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CD95 (Fas) Ligand-Expressing Vesicles Display Antibody-Mediated, FcR-Dependent Enhancement of Cytotoxicity

Satoshi Jodo,* Andreas M. Hohlbaum, † Sheng Xiao,* Derek Chan,* David Strehlow,* David H. Sherr,‡ Ann Marshak-Rothstein,† and Shyr-Te Ju2*†

Bioactive Fas ligand (FasL)-expressing vesicles were generated (vesicle preparation, VP) from two cell lines overexpressing FasL. The effect of NOK-1 anti-FasL mAb (mouse IgG1) on the cytotoxicity of FasL VP against various targets was determined. At high concentrations (1–10 μg/ml), NOK-1 inhibited the cytotoxicity. By contrast, NOK-1 in the dose range of 1–100 ng/ml significantly enhanced cytotoxicity against the FcR+ LB27.4, M59, and LF+ targets, but not the FcR− Jurkat and K31H28 hybridoma T cell targets. The ability to enhance FasL VP-mediated cytotoxicity could be blocked by the FcR-specific mAb 2.4G2. Enhancement was also observed with FcR+ A2O B lymphoma but not with the FcR− A20 variant. Enhancement of FasL VP cytotoxicity was observed with five IgG anti-FasL mAbs, but not with an IgM anti-FasL mAb. Inhibition was observed with high doses of all mAb except the IgG anti-FasL mAb G247-4, which is specific to a segment outside the FasL binding site. Interestingly, under identical conditions but in the presence of 2.4G2, G247-4 inhibited the cytotoxicity of FasL VP. In addition, G247-4 inhibited the FasL VP-mediated killing of FcR− Jurkat. The data demonstrate that FasL-expressing bioactive vesicles display a property heretofore unknown in bioactive agents that express FasL-mediated cytotoxicity. The mechanism of the Ab-mediated, FcR-dependent enhancement of cytotoxicity of bioactive vesicles and its physiological significance are discussed. The Journal of Immunology, 2000, 165: 5487–5494.

The CD95 (Fas) ligand (FasL) is a type II transmembrane protein and a member of the TNF superfamily (1, 2). FasL is expressed on various types of cells including activated T cells (1, 2). When it interacts with Fas-expressing cells, cell-associated FasL cross-links Fas, and the subsequent aggregation of Fas activates an apoptotic program leading to the death of the Fas-expressing cells (3, 4). In addition to cell-associated FasL, a soluble form of FasL (sFasL) is produced as a result of metallo-proteinase digestion (5–7). The sFasL has a limited target range. Under certain experimental conditions, sFasL can be inhibitory to cell-associated FasL (6, 7).

FasL-expressing cells also produce microvesicles (FasL vesicle preparation (VP)) that bear FasL and display FasL function (7, 8–10). We have recently shown that the FasL VP prepared from hFasL-PA317 cells (a retroviral packaging cell line for the human FasL gene) are highly cytotoxic and capable of killing sensitive targets within a 5-h time period (9, 10). In contrast to cell-associated FasL and sFasL, the FasL VP-mediated killing of the B lymphoma target LB27.4 can be enhanced by low doses of anti-FasL mAb (NOK-1, mouse IgG1), whereas high doses of NOK-1 inhibit the activity of all three forms of FasL (10). Cross-linking of FasL VP by a low dose of NOK-1 could work by generating complexes that consist of multiple bioactive vesicles. Such complexes could interact more effectively with the target cells. Alternatively, FcR+ expressed by the LB27.4 cells could bind the NOK-1/FasL VP complexes and increase the extent of interaction between FasL VP and target cells. The latter explanation implies that the enhancement of FasL VP-mediated cytotoxicity depends on the subclass of anti-FasL mAb and the FcR expression level of the targets. Here, we examined six anti-FasL mAbs, seven target cell lines, and FasL VP prepared from cell lines expressing either human (h) FasL or murine (m) FasL. The data presented herein establish that an FcR-dependent mechanism is responsible for the anti-FasL-mediated enhancement of FasL VP cytotoxicity. The anti-FasL mAb-mediated, FcR-dependent enhancement of FasL VP cytotoxicity represents a novel form of cytotoxicity previously unknown in the immune system. The significance, implications, and use of the novel cytotoxic mechanism are discussed.

Materials and Methods

Production of FasL-expressing cell lines of hFasL-PA317, N2-mFasL, and L5-mFasL

A retroviral packaging cell line carrying the hFasL gene (hFasL-PA317) was prepared according to the method described by A. D. Miller (11). The hFasL cDNA (provided by Dr. S. Nagata, Osaka University Medical School, Japan) was cloned into pLXSN (A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA; accession no. M28248), and the construct was transfected into PES01 cells (obtained from A. D. Miller) with lipofectamine. The virus-laden supernatant was used to infect PA317 cells (American Type Culture Collection (ATCC), Manassas, VA) for 24 h followed by selection with G418 (0.4 mg/ml)-containing culture medium.
Six cloned G418-resistant cell lines were derived. One clone was expanded and used in this study. A similarly prepared packaging cell line carrying the human krox gene (krox-PA317) was used as control throughout the study. The N2-mFasL cell line was prepared by electroporating the Neuro-2a tumor cell line (ATCC) with a mouse FasL gene whose metalloproteinase-sensitive site had been deleted (13). We tested 20 G418 (0.8 mg/ml)-resistant clones and found 19 of them to express strong cell-mediated cytotoxicity. A clone was expanded for use in this study. The L5-mFasL cell line, derived by transfecting L5178Y (ATCC) cells with the same mouse FasL gene, has also been described (13).

**FasL VP**

Cloned hFasL-PA317 and N2-mFasL cells were maintained in a 150 mm × 25 mm culture dish in 30 ml of G418-containing culture medium. When the cells reached ~70% confluence, fresh culture medium without G418 was used to replace the G418-containing medium. Supernatants were collected 48 h later and centrifuged at 13,000 rpm in a Sorvall Superspeed centrifuge (Newton, CT) at 5°C for 30 min to remove cell debris. To generate human FasL VP, the cell-free supernatants were centrifuged for 3 h at 5°C at 25,000 rpm in a Beckman ultracentrifuge (Beckman Coulter, Fullerton, CA) using an SW25 rotor. The pellet was suspended with culture medium to 7% of the original volume and passed through a 0.45-μm sterile filter. Less than 10% of cytotoxic activity was lost after the filtration, which also defines the upper size limit of the bioactive vesicles. To generate human sFasL, the cell-free supernatants were centrifuged for 18 h at 5°C at 25,000 rpm in a Beckman ultracentrifuge using an SW25 rotor, and the top 25% of the supernatant was collected. The biologic activities and the physiochemical properties of FasL VP and sFasL have been characterized in previous studies (9, 10). Both human FasL VP and human sFasL express cytotoxicity against LB27.4 target cells. The FasL VP have a density of 1.14–1.16 g/ml, contain full-length FasL, and are retained by a filter (Centriprep 500; Millipore, Burlington, MA) that excludes particles with a molecular mass less than 50 kDa (Centriprep-50; Millipore). A previous study has shown that some sFasL exist as aggregates of 300 kDa (14).

The same procedures were used to prepare FasL VP from N2-mFasL cells that overexpress mouse FasL lacking the metalloproteinase-sensitive site. Following ultracentrifugation, the murine FasL VP displayed strong FasL-specific cytotoxicity, and full-length FasL could be detected by Western blot using polyclonal rabbit anti-mouse FasL Abs. Cytotoxic activity was not detected in the supernatant obtained following ultracentrifugation. In addition, sFasL was not detected in the supernatant by Western blot analysis. As described in a previous study (13), Western blot analysis detected sFasL in supernatants that were prepared from a cell line overexpressing the wild-type FasL (data not shown).

**Cytotoxicity assays**

Target cells, LB27.4 (B lymphoma hybridoma; ATCC), LF1° (T lymphoma; Ref. 15), K31H28 T cell hybridoma (K3; Ref. 16), M59 (macrophage hybridoma; Ref. 17), FcR° A20, FcR° A20 (kindly provided by Dr. C. Janeway, Yale University, New Haven CT; Ref. 18), or Jurkat (T lymphoma, obtained form Dr. R. Tepper, Massachussetts General Hospital, Boston, MA) were labeled with Na251CrO4 as previously described (19). Various amounts of the FasL VP were cultured with 2 × 102 target cells, in a total of 0.2 ml in each well of a 96-well plate. At 5 or 16 h after culture, supernatants were removed and counted with a gamma-scintillation counter (LKB, Turku, Finland). Use of the 5-h and 16-h cytotoxicity assays was based on the sensitivity of the various target populations under the specific assay conditions. Target cells cultured in the absence of FasL VP were used as background release. Target cells lysed by 0.5% Nonidet P-40 were used as total release, which represents 100% cell death. Cytotoxicity was expressed as percentage specific 51Cr release according to the formula: 100 × (experimental release − background release)/total release − background release). The effect of anti-FasL mAb was studied by adding various dilutions of the mAb at the beginning of culture. The anti-human FasL mAb tested were NOK-1 (mouse IgG1; PharMingen, San Diego, CA), NOK-2 (mouse IgG2a; PharMingen), NOK-3 (mouse IgM; provided by K. Ozeremenko) (5), G242 anti-FasL mAb (mouse IgG1; PharMingen), and Alf-1.2 (mouse IgG; provided by D. Kaplan, Case Western Reserve University, Cleveland, OH) (20). The isotype of Alf-1.2 has not been established. The anti-mouse FasL mAb Kay-10 was also used in these studies (mouse IgG2b; PharMingen) (21). Normal mouse isotypes were used as controls. The preparation of Fas-IgG1, a mouse Fas and human IgG1 fusion protein, has been described (16). To determine the role of mouse FcR, 2.4G2 anti-FcR mAb (rat IgG2b; PharMingen) (22) or normal rat IgG2b (PharMingen) control was added together with the anti-FasL mAb at the beginning of culture. None of these Abs display detectable cytotoxicity against various targets used in this study. All experiments were conducted in duplicate and conducted two times or more.

**FcR expression**

Cell surface FcR expression on target cells was determined by flow cytometry using biotin-conjugated 2.4G2 mAb. Cell-bound 2.4G2 mAb was measured with PE-streptavidin. Biotin-conjugated rat IgG2b was used as a specificity control.

**Results**

Enhancement but not inhibition of FasL VP-mediated cytotoxicity is target cell dependent

We determined the ability of NOK-1 anti-human FasL mAb to modulate the cytotoxicity mediated by cell-associated human FasL, FasL VP, and sFasL prepared from the hFasL-PA317 cell line. Similar preparations from the control krox-PA317 cell line were used to establish the specificity of these reagents (9, 10). Five targets (LB27.4, LF1°, M59, Jurkat, and K3) frequently used in our laboratory were compared. We first tested the effect of NOK-1 on FasL VP cytotoxicity (Fig. 1). For each target we used a concentration of FasL VP that induced between 40 and 60% specific lysis of that target for the analysis. In the presence of high concentrations of NOK-1 (1–10 μg/ml), target death induced by FasL VP was strongly inhibited. As the NOK-1 concentration decreased, a dose-dependent reduction of inhibition was observed. Interestingly, as the concentration decreased further, a significant enhancement of killing was observed with LB27.4, LF1°, and M59, but not with Jurkat and K3 target cells. The latter observation indicates that cross-linking of FasL VP by NOK-1 is not sufficient to enhance cytotoxicity. The “bell-shaped” enhancement response was based on the sensitivity of the various target populations under the specific assay conditions. Target cells cultured in the absence of FasL VP were used as background release. Target cells lysed by 0.5% Nonidet P-40 were used as total release, which represents 100% cell death. Cytotoxicity was assessed in either a 5- or 16-h cytotoxicity assay, as dictated by target sensitivity to FasL VP. FasL VP was used at a concentration capable of inducing between 40 and 60% specific lysis of each target population. Various amounts of NOK-1 mAb were added at the beginning of the cytotoxicity assays. Mouse Ig control did not affect the cytotoxicity in all cases (not shown for clarity purposes). All assays were conducted in duplicate. All experiments were conducted between 3 and 10 times with similar results.
was observed in a dose range between 1 and 100 ng/ml. Under identical conditions, no effect on cytotoxicity was observed with isotype control Abs (not shown).

We have also prepared FasL VP from N2-mFasL and L5-mFasL cell lines that expresses a form of mouse FasL in which the metalloproteinase-sensitive site has been deleted (13). The cytotoxicity of FasL VP and supernatant (sup.) following ultracentrifugation was prepared from N2-mFasL and L5-mFasL cells as described in Materials and Methods. Cytotoxicity of each preparation was assessed on LB27.4 target for cell-associated FasL (a), supernatants (b), and FasL VP (c). In d, the ability of Kay-10 anti-FasL mAb to modulate the cytotoxicity of FasL VP prepared from N2-mFasL was assessed on LB27.4 and Jurkat targets.

Anti-FasL Ab-mediated enhancement of cytotoxicity is unique to FasL VP

In an early study, we examined the ability of NOK-1 to modulate the cytotoxicity of cell-associated FasL and sFasL against LB27.4
target cells (10). We found that 1–100 ng/ml NOK-1 weakly enhanced the cytotoxicity of hFasL-PA317 cells. A similar result was obtained with N2-mFasL (data not shown). Because both cell lines efficiently produce FasL-expressing bioactive vesicles, the weak enhancement may not be the property of cell-associated FasL. We have examined a panel of FasL-expressing cell lines and found two (hFasL-3T3 and L5-mFasL) that did not produce a significant level of FasL VP during the 5-h cytotoxicity assay. Therefore, the ability of NOK-1 and Kay-10 to modulate the cytotoxic activity of hFasL-3T3 and L5-mFasL was determined (Fig. 3). The results were similar in both cases. Inhibition of cytotoxicity was observed with mAb concentration between 1 and 10 μg/ml. In contrast to the enhancement observed with FasL VP, the anti-FasL mAb, in a range between 1 and 100 ng/ml, did not enhance the cell-mediated cytotoxicity against LB27.4 target cells. In addition, as previously described, inhibition (at the high dose range) but no enhancement (at the low dose range) of human sFasL cytotoxicity was observed with NOK-1 mAb (Fig. 3). These results indicate that only FasL VP have the capacity to work in concert with anti-FasL mAb and result in enhancement of cytotoxicity.

Anti-FasL Ab-mediated enhancement of FasL VP cytotoxicity depends on FcR expression on targets

Because previous studies have shown that Jurkat cells do not express the FcR (23), we hypothesized that the enhancement of FasL VP cytotoxicity by anti-FasL mAb was mediated by Fc/FcR interactions that focus bioactive vesicles on the target cells. Whether the enhancement of cytotoxicity correlated with target FcR expression was determined by fluorescent staining using the FITC-conjugated 2.4G2 anti-FcR mAb. The results showed a specific and strong staining of LB27.4, LF⁺, and M59 cells (target populations that exhibit “enhanceable cytotoxicity”). In contrast, staining of K3 cells, the “nonenhanceable target population,” was extremely weak (Fig. 4a). To further demonstrate that the enhancement of FasL VP cytotoxicity depends on FcR expression, we compared the effect of NOK-1 mAb on the cytotoxicity of FasL VP against FcR⁺ A20 and FcR⁻ A20 target populations. As shown in Fig. 4b, FasL VP killed both targets, and the sensitivity of the two targets was comparable. In the presence of 10 ng/ml NOK-1 mAb, killing of FcR⁺ A20 was significantly enhanced. The enhancement was observed over a wide range of the FasL VP concentrations examined. In contrast, killing of the FcR⁻ A20 target by FasL VP was inhibited (Fig. 4b). We also determined the effect of various doses of NOK-1 mAb on FasL VP cytotoxicity. Although a dose-dependent inhibition was observed with the FcR⁻ A20 target, the killing of FcR⁺ A20 was enhanced by low doses of NOK-1 mAb and inhibited by high doses of NOK-1 mAb (Fig. 4c).

Enhancement but not inhibition of cytotoxicity is blocked by 2.4G2 anti-FcR mAb

To directly establish the role of target FcR in the enhancement of FasL VP-mediated cytotoxicity, we used the 2.4G2 anti-FcR mAb to determine whether enhancement of cytotoxicity could be blocked (Fig. 5). As shown in Fig. 5a, 10 ng/ml of NOK-1 mAb increased the FasL VP-mediated cytotoxicity against ⁵¹Cr-labeled LB27.4 cells from 19 to 72%. The presence of 2.4G2 mAb inhibited this enhancement of cytotoxicity in a dose-dependent manner. The enhancement was completely blocked by 100 ng/ml of 2.4G2 mAb, while a significant blocking was still observed with 2.4G2 mAb at a concentration of 10 ng/ml. No effect was observed with a normal rat IgG2b control mAb. Next, we determined the ability of various doses of NOK-1 to modulate the cytotoxicity of FasL VP in the presence of excess 2.4G2 mAb (500 ng/ml). The results show that the enhancing effect of NOK-1 mAb was completely inhibited by 2.4G2 anti-FcR mAb at 10 μg/ml, while a partial inhibition was observed with a low dose of 2.4G2 mAb at 1 μg/ml. The results indicate that the enhancement of FasL VP cytotoxicity by anti-FasL mAb is dependent on FcR expression on targets.

**FIGURE 4.** Enhancement of FasL VP-mediated cytotoxicity depends on target FcR expression. a. Various targets were stained with biotinylated 2.4G2 anti-FcR mAb (bold line) or control IgG2b (light line) followed by PE-conjugated streptavidin (FL2). Stained cells were analyzed with a FACScan flow cytometer. The experiments were repeated twice with similar results. b. Various doses of FasL VP were used to kill FcR⁺ A20 and FcR⁻ A20 targets in the presence or absence of NOK-1 mAb (10 ng/ml). c. The ability of various doses of NOK-1 mAb to modulate FasL VP cytotoxicity is compared between the FcR⁺ A20 and FcR⁻ A20 targets.
blocked, so that the entire peak of enhancement was eliminated. In contrast, the ability of NOK-1 mAb to inhibit cytotoxicity was not blocked (Fig. 5b). In addition to eliminating the enhancing effect of NOK-1 mAb, 2.4G2 appeared to facilitate the ability of NOK-1 mAb to inhibit cytotoxicity. This is shown by the ability of NOK-1 in the low dose range (1–100 ng/ml) to effectively inhibit cytotoxicity in the presence of 2.4G2 mAb.

**Influence of anti-FasL Ab subclasses and specificity on FasL VP-mediated cytotoxicity**

We tested a panel of anti-FasL mAb to determine the effect of anti-FasL mAb subclass and specificity on FasL VP cytotoxicity against the FcR1 LB27.4 target cells (Fig. 6). The same response pattern, i.e., enhancement with low concentrations and inhibition with high concentrations, was observed with NOK-2 and Alf-1.2 mAb, as had been seen with NOK-1 mAb (Fig. 1). An IgM anti-FasL mAb (NOK-3) did not enhance cytotoxicity at the low dose range but did inhibit cytotoxicity at the high dose range, consistent with the fact that the enhancement was dependent on the Fc of the IgG and not the IgM class of the FasL-specific mAb. Interestingly, the IgG1 mAb, G247-4, enhanced the FasL VP-mediated cytotoxicity even at the high dose range. Under identical conditions, G247-4 inhibited the FasL VP-mediated cytotoxicity against Jurkat target cells (Fig. 7a). Next, we determined whether the G247-4-mediated enhancement of FasL VP cytotoxicity against LB27.4 target cells could be blocked by 2.4G2 mAb. The presence of 2.4G2 not only eliminated the enhancement but also allowed G247-4 to inhibit the cytotoxicity against LB27.4 (Fig. 7b). The conversion from enhancement to inhibition was not observed with rat IgG2b control. The presence of 2.4G2 did not influence the ability of G247-4 to block the killing of Jurkat cells (Fig. 7a). It was noted that the ability of G247-4 to inhibit cytotoxicity was weaker than the other mAb (compare with Fig. 6). G247-4 binds to a region near the “self-association” site (14). Such binding may not be as effective as binding to the FasL binding site for blocking cytotoxicity. It is also possible that G247-4 has a low affinity for FasL. The data nevertheless demonstrate that both the subclass and the fine specificity of the anti-FasL mAb are important parameters for modulating the cytotoxicity of FasL VP.

**Effect of Fas-IgG1 fusion protein on FasL VP-mediated cytotoxicity**

The Fas moiety on Fas-IgG1 fusion protein is a dimer rather than the trimer of natural Fas. Therefore, it interacts with the FasL binding site with a lower affinity than cell-associated Fas. Fas-IgG1 could be used to determine unambiguously whether enhancement of FasL VP-mediated cytotoxicity could be achieved with a molecule that binds to FcR (through IgG1 Fc) and to the FasL binding site (through the Fas dimer). Therefore, we determined the effect of Fas-IgG1 on the cytotoxicity mediated by FasL VP against Jurkat and LB27.4 targets. The results shown in Fig. 8 indicate that Fas-IgG1 is a strong inhibitor of FasL VP when cytotoxicity was assessed on Jurkat target cells. In contrast, strong enhancement but no inhibition of cytotoxicity was observed with LB27.4 target cells. Like G247-4, enhancement of cytotoxicity was observed with high concentrations of Fas-IgG1. Moreover, 2.4G2, but not a control Ab, converted the Fas-IgG1-mediated enhancement of cytotoxicity to inhibition of cytotoxicity. Cytotoxicity was inhibited more than 50% with 100 ng/ml of Fas-IgG1, and complete inhibition of cytotoxicity was obtained with 1–10 µg/ml of Fas-IgG1. Taken together, the data demonstrate that FcR-dependent enhancement of FasL VP-mediated cytotoxicity significantly reduces the inhibitory ability of Fas-IgG1, even though the Fas-IgG1 interacts directly with FasL binding sites.
FIGURE 7. G247–4 anti-FasL mAb possesses the ability to inhibit FasL VP-mediated cytotoxicity. Various doses of G247–4 anti-FasL mAb were tested for their ability to modulate the cytotoxicity of FasL VP in a 5-h assay against Jurkat target cells (a) or LB27.4 target cells (b). The ability of G247–4 mAb to inhibit FasL-mediated cytotoxicity against LB27.4 was determined by adding 2.4G2 mAb to block FcR-mediated enhancement. Cytotoxicity assay was conducted in the absence or presence of 0.5 μg/ml of 2.4G2 mAb. Rat IgG2b was used as a specificity control.

Discussion
This study describes the novel effect of a panel of IgG anti-FasL mAb (NOK-1, NOK-2, Alf-1.2, and Kay-10) that can significantly enhance the cytotoxicity of FasL VP. We found that although most of these Abs inhibited FasL VP cytotoxic activity at high concentrations, they enhanced cytotoxicity when used at low concentrations with target cells that were FcR+ FasL+ cells. The enhancement could be specifically blocked with the 2.4G2 anti-Fc mAb. In addition, the enhancing effect of these mAb was observed with FasL VP but not with sFasL or cell-associated FasL. The data establish that the enhancement is mediated by the interactions between the Fc of the anti-FasL mAb and the FcR on the target cell surface. Presumably, this interaction focuses the FasL-expressing bioactive vesicles onto the target cells, thereby increasing the cytotoxicity of FasL VP.

The fact that enhancement of cytotoxicity was observed only with FasL VP and at a relatively low concentration of anti-FasL mAb (1–100 ng/ml) indicates that this phenomenon depends on the unique physical properties of the FasL VP and on a critical concentration of anti-FasL that facilitates association between FasL VP and the target population but does not block all FasL binding sites. The small size of FasL VP as microvesicles (<0.45 μm in diameter) in comparison to cells suggests that binding of a few anti-FasL mAb molecules would allow the focusing of FasL VP to LB27.4 through Fc/FcR binding. Increasing the concentration of the anti-FasL mAb blocks most or all FasL binding sites and results in inhibition of cytotoxicity.

There may be several possible reasons why mAb-mediated enhancement was not observed with cell-associated FasL. First, effector cells may use other interaction molecules such as integrins to facilitate their engagement with targets. Second, the interaction between vesicles and cells may rely more on Brownian movement. Brownian movement, coupled with the FcR-mediated focusing onto target cells, could provide faster and more effective Fas/FasL interaction in the latter case. Third, the amount of anti-FasL mAb needed to link an effector cell with a target may be so large that it is within the dose range that inhibits FasL VP cytotoxicity.

The results obtained with sFasL, are consistent with the idea that multivalency of the FasL, is a critical factor. It has been suggested that recombinant sFasL, consisting of the entire extracellular domain, could exist in a multimeric form as ~300-kDa aggregates, or about four trimeric units of FasL (14). We have shown that natural sFasL is heterogeneous and can be separated by filtration into two fractions, one with molecular masses between 100 and 500 kDa and the other between 50 and 100 kDa (10). Thus, sFasL may have limited valency. If so, it is not surprising that binding of NOK-1 anti-FasL mAb to natural sFasL could not effectively focus a significant level of “noninhibited” sFasL on LB27.4 to enhance cytotoxicity. In addition to NOK-1, inhibition but not enhancement of sFasL cytotoxicity was observed with NOK-2, NOK-3, and Alf-1.2 (data not shown). These results suggest that FasL VP particles are more multivalent than sFasL and display the appropriate physical form necessary to enhance cytotoxicity against FcR+ targets.

Once bound to FcR on target cells, there are two possible mechanisms whereby FasL VP might deliver its apoptotic signal. The focused FasL VP could either cross-link Fas on the very cell to which they attached or they could cross-link Fas on a neighboring bystander. In preliminary studies, enhancement by NOK-1 mAb was observed when cytotoxicity was conducted with 51Cr-labeled FcR+ A20 in the presence of either unlabeled FcR+ A20 or unlabeled FcR− A20 cells. Enhancement was not observed when cytotoxicity was conducted with 51Cr-labeled FcR− A20 in the presence of unlabeled FcR+ A20. These observations strongly suggest that NOK-1 mAb only enhances FasL VP-mediated cytotoxicity on target cells with functional FcR and that the bound FasL VP does not enhance killing of neighboring bystanders. FcR may mediate enhancement simply by binding the Fc of NOK-1 mAb and thereby increasing the effective concentration of FasL VP at the cell surface. Alternatively, cross-linking FcR may transduce a signal that sensitizes the Fas-mediated death pathway.
However, FcR mediates an inhibitory signal in B cells but an activational signal in T cells and macrophages (24), yet enhancement of cytotoxicity is observed in FcR⁺ target independent of this dichotomy. In addition, enhancement by NOK-1 mAb was not observed with sFasL and cell-associated FasL. Furthermore, a high dose of genistein (0.2 mM) known to inhibit protein tyrosine phosphorylation did not affect FasL VP cytotoxicity against LB27.4 target cells and had no effect on the enhancement of FasL VP cytotoxicity by NOK-1 mAb (data not shown).

Distinct protocols were used to generate the panel of anti-FasL mAb used in this study. The fine specificity of some of these mAb has been analyzed (14). NOK-1 and G247-4 are thought to recognize the FasL binding site (the C-terminal region) and a segment near the “self-association” site (amino acid residues 103 to 136, nonbinding site), respectively (14). The observation that high concentrations of G247-4 enhance the cytotoxicity of FasL VP suggests that the enhancement of FasL VP activity is dependent on the fine specificity or the affinity of the anti-FasL mAb. The latter interpretation was supported by similar results obtained with Fas-IgG1, which displays Fas as dimer and interacts with FasL binding site with a lower affinity than natural Fas. It should be noted, however, that both binding of G247-4 to the non-FasL binding site and binding of Fas-IgG1 to the FasL binding site resulted in inhibition of cytotoxicity if the FcR binding was blocked by 2.4G2 or if cytotoxicity was assayed on the FcR⁻ Jurkat cells. These data suggest that reagents specific for either binding site or nonbinding site epitopes of FasL possess the ability to enhance and inhibit cytotoxicity of FasL VP.

Although we could not demonstrate a significant enhancing effect of NOK-1 anti-FasL mAb on the cytotoxicity of cell-associated FasL, several observations suggest that FcR-mediated interactions reduce the ability of anti-FasL mAb to efficiently block cytotoxicity. First, the amount of the NOK-1 anti-FasL mAb needed to inhibit the killing of Jurkat cells was less than that needed to inhibit the killing of LB27.4 target (Figs. 1 and 3). Second, the amount of NOK-1 anti-FasL mAb needed to inhibit the killing of LB27.4 was significantly reduced in the presence of 2.4G2 (compare Fig. 1 and Fig. 5). Third, 2.4G2 mAb converted enhancement to inhibition when cytotoxicity assays were conducted in the presence of G247-4, i.e., the inhibition was observed in the dose range that enhanced cytotoxicity in the absence of 2.4G2 (Fig. 7b). Finally, we observed that the ability of NOK-1 anti-FasL mAb to inhibit the killing of LB27.4 by hFasL-3T3 was enhanced in the presence of 2.4G2 mAb (data not shown).

The demonstration of Ab-mediated, FcR-dependent enhancement of cytotoxicity represents a newly identified cytotoxic mechanism of the immune system. In addition to understanding the properties of the FasL-expressing bioactive vesicles, sFasL and cell-associated FasL, the enhancement of FasL VP-mediated cytotoxicity offers a very sensitive assay for detecting anti-FasL mAb. We have used this assay to test a panel of IgG anti-FasL mAb. Three mAb were able to enhance cytotoxicity at 0.01–1 ng/ml. There may be a physiological significance to this observation. It has been shown that a significant percentage (~30%) of sera from patients with systemic lupus erythematosus contains autoantibodies against FasL (25). It has also been shown that FasL bioactive vesicles can be produced from activated T cells (8), tumor cells, (26), and a number of transfected cell lines overexpressing FasL (9, 10). In view of the powerful enhancing ability of anti-FasL mAb (below nanogram/milliliter level), it is highly possible that anti-FasL-mediated enhancement of cytotoxicity against FcR⁺ targets could take place in patients with systemic lupus erythematosus or other autoimmune diseases.

Finally, our demonstration that the IgG anti-FasL mAb can focus FasL VP onto target cells through Fc/FcR interactions is reminiscent of several earlier studies of viral replication in FcR⁻ cells (27–29). Peiris et al. reported Ab-dependent enhancement of virus replication in FcR⁻ cells, and they demonstrated that the enhancement was blocked by anti-FcR Ab (27). Homsy et al. have shown that anti-HIV Ab enhance HIV infection in human cells, and the enhancement of HIV infection was mediated through FcR and not CD4 (28). It is also known that neutralized Dengue virus displays residual infectivity mediated through high-affinity IgG FcR (29). Our study extends these observations from viral replication to the expression of apoptotic function of the FasL VP. In this respect, the FcR-dependent, Ab-mediated interaction between retroviral particles and target cells may be exploited for the enhancement of gene transfer in FcR⁻ cells. Our study also raises concerns on the potential effects of IgG/FcR interactions in using IgG fusion proteins and mAb (e.g., anti-Fasl, anti-TNF-α, etc.) as modulating agents in general. More studies are needed to determine the physiological significance of this phenomenon and how to exploit the Ab-mediated, FcR-dependent enhancing effect on bioactive vesicles and virus for practical use.

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