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Involvement of TNF-Related Apoptosis-Inducing Ligand in Human CD4⁺ T Cell-Mediated Cytotoxicity¹

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TNF-related apoptosis-inducing ligand (TRAIL) has been identified as a member of the TNF family that induces apoptosis in a variety of tumor cells, but its physiological functions are largely unknown. In the present study, we examined the expression and function of TRAIL in human CD4⁺ T cell clones by utilizing newly established anti-human TRAIL mAbs. Human CD4⁺ T cell clones, HK12 and 4HM1, exhibited perforin-independent and Fas ligand (FasL)-independent cytotoxicity against certain target cells, including T lymphoma (Jurkat) and keratinocyte (HaCaT) cell lines, which are susceptible to TRAIL-mediated cytotoxicity. In contrast to FasL, the expression of which was inducible upon anti-CD3 stimulation, TRAIL was constitutively expressed on HK12 and 4HM1 cells, and no further increase was observed after anti-CD3 stimulation. Spontaneous cytotoxic activities of resting HK12 and 4HM1 cells were abolished by the combination of anti-TRAIL and anti-FasL mAbs. These results indicate a differential regulation of TRAIL and FasL expression on human CD4⁺ T cell clones and that TRAIL constitutes an additional pathway of T cell-mediated cytotoxicity. The Journal of Immunology, 1999, 162: 2639–2647.

Members of the TNF family include physiological death factors and play important roles in regulating immune responses. It has been well characterized that TNF-α and Fas ligand (FasL) induce apoptotic cell death via their specific receptors (TNFR-I and Fas/CD95, respectively), which contain structurally and functionally homologous death domains (1, 2). TNF-related apoptosis-inducing ligand (TRAIL)/APO-2L is a recently identified type II integral membrane protein belonging to the TNF family and induces apoptosis in various tumor cell lines (3, 4). TRAIL mRNA were detected in a variety of tissues and cells as estimated by Northern blot analysis (3, 4). Recent molecular cloning of the TRAIL receptors (TRAIL-Rs) elucidated that TRAIL binds to at least four receptors, TRAIL-R1/DR4, TRAIL-R2/DR5/TRICK2/killer, TRAIL-R3/TRID/DR5/LIT, and TRAIL-R4/TRUNDD/DecR2, with similar affinities (5–16). These receptors could be classified into two groups, death-inducing receptors (TRAIL-R1 and R2) and death-inhibitory receptors (TRAIL-R3 and R4). Both TRAIL-R1 and TRAIL-R2 contain the death domain homologous to that in TNFR-I, DR3/TRAMP/HAPO-3/WSL-1, or Fas and appear to be responsible for the TRAIL-induced apoptotic cell death in a variety of tumor cells (5–8, 10). In contrast to these death-inducing receptors, TRAIL-R3 is devoid of cytoplasmic domain and exists as a glycospholipid-anchored protein on the cell surface. It has been suggested that TRAIL-R3 competes with the death-inducing TRAIL-Rs for TRAIL binding and may work as a decoy receptor (9–13). Since TRAIL-R3 mRNA was preferentially found in normal cells but not in transformed cells, it is thought that TRAIL-R3 might be responsible for the cellular resistance of normal cells to TRAIL-mediated cytotoxicity (9, 13). TRAIL-R4 has the cytoplasmic domain containing a truncated death domain that cannot transmit a death signal but can activate NF-κB, which may protect the cells from TRAIL-mediated apoptosis (14–16). These findings suggested a complex regulation of cellular susceptibility to the TRAIL-mediated apoptosis at the level of multiple receptor expression. In contrast to this progress in the receptors, little is known about the expression of TRAIL at the protein level and its physiological functions.

T cell-mediated cytotoxicity not only plays an important role in immune surveillance against virus-infected or transformed cells but also has been implicated in the tissue damage associated with diseases, such as graft-vs-host disease and organ-specific autoimmune diseases. It has been generally accepted that CTL kill target cells via two major effector pathways, perforin- and FasL-mediated pathways (17–19). The effector molecules in the former, such as perforin and granzymes, are secreted from cytoplasmic granules of CTL into an intercellular space after contact with target cells. Locally secreted perforin molecules form a channel in the target cell membrane, resulting in osmotic lysis and influx of granzymes that activate caspases and lead to nuclear disintegration (20, 21). In the latter pathway, the intercellular interaction between FasL on CTL and its receptor, Fas, on target cells, induces apoptotic target cell death via the death domain-mediated recruitment of the caspases (1, 2). It has been demonstrated that these two pathways are required for the clearance of certain pathogens and tumors (22–25). It has also been indicated that these pathways are involved in the pathogenesis of hepatitis and graft-vs-host disease (26–30). In addition to these two pathways, however, previous
studies have suggested the possible existence of some other effector mechanisms for CTL cytotoxicity. For example, Braun et al. demonstrated that CTL from mice deficient in both FasL and perforin lysed some kinds of tumor cells in an in vitro assay (30). Furthermore, it has also been reported that when perforin-knockout mice were injected with a low number of syngeneic Fas-negative tumor cells, these mice could reject them like wild-type mice (24). In our preliminary studies, we also found that some CD4+ T cell clones spontaneously lysed certain target cells in a FasL- and perforin-independent manner. The molecules on effector and target cells responsible for such a FasL- and perforin-independent cytotoxicity have not been defined. It is known that membrane-bound or soluble TNF-α could contribute to the cytotoxicity exerted by some CTL or lymphokine-activated killer cells (31–33). However, the TNF-α-mediated cytotoxicity appeared not to be relevant to the cytotoxicity reported by Braun et al., since neutralizing anti-TNF-α Abs only partially blocked this cytotoxicity (30).

In the present study, to elucidate the physiological expression and function of TRAIL in immune system, we generated neutralizing mAbs against human TRAIL (hTRAIL) and estimated the toxicity and a differential regulation of TRAIL and FasL expression in human TRAIL-mediated apoptosis. In contrast, FasL was inducible upon anti-CD3 stimulation, and bystander cytotoxicity by activated CD4+ T cell clones was mediated by both TRAIL and FasL. These results indicated the involvement of TRAIL in T cell cytotoxicity and a differential regulation of TRAIL and FasL expression in human CD4+ T cell clones.

Materials and Methods

Cells

A mouse B lymphoma cell line, 2PK-3, was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 containing 10% FCS, 100 μg/ml streptomycin and penicillin, and 2 mM glutamine (culture medium). The human T lymphomas Jurkat and HUT78, a chronic myelogenous leukemia K562, and a monocytic leukemia THP-1 (Kanebo, Osaka, Japan) and maintained in culture medium. A human T lymphoma, PEER, was kindly provided by Dr. J. Minowada (Hayashibara Biochemical Laboratories, Fukuji, Japan) and maintained in culture medium. A spontaneously transformed human keratinocyte cell line, HaCat (34), was kindly provided by Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany) and cultured in DMEM containing 10% FCS, 100 μg/ml streptomycin and penicillin, and 2 mM glutamine. Human CD4+ T cell clones (HK12, 4HM1, HK1, HK11, HK15, and HK101) that had been established from PHA blasts as described before (35) were provided by Dr. M. Azuma (National Children’s Medical Research Center, Tokyo, Japan) and maintained in culture medium supplemented with 10 U/ml of recombinant IL-2. The Ag specificity of these clones has not been determined. For the preparation of anti-CD3-activated CD4+ T cell clones, these cells (2 × 10^6) were cultured for 3 h on six-well plates precoated with 10 μg/ml anti-CD3 mAb (OKT-3) and then washed twice with culture medium.

Reagents

A hydroxamic acid-based metalloprotease inhibitor (KB-R8301) that inhibits the processing of FasL (36) was provided by Dr. K. Yoshino (Osaka, Japan). PMA and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO). OKT-3 was prepared from the hybridoma obtained from ATCC. An anti-human Fas mAb (NOK-2) was prepared as described before (36). A heteroconjugated bispecific Ab (OCT-3Xanti-Fas) was prepared by chemically conjugating OKT-3 and an anti-nitrophthol (NP) hapten mAb with N-succinimidyl-3-(2-pyridyldithiol)propionate (Pharmacia, Tokyo, Japan) as described previously (38).

Construction and preparation of DR5-Ig chimera protein

A cDNA fragment encoding the extracellular domain (amino acids 1–183) of human DR5/TRAIL-R2 was amplified by PCR from the full-length human DR5 cDNA kindly provided by Dr. J. Ni (Human Genome Science, Rockville, MD) (13) using ATGGACAAACCGGGACAC as the 5’ primer and GCCGTGATCTTGTGTCGAC as the 3’ primer. The 5’ and 3’ primers were tagged with an EcoRI and a BamHI site, respectively. The 550-bp PCR product was digested with EcoRI and BamHI and then introduced into the EcoRI and BamHI sites of pBluescript II carrying the human Cyl genomic sequence as described previously (39). This results in-frame fusion of the extracellular region of DR5 to the Fc portion of human IgG1 (DR5-Ig). After confirmation of the nucleotide sequence, the EcoRI- and NotI-digested fragment encoding DR5-Ig was transferred into the EcoRI and NotI sites of the PSG5 expression vector (Strategene, La Jolla, CA). COS7 cells were transfected with DR5-Ig/PSG5 as described before (40). At 16 h after transfection, the culture medium was changed to a serum-free medium (ASF-104, Ajinomoto, Tokyo, Japan) and further cultured for 96 h. DR5-Ig fusion protein in the supernatant was purified with a protein G column as described before (41).

Preparation of hTRAIL or FasL transfectants

hTRAIL cDNA was prepared by RT-PCR amplification of total RNA from anti-CD3-activated 4HM1 cells, with an oligonucleotide primer corresponding to the first six codons as the 5’ primer and that corresponding to the last six codons as the 3’ primer, according to the published sequence (3, 4). The 5’ and 3’ primers were tagged with a XhoI and a NotI site, respectively. After XhoI and NotI digestion, the PCR product of 850 bp was subcloned into pBluescript II SK(+) and the nucleotide sequence was confirmed by using an Applied Biosystems (Foster City, CA) 373A automated sequencer and fluoresceinylated dye terminator cycle sequencing method. The 850-bp cDNA was then transferred into the XhoI and NotI sites of the MKITneo expression vector, kindly provided by Dr. K. Ma-ruyama (Tokyo Medical and Dental University, Tokyo, Japan). For generating hTRAIL transfectants, hTRAIL/PMKTneo was transfected into 2PK-3 cells by electroporation (290 V, 960 μF) using a Gene Pulser (Bio-Rad, Hercules, CA). After selection with 1 mg/ml G418 and cloning by limiting dilution, a stable transfectant (hTRAIL/2PK-3) expressing a high level of TRAIL was selected by staining with DR5-Ig. In a similar way, BHK cells stably expressing hTRAIL (hTRAIL/BHK) were prepared. Generation of human FasL (hFasL)-expressing 2PK-3 cells (hFasL2PK-3) was performed as described before (36).

Generation of anti-hTRAIL mAbs

Six-week-old female BALB/c mice (Cleargen, Tokyo, U.S.A.) were immunized by i.p. injection of hTRAIL/2PK-3 (1 × 10^7 cells) several times at 10-day intervals. Three days after the final immunization, the spleno-cytes were fused with P3U1 mouse myeloma cells as described previously (39). After HAT (hypoxanthine/aminopterin/thymidine) selection, the Abs that inhibit cytotoxic activity of hTRAIL/2PK-3 against Jurkat cells were screened. Two anti-RK1 Abs (R1K-1 and R1K-2) were identified by their strong inhibitory effects and cloned by limiting dilution. RK-1 and RIK-2 (both mouse IgG1κ) were purified from culture supernatant with the protein G column.

Cytotoxic assays

A [3H]Tdr release assay was performed as described previously (18) with [3H]Tdr-labeled Jurkat cells (1 × 10^7) and effector cells at the indicated E:T ratios. After 8 h, intact nuclei were harvested using a Micro 96 harvester (Skatron, Lier, Norway), and radioactivity was measured on a microplate beta counter (Micro β Plus; Wallac, Turku, Finland). The percentage TdR release was calculated as follows: [%cpm with effector/cpm without effector] × 100.

A 51Cr release assay was performed as described previously (41). Briefly, 51Cr-labeled target cells (1 × 10^7) and effector cells were mixed in the U-bottomed wells of a 96-well microtiter plate at the indicated E:T ratios. After 8 h of incubation, cell-free supernatants were collected and counted in a gamma counter. The percentage specific 51Cr release was calculated as described before (38). In some experiments the effector cells were pretreated with 20 nM CMA for 2 h to inactivate perforin (37). Anti-FasL mAb (NOK-2) and/or RIK-2 were added to be a final concentration of 10 μg/ml at the start of the cytotoxic assay. In some experiments, cytotoxicity of CD4+ T cell clones against NP-modified HaCaT cells was tested in the presence of 1 μg/ml OKT-3xanti-NP in an 8-h 51Cr release assay. NP modification of target cells was conducted with NP-O-succinimide as described previously (38).
Flow cytometric analysis

2PK-3, hTRAIL/2PK-3, and hFasL/2PK-3 cells (1 x 10^6) were incubated with 1 μg of biotinylated mAbs or DR5-Ig for 1 h at 4°C followed by phycoerythrin (PE)-labeled avidin or PE-labeled anti-human IgG (PharMingen, San Diego, CA), respectively. After washing with PBS, the cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA), and data were processed by using the CELLQuest program (Becton Dickinson). Expression of TRAIL and FasL on resting or anti-CD3-activated CD4^+ T cells was analyzed in a similar way with biotinylated anti-TRAIL (RIK-2) or anti-FasL (NOK-2) mAbs. For the analysis of FasL expression, 10 μM KB-R8301 was added to the stimulation culture to avoid the release of FasL from the cell surface.

Results

Characterization of hTRAIL transfectants

To generate and characterize the mAbs to hTRAIL, we first established stable cDNA transfectants expressing full-length hTRAIL. Mouse B lymphoma 2PK-3 cells, which were totally resistant to recombinant TRAIL (rTRAIL) (unpublished data), were transfected with hTRAIL cDNA in the pMKITneo expression vector by electroporation to make hTRAIL/2PK-3. After G418 selection, cell surface expression of TRAIL on the hTRAIL/2PK-3 cells was verified by staining with DR5-Ig. hTRAIL/2PK-3 cells, but not 2PK-3 or hFasL/2PK-3 cells, were stained by DR5-Ig (Fig. 1A). An anti-hFasL mAb (NOK-2) that we previously generated (36) stained hFasL/2PK-3, but not hTRAIL/2PK-3, indicating that NOK-2 does not cross-react with TRAIL.

It has been demonstrated that Jurkat cells were highly sensitive to both rTRAIL-mediated and anti-Fas mAb-mediated apoptosis (3, 4, 42). Consistent with this, we observed that the hTRAIL/2PK-3 cells and the hFasL/2PK-3 cells efficiently lysed Jurkat cells in an 8-h [3H]TdR-release assay, while no significant cytotoxicity was observed with the parental 2PK-3 cells (Fig. 1B). Similarly, hTRAIL/BHK cells, but not BHK cells, exhibited potent cytotoxicity against Jurkat cells (not shown).

Previously, we and others have reported that some other members of the TNF family, such as TNF-α and FasL, undergo processing by some metalloproteinase(s) and are released from the cell surface in a soluble form (36, 43–46). We again observed that culture supernatant of the hFasL/2PK-3 cells exhibited specific cytotoxicity against FasL-transfected cells (not shown), as we previously demonstrated for hFasL/BHK cells (36). In contrast, no detectable level of cytotoxic activity against Jurkat cells was found in the supernatant of hTRAIL/2PK-3 or hTRAIL/BHK cells (not shown).

We also tested the cytotoxic activities of hTRAIL/2PK-3 and hFasL/2PK-3 cells against various cell lines (Fig. 1C). A human keratinocyte cell line, HaCaT, was sensitive to both TRAIL and FasL, as was the Jurkat cell line. A T lymphoma cell line, PEER, was sensitive to TRAIL but not to FasL. A T lymphoma cell line, HUT78, was sensitive to FasL but not to TRAIL. A chronic myelogenous leukemia cell line, K562, and a monocytic leukemia cell line, THP-1, were resistant to both TRAIL and FasL.

Characterization of anti-hTRAIL mAbs

To characterize the expression and function of hTRAIL, we generated two mAbs that specifically bind to hTRAIL and block the cytotoxic activity. Hybridomas were prepared from splenocytes from mice immunized with the hTRAIL/2PK-3 cells. Two hybridomas, producing RIK-1 and RIK-2 mAbs (both mouse IgG1k), were selected by their strong ability to block the hTRAIL/2PK-3 cytotoxicity against Jurkat cells. Both RIK-1 and RIK-2 reacted with hTRAIL/2PK-3, but not with 2PK-3 or hFasL/2PK-3 cells, as estimated by cell surface staining (Fig. 2A). These mAbs also reacted with hTRAIL/BHK but not with BHK cells (not shown).

FIGURE 1. Characterization of hTRAIL and FasL transfectants. A, Cell surface staining with DR5-Ig and anti-FasL mAb (NOK-2). 2PK-3, hTRAIL/2PK-3, and hFasL/2PK-3 cells were stained with DR5-Ig or biotinylated NOK-2 followed by PE-labeled anti-human IgG or PE-labeled avidin, respectively (white histograms). Black histograms indicate background staining with control Ig and PE-labeled secondary reagents. B, Cytotoxic activity of TRAIL or FasL transfectants against tumor cell lines. Cytotoxic activity of hTRAIL/2PK-3 (○), hFasL/2PK-3 (●), or 2PK-3 (△) was tested against Jurkat cells in an 8-h [3H]TdR release assay at the indicated E:T ratios. Data represent means ± SD of triplicate samples. Similar results were obtained in two independent experiments. C, Cytotoxic activity of TRAIL or FasL transfectants against tumor cell lines. Cytotoxic activity of hTRAIL/2PK-3 (black bars), hFasL/2PK-3 (hatched bars), or 2PK-3 (white bars) was tested against the indicated target cells in an 8-h [3H]Cr release assay at an E:T ratio of 10. Data represent means ± SD of triplicate samples. Similar results were obtained in two independent experiments.
Previously, we demonstrated that the treatment with a hydroxamic acid-based metalloproteinase inhibitor, KB-R8301, led to accumulation of membrane FasL on the cell surface of FasL transfectedants (36). This was again observed with the hFasL/2PK-3 cells (not shown). In contrast, the same treatment with 10 μM KB-R8301 and various inhibitors of other proteases, including metalloendopeptidases (phosphoramidon, 100 μM), serine proteases (aprotinin, 10 μM), serine/cysteine proteases (leupeptin, 100 μM), cysteine proteases (E64, 100 μM), aspartate proteases (pepsstatin, 100 μM), and aminopeptidases (Bestatin, 100 μM), did not affect the expression of TRAIL on the hTRAIL/2PK-3 and hTRAIL/BHK cells (data not shown).

We next compared the blocking activities of RIK-1 and RIK-2 with that of DR5-Ig. As represented in Fig. 2, both RIK-1 and RIK-2 inhibited the hTRAIL/2PK-3 cytotoxicity against Jurkat cells as efficiently as DR5-Ig in the 8-h [3H]TdR release assay.

**CD4⁺ T cell clones spontaneously lysed certain target cells via a FasL- and perforin-independent pathway**

CD4⁺ T cells exhibit cytotoxic activity against certain target cells in an Ag-specific or -nonspecific manner (18, 41, 47–50). As represented in Fig. 3, we observed that human CD4⁺ T cell clones (HK12 and 4HM1) exhibited spontaneous cytotoxicity against Jurkat, HaCaT, and PEER cells but not against HUT78, THP-1, or K562 cells in an 8-h [51Cr] release assay without an anti-CD3 stimulation. A similar target specificity was also observed for all of the other CD4⁺ T cell clones tested in the present study (a total of six clones from two healthy donors; data not shown). None of these CD4⁺ T cell clones expressed a detectable level of perforin (not shown), and the treatment with CMA, which has been successfully used to inhibit the perforin-mediated cytotoxicity (37), failed to inhibit the spontaneous cytotoxicity of these clones (Fig. 3). Furthermore, the addition of a neutralizing mAb against hFasL (NOK-2) hardly inhibited the cytotoxicity exerted by these cells (Fig. 3). These results suggest the contribution of a perforin- and FasL-independent cytotoxic pathway against these target cells. Since all the CD4⁺ T cell clones used in the present study originated from PHA blasts, their antigenic specificities are unknown. However, these CD4⁺ T cell clones efficiently lysed MHC class II-negative targets (Jurkat and PEER cells), and the addition of anti-CD3 failed to inhibit the cytotoxicity (not shown), suggesting that they lysed the targets in a TCR/CD3-independent and MHC-unrestricted manner.

We noted that the tumor cell lines, which were efficiently lysed by the CD4⁺ T cell clones (Jurkat, HaCaT, and PEER cells), were also sensitive to hTRAIL/2PK-3-mediated cytotoxicity (Fig. 1C). In contrast, HUT78, K562, and THP-1 cells, which were not lysed by the CD4⁺ T cell clones, were resistant to the hTRAIL/2PK-3-mediated cytotoxicity. These results suggest a possible involvement of TRAIL in the CD4⁺ T cell-mediated cytotoxicity against Jurkat, HaCaT, and PEER cells.

**Expression of TRAIL on CD4⁺ T cell clones**

Although the expression of TRAIL at the mRNA level has been demonstrated in various tissues and cells, including activated T cells (3, 4, 51), little is known about its expression and regulation at the protein level. Therefore, we examined the cell-surface expression of TRAIL on CD4⁺ T cell clones by flow cytometry using the RIK-2 mAb. As represented in Fig. 4, substantial expression of TRAIL was detected on the surface of resting CD4⁺ T cell clones without anti-CD3 stimulation. We previously demonstrated that the expression of FasL was inducible on the T cell surface in response to cross-linking of TCR/CD3 or stimulation with PMA + ionomycin (36, 41). Consistent with this, substantial levels of FasL expression were observed on HK12 and 4HM1 cells 3 h after the anti-CD3 stimulation, while resting HK12 and 4HM1 cells expressed only marginal levels of FasL on the surface (Fig. 4). In contrast to FasL, the anti-CD3 stimulation hardly affected the expression level of TRAIL as compared with the resting state (Fig. 4).

**Involvement of TRAIL in CD4⁺ T cell-mediated cytotoxicity**

We finally addressed whether TRAIL is involved in the spontaneous cytotoxic activity of CD4⁺ T cell clones against Jurkat, HaCaT, and PEER cells. As represented in Fig. 5, A through C, spontaneous cytotoxicity exerted by resting HK12 cells against
Jurkat cells (Fig. 5A), HaCaT cells (Fig. 5B), and PEER cells (Fig. 5C) was almost completely abrogated by the addition of the anti-TRAIL mAb (RIK-2) alone. In contrast, only marginal inhibition was observed in the presence of anti-FasL mAb (NOK-2). A similar inhibition by RIK-2 but not by NOK-2 was also observed for all of the other CD4<sup>+</sup> T cell clones tested (data not shown). These results clearly indicated that the TRAIL expressed on these CD4<sup>+</sup> T cell clones (Fig. 4) was fully responsible for their spontaneous cytotoxic activity against Jurkat, HaCaT, and PEER target cells.

We also examined the contribution of TRAIL and FasL to the cytotoxic activity of anti-CD3-activated CD4<sup>+</sup> T cell clones that express both TRAIL and FasL, as indicated in Fig. 4. As represented in Fig. 5, A and B (bottom), NOK-2 alone, but not RIK-2 alone, partially inhibited the cytotoxicity, and the combination of RIK-2 and NOK-2 almost completely abrogated the cytotoxic activities of anti-CD3-activated HK12 cells against Jurkat (A) and HaCaT (B) target cells. In contrast, when a FasL-resistant cell line, PEER, was used as the target, the addition of RIK-2 alone but not NOK-2 alone almost completely abrogated the cytotoxic activity of anti-CD3-activated HK12 cells (Fig. 5C). Similar results were obtained with all of the other CD4<sup>+</sup> T cell clones tested (data not shown). These results indicated that both the TRAIL and the FasL expressed on the anti-CD3-activated CD4<sup>+</sup> T cell clones (Fig. 4) were responsible for mediating the cytotoxic activities against Jurkat and HaCaT target cells and that TRAIL, but not FasL, mediated cytotoxic activity of these cells against PEER target cells.

We finally examined whether TRAIL can participate in the Ag-specific cytotoxicity of CD4<sup>+</sup> T cell clones. Since the antigenic specificities of the T cell clones used in the present study were unknown, we used the (OKT-3Xanti-NP)-redirected cytotoxicity against NP-labeled HaCaT cells to mimic the TCR/CD3-mediated cognate interaction with target cells. As represented in Fig. 5D,
RIK-2 and NOK-2, each used alone, partially inhibited the cytotoxic activity, and the combination of RIK-2 and NOK-2 almost completely abrogated the redirected cytotoxic activity of HK12 cells against HaCaT cells. These results suggest that when HK12 cells encounter the TRAIL- and FasL-sensitive target bearing specific Ag, both TRAIL and FasL can participate in the Ag-specific cytotoxicity.

Discussion

In the present study, we investigated the expression and function of TRAIL in human CD4+ T cell clones by using newly established neutralizing mAbs against hTRAIL. Unexpectedly, TRAIL was constitutively expressed on the surface of resting CD4+ T cell clones and was responsible for their spontaneous cytotoxicity against certain target cells, such as Jurkat and HaCaT cells. In contrast, FasL expression was solely inducible upon anti-CD3 stimulation, and the activated CD4+ T cell clones lysed the Jurkat and HaCaT target cells by utilizing both TRAIL and FasL. These results are the first indication that the expression of TRAIL and FasL is differentially regulated in T cells and that TRAIL is involved in T cell-mediated cytotoxicity.

Recently, Jeremias et al. reported the expression of TRAIL mRNA in human peripheral blood T cells following activation with anti-CD3 mAb or PMA + ionomycin, but they did not address the protein expression and function (51). More recently, Mariani and Krammer demonstrated expression of the TRAIL protein on murine activated T cells by utilizing a rabbit polyclonal Ab to a peptide sequence in the extracellular region of hTRAIL (52). In the present study, we observed that all six human CD4+ T cell clones that we tested constitutively expressed TRAIL on cell surface. A very recent study by Thomas and Hersey (53) demonstrated that each of four human CD4+ T cell clones that had been raised against autologous melanoma cells exhibited TRAIL-mediated cytotoxicity against Jurkat cells in an Ag-nonspecific and MHC-unrestricted manner, as we observed with our T cell clones in the present study. Although they did not examine the possibly constitutive expression of TRAIL on their T cell clones, their results also suggest that TRAIL can be generally expressed on long-term cultured human CD4+ T cell clones without specific Ag stimulation. In our ongoing study with RIK-2 mAb, surface expression of TRAIL is not detectable on freshly isolated peripheral blood T cells but is inducible upon continuous stimulation with anti-CD3, especially in the presence of IL-2, suggesting a critical contribution of cytokines (unpublished data). Therefore, it is possible that the constitutive expression of TRAIL observed on the CD4+ T cell clones was due to a continuous stimulation with IL-2 supplemented to the culture. Further studies are now under way to characterize the cytokines that regulate the TRAIL expression.

The apparently differential regulation of FasL and TRAIL expression on T cells may be relevant to their differential roles in the immune system. It is well known that FasL plays critical roles, not only in mediating T cell cytotoxicity but also in maintaining T cell homeostasis and tolerance (1, 17–19). Preactivated T cells up-regulate Fas expression and undergo apoptosis in response to FasL expressed on neighboring T cells or APCs. In contrast, preactivated T cells appear to be highly resistant to TRAIL-induced apoptosis. Jeremias et al. reported that peripheral blood T cells remained resistant to rTRAIL even after prolonged activation with PHA and IL-2 (51). We also observed that neither T cell blasts nor the CD4+ T cell clones used in this study were sensitive to the TRAIL transfectant- or rTRAIL-mediated cytotoxicity (our unpublished data). Moreover, the addition of NOK-2, but not RIK-2, almost completely inhibited the activation-induced cell death (AICD) of anti-CD3-stimulated HK12 cells, suggesting little if any contribution of TRAIL to AICD. Some other possible contributions of TRAIL to immune responses are now under investigation.

Possibly another way of differential regulation of TRAIL and FasL at the posttranslational processing has been recently suggested by Mariani and Krammer (54). They reported that hTRAIL
might be processed to be a 19–20-kDa soluble form by leupeptin- and E64-sensitive cysteine proteases, in contrast to FasL, which is processed by E64-resistant metalloproteases, and that cell surface staining with a rabbit anti-TRAIL polyclonal Ab was enhanced by treatment of Jurkat cells with the cysteine protease inhibitors (54). However, they did not address cytotoxic activity of the naturally processed soluble TRAIL. In reference to their results, we examined the effect of various protease inhibitors, including leupeptin, E64, and a metalloprotease inhibitor (KB-R8301) that inhibited the processing of TNF-α and FasL (36), but we could not detect the enhancement of surface TRAIL expression on our hTRAIL transfectants or human CD4+ T cell clones (data not shown). We also could not detect cytotoxic activity against Jurkat or HaCaT cells in supernatants of the TRAIL transfectants or the human CD4+ T cell clones (data not shown), but this does not formally exclude the presence of weakly cystotoxic soluble TRAIL, as has been observed with soluble FasL that could not efficiently lyse Jurkat cells (42, 55, 56). Therefore, while further biochemical analysis will be needed to characterize the processing, TRAIL appears to act as a proapoptotic ligand primarily in the membrane-bound form, as does FasL.

Our present study supplements TRAIL as an additional effector molecule mediating T cell cytotoxicity. It has been suggested that perforin and FasL constitute two predominant pathways of T cell-mediated cytotoxicity (17–19). TNF-α and lymphotixin also participate in the T cell cytotoxicity against certain target cells (31–33). The relative contribution of these multiple effector molecules appears to be variable depending on their expression in the effector T cells and susceptibility of the target cells to these molecules. Perforin can be expressed in almost all CD8+ T cells and a minor population of CD4+ T cells (38). FasL can be expressed on almost all CD4+ T cells and CD8+ T cells, except for some Th2 clones (57, 58). The possibly differential expression of TRAIL among T cell subpopulations remains to be determined. With respect to target susceptibility, perforin can potentially lyse any target cells by making pores in the plasma membrane. In contrast, FasL and TRAIL require expression of the death-inducing receptors on the target cells. FasL induces target cell apoptosis via its receptor, Fas, which recruits FADD (Fas-associated death domain) and activates FLICE (FADD-like IL-1β-converting enzyme; caspase 8) and the subsequent caspase cascade (1, 2). However, it is known that solely the expression of Fas does not always determine the susceptibility to FasL-induced apoptosis, which may be explained by the expression of antiapoptotic molecules such as Bcl-2 or FLIP, which antagonizes FLICE (1, 2, 59, 60). The TRAIL case is more complicated. TRAIL induces target cell apoptosis via TRAIL-R1 and TRAIL-R2, which recruit FADD or a FADD-like adapter and activate FLICE or FLICE-2 (caspase-10) and the subsequent caspase cascade, as does Fas. However, it has been shown that TRAIL-R1 and TRAIL-R2 also recruit TRADD and activate NF-κB, which may act protectively against apoptosis (61). Moreover, TRAIL also binds to antagonistic receptors, including TRAIL-R3, which lacks a cytoplasmic domain, and TRAIL-R4, which does not induce apoptosis but activates NF-κB and protects against TRAIL-R1- and TRAIL-R2-mediated apoptosis (9–16). Therefore, the susceptibility to TRAIL would be determined primarily by the relative expression of these agonistic and antagonistic receptors, and secondarily by cellular resistance to the FADD-mediated apoptosis pathway, as in the case of FasL. RT-PCR analysis for expression of the TRAIL-R mRNA in the TRAIL-susceptible target cell lines used in this study showed that Jurkat cells expressed TRAIL-R2 and TRAIL-R3 but not TRAIL-R1 or TRAIL-R4, and that both HaCaT and PEER cells expressed TRAIL-R2, TRAIL-R3, and TRAIL-R4 but not TRAIL-R1 (data not shown). The relative contribution of each agonistic or antagonistic receptor to TRAIL sensitivity remains to be determined by using the mAb specific to each receptor.

It has been suggested that TRAIL induces apoptosis preferentially in transformed cells but not in normal cells, possibly because of preferential expression of TRAIL-R3 in the latter (9, 13). Consistent with this, we also found that cells of the transformed keratinocyte cell line HaCaT, but not normal keratinocytes, were sensitive to TRAIL (unpublished data). Therefore, it is possible that FasL and TRAIL may act complementarily to eliminate a variety of transformed cells in an immune surveillance system. In this respect, it is noteworthy that TRAIL-R2 has been also identified as a p53-inducible tumor suppressor gene (6), suggesting a contribution of TRAIL to the elimination of DNA-damaged cells. In the present study, we showed that TRAIL can mediate CD4+ T cell cytotoxicity against a FasL-resistant lymphoma cell line, PEER. Furthermore, a recent study demonstrated that 7 of 10 FasL-resistant human melanoma cell lines were susceptible to TRAIL-induced apoptosis and that TRAIL at least partly mediated Ag-specific cytotoxicity of human CD4+ T cell clones against FasL-resistant melanoma cells (53). Therefore, it is possible that the TRAIL-mediated cytotoxicity by CD4+ T cells might play an important role, especially in the elimination of such FasL-resistant tumor cells.

Physiological and pathological roles of TRAIL remain to be determined. The TRAIL-mediated T cell cytotoxicity may be involved in the clearance of virus- or intracellular pathogen-infected cells and the rejection of allografts, which were independent of either perforin or FasL (22, 23, 62–64). It is also interesting to note that TRAIL may be involved in the pathogenesis of AIDS. Recently, Jeremias et al. (51) reported that peripheral blood T cells from HIV-infected patients were susceptible to TRAIL-induced apoptosis, and Katsikis et al. (65) reported that anti-CD3-induced AICD of peripheral blood T cells from some HIV-infected patients was inhibited by an anti-TRAIL mAb but not by anti-FasL mAb. The anti-TRAIL mAbs generated in the present study will be useful for investigating further the expression and function of TRAIL in HIV infection.

The Ag specificity of T cell-mediated cytotoxicity is a favorable feature that can selectively eliminate the Ag-presenting target cells without damaging bystander normal cells. However, it is well known that Ag-specific CTL can cause bystander lysis of certain target cells once activated by specific target cells (33, 66). We previously demonstrated that the perforin-mediated cytotoxicity is highly Ag-specific, but the FasL-mediated cytotoxicity participates in both Ag-specific and bystander cytotoxicities (33). In the present study, we also demonstrated that TRAIL can also participate in both Ag-specific and bystander T cell cytotoxicities against TRAIL-sensitive target cells but apparently without stimulation with specific Ag. This difference results from the constitutive nature of TRAIL expression on the T cell clones, which appears to be primarily regulated by cytokines. It has been also shown by others that Ag-specific CD4+ T cell clones exhibited Ag-nonspecific and MHC-unrestricted cytotoxicity against certain target cells when cultured with IL-2, some of which might be partly mediated by FasL (67). Such an Ag-nonspecific cytotoxicity mediated by TRAIL and FasL may be relevant to unfavorable consequences of chronic activation of CD4+ T cells that lead to inflammatory tissue damages in pathological situations, including some autoimmune diseases and infections. On the other hand, the TRAIL-mediated MHC-unrestricted cytotoxicity of CD4+ T cells may be more useful for the eradication of MHC class II-negative tumor cells than...
the FasL-mediated cytotoxicity, because the latter can cause a serious liver damage, whereas the former does not (56). Further studies on the regulation of TRAIL expression in T cells will provide new insights into pathological and physiological roles of TRAIL-mediated cytotoxicity and its potential application to tumor immunotherapy.

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References


