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[6,14], transcription inhibitors[6,14,46], 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

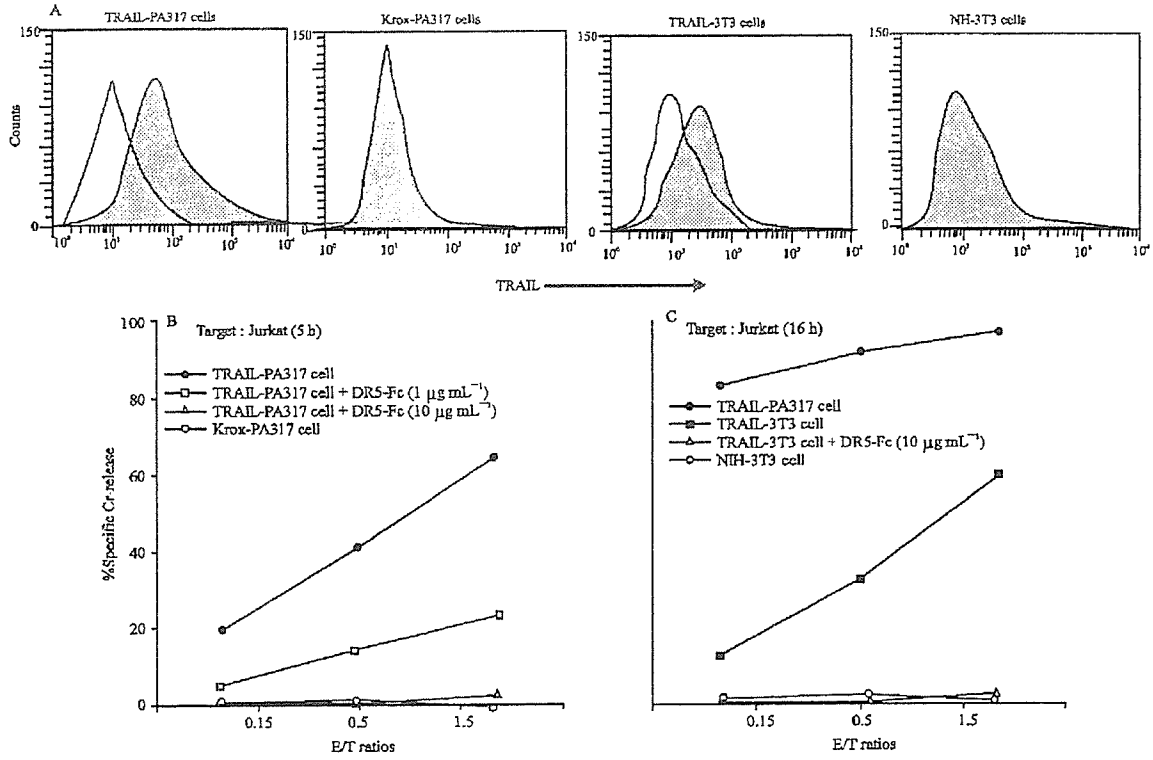

Fig. 1: TRAIL-expressing cell lines express surface TRAIL protein and induce cell death of sensitive target cells in a TRAIL-specific fashion.

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

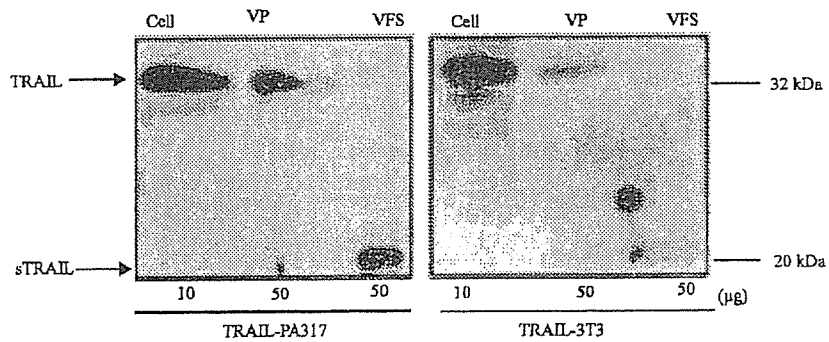

Fig. 2: Molecular structures of cell-bound TRAIL, vTRAIL and sTRAIL
Western analyses were carried out using a biotinylated anti-human TRAIL polyclonal antibody against preparations are indicated. Protein amounts loaded are also indicated. The size of anti-TRAIL-reactive bands was estimated based on molecular 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

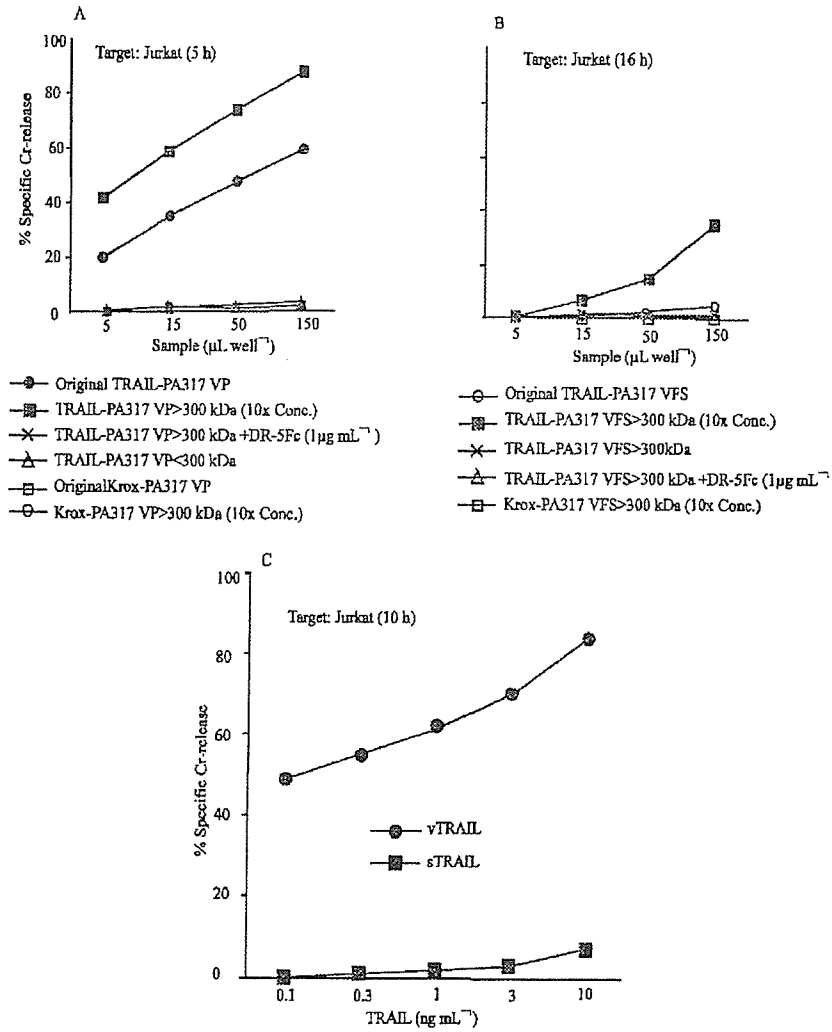

Fig. 3: Cytotoxicity of VP and VFS obtained from culture supernatants of TRAIL-PA317 and Krox-PA317 cells.
A) Various amounts of VP were mixed with ^{51}Cr-labeled Jurkat target cells and cytotoxicity were determined 5 h after culture in the absence or presence of DR5-Fc
B) Various amounts of VFS were 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[40]. 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.

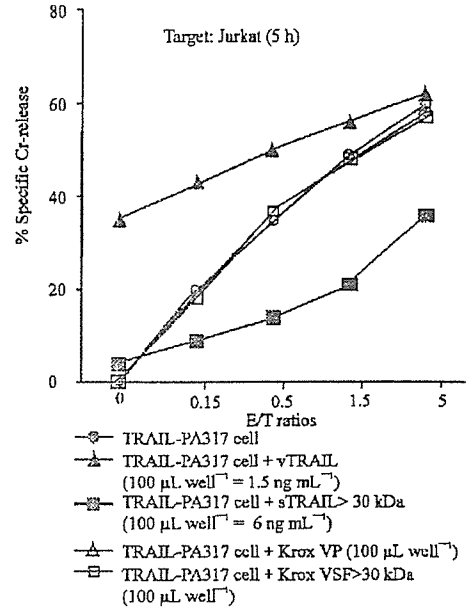

Fig. 4: Different effects of sTRAIL and vTRAIL on cell-mediated cytotoxicity.
Cell-mediated cytotoxic assays were carried out against ^{51}Cr-labeled Jurkat target cells with various E/T ratios of TRAIL-PA317 cells, in the absence or presence of sTRAIL or vTRAIL. Cytotoxicity was determined 5 h after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

Protective effect of pravastatin on vascular endothelium in patients with systemic sclerosis: a pilot study

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In patients with systemic sclerosis (SSc), endothelial cell activation or damage in small vessels is followed by intimal hyperplasia and peripheral ischaemia.[1] Raised levels of plasma von Willebrand factor (vWF), thrombomodulin (TM), and other endothelial/thrombotic markers have been found in patients with SSc[2–5]; vWF is increased in plasma from patients with SSc with diffuse skin involvement and with severe disease, presumably correlating with disease activity.[3] Besides a cholesterol lowering effect, statins exert non-lipid related mechanisms, so-called "pleiotropic effects", which may contribute to reducing risks of cardiovascular events. In this study the effect of a low dose pravastatin on markers of endothelial cell activation/injury and coagulation was investigated in patients with SSc.

This clinical trial was approved by the ethical committee of Hokkaido University Graduate School of Medicine, and all the patients gave their informed consent. The study comprised 18 patients with SSc without hyperlipidaemia (16 women, two men) attending the connective tissue disease clinic of Hokkaido University Hospital (mean (SD) age 52.3 (12.5)). Diagnosis of SSc was based on the American College of Rheumatology criteria.[6] Patients with morphoea were not included in the study.

Nine patients were treated with low dose (10 mg/day) pravastatin for 8 weeks and the other nine patients were not treated. No differences in age, sex, treatment or severity were found between the groups. Three patients from the pravastatin group and four from the non-treatment group were receiving low dose steroids. Ten disease-free subjects matched for age and sex served as healthy controls. Lipid profiles (total cholesterol, low density lipoprotein cholesterol, and high density lipoprotein cholesterol), vWF activity, TM, thrombin-antithrombin complex (TAT), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), and P-selectin were measured on days 0 and 56. Fisher's exact test was used for statistical analysis. Values of $p<0.05$ were regarded as significant.

Data before pravastatin treatment were compared between the patient groups and the healthy control group. vWF, TM, TAT, sICAM-1, and sVCAM-1 were significantly increased in the patient groups compared with the healthy controls (fig 1). Before pravastatin administration, no significant difference was found in any measures between the pravastatin group and non-pravastatin group. In the pravastatin group, plasma activity of vWF and TAT on day 56 were significantly reduced compared with those before treatment (table 1). vWF activity in the pravastatin group was significantly lower than in the non-treatment group ($p = 0.024$) on day 56. During the follow up period, low dose pravastatin did not have significant effect on the other measures, including lipid profiles.

vWF is synthesised by endothelial cells and its secretion is triggered by inflammatory/thrombotic mediators, thus establishing it as a marker of endothelial activation/injury. Although TAT is not a generally accepted marker of endothelial injury, it reflects thrombin generation induced by endothelial perturbation. Statins induce an increment in plasminogen activator synthesis and release, a decrease in plasminogen activator inhibitor-1 activity and endothelin-1 expression, and an up regulation of nitric oxide synthase, thus diminishing procoagulant activity and vasoconstriction.[7 8]

In this pilot study, we have shown that low dose pravastatin reduces plasma vWF activity and TAT without affecting lipid profiles and has a protective effect against perturbation of endothelial cells, leading to some beneficial effects in the affected patients.

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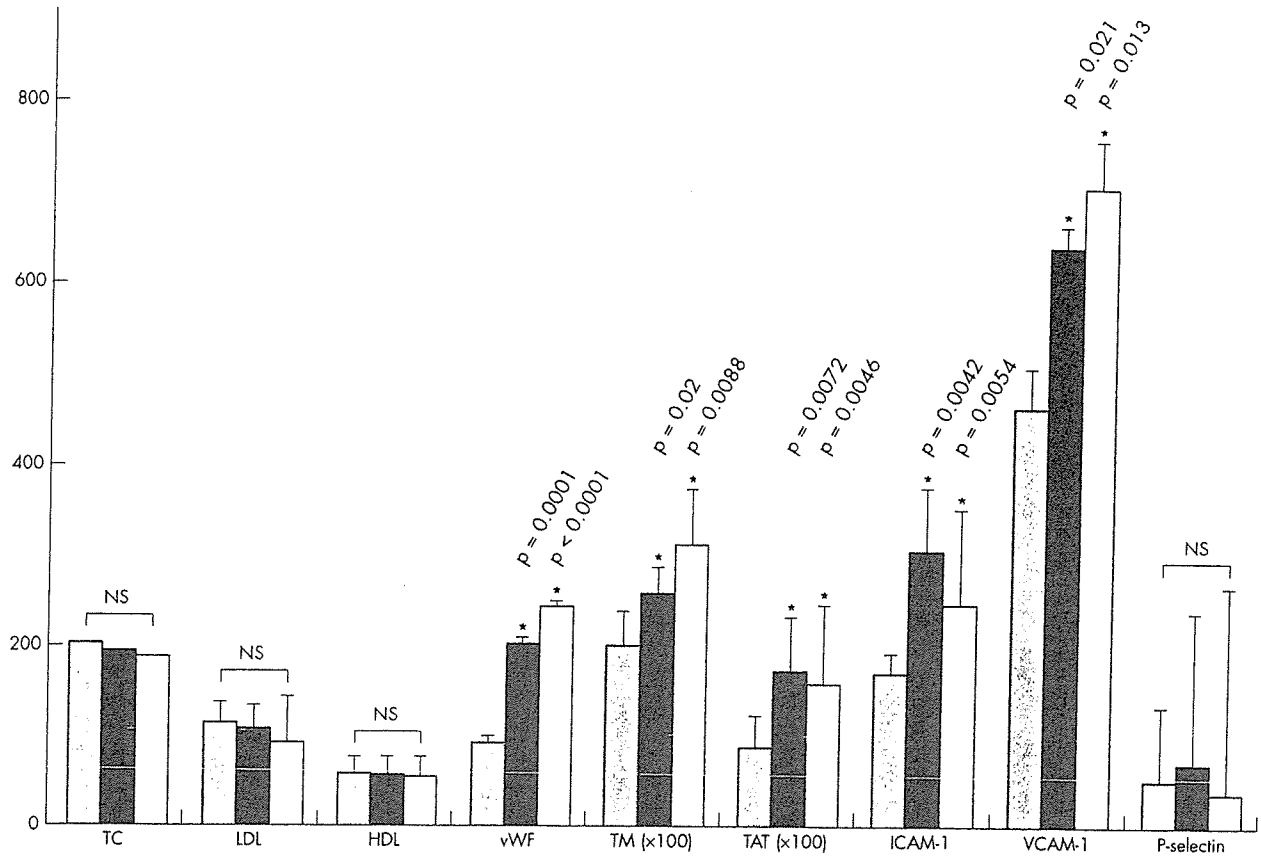

Figure 1 Laboratory data in healthy controls (grey bars), the pravastatin treated group (black bars), and the non-treated group (white bars) before pravastatin treatment. Asterisks indicate that the variable is significantly higher in the treatment group than in the healthy control group. TC, total cholesterol (mg/dl); LDL, low density lipoprotein cholesterol (mg/dl); HDL, high density lipoprotein cholesterol (mg/dl); vWF, von Willebrand factor activity (%); TM, thrombomodulin (fU/ml); TAT, thrombin-antithrombin complex (μg/l); ICAM-1, soluble intercellular adhesion molecule-1 (ng/ml); VCAM-1, soluble vascular cell adhesion molecule-1 (ng/ml); and P-selectin (ng/ml). Error bars indicate standard deviations.

Table 1 Laboratory data in patients with SSc at days 0 and 56

Data	Pravastatin	Day 0	Day 56	p Value
TC (mmol/l)	(+)	5.00 (0.65)	4.50 (1.10)	NS
	(−)	4.85 (1.35)	4.75 (1.20)	NS
LDL (mmol/l)	(+)	2.80 (0.55)	2.35 (1.00)	NS
	(−)	2.40 (0.60)	2.55 (1.10)	NS
HDL (mmol/l)	(+)	1.60 (0.50)	1.45 (0.35)	NS
	(−)	1.40 (0.35)	1.35 (0.25)	NS
vWF (%)	(+)	201.4 (30.9	163.8 (32.9)	0.0053
	(−)	226.9 (33.5	250.8 (66.1)	NS
TM (fU/ml)	(+)	2.6 (0.6)	2.5 (0.9)	NS
	(−)	3.1 (0.9)	3.2 (0.9)	NS
TAT (mg/l)	(+)	1.7 (0.7)	0.9 (0.3)	0.0052
	(−)	1.7 (1.0)	1.6 (1.3)	NS
ICAM-1 (ng/ml)	(+)	304.6 (90.2)	294.1 (72.0)	NS
	(−)	245.0 (51.1)	253.6 (48.2)	NS
VCAM-1 (ng/ml)	(+)	641.4 (191.4)	691.3 (244.8)	NS
	(−)	702.6 (228.2)	641.4 (191.4)	NS
P-selectin (ng/ml)	(+)	74.1 (37)	75.1 (32)	NS
	(−)	55.6 (35)	56.2 (36)	NS

NS, not significantly different when the values were compared between days 0 and 56.
There were no significant differences in the measures between the pravastatin group and the non-treatment group.
Cholesterol (mmol/l ÷ 0.025084 = mg/dl).