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## Bioactivities of Fas Ligand-Expressing Retroviral Particles

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# Bioactivities of Fas Ligand-Expressing Retroviral Particles<sup>1</sup>

Satoshi Jodo, David Strehlow, and Shyr-Te Ju<sup>2</sup>

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  $\geq 500$  kDa and a fraction between 50 and 500 kDa. Following ultracentrifugation, the activity in the  $\geq 500$ -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-packaging 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.

**C**D95 (Fas) is a type I transmembrane protein expressed by a variety of nucleated cells (1–4). Cross-linking of Fas by anti-Fas mAb induces cells to undergo apoptosis (5, 6). Under a defined experimental condition, transplanted tumor cells expressing surface Fas can be eliminated by monoclonal anti-Fas Abs (5). Mice bearing a defective Fas gene fail to maintain self-tolerance and develop autoimmune lymphoproliferative disease (1–4). Thus, Fas is a cell surface molecule critically involved in the development of autoimmune diseases. The physiological ligand for Fas (FasL)<sup>3</sup> is a type II transmembrane protein, which is induced when T cells are strongly activated (7–10). Cells expressing FasL are cytotoxic to Fas<sup>+</sup> targets (9, 10). A soluble form of human FasL (sFasL), produced by metalloproteinase digestion, has also been identified, and it is cytotoxic against some Fas<sup>+</sup> targets (11, 12). However, sFasL is less potent and often counteractive against FasL expressed by activated T cells (12).

In addition to cell-associated FasL, sFasL, and anti-Fas mAb, bioactive vesicles expressing FasL has also been described (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 those 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).

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<sup>3</sup> Abbreviations used in this paper: FasL, Fas ligand; sFasL, soluble FasL; VP, vector preparation; hFasL, human FasL; VFS, vector-free supernatant; CFS, cell-free supernatant.

### 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 cell debris 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 Centricon-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 Centricon-100 and Millipore Centricon-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 human 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<sup>+</sup> and LF<sup>-</sup> 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 *lpr*-derived T cell hybridoma 5D5, which lacks Fas, was also used for specificity control (19). A macrophage hybridoma, m $\phi$ 59 (20), and a T cell hybridoma, 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 ( $2 \times 10^4$ ), labeled with Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> as previously described (22), were mixed with various cytotoxic reagents including activated 5D5 T cell hybridoma cells, 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 <sup>51</sup>Cr-release, was determined by the formula: [(cpm of sample - cpm of background)/(cpm of total release - cpm of background)]  $\times$  100%. In some experiments, the effect of enhancers or inhibitors on cytotoxicity was tested by the addition of inhibitors at the beginning of 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 <sup>137</sup>Cs 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 resistance to NIH-3T3 cells and the ability to kill Fas<sup>+</sup> targets. Both the FasL VP not subject to irradiation and the FasL VP incubated in a 37°C water bath were examined in parallel. The infection activity was determined by counting the number of growing G418-resistant colonies according to the method of Miller et al. (16). The results were expressed as total CFU per sample preparation. Cytotoxicity of each sample was determined as described above and expressed as lytic units per sample preparation. A lytic unit is defined as the amount of cytotoxic materials, volume, or cell number required to cause 20% specific lysis of LB27.4 target in the 5-h <sup>51</sup>Cr-release assay.

### Effect of NOK-1 anti-FasL mAb on FasL VP cytotoxicity and infectivity

The dose of FasL VP that induced 35–55% killing of LB27.4 in a 5-h assay was used to determine the effect of NOK-1 anti-FasL mAb (IgG1 isotype, PharMingen) on FasL VP cytotoxicity. The experiments were conducted by adding various doses of NOK-1 at the beginning of the assays. An irrelevant hybridoma protein of the IgG1 isotype was included as a control. The effect of NOK-1 on viral infectivity was determined as follows: We titrated the FasL VP to determine the amount capable of generating ~70 colonies in a well (6-well plate) plated with  $2 \times 10^4$  NIH-3T3 cells 1 day earlier. Various concentrations of NOK-1 or control isotype were cultured with FasL VP (50  $\mu$ l) during the 6 h of infection. Wells were washed three

times 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*

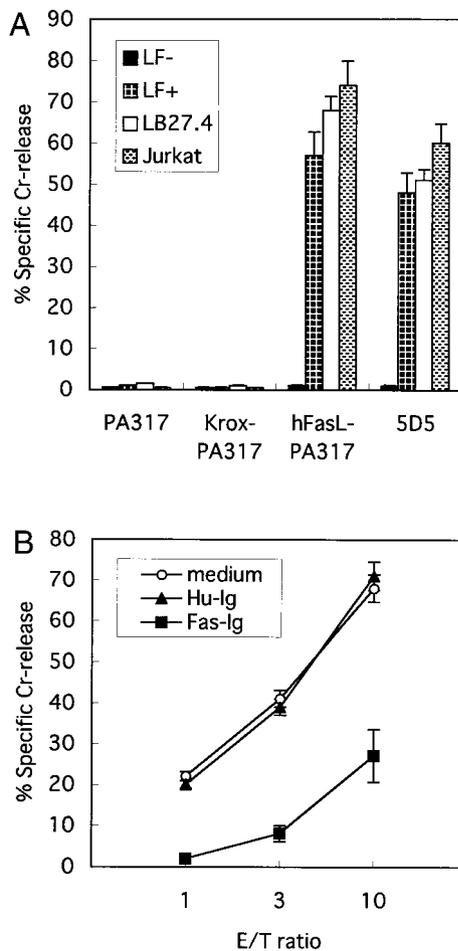
The retroviral packaging cell line, hFasL-PA317, which carries the human FasL gene, was tested for FasL-mediated cytotoxicity against a panel of targets including both human (Jurkat) and mouse (LB27.4 and LF<sup>+</sup>) tumor cells. The parental PA317 and the control Krox-PA317 packaging cells were 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<sup>+</sup> targets that express Fas (Fig. 1A). LF<sup>-</sup> 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 leukemia 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<sup>+</sup> 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 metalloproteinase cleavage, and the third is FasL associated with the FasL VP. To demonstrate the various forms of FasL-mediated cytotoxicity, a graded number of the hFasL-PA317 cells were cultured 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 supernatant preparations. 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.

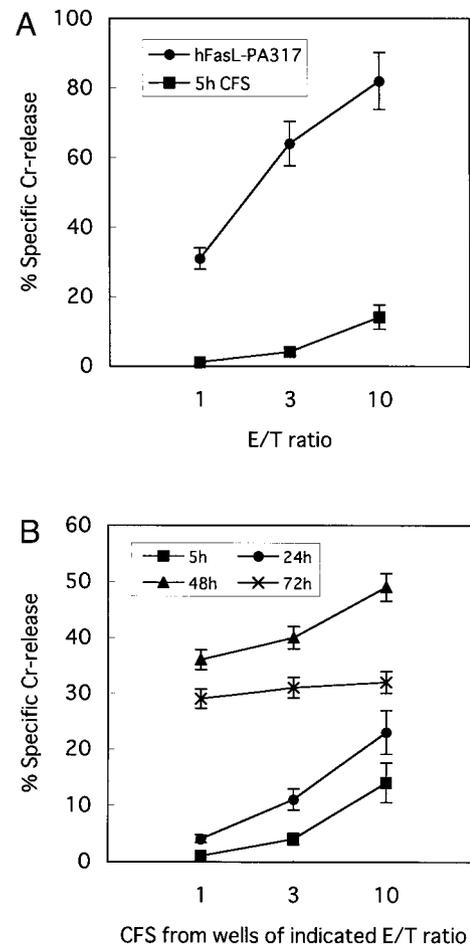
For large quantity preparations, cells were cultured in large petri dishes (150 mm  $\times$  25 mm; Falcon, Lincoln Park, NY). At 70% confluence, culture medium was replaced with medium without G418. Culture supernatant was collected 48 h later, and cell debris



**FIGURE 1.** hFasL-PA317 cells express Fas-specific cytotoxicity. *A*, Various  $^{51}\text{Cr}$ -labeled targets were mixed with hFasL-PA317 cells or activated 5D5 hybridoma T cells and cultured in a 37°C, 10%  $\text{CO}_2$  incubator at an E:T ratio of 10. Activation of 5D5 cells by plate-bound 143-2C11 anti-CD3 mAb was conducted as previously described (19). *B*, Fas-Ig, but not human IgG1 myeloma protein (Hu-Ig), inhibited the killing of LB27.4 by hFasL-PA317 cells. Cytotoxicity was conducted as described above at various E:T ratios. The concentration of the inhibitors tested was 10  $\mu\text{g}/\text{ml}$ . Cytotoxicity was determined after 5 h of culture. Nonspecific release was <25% in all cases. All experiments were conducted in duplicate and repeated three times.

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 pellet (FasL VP) was suspended to 7% of the original volume, filtered through a 0.45-micron membrane, and tested for infectivity.

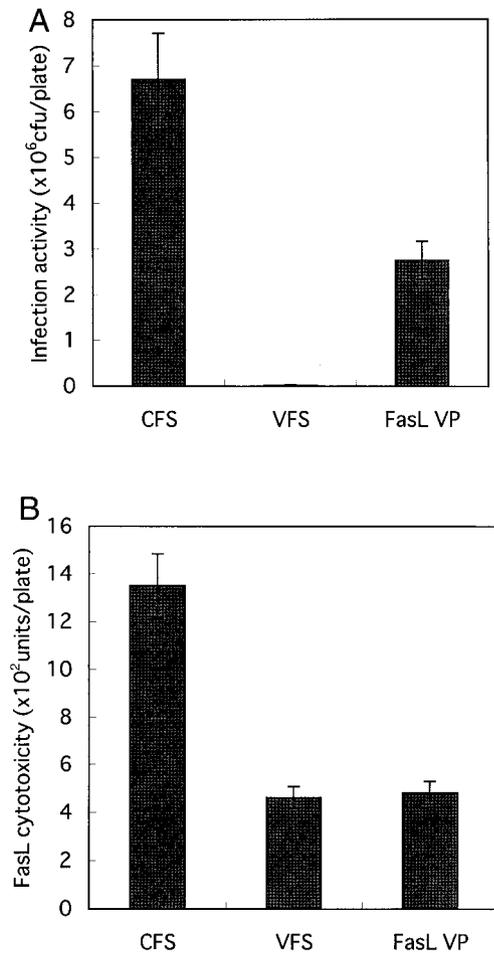
The FasL VP retained the ability to transfer G418-resistance to NIH-3T3 cell (Fig. 3A). The low (40%) recovery of the ability to infect NIH-3T3 cells was presumably due to retroviral inactivation during ultracentrifugation or loss during filtration. These preparations were also tested for FasL-mediated 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



**FIGURE 2.** hFasL-PA317 expressed cell-mediated cytotoxicity and produced cell-free cytotoxic materials. *A*, Various numbers of hFasL-PA317 cells were cultured with  $^{51}\text{Cr}$ -labeled LB27.4 cells for 5 h and cytotoxicity was determined (●). A replica plate was prepared without the addition of LB27.4 and cultured under identical condition. After incubation for 5 h, supernatants (150  $\mu\text{l}$ ) of the replica plate was removed and cocultured with LB27.4 target. Cytotoxicity was determined after 5 h of culture (■). *B*, Supernatants of three additional replica plates were collected at various times after culture and tested in a 5-h cytotoxicity against  $^{51}\text{Cr}$ -labeled LB27.4. The cytotoxicity of supernatant was slightly underestimated because 150  $\mu\text{l}$  instead of 200  $\mu\text{l}$  were collected for assays.

be noted that the total lytic units present in the CFS is ~15% of that associated with hFasL-PA317 cells (Fig. 3 legend).

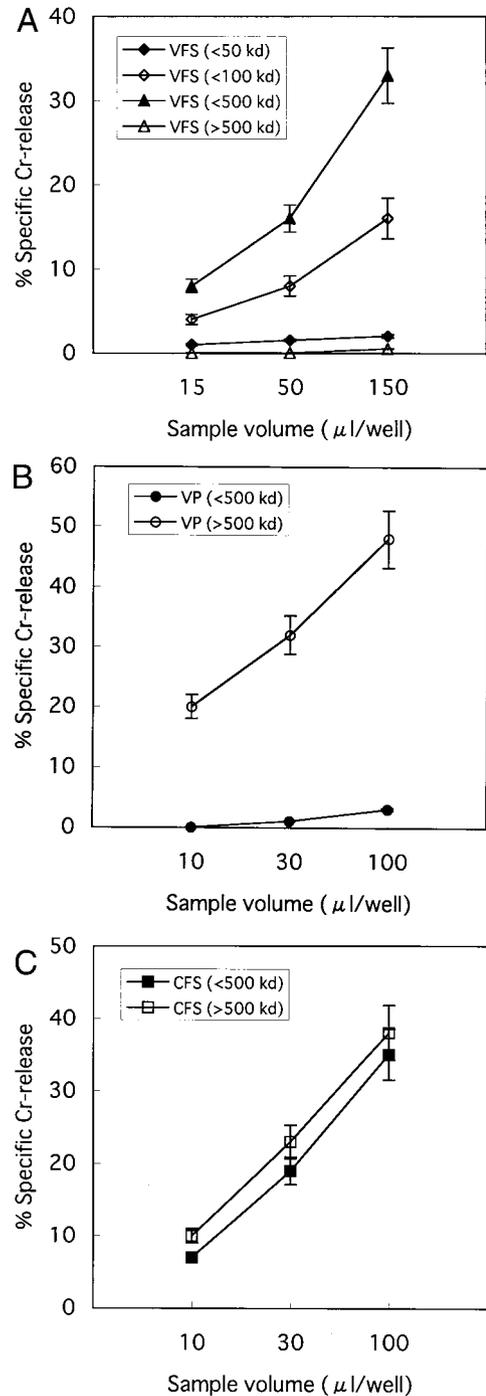
To further characterize the FasL activity, the CFS, the VFS, and the FasL VP were fractionated with Millipore Centricon-500, which retains components larger than 500 kDa. The results indicate that most of the cytotoxic activity in the VFS was present in the components <500 kDa, i.e., sFasL (Fig. 4A). Further fractionation with Centricon-100 and Centricon-50 indicates nearly 30% of the activity was confined between 50 and 100 kDa, and the remaining activity was between 100 and 500 kDa. By contrast, the majority of the cytotoxicity of the FasL VP 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).



**FIGURE 3.** Cytotoxicity of culture supernatant is detected in vector-free fraction and in vector-containing fraction. CFS, VFS, and VP were prepared as described in *Materials and Methods*. Infectivity (CFU) (A) and cytotoxicity (units) (B) were expressed as total activity in a plate of confluent hFasL-PA317 cells. A confluent plate contains  $25 \times 10^6$  cells, and the lytic units of the hFasL-PA313 cells are 10,000 (data not shown).

#### *Dissociating FasL VP infectability from FasL-mediated cytotoxicity*

Because FasL VP possesses the ability to transfect 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 Fas<sup>+</sup> target cells (Fig. 5, B 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

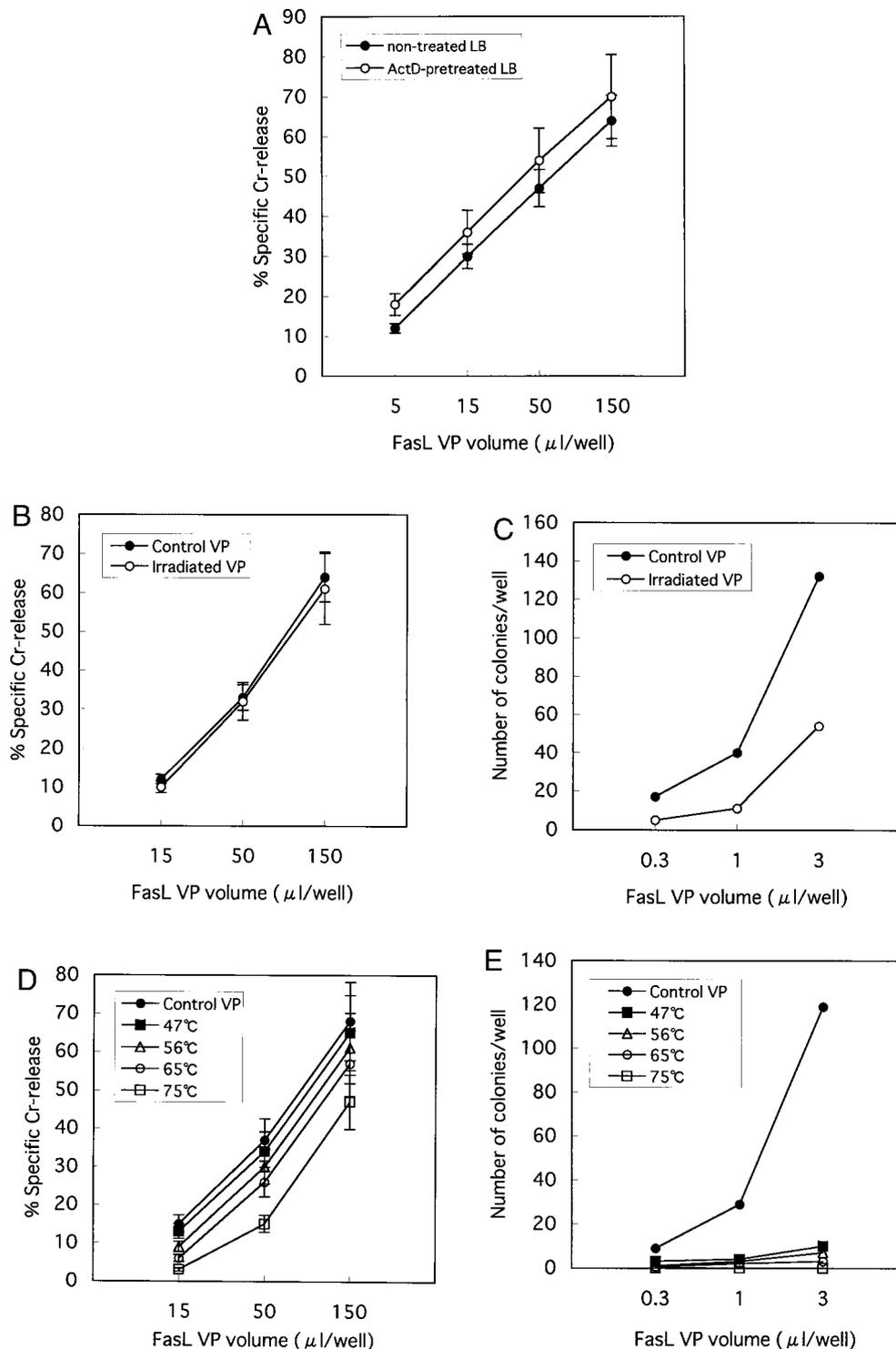


**FIGURE 4.** Molecular size fractionation of culture supernatant for FasL cytotoxicity. VFS were fractionated with Centricon-50, -100, and -500. Filtrates were examined against <sup>51</sup>Cr-labeled LB27.4 target in a 5-h cytotoxicity assay (A). The VP fraction (B) and the CFS fraction (C) were separated by Centricon-500 into a fraction larger than 500 kDa (>500 kDa) and a fraction smaller than 500 kDa (<500 kDa) and cytotoxicity was determined.

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*

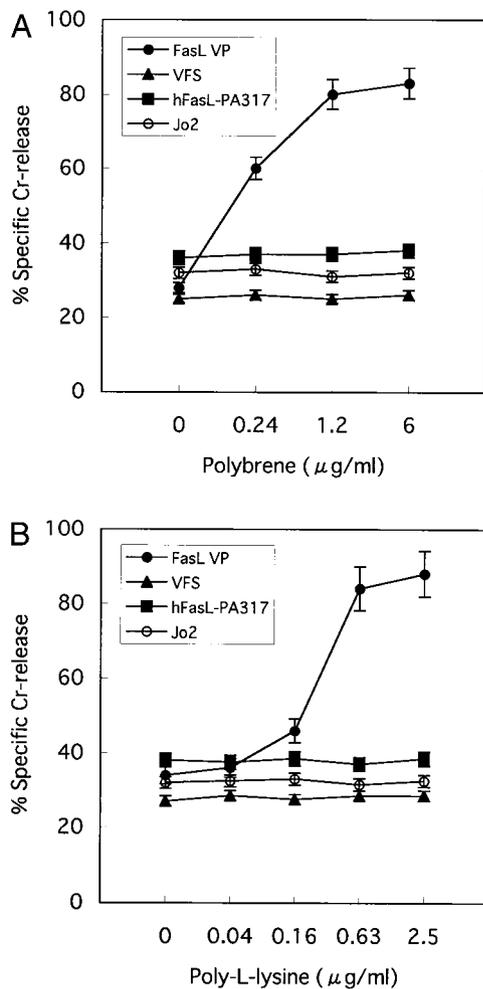
A well-established property of retroviral vector is the enhanced efficiency of gene transfer by the presence of Polybrene, presumably by increasing the contact between the viral vectors and host



**FIGURE 5.** Dissociation of infectivity from FasL cytotoxicity. LB27.4 cells were either treated with actinomycin D or untreated for 45 min as described in *Materials and Methods* and then used as targets for FasL VP in a 5-h cytotoxicity assay (A). FasL VP was irradiated with 10,000 rads or untreated and then tested for cytotoxicity and infectivity (B and C). FasL VP was heat shocked at various temperatures for 30 min and then tested for cytotoxicity and infectivity. FasL VP treated at 37°C was used as a control (D and E).

cells. 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  $\mu\text{g}/\text{ml}$ . The optimal

enhancement of cytotoxicity was observed with Polybrene concentrations ranged between 1.2 and 6  $\mu\text{g}/\text{ml}$ . 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

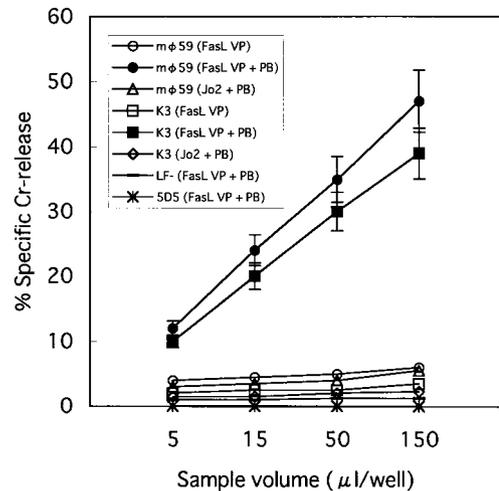


**FIGURE 6.** Selective enhancement of the cytotoxicity of FasL VP by Polybrene (A) and poly-L-lysine (B). Various preparations (FasL VP, VFS, hFasL-pA317 and Jo2 anti-Fas mAb), capable of killing 20–40% of the labeled LB27.4 target in a 5-h cytotoxicity assay, were used. Various concentrations of Polybrene and poly-L-lysine were added at the beginning of culture.

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 mφ59 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 mφ59 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, LF<sup>-</sup> and 5D5 (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,

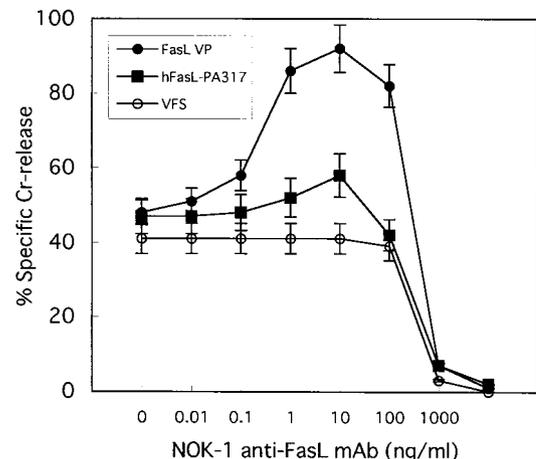


**FIGURE 7.** Polybrene enables FasL VP to kill targets that are highly resistant to FasL VP and anti-FasL mAb. FasL VP and Jo2 anti-Fas mAb (20 μg/ml) were used to kill various targets in a 5-h cytotoxicity assay without or with the addition of 6 μg/ml of Polybrene (PB). Two Fas<sup>+</sup> targets, mφ59 and K31H28 (K3) and two Fas<sup>-</sup> targets, LF<sup>-</sup> and 5D5, were used to demonstrate the specificity of the enhancement of cytotoxicity. Polybrene was not cytotoxic and did not increase background release in all targets tested.

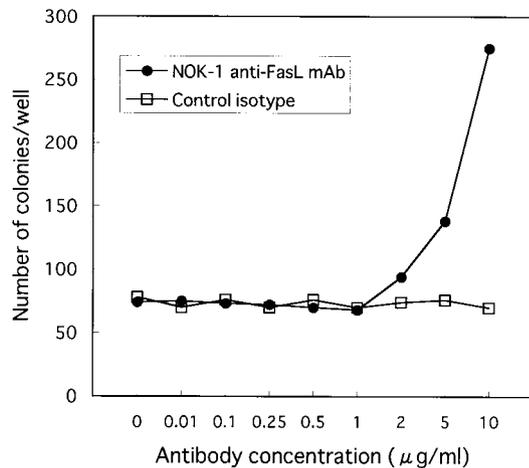
and VFS, which contains sFasL, were included as controls. Polybrene was not used in this experiment. The results are shown in Fig. 8. When high doses of NOK-1 were used, the cytotoxicity of all three samples was strongly inhibited. However, when low doses of NOK-1 were used, the cytotoxicity of FasL 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



**FIGURE 8.** Effects 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, pre-tested and capable of killing 40–50% of the <sup>51</sup>Cr-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).



**FIGURE 9.** Effect of NOK-1 anti-FasL mAb on vector infectivity. NIH-3T3 ( $2 \times 10^4$  cells/well) were plated in 6-well plates and used 24 h later. FasL VP (50  $\mu$ l), mixed with various amounts of NOK-1 mAb or control isotype, was then added to individual wells in a total volume of 4 ml. Polybrene was not used. After culture for 6 h, supernatants were removed. After washing with culture medium, individual wells were cultured in 4 ml of medium containing G418 (0.75 mg/ml). The G418-resistant colonies were determined 10 days later.

of NOK-1 (Fig. 9). Interestingly, the ability to transfer G418-resistance was significantly enhanced when infection was conducted in the presence of 2–10  $\mu$ g/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 express 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 cell-associated FasL, sFasL, or anti-Fas mAb. FasL cytotoxicity could be dissociated from the vector's ability to infect target cells. Moreover, NOK-1 anti-FasL mAb modulates the cytotoxicity of FasL VP and enhances FasL VP infectivity. These observations indicate that the FasL VP represents a novel, powerful, and versatile biological agent. In addition, the data raise the possibility that the retroviral vector technology could be used to generate 1) novel viral vectors whose behavior could be regulated by the target protein and 2) powerful bioactive vesicles that express predetermined effector functions of the target genes.

Because the retrovirus was generated from a packaging cell line, which carries the human FasL gene, it is conceivable that Fas-specific 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 maybe 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-FasL 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.

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 Fas-expressing cells, the target range for FasL gene transfer 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<sup>+</sup> targets. By contrast, when NIH-3T3 and m $\phi$ 59 cells were treated with FasL VP, G418-resistant cells were generated. In contrast to their parental cells (NIH-3T3 and m $\phi$ 59), 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

not shown), indicating that the production of bioactive vesicles expressing FasL cytotoxicity is not dependent on the production of viral particles. The FasL cytotoxicity of the vesicles produced by the G418-resistant NIH-3T3 cells was about 10% of that produced by the hFasL-PA317 packaging cells, raising the possibility that active production of virus particles facilitates the production of functional bioactive vesicles. In this respect, it should be noted that the FasL cytotoxicity of FasL VP prepared from hFasL-PA317 cells is 1000 times stronger than that produced by PHA-activated Jurkat cells (data not shown), which has been reported to produce bioactive vesicles expressing FasL function (14).

The generation of bioactive vesicles from the hFasL-PA317 packaging cell line implies that other specific functions could also be produced. We have successfully generated similar retroviral vector cell line (TRAIL-PA317) carrying the human TRAIL protein. TRAIL VP prepared from TRAIL-PA317 kills Jurkat target in a TRAIL-specific fashion (29). Retroviral packaging cell lines, which carry other types of cell membrane proteins, are now being made to determine whether their vector preparations are capable of carrying out the specific functions of the proteins encoded by the target genes. For example, one could produce vector preparations from packaging cell lines carrying TNF or CD40 ligand and determine whether they can modulate fibroblast function or stimulate B cells, respectively (23, 24). In contrast to this protocol, modifications of the *env* gene have been used to express new functions on the retroviral particles, including redirecting target range and increase of virus/cell interaction (25, 26). It should be possible to use this approach to generate FasL VP with high target specificity. It is relevant that the vector's ability to infect host cells could be eliminated completely by heat shock treatment, allowing the vector preparation to specifically and directly express the desired effector function of the protein in the absence of gene transfer.

The ability of NOK-1 to enhance the infectivity of FasL VP is of great significance because it indicates that a cell-derived protein (FasL) can have an effect on gene transfer when present in the vector preparation. The ability of anti-HIV Abs to enhance HIV infection in human cells has been demonstrated and the enhancement was mediated through the Fc receptor (27). How NOK-1 anti-FasL mAb enhances infectivity of the FasL VP remains to be elucidated. Because fibroblasts express little FcR (28), it seems unlikely that the enhancement by NOK-1 mAb is mediated through FcR. One possibility is that anti-FasL protects NIH-3T3 cells from FasL VP cytotoxicity, and hence increases the overall frequency of infected cells. If this interpretation is correct, then the actual infection efficiency of the FasL VP will be underestimated. However, FasL VP was not apparently cytotoxic to NIH-3T3 cells when cultured together for 5 h. We favor the interpretation that cross-linking of FasL VP by NOK-1 enhances the interaction between vectors and cells. This interpretation is consistent with the results obtained with Polybrene treatment, which enhances the infectivity and modulates the FasL cytotoxicity of FasL VP. The significance of our finding is that a protein encoded by the nonviral gene could be used to regulate the behavior of retroviral vectors, thus having important implications in studies using gene transfer and gene therapy.

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