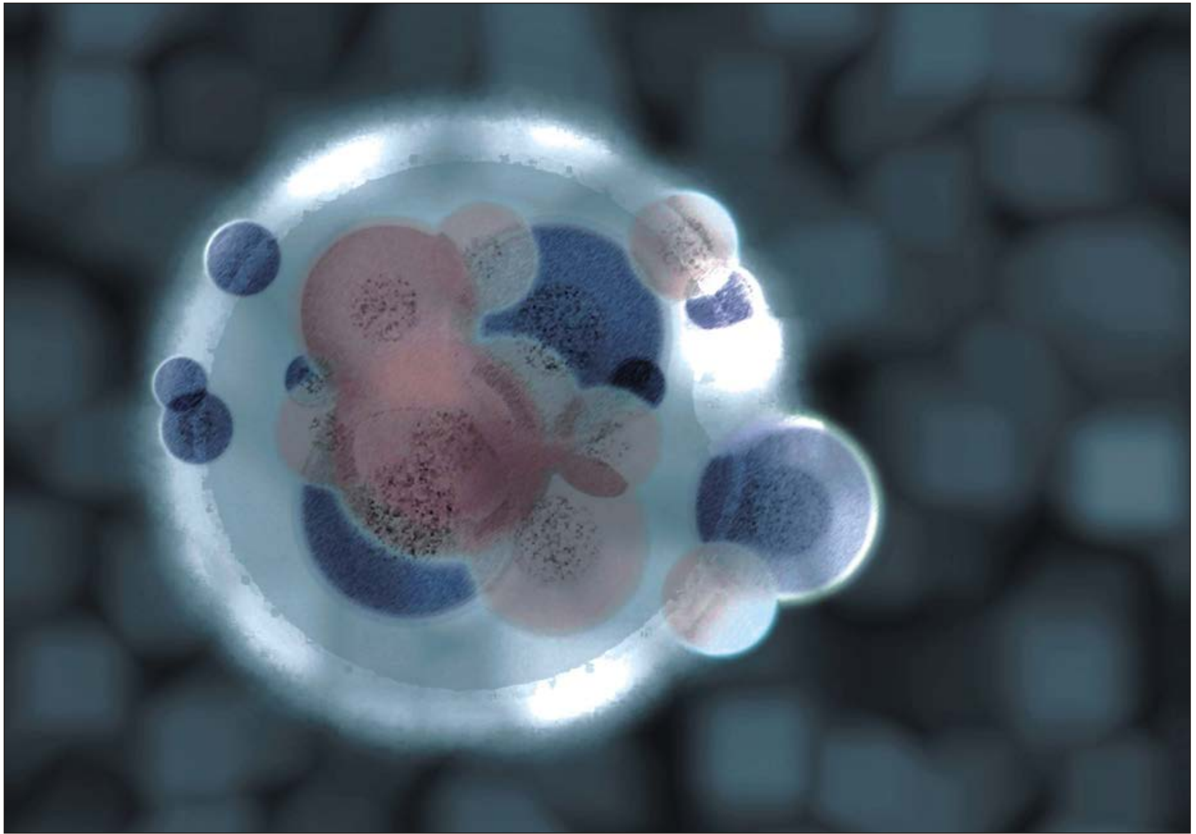

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TRAIL-mediated Cytotoxicity: Impacts of sTRAIL and vTRAIL Microvesicles

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Abstract: Previous studies have shown that FasL-expressing cells produced nearly equal amount of soluble FasL (sFasL) and microvesicle-associated FasL (vFasL) under regular tissue culture condition. Here, we studied the ability of TRAIL-expressing cells to produce sTRAIL and vTRAIL and compared their impact on TRAIL-mediated cytotoxicity. We found that TRAIL-expressing cells produced extremely low level of vTRAIL. It indicates that the ability of TRAIL-expressing cells to produce vTRAIL but not sTRAIL is significantly different from FasL-expressing cells and that the ability to produce vTRAIL and vFasL is a property intrinsic to the protein itself. Our study also shows that the microvesicles, containing full-length TRAIL, express strong cytotoxicity against a commonly used Jurkat target cells whereas the cytotoxicity of sTRAIL was nearly undetectable. We concluded that sTRAIL is efficiently produced so much so that it can be inhibitory for the cytotoxicity expressed by the TRAIL-expressing cells. The significance of the findings is discussed.

Key words: TRAIL, soluble TRAIL, TRAIL microvesicles, cytotoxicity, apoptosis

INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type II membrane protein that belongs to the TNF family[1,2]. Among members of this family, the extracellular portion of TRAIL is highly homologous to that of the CD95 ligand (FasL)[3]. Like FasL, TRAIL also induces apoptosis of target cells. In humans, TRAIL can bind to two death-inducing receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5)[4-7], leading to receptor-clustering followed by activation of the caspase-cascade and induction of apoptosis in TRAIL-sensitive cells[8-11].

TRAIL is expressed in activated human T cells[12-14], monocytes/macrophages[15], natural killer cells[16-20] and dendritic cells[21-23]. TRAIL-mediated apoptosis was reported to contribute to the activation-induced death of human peripheral blood T cells. Like the Fas/FasL system, TRAIL-based immune regulation may play a role in autoimmune diseases and the maintenance of immune privilege sites[24-28]. In addition, TRAIL is reported to preferentially kill virus-infected[29] and malignant tumor cells[1,2,8,30,31], but not normal cells[32]. Therefore, TRAIL has been proposed to be a potentially useful therapeutics. Many investigators have attempted to make agonistic recombinant soluble TRAIL as well as agonistic anti-TRAIL receptor antibodies. These reagents can be used to kill cancer cells either by themselves or in combination with anti-tumor drugs[14,33-39].

Cells that express TRAIL express killing activity against TRAIL-sensitive target cells. Like FasL-expressing cells, TRAIL-expressing cells also release a soluble form of TRAIL (sTRAIL) thought to be cleaved by protease(s)[24] that is yet to be identified. In addition to sTRAIL, human TRAIL-expressing cells also secrete microvesicles that contain TRAIL (vTRAIL) and vTRAIL are cytotoxic against TRAIL-sensitive target cells[40-42]. While production of sTRAIL and sFasL is dependent on the presence of sensitive sites on the extracellular domains of TRAIL/FasL, the requirement for the production of vTRAIL and vFasL is not well understood. In the present study, we quantitatively investigated the amount of sTRAIL and vTRAIL produced by TRAIL-expressing cells. Present study suggests that TRAIL-expressing cells preferentially secrete sTRAIL but not vTRAIL. The ability to produce vTRAIL appears to be an intrinsic property of the TRAIL itself. Moreover, the high level of sTRAIL in the culture supernatant could act as feedback inhibitor for cell-mediated, TRAIL-dependent cytotoxicity. This study characterized TRAIL turnover system and defined the functional significance of sTRAIL and vTRAIL.

MATERIALS AND METHODS

TRAIL-expressing cell lines: Human TRAIL cDNA, provided by Dr. J. Tschopp, was cloned into Moloney

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leukemia virus-derived pLXSN (GenBnk accession No. M28248)[43]provided by Dr. A.D. Miller. The TRAIL construct, pMLV-hTRAIL, was used to transfect the packaging cell line PE501 (a gift from Dr. A.D. Miller) using lipofectamine (GIBCO). Viral-laden supernatants from transfected cell line were recovered at 48 h after transfection and were used to infect the PA317 packaging cell line that was provided by Dr. S.T. Ju. Clones were selected with 0.75 mg mL^{-1} of G418 (GIBCO) and the cell line TRAIL-PA317 that expressed the strongest cytotoxicity was used in the present study. A similarly prepared packaging cell line Krox-PA317 carrying the human *ckrox* gene[44] was used for the preparation of various controls throughout the study. The generation of both TRAIL-PA317 and Krox-PA317 cells has been described previously[42]. We also established TRAIL expressing NIH-3T3 cells by transferring the human *trail* gene to NIH-3T3 cells. Various amounts of vector prepared from packaging cells were cultured with NIH-3T3 cells (2×10^3 cells $well^{-1}$ in 24-well plates) in the presence of 6 μg mL^{-1} polybrene (Sigma). Medium was replaced 24 h later with fresh medium containing 0.75 mg mL^{-1} G418. Cell populations that survived the G418 selection were examined for TRAIL-mediated cytotoxicity. One cell line (TRAIL-3T3) that expresses strongest cytotoxicity was used in this study.

Target cells: A20, CEM, Jurkat, HCT-15 and Hep G2 cells were purchased from ATCC. A-172, MOLT-4 and U-937 cells were provided by Dr. K. Nishimura (Division of Immunoregulation, Institute for Genetic Medicine, Hokkaido University).

Preparation of sTRAIL and vTRAIL: TRAIL-expressing cells (80% confluence) were maintained in 150×25 mm Petri dishes (FALCON) in 30 mL of culture media. Culture supernatants and cells were harvested 24 h later. The cell number harvested was ~ 30×10^6/dish. Supernatants were centrifuged at 5°C for 30 min at 15,000 rpm in a Beckman centrifuge (Avanti™ 30) using an F0650 rotor to remove cell debris. To prepare sTRAIL, the Cell-free Supernatants (CFS) were centrifuged at 5°C for 16 h at 25,000 rpm in a Beckman ultracentrifuge (L8-M) using a SW28 rotor. The top 10% volume was collected in order to avoid potential contamination of vesicles. This Vesicle-free Supernatant (VFS) was passed through a 0.45 μm sterile filter and stored at 4°C. The Vesicle-containing Pellet (VP) was suspended with culture medium to 3% of the original volume. The suspension was passed through a 0.45 μm sterile filter and stored at 4°C.

Filtration based on molecular size was used to characterize various TRAIL-containing fractions. The filtration/concentration apparatus used were MACROSEP 300K OMEGA (Pall Life Sciences) which retain components larger than 300 kDa and Centricon Plus-20 PL-30 (Millipore) which retains components larger than 30 kDa. The former was used for concentrating vTRAIL (present in microvesicles) and the latter was used to concentrate sTRAIL (~60 kDa as a trimer).

Flow cytometric analysis: TRAIL-PA317, TRAIL-3T3, Krox-PA317 and NIH-3T3 cells were incubated with 0.1 μg of Phycoerythrin (PE)-conjugated mouse anti-human TRAIL mAb (RIK-2, mouse IgG1/κ) and PE-conjugated control mouse IgG1 (DAKO) for 30 min on ice. After washing with PBS containing 0.2% BSA, the cells were analyzed on a FACS Calibur™ (Becton Dickinson) and data were processed using Cell Quest™ software (Becton Dickinson).

Quantification of TRAIL: Protein concentrations of TRAIL were determined using a capture ELISA (OptEIA™ Human TRAIL set, Pharmingen). This assay measures both soluble TRAIL and transmembranous TRAIL, because the mAbs used recognize epitopes present on soluble TRAIL and full-length TRAIL. To measure cellular TRAIL, cells (4×10^6) were washed with PBS, then treated with 1 mL of Cell Lysis Buffer (Pharmingen) with Protease inhibitor Cocktail (Pharmingen), according to the instruction in the kit. VP tested in this study obtained from different cells were re-suspended and filled with PBS, then pelleted by ultracentrifugation once more, re-suspended again with PBS, then treated with Cell Lysis Buffer and Protease inhibitor Cocktail at the recommended ratio. VFS obtained from different cells were analyzed without using lysis buffer. All samples were diluted with Assay Dilute (Pharmingen) and immediately assayed. Standard curves were generated with various concentrations of recombinant TRAIL provided with the kit.

Cytotoxicity assays: Target cells were labeled with $Na_2{}^{51}CrO_4$ as described[45]. The effector samples included TRAIL-expressing cells, VP and VFS. Various amounts of samples were cultured with 2×10^4 target cells in a total of 0.2 mL in individual wells of a 96-well plate. In some experiments, inhibitor was added to the mixtures at the beginning of culture to determine their effect on cytotoxicity. The inhibitor used was DR5-Fc (Alexis) that consists of the extracellular domain of the human TRAIL receptor DR5 fused with the Fc portion of human IgG and it was seen to specifically block TRAIL-induced apoptosis. Supernatants of cytotoxicity assays were removed at an appropriate period after culture and

radioactivity was determined using a γ-scintillation counter (Autowell gamma system ARC-380, Aloka). Background release was determined by culturing target cells in the absence of cytotoxic effectors. Target cells treated with 0.5% NP-40, were used to determine total release of radioactivity, which represented 100% cell death. Background release was routinely 8-25% of the total release throughout this study. Cytotoxicity is expressed as percent specific ^{51}Cr release, which is determined by the formula, 100% x (experimental release – background release)/(total release – background release).

Although some target cells could become more sensitive to TRAIL-mediated cell death when treated with protein synthesis inhibitors[8,14], transcription inhibitors[8,14,46], or inhibitors of NFκB pathways[23,47], we did not treat target cells with any drugs to increase their susceptibility to TRAIL in present studies.

Western blotting: Detection of TRAIL protein in cell lysates, VP lysates and VFS was carried out using a biotinylated anti-human TRAIL polyclonal antibody (Genzyme Techne). Cells were harvested and washed with PBS. Cells (10^7 cells) were lysed in 100 μL of ice-cold lysis buffer (1 M Tris, 1% NP-40, 5 M NaCl, 1 mM PMSF, 20 μg mL^{-1} leupeptin, 20 μg mL^{-1} aprotinin, 500 mM EDTA, 1 mM NaF). VP obtained from different cells was suspended with PBS and ultra-centrifuged. Pellets were lysed in the same manner as described for cell pellets. VFS obtained from different cells was concentrated by Centricon Plus-20 PL-30 and analyzed immediately.

Samples were subject to Western blotting analysis using a 15% SDS polyacrylamide gel. Membranes were blocked with milk proteins for 20 h and then incubated with 0.2 mg mL^{-1} biotinylated anti-human TRAIL antibody for 20 h at 4°C. Membranes were washed three times and then incubated with streptavidin-conjugated horseradish peroxidase (RPN-1231, Amersham, diluted 3000 folds). Specific bands were revealed using the ECL™ Western blotting detection reagent (RPN-2109, Amersham). Molecular weight standard was included to determine the size of TRAIL in various samples.

RESULTS

TRAIL-expressing cell lines express surface TRAIL protein and induce cell death of sensitive target cells in a TRAIL-specific fashion: We first determined our panel of transfectants for cell surface expression of TRAIL protein using Flow Cytometric Analysis. As represented in Fig. 1A, anti-TRAIL mAb but not isotype control stained both TRAIL-PA317 and TRAIL-3T3 cells. Under the same staining condition, staining was not observed with Krox-PA317 and NIH-3T3 cells.

We examined a panel of target cells for sensitivity to TRAIL using the TRAIL-PA317 cells as effectors. Various ^{51}Cr-labeled targets were mixed with TRAIL-PA317 cells and cultured at various effector/target cell ratios (E/T ratios). The results indicated that TRAIL-PA317 cells expressed cytotoxicity against many target cells. Among them, Jurkat, MOLT-4, Hep G2, A-172 and HCT-15 cells were highly sensitive and A20, CEM and U-937 cells were relatively more resistant (data not shown). Jurkat cells were used as target for most of the study because they are the most sensitive target cells. TRAIL-PA317 cells effectively killed Jurkat cells in a 5 h assay (Fig. 1B). TRAIL-3T3 cells that expressed a lower cell surface TRAIL level modestly killed target in this short assay (data not shown). However, strong killing was obtained in a 16 h assay (Fig. 1C). Krox-PA317 and NIH-3T3 cells that lack TRAIL did not kill Jurkat cells (Fig. 1B and C).

Inhibition of cytotoxicity was used to further characterize the cytotoxicity of TRAIL-PA317 and TRAIL-3T3 cells. The cytotoxicity of both TRAIL-expressing cells was completely abrogated by 10 μg mL^{-1} of DR5-Fc (Fig. 1B and C). Nonspecific human IgG1 myeloma protein had no effect (data not shown). Cytotoxicity was also inhibited by DR5-Fc when MOLT-4, Hep G2, A172 and HCT-15 cells were used as target cells (data not shown).

Molecular characterization of cell-associated TRAIL, vTRAIL and sTRAIL: We used Western blotting assays to determine the molecular property of TRAIL present in the culture supernatant. The fractions that contain secreted vesicles (VP) and Vesicle-free Supernatant (VFS) were prepared as described in Materials and Methods. In addition, TRAIL present in cell lysates was included for comparison. Samples from TRAIL-PA317, Krox-PA317, TRAIL-3T3 and NIH-3T3 cells were analyzed. As shown in Fig. 2, the anti-TRAIL Ab recognized a band with a molecular mass of 32 kDa in the lysates of TRAIL-PA317 and TRAIL-3T3 cells. This band corresponds to the full-length TRAIL protein. The same band was detected in the VP prepared from TRAIL-PA317 and TRAIL-3T3, indicating that TRAIL protein was present in vTRAIL was a full-length TRAIL incorporated in the microvesicles that are eventually secreted into culture supernatant. By contrast, anti-TRAIL Ab did not detect the full-length TRAIL in the VFS but recognized a protein with a molecular mass of 20 kDa, a size previously reported for sTRAIL[24].

In all cases, no band was detected in any sample prepared from Krox-PA317 and NIH-3T3 cells (data not shown).

TRAIL-expressing cells produce high levels of sTRAIL but little vTRAIL: Human TRAIL-specific ELISA was

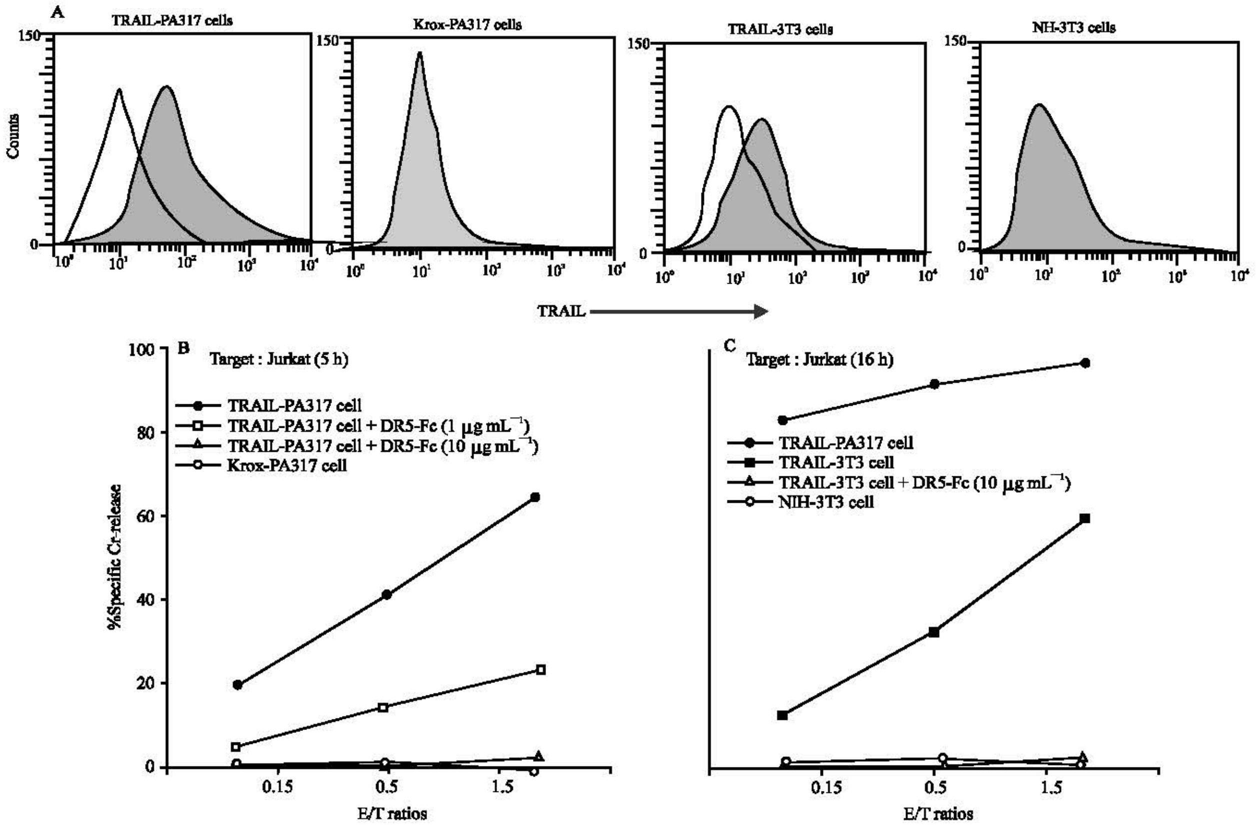

Fig. 1: TRAIL-expressing cell lines express surface TRAIL protein and induce cell death of sensitive target cells in a TRAIL-specific fashion.

A) Surface expression of TRAIL protein on TRAIL-PA317, Krox-PA317, TRAIL-3T3 and NIH-3T3 cells. Four groups of cells were stained with Phycoerythrin (PE)-conjugated mouse anti-human TRAIL mAb (RIK-2) (grey) and with PE-conjugated mouse IgG1 (white, background staining)

B) Cytotoxicity of TRAIL-PA317 and Krox-PA317 cells against ^{51}Cr-labeled Jurkat target cells at various E/T ratios in the absence or presence of DR5-Fc. Cytotoxicity was determined 5 h after culture

C) Cytotoxicity of TRAIL-PA317, TRAIL-3T3 and NIH-3T3 cells against ^{51}Cr-labeled Jurkat target cells at various E/T ratios in the absence or presence of DR5-Fc. Cytotoxicity was determined 16 h after culture

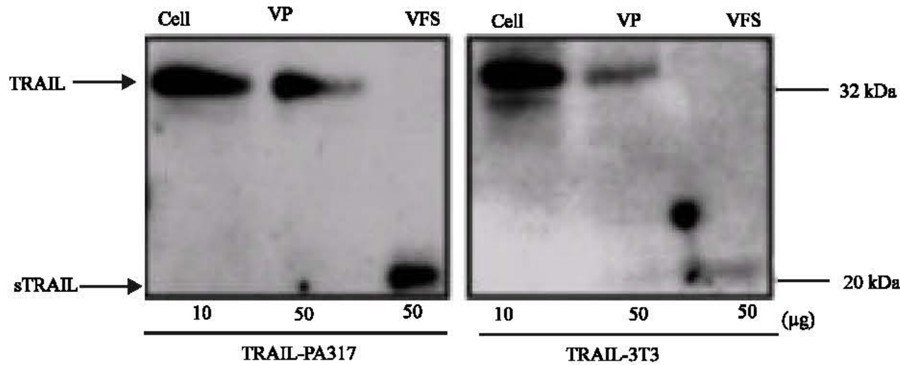

Fig. 2: Molecular structures of cell-bound TRAIL, vTRAIL and sTRAIL

Western analyses were carried out using a biotinylated anti-human TRAIL polyclonal antibody against preparations are indicated. Protein amounts loaded are also indicated. The size of anti-TRAIL-reactive bands was estimated based on molecular mass standard. The 32 kDa band corresponded to the full-length TRAIL and the 20 kDa band corresponded to sTRAIL

Table 1: Total amounts of vTRAIL and sTRAIL protein secreted in culture supernatants of TRAIL-expressing cells

Cells	Cell lysate[a]	vTRAIL[b]	sTRAIL[b]
TRAIL-PA317	51.2	2.6	37.8
TRAIL-3T3	22.5	0.8	8.7

[a]Total amounts of TRAIL protein (ng) in cell lysate of 30×10^{6} TRAIL-expressing cells cultured in 150×25 mm dishes.
[b]Total amounts of vTRAIL and sTRAIL protein (ng) accumulated in 24 h culture supernatants

used to determine the TRAIL levels produced as vTRAIL and sTRAIL during a 24 h culturing period. Assays were done three times with similar results and data represented in Table 1. High levels of TRAIL protein were measured in the lysates of TRAIL-PA317 and TRAIL-3T3 cells, although TRAIL protein level in TRAIL-PA317 was 2.5 times more than that present in TRAIL-3T3. This is

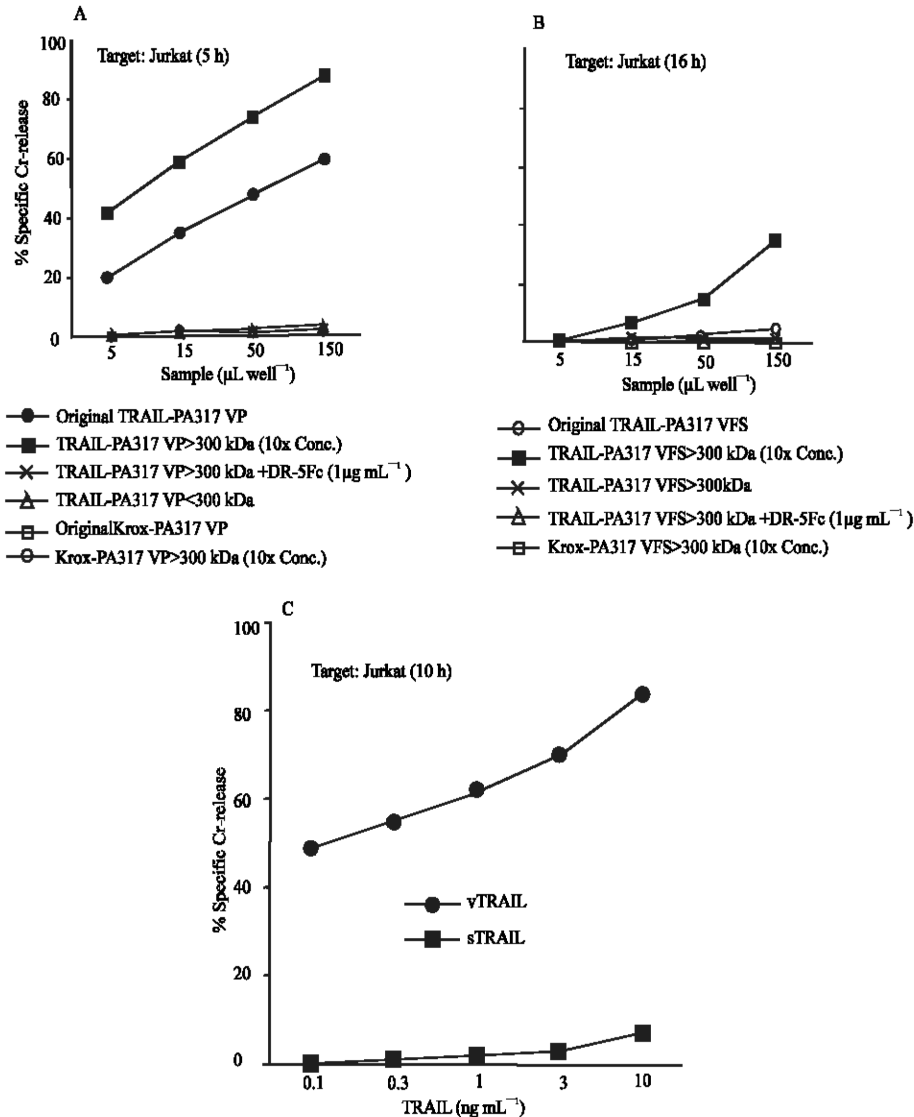

Fig. 3: Cytotoxicity of VP and VFS obtained from culture supernatants of TRAIL-PA317 and Krox-PA317 cells.
A) Various amounts of VP were mixed with ^{51}Cr-labeled Jurkat target cells and cytotoxicity were determined 5 h after culture in the absence or presence of DR5-Fc
B) Various amounts of VFS were mixed with ^{51}Cr-labeled Jurkat target cells and cytotoxicity was determined 16 h after culture in the absence or presence of DR5-Fc
C) Quantitative comparison of cytotoxicity of sTRAIL and vTRAIL prepared from culture supernatants of TRAIL-PA317 cells. Cytotoxicity of sTRAIL and vTRAIL against Jurkat target cells was determined 10 h after culture, based on the TRAIL protein concentrations, which were measured using ELISA

consistent with both the higher cell surface expression of TRAIL (Fig. 1A) and the stronger cell-mediated cytotoxicity of TRAIL-PA317 cells (Fig. 1C). The total amount of TRAIL released as vTRAIL was 2.6 ng for TRAIL-PA317 cells and 0.8 ng for TRAIL-3T3 cells. In sharp contrast, the total amount of sTRAIL was 37.8 ng for TRAIL-PA317 cells and 8.7 ng for TRAIL-3T3 cells. The amount of TRAIL protein released as sTRAIL is about 15 times (in molar ratio) more than vTRAIL and this is observed in both TRAIL-PA317 and TRAIL-3T3 cells. This sharp contrast is significant in two aspects. First, the efficient release of sTRAIL suggests a faster TRAIL turnover and a more important role in the down-regulation of cell surface expression and bioactivity of TRAIL. Second, the inefficient expression of TRAIL in microvesicles suggests that TRAIL sorting to this fraction may be an intrinsic property of TRAIL. Alternatively, the efficient release of sTRAIL may prevent TRAIL from taking the vesicle pathway.

Impact of vTRAIL and sTRAIL on TRAIL-mediated cytotoxicity: We compared the cytotoxicity of vTRAIL and sTRAIL secreted from TRAIL-PA317 cells. Because vTRAIL was low in the VP, advantage was taken to concentrate vTRAIL 10 times using a filtration system that retains materials that are larger than 300 kDa. As shown in Fig. 3A, the original VP had moderate killing activity and 10 times increase in cytotoxicity was observed for the concentrated fraction, indicating that most if not all vTRAIL is associated with microvesicles. Indeed, no cytotoxicity was detected in the filtrate. Killing by vTRAIL is completely inhibited by DR5-Fc. Thus, concentration by filtration is a simple method to prepare more potent apoptosis-inducing vesicles. This method is particularly useful when apoptosis-inducing ligand is weakly expressed as is the case for vTRAIL. In parallel experiments, cytotoxicity was not observed with samples similarly prepared from Krox-PA317 (Fig. 3A).

Monomeric sTRAIL was reported to be a 19-20 kDa molecule[24]. Once secreted, sTRAIL forms a homo-trimer (~60 kDa) that is thought to have functional activity[48]. Therefore, we used a filtration system that retains materials with sizes larger than 30 kDa to concentrate sTRAIL. In spite of high concentration of sTRAIL in the concentrated fraction as determined by ELISA, no cytotoxicity was observed in the 5 or 10 h cytotoxicity assays, indicating that the cytotoxicity of sTRAIL is extremely weak. However, the concentrated fraction did induce a weak killing of Jurkat cells in the 16 h assay (Fig. 3B). The killing was completely inhibited by DR5-Fc. In addition, killing was not detected with the concentrated fraction obtained from Krox-PA317 culture supernatant.

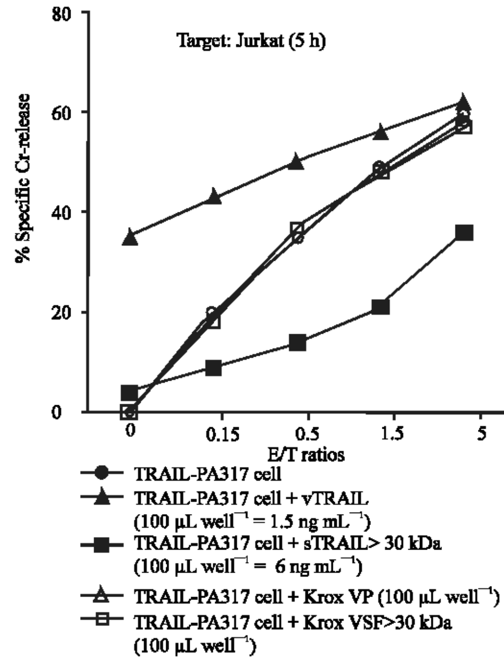

Fig. 4: Different effects of sTRAIL and vTRAIL on cell-mediated cytotoxicity.
Cell-mediated cytotoxic assays were carried out against ^{51}Cr-labeled Jurkat target cells with various E/T ratios of TRAIL-PA317 cells, in the absence or presence of sTRAIL or vTRAIL. Cytotoxicity was determined 5 h after culture. Identically prepared VP and VFS obtained from Krox-PA317 cells were also examined

Although cytotoxicity was observed with sTRAIL in concentrated fraction, the significance of this cytotoxicity is weak. First, it is not clear if this cytotoxicity is caused by microaggregates of sTRAIL resulted from the concentrating process. Second, the amount of sTRAIL is much higher than vTRAIL in microvesicles. Indeed, when cytotoxicity was measured based on protein concentration, the cytotoxicity of vTRAIL is >150 times stronger than sTRAIL (Fig. 3C).

sTRAIL can inhibit TRAIL-PA317-mediated cytotoxicity: Because of the high level of secreted sTRAIL and its weak cytotoxicity, sTRAIL in the supernatant may act as inhibitor of TRAIL-PA317-mediated cytotoxicity. Indeed, VFS obtained from TRAIL-PA317 cell culture was able to inhibit TRAIL-PA317 cell-mediated killing of Jurkat target, especially at low E/T ratios (data not shown). To illustrate the inhibiting power of sTRAIL in the 5 h cytotoxicity assay, we used 6 ng mL^{-1} of sTRAIL as an inhibitor for TRAIL-PA317 cell-mediated killing of Jurkat at various E/T ratios. In addition, we determined the effect of various

concentrations of sTRAIL on the cytotoxicity of TRAIL-PA317 cells at a fixed E/T ratio. As shown in Fig. 4, sTRAIL itself did not kill Jurkat target cells but was able to inhibit the cytotoxicity of TRAIL-PA317 cells at all E/T ratios tested. Control VFS obtained from Krox-PA317 cells had no effect on cell-mediated cytotoxicity. At the fixed E/T ratio, sTRAIL inhibited cytotoxicity in a dose-dependent manner (data not shown). It indicates that sTRAIL could be a natural inhibitor of TRAIL-mediated cytotoxicity in a feedback manner.

In contrast, as shown in Fig. 4, vTRAIL killed Jurkat targets in the absence of TRAIL-PA317 cells. A combination of vTRAIL and TRAIL-PA317 cells killed target cells more effectively compared with TRAIL-PA317 or vTRAIL alone. vTRAIL was able to augment cell-mediated cytotoxicity in a dose-dependent fashion (data not shown). Control VP obtained from Krox-PA317 cells had no effect on cell-mediated cytotoxicity.

DISCUSSION

Four major points are made in the present study. First, it demonstrates that TRAIL-expressing cells efficiently shed sTRAIL but secret microvesicles that contain little TRAIL. Second, despite of low level expression of vTRAIL, convincing evidence are presented to indicate that vesicles produced by TRAIL-expressing cells retain cytotoxic activity against TRAIL-sensitive cells. We provided molecular characterization, standard and traditional 5 h acute cytotoxicity analyses and vigorous specificity controls to firmly establish this point. Third, sTRAIL is efficiently shed but fails to retain cytotoxicity against TRAIL-sensitive targets. Fourth, the efficient shedding of sTRAIL is sufficient to provide a feedback-like mechanism to inhibit cytotoxicity mediated by the parent cells.

Unlike earlier studies, the present study provides quantitative measurement as well as bioactivity comparison among TRAIL associated with cells, sTRAIL shed from TRAIL-expressing cells and vTRAIL in secreted microvesicles. It is appropriate to discuss this study, particularly those of TRAIL-PA317 cells, in the context of our earlier work on FasL-PA317 cells. For TRAIL-PA317 cells, the amount of TRAIL protein released as soluble TRAIL (sTRAIL) were over 10 times greater than that produced as microvesicle-associated TRAIL (vTRAIL). For FasL-PA317 cells, the amount of sFasL produced was comparable to the amount of FasL present in the VP[49-51]. It has been shown that FasL are released into supernatant as exosomes from multi-vesicular bodies through exocytosis[52,53]. Partition of FasL toward the exosome pathway is regulated by FasL cytoplasmic domain (V. Pidiyar and S-T Ju, unpublished observation). TRAIL cytoplasmic tail apparently lacks the critical motifs that sort TRAIL to exosome pathway, resulting in weak expression of TRAIL in the secreted microvesicles. It is important to emphasize that despite the low expression of vTRAIL, we were able to prepare potent TRAIL-containing microvesicles by filtration-based concentration and used them to firmly establish that vTRAIL is full-length and unlike sTRAIL, retains the ability to mediate cytotoxicity against TRAIL-sensitive targets.

It has been suggested that sFasL, while losing cytotoxic strength, becomes inhibitor of FasL-mediated cytotoxicity, presumably by competing for Fas on target cells[54,55]. As the expression of FasL by most physiological FasL-expressing cells such as activated T cells is transient, the shedding of sFasL itself is an effective way to down-regulate FasL-mediated cytotoxicity[56]. However, whether the secreted sFasL has sufficient concentration to inhibit FasL-mediated cytotoxicity is difficult to assess. In view of the efficient production of sTRAIL, we determined if the sTRAIL shed to the supernatant was sufficient to inhibit TRAIL-mediated cytotoxicity. The result clearly indicates that sTRAIL shed to the local environment may accumulate to a level that is sufficient to inhibit TRAIL-mediated cytotoxicity. Thus, in addition to remove functional cell surface TRAIL, sTRAIL itself may reduce TRAIL-mediated cytotoxicity in a feedback manner.

Present analysis of the molecular property of sTRAIL and vTRAIL provides evidence for structure-function relationship. Our Western blotting analysis and inhibition of cytotoxicity indicate that sTRAIL is capable of binding to TRAIL receptors but not able to generate clusters of receptors like TRAIL on cells and vesicles. Like FasL-induced cell death, strong clustering of TRAIL receptors will lead to functional TRAIL DISC that is required for the transmission of death signals[11]. Binding of sTRAIL to TRAIL receptors will inhibit clustering of TRAIL receptor by TRAIL-PA317 cells and competitively inhibits the cytotoxicity mediated by TRAIL-PA317 cells. However, TRAIL signaling system is complicated by the variable presence of decoy receptors on target cells that may have different affinity and different threshold for signal transduction for full-length TRAIL and sTRAIL. We found a few targets that are sensitive to sTRAIL but resistant to vTRAIL (unpublished observation).

It has been reported that human malignant melanoma cells do not express cell surface FasL but produced FasL-expressing vesicles that are able to induce death of activated T cells and in so doing escaping from the attack of cytotoxic T cells[57]. Present study suggests that

tumor cells over-expressing TRAIL may not use TRAIL-expressing microvesicles to help escape from immune attack because TRAIL is not efficiently incorporated in the microvesicles secreted from TRAIL-expressing cells. On the other hand, sTRAIL produced by TRAIL-expressing tumors may interfere with normal TRAIL/TRAIL receptor interaction, perturbing immune surveillance system and tipping the balance of immune regulation. In this respect, it was reported that TRAIL is a potent inhibitor of autoimmune inflammation as it blocks cell cycle progression[26]. Perhaps, not only cell surface TRAIL, but also sTRAIL may well impact inflammation through TRAIL/TRAIL receptor interactions.

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