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Bioactivities of Fas Ligand-Expressing Retroviral Particles

Satoshi Jodo, David Strehlow, and Shyr-Te Ju

Culture supernatants from retroviral packaging cells carrying the human Fas ligand (FasL) gene killed both human (Jurkat) and mouse (LB27.4) targets within 5 h of incubation. Cytotoxicity was found both in a fraction ≥500 kDa and a fraction between 50 and 500 kDa. Following ultracentrifugation, the activity in the ≥500-kDa fraction was concentrated in the pellet (FasL vector preparation (VP)), which was also infective when added to NIH-3T3 cells. Both Polybrene and poly-L-lysine significantly enhanced the cytotoxicity of FasL VP but not anti-Fas mAb, soluble FasL (sFasL), and cell-associated FasL. In the presence of Polybrene, FasL VP killed targets that are resistant to anti-Fas mAb and sFasL. The infectivity but not FasL cytotoxicity of FasL VP was sensitive to irradiation and heat shock. By contrast, cytotoxicity of FasL VP could be enhanced or inhibited depending on the doses of anti-FasL mAb. Interestingly, the infectivity of FasL VP was specifically enhanced by anti-FasL mAb, suggesting that a nonviral gene product could be used to regulate the behavior of the retroviral vector. Thus, in addition to expressing potent FasL cytotoxicity, the FasL VP exhibits unique properties heretofore not attributed to anti-Fas mAb, sFasL, and cell-associated FasL. Our study raises the possibility of using the retroviral gene-packing technology to make powerful, versatile, and regulatable bioactive vesicles expressing a predetermined function of the protein encoded by the target gene. The Journal of Immunology, 2000; 164: 5062–5069.

The retroviral vector for human FasL gene was produced according to the method of Miller et al. (16). The human FasL cDNA was cloned into pLXSN (American Type Culture Collection (ATCC), Manassas, VA), transfected into PE501 cells by Lipofectamine (Life Technologies-MRL, Gaithersburg, MD). PE501 cells were obtained from Dr. A. D. Miller (Fred Hutchinson Cancer Institute, Seattle, WA). After culturing the transiently transfected cells for 24 h, culture supernatants laden with ecotropic virus were obtained and used to infect the PA317 Moloney murine leukemia virus packaging cell line (PA317 MoMLV, also from A. D. Miller) to generate the amphotropic vectors. Clones were selected with culture medium containing G418 (0.4 mg/ml). Six clones were tested and all expressed strong FasL-mediated cytotoxicity (13–15). In most cases, the vesicles expressed extremely weak FasL-mediated cytotoxicity (13, 14). We have observed a potent FasL-mediated cytotoxicity in the retroviral vector prepared from a packaging cell line that carries the human FasL gene (29). The FasL vector preparation (FasL VP) is made of a family of particles including active vectors, inactive vectors, and vesicles. Because the packaging cell line over-expresses both the viral proteins and the FasL proteins, FasL VP is expected to express these proteins as well. Our initial study using Western blotting and immuno-adsorption has demonstrated the presence of FasL protein and Fas-specific cytotoxicity in the FasL VP, including the expression of FasL protein by the active vectors (29). The data suggest that the retroviral gene transfer technology can be used to produce bioactive vesicles displaying predetermined apoptotic function encoded by the target gene, i.e., fasL. In the present study, we demonstrate that FasL VP exhibited several distinctive properties heretofore not attributed to cell-associated FasL, sFasL, and anti-Fas mAb. We show that the gene transfer function of the FasL VP could be dissociated from the cytotoxicity mediated directly by FasL VP. Although these bioactivities of FasL VP could be dissociated, the infectivity of retroviral vector was significantly enhanced by the presence of anti-FasL mAb. Thus, by introducing a cell-associated protein into retroviral vector, it may be possible to regulate the vector function and behavior through the protein encoded by the nonviral gene. Our study has significant implication in gene transfer technology and gene therapy.

Materials and Methods

Production of retroviral packaging cell lines carrying the human FasL gene

The retroviral vector for human FasL gene was produced according to the method of Miller et al. (16). The human FasL cDNA expression construct was kindly provided by Dr. S. Nagata (Osaka University Medical Center, Osaka, Japan; Ref. 7). The human FasL (hFasL) cDNA was cloned into pLXSN (American Type Culture Collection (ATCC), Manassas, VA), transfected into PE501 cells by Lipofectamine (Life Technologies-MRL, Gaithersburg, MD). PE501 cells were obtained from Dr. A. D. Miller (Fred Hutchinson Cancer Institute, Seattle, WA). After culturing the transiently transfected cells for 24 h, culture supernatants laden with ecotropic virus were obtained and used to infect the PA317 Moloney murine leukemia virus packaging cell line (PA317 MoMLV, also from A. D. Miller) to generate the amphotropic vectors. Clones were selected with culture medium containing G418 (0.4 mg/ml). Clones were expanded using the same selection medium. Six clones were tested and all expressed strong FasL cytotoxicity. One line that expressed stronger cytotoxicity than the others was selected for further study. Experiments periodically conducted with other clones are consistent with these described herein. A similarly prepared packaging cell line carrying the human cKrox gene (Krox-PA317), which codes for the transcription factor Krox, was used as control throughout the study (17).
Preparation of vector-containing and vector-free fractions

The hFasL-PA317 and the Krox-PA317 cells were first cultured in the presence of G418 to 70% confluence. The medium was replaced with culture medium without G418 and cultured for 2 days. The supernatant was collected for further study, and the cells were used as effectors in cytotoxicity assays. The supernatant was centrifuged at 13,000 rpm for 30 min to remove cells and then centrifuged at 25,000 rpm for 17 h with a Beckman ultracentrifuge (Beckman, Fullerton, CA). The top 80% volume of the supernatant was collected as vector-free supernatant (VFS). The pellet, used as FasL VP, was obtained in parallel by a 3-h centrifugation under otherwise identical condition. The vector-containing pellet was suspended in medium and then sterilized by passing through a 0.45-micron filter. The sFasL was also obtained by fractionation of cell-free supernatant (CFS) using a Millipore Centricron-500 (Millipore, Bedford, MA) that filters through proteins with a m.w. <500 kDa. The molecular size of the sFasL fraction was estimated with Millipore Centricron-100 and Millipore Centricron-50, which separate proteins with a m.w. <100 kDa and 50 kDa, respectively.

FasL cytotoxicity

Seven tumor cell lines were used as targets for FasL cytotoxicity. The human T lymphoma cell line Jurkat expresses mouse Fas and is sensitive to FasL. LB27.4 is a mouse B lymphoma hybridoma (ATCC), which expresses mouse Fas and is sensitive to both mouse and human FasL (Ref. 18, and this study). LF+ and LF− targets are a pair of mouse T lymphomas that have been transfected with mouse Fas gene and antisense of Fas gene such that the former is sensitive and the latter is completely resistant to FasL (9). The Ipr-derived T cell hybridoma 5D5, which lacks Fas, was also used for specificity controls. The cell line Krox-PA317 (Millipore, Bedford, MA) that filters through proteins with a m.w. <500 kDa, K31H28 (21), both highly resistant to Jo2 anti-Fas mAb (PharMingen, San Diego, CA), were used to demonstrate the potency of FasL VP as a cytotoxic agent. Target cells (2 × 10^4), labeled with Na^131I, as previously described (22), were mixed with various cytotoxic reagents including activated 5D5 T cell hybridomas, which express cytotoxic FasL following incubation with plate-bound anti-CD3 mAb (19). After culture for 5 h, culture supernatants were collected and the radioactivity released to the supernatant was counted with a gamma-counter. Cells cultured in the absence of cytotoxic reagent were used as background controls. The radioactivity released by cells cultured in the presence of 0.5% Nonidet P-40 was used as a reference for total cell death. The cytotoxic activity, expressed as % specific 51Cr-release, was determined by the formula: [(cpm of sample − cpm of background)/(cpm of total release − cpm of background)] × 100%. In some experiments, the effect of enhancers or inhibitors on cytotoxicity was tested by the addition of inhibitors at the beginning of the culture. The enhancers tested were Polybrene and poly-l-lysine (Sigma, St. Louis, MO). The inhibitors tested were Fas-Ig (21) and NOK-1 anti-FasL mAb (PharMingen).

Effect of irradiation and heat shock on virus infectivity and FasL cytotoxicity

FasL VP was either treated with 10,000 rads by gamma-irradiation with a 137Cs irradiator or heat-shocked at various temperatures for 30 min in a water bath. Treated FasL VP was then tested for the ability to transfer G418. Culture supernatant was collected 48 h later, and cell debris was washed with medium and then cultured in medium containing 0.75 mg/ml of G418. The number of growing G418-resistant colonies was determined 10 days later by Giemsa stain (Sigma). The results were expressed as number of colonies per well.

Results

hFasL-PA317 expresses FasL-mediated cytotoxicity

The retroviral packaging cell line, hFasL-PA317, which carries the human FasL gene, was tested for FasL-mediated cytotoxicity against a panel of targets including both human (Jurkat) and mouse (LB27.4 and LF+ ) tumor cells. The parental PA317 and the control Krox-PA317 packaging cells were used for comparison. In addition, the mouse T cell hybridoma 5D5 (19), which lacks Fas and expresses FasL-mediated cytotoxicity upon stimulation with immobilized anti-CD3, was included as a positive control. The results indicate that hFasL-PA317 cells express potent cytotoxicity against Jurkat, LB27.4, and LF+ targets that express Fas (Fig. 1A). LF+ targets, which lack surface Fas, were resistant (Fig. 1A). In contrast, cytotoxicity was not observed with the Krox-PA317 cells. Soluble Fas-Ig but not a nonspecific human IgG1 myeloma protein inhibited the cytotoxicity against LB27.4 targets (Fig. 1B). The cytotoxicity expressed by hFasL-PA317 cells was comparable to or stronger than 5D5 T cells that were activated by plate-bound anti-CD3 (Fig. 1A). Other sensitive targets included human T lymphoma cells MOLT-4 and U266B1 myeloma (ATCC) (data not shown). These observations demonstrate that hFasL-PA317 cells express FasL-mediated cytotoxicity against a large panel of Fas+ targets of both human and murine origins.

FasL cytotoxicity expressed by cells, CFS, FasL VP, and VFS

We reasoned that there could be three components in the hFasL-PA317 cells capable of expressing FasL-mediated cytotoxicity. One is the FasL expressed on the cells, the second is sFasL released by metalloprotease cleavage, and the third is FasL associated with hFasL-PA317 cells. Krox-PA317 cells express FasL-mediated cytotoxicity upon stimulation with immobilized anti-CD3 mAb (19). The data demonstrate that under the defined experimental conditions, target cells were killed by FasL associated with hFasL-PA317 cells in a 96-well plate overnight. The adherent cells were washed with medium, and target cells were added. The mixtures were cultured for 5 h, and then the cytotoxicity was determined. Four replica plates were also made but without the addition of labeled target cells. Supernatants collected from one replica plate after a 5-h culture were mixed with the labeled LB27.4 target and cultured for an additional 5 h. There was little or no cytotoxicity detected with the supernatants. In contrast, strong killing of the same target cells was observed with the hFasL-PA317 cells in a manner that was dependent on E/T ratios (Fig. 2A). The data demonstrate that under the defined experimental conditions, target cells were killed by FasL associated with hFasL-PA317 and that, within a 5-h culture period, any cytotoxic component that may have been released was insufficient to account for the cytotoxicity of hFasL-PA317 cells.

To determine the presence of cell-free cytotoxic components, supernatants were generated from the remaining three replica plates after cells were cultured for 24, 48, and 72 h. There was a steady increase of cytotoxic activity in the culture supernatants, which was maximal at 48 h of culture. The cytotoxicity of the 72-h culture supernatant was lower, possibly due to exhaustion of nutrients and/or further digestion by the matrix metalloproteinase (Fig. 2B). Therefore, the FasL VP prepared from 48-h culture supernatants were used throughout the rest of this study.

For large quantity preparations, cells were cultured in large petri dishes (150 mm × 25 mm; Falcon, Lincoln Park, NY). At 70% confluence, culture medium was replaced with medium without G418. Culture supernatant was collected 48 h later, and cell debris
was removed by centrifugation at 13,000 rpm for 30 min using a Sorvall (Wilmington, DE) SS-34 rotor. The CFS collected was further centrifuged at 25,000 rpm for 17 h at 5°C using SW-30 rotor in a Beckmann ultracentrifuge. The top 80% of the supernatant was carefully removed and used as VFS. This preparation lacked the viral vector as determined by the inability of the supernatant to render the NIH-3T3 cells resistant to G418 through viral infection (Fig. 3A). The low (40%) recovery of the ability to infect NIH-3T3 cells was presumably due to retroviral inactivation during ultracentrifugation or loss during filtration. These preparations were also tested for FasL-mediated cytotoxicity (Fig. 3B). All fractions were able to kill LB27.4 targets in a 5-h assay. The total cytotoxic activity present in the VFS was about the same as the FasL VP. The combined cytotoxic activity of VFS and FasL VP accounts for 65–80% of the activity of the original CFS. It should be noted that the total lytic units present in the CFS is ~15% of that associated with hFasL-PA317 cells (Fig. 3 legend).

To further characterize the FasL activity, the CFS, the VFS, and the FasL VP were fractionated with Millipore Centricon-500, which retains components larger than 500 kDa. The results indicate that most of the cytotoxic activity in the VFS was present in the compartment that retained components larger than 500 kDa (Fig. 4B). A comparable level of cytotoxic activity was observed in both compartments when CFS was fractionated with Centricon-500 (Fig. 4C). However, the ability to transfer G418 resistance was detected only in the fraction containing components larger than 500 kDa (data not shown).
Dissociating FasL VP infectability from FasL-mediated cytotoxicity

Because FasL VP possesses the ability to transfet cells as well as to kill the targets, it is conceivable that an efficient FasL gene transfer could have caused or contributed to the expression of FasL cytotoxicity. We used three methods to rule out this possibility (Fig. 5). First, targets were treated with actinomycin D for 45 min and then examined for sensitivity to FasL VP. This treatment essentially inhibited to completion the gene transcription in target cells (18) but had no inhibitory effect on their sensitivity to FasL VP (Fig. 5A). Next, we irradiated FasL VP with 10,000 rads and then examined FasL cytotoxicity and the ability to transfer G418 resistance to NIH-3T3 cells. The results indicate that the gamma-irradiation inhibited 64% the ability of the viral vector to transfer G418 resistance, but had no effect on its ability to kill Fas1 target cells (Fig. 5B and C). The third method we tested was heat-shock treatment. FasL VP were treated for 30 min at temperature between 47°C and 75°C. FasL VP treated at 37°C was used as control. The results showed that treatments at temperature below 75°C had little effect on FasL VP cytotoxicity (Fig. 5D). A moderate inhibition was observed with FasL VP treated at 75°C. In sharp contrast, the heat-shock treatment completely eliminated the ability of the virus to infect NIH-3T3 cells and transfer of G418 resistance (Fig. 5E). The data clearly demonstrate that infection and FasL-mediated cytotoxicity could be dissociated and that FasL gene transfer is not required for the FasL VP to express FasL-mediated cytotoxicity.

Distinct properties of FasL VP

A well-established property of retroviral vector is the enhanced efficiency of gene transfer by the presence of Polybrene, presumably by increasing the contact between the viral vectors and host
Therefore, the effect of Polybrene on the FasL-mediated cytotoxicity by FasL VP was examined. The results showed that the cytotoxicity of FasL VP was significantly increased by Polybrene in a dose-dependent manner (Fig. 6A). Under the experimental conditions, the cytotoxicity of FasL VP was increased as the concentrations of Polybrene raised from 0 to 1.2 μg/ml. The optimal enhancement of cytotoxicity was observed with Polybrene concentrations ranged between 1.2 and 6 μg/ml. The enhancing activity of Polybrene was not observed with the cytotoxicity of VFS, hFasL-PA317, or Jo2 anti-Fas mAb (Fig. 6A). The ability of Polybrene to increase the cytotoxicity of FasL VP appears to be due to its multiple cationic characteristics because the same results
were obtained with poly-L-lysine (Fig. 6B). Similar to Polybrene, poly-L-lysine enhanced the cytotoxicity of FasL VP but not VFS, hFasL-PA317, or Jo2 anti-Fas mAb. The enhancement of cytotoxicity varies among different targets. At the extreme situation, targets (K31H28 T cell hybridoma and m659 hybridoma) that are resistant to high doses of Jo2 anti-Fas mAb and the FasL VP became sensitive to FasL VP when Polybrene was added to the culture (Fig. 7). Both K3 and m659 were moderately sensitive to hFasL-PA317, Polybrene did not enhance the killing (data not shown). Moreover, even in the presence of Polybrene, FasL VP-mediated cytotoxicity could not be against two Fas-negative targets, LF5 and SD5 (Fig. 7). These observations indicate that the Polybrene-enhanced cytotoxicity was mediated through Fas receptor, but the cytotoxicity of FasL VP can be regulated in a fashion distinct from that of sFasL, Jo2 anti-Fas mAb, and the membrane FasL associated with cells.

**Effect of anti-FasL mAb**

The anti-FasL mAb NOK-1 has been shown to inhibit FasL-mediated cytotoxicity (11); therefore, its effect on the FasL VP-mediated cytotoxicity and transfer of G418 resistance was determined. The FasL-PA317 cells, which express cell-associated FasL, and VFS, which contains sFasL, were included as controls. Polybrene was not used in this experiment. The results are shown in Fig. 8. When high doses of NOK-1 were used, the cytotoxicity of all three samples was strongly inhibited. However, when low doses of NOK-1 were used, the cytotoxicity of FasL VP, but not hFasL-PA317 or VFS, was significantly enhanced.

**Regulation of vector behavior through FasL associated with FasL VP**

The effect of NOK-1 on gene transfer was studied in parallel. The assays were conducted in the absence of Polybrene. The ability to infect NIH-3T3 was not affected by the presence of 0.01–1 µg/ml of NOK-1 anti-FasL mAb on FasL VP cytotoxicity. FasL VP and VFS were prepared from 48-h culture supernatant as described in text. FasL VP, VFS, and hFasL-PA317 cells, pretested and capable of killing 40–50% of the 51Cr-labeled LB27.4 cells in a 5-h assay, were used. Various concentrations of NOK-1 were added in the beginning of assay to determine its effect on cytotoxicity. Under identical condition, isotype control did not affect cytotoxicity (data not shown).
of NOK-1 (Fig. 9). Interestingly, the ability to transfer G418-resistant cells was significantly enhanced when infection was conducted in the presence of 2–10 µg/ml of NOK-1. No effect on infectivity was observed when experiments were conducted with an isotype control mAb. Under the experimental condition, the FasL VP did not induce death of NIH-3T3 cells in a 5-h cytotoxicity assay, suggesting that the enhancement was unlikely due to protection of cell death by NOK-1. The data are consistent with the interpretation that FasL vectors were cross-linked by NOK-1 and became more effective presumably because of increased interactions between the cross-linked vectors and cells. The results suggest strongly that the FasL of the vector preparation can be used as a target to regulate viral infectivity and gene transfer function.

**Discussion**

This study describes the unique properties of a novel biological agent, FasL VP, which is prepared from the hFasL-PA317 packaging cell line that carries the human FasL gene. FasL VP directly expresses FasL cytotoxicity. We have shown that the hFasL-PA317 cells expresses cytotoxicity mediated by cell-associated FasL, sFasL, and FasL VP. FasL VP enriched by ultrafiltration or ultracentrifugation directly expresses FasL-mediated cytotoxicity without the need for transferring the FasL gene to target cells. FasL VP, which contains concentrated vectors and vesicles, expresses highly potent FasL cytotoxic activity capable of killing targets that are resistant to anti-Fas mAb and sFasL. Both Polybrene and poly-L-lysine enhance the cytotoxicity of FasL VP but not sFasL or FasL on hFasL-PA317, whereas high doses inhibit the cytotoxicity of all three. Several anti-FasL mAbs have been shown to inhibit cell-mediated cytotoxicity (11). We have found four different mAbs to be able to enhance cytotoxicity of FasL VP (S. Jodo, unpublished observation). The extremely low concentration of the mAb (0.1–10 ng/ml) that was needed for enhancement is remarkable. It suggests that this enhancement is unique to FasL VP. The enhancement is consistent with the interpretation that a proper cross-linking of vectors by NOK-1 increases the interactions between vectors and target cells. However, additional factors must be involved because NOK-1 mAb recognizes both sFasL and FasL on hFasL-PA317 cells because their activity could be inhibited by a high dose of NOK-1 (Fig. 8). Yet enhancement of cytotoxicity by lower doses of NOK-1 was not observed.

The association of FasL cytotoxicity with the FasL VP also poses a unique situation with respect to gene transfer, which is the original purpose for generating such a vector. Because it kills FasL-expressing cells, the target range for FasL gene is limited to cells that either are more resistant to the FasL cytotoxicity or can generate FasL-resistance rapidly through selection. Consistent with the former possibility, we have been unable to generate G418-resistant cells by standard vector-mediated gene transfer experiments using LB27.4, Jurkat, and LF+ targets. By contrast, when NIH-3T3 and m059 cells were treated with FasL VP, G418-resistant cells were generated. In contrast to their parental cells (NIH-3T3 and m059), the G418-resistant cells express FasL cytotoxicity (indicating FasL VP contains functional vectors for FasL gene), greatly reduce cell surface Fas expression, and become highly resistant to FasL VP. The G418-resistant, FasL-expressing NIH-3T3 cells produce FasL-expressing vesicles but not viral particles (data because the retrovirus was generated from a packaging cell line, which carries the human FasL gene, it is conceivable that Fasspecific cytotoxicity is the result of gene transfer-induced expression of FasL in target cells, which in turn kills the Fas-expressing cells. Three pieces of evidence support the conclusion that the FasL VP directly expresses FasL-mediated cytotoxicity without the need for FasL gene transfer. First, inhibition of target cell transcription with actinomycin D did not inhibit the cytotoxicity of FasL VP. Second, both irradiation and heat-shock treatment inhibited the ability of the FasL VP to infect the host cells, which are more resistant to FasL (discussed below), but not its ability to kill sensitive targets. Third, full-length FasL protein was demonstrated in the FasL VP by Western blotting (29). However, under different conditions, FasL VP could transfer the FasL gene and to endow the recipient cells with FasL cytotoxicity (discussed below).

A unique property of FasL VP is its distinct response to polycationic molecules. Polybrene has been widely used to promote the efficiency of retroviral infection. Both Polybrene and poly-L-lysine enhance the cytotoxicity of FasL VP. The mechanism of the enhancement appears to be dependent on the interactions between FasL VP and target cells, which is increased by these polycationic molecules. The inability of sFasL to express higher cytotoxicity in the presence of Polybrene or poly-L-lysine is consistent with this interpretation. The inability of Polybrene to increase the cytotoxic activity of hFasL-PA317 suggests that the size of FasL-expressing membrane particles may be another influencing factor. It is possible that hFasL-PA317 cells are sufficiently large such that they are unable to be cross-linked by Polybrene. Another explanation is that cellular FasL are more efficient in cross-linking once cell (E:T) interaction begins because of the higher number of FasL and the fluidity of the cell membrane in comparison to the small retroviral particles.

An interesting observation is the ability of low concentrations of NOK-1 anti-Fas mAb to enhance the cytotoxicity of FasL VP but not sFasL or FasL on hFasL-PA317, whereas high doses inhibit the cytotoxicity of all three. Several anti-FasL mAbs have been shown to inhibit cell-mediated cytotoxicity (11). We have found four different mAbs to be able to enhance cytotoxicity of FasL VP (S. Jodo, unpublished observation). The extremely low concentration of the mAb (0.1–10 ng/ml) that was needed for enhancement is remarkable. It suggests that this enhancement is unique to FasL VP. The enhancement is consistent with the interpretation that a proper cross-linking of vectors by NOK-1 increases the interactions between vectors and target cells. However, additional factors must be involved because NOK-1 mAb recognizes both sFasL and FasL on hFasL-PA317 cells because their activity could be inhibited by a high dose of NOK-1 (Fig. 8). Yet enhancement of cytotoxicity by lower doses of NOK-1 was not observed.
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