et al., 2001; Wang and El-Deiry, 2003). Till date, three signalling pathways have been implicated in TRAIL-induced apoptosis, caspase-dependent, mitochondrial-dependent, and caspase-independent pathways. The impairment of mitochondrial metabolism is a key event upstream of the bifurcation of caspase-dependent and -independent cell death pathways. Mitochondrial damage could occur as a consequence of activation of the cell death pathway and/or production of reactive oxygen species (ROS). This damage is associated with a decrease in mitochondrial membrane potential and results in secondary excessive mitochondrial ROS generation, release of apoptotic-inducing factors (AIF) and caspase activation (Wettsman et al., 2003; Ravagnan et al., 2002; Desagher and Martinou, 2000). Numerous pro-apoptotic signaling molecules act to change the permeability of the mitochondrial outer membrane, thereby triggering the release of potentially toxic proteins, such as apoptosis-inducing factor (AIF), a phylogenetically old flavoprotein. In healthy cells, AIF is confined to the mitochondrial intermembrane space. Upon apoptosis signaling, AIF is released into they cytosol and translocates to the nucleus.

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Once there, AIF binds to DNA and initiates transcription of genes involved in phosphatidylserine (PS) externalization, chromatin condensation, and decreases in mitochondrial membrane potential (Candé et al., 2002; Bidere et al., 2003). Recent studies suggest that AIF is a major factor determining caspase-independent neuronal death, emphasizing the central role of mitochondria in the control of physiological and pathological cell death (Murahashi et al., 2003).

There is increasing evidence that conventional chemotherapeutic agents, and inhibitors of protein/RNA synthesis, sensitize characterized tumor cells to TRAIL-induced apoptosis via a mitochondrial-dependent pathway. Glazyrin et al. reported that actinomycin D (Act D), an RNA synthesis inhibitor, sensitized pancreatic cancer cells to Fas-mediated apoptosis through abrogation of the DNA binding activity of NF-κB, rather than through the PI3-kinase/Akt pathway (Glazyrin et al., 2002). Suzuki et al. demonstrated that HepG2 cells showed Fas-mediated cell death only in the presence of Act D (Suzuki et al., 1998). Quirk et al. showed that, in mouse granulosa cells, treatment with the protein synthesis inhibitor cycloheximide (CHX) potentiates Fas-mediated cell death (Quirk et al., 1998). We have recently demonstrated that the neuroblastoma SH-SY5Y cell line is resistant to rsTRAIL-induced apoptosis. These results suggest that there might be unknown mechanisms disabling the apoptosis machinery in cancer cells exhibiting resistance to chemotherapeutics. In the present study, we investigated the molecular mechanisms by which Act D and rsTRAIL regulate apoptosis in SH-SY5Y cells.

2. Materials and methods

2.1. Materials

Human neuroblastoma cell line SH-SY5Y was purchased from ATCC. Cell culture medium and fetal bovine serum (FBS) were purchased from Hyclone, and geneticin (G418) was purchased from Invitrogen. Rabbit polyclonal antibodies against caspase-3, caspase-9, caspase-7, and AIF were purchased from Santa Cruz Co., and the antibodies against Bcl-X_L (goat, polyclonal), Bcl-2 (rabbit, polyclonal), and β-actin (mouse, monoclonal) were from Sigma Chemical Co. Horseradish peroxidase-conjugated antibody complex against mouse, goat, and rabbit IgG were purchased from R&D System (Minneapolis, MN, USA). Caspase peptide family inhibitor z-VAD-fmk was purchased from Biovision Co., the enhanced chemiluminescence (ECL) reagent was from Amersham Biosciences, Lipofect FuGENE 6 was from Roche (Mannheim, Germany), and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] and JC-1 (5,5′,6,6-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine) were from Sigma Chemical Co. Plasmids of pcDNA3.0 and pcDNA3.0-bcl-xl were gifts from Dr. Shimin Hu (University of Pennsylvania, USA). The siRNA expression vector pAVU6+27 was provided by Dr. Good (University of Michigan, USA). Recombinant soluble TRAIL (rsTRAIL) was prepared by Shi et al. (2003).

2.2. Cell culture and apoptosis assay

The SH-SY5Y cells were cultured in RPMI-1640 media supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2 incubator. The cells were pretreated with Act D at 0.5 μg/ml for 2 h. Cells were treated with caspase-family inhibitor z-VAD-fmk at 5 μg/ml and rsTRAIL at 0.5 μg/ml for 24 h, or left untreated. Cell death by apoptosis was evaluated using the Annexin V-FITC apoptosis detection kit, according to the manufacturer's instructions (BD Biosciences Pharmingen). Briefly, 2×10^5 cells were washed once and resuspended with ice-cold phosphate-buffered saline (PBS, pH 7.4). Cells were then incubated with Annexin V-FITC for 30 min, followed by propidium iodidement (PI) for 5 min at room temperature. The apoptotic cells were analyzed with flow cytometry (FACS Calibur, Becton Dickinson). Each experiment was performed at least three times with at least three independent wells each time. The significance of the difference was calculated by the Student's-test. Results of representative experiments are given as the mean ± S.D. and multiple experiments as the mean ± S.E.

2.3. Cell transfection

SH-SY5Y cells stably expressing Bcl-X_L, Bcl-xl-siRNA, or AIF-siRNA were generated as described by Bian et al. (2001) and maintained in RPMI-1640 media supplemented with 400 μg/ml of G418. Cells were transfected using Lipofect FuGENE 6 according to the manufacturer's instructions. Briefly, cells were seeded in tissue culture plates and transfected when cells reached 50% confluence. Three days after transfection, cells were fed with fresh media containing 400 μg/ml of G418 to generate stably transfected cells

2.4. SDS-PAGE and Western blot

The treated SH-SY5Y cells were washed twice with cold phosphate-buffered saline (PBS), lysed in lysis buffer (1% NP40, 10 mM Tris Cl, pH 8.0, 150 mM NaCl, 10 mM NaF, 1 mM $NaVO_3$, 2 mM EDTA, pH 8.0, 1 mM DTT, 1 mM PMSF, and protease inhibitors pepstatin A, leupeptin, and aprotinin), and centrifuged at 12,000 rpm at 4 °C to remove cellular debris. Cell lysate aliquots containing 100 μg of total protein were resolved by 12% SDS-PAGE. The proteins in the gel were transferred to PVDF membranes (Amersham Biosciences Inc.). The membranes were blocked in TBS–Triton-X 100 (0.2%, v/v, pH 7.4) containing 5% milk for 2 h at room temperature, followed by incubation at 4 °C overnight with specific antibodies, according to the manufacturer's instructions. The PVDF membranes were then incubated with anti-mouse-, or anti-goat-, or anti-rabbit-horseradish peroxidase secondary antibodies. Proteins were visualized using the ECL detection system (Amersham Biosciences Inc.) and exposed to X-ray film at multiple time points

2.5. Measurement of mitochondrial membrane potential

Variations in mitochondrial transmembrane potential ($\Delta\psi m$) were measured using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-ethylbenzimidazolylcarbocyanine iodide) as a probe. Cells were plated in six-well plates at 1×10^5 cells/well in a volume of 2 ml and incubated in RPMI-1640 media overnight. The cells were treated with Act D at 0.5 μg/ml for 2 h, and then cultured in fresh media with or without rsTRAIL at 0.5 μg/ml for 24 h. The treated cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 1 ml PRMI-1640 media containing 10% FBS and JC-1 (20 μg/ml), then incubated for 30 min at room temperature in the dark. The cells were washed twice with PBS, and resuspended in 0.5 ml of PBS, immediately followed by flow cytometry.

2.6. Preparation of cytosolic fraction of SH-SY5Y cells and AIF detection

SH-SY5Y cells (5×10^6) were treated as described above, and harvested by centrifugation at 1000 rpm for 5 min. The cytosolic fraction was prepared as described by Yu et al. (2000). Briefly, after washing once with ice-cold PBS, the cells were resuspended in ice-cold buffer A (20 mM HEPES-KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol and 0.1 mM PMSF) supplemented with protease inhibitors (5 μg/ml pepstatin A, 10 μg/ml leupeptin and 2 μg/ml aprotinin) and 250 mM sucrose, followed by incubation on ice overnight. The cells were homogenized, followed by centrifugation at 12,000 × *g* for 5 min at 4 °C. The supernatants were collected and centrifuged again at 100,000 × *g* at 4 °C for 30 min to collect the cytosolic fraction. An aliquot of the cytosolic fraction containing about 100 μg total protein was resolved by SDS-PAGE and Western blot assay.