Apoptosis-inducing Membrane Vesicles
A NOVEL AGENT WITH UNIQUE PROPERTIES*

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The CD95 ligand (FasL) transmembrane protein is found on activated T cells and cells outside the immune system. A well-known turnover process of membrane FasL is mediated by matrix metalloproteinase, which generates soluble FasL (sFasL). Here, we demonstrate that membrane FasL turnover occurs effectively through the release of membrane vesicles. Quantitative analysis indicates that this process is as effective as sFasL release for FasL-3T3 cells but somewhat less effective for FasL-expressing T cells. The apoptosis-inducing membrane vesicles display unique properties not found in FasL-expressing cells and sFasL. Unlike sFasL, vesicle-associated FasL remained bioactive, killing the same panel of targets that are susceptible to FasL-expressing cells. In contrast to FasL-expressing T cells, FasL-mediated killing by vesicles do not involve LFA-1/ICAM interaction and do not depend on de novo protein synthesis. These observations indicate that the release of FasL-bearing vesicles contributes to the turnover of cell-associated FasL, but the impact of the bioactive FasL-expressing vesicles on the function of cell-associated FasL is different from that of sFasL.

CD95 (Fas) is a type I transmembrane protein expressed by a variety of nucleated cells (1). The physiological ligand for Fas (FasL) is a type II transmembrane protein expressed by activated T cells and non-T cells under a variety of conditions (2–11). Cross-linking of Fas induces cells to undergo apoptosis (12–14). This apoptosis pathway has been implicated in immune response regulation, self-tolerance, graft rejection, tumor escape of immune surveillance, and maintenance of the immune privileged sites (2–6, 15–28).

Regulation of FasL expression has been demonstrated at the transcriptional and post-transcriptional levels (4–6, 29–32).

Recent studies have suggested that the level of cell surface FasL is regulated by a mechanism involving matrix metalloproteinase (MMP) cleavage that releases from cells soluble FasL (sFasL) lacking the transmembrane and cytoplasmic domains (29–32). Compared with cell-associated FasL, sFasL is a relatively poor mediator of cytotoxicity. Indeed, under certain conditions, sFasL can actually inhibit the cytotoxicity of FasL-expressing cells (30, 31). Thus, sFasL release effectively down-regulates the function of cell-associated FasL. Here, we demonstrate that there is a second mechanism responsible for FasL turnover. This mechanism involves the release of cell surface FasL in the form of vesicles, which contain full-length FasL and are bioactive. Although the presence of FasL-bearing vesicles was implicated in previous studies (33, 34), the FasL expression level was so low that a quantitative study to determine its contribution to cell membrane FasL turnover was difficult. We have generated retroviral packaging cell lines that produce large amounts of FasL-expressing vesicles; however, it is not clear whether the retroviral packaging process has influenced the production of FasL membrane vesicles (35–37).

To determine whether normal FasL-expressing cells produce apoptosis-inducing vesicles, we generated FasL-expressing 3T3 cells that do not produce virus. We generated T cells that produce high levels of membrane FasL upon activation. In addition, we studied normal T cells for cell membrane FasL turnover upon activation. We demonstrated that these cells release of FasL-bearing vesicles capable of inducing apoptosis in target cells. Our quantitative analysis indicated that release of vesicles contributes to the turnover of cell-associated FasL, but the extent of contribution varies in different cell lines examined. Interestingly, the apoptosis-inducing vesicles display unique properties. In contrast to sFasL, FasL-bearing vesicles fully retained the target range of the FasL-expressing cells. However, there is a reduction of specific activity in comparison with cell-associated FasL. These observations suggest that release of vesicles is a physiologically significant process regarding both the turnover of cell-associated FasL and the impact on FasL function of the FasL-expressing cells.

EXPERIMENTAL PROCEDURES
Production of FasL-expressing Cell Lines—We obtained an hfasl cDNA construct from Dr. S. Nagata (Osaka University Medical School, Japan). A retroviral packaging cell line (hfasl-PA317) carrying the hfasl gene and control packaging cell line Krox-PA317 carrying the human krox were prepared according to the method of A. D. Miller (38) and have been described previously (36, 39). A vesicular stomatitis virus G glycoprotein pseudotyped, vector-packaging cell line carrying the hfasl gene was prepared according to the method described by Burns et al. (40). Various amounts of vector prepared from packaging cell lines were cultured with NIH-3T3 cells (2 × 10⁶ cells/well in a 24-well plate) in the presence of 6 μg/ml Polybrene (Sigma Chemical Co., St. Louis, MO). Medium was replaced 24 h later with fresh medium containing 0.75 mg/ml G418 (Life Technologies, Inc., Gaithersburg, MD).
Activation of T Cells for FasL Expression—Peripheral blood T (PBT) cells (5 × 10^6/ml) were obtained from healthy individuals and were activated by phorbol ester (PMA) (Sigma) and 0.5 μg/ml ionomycin (Sigma) for 24 h. Culture supernatants were collected for vesicle preparation. Activated T cells were purified by centrifugation through a Ficoll-Hypaque gradient centrifugation and then activated by 20 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) for 3–4 days with daily splits of culture. The IL-2–maintained T cells (2 × 10^6/ml) were purified by Ficoll-Hypaque gradient centrifugation and then activated by 20 ng/ml PMA (Sigma) and 0.5 μg/ml ionomycin (Sigma) for 24 h. Culture supernatants were collected for vesicle preparation. Activated T cells were purified by centrifugation through a Ficoll-Hypaque gradient, washed, and then examined for FasL-mediated cytotoxicity in the presence of 6 mM EGTA and 3 mM MgCl_2 (42). The PMA plus ionomycin (P/I)-activation protocol was also used to prepare activated Jurkat T cells and P/L-2-hFasL-EL-4 T cells.

Preparation of Vesicles and sFasL—Adherent cells (80% confluence) were maintained in 150- or 25-mm Petri dishes in 25 ml of culture medium. Non-adherent cells were cultured at 10^6/ml. Cells were cultured for 24 h. The cell number harvested was ~25 × 10^6/ml dish. Supernatants were centrifuged at 13,000 rpm in a Sorvall RC-5B centrifuge (Newton, CT) at 5 °C for 30 min to remove cell debris. The cell-free supernatants were then centrifuged for 3 h at 5 °C at 25,000 rpm in a Beckman ultracentrifuge (Model L8 m-55, Beckman Coulter, Fullerton, CA) using an SW25 rotor. For P/I-treated cells, the vesicles were washed once with 25 ml of medium by ultracentrifugation. The vesicle-containing pellet (VP) was suspended with 1.5 ml of culture medium and passed through a 0.45-μm sterile filter before use. To prepare sFasL, cell-free supernatants were centrifuged at 25,000 rpm for 16 h and the top 10% volume (to avoid potential contamination of vesicles) was collected for analysis.

Quantification of FasL—Human FasL concentrations were determined using a capture ELISA kit (Oncogene, Boston, MA). This assay measures both sFasL and intact FasL, because the mAb used are specific to epitopes present on both sFasL and intact FasL. To measure cell-associated FasL, cells (10 × 10^6) were washed, resuspended, and then treated with antigen-extraction buffer that was provided with the kit. To standardize the effect of this treatment, antigen-extraction buffer was also added to the vesicle and sFasL preparations. All samples were diluted with sample dilution buffer (provided with the kit) and immediately assayed. Standard curves were generated with various molar concentrations of recombinant soluble FasL (rsFasL) provided with the kit. The molecular mass of the rsFasL is 35,000 Da. A different source of rsFasL, was obtained from Upstate Biotechnology Inc., Lake Placid, NY. This rsFasL is made of the extracellular region of FasL (from position 103 to 281) with a FLAG-tagged peptide, and it has a molecular mass of 35,000 Da as determined by SDS-polyacrylamide gel electrophoresis. Essentially identical standard curves were established with both standards. To determine the concentrations (nanomolar) or amounts (nanograms) of the physiologically derived sFasL on the full-length FasL, the molecular masses used for calculation were 27,000 and 40,000 Da, respectively (29).

Cytotoxicity Assays—Target cells were labeled with Na_2^{31}CrO_4 (PerkinElmer Life Sciences) as previously described (43). The effector samples included FasL-expressing cells, vesicle preparations, and sFasL preparations. Various targets were used to test the target range of these preparations. Target populations included LB27.4, A20, HA1.6 (a Fe^3+–reconstituted A20) (44), Jurkat, and WEHI-279 (kindly provided by Dr. D. Scott, American Red Cross, Rockville, MD). Various amounts of each sample were cultured with 2 × 10^4 target cells in a total of 0.2 ml in each well of a 96-well plate. In some experiments, inhibitors were added to the mixtures to determine their effect on cytotoxicity. Supernatants were removed at 5 h after culture and were determined with a gamma scintillation counter (LKB, Turku, Finland). Background release was determined by culturing target cells in the absence of test samples. Target cells, treated with 0.5% Nonidet P-40, were used to determine total release, which represented 100% cell death. Background release was routinely 6–15% of total release. Cytotoxicity is expressed as percent specific ^{31}Cr release, which is determined by the formula, 100% × (experimental release – background release)/total release – background release). Specific experimental conditions were described in detail under “Results.” All experiments were carried out in duplicate and conducted two times or more. The Fas-dependent nature of the cytotoxicity of cells, vesicle preparations, and sFasL has been rigorously demonstrated in previous studies (35–37).

RESULTS

Comparison of Cytotoxicity Mediated by Vesicles Prepared from Various FasL-Expressing Cell Lines—To study the bioactivity of FasL-bearing vesicles and sFasL, we transfected several tumor cell lines with a human fas gene. One FasL-expressing cell line is the hFasL-PA317 packaging cell line, which we have recently characterized (35–37). The second cell line is hFasL-3T3, which was derived by transfection with the vesicular stomatitis virus G glycoprotein pseudotyped vector. We also generated a hFasL-expressing EL-4 T cell line (pIL-2-hFasLEL-4) by transfection with an expression vector in which the hfasl gene is under the control of a 1.9-kb mouse IL-2 enhancer. This cell line was generated to determine the release of FasL-bearing vesicles during T cell activation, because activated T cells are the major source of FasL under physiological conditions of antigen stimulation. The fourth cell line was Jurkat T lymphoma, which has been reported to produce FasL vesicles (34). Finally, we prepared activated peripheral blood T cells, because they are the physiologically relevant cells that express FasL.

Both FasL-PA317 cells and hFasL-3T3 cells constitutively expressed potential cytotoxicity in a short-term 5-h cytotoxicity assay (Fig. 1a). In contrast, P/L-2-hFasLEL-4 cells were not constitutively cytotoxic. However, when stimulated by PMA plus ionomycin and then washed with medium, the activated P/L-2-hFasLEL-4 cells expressed strong FasL-mediated cytotoxicity (Fig. 1a). Optimal expression of cytotoxicity was observed when cells were treated for 2–24 h (data not shown). We used cells treated for 24 h so that their cell FasL turnover could be determined under the optimal and standard conditions of FasL expression. For comparison, activated human peripheral blood T cells (PB T) expressed only moderate cytotoxicity (Fig. 1a). The cytotoxicity of activated Jurkat T cells could only be detected in a 16-h assay (data not shown) but not in the 5-h assay.

The cytotoxicity of vesicles isolated from 24-h culture supernatants of each of the above cell lines was compared. Vesicles
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FasL protein levels of various cell lines, VP, and sFasL fractions

The levels of hFasL in various samples were determined in duplicate assays by ELISA as described under “Experimental Procedures.” The data expressed are based on 25 × 10^6 cells and VP or sFasL prepared from 25 ml of culture supernatants obtained after 24 h of culture of 25 × 10^6 cells. The data presented are averages of two to three experiments of similar results. The different lower limits of detection for cell FasL (<0.02 ng), vesicle FasL (<0.05 ng), and sFasL (<0.4 ng) are due to the dilution factors used to prepare samples for the ELISA and the variations of sample size.

| Cell line | II FasL | III Vesicle FasL | IV sFasL |
|-----------|---------|-----------------|---------|
| 3T3       | <0.02   | <0.05           | <0.4    |
| hFasL-3T3 | 616     | 1765            | 1178    |
| Krox-PA317| <0.02   | <0.05           | <0.4    |
| hFasL-PA317| 456    | 771             | 333     |
| pIL-2-hFasL-EL-4 | <0.02 | <0.05 | <0.4 |
| P/I-pIL-2-hFasL-EL-4 | 103 | 24 | 112 |
| Jurkat    | <0.02   | <0.05           | <0.4    |
| Human PBT | 0.7     | 0.5             | 2.1     |
| Human PBT | 5       | 0.3             | <0.4    |
| Jurkat    | 7       | 1.7             | 12      |

a Cells were stimulated with PMA (20 ng/ml) and ionomycin (0.5 μM) for 24 h.

b The PBT cells were generated by using recombinant IL-2 to expand PHA-activated T cells. These cells contained FasL, but little vesicle-associated FasL and sFasL were detected, suggesting that the turnover of cell-associated FasL requires further activation.

c Human PBT cells were activated with PHA for 3 days followed by expansion with rIL-2 for 3 days and then cultured in the presence of PMA and ionomycin for 24 h.

Prepared from hFasL-PA317 and hFasL-3T3 cell lines displayed strong cytotoxicity (Fig. 1b). Vesicles prepared from PBT or Jurkat cells displayed undetectable cytotoxicity (Fig. 1b). Interestingly, vesicles prepared from the P/I-activated pIL-2-hFasL-EL-4 cells, which expressed strong cytotoxicity, were only weakly cytotoxic. This is largely due to the absence of LFA-1/ICAM interactions between FasL-expressing vesicles and target cells (see below).

**FasL Protein Expression Levels Correlate with Cytotoxicity of FasL-expressing Vesicles**—A highly sensitive hFasL-specific ELISA was used to determine the protein levels among various vesicle preparations (Table I, column III). The specificity of the assay was demonstrated by the lack of detectable hFasL in vesicles prepared from 3T3 cells, Krox-PA317 cells, and pIL-2-hFasL-EL-4 cells. Remarkably, vesicles preparations from both the hFasL-PA317 and hFasL-3T3 cell lines contained a very high level of hFasL. Among various T cell lines tested, the P/I-activated pIL-2-hFasL-EL-4 cells produced the highest level of hFasL. In contrast, little FasL protein was detected in the vesicles prepared from activated PBT cells. Activated Jurkat cells produced the lowest level of vesicle FasL, barely above the detectable level of the ELISA. The data demonstrate a strong correlation between hFasL protein content and cytotoxicity of the vesicle preparations.

**Rapid Turnover of Cell-associated FasL**—It is important to note that factors such as copies of transfected gene and cell size contributed to the variable expression of cell-associated FasL, which affects the levels of FasL associated with vesicles. Therefore, the level of cell-associated FasL was also determined (Table I, column II). Both hFasL-PA317 and hFasL-3T3 cell lines contained a large amount of hFasL. Comparatively, only a moderate amount was present in the P/I-activated pIL-2-hFasL-EL-4 cells. The moderate FasL level expressed by the P/I-activated pIL-2-hFasL-EL-4 cells may account for its modest production of vesicle-associated FasL. It is interesting that the P/I-activated pIL-2-hFasL-EL-4 cells displayed a higher level of cell-mediated cytotoxicity than hFasL-3T3 cells (Fig. 3). Thus, in contrast to the results obtained from FasL-bearing vesicles, FasL protein expression levels of cells do not necessarily correlate with the cytotoxicity of FasL-expressing cells. This is due to the fact that the P/I-activated pIL-2-hFasL-EL-4 cells but not the hFasL-PA317 and hFasL-3T3 effectively utilized LFA-1/ICAM to facilitate cell-mediated killing (45, 46; see Fig. 4 below). The total FasL accumulated in the vesicles over the 24-h culture period was 1.7 and 2.9 times that of FasL constitutively expressed by hFasL-PA317 and hFasL-3T3 cells, respectively. In contrast, the total vesicle-associated FasL accumulated for activated pIL-2-hFasL-EL-4, PBT, and Jurkat was 23%, 24%, and 71%, respectively, that of the respective cell source. The data suggest that activated T cells may not release FasL-containing vesicles as efficiently as hFasL-PA317 and hFasL-3T3 cells.

Previous studies have shown that the MMP-mediated release of sFasL is an effective turnover mechanism of cell membrane FasL (29–32). To determine its contribution to FasL turnover relative to the release of FasL-containing vesicles, the sFasL accumulated in 24-h culture supernatants was determined (Table I, column IV). The data showed that the sFasL levels accumulated were high, but higher amounts of FasL were observed with vesicles in the culture supernatants of hFasL-PA317 cells and hFasL-3T3 cells, indicating that both mechanisms strongly contributed to FasL turnover. In contrast, a higher level of sFasL than vesicle-associated FasL was observed for the P/I-activated pIL-2-hFasL-EL-4 cells, PBT cells, and Jurkat cells, indicating that the release of sFasL contributed to FasL turnover in T cells more than FasL released as vesicles. Interestingly, IL-2 maintained PBT contained a modest amount of hFasL but did not produce detectable levels of vesicle-associated FasL and sFasL, suggesting that further activation, as demonstrated with P/I treatment, is required.

**Target Range of Vesicle-associated FasL**—Once released from cells, sFasL lost its activity against many targets that are sensitive to cell-associated FasL (30–32). The target range of vesicle-associated FasL was compared with sFasL using four targets that are sensitive to hFasL-PA317, hFasL-3T3, and P/I-activated pIL-2-hFasL-EL-4 T cells (data not shown). The comparison is based on the molar concentrations, which were calculated from the FasL levels determined by ELISA using 27,000 Da for sFasL and 40,000 Da for the full-length FasL. As shown in Fig. 2, vesicles prepared from hFasL-3T3 cells effectively killed A20, IIA1.6, Jurkat, and WEHI-279 targets. In contrast, sFasL killed only A20 but not the other three targets. It has been reported that few targets are sensitive to sFasL and many targets are resistant (30–32). The selective killing of A20 but not other targets by sFasL suggests that this killing is controlled by factors uniquely associate with A20 cells. Indeed, Jurkat and WEHI-279 that are more sensitive to vesicle-associated FasL than A20 are resistant to sFasL. The data indicated that vesicle-associated FasL but not sFasL retained the target range of FasL-expressing cells.

**Vesicle Preparations from Different FasL-expressing Cell Lines Display Identical Target Range and Specific Activity**—We prepared vesicles from three different cell lines (hFasL-PA317, hFasL-3T3, and P/I-activated pIL-2-hFasL-EL-4 T cells), measured their FasL protein levels, and tested them against three different targets to determine whether these FasL VP display identical target range and specific activity (Fig. 3). FasL VP prepared from these cell lines showed comparable cytotoxicity against A20, Jurkat, and WEHI-279 targets when the same level of FasL was assayed, i.e. they have the same target range and specific activity. The data indicate that vesicle-associated FasL with identical target range and specific activity.
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specific activity can be produced from different FasL-expressing cell lines.

The Power of Cell-associated FasL—We used various concentrations of FasL to compare the cytotoxicity of cell-associated FasL with vesicle-associated FasL (Fig. 4). We found that the cytotoxicity expressed by former was significantly stronger than the latter. The observation is valid because vesicle preparations from three different cell lines showed comparable cytotoxicity. The dramatic difference between vesicle-associated FasL and cell-associated FasL was observed not only for P/I-activated pIL-2-hFasL-EL-4 cells (Fig. 4, a and d) but also for hFasL-PA317 and hFasL-3T3 cell lines (Fig. 4, a and b). Three possible factors were considered. First, FasL-mediated cytotoxicity by cells involves cell interaction molecules, and the extent of this interaction varies among cell types. Second, cells may contain stored FasL, which are rapidly released to cell membrane upon activation (47). Third, the de novo synthesized FasL (during the 5-h cytotoxicity assay) may have provided an additional resource for cell-associated FasL that was not considered by the ELISA, which determines FasL expression on a fixed time point.

To address the role of cell interaction molecules in FasL-mediated cytotoxicity, we determined the ability of anti-LFA-1 mAb to block FasL-mediated cytotoxicity (Fig. 5). In contrast to control rat IgG, anti-LFA-1 mAb effectively inhibited the killing of P/I-activated pIL-2-hFasL-EL-4 cells. Under the same condition, killing by hFasL-3T3 cells was not inhibited. The data indicate the former but not the latter FasL-expressing cells effectively used LFA-1/ICAM interaction to facilitate the cytotoxicity. Indeed, P/I activation has been shown to effectively enhance LFA-1 and ICAM interaction between effector T cells and target cells (48). Interestingly, cytotoxicity of FasL VP, including those prepared from P/I-activated pIL-2-hFasL-EL-4 cells was not inhibited by anti-LFA-1 mAb under the same condition. The data suggest that FasL-bearing vesicles do not co-express LFA-1 and therefore display weaker specific cytotoxicity than cell-associated FasL. Thus, for cell-mediated cytotoxicity that depends on LFA-1/ICAM interaction, release of FasL as vesicles is an effective way to down-regulate the activity of cell-associated FasL.

To determine whether the de novo synthesis of FasL contributes to the 5-h cell-mediated cytotoxicity, we compared the cytotoxicity mediated by hFasL-3T3 cells and FasL VP in the presence of 20 µg/ml cycloheximide (Chx), which inhibited more than 98% de novo protein synthesis (49). The result demonstrated that Chx significantly inhibited the killing mediated by hFasL-3T3 cells (Fig. 6a). However, complete inhibition was not obtained even when the effector/target ratios were low, consistent with the pre-existing FasL on hFasL-3T3 cells. In contrast, Chx did not inhibit the killing by FasL-expressing vesicles, which only have pre-existing FasL and do not have the machinery for de novo protein synthesis (Fig. 6b). There was a slight enhancement of killing, which could be due to inhibition of anti-apoptotic molecules in the target cells. Significantly, Chx completely inhibited cell-mediated killing when added to pIL-2-hFasL-EL-4 cells 30 min prior to activation by P/I, indicating that the dose of Chx is enough to completely inhibit the de novo synthesis of FasL (Fig. 6c). The inhibition of FasL expression was confirmed by ELISA assay (data not shown).

FIG. 2. Quantitative comparison of cytotoxicity expressed by vesicle-associated FasL and sFasL prepared from hFasL-3T3 cells. Various concentrations of samples (nM) were examined for cytotoxicity against various targets in a 5-h assay. The targets tested are: (a) A20, (b) IIA1.6, (c) Jurkat, and (d) WEHI-279. Background release was less than 15% in all cases.

Previous studies have demonstrated that a major pathway of FasL turnover is the release of sFasL resulting from MMP digestion. Here, we showed that FasL-expressing cells also release cell membrane FasL as vesicles capable of inducing apoptosis of target cells. The biological significance of releasing cell membrane FasL as vesicles is unclear. This process could down-regulate membrane expression of FasL. On the other hand, FasL presented by vesicles could be functional and could have an important biological function (33–37). Using gene-transfected hFasL-PA317 and hFasL-3T3 cell lines and conducting quantitative analyses of FasL protein expression levels, we have shown that FasL is released from cells in the form of vesicles, and this process is as effective as the MMP-mediated release of sFasL. In contrast, FasL-expressing T cells release lower levels of FasL-containing vesicles and thus contribute modestly to cell FasL turnover. Unlike sFasL, which is unable to kill many of the targets sensitive to FasL-expressing cells, vesicle-associated FasL retained the ability to kill these targets. These observations suggest that the rapid release of FasL-bearing vesicles may serve a more complex role than just simply down regulating the cell-associated FasL as is the case of sFasL release.

Our analyses based on quantitative hFasL-specific ELISA demonstrated that release of FasL-bearing vesicles is as effective a turnover mechanism as sFasL release, because large amounts of FasL accumulated as vesicles in the supernatants.
of hFasL-PA317 and hFasL-3T3 cells. The FasL accumulated in vesicles was comparable to or slightly more than the amount of sFasL released. In contrast to hFasL-PA317 and hFasL-3T3 cell lines, the P/I-activated Jurkat, PBT, and pIL-2-hFasL-EL-4 T cells expressed variable levels of FasL from very low to moderate. Taking this factor into consideration, the FasL levels of vesicles generated by these activated T cells ranged from 23% to 71% of the cell-associated FasL (Table I), whereas the sFasL levels were significantly higher. This indicates that the release of FasL-bearing vesicles contributes modestly to the FasL turnover in activated T cells. The modest accumulation of FasL-bearing vesicles is not likely due to an overactive MMP that degrades the full-length FasL of vesicles, because the same high level of vesicle-associated FasL was obtained when hFasL-3T3 cells were cultured with activated pIL-2-hFasL-EL-4 cells (data not shown). In an earlier study, sFasL was shown to be the major species detected in culture supernatants of T lymphoma cells that overexpress hFasL (29). Our results obtained from three additional and different FasL-expressing T cell populations not only support the previous observation but also indicate that the release of vesicles contributes modestly to T cell FasL turnover. Whether different types of cells utilize different mechanisms, i.e. membrane shedding or secretion of exosomes (50), for the release of FasL-containing vesicles remains to be established.

T cell-mediated cytotoxicity depends on cell interaction forces. Antibodies against LFA-1 and ICAM are effective inhibitors of FasL-mediated cytotoxicity of T cells (45, 46). In the present study we showed that the FasL-mediated cytotoxicity of the P/I-activated pIL-2-hFasL-EL-4 cells but not hFasL-3T3 cells depended on LFA-1/ICAM interaction. Because of this cell interaction, the former cells are more potent than the latter cells even though their FasL is only one-sixth of the latter cells. In contrast to the P/I-activated pIL-2-hFasL-EL-4 cells, the cytotoxicity of vesicles prepared from the same cells was not inhibited by anti-LFA-1 mAb. Thus, release of FasL-containing vesicles could effectively down-regulate FasL function of T cells. Although we have clearly shown that vesicles contain FasL, whether or not LFA-1 could be released in the form of vesicles and whether a vesicle co-expresses both FasL and LFA-1 have not been firmly established.

One reason that sFasL release has been considered a down-
regulatory mechanism for cell-associated FasL is that sFasL is unable to kill many targets that are sensitive to the FasL-expressing cells. In addition, affinity-purified and concentrated FasL was shown to inhibit killing mediated by cell-associated FasL (30, 31). The FasL prepared from hFasL-3T3 cells also inhibited killing mediated by FasL-bearing vesicles or FasL-expressing cells. This loss and gain of function was not observed for FasL-bearing vesicles, which do not inhibit the killing mediated by FasL-expressing cells (data not shown). Moreover, vesicle-associated FasL retained the ability to kill a panel of targets that are sensitive to the FasL-expressing cells from which the vesicles were derived. This suggests that release of FasL-bearing vesicles may have a different biological function than the MMP-mediated release of sFasL. For example, certain tumor cells (25–27), cells in the immune privileged sites (24, 28), and cells outside the immune system (51–53) constitutively express FasL under specific conditions. Their FasL is likely maintained at a steady level despite continuous release of vesicles that bear FasL. The accumulated FasL-bearing vesicles could provide additional killing power and could function beyond the local area controlled by FasL-expressing cells. Active release of FasL-bearing vesicles may help FasL-expressing cells mediate its functions rather than the inhibition as observed with sFasL. FasL-bearing vesicles may have long-lasting apoptosis-inducing power, because their expression is no longer dependent on de novo protein synthesis. A recent study suggests sFasL bind to extracellular matrix and display an enhanced cytotoxicity against Jurkat target (54). However, the loss of cytotoxicity against various targets associated with sFasL release would significantly reduce the role of FasL as a general cytotoxic mediator in vivo. Given that multiple roles of FasL in the immune system have been demonstrated, the present study indicates that release of FasL-bearing vesicles, like sFasL release, is an important factor to consider because of its distinct influence on the expression and function of cell-associated FasL. In this respect, our ongoing study has shown that FasL-bearing vesicles display two bioactivities in vivo, i.e. they act as a chemotactic factor for neutrophils when injected intraperitoneally, and they induce lethal fulminant hepatitis when injected intravenously.2

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