Inhibition of Metalloproteinase Cleavage Enhances the Cytotoxicity of Fas Ligand[1]

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The Fas ligand (FasL)/Fas receptor (CD95) pathway is an important mediator of apoptosis in the immune system and can also mediate cancer cell death. Soluble FasL (sFasL), shed from the membrane-bound form of the molecule by a putative metalloproteinase (MP), may function to locally regulate the activity of membrane-bound FasL. Using a replication-defective recombinant adenovirus-expressing FasL (RAdFasL), we identified a variable ability of different carcinoma cells to respond to FasL-induced cytotoxicity and to shed sFasL. Blockade of FasL cleavage with an MP inhibitor significantly enhanced RAdFasL-induced apoptosis suggesting that sFasL may antagonize the effect of membrane-bound FasL. In support of this concept, a recombinant adenovirus expressing a noncleavable form of FasL (RAdD4) was found to be a potent inducer of apoptosis even at very low virus doses. Our results highlight the therapeutic potential of noncleavable FasL as an antitumor agent and emphasize the important role of MP via the production of sFasL in regulating the response of the Fas pathway. Moreover, these findings have general implications for the therapeutic exploitation of TNF family ligands and for the possible impact of MP-based therapies on the normal physiology of Fas/TNF pathways. ***The Journal of Immunology*****, 2003, 170: 677–685.**

Fas ligand (FasL[3]; CD95L) is a member of the TNF family which can trigger apoptotic cell death following binding to its receptor Fas (1–2). This interaction is central to lymphocyte homeostasis and also provides a mechanism by which cytotoxic T lymphocytes can destroy target cells (3–6). However, a fine balance exists between the removal of legitimate T cell targets, such as virally infected or cancerous cells, and tissue destruction which results when the Fas/FasL pathway is inappropriately activated. Thus, FasL has been implicated in a number of autoimmune diseases such as hepatitis, insulin-dependent diabetes, thyroiditis, and uveitis (7–11).

Expression of Fas and FasL is not restricted to cells of the lymphoid system. Fas is ubiquitously expressed on a number of different cell types including tumor cells (12–14). Likewise, FasL expression is not restricted to activated T cells and NK cells (15–17) but is also expressed at immune privileged sites such as eyes and testis and can be up-regulated in response to UV irradiation or cytotoxic drug treatment in cells of lymphoid or epithelial origin (18–22). These observations have led to attempts to harness Fas/FasL interactions to protect allografts from immune attack and to induce antitumor responses. Thus, myoblasts engineered to express FasL were found to prolong the survival of cotransplanted islet allografts in diabetic mice (23) and allogeneic fibroblasts engineered to express FasL abolished tumor growth and induced specific protective immunity when mixed with neoplastic cells before implantation in vivo (24). However, the action of FasL is not always confined to Fas-positive target cells, as locally expressed FasL has also been shown to act on Fas-negative tumor cells to induce neutrophil-mediated tumor rejection and elicit systemic protective immunity (25). A number of studies have described recombinant adenovirus-mediated transfer of FasL to tumor cells with beneficial results. Thus a replication-defective recombinant adenovirus (RAd) expressing FasL (RAdFasL) was found to induce apoptosis in human prostate cell lines (26) and in glioma cells (27). Moreover, RAdFasL treatment of established tumors resulted in tumor regression through apoptosis and inflammation in syngeneic murine models of renal carcinoma (28).

Recently, a metalloproteinase (MP) which specifically cleaves TNF-α has been identified as a member of the ADAM MP family (29–30). The TNF-homologous portion of membrane-bound FasL (mFasL) is also processed and shed as a soluble 26-kDa molecule (sFasL) from the surface of cells (31–32). Although recent data demonstrates that this function is fulfilled in glandular epithelial cells by the MP matrilysin, the MPs responsible for FasL cleavage in other cell types remain to be identified (33). Significant levels of sFasL have been detected in the serum of patients with large granular leukemia of T or NK type, or NK lymphomas (15). The exact function of sFasL is still unclear as although human sFasL is functional in inducing apoptosis in some mouse Fas-positive cells (34) in mice it can induce apoptosis in only presensitized cells at high doses (35). The shedding of sFasL has been shown to down-regulate the apoptotic and inflammatory activity of its membrane-bound counterpart, suggesting that mFasL is the functional form and that shedding of sFasL acts to regulate mFasL cytotoxic activity (36–39).

The potential use of FasL as a suicide gene prompted us to develop recombinant adenoviruses to deliver FasL to Fas-positive ovarian and cervical carcinoma cells. We found variability among these cell lines in their sensitivity to FasL-induced cytotoxicity and

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[3] Abbreviations used in this paper: FasL, Fas ligand; RAd, replication-defective recombinant adenovirus; RAdFasL, replication-defective recombinant adenovirus expressing FasL; MP, metalloproteinase; mFasL, membrane-bound FasL; sFasL, soluble FasL; MPi, MP inhibitor; moi, multiplicity of infection; CHX, cycloheximide.

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in their ability to generate sFasL. Blockade of sFasL generation, either by an MP inhibitor (MPi) or by mutation of mFasL, resulted in greatly enhanced cell killing. These data have implications both for the therapeutic exploitation of FasL and, more broadly, for the physiological regulation of this and other TNF family members.

Materials and Methods

Cell lines and culture

MG60 and MG79 ovarian tumor cell lines were derived from ascitic fluid of patients with recurrent ovarian carcinoma, as previously described (40). Briefly, freshly drained ascitic fluid was obtained with patient consent and tumor cells were separated on discontinuous Ficoll gradients and were cultured in DMEM (MG60) or RPMI 1640 (MG79) with 10% FCS, L-glutamine, 1% nonessential amino acids (Life Technologies, Grand Island, NY), and 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO). All other cell lines were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 with 10% FCS and L-glutamine, with the exception of SKOV-3, ME-180, and SiHa which were maintained in DMEM, with 10% FCS, and L-glutamine.

Reagents

The CH11 anti-Fas mAb (Immunotech, Westbrook, ME) was used at increasing concentrations (1–100 ng/ml) as indicated in the presence of 10 μg/ml cycloheximide (CHX). The MPi KB8301 (BD PharMingen, San Diego, CA) was dissolved in DMSO to 10 mM and was used at varying concentrations to block cleavage of sFasL in cultures of cells following RAdFasL infection (41). Treatment of cells with MPi alone had no significant cytotoxic effects. The broad spectrum caspase inhibitor zVAD-fmk (Z-Val-Ala-Asp(OMe)-fluoromethylketone; Alexis, San Diego, CA) was dissolved in DMSO to 10 mM and was used at 10 μM. zVAD was added to cultures during and after virus infection. sFasL was detected in cell culture supernatants using a sFasL ELISA kit (MBL, Nagoya, Japan). Culture supernatants of 200 μl from typically 5×10^3 RAd-infected cells were collected at 48-h postinfection and were used immediately or stored at −80°C before use. zVAD-fmk was added to culture medium during and after virus infection to obtain a measurement of sFasL release from live infected cells. Cell counts were performed before virus infection and data was expressed as sFasL release from 1×10^3 cells. Cell supernatants and caesium chloride banded virus were diluted 1/1 with buffer before assay according to manufacturer's instructions.

Adenovirus vectors

RAdFasL virus was generated by homologous recombination between the Ad5-based *dl309* plasmid pJM17 (kind gift from F. Graham, McMaster University, Ontario, Canada) and an adenovirus transfer vector pMC3, into which human FasL cDNA or noncleavable D4 mutant cDNA (Ref. 37; kindly provided by Dr. S. Nagata, Osaka University Medical School, Osaka, Japan) was cloned under the control of the CMV immediate early promoter. Virus rescue was performed in the AdE1a and SV40 large T-transformed human renal epithelial cell line 293T by cotransfecting pMC3-FasL and pJM17. Following homologous recombination between the plasmids, plaques of recombinant adenovirus were visible from day 6 posttransfection. Following several rounds of plaque purification on 911 cells, the presence of recombinant virus was verified by RT-PCR in infected target cells, using primers for FasL in RNA samples from RAdFasL-infected cells. RAd35 virus (a kind gift from G. Wilkinson, University of Wales College of Medicine, Cardiff, U.K.) containing the *lacZ* gene was previously constructed as described above and was used as a control virus in all infection experiments.

RT-PCR

RNA isolation and reverse transcription was performed as previously described (42). FasL cDNA amplification was performed in a 25-cycle PCR with denaturation at 94°C for 30 s, annealing at 54°C for 50 s, extension at 72°C for 50 s and a final extension at 72°C for 5 min. The sequences of the primers used were FasL forward, 5′-GGTCCATGCCTCTGGAATGG-3′; FasL reverse, 5′-CACATCTGCCCAGTAGTGCA-3′, to generate a 249-bp product. Amplification for GAPDH was simultaneously conducted to normalize for FasL expression using the following primers: GAPDH forward, 5′-CCACCCATGGCAAATTCCATGGCA-3′; GAPDH reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′, with 30 cycles of denaturation at 94°C for 45 s, annealing at 66°C for 30 s, extension at 72°C for 50 s, and a final extension at 72°C for 5 min, to generate a 597-bp fragment. All appropriate control experiments were performed, including amplification of DNase I-treated RNA samples for detection of residual DNA contamination and amplification of water control samples. PCR products were analyzed on a 1.5% agarose gel, transferred to a Hybond-N^+ membrane and hybridized with the following gene-specific oligo-probes: FasL probe, 5′-ATGAGGAACTCTAAGTATCC-3′; GAPDH probe, 5′-TGAGAAGTATGACAACAGCC-3′.

Cytotoxicity and apoptosis assays

FasL-induced cytotoxicity was determined using the MTT or WST-1 colorimetric assay (43). Briefly, cells were plated at densities of $4-6 \times 10^3$, according to cell line, in a flat-bottom 96-well plate and left to adhere overnight. The following day a sample well was trypsinized to estimate cell number and cells were then infected with adenovirus at varying multiplicities of infection (moi). At various time points following infection, 20 μl of 5 mg/ml MTT (Sigma-Aldrich) in PBS were added to each well. After 4 h at 37°C, liquid was carefully removed from the wells and the remaining formazan crystals were dissolved in DMSO. OD_{590} was recorded on a microplate autoreader (Bio-Tek Instruments, Winooski, VT). For experiments using the CH11 anti-Fas mAb, cells were treated with increasing concentrations of CH11 for 6 h and then cocultured with CHX (10 μg/ml) for 24 h before being analyzed by MTT assay. For experiments using sFasL, cells were treated with 0.5 μg/ml sFasL (Alexis) for 24 h, then 10 μl of WST-1 (Roche, Basel, Switzerland) were added and OD_{450} was recorded. In some experiments, a cell death ELISA kit (Roche) was used to confirm the MTT results. Electrophoretic examination of DNA fragmentation (DNA ladders) was performed as previously described (44).

Chromium release assays

Fas-positive target cells (293T) were incubated with $[^{52}Cr]O_4$ for 1–2 h, washed, and incubated in 96-well plates at 1×10^5 cells per well in 100 μl of growth medium. Effector cells (MG79) infected with Rad-FasL or control RAd35 virus were then added (100 μl/well) at a known E:T ratio. To measure spontaneous cell lysis, labeled 293T cells were incubated with growth medium alone. After 12 h of coculture, 100 μl of culture supernatant was harvested from each well and levels of $[^{52}Cr]O_4$ were measured using a gamma counter (Packard Instrument, Berks, U.K.). All tests were conducted in triplicate and the percentage of specific lysis was calculated as follows: percent specific lysis = (lysis in the presence of effectors − spontaneous lysis) × 100.

Results

FasL delivery in cervical and ovarian carcinoma cell lines results in cytotoxicity

RAdFasL, a recombinant E1a-deleted adenovirus into which human FasL cDNA was cloned under the control of the CMV immediate early promoter, was used to deliver FasL to ovarian and cervical carcinoma cell lines. FasL expression was detected in RAdFasL-infected SiHa carcinoma cells by immunofluorescence (Fig. 1). RT-PCR analysis was used to confirm FasL expression in RAdFasL-infected SiHa, MG79, SKOV-3, and IGROV-1 cell lines with similar FasL mRNA levels being observed in the different infected lines (Fig. 2). FACS analysis of the panel of ovarian and cervical carcinoma cell lines confirmed the expression of Fas at high levels on the cell surface, with no significant differences in the levels of expression between cell lines (Table I). Cytotoxicity assays revealed that following RAdFasL infection, a number of carcinoma cell lines showed a significant decrease in cell survival. This effect was particularly marked in the early passage ovarian tumor cell lines MG60 and MG79 (Fig. 3, *A* and *B*) and was not dependent on the concomitant inhibition of protein synthesis, a requirement for efficient Fas-induced apoptosis in response to treatment with the agonistic CH11 Ab (Fig. 3*C*). Virus infection at increasing moi resulted in corresponding increases in cytotoxicity, with 18% cell death induced in the MG79 cell line following infection with RAdFasL at moi 10, compared with 61% cell death following infection at moi 100 (Fig. 3*A*). Although the established cell line CaSki showed significant levels of cytotoxicity following RAdFasL infection, other cell lines such as IGROV-1, SiHa, and SKOV-3 consistently showed little or no change in survival after RAdFasL infection (Fig. 3*B*). This differential response was not

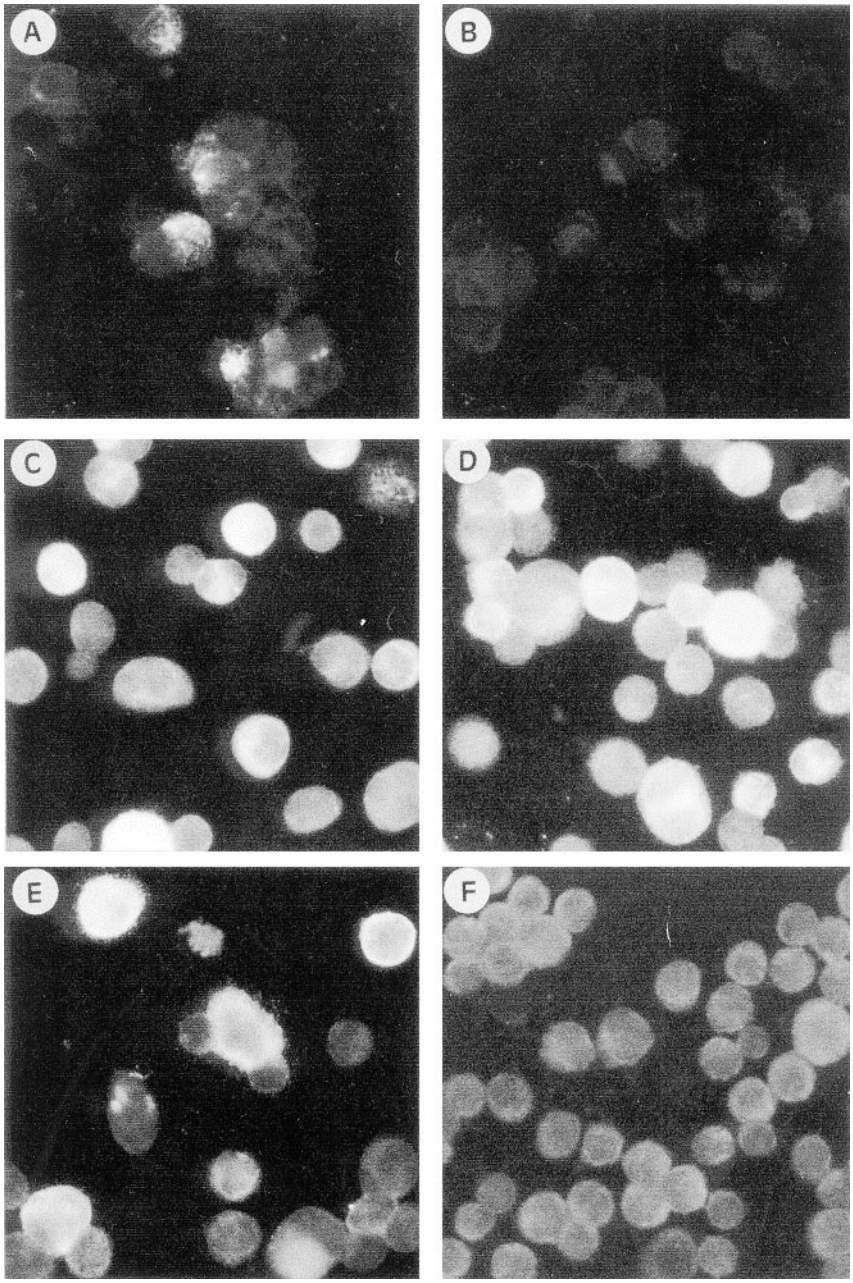

FIGURE 1. FasL expression at the protein level in RAdFasL-infected carcinoma cell lines. Immunofluorescence staining of RAdFasL-infected SiHa cervical carcinoma cells. 293 cells were transfected with the pMC3-FasL plasmid (*A*) or a control construct (*B*) and stained with the anti-FasL Ab Q20 (Santa Cruz Biotechnology, Santa Cruz, CA). SiHa cells were infected at a moi of 100 and stained for FasL expression at 24 (*C*), 48 (*D*), and 72 (*E*) h postinfection. Cells infected with RAd35 show only background staining for FasL (*F*). An isotype control Ab was also used to verify the specificity of staining (data not shown).

due to variable levels of Fas expression (Table I) or to differences in either adenovirus infectivity (40) or FasL expression (Fig. 2*B*). Treatment of this panel of carcinoma cell lines with anti-Fas Ab CH11, in the presence of the protein synthesis inhibitor cycloheximide, revealed that the cell lines SKOV-3 and SiHa were relatively resistant to Ab Fas-induced apoptosis compared with the MG60 and MG79 cell lines (Fig. 3*C*). This observation, together with data from RAdFasL infection assays, suggests that SiHa and SKOV-3 may be partly impaired in their ability to trigger cell death through the Fas pathway.

The apoptotic nature of the effects of RAdFasL on cell survival was confirmed by UV microscopy of propidium iodide-stained cells which revealed characteristic apoptotic nuclear fragmentation in infected carcinoma cells (data not shown) and DNA laddering (Fig. 4*A*). The ability of the broad spectrum caspase inhibitor zVAD-fmk to inhibit cytotoxicity when added to RAdFasL-infected cells also confirmed the apoptotic nature of RAdFasL-induced effects (Fig. 4, *A* and *B*). To confirm that surface expression of FasL in RAdFasL-infected carcinoma cells was responsible for the cytotoxicity and that this could elicit bystander cell death in noninfected cells, RAdFasL-infected MG79 cells were used as effector cells in chromium release assays with Fas-positive 293T target cells (Fig. 5). Significant cell death was observed even at an E:T ratio of 0.5:4 and this effect was blocked by treatment with either anti-FasL Ab or the zVAD caspase inhibitor (Fig. 5).

Inhibition of sFasL production potentiates RAdFasL-induced cytotoxicity

The inability of RAdFasL to induce significant levels of cell death in SiHa and SKOV-3 carcinoma cells, coupled with reported down-regulating effects of sFasL on mFasL-mediated apoptosis (36–38), prompted us to investigate the levels of sFasL secreted from RAdFasL-infected cells. Following infection with RAdFasL virus, tumor cell lines were found to secrete sFasL at levels proportional to the level (moi) of infection (Fig. 6*A*). Variation in the amount of sFasL released from cells infected at the same moi

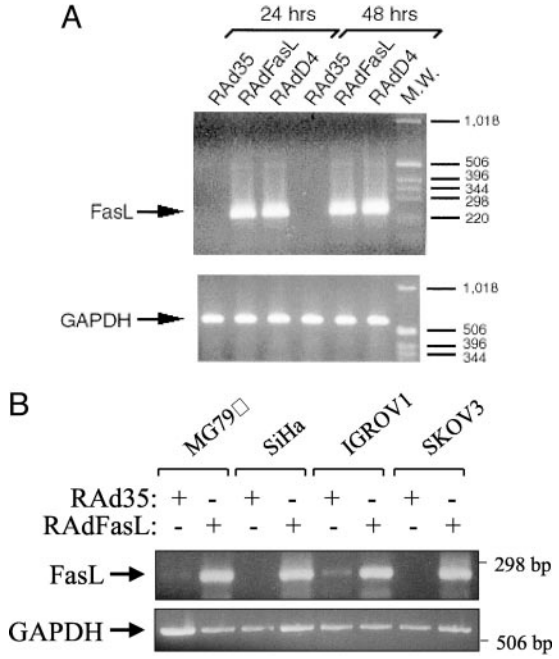

FIGURE 2. FasL expression at the transcriptional level in RAdFasL-infected carcinoma cell lines. *A*, RT-PCR analysis of FasL transcription in MG79 cells infected for 24 and 48 h with either RAdFasL or the noncleavable RAdD4 FasL virus. The extraction of RNA and RT-PCR analysis are described in *Materials and Methods*. *B*, RT-PCR analysis of FasL transcription was also performed in SiHa, SKOV-3, and IGROV-1 cells infected for 48 h with RAdFasL. In both *A* and *B*, GAPDH was used as a normalization control.

could not be accounted for by differences in RAd infectivity, or by variation in levels of FasL expression as determined by RT-PCR (Fig. 2). Interestingly, MG79 cells, which were among the most susceptible to RAdFasL-induced killing, secreted the lowest levels of sFasL. Although SKOV-3 ovarian carcinoma cells secreted higher levels of sFasL than MG79, SiHa cells consistently released the highest levels of sFasL following RAdFasL infection. Intermediate levels of sFasL secretion (around 3–6 ng/ml) were produced in response to infection with 100 moi of RAdFasL in MG60, IGROV-1, CaSki, and ME-180 cell lines. To confirm the specificity and mechanism of the release of sFasL, we used the MPi KB8301 to inhibit sFasL release from RAdFasL-infected cells. sFasL ELISA confirmed that following treatment with MPi, release of sFasL from RAdFasL-infected MG79 and SKOV-3 tumor cell lines was blocked (Fig. 6*B*); similar blockade to that seen with MG79 was observed for the MG60 cell line (data not shown).

To examine whether inhibition of FasL cleavage would affect the subsequent proapoptotic activity of mFasL, cells were treated with MPi following virus infection. Analysis of cell survival by MTT assay revealed that RAdFasL-infected carcinoma cells treated with MPi showed a further increase in cytotoxicity compared with cells infected with virus alone (Fig. 7, *A* and *B*). This effect was particularly apparent in MG60 ovarian tumor cells where addition of MPi resulted in an increase in cytotoxicity from 55 to 92% at a moi of 100 (Fig. 7*A*). Furthermore, inhibition of MP activity significantly enhanced the ability of low doses of RAdFasL to induce cytotoxicity (Fig. 7*A*). The enhancing effect of MPi treatment was also evident in the relatively Fas-resistant SKOV-3 cell line (Fig. 7*B*).

Table I. *Expression of Fas (CD95) in ovarian and cervical carcinoma cell lines*[a]

Cell Line	% Positive Cells (±SD)	Mean Fluorescence Intensity (±SD)
MG60	86.5 ± 0.7	2.45 ± 0.6
MG79	88.5 ± 13.3	2.2 ± 1.1
IGROV-1	85.1 ± 8.1	2.8 ± 0.3
SKOV-3	70.5 ± 6.4	2.25 ± 0.2
CaSki	92.7 ± 1.8	5.3 ± 1.2
ME-180	73.7 ± 17	2.15 ± 0.9
SiHa	75.3 ± 18.2	1.8 ± 0.3

[a] Values represent the mean ± SD from triplicate determinations.

Recombinant adenovirus delivering a noncleavable form of FasL induces a potent cytotoxic effect

As a result of our findings concerning the impact of inhibiting sFasL cleavage on cell survival, we developed a second recombinant adenovirus (RAdD4) expressing a truncated version of FasL. This mutant (D4) carries a deletion (aa 111 to 133) which spans the cleavage site for sFasL (amino acid sequence EKQI at position 128–131) and has been previously used to generate transfectants of mouse WR19L cells which lack the ability to shed sFasL (37). Expression of D4FasL after infection of carcinoma cell lines was confirmed using immunofluorescence (data not shown) and RT-PCR analysis (Fig. 2). High levels of cytotoxicity proportional to virus dose were induced in MG79 cells following infection with RAdD4 with a dose of 1 moi resulting in a significant reduction in cell survival (Fig. 8*A*, compare with RAdFasL infection in Fig. 3*A*). Comparison of both FasL viruses (D4 and full-length FasL) revealed that RAdD4 was up to 5-fold and 13-fold more potent in inducing cell death in MG60 and ME-180 carcinoma cells, respectively, than its full-length RAdFasL counterpart (Fig. 8*B*). Although RAdD4 infection induced greater cytotoxicity in SKOV-3 cells than RAdFasL, no such effect was observed in the relatively FasL-resistant IGR0V-1 and SiHa cell lines suggesting that mechanisms other than the generation of sFasL are responsible for the refractory nature of these cells to Fas-induced apoptosis (Fig. 8*B*). RAdD4 induced a more extreme and rapid cytotoxicity as compared with RAdFasL and the apoptotic nature of this effect was confirmed using zVAD (Fig. 4*B*). To confirm the mechanism responsible for the enhanced cytotoxic effect of RAdD4 infection, the effect of MPi treatment on RAdD4 and RAdFasL-infected cells was compared. Consistent with the contribution of FasL cleavage to the observed effects, MP inhibition enhanced the cytotoxic activity of RAdFasL while having no effect on either RAdD4-induced cell death (Fig. 9*A*) or that resulting from treatment with *cis*-platin (Fig. 9*B*). Taken together these data suggest that sFasL is capable of blocking cell death induced by membrane-bound FasL. To confirm this effect, infection of MG60 cells with RadD4 at an moi of 1 was performed in the presence or absence of 0.5 μg/ml recombinant human sFasL. The presence of sFasL resulted in a 2-fold increase in cell viability following infection with RadD4 (Fig. 10).

Discussion

The ability of Fas to mediate apoptosis is well-established, but it is only recently that attempts have been made to harness this pathway for use in therapeutic approaches to cancer (26–28). Ligating Fas in a therapeutic setting has the advantage of directly activating the Fas apoptotic pathway, which is often retained even in advanced tumors. Replication-defective adenoviruses are among the most efficient vectors for transient transduction of replicating and nonreplicating eukaryotic cells (45). In this study, we developed a

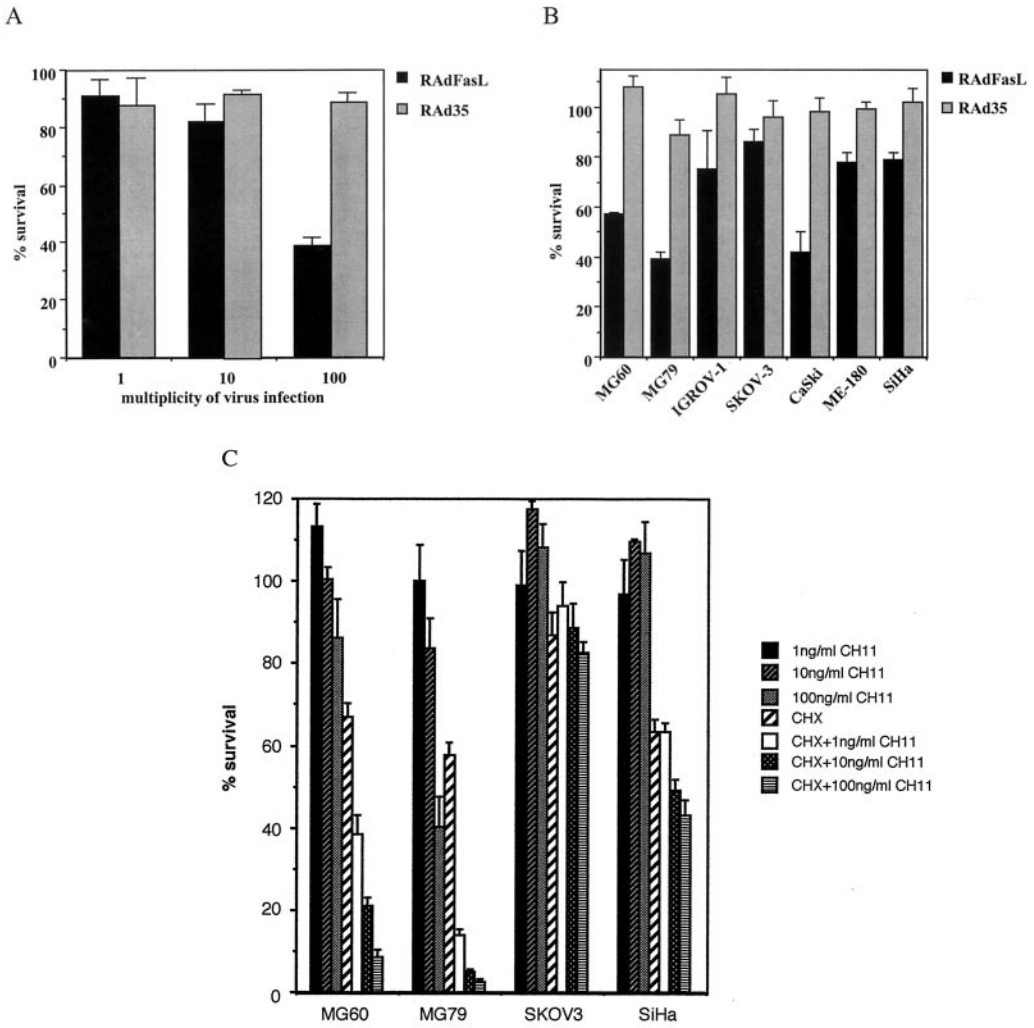

FIGURE 3. The effect of RAdFasL infection or anti-Fas Ab on carcinoma cell survival. *A,* MG79 early passage ovarian carcinoma cells were infected at varying moi with RAdFasL or RAd35 and cell survival was assessed at 48 h postinfection by MTT assay. *B,* Ovarian and cervical carcinoma cell lines were infected with RAdFasL or RAd35 at a moi of 100. Cell survival was assessed by MTT assay at 48 h postinfection and values are expressed relative to uninfected control cells. *C,* The carcinoma cell lines were treated with increasing concentrations of the CH11 anti-Fas mAb as indicated for 6 h and then cocultured with CHX (10 μg/ml) for 24 h before being analyzed by MTT assay. Values are expressed relative to untreated control cells. All data is representative of at least three independent experiments and the values are presented as the mean ± SD of triplicate determinations.

recombinant adenovirus to deliver FasL to Fas-expressing ovarian and cervical carcinoma cells. Infection of target tumor cell lines with the RAdFasL virus resulted in significant levels of cell death in the majority of cell lines studied. Incubation of RAdFasL-infected cells with general caspase inhibitor confirmed the apoptotic nature of the observed effects. Interestingly, our data show that RAdFasL-induced cytotoxicity was most pronounced in several recently established ovarian carcinoma cell lines. As these cells have been cultured in vitro for around 20 passages, they are likely to mimic tumor cells in vivo more closely than ovarian cell lines growing in culture for many years, thus providing a more accurate model for gene therapy of ovarian cancer. Furthermore, this observation suggests that the establishment of carcinoma cells in culture involves selection against the activity of apoptotic pathways.

Previous studies on the effects of ligating Fas on tumor cell lines using Abs have relied on including the protein synthesis inhibitor cycloheximide to observe an apoptotic effect and we have confirmed this requirement (46). The mechanism behind this observation is still unclear but it is possible that some cells may synthesize de novo a protein that protects against apoptosis. Our studies using RAdFasL have been performed in the absence of cycloheximide yet a potent cytotoxic effect is seen suggesting that activating the Fas pathway using membrane-bound FasL rather than cross-linking Ab may provide a more potent apoptotic stimulus. This observation supports previous work demonstrating that extensive aggregation of Fas molecules is required to induce apoptosis and that this is more readily achieved by membrane-bound FasL rather than Fas-specific Abs (47). Ligating Fas on cervical and ovarian cell lines using anti-Fas Ab in the presence of cycloheximide confirmed that in early passage ovarian tumor cell lines and some longer established lines, Fas-induced apoptotic pathways are intact. Indeed, exposure of these cells to RAdFasL induced a dramatic cytotoxic effect. However, SKOV-3 ovarian and SiHa cervical cell lines showed minimal response to either Ab or RAdFasL virus, suggesting that these cell lines may be partially impaired in their ability to respond to Fas ligation. Recently, a number of studies have proposed mechanisms for tumor cell resistance to Fas-mediated apoptosis (48). The shedding of FasL in soluble form from the membrane has been shown to act as a mechanism for down-regulating at least part of its killing activity (36–38). Additionally, a study of colon carcinoma cell lines revealed that during malignant transformation, colonocytes acquire different mechanisms to escape Fas-mediated apoptosis, including down-regulation of Fas expression,

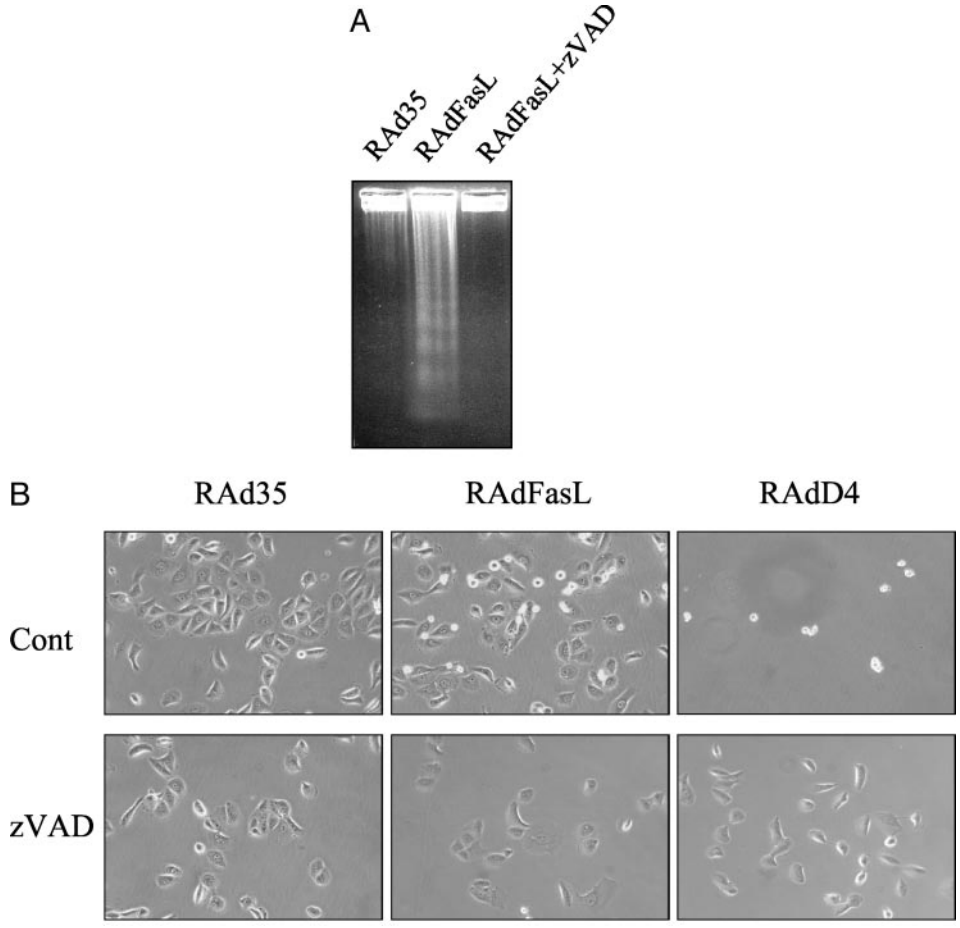

FIGURE 4. RAdFasL treatment induces apoptosis which is blocked by the broad spectrum caspase inhibitor zVAD. *A*, RAdFasL infection of MG79 cells induces DNA laddering which is blocked in the presence of 10 μM zVAD. *B*, MG79 ovarian carcinoma cells were infected with either RAdFasL, RAdD4 expressing noncleavable FasL, or control RAd35 RAds at a moi of 100 in the presence of 10 μM zVAD and photographed at 24 h postinfection. Rounded cells indicative of apoptosis are present in both RAdFasL and RAdD4-infected cultures but are not evident in the presence of zVAD. The more extreme cytotoxicity of RAdD4 as compared with RAdFasL is clearly evident.

inhibition of Fas capping, and activation of antiapoptotic pathways (49). The amplified expression of a soluble decoy receptor, DcR3, that binds to FasL inhibiting its ability to induce apoptosis has also been detected in primary colon and lung carcinomas (50).

Recently, a number of studies have examined the potential of using FasL to induce cell death in vitro and tumor rejection in vivo. Gene transfer of FasL by retrovirus or adenovirus to glioma cells was shown to induce apoptosis in vitro (27). Adenoviral delivery of FasL has also been tested in vivo where it inhibited tumor cell growth and led to tumor regression (28). In addition to its role as an inducer of apoptosis, FasL has been implicated in the induction of immune responses effective against tumors. Locally produced FasL was effective in generating neutrophil-mediated tumor rejection and tumor-specific immunity in vivo (51, 52). However, this effect is likely to be Fas-independent, as Fas-negative tumors have been shown to respond to FasL delivery through a neutrophil-based inflammatory response leading to tumor rejection (25). Peritoneal exudate cells in FasL-treated IL-1β knockout mice did not show neutrophil infiltration, implicating IL-1β in the generation of this effect (51).

RAdFasL-infected cells secrete sFasL at levels proportional to the multiplicity of viral infection and previous studies have established that this cleavage involves a MP (32, 33, 37). Recent studies have alluded to a complex role for sFasL in down-regulating the apoptotic function of the membrane-bound form of the molecule (36–39). To address this issue, RAdFasL-infected carcinoma cells were incubated in the presence of a MPi KB8301. Carcinoma cell lines treated with this compound showed enhanced cytotoxicity compared with controls infected with virus in the absence of KB8301. Similarly, mouse WR19 cells expressing FasL incubated in the presence of another MPi showed enhanced cytotoxicity against Fas-expressing Jurkat targets compared with controls without MPi (37). Interestingly, in SKOV-3 ovarian tumor cells which showed a minimal apoptotic response following RAdFasL infection, subsequent treatment with MPi resulted in increased levels of cell death compared with cells treated with virus or inhibitor alone. This suggests that the inhibition of sFasL production from RAdFasL-infected SKOV-3 cells enhances the ability of FasL to induce apoptosis, even in a situation where the Fas pathway is relatively

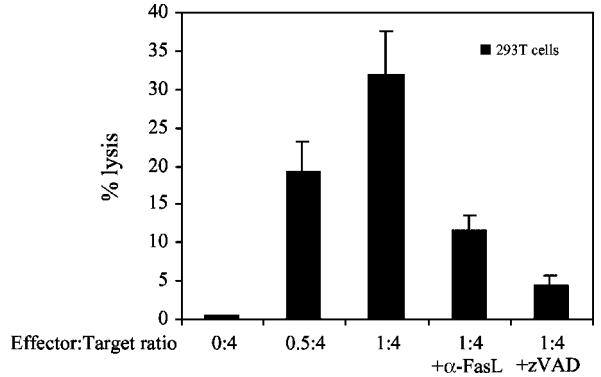

FIGURE 5. FasL expression on the surface of RAdFasL-infected carcinoma cells induces bystander cytotoxicity. MG79 cells were infected with 100 moi of RAdFasL for 48 h before being incubated at different ratios with target Fas-positive 293T cells. Cytotoxicity was determined in chromium release assays and the values are presented as the mean ± SD of triplicate determinations. The data is representative of at least three independent experiments.

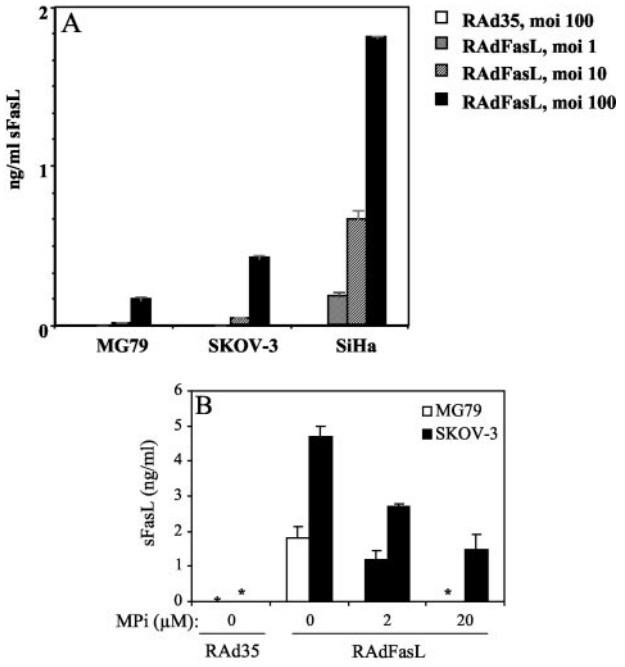

FIGURE 6. RAdFasL-infected cells secrete sFasL which can be inhibited by a MPi. *A*, Release of sFasL from RAd-infected ovarian and cervical cell lines, measured by ELISA. Cells were infected at varying moi and supernatant was collected at 48 h postinfection for use in ELISA. *B*, The release of sFasL in response to RAdFasL infection of MG79 and SKOV-3 cells is inhibited in a dose-dependent manner by the MPi KB8301. Cells were infected at 100 moi and supernatant was collected at 48 h postinfection for use in ELISA. All data is representative of at least three independent experiments and the values are presented as the mean ± SD of triplicate determinations. ∗, Values below the detection limit of this ELISA.

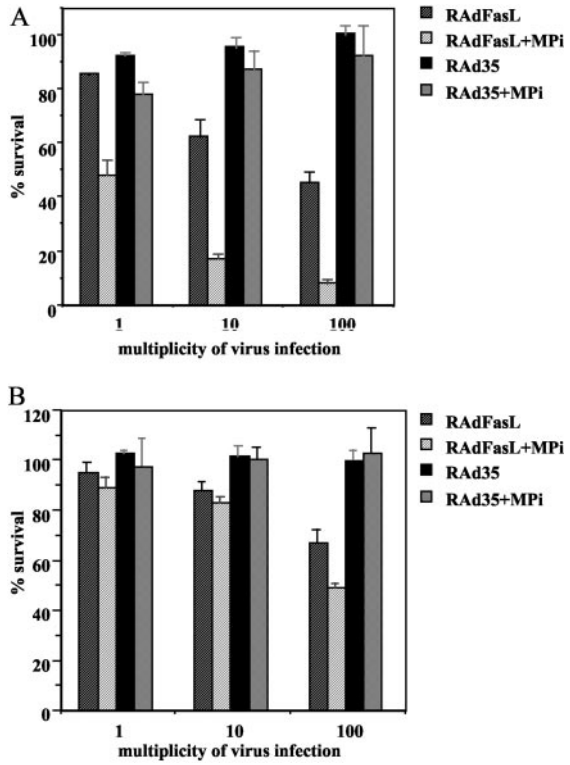

FIGURE 7. Inhibition of MP activity enhances RAdFasL-induced cytotoxicity in carcinoma cells. MG60 (*A*) or SKOV-3 (*B*) cells were infected with RAdFasL or RAd35 and incubated postinfection in the presence or absence of 10 μM MPi KB8301. Cell survival was assessed by MTT assay at 48 h postinfection and values are expressed relative to uninfected control cells. All data is representative of at least three independent experiments and the values are presented as the mean ± SD of triplicate determinations.

inefficient. However, this enhanced apoptotic effect was not observed in another cell line SiHa where the Fas pathway also appears to be defective. Interestingly, the SiHa cell line produced abundant levels of sFasL in response to RAdFasL infection, suggesting that this cell line has high levels of MP activity which may not be efficiently blocked by the MPi. This highlights the need to develop an alternative strategy to block sFasL release. One such approach has involved a mutant of FasL carrying a deletion in the MP cleavage site (D4) and in vitro, this showed increased cytotoxicity against Fas-positive Jurkat cells compared with wild-type controls (37). Thus, we generated a recombinant adenovirus expressing the noncleavable D4 mutant (RAdD4) and found that this virus was a much more potent inducer of cell death than virus carrying wild-type FasL. Although the relatively Fas-resistant SKOV-3 cell line was susceptible to RAdD4-induced cytotoxicity, this was not the case with either the IGROV-1 or SiHa cell line

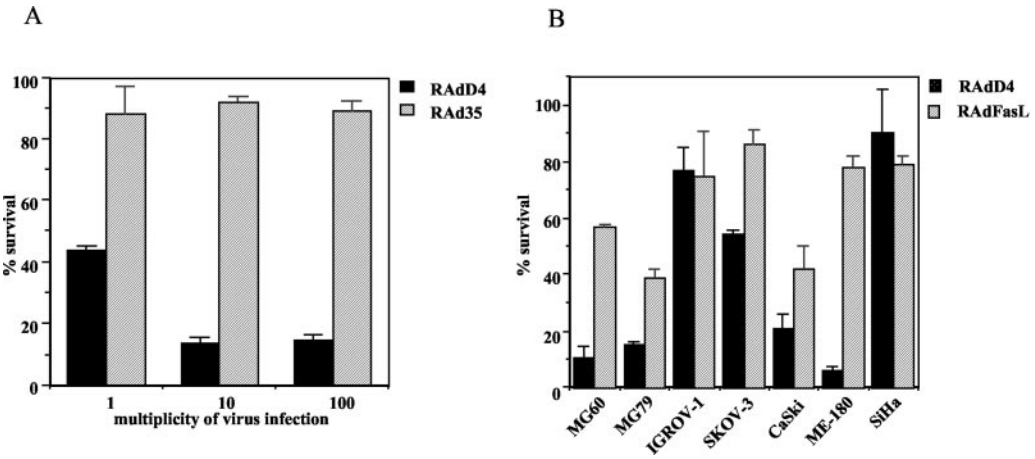

FIGURE 8. Infection of carcinoma cells with the noncleavable FasL-expressing adenovirus (RAdD4) results in enhanced cytotoxicity when compared with RAdFasL. *A*, MG79 ovarian carcinoma cells were infected at varying moi with RAdD4 or RAd35 and cell survival was assessed at 48 h postinfection by MTT assay. *B*, Ovarian and cervical cell lines were infected with RAdD4 or RAdFasL at a moi of 100. Cell survival was assessed by MTT assay at 48 h postinfection. Values are expressed relative to uninfected control cells. All data is representative of at least three independent experiments and the values are presented as the mean ± SD of triplicate determinations.

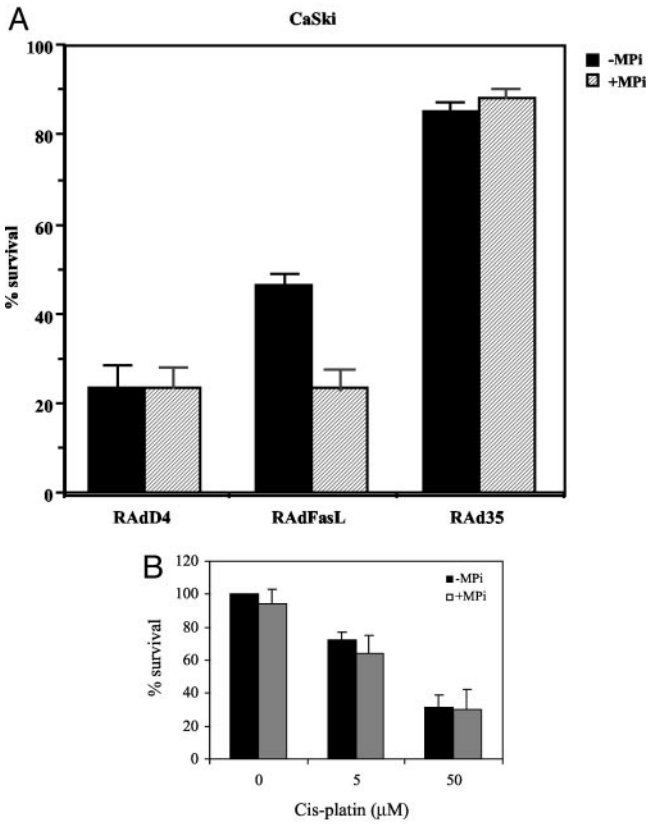

FIGURE 9. MP inhibition has no effect on cytotoxicity induced by either RAdD4 infection or *cis*-platin treatment. *A*, CaSki cells were infected with a 100 moi of either RAdFasL, RAdD4, or control RAd35 and incubated postinfection in the presence or absence of 10 μM MPi KB8301. *B*, MG79 cells were treated with 5 or 50 μM *cis*-platin for 48 h in the presence or absence of 10 μM MPi KB8301. Cell survival was assessed by MTT assay at 48 h postinfection and values are expressed relative to uninfected control cells. All data is representative of at least three independent experiments and the values are presented as the mean ± SD of triplicate determination

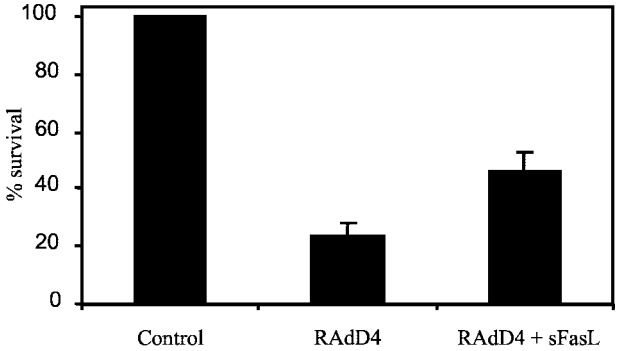

FIGURE 10. The presence of sFasL reduces cytotoxicity initiated by RAdD4 infection. MG60 ovarian carcinoma cells were infected with RAdD4 at a moi of 1, in the presence and absence of 0.5 μg/ml sFasL. Cell survival was assessed 24 h postinfection by WST-1 assay; values are presented as the mean ± SD of triplicate determinations and are representative of two independent experiments.

suggesting that alternative pathways are responsible for the resistance of these cell lines to Fas-induced apoptosis. Our data demonstrate that removal of the MP cleavage site in FasL generates a highly cytotoxic ligand which apart from efficiently inducing apoptosis may also produce more robust anti-tumor immune responses. This possibility is supported by recent work demonstrating a correlation between the cytotoxicity of various forms of FasL and the ability to induce an inflammatory response (39). Thus, the D4 form of FasL could be exploited for more efficient approaches to the protection of allografts from immune rejection and to the development of antitumor therapies.

Of broader significance is the relative contribution of soluble vs membrane-bound FasL to the regulation of the Fas pathway. The ability of sFasL to block membrane-bound FasL-induced apoptosis and inflammation has been reported and our work serves to emphasize the crucial role of MP in regulating the cytotoxicity of FasL (37, 39). Thus, the generation of sFasL is likely to be important in local microenvironments where FasL-expressing cells are engaging Fas-positive target cells and is thus relevant to the contribution of the Fas pathway to diverse regulatory effects such as those governing the immune response, skin homeostasis, and tissue regeneration (6, 21, 53). Our work suggests that the MP-mediated cleavage of other ligands of the TNF family may also be important in regulating the response of target cells to these key mediators of cell growth and death. Consequently, the factors regulating MP expression and activity would be expected to have significant effects on the activation of the TNFR family and thereby influence the regulation of diverse cell systems including those (such as the Fas pathway and the recently described APRIL and BAFF ligands, Ref. 54) involved in the control of immune responses and of cell proliferation. This work also raises concerns about the side effects of those MPi currently undergoing clinical trials for the treatment of cancer, arthritis, and various forms of tissue injury (55–57). The potential role of MP in regulating responses to TNF family ligands suggests that such inhibition would have profound effects on immune responses and cell growth/survival.

In conclusion, our work highlights the important role of MP cleavage in regulating the activity of FasL. Although clearly of significance in the normal control of the Fas pathway, the development of a potent noncleavable FasL-expressing recombinant adenovirus is of obvious clinical interest. A similar approach (i.e., inhibition of membrane-bound ligand cleavage) applied to other potentially therapeutic members of the TNF ligand family (e.g., CD40L, TRAIL) is an important area for future research into clinical application of these agents (58–60).

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