Inducible Release of TRAIL Fusion Proteins from a Proapoptotic Form for Tumor Therapy

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can selectively kill neoplastic cells and control of its activity could enhance tumor therapy. We have developed means to control the secretion of a novel recombinant (r) TRAIL fusion protein using a viral protease. This system uses the endoplasmic reticulum (ER) as a storage depot for rTRAIL, because TRAIL acts by binding to its cognate receptors on the cell surface. We have engineered two TRAIL variants: (a) a secretable form that enhances apoptosis via a bystander effect; and (b) an ER-targeted TRAIL that is retained in the ER until selectively released by the viral protease. Gene delivery can be monitored in vivo by systemic administration of a near infrared fluorescent (NIRF) probe activated by the protease. This study serves as a template for design of recombinant proteins to enhance and control apoptosis of tumor cells via specific viral proteases and for use of viral proteases as in vivo reporters for cancer therapy.

INTRODUCTION

Safety and efficacy of therapeutic protein therapy for cancer can be enhanced by gene delivery using regulatory systems that control timing of release and activity within tumors. We and others have shown that delivery of both the full-length and a truncated secretable recombinant (r) tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce apoptosis in human glioma cells (1–3). Here, we describe the engineering of a novel secretable TRAIL fusion protein that has higher apoptosis-inducing ability than native TRAIL and can be activated selectively by delivering a viral protease to control its secretion. The protease, in turn, can be imaged in tumors in vivo by real time near infrared fluorescent (NIRF) imaging. One particularly attractive feature of TRAIL is that it can selectively induce apoptosis in tumor cells, including glioma cells of diverse origins (4), both in culture and in vivo, while sparing most normal cells (5, 6). TRAIL induces apoptosis by binding to death domain-containing receptors, TRAIL-R1 (7) and TRAIL-R2 (8), on the cell surface, thereby initiating a cascade of signaling events leading to activation of caspases. TRAIL can also bind to two decoy receptors, TRAIL-R3 (9) and TRAIL-R4 (7), with TRAIL-R3 lacking a death domain (10) and TRAIL-R4 having a nonfunctional death domain (11). These decoy receptors do not transduce apoptotic signals and are believed to compete with the death receptors for ligand binding and thereby inhibit ligand-induced apoptosis in normal cells (4). These receptors are active on the surface of the cells and not in other cell compartments, such as the endoplasmic reticulum (ER; Refs. 10 and 12).

The effectiveness of TRAIL can be increased by providing sufficient amounts to receptors on the surface of the tumor cells. We have engineered a secretable form of rTRAIL, S-TRAIL, consisting of an NH2-terminal fusion of the extracellular domain of Flt3L, a ligand for Flt3 tyrosine kinase receptor, with extracellular domain of TRAIL. Flt3L is involved in the growth and differentiation of primitive hematopoietic cells (13) and in innate and specific immune responses (14). The extracellular domain of Flt3L can aid in the secretion of various proteins from cells (15), and studies in both animals and humans have demonstrated that recombinant Flt3L, if administered either alone or in combination with other cytokines, can inhibit effectively the growth and metastasis of malignancies of liver, lung, and breast in murine models (16). Therefore, we reasoned that S-TRAIL would have increased apoptosis-inducing ability by combining the therapeutic potential of the extracellular domains of both Flt3L and TRAIL. The potential for regulating TRAIL-mediated apoptosis was explored by retaining S-TRAIL in an inactive form in the ER (Figs. 1 and 2C) and then releasing it in an active form by expressing a viral protease that specifically cleaves a peptide substrate sequence between the ER retention sequence and S-TRAIL sequence and itself serves as an imaging marker for gene delivery (Figs. 1 and 2, F–H).

The mode of cancer therapy described in this study has three important advantages: (a) design of an enhanced apoptosis-inducing secretable form of TRAIL with a bystander effect; (b) control of the conversion of this S-TRAIL from a nonapoptotic resident of the ER to an apoptosis-inducing protein by selective protease activation; and (c) in vivo imaging of protease activity using a NIRF probe. Release of S-TRAIL in tumors can be controlled by coinjection of viral vectors encoding ER-S-TRAIL and the viral protease and should be compatible with clinical trials for accessible tumor foci.

MATERIALS AND METHODS

Generation of TRAIL Amplicons. Amplicons, pHGCX bearing an expression cassette for enhanced green fluorescent protein (GFP) under immediate early 45S promoter (from Dr. Yoshimura Saeki, Massachusetts General Hospital) and pKSR2 (generated by Dr. Shah) and pHZCX (from Dr. Saeki) derived from it by replacing GFP sequences with those for either the Disco-soma red fluorescent protein (DsRed2; Clontech) or lucZ (Clontech), respectively, were used as the plasmid backbones (17). For creating S-TRAIL, the cDNA sequence encoding amino acids (a.a.) 114–281 of TRAIL was amplified by PCR using the pORF-TRAIL vector (Invitrogen, San Diego, CA) as a template with primers EcoRI-TrAIL (5′-CGCGGAAAATTCGTAGAGAAA-GAGGTCCCTCAGAGA 3′) and BamHI-TRAIL (5′-CGCGGATCCTTAGCA-ACTAAGAAGGCC-CC 3′). The resulting 0.52-kb fragment was digested with EcoRI and BamHI, ligated in-frame with a HindIII/EcoRI-digested, 0.7-kb cDNA fragment encoding the extracellular domain of Flt3L (a.a. 1–81) in-frame with the isoelucine zipper domain in the pFETZ vector (kindly provided by Dr. Yukai He; Ref. 13) and ligated into the HindIII/BamHI-digested pKSR2, resulting in the S-TRAIL construct. The cDNA sequence encoding the herpes simplex virus (HSV)-1 protease (PR) sequence (HSV-1PR; UL26 gene; Ref. 18; a.a. 1–740) in the PGX-18 vector (kindly provided by Dr. David Knipe, Department of Virology, Harvard Medical School) was fused at its COOH terminus to the ER retention sequence, KDEL (19), by annealing two oligonucleotides 5′-GGATCCCTGGTGCTGCGACAGCAAGCAGCA-GCTTGCGACGCGCAGAAGACAGCCCTT3′ and 5′-GGCGAATTTCAG-CCTGCTTCGGCGTGCGCTGC-3236

Received 11/10/03; revised 1/26/04; accepted 2/26/04.

Grant support: The American Brain Tumor Association (K. Shah), Goldhirsh Medical Foundation (X. Breakefield and K. Shah), National Cancer Institute (NCI) CA109246 (X. Breakefield), NCI CA86355 (R. Weissleder and X. Breakefield), and NCI CA92782 (X. Breakefield).

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CGAGGCTGTGCGCAGACCCAGGATCC 3' in annealing buffer at 65°C for 15 min and at room temperature for 1 h. The resulting sequence was digested with BamHI and EcoRI and then ligated into the BamHI/EcoRI-digested S-TRAIL construct. This resulted in ER-S-TRAIL plasmid. For constructing the GFP fusion versions of S-TRAIL and ER-S-TRAIL, the cDNA for the extracellular domain of Flt3 fused to COOH terminally to KDEL was isolated and ligated into pXG-18. All amplicon plasmid constructs were packaged as HSV amplicon vectors in African green monkey kidney Vero 2–2 cells (from Dr. David Baltimore, Massachusetts Institute of Technology), and Gli36 human primary glioma cells (from Dr. Anthony Campagnoni, University of California at Los Angeles, CA) were grown in DMEM with 10% fetal bovine serum (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere with 5% CO₂ and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY).

**Western Blotting.** Cells were lysed 24 h after infection with amplicon vectors at multiplicity of infection (MOI) of 1 tu/cell in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, and 1% NP40, supplemented with proteinase inhibitor (Complete-Mini cocktail; Boehringer-Mannheim, Indianapolis, IN) and centrifuged at 40,000 g for 30 min at 4°C. Equal amounts of total cell protein (30 μg) were denatured, separated by SDS-PAGE, and transferred to nitrocellulose membrane (Inmobilon) in transfer buffer [25 mM Tris and 192 mM glycine (pH 8.3)] by using a Bio-Rad Transblot Cell for 1 h at 0.5 mA at 4°C. The membranes were washed in Tris-buffered saline (TBS)-T buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] and blocked with 10% nonfat dried milk in TBS-T for 3 h at room temperature, washed with

**Fig. 1.** Schematic view of apoptosis and imaging after infection of cells with vector encoding endoplasmic reticulum (ER)-S-tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) followed by vector encoding ER-hesper simplex virus (HSV)-1PR and incubation with the NIRF probe. S-TRAIL will be retained in an inactive form in the ER of the glioma cell and then released in an active form only after coexpression of a viral protease that specifically cleaves the protease substrate sequence interposed between the ER retention and S-TRAIL sequences. This protease also serves as NIRF imaging marker for vector-mediated gene expression.
Cells were blocked in PBS with PBS, the cells were mounted, and microscopy was performed. For the incubated with an antigoat Alexa dye-conjugated secondary antibody (Molecular permeabilized in PBS 20 min at room temperature. The cells were then washed with PBS and reader (Molecular Devices), and the data were analyzed by SOFTMAX. polyclonal antibody. Plates were read at 450 nm using an absorbance plate human rTRAIL expressed in International, Camarillo, CA) according to manufacturer’s protocol using of infected cells was measured with the TRAIL Immunoassay Kit (Biosource control media and WST reagent. TRAIL protein concentration in the medium addition of WST reagent using a V_max kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Background absorbance was subtracted using the control media and WST reagent. TRAIL protein concentration in the medium of infected cells was measured with the TRAIL Immunoassay Kit (Biosource monoclonal antibody and soluble biotinylated TRAIL polyclonal antibody. Plates were read at 450 nm using an absorbance plate reader (Molecular Devices), and the data were analyzed by SOFTMAX. Statistical evaluation was performed using the two-tailed Student t test, and a P of <0.05 was considered statistically significant.

**Immunocytochemistry and Propidium Iodide Staining.** Twelve h after infecting Gil36 cells on coverslips in a 24-well plate with S-TRAIL amplicon vectors, the cells were washed with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were then washed with PBS and permeabilized in PBS + 0.1% Triton X-100 for 20 min at room temperature. Cells were blocked in PBS + 1% BSA for 1 h and incubated with a goat anti-caspase-3 antibody (Cell Signaling) for 1 h, washed with PBS, and then incubated with an antigen Alexa dye-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 1 h. After hybridization and subsequent washing with PBS, the cells were mounted, and microscopy was performed. For the bystander effect of S-TRAIL, 0.2 × 10^6 Gil36 cells were infected with S-TRAIL amplicon vector at an MOI = 1, and 6 h later, cells were washed, and fresh medium was added. Eighteen h later, 150 μL of the medium from the infected cells were collected, mixed with 150 μL of fresh medium, and added to uninfected Gil36 cells, primary human skin fibroblasts, primary rat neuronal cultures (kindly provided by Dr. Norey, Massachusetts General Hospital), and immortalized mouse neuronal CAD cells, growing on coverslips. Six h later, the cells were either stained with propidium iodide (1.5 μg/ml PBS/Molecular Probes) for 30 min at 37°C or fixed and stained with anticaspase-3 antibody as described above. Twenty-four h after incubating cells with S-TRAIL-conditioned medium, WST assays were performed as described above. For ER localization of TRAIL, 293T/17 were transfected with ER-S-TRAIL-GFP amplicon fixed 48 h later, permeabilized and blocked as above, incubated with a goat anti-protein disulfide isomerase (PDI) antibody (Stressgen, San Diego, CA) for 1 h, washed with PBS, and then incubated with an antigen Alexa dye conjugated secondary antibody (Molecular Probes) for 1 h. Cells were examined by confocal microscopy using a laser scanning microscope SPASCAL (Carl-Zeiss, GMBH, Germany). A ×40 oil immersion objective (numerical aperture 1.3) was used for scanning cells with step (pixel) size of 101 μm in the X plane and 7.4 μm in the Y plane. The pinhole setting was 60 μm, which yielded a theoretical thickness (full width at half maximum) of ~0.05 μm.

**NIRF Probe Synthesis and Modeling.** The HSV-1PR-selective NIRF probe was based on a specific peptide substrate LVLAnSASSFGYCOH (= cleavage site). The HSV-1PR recognizes this sequence and cleaves the peptide between alanine and serine. Peptide substrate was conjugated to the lysine side chain of a partially methoxy polyethylene glycol-protected poly-L-lysine graft copolymer through a thiol ether linkage (21). The NIRF probe was tested in cell culture using Gil36 cells infected with HSV-1PR amplicon vectors at MOI = 1. Cells were grown to confluency in a 24-well dish, and the medium was replaced with 200 μl of fresh medium containing 0.2 μM HSV-1PR-specific NIRF probe. After incubation for 1 h at 37°C, cells were washed three times and then visualized under the fluorescence microscope (Axiovert).

**Tumor Model, Amplicon Vector Injections, and Imaging in Vivo.** Gil36 tumor cells in midlog phase were harvested by trypsinization, and single cell suspensions of 5 × 10^6 cells in 100 μl of DMEM were injected s.c. into the upper lateral abdomen on both sides of 4–5-week-old nude mice. Five to 7 days later, when tumors had grown to 2–7 mm in diameter, mice received intratumoral injections of either ER-HSV-1PR amplicon vector (3.5 × 10^5 tu/ml) or control amplicon vector, HGCX (2.4 × 10^5 tu/ml), in a total volume of 20 μl, with intratumoral manipulation of the needle to ensure spread of virus. Tumor-bearing mice (n = 6) were anesthetized with ketamine/xylazine (90/10 mg/kg body weight) i.p. and imaged for 2 min, 24 h after i.v. injection of 2.5 nmol ER-HSV-1PR-specific NIR fluorochrome (corresponding to 2.5 mg probe/kg). The imaging system consisted of a light-tight imaging chamber equipped with a 150-W halogen white light and an excitation bandpass filter (610–650 nm; Omega Optical, Brattleboro, VT) to excite Cy5.5 fiberoptic cables and light diffusers, resulting in a relatively spatially homogenous photon source over the imaging area. A charged cooled device camera equipped with an f/1.2 12.5–75-mm zoom lens and emission long pass filter at 700 nm (Omega Optical) was used to detect the fluorescence. Image analysis was performed using the in-house CMIR Image program.

**RESULTS**

The plasmid constructs used to generate HSV amplicon vectors in this study are diagrammed in Fig. 2. A secretable form of TRAIL was created by fusing coding sequences for the extracellular domain Flt3L (a.a. 1–81) and an isoleucine zipper with the NH2-terminal a.a.114–281 of TRAIL (Fig. 2B). To determine the expression of recombinant TRAIL proteins, cells were infected with TRAIL amplicon vectors, and 24 h later, cells were visualized for signs of apoptosis by light microscopy (Fig. 3A). Thirty-six h after infection, total protein was isolated from tumor cells, fractionated by denaturing SDS-PAGE, and immunoblotted using antisera against TRAIL. Immunoreactive proteins, corresponding to the size of TRAIL and S-TRAIL, were present in the respective amplicon vector-infected cells (Fig. 3B) and not in noninfected cells (data not shown).

WST viability assays performed 24 h postinfection revealed >70% apoptosis with both TRAIL amplicon vectors. S-TRAIL vector-infected Gil36 cells had significantly lower cell viability as compared with TRAIL vector-infected cells (P < 0.005; Fig. 3C). Immunocytochemistry of cells infected with S-TRAIL amplicon vectors (Fig. 3D) or cells exposed to conditioned medium from S-TRAIL-infected Gil36 cells (Fig. 3E) using antibody for caspase-3 confirmed TRAIL-induced apoptosis. The apoptosis-inducing ability of the medium from the S-TRAIL-infected cells and the increased number of apoptotic cells (green) compared with infected cells (red) in S-TRAIL-infected cultures supported the bystander effect of S-TRAIL. To test the potential toxicity of S-TRAIL on normal cells, we exposed primary human skin fibroblasts and primary rat neuronal cultures, as well as immortalized (nontumorigenic) mouse neuronal CAD and tumorigenic human glioma cells to conditioned medium from S-TRAIL-infected Gil36 cells. Immunocytochemistry with caspase-3 antibodies and propidium iodide staining showed that only the tumorigenic glioma cells were susceptible to TRAIL-induced apoptosis (Figs. 3E and 4). Together, these results show that S-TRAIL is secreted from vector-infected cells, has a bystander effect, and is more effective than the TRAIL protein itself in inducing apoptosis in cultured glioma cells. Furthermore, S-TRAIL is not toxic to normal cells in culture.

TRAIL is a type II membrane protein, with its targeting and insertion into the ER during protein translation and post-translational processing being different from the type I receptor proteins (22). Insertion of type I membrane proteins is initiated by an NH2-terminal, cleavable signal sequence that directs the transfer of protein across the ER membrane with subsequent anchoring of the protein in the plasma
membrane by a hydrophobic sequence with an exoplasmic NH2 terminus and a cytoplasmic COOH terminus (Nexo/Ccyt orientation) or being secreted if it lacks membrane-spanning domain. Type II signal anchors lie within proteins and initiate translocation of COOH-terminal sequences into the ER, eventually generating an Nexo/Ccyt orientation of the protein within the plasma membrane. S-TRAIL has been engineered to become a type I protein that is secreted from cells. TRAIL and S-TRAIL bind to either the DR4 or DR5 death receptors at the cell surface and induce apoptosis in tumor cells via a caspase-3 signaling pathway (23). These receptors are not coupled to this signaling pathway in the ER, and thus, this compartment offers a potential reservoir to store TRAIL in an inactive form. To explore this potential, we included an ER retention sequence, KDEL in the COOH terminus of S-TRAIL separated from each other by an HSV-1 protease-specific substrate sequence (see ER-S-TRAIL, Fig. 2). WST viability assays performed 24 h postinfection of Gli36 cells with HSV amplicon vectors expressing various forms of TRAIL revealed that the ER-S-TRAIL protein induced significantly less apoptosis as compared with the S-TRAIL protein (Fig. 5, A and B). To determine whether the KDEL-bearing TRAIL protein was localized in the ER of cells, we transfected 293T/17 cells with the ER-S-TRAIL-GFP amplicon construct (Fig. 2E). Total protein from transfected cells was isolated 36 h later, fractionated by denaturing SDS-PAGE, and immunoblotted using antiserum against GFP. An immunoreactive protein corresponding to the size of ER-S-TRAIL-GFP (Mr ~76,000) was expressed in cells transfected with this amplicon, whereas a Mr 28,000 GFP protein was expressed in pHGCX control amplicon-transfected cells, as predicted (Fig. 5E). In addition, GFP fluorescence conferred by ER-S-TRAIL-GFP colocalized in cells with the ER marker, PDI suggesting residence in the ER (Fig. 5D). Immunoblot analysis with an anticaspase-9 antibody showed the presence of an activated Mr 30,000 band only in the TRAIL and S-TRAIL amplicon vector-infected cells and not in noninfected and ER-S-TRAIL-infected cells (Fig. 5C). Similarly, immunoblotting with the anticleaved-PARP antibody showed the presence of a strong Mr 89,000 cleaved-PARP fragment in the TRAIL expressing cells, whereas this protein was absent in noninfected and very faint in ER-S-TRAIL-infected cells (Fig. 5C). In addition, immunoblotting with the anti-PARP antibody showed the presence of strong Mr 116,000 and 26,000 protein bands in the TRAIL- and S-TRAIL-expressing cells, whereas these protein bands are either absent or much fainter in controls (Fig. 5C). These results reveal that ER retention sequences direct the ER-S-TRAIL fusion protein into the ER and block TRAIL-mediated apoptosis in glioma cells.

To explore the possibility of using a viral protease, HSV-1PR to both release the ER-targeted TRAIL and serve as an imaging marker, different versions of HSV-1PR coding sequences tagged with the FLAG antigenic marker were cloned into HSV amplicons (Fig. 2, F–H). To determine the expression of recombinant HSV-1PRs, Gli36 cells were infected with HSV-1PR amplicon vectors, and 36 h later, total protein was resolved by SDS-PAGE and immunoblotted using antiserum against the FLAG-tag. Immunoreactive proteins, corresponding to the predicted size of PR fusion proteins, were present in
amplicon vector-infected cells (Fig. 6A) and not in noninfected cells (data not shown). Tumor cells coinfected with ER-S-TRAIL and ER-HSV-1PR amplicon vectors had a greater extent of cell death, as assessed by WST viability assays, as compared with those infected with either vector alone (Fig. 6B and C). ELISAs were performed to determine whether the extent of apoptosis correlated with the amount of secreted TRAIL from the vector-infected cells. The amount of secreted TRAIL was considerably reduced in cells infected with the ER-S-TRAIL vector as compared with those infected with the S-TRAIL vector (Fig. 6D). There was no marked increase in S-TRAIL secretion when the cells were coinfected with ER-S-TRAIL and HSV-1PR or LY-HSV-1PR (data not shown). These results indicate that the ER-targeted TRAIL fusion protein can be released from the ER and secreted from cells expressing ER-targeted HSV-1PR with subsequent induction of apoptosis in glioma cells.

To explore the potential of HSV-1PR as a marker for in vivo imaging, we designed an HSV-1PR-selective NIRF probe based on a specific peptide substrate LVLA/H11569SSSFYG-OH conjugated to a delivery graft copolymer. This probe was first tested in cultured Gli36 cells infected with HSV-1PR amplicon vectors at an MOI = 1 and incubated with the NIRF probe 36-h postinfection. The cells infected

Fig. 6. Effect of herpes simplex virus (HSV)-1PR on endoplasmic reticulum (ER)-targeted S-TRAIL-induced apoptosis. In A, Gli36 cells were infected with HSV-1PR, ER-HSV-1PR, or LY-HSV-1PR amplicon vectors or control amplicon vector, HGCX (multiplicity of infection = 1), and lysed 36 h later. Proteins were resolved by SDS-PAGE and immunoblotted with anti-FLAG-tag antibody. Lane 1, HSV-1PR-infected cells; Lane 2, ER-HSV-1PR-infected cells; Lane 3, LY-HSV-1PR-infected cells. Gli36 cells were also infected with either ER-HSV-1PR, S-TRAIL, and ER-S-TRAIL vectors with and without coinfection with ER-HSV-1PR vector (multiplicity of infection = 1), and 24 h later, cell viability was determined either microscopically (B) or by WST assays (C), or immunoreactive TRAIL protein concentration in the medium of the infected cells was determined by ELISA (D).
with all three protease-encoding vectors showed a markedly higher NIFR fluorescence as compared with the noninfected cells (Fig. 7A), indicating that this HSV-1PR substrate-NIFR probe can be activated in cells by lysosomal, ER, and cytoplasmic-targeted HSV-1PR, in order of their decreasing intensity. Nude mice bearing bilateral s.c. Gli36 gliomas (5–7 mm in diameter) were injected in the left tumor with ER-HSV-1PR amplicon vector (3.5 × 10^5 tu in 20 μl) and in the right tumor with control HGCX vector (2.6 × 10^5 tu in 20 μl); 36 h later, HSV-1PR-NIFR probe (40 nmol in 150 μl) was administered i.v. Twenty-four h later, NIFR signal was apparent in HSV-1PR-infected tumors and not in HGCX-infected tumors (Fig. 7B). These results show that HSV-1PR delivered via viral vectors can be imaged in live animals.

DISCUSSION

The ability of TRAIL to selectively induce apoptosis in transformed cells makes it attractive as an apoptosis-inducing agent for cancer therapy (23) as compared with other proapoptotic proteins, such as FasL and tumor necrosis factor-α, which can have severe toxic side effects on normal cells. Previous studies with rTRAIL have revealed that the extracellular domain of TRAIL, termed soluble TRAIL, has a high degree of potency for experimental tumors (8, 23). In this study, we have generated a fully secretable version of TRAIL (S-TRAIL) and targeted this secretable version for sequestration in the ER of the cell. We have shown that S-TRAIL is effective in killing both the producing and surrounding tumor cells and that TRAIL-induced apoptosis can be regulated by reversibly retaining S-TRAIL in the ER. An ER-targeted viral protease is effective in the dual functions of controlling the release of S-TRAIL from the ER and marking gene delivery/expression in vivo. Thus, in this system, the ER fulfills a function similar to that performed by secretory granules in specialized secretory cells, with the physiological stimulus that normally triggers exocytosis of secretory granules being replaced by an ER-targeted viral protease that activates S-TRAIL by allowing release through the constitutive secretory pathway. Coinfection of tumor cells with amplicon vectors encoding ER-S-TRAIL and HSV-1PR can thus serve to simultaneously reduce tumor mass by apoptosis and image gene delivery.

In this study, we have shown that the NH2-terminal fusion of the extracellular domain of Fli3L to that of TRAIL results in a secreted form of S-TRAIL with more potent apoptotic effects as compared with TRAIL itself. In addition, S-TRAIL has a bystander effect demonstrated by the fact that cells infected with S-TRAIL amplicon vector encoding DsRed2 and S-TRAIL not only underwent apoptosis themselves (positive staining for caspase-3) but also induced apoptosis in surrounding noninfected cells (Fig. 3). It has been demonstrated that TRAIL trimerization enhances death signaling on binding to its receptor (12). The addition of the isoleucine zipper (a variant of the leucine zipper, which exhibits higher trimer folding, as compared with the leucine zipper, which favors dimeric or tetrameric folding; Ref. 24) to the NH2 terminus of the TRAIL extracellular domain should serve to enhance its trimerization and thus may also contribute to the marked apoptotic effect of S-TRAIL.

Of the five TRAIL receptors, two of them contain a region with a significant homology to the death domains of tumor necrosis factor receptor 1 and CD95, which trigger the signal cascade for apoptosis. These receptors are present on the cell surface and are not thought to be active in the ER (12). In our studies, glioma cells infected with ER-S-TRAIL amplicon vector showed increased viability as compared with those infected with the S-TRAIL amplicon vector, indicating that sequestration of S-TRAIL in the ER prevents its activity. The death of some of the cells infected with the ER-S-TRAIL vector may result from escape of the S-TRAIL protein from ER and binding to TRAIL receptors at the surface of the tumor cells. The association of apoptotically related proteins and changes in expression of TRAIL receptors on the cell surface in the presence of S-TRAIL are not known. The combined use of proteasome inhibitor (PS-341) and TRAIL has been shown to overcome the block to mitochondrial participation in the apoptotic process mediated by overexpression of Bel-xL (25). The putative mechanism underlying this effect appears to be an increase of both DR5 and DR4 receptor levels, leading to enhanced cleavage of caspase 8 and Bid and increased release of SMAC and cytochrome c (25). Furthermore, the up-regulation of proapoptotic Bak in TRAIL-mediated apoptosis in culture, as well as in vivo in IFN-γ-sensitized cells, has been demonstrated (26). Thus, we would anticipate changes in these apoptotically related proteins and death receptors in response to S-TRAIL.

Activation of recombinant proteins can be regulated using specific protease/peptide substrate combinations and proteases that are not expressed in mammalian cells, such as viral proteases like HIV-1PR (27). This approach offers the potential to express viral proteases in the cells via different vehicles, such as viral vectors, and use them to activate recombinant proteins generated by mammalian cells. Our results show that HSV-1PR and its designated peptide substrate can...
act specifically in glioma cells to release inactive ER-S-TRAIL from the ER in an active form secreted from cells.

The ability to image gene expression in vivo is critical to assess the efficacy of gene delivery and can potentially be used to quantitate therapeutic effects. In previous work, we have developed protease-specific, NIRF-imaging probes, which are activated by endogenous proteases and caspases that are up-regulated in tumors (28) and cardiovascular disease (29), and evaluated their specificity by treatment with protease inhibitors (30). To directly image gene delivery, however, it is important to develop protease-substrate combinations that are not endogenously active in mammalian systems. We have shown that the HIV-1PR NIRF probe was not activated by any endogenous proteases present in human glioma cells but was activated by infection of these cells with vectors encoding HIV-1PR (27) both in culture and in tumors in mice.

The use of the ER as a release valve for therapeutic proteins is not unique to this study. Rivera et al. (31) generated a dimeric protein, one subunit of which was retained by the ER and the other resected by a drug that induced separation of the subunits. One of the limitations to this approach in normal cells is that overexpression of ER proteins, especially recombinant ones, can cause a physiological response in cells, which changes transcriptional/translational activities (32). In the present study, a therapeutic protein is retained in the ER by the KDEL sequence with release being conferred by expression of a nonmammalian protease that specifically cleaves a peptide sequence between the KDEL and protein sequences. Although such a recombinant protein might still trigger physiological changes, it is done in the context of killing tumor cells, where associated toxic effects could be advantageous. If the vectors expressing the ER-targeted therapeutic protein and protease infect normal cells near the tumor cells, they may suffer some toxicity but will be able to release a protein selectively toxic to tumor cells and thus be recruited into the therapeutic paradigm.

In conclusion, we have engineered an apoptotic ER-S-TRAIL protein that has an enhanced apoptosis-inducing ability with a bystander effect and created a system that controls conversion of ER-S-TRAIL from a nonapoptotic ER resident to a secreted, apoptosis-inducing protein by selective protease activation. Future improvements in this system could include use of neuroprecursor cells, which migrate to tumors (33), and incorporation of ER-targeted S-TRAIL and viral protease sequences in the same vector, with the protease being under a drug-regulated promoter (34). Such vector delivery protein expression systems are fully compatible with other current modes of cancer therapy.

ACKNOWLEDGMENTS

We thank Suzanne McDavitt for skilled editorial assistance.

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*Cancer Res* 2004;64:3236-3242.

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