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Original Contribution

Mitochondrial superoxide mediates mitochondrial and endoplasmic reticulum dysfunctions in TRAIL-induced apoptosis in Jurkat cells

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ABSTRACT

Reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), have been reported to be important mediators of the apoptosis induced by death ligands, including Fas, tumor necrosis factor-α, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Conversely, there is evidence that H_2O_2 and prooxidative conditions are protective. Therefore, the roles of ROS in death ligand-induced apoptosis are a matter of debate. In this study, we attempted to define the oxidant species mediating TRAIL-induced apoptosis in human tumor cells. The generation of intracellular $O_2^{\bullet-}$, but not H_2O_2, was correlated with apoptosis in the cells. TRAIL treatment resulted in increased mitochondrial $O_2^{\bullet-}$ generation and the oxidation of cardiolipin. The $O_2^{\bullet-}$-selective scavenger MnTBaP [Mn(III) tetrakis (4-benzoic acid) porphyrin chloride] specifically blocked TRAIL-induced apoptosis and proapoptotic events including mitochondrial membrane collapse and caspase-3/7 activation. TRAIL also induced endoplasmic reticulum (ER) stress responses including caspase-12 activation, while inhibition of caspase-12 prevented the apoptosis. In addition, increased mitochondrial $O_2^{\bullet-}$ generation by uncoupling of oxidative phosphorylation or inhibition of the electron transport chain amplified the TRAIL-induced apoptosis and proapoptotic events. This amplification was also significantly abolished by MnTBaP treatment. Our data indicate that mitochondrial $O_2^{\bullet-}$ mediates mitochondrial and ER dysfunctions during TRAIL-induced apoptosis in Jurkat cells. The present findings suggest that pharmacological agents increasing mitochondrial $O_2^{\bullet-}$ may serve as clinical drugs that amplify TRAIL effectiveness toward cancer cells.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, selectively induces cell death by binding to two death receptors, TRAIL-R1 and TRAIL-2 [1,2]. Binding of TRAIL to TRAIL-R1 or TRAIL-R2 expressed on the cell surface induces the extrinsic apoptotic pathway, in which caspase-8 plays a key role [3,4]. Active caspase-8 directly activates the effector caspases (caspase-3, -6, and -7). Caspase-8 can also engage the intrinsic apoptotic pathway by cleaving and activating the proapoptotic Bcl-2 family molecule Bid [5]. Truncated Bid activates Bax and Bak, leading to their oligomerization and pore formation in the outer mitochondrial membrane through which cytochrome *c* is released into the cytosol. The released cytochrome *c* then binds to another proapoptotic protein, Apaf-1, leading to the assembly of the apoptosome and the resulting activation of caspase-9 [6]. Caspase-9 also activates the effector caspases-3/6/7, thereby providing a positive feedback loop to the caspase-8-induced apoptotic events.

Reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals, are products of normal metabolism in virtually all aerobic organisms. Low physiological levels of ROS function as second messengers in intracellular signaling and are required for normal cell function, while excessive ROS cause damage to multiple macromolecules, impair cell function, and promote apoptotic or necrotic cell death [7,8]. Generation of ROS is associated with the apoptosis induced by death ligands such as Fas [9–13] and TNF-α [14,15]. Moreover,

Abbreviations: DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate; DHE, dihydroethidine; $\Delta\Psi_m$, mitochondrial membrane potential; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FITC, fluorescein isothiocyanate; GRP78, glucose-related protein 78; H_2O_2, hydrogen peroxide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; MitoSOX, 3,8-phenanthridinediamine, 5-(6′-triphenyl-phosphoniumhexyl)-5,6-dihydro-6-phenyl; MnTBaP, Mn (III) tetrakis (4-benzonic acid) porphyrin chloride; NAO, 10-*N*-nonyl acridine orange; ROS, reactive oxygen species; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; Tg, thapsigargin; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; PI, propidium iodide; XBP-1, X-box- binding protein-1.

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direct application of H_2O_2 alone induces apoptosis [16,17] or potentiates death ligand-induced apoptosis in different cell types [18]. In addition, various antioxidants such as *N*-acetyl-L-cysteine and manganese superoxide dismutase (SOD) and catalase block TNF-α- and Fas-induced apoptosis [19,20]. These observations suggest that ROS are important mediators of death ligand-induced apoptosis. Both NADPH oxidase [9,13,15] and mitochondria [10,14] have been implicated as the cellular sources of ROS generated by TNF-α- or Fas-mediated signaling in various cell types. Conversely, ROS or prooxidative conditions are protective under certain circumstances [21–23]. Glutathione depletion inhibits Fas-mediated caspase-8 activation and apoptosis by affecting the formation of the death-inducing signaling complex [21]. Thus, the issue of whether ROS act as proapoptotic or antiapoptotic factors in death receptor-mediated cell death remains controversial [23].

In the present study, we measured the intracellular levels of $O_2^{\bullet-}$ and H_2O_2 in human tumor cells treated with TRAIL using oxidant-selective fluorescent probes. We found that the generation of intracellular $O_2^{\bullet-}$, but not H_2O_2, was correlated with TRAIL-induced apoptosis in Jurkat cells. Further analyses suggested that mitochondrial $O_2^{\bullet-}$ mediated mitochondrial and endoplasmic reticulum (ER) dysfunctions during TRAIL-induced apoptosis in the cells.

Methods

Reagents

Soluble recombinant human TRAIL was obtained from Enzo Life Sciences. (Farmingdale, NY, USA). SOD, catalase, thapsigargin (Tg), rotenone, antimycin A, oligomycin, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Peroxynitrite ($ONOO^-$) in NaOH solution was purchased from Dojindo Laboratories (Kumamoto, Japan). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), dihydroethidine (DHE), [3,8-phenanthridinediamine, 5-(6′-triphenyl-phosphoniumhexyl)-5,6-dihydro-6-phenyl] (MitoSOX Red; MitoSOX), and 10-*N*-nonyl acridine orange (NAO) were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBaP), pan caspase inhibitor z-VAD-fluoromethylketone (fmk), caspase-3/7-specific inhibitor z-DEVD-fmk, caspase-8-specific inhibitor z-IETD-fmk, and caspase-9-specific inhibitor z-LEHD-fmk were purchased from Merck Japan (Tokyo, Japan). The caspase-12-specific inhibitor z-ATAD-fmk, caspase-4-specific inhibitor z-LEVD-fmk, and polyclonal anti-caspase-12 antibody were purchased from BioVision (Mountain View, CA, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Molecular Probes (Eugene, OR, USA). The reagents were dissolved in dimethyl sulfoxide and diluted with Hank's balanced salt solution (HBSS) to a final dimethyl sulfoxide concentration of $< 0.1\%$ before use. Monoclonal anti-human TRAIL-R1 and TRAIL-R2 antibodies, mouse-IgG_1 and mouse-IgG_{2b} isotype control antibodies, and phycoerythrin (PE)-conjugated goat $F(ab')_2$ anti-mouse IgG were purchased from R&D Systems (Minneapolis, MN, USA). Polyclonal antibodies against X-box-binding protein (XBP)-1 and glucose-related protein 78 (GRP78) and a monoclonal anti-β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human Jurkat leukemic cells were obtained from RIKEN BioResource Center Cell Bank (Tsukuba, Japan) and grown in high glucose-containing RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) in a 5% CO_2-containing atmosphere. Human melanoma A375 cells were obtained from RIKEN BioResource Center Cell Bank and grown in high glucose-containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS in a 5% CO_2-containing atmosphere. A375 cells were harvested by incubation in 0.25% trypsin-EDTA medium (Gibco-Invitrogen, Carlsbad, CA, USA) for 5 min at 37 °C.

Determination of surface TRAIL-R1/R2 expression

The expression levels of TRAIL-R1 and TRAIL-R2 on the cell surface were determined by flow cytometry. Cells (5×10^5 cells/100 μl) were incubated with the anti-TRAIL-R1, anti-TRAIL-R2 or isotype-matched control antibodies for 30 min at 4 °C. The cells were then centrifuged into a pellet, resuspended in PBS, and incubated with PE-conjugated goat $F(ab')_2$ anti-mouse IgG for 30 min at 4 °C. The fluorescence was measured using the FL-2 channel of a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) and analyzed using CellQuest software (Becton-Dickinson).

Determination of apoptotic cell death

Apoptotic cell death was quantitatively assessed by double staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) as previously described [24]. Briefly, cells in 24-well plates (2×10^5 cells/ml) were incubated with the agents to be tested for 20 h in 10% FBS-containing RPMI 1640 medium at 37 °C. Subsequently, the cells were stained with FITC-conjugated annexin V and PI using a commercially available kit (Annexin V FITC Apoptosis Detection Kit I; BD PharMingen, San Diego, CA, USA) according to the manufacturer's instructions.The stained cells were evaluated in the FACSCalibur and analyzed using CellQuest software. Four cellular subpopulations were evaluated: viable cells (annexin V^-/PI^-); early apoptotic cells (annexin V^+/PI^-); late apoptotic cells (annexin V^+/PI^+); and necrotic/damaged cells (annexin V^-/PI^+). Annexin V^+ cells were considered to be apoptotic cells.

Measurements of intracellular ROS

Intracellular ROS production was measured by flow cytometry using the oxidation-sensitive dyes DCFH-DA, DHE, and MitoSOX and the signals were calibrated as previously described [25]. Briefly, cells (5×10^5 cells/500 μl) suspended in HBSS were incubated with the agents to be tested for various times at 37 °C followed by incubation with 5 μM each of DCFH-DA, DHE, or MitoSOX for 15 min at 37 °C. The cells were then washed, resuspended in HBSS on ice, and centrifuged at 4 °C. The green fluorescence (DCFH-DA) and red fluorescence (DHE and MitoSOX) were measured using the FL-1 and FL-2 channels, respectively, of the FACSCalibur and analyzed using CellQuest software. The data were expressed as F/F_0, where F_0 is the fluorescence in unstimulated cells and F is the fluorescence in stimulated cells.

Measurements of caspase-3/7 activation and mitochondrial membrane potential ($\Delta\Psi_m$)

Activation of caspase-3/7 and changes in $\Delta\Psi_m$ were simultaneously measured as previously described [24]. Briefly, cells in 24-well plates (2×10^5 cells/ml) were treated with the agents to be tested for 20 h in 10% FBS-containing RPMI 1640 medium at 37 °C, and then stained with the dual sensor MitoCasp (Cell Technology Inc., Mountain View, CA, USA) according to the manufacturer's protocol. Caspase-3/7 activation and $\Delta\Psi_m$ were evaluated using