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TRAIL Causes Cleavage of Bid by Caspase-8 and Loss of Mitochondrial Membrane Potential Resulting in Apoptosis in BJAB Cells

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A new member of the TNF family, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), has been shown to induce apoptosis. However, the mechanism for TRAIL-induced apoptosis remains to be clarified. SDS-PAGE and Western blot analysis showed that cleavage of Bid was induced by a 1-h incubation of BJAB cells with TRAIL and was blocked by a caspase-8 inhibitor. Flow cytometry demonstrated that loss of mitochondrial membrane potential in BJAB cells began about 1.5 h after the treatment with TRAIL and was apparent at 2 h in comparison with the control. DNA ladder formation, which is characteristic for apoptosis, in the cells treated with TRAIL was detected at 2 h and observed most effectively at 3 h. The time course study suggests that TRAIL causes cleavage of Bid via activation of caspase-8, subsequently the loss of mitochondrial membrane potential, resulting in apoptosis in BJAB cells.

Members of the tumor necrosis factor (TNF) ligand and receptor play an important role in regulating many biological processes, including apoptosis, cytokine production, and cell activity (1). A new member of the TNF ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), has been shown to induce apoptosis in many tumor cells upon binding to its death domain-containing receptors, DR4 and DR5 (1–5). Two additional TRAIL receptors, DcR1, and DcR2, lack functional death domains and function as decoy receptors for TRAIL (5). DR4 and DR5 are expressed in many types of tumor cells as well as normal tissues, whereas DcR1 and DcR2 are expressed frequently in normal tissues but infrequently in tumor cells (6). Recent studies have demonstrated that TRAIL dose not seem to be cytotoxic to normal cells *in vitro* (2, 7), and may have potential as a cancer therapeutic cytokine (8). Within TNF family, TRAIL shares the highest similarity at the amino acid level with Fas ligand, and these ligands show a similar potency in inducing apoptosis (2). The mechanism of Fas-induced apoptosis is competitively best understood, whereas mechanism of TRAIL-induced apoptosis remains to be clarified.

Central to the apoptotic pathway is a family of cysteine proteases, termed caspases (9). Caspase-8 has been demonstrated to play an important role in mediating Fas-induced apoptosis (9–13). Activated caspase-8 mediates downstream apoptotic events that include mitochondrial dysfunction, cytochrome *c* release and caspase-3 activation (12, 14, 15). Recently, Bid, a BH3 domain-containing proapoptotic member of Bcl-2 family that heterodimerize with either agonists or antagonists has been identified (16–18). In Fas-induced apoptosis, activated caspase-8 cleaves Bid into the COOH-terminal part, 15 kDa fragment, which is translocated to mitochondria where it can cause mitochondrial dysfunction such as loss of membrane potential and the permeability transition (13, 16, 19–23). Subsequently, cytochrome *c* released from mitochondria to cytosol has been shown to initiate the activation of caspase-3 (24), and then the downstream caspases cleave the death substrates that are central to apoptotic events such as morphological changes and DNA fragmentation (25, 26).

In order to clarify the mechanism of TRAIL-induced apoptosis, we have investigated by using SDS-PAGE and Western blotting methods whether Bid is cleaved by TRAIL. We have also examined the change of mitochondrial membrane potential in BJAB cells treated with TRAIL by flow cytometry. Our results suggested that TRAIL caused cleavage of Bid via activated caspase-8, subsequently the loss of mitochondrial membrane potential, and then induced apoptosis in human B lymphoma cell line BJAB.

MATERIALS AND METHODS

Materials. TRAIL was obtained from R & D systems Inc. Proteinase K was from Merck (Darmstadt, Germany). The peptide inhibitor

Abbreviations used: Ac-IETD-CHO, acetyl-L-isoleucyl-L-glutamyl-L-threonyl-L-aspart-1-aldehyde; Ac-DMQD-CHO, acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspart-1-aldehyde; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; CHX, cycloheximide; FCS, fetal calf serum; FACS, fluorescence-activated cell sorting.

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for caspase-8, Ac-Ile-Glu-Thr-Asp-H (Ac-IETD-CHO) and for caspase-3, Ac-Asp-Met-Glu-Asp-H (Ac-DMQD-CHO) was from Peptide Institute, Inc., Osaka, Japan. 3,3′-Dihexyloxacarbocyanine iodide [$DiOC_6(3)$] was from Molecular Probes, Inc. An affinity-purified goat polyclonal antibody of the carboxy terminus of human Bid, BID (C-20) and secondary antibody, anti-goat IgG antibody were from Santa Cruz Biotechnology, Inc. ECL western blotting detection reagents were from Amersham Pharmacia Biotech.

Cell culture and treatment with TRAIL. The human B lymphoma cell line BJAB was cultured in RPMI1640 medium supplemented with 6% heat-inactivated FCS at 37°C under 5% CO_2 in a humidified atmosphere. The cell viability was greater than 90% in each cell preparation used. Cells (10^6 cells/ml) were exposed to 50 ng/ml TRAIL in the presence of 0.2 μg/ml cycloheximide (CHX) for individual incubated time throughout this experiment. On the experiments for competitive inhibition, cells were preincubated with 100 μM Ac-IETD-CHO or Ac-DMQD-CHO for 30 min.

SDS-PAGE and Western blot analysis. Cells (10^6 cells/ml) were incubated for different periods of time with TRAIL in the presence or absence of the peptide inhibitor for caspase as the above-mentioned method. After washing three times with cold PBS, cells were suspended with sample buffer (10^6 cells/30 μl) containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue. The samples were boiled for 5 min and analyzed by 15% SDS-PAGE under nonreducing conditions. After electrophoresis, proteins were transferred to the PVDF membrane by semi-dry electroblotting. Briefly, the membrane was blocked with 5% skim milk in T-TBS [10 mM Tris (pH 7.4), 100 mM NaCl, and 0.05% Tween 20] for 1 h at 37°C. Then, the membrane was incubated with an affinity-purified goat polyclonal antibody of the carboxy terminus of human Bid in T-TBS (dilution 1:500) for 1 h at room temperature and washed three times with T-TBS. The membrane was subsequently incubated with anti-goat IgG antibody in T-TBS (dilution 1:2000) for 30 min and washed twice with T-TBS, once with TBS and then the Bid protein was detected using ECL Western detection reagents.

Flow cytometric detection of mitochondrial membrane potential. To assess the change in mitochondrial membrane potential, after the incubation with TRAIL as the above-mentioned method, cells were incubated with 40 nM $DiOC_6(3)$ for 15 min at 37°C. Then, the cells were washed with PBS twice, suspended in PBS and then were analyzed with a flow cytometer (FACScan, Becton Dickinson) (27). Dead cells and debris were excluded from the analysis by electronic gating of forward and side scatter measurements.

Detection of DNA ladder formation induced by TRAIL. After the incubation for individual times with TRAIL in the presence or absence of the peptide inhibitor for caspase as the above-mentioned method, the medium was removed, and the cells were washed with PBS. The cells (2×10^6 cells) were suspended in 1 ml of cytoplasm extraction buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, and 0.5% Triton X-100] and centrifuged 1000 g for 5 min at 4°C. The pellet was resuspended with lysis buffer [10 mM Tris (pH 7.5), 400 mM NaCl, 1 mM EDTA, and 1% Triton X-100]. After sitting on ice for 10 min, the cell lysate was centrifuged 12000 g for 10 min at 4°C. The supernatant was treated with 0.2 mg/ml RNase overnight at room temperature, followed by treatment with proteinase K according to the method as described previously (28). DNA ladder formation, which is characteristic for apoptosis, was analyzed by conventional electrophoresis.

RESULTS

Cleavage of Bid in BJAB cells by TRAIL. To examine the cleavage of Bid in BJAB cells treated with TRAIL, we performed Western blots using an anti human Bid antibody. Bid, which was expressed as the 23 kDa protein (16), began to be cleaved to a 15-kDa fragment at 1 h after the treatment with TRAIL in the presence of CHX (Fig. 1). The cleavage of Bid was inhibited by 100 μM Ac-IETD-CHO, a caspase-8 inhibitor, (data not shown). These results suggest that TRAIL can cause cleavage of Bid via activated caspase-8 in BJAB cells.

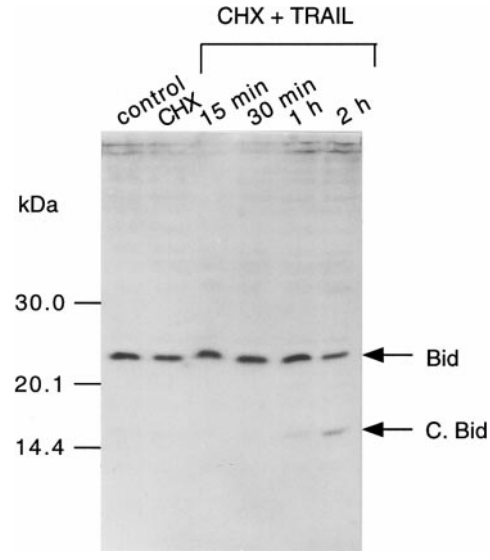

FIG. 1. Cleavage of Bid in BJAB cells was induced by TRAIL. After incubation for indicated time, the cells were analyzed by 15% SDS-PAGE and Western blots were performed using an anti human Bid antibody as described in Materials and Methods. Bid was expressed as the 23 kDa protein (Bid) and was cleaved to a 15 kDa fragment (C. Bid) from the 1-h incubation with TRAIL in the presence of CHX.

Loss of mitochondrial membrane potential by TRAIL. A change on standing of mitochondrial membrane potential in BJAB cells treated with TRAIL was measured by a flow cytometer. As shown in Fig. 2, loss of mitochondrial membrane potential in BJAB cells began about 1.5 h after the treatment with TRAIL in the presence of CHX and was apparent at 2 h. On the other hand, in the cells treated with only CHX, mitochondrial membrane potential was not altered at 2.5 h. These results suggest that TRAIL can cause loss of mitochondrial potential in BJAB cells, following activation of caspase-8.

The effects of caspase-3 and caspase-8 inhibitors on DNA ladder formation in BJAB cells by TRAIL. The DNA ladder formation, which is characteristic for apoptosis, was shown at 3 h in the cells treated with TRAIL in the presence of CHX, but in the control and in only CHX-treated cells, the ladders were not detectable at 3 h (Fig. 3A). When the caspase-8 inhibitor, Ac-IETD-CHO, and the caspase-3 inhibitor, Ac-DMQD-CHO, were added, the DNA ladders were not detected at 3 h (Fig. 3B). These results have confirmed that TRAIL induces apoptosis in BJAB cells, and suggested that caspase-8 and caspase-3 are involved in TRAIL-induced apoptosis.

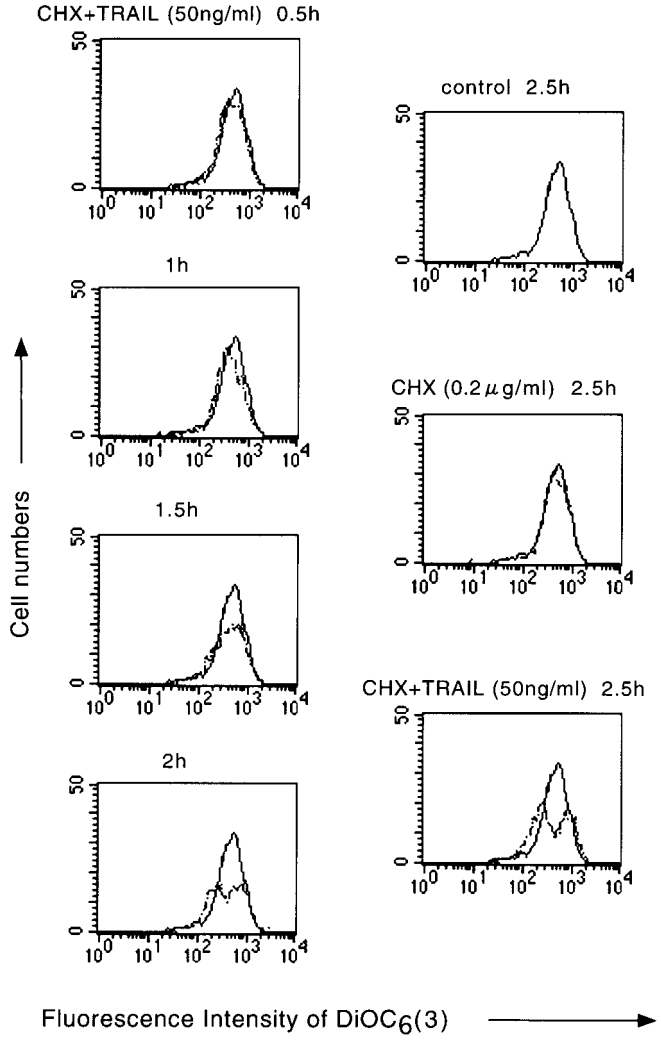

FIG. 2. Change of mitochondrial membrane potential in BJAB cells treated with TRAIL in the presence of CHX. After incubation for indicated time, the cells were incubated with 40 nM $DiOC_6(3)$ for 15 min at 37°C, and then the cells were analyzed with a flow cytometer (FACScan). The horizontal axis shows the relative fluorescence intensity, and the vertical axis shows the cell numbers. Data shown as follows: solid curves, mitochondrial membrane potential of the control cells incubated without TRAIL at 2.5 h; dotted curves, mitochondrial membrane potential of cells incubated with TRAIL at 0.5–2.5 h.

DISCUSSION

The present study demonstrated that TRAIL caused cleavage of Bid via activated caspase-8 in apoptotic pathway of BJAB cells. Bid possesses only the BH-3 domain, lacks a carboxyl-terminal signal-anchor segment, and is found in both cytosolic and membrane locations (16). Recent reports demonstrate that Bid was cleaved into the COOH-terminal part, major 15 kDa fragment and minor 13 kDa and 11 kDa fragments, by activated caspase-8 in pathway of Fas-induced apoptosis (20). In our study, Bid was cleaved to a 15 kDa fragment in the cells treated with TRAIL in the presence of CHX at 1–2 h. Furthermore, the loss of mitochondrial membrane potential in BJAB cells began about 1.5 h after the treatment with TRAIL in the presence of CHX and was apparent at 2 h. Our results are a good agreement with the results of recent studies as for pathway of Fas-induced apoptosis. Our results and previous literature demonstrated that activated caspase-8 cleaved Bid into the COOH-terminal part, 15 kDa fragment (20), which was translocated to mitochondria where it triggered changes in mitochon-

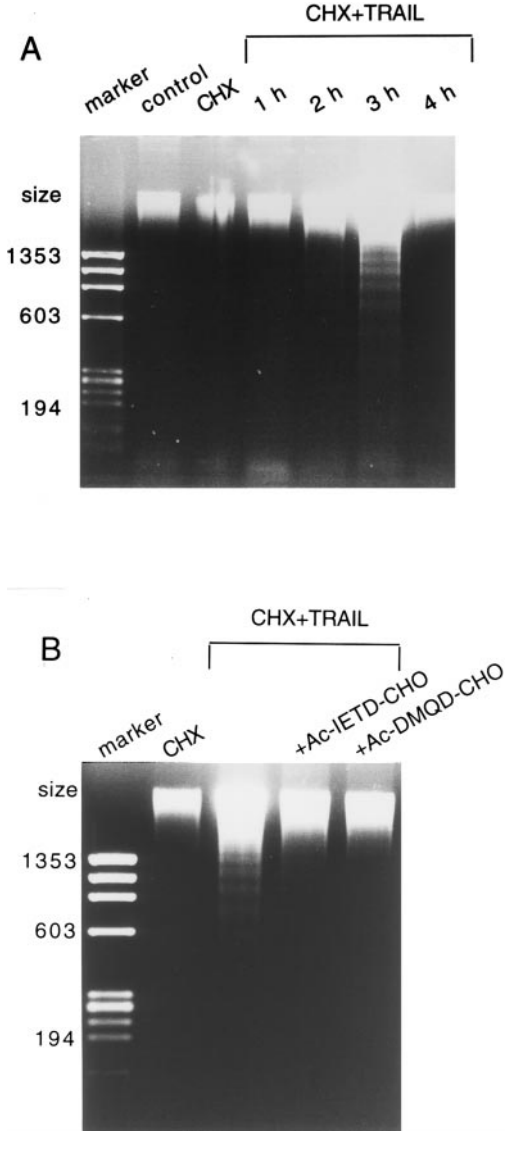

FIG. 3. Detection of DNA ladder formation induced by TRAIL. (A) The cells were incubated with or without TRAIL in the presence or absence of CHX as described in Materials and Methods. The samples of the control and CHX were incubated at 37°C for 3 h, and the samples of CHX plus TRAIL were incubated for indicated time. (B) The cells were incubated with or without TRAIL (100 ng/ml) in the presence of CHX (0.1 μg/ml) for 3 h. The samples of CHX plus TRAIL plus Ac-IETD-CHO, or Ac-DMQD-CHO were preincubated with Ac-IETD-CHO and Ac-DMQD-CHO for 30 min as described in Materials and Methods. Marker lane: size marker DNA (ΦX174/*Hae* III digest).

drial membrane permeability and loss of membrane potential (13, 16, 19–23).

In our study, the DNA ladder formation in the cells treated with TRAIL in the presence of CHX was investigated. Additionally, we investigated effects of caspase-8 and caspase-3 inhibitors on DNA ladder formation induced by TRAIL. The DNA ladder formation had been detectable at 2 h, and showed the most induction at 3 h. When the caspase-8 inhibitor, Ac-IETD-CHO and the caspase-3 inhibitor, Ac-DMQD-CHO were added, the DNA ladders were blocked at 3 h, suggesting that caspase-8 and caspase-3 are involved in TRAIL-induced apoptosis in BJAB cells. Caspase-8 is the upstream enzyme which is recruited to death-inducing signal complex by binding to a Fas-associated adapter protein (29). In Fas-induced apoptosis, activated caspase-8 mediates downstream apoptotic events (12, 14, 15, 30, 31). Recently, Imai *et al.* identified a new protein, FLASH, which contains a domain that interacts with a death-effector domain in caspase-8 or in the adapter protein (32). Therefore, it is considered that in apoptotic pathway of TRAIL as well as Fas ligand, activated caspase-8 mediated downstream apoptotic events. Finally, loss of mitochondrial membrane potential resulted in the activation of caspase-3 (28), and activated caspase-3 cleaved DNA fragmentation factor, which is a human homologue of the mouse inhibitor of caspase-activated deoxyribonuclease, resulting in DNA ladder formation (25, 26).

In summary, TRAIL as well as Fas ligand causes cleavage of Bid via activated caspase-8, subsequently the loss of mitochondrial membrane potential and activation of caspase-3, resulting in apoptosis, in TRAIL-induced apoptotic pathway in human B lymphoma cell line BJAB. TRAIL and Fas ligands show a similar potency in inducing apoptosis (2). Moreover, TRAIL induces apoptosis in the cells, which resist inducing apoptosis by Fas ligand (33). Since TRAIL may have more effective potential as a cancer therapeutic cytokine than Fas ligand, further research is necessary to clarify the mechanism of TRAIL-induced apoptosis.

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