In Vivo Inhibition of Fas Ligand-Mediated Killing by TR6, a Fas Ligand Decoy Receptor

KEVIN CONNOLLY, YLIN HEE CHO, ROXANNE DUAN, JAMES FIKES, THERESA GREGORIO, DAVID W. LAFLEUR, ZEBEDEE OKOYE, THEODORA W. SALCEDO, GEMMA SANTIAGO, STEPHEN ULLRICH, PING WEI, KATHLEEN WINDLE, ELING WONG, XIAO-TAO YAO, YA-QIN ZHANG, GRACE ZHENG, and PAUL A. MOORE

Human Genome Sciences Inc., Rockville, Maryland
Received January 23, 2001; accepted March 20, 2001

This paper is available online at http://jpet.aspetjournals.org

Abstract

TR6, a member of the tumor necrosis factor (TNF) receptor superfamily, has recently been shown to bind to Fas ligand (FasL) and inhibit FasL-mediated cell killing in vitro. In the current study, we demonstrate that TR6 can block the lethal activity of FasL in multiple in vitro systems, and extend this finding to an in vivo model of hepatitis. The binding of human TR6 to human FasL was verified with BLAcore chip technology. Human primary hepatocytes, HT-29 cells and Jurkat cells were assayed for viability to demonstrate TR6 inhibition of FasL-mediated cytotoxicity in vitro. Human TR6 was also shown to cross-react with membrane-bound mouse FasL, since the in vitro cytotoxic activity of L929 cells transfected with murine Fasl was inhibited in the presence of human TR6. In vivo, Fasl-induced acute, lethal, fulminant hepatic apoptosis resulting in death within 2 h of intravenous injection into Fas–/– mice, but not Fas ligand knockout mice. Pretreatment of mice with TR6 blocked Fasl-induced mortality, presumably by attenuating Fasl-induced hepatic apoptosis. Thus, in both in vitro and in vivo systems, TR6 acts as a functional FasL decoy receptor and may be clinically useful in the treatment of hepatitis and other diseases associated with FasL-mediated tissue injury.

Fas receptor and Fas ligand are pro-apoptotic mediators of the TNF receptor and ligand family that induce apoptosis upon receptor/ligand engagement. Fas/FasL-mediated apoptosis is a normal and important homeostatic mechanism useful in the down-regulation of hyperimmune responses and the deletion of activated lymphocytes (Van Parijs and Abbas, 1996). Fas/FasL-induced apoptosis is also important in host protection and surveillance, preventing damage to immune-privileged sites, and eliminating virus-infected or transformed cells (Nagata, 1997). While necessary for normal physiological processes, unregulated apoptosis is implicated in organ-specific tissue injury and autoimmune disease both in experimental animal models and several human disease states (Maggi, 1998).

It is becoming increasingly clear that there is a link between dysregulation of apoptosis and liver disease (Patel et al., 1998). In mice, lethal hepatic injury occurs following injection of cross-linked FasL (Schneider et al., 1998). Monomeric FasL appears much less efficient in inducing apoptosis (Hohlbaum et al., 2000). However, in the absence of FasL, hepatic apoptosis can be triggered by injecting agonistic monoclonal antibodies that bind and cross-link the Fas receptor (Ogasawara et al., 1993). This triggers the intracellular death domain of the Fas receptor and initiates the cascade of caspase activity which, if unimpeded, ultimately leads to the death of the cell (Waring and Mullbacher, 1999).

Another mouse model, concanavalin A induced hepatitis, is somewhat more complicated, since the histopathology shows evidence of hepatic necrosis as well as liver apoptosis, caused by at least three putative contributors, FasL (Tagawa et al., 1998), TNF (Ksontini et al., 1998) and perforin (Watanabe et al., 1996).

FasL/Fas interaction has been implicated in several human liver diseases such as alcoholic cirrhosis (Nanji, 1998), hepatitis B and C (Lau et al., 1998) and acute fulminant hepatitis (Galle and Krammer, 1998). The evidence is based, in part, on an increase in Fas, FasL and apoptotic cells in the liver. Similar circumstantial evidence supporting the role of the Fas/FasL system in the pathogenesis of other human diseases, especially those with an autoimmune component, has been reported in several clinical studies (Maggi, 1998). The neutropenia associated with patients who have large granular lymphocytic leukemia has been attributed to the high levels of circulating soluble Fasl in the blood of these patients (Liu et al., 2000). In patients with myelodysplastic syndrome, their cytopenia appears to be mediated in part by FasL-mediated destruction of hematopoietic bone marrow stem cells, since there is evidence of up-regulated Fas expres-

ABBREVIATIONS: FasL, Fas ligand; FACS, fluorescence activated cell sorter; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; TR6, TNF-like receptor six; Eu-DTPA chelate, Europium Titriplex V, diethylene-triamine-pentaacetic acid; Ab, antibody.
sion in the bone marrow of these patients (Bouscary et al., 1997). Conversely, a compromised FasL/Fas system could allow tumor cells to elude destruction, enhancing the cancer risk to the host (Maggi, 1998).

The importance of maintaining the proper degree of FasL-mediated apoptosis in the normal host suggests the existence of critical regulatory mechanisms. Putative regulators are believed to include soluble forms of both FasL and Fas, in addition to antagonistic decoy receptors. One potential decoy receptor for the Fas/FasL system, known as DcR3 or TR6, was recently identified by searching expressed sequence tag databases for novel TNF receptor family members (Pitti et al., 1998). TR6 lacks an obvious transmembrane domain, suggesting that it may be a secreted soluble receptor for a ligand in the TNF family. TR6 does in fact bind the TNF family member, FasL, inhibiting apoptosis mediated by this ligand in vitro (Pitti et al., 1998). It also binds another TNF family member, LIGHT (Zhi et al., 1998; Nauri et al., 1998), inhibiting apoptosis mediated by this protein (Yu et al., 1999).

Although TR6 has been shown to inhibit FasL-mediated killing in vitro (Pitti et al., 1998), TR6-mediated inhibition of FasL activity in an in vivo model has not yet been demonstrated. Here we describe the synthesis and in vivo biological activity of a TR6 fusion protein produced using the full-length coding region of TR6 and an Fc domain of IgG1. Biochemical and biological characterization of this TR6-Fc form revealed it not only to bind FasL and inhibit apoptosis in vitro, but also to block mortality and greatly attenuate the hepatic apoptosis associated with intravenous injection of cross-linked FasL into Fas+ mice. These in vivo results suggest that there is therapeutic potential for use of TR6 in diseases such as hepatitis where Fas/FasL is implicated in mediating organ damage.

Materials and Methods

Animals. Female BALB/c mice (20–25 g) were obtained from Charles River Laboratories (Raleigh, NC). Female MLR/lpr mice (30–35 g) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed five per cage, and kept under standard conditions for one week before being used in experiments. The animals were maintained according to National Research Council standards for the care and use of laboratory animals. The animal protocols used in this study were reviewed and approved by the Human Genome Sciences Institutional Animal Care and Use Committee.

Human TR6, TR6-Fc, and Fas-Fc Expression Vectors. Full-length TR6 was amplified by the polymerase chain reaction (PCR) and subcloned into a baculovirus expression vector. To generate a TR6-Fc fusion, the full-length TR6 open reading frame was amplified by PCR and translationally fused to the N-terminus of the Fc domain of IgG1 including the hinge region in a baculovirus expression vector. To generate a Fas-Fc expression vector, the extracellular region of Fas was amplified by PCR and translationally fused to the IgG1 hinge and Fc domain in a mammalian expression vector.

Cells infected with baculovirus (Rooney et al., 2000) clone pA2TR6Fc were grown in media containing 1% ultra-low IgG serum. Conditioned culture supernatant (20 liters) was adjusted to pH 7.0, filtered through a 0.22-μm filter, and loaded on a Protein A column previously conditioned with 20 mM phosphate buffer with 0.5 M NaCl, pH 7.2. The column was washed with 15 column volumes of 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.2, and followed by 5 column volumes of 0.1 M citric acid (pH 5.0). TR6-Fc, eluted with 0.1 M citric acid (pH 2.4)/20% glycerol, and fractions were neutralized with 1 M Tris-HCl, pH 9.2. The human TR6-Fc-positive fractions were determined by SDS-polyacrylamide gel electrophoresis (PAGE). The peak fractions were pooled and concentrated using an Amicon concentrator. The TR6-Fc concentrate was then loaded onto a Superdex 200 column containing phosphate-buffered saline (PBS) and 0.5 M NaCl. The included TR6-Fc-positive fractions were identified using nonreducing SDS-PAGE. The TR6-Fc-positive fractions eluting as disulfide-linked dimers were pooled and further concentrated with CentriPlus 10,000 mol. wt. cutoff spin concentrators.

To confirm purity, TR6-Fc protein was blotted to a ProBlott membrane cartridge (PE BioSystems, Inc., Foster City, CA). After staining with Ponceau S (0.2% in 4% acetic acid), the membrane was placed in a “Blot Cartridge” and subjected to N-terminal amino acid sequence analysis using a model ABI-494 sequencer (PE BioSystems, Inc.) and the gas-phase blot cycles. Proteins were subject to reverse phase high-performance liquid chromatography analysis to assess purity. In the case of Fas-Fc, the N terminus was unblocked using pyroglutamate aminopeptidase followed by N-terminal sequence analysis. TR6 protein was expressed in the baculovirus system as described above.

Human Fas-Fc fusion protein was purified by removing conditioned media from cultured Chinese hamster ovary cells and capturing protein on a Poros 50 Protein A affinity column with elution at 0.1 M citrate, pH 2.0, as described for TR6-Fc. Further purification was effected by size separation on a Superdex 200 gel filtration resin in PBS/glycerol. N-Terminal sequence of Fas-Fc was blocked and protein identity was confirmed after digestion with pyroglutamate aminopeptidase to unblock the N terminus and 16% SDS-PAGE, respectively. The protein behaved as a disulfide-linked dimer, as expected for an Fc fusion protein.

Synthesis of Human FasL and FLAG-FasL. The extracellular region of FasL (amino acid Q130-L177) was amplified by PCR and fused at its N terminus to the FLAG epitope and the preprotryptsin signal peptide in the cytomegalovirus-FLAG vector (Sigma, St. Louis, MO). The FLAG-FasL vector was transiently expressed in HEK293T cells, and 1 liter of conditioned media was collected. Conditioned media from 293T cells transiently transfected with FLAG-FasL expression vector were used for purification. FLAG-FasL was affinity-purified using an anti-FLAG M1 column (Sigma). The purified protein was analyzed by size exclusion chromatography on a Superdex 200 column equilibrated in PBS and 10% glycerol. The relative molecular size was estimated using molecular mass standards (Bovine serum albumin, 66 kDa; soybean trypsin inhibitor, 21 kDa; and aprotinin, 6 kDa). The fractions corresponding to FasL trimer were pooled.

BIACore Chip Preparation and Analysis. TR6, TR6-Fc, and Fas-Fc proteins were covalently immobilized to the BIACore sensor chip (CM5 chip) via an amine group on a N-ethyl-N-(dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide chemistry. Various dilutions of FLAG-FasL were passed over the receptor-derivatized flow cells at 15 μl/min. The amount of bound protein was determined during washing of the flow cell with HEPES buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant P20). Binding specificity to receptor was determined by competition with a soluble competitor in the presence of FLAG-FasL. The flow cell surface was regenerated by washing with 20 μl of 10 mM glycine-HCl buffer, pH 2.3, to displace bound protein. For kinetic analysis, the flow cells were tested at different flow rates, with the low receptor density and the on/off rates determined using a kinetic BIA-3 evaluation program, wherein a binding curve with a χ2 < 10 was considered a good fit. The χ2 values resulting from the flow cells described above were all below 6, using a 1:1 binding model.

In Vitro Soluble Human FasL-Mediated Cytotoxicity. Fresh human hepatocytes were prepared from nontransplantable livers and supplied preplated into collagen coated 96-well plates by In Vitro Technologies, Inc. (Baltimore, MD). Hepatocytes were treated with increasing amounts of FLAG-FasL in the presence of 1 μg/ml anti-FLAG antibody (Alexis, San Diego, CA). To determine the ability of
TR6-Fc to inhibit FasL-mediated killing of human hepatocytes, some wells received 1 μg/ml of TR6-Fc or 1 μg/ml of an irrelevant Fc fusion protein, TR11-Fc. Final well volume was 200 μl. The plates were incubated for 18 h at 37°C before assessing final cell viability using AlamarBlue (BioSource International, Camarillo, CA) for an additional 4 h per kit instructions. AlamarBlue is a vital dye that is taken up by viable intact cells. After incubation, cells are washed to remove excess dye and solubilized with detergent. As cell viability falls, the optical density will also decrease proportionately. The plates were read in a CytoFluor fluorescence plate reader using excitation of 530 nm and emission of 590 nm. Each assay point was performed in triplicate, and the mean and standard error of the mean were calculated.

The HT-29 cell line, a human colon adenocarcinoma cell line obtained from the American Type Culture Collection (ATCC HTB-38), is sensitive to FasL-mediated cytotoxicity, presumably through activation of its Fas receptor. HT-29 cells were grown in Dulbecco’s minimum essential media with 10% fetal bovine serum, 2 mM glutamine/penicillin/streptomycin. To measure FLAG-FasL-induced cytotoxicity, target cells were trypsinized, washed, and plated in a 96-well plate at 50,000 cells/well. HT-29 cells were treated with cross-linked FLAG-FasL plus FLAG antibody (1 μg/ml), or with cross-linked FLAG-FasL in combination with Fas-Fc, or TR6. Because antibody cross-linking of FasL via its FLAG domain significantly enhances the ability of FasL to mediate cell death, the FLAG antibody was included. The final volume in each well was 200 μl. After 5 days of culture, the plate was harvested and 20 μl of AlamarBlue reagent added. To assess final viability, cells were incubated for 4 h and the plate analyzed in a CytoFluor fluorescence plate reader with excitation of 530 nm and emission of 590 nm. Each assay point was performed in triplicate, and the mean and standard error of the mean were calculated.

The Jurkat human T cell line, which also expresses the Fas receptor and is sensitive to FasL (Pitti et al., 1998), was tested in an in vitro cytotoxicity assay similar to that used on HT-29 cells. In addition, Jurkat cells were evaluated by fluorescence-activated cell sorter (FACS) analysis in an apoptosis assay. Jurkat cells (RPMI + 5% fetal bovine serum) were seeded at 50,000 cells/well and then treated with FLAG-FasL and FLAG mouse monoclonal antibody (200 ng/ml) before being incubated at 37°C for 16 h to induce apoptosis. When TR6 or Fas-Fc was included in the assay, the decoy receptor protein was preincubated with FLAG-FasL plus FLAG antibody for 15 min. To determine the degree of apoptosis, cells were harvested, stained with annexin-V and propidium iodide, and evaluated using FACS analysis.

In Vitro Membrane-Bound Murine FasL-Mediated Cytotoxicity. To analyze the in vitro killing of Fas+ target cells by murine FasL, murine effector L929 cells (2.5 × 10⁵ cells/well) transfected with murine FasL were incubated with Fas+ murine A20 target cells (5 × 10⁴ cells/well) labeled with Europium Titriplex V, diethylene-triamine-pentaacetic acid (Eu-DTPA chelate; Wallac Oy, Turku, Finland).

A-20 target cells were labeled with Eu-DTPA chelate in the presence of dextran sulfate, which made the cells permeable to the chelate. The cell wall was closed by washing cells in the presence of CaCl₂ and glucose. Following an 18 h incubation with effector L929 cells at an effector/target cell ratio of 50:1, Eu-DTPA chelate released from the A-20 target cell was detected in supernatant using an enhancer solution that favors dissociation of the Europium from the chelate that was measured using fluorescence. Each point was measured in triplicate and the mean percentage of cell death calculated as

\[
\text{Mean experimental Eu release} - \text{Spontaneous Eu release} / \text{Mean maximum Eu release} - \text{Spontaneous Eu-release} \times 100.
\]

Maximum release was measured by mean amount of dye released from cells upon lysis with detergent.

In Vivo FasL-Mediated Killing. Soluble human FLAG-FasL was synthesized at Human Genome Sciences Inc. (Rockville, MD). To induce cross-linking, FasL was incubated with FLAG antibody (Sigma) and injected intravenously into mice following a variation of the procedure used by Schneider et al. (1998). Test proteins were injected i.v. or s.c. at various time points before FasL injection, and mortality was recorded over time.

In liver biopsy experiments, 1-cm² liver samples were fixed in 10% neutral-buffered formalin for 24 h and then transferred to 70% methanol until it was time for embedding in paraaffin. Liver sections were stained with hematoxylin and eosin and scored in a blinded fashion for the severity of hepatocellular apoptosis. The results were expressed as the mean of the grading scale for each group where 0 = <1 apoptotic cell per five 10² fields; 1 = 1 apoptotic cell per two to five 10² fields; 2 = 1 to 4 apoptotic cells per one 10² field; 3 = 5 to 20 apoptotic cells per one 10² field; and 4 = >20 apoptotic cells per one 10² field.

Statistics. Statistical difference between groups was determined using an unpaired, two-tailed Student’s t test. Error bars represent S.E.M.

Results

BIAcore Analysis of Receptor Binding to FasL. The purified receptor proteins were subject to BIAcore analysis to determine the relative affinities of human TR6, TR6-Fc, and Fas-Fc toward human FasL. The purified receptor proteins were immobilized onto the BIAcore flow cell surface, and the rates of association and dissociation of soluble FLAG-FasL were determined and used to calculate K₅₀ values. The various kinetic values for FasL binding are shown in Table 1. The K₅₀ for binding of FasL to TR6, 17.2 nM, was approximately 4-fold higher than the 4.6 nM value determined for TR6-Fc. The K₅₀ value for Fas-Fc binding to FasL was 7.4 nM, similar to that of TR6-Fc. The BIAcore data indicate that all the purified receptors show relatively high-affinity binding toward FLAG-FasL, although for reasons unknown, the affinity of TR6 is somewhat less than that of TR6-Fc.

<table>
<thead>
<tr>
<th>Soluble Receptor</th>
<th>ka (1/Ms)</th>
<th>kd (1/s)</th>
<th>K₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR6-Fc</td>
<td>1.63 × 10⁵</td>
<td>7.5 × 10⁻⁴</td>
<td>4.61</td>
</tr>
<tr>
<td>TR6</td>
<td>3.3 × 10⁵</td>
<td>5.74 × 10⁻³</td>
<td>17.20</td>
</tr>
<tr>
<td>Fas-Fc</td>
<td>1.57 × 10⁵</td>
<td>1.17 × 10⁻³</td>
<td>7.44</td>
</tr>
</tbody>
</table>

ka, rate of association; kd, rate of dissociation.

In Vivo FasL-Mediated Killing of Primary Human Hepatocytes, HT-29 Cells, and Jurkat Cells. Since the liver is one of the major target organs of FasL-mediated apoptosis (Patel et al., 1998), it was important to determine whether TR6 could block FasL killing of human hepatocytes in vitro. Primary human hepatocytes were incubated with increasing amounts of FLAG-FasL in the presence of 1 μg/ml of cross-linking anti-FLAG antibody (Alexis Biochemicals). At a concentration of approximately 10 ng/ml, FasL exhibited maximal cytotoxicity, killing >75% of the human hepatocytes (Fig. 1). Incubation with 1 μg/ml of TR6-Fc (but not TR11-Fc) completely abrogated killing, until the FasL concentration was increased 100-fold to 1000 ng/ml.

Figure 2 illustrates the ability of TR6 to block FasL-mediated killing...
Materials and Methods

TR11-Fc. Results represent the mean \( p \) between activity of the FasL control and that of the treatment groups. **

Effect of soluble receptors on human FasL-mediated killing of primary human hepatocytes. FasL-mediated cytotoxicity was measured in an AlamarBlue viability assay, using a fluorescence plate reader with an excitation wavelength of 530 nm and emission wavelength of 590 nm. An unpaired, two-tailed Student’s test was used to evaluate the statistical difference between activity of the FasL control and that of the treatment groups. *** \( p < 0.001 \); ** \( p < 0.01 \); * \( p < 0.05 \); † \( p < 0.1 \).

In Vivo Lethality of FasL. To determine its minimum lethal dose, 1, 3, 6, or 13 \( \mu \)g of FLAG-FasL was mixed with 4, 12, 25, or 50 \( \mu \)g of cross-linking FLAG antibody and injected intravenously into BALB/c mice (n = 3). Only the mice injected with the lowest, 1-\( \mu \)g dose of FasL survived. Thus, the minimum lethal dose of cross-linked FasL appeared to be approximately 3 \( \mu \)g/mouse.

To establish that the in vivo mechanism of FasL-induced lethality was due to binding of FasL to its cell-bound Fas receptor, 5 mg of FLAG-FasL was mixed with 19 mg of FLAG antibody and injected into either Fas-/- MRL/lpr -/- mice or Fas+ MRL/lpr +/- control mice. Within 30 min, all the Fas-/- MRL/lpr -/- mice were dead, while all the Fas+ MRL/lpr +/- mice survived. This indicated that in vivo FasL killing was dependent on the expression of Fas receptor on the target cells.

In Vivo Effect of Soluble Receptors on FasL-Induced Mortality in Mice. To determine whether TR6-Fc could block a high dose of FasL, female BALB/c mice (n = 20) were injected intravenously with 13 \( \mu \)g of FLAG-FasL mixed with 50 \( \mu \)g of murine antibody (Ab) to FLAG. Half of the mice also received an intravenous injection of 96 \( \mu \)g of TR6-Fc 1 h before administration of FasL. TR6-Fc has a molecular weight of about 60,000, compared with 18,500 for FasL, which resulted in a TR6-Fc/FasL molar ratio of 2.3:1, with each molecule of TR6-Fc capable of binding two molecules of FasL. Within 1 h of FasL injection, all the mice injected only with cross-linked FLAG-FasL were dead. There were no deaths in the cross-linked FasL group treated with 96 \( \mu \)g of TR6-Fc. In a third group of mice injected with FLAG-FasL...
not cross-linked by FLAG antibody, there were no deaths (data not shown), in keeping with other reports on the poor activity of soluble, monomeric FasL (Schneider et al., 1998).

In a dose-response experiment, TR6-Fc was injected intravenously at a dose of 2, 8, or 24 μg/mouse 1 h before intravenous injection with FLAG-FasL (4 μg/mouse) mixed with 12 μg of FLAG antibody (Table 2). This results in TR6-Fc/FasL molar ratios of approximately 1:6, 1:2, and 2:1, respectively. In the FasL control group, all but 1 of the 10 mice died within 2 h. The low dose of TR6-Fc (2 μg) had no protective activity, and all the animals died within 2 h. The middle dose of TR6-Fc (8 μg) prolonged life an additional 2 h. The high, 24-μg dose of TR6-Fc was most protective, with 7 of the 10 mice surviving without weight loss to the end of the experiment on day 7. This indicates not only that TR6-Fc was efficacious at a molar ratio of 2:1, but also that its protective effect was not lost over time.

In a separate experiment, TR6-Fc, when injected 10 min before cross-linked FasL, completely blocked FasL-induced mortality with a minimum effective dose of 7 μg/mouse (data not shown). In the same experiment, intravenous injection of 70 μg/mouse of monomeric TR6 with no Fc completely blocked FasL-induced mortality when injected 10 min before FasL, but it was not protective when given an hour before FasL, presumably because of its shorter half-life compared with TR6-Fc.

To determine whether TR6-Fc exhibited protective activity when injected subcutaneously as opposed to intravenously, 350 μg of TR6-Fc was injected s.c. 1, 3, or 5 h before i.v. injection of 4 μg of FLAG-FasL mixed with 15 μg of FLAG antibody (Table 3). Even at the receptor/ligand molar ratio of 27:1, none of the animals injected subcutaneously with 350 μg of TR6-Fc survived for more than 2 h, whereas all animals injected intravenously with 93 μg of TR6-Fc or Fas-Fc 1 h before FasL were protected. In this experiment, a different member of the TNF receptor superfamily, TR-11 (93 μg/mouse), was used as a negative control, and it failed to protect any animals from FasL-induced death.

The role of hepatocellular apoptosis in FasL-induced mortality was not specifically investigated in this series of experiments. The rapid onset of mortality after FasL treatment suggests that the cause of death was probably related to a perturbation in cardiovascular and/or pulmonary function. When the mice were necropsied, the heart appeared blanched, almost white, while the liver, apparently suffused with blood, looked almost black. The presumed hypotension in the moribund FasL control animals just before death was so great that no blood could be obtained from intracardiac puncture. The extreme degree of hepatic apoptosis depicted in Table 4 clearly correlates with mortality. The severe apoptosis may have precipitated hepatic vascular changes, setting
off a cascade of systemic events leading to probable cardio-
pulmonary collapse and death.

**Histological Evaluation of Livers from Mice Injected with FasL.** Since hepatocytes seem to be particularly sensitive to the cytotoxic effects of FasL (Ogasawara et al., 1993; Patel et al., 1998; Schneider et al., 1998), histologic evaluation was made of livers from mice injected intravenously with 1) cross-linked-FasL (4 μg/mouse); 2) TR6-Fc (93 μg/mouse) 1 h before FasL; and 3) normal vehicle-injected controls. One to 2 h after FasL injection, mice were euthanized by cervical dislocation. Liver tissue was immediately removed, fixed in 10% neutral-buffered formalin, processed, embedded, and sectioned by routine methods, and then stained with hematoxylin and eosin for light microscopy. Liver sections were scored in a blinded fashion for the severity of hepatocellular apoptosis. The results were expressed as the mean of the grading scale for each group, where 0 = <1 apoptotic cell per five 10× fields; 1 = 1 apoptotic cell per two to five 10× fields; 2 = 1 to 4 apoptotic cells per one 10× field; 3 = 5 to 20 apoptotic cells per one 10× field; and 4 = >20 apoptotic cells per one 10× field. The data summarized in Table 4 indicate that injection of cross-linked FasL alone resulted in a score of 3.9, whereas pretreatment with TR6-Fc significantly reduced that score to 1.3.

In FasL-treated mice, marked hepatocellular apoptosis was present in the midzonal to periportal regions (Fig. 5A). A narrow plate of periportal hepatocytes and bile duct epithelial cells remained unaffected, as did centrilobular hepatocytes. Apoptosis was characterized by contiguously arranged hepatocytes with cellular shrinkage, nuclear fragmentation, cytoplasmic condensation and hypereosinophilia, and occasional apoptotic body formation. Sinusoids were mildly to severely dilated with blood. In contrast, prior treatment with TR6-Fc greatly attenuated FasL-induced apoptosis. In TR6-Fc-pretreated mice, hepatocellular changes consisted of only minimal to mild increases in hepatocellular apoptosis (Fig. 5B). Apoptotic cells occurred singularly and in small clusters (2–5 cells) in midzonal, and occasionally in periportal, areas, and they were characterized by hepatocellular shrinkage, nuclear fragmentation, cytoplasmic condensation and intense hypereosinophilia, and apoptotic body formation. Only

---

**TABLE 2**

Dose-dependent effect of TR6-Fc (i.v.) on FasL-induced mortality

TR6-Fc was injected i.v. at a dose of 2, 8, or 24 μg/mouse (n = 10), 1 h before i.v. injection with FLAG-FasL (4 μg/mouse) mixed with 12 μg of FLAG antibody, as described under **Materials and Methods.** The results were expressed as the mean of the grading scale for each group, where 0 = <1 apoptotic cell per five 10× fields; 1 = 1 apoptotic cell per two to five 10× fields; 2 = 1 to 4 apoptotic cells per one 10× field; 3 = 5 to 20 apoptotic cells per one 10× field; and 4 = >20 apoptotic cells per one 10× field. The data summarized in Table 4 indicate that injection of cross-linked FasL alone resulted in a score of 3.9, whereas pretreatment with TR6-Fc significantly reduced that score to 1.3.

---

**TABLE 3**

Effect of soluble receptors on FasL-induced mortality

TR6-Fc was injected s.c. 1, 3, or 5 hours before i.v. injection of 4 μg of FLAG-FasL, mixed with 15 μg of FLAG antibody. As a positive standard, TR6-Fc or FasFc at 93 μg/mouse was injected i.v. 1 h before FasL. The negative control, TR-11 (93 μg/mouse, i.v.), was also injected 1 h before FasL injection. For details of the procedure, see **Materials and Methods.**

---

**Fig. 4.** Effect of soluble receptors on membrane-bound murine FasL-mediated cytotoxicity. Mouse L929 cells transfected with murine FasL were incubated with murine A-20 cells containing Eu-DTPA, as described under **Materials and Methods.** TR2-Fc was used as a negative control protein. Each point was measured in triplicate, and the mean percentage of cell death was calculated as follows: (Mean experimental Eu release – Spontaneous Eu release)/Mean maximum Eu release × 100. An unpaired, two-tailed Student’s t test was used to evaluate the statistical difference between activity of the FasL control and that of the treatment groups. **p < 0.01; ***p < 0.001. TR6-Fc; ▲, TR8; ○, TR2-Fc; ○, Fas-Fc. Results represent the mean ± S.E.M. For details, see **Materials and Methods.**
rare apoptosis (1 per liver section) was noted in the vehicle-treated control mice (Fig. 5C).

**Discussion**

Programmed cell death, or apoptosis, is the result of a series of specific enzymatic reactions leading to nuclear condensation and a characteristic pattern of DNA fragmentation (Ashkenazi and Dixit, 1998). Under normal conditions, apoptosis is a well-defined, controlled series of events, driven by the need to remove unwanted or unneeded cell populations (Lynch et al., 1995). However, the process becomes pathological when some as yet undetermined signal triggers the body to turn upon itself, causing autoimmune disease (Thompson, 1995). A major contributor to this apoptotic process is the interaction of FasL with the Fas receptor of the target cell. FasL hyperactivity has been implicated in a number of autoimmune diseases including graft versus host disease (Baker et al., 1997) and diabetes (Stassi et al., 1997). Modulation of the FasL/Fas pathway, therefore, may provide a potential avenue of treatment for a range of human diseases.

In the current study, we expand the characterization of TR6, a recently identified natural soluble decoy receptor of FasL also called DcR3 (Pitti et al., 1998). First, using BIAcore technology, we demonstrate that the association between FasL and the human recombinant fusion protein, TR6-Fc, is of high affinity and is similar to the affinity FasL has for Fas-Fc (Table 1; $K_d = 4.61$ versus 7.44 nM, respectively). The reason for the difference in potency between Fas-Fc and TR6-Fc in the in vitro FasL-mediated cytotoxicity assays is not clear. Both Fc molecules have two FasL receptors. It is possible that differing stereochemistries between TR6-Fc and Fas-Fc could confer increased binding avidity on TR6-Fc. The $K_d$ derived from the BIAcore assay is a measurement of the binding affinity of a single receptor to its ligand. It is not capable of distinguishing whether the receptor at the binding site is a monomer or part of a multimer; therefore, binding data may not directly correlate with activity in a cellular assay.

In terms of in vitro assays, using soluble FasL as the cytotoxic agent, we demonstrate that decoy receptors can block FasL-mediated apoptosis in target cells including primary hepatocytes (Fig. 1), HT29 cells (Fig. 2), and Jurkat cells (Fig. 3, A and B). Its efficacy against an increasing concentration of FasL indicates that TR6-Fc consistently inhibited FasL-induced cytotoxicity even when the concentration of FasL was increased to 100 ng/ml. In contrast, TR6 blocked FasL-mediated killing of Jurkat cells up to a FasL concentration of 10 ng/ml, and it was much less effective in protecting HT29 cells, losing efficacy when the FasL concentration passed 0.1 ng/ml. This cell-dependent difference in TR6 efficacy may not be entirely due to the level of Fas protein expression on the target cell, but it may also involve intracellular mediators that regulate the apoptotic cascade. Historically, HT29 cells have proved to be more sensitive than Jurkat cells to the cytotoxic effects of FasL. Thus, the sensitivity to FasL differs between cell lines, the amount of TR6 inhibitor protein needed to block FasL-mediated killing may also differ. In addition, HT29 cell death is typically evaluated in a 5-day assay, while the Jurkat cell assay lasts 2 days. It is possible that HT29 cells, being more sensitive to FasL killing, and being exposed to the ligand for a longer time, would require more TR6 for protection. Also, the HT29 cell line is adherent, while the Jurkat line is a suspension culture, making it difficult to compensate for such variables.

**Table 4**

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal, Vehicle Control $n = 10$</th>
<th>FasL control (4 mg/mouse, i.v.) $n = 15$</th>
<th>FasL (4) + TR6-Fc (93 mg/mouse, i.v.) $n = 15$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis score</td>
<td>0.1±0.1***</td>
<td>3.9±0.07</td>
<td>1.3±0.25***</td>
</tr>
</tbody>
</table>

**Fig. 5.** Histologic features of hepatocytes from the livers of mice injected with 1) cross-linked-FasL (4 mg/mouse, i.v.); 2) TR6-Fc, 93 mg/mouse, i.v., 1 h before FasL; and 3) normal vehicle-injected controls. One to 2 h after FasL injection, liver tissue was removed, fixed in 10% neutral-buffered formalin, processed, embedded, and sectioned by routine methods, and stained with hematoxylin and eosin for light microscopy. Microscopic examination showed massive hepatic apoptosis (severity score 5). A portal area is positioned on the left side of each photomicrograph. Microscopic examination showed massive hepatic apoptosis (severity score 5). A portal area is positioned on the left side of each photomicrograph. Microscopic examination showed massive hepatic apoptosis (severity score 5). A portal area is positioned on the left side of each photomicrograph.
as rate of proliferation and amount of exposed cell surface area. Using an in vitro murine model of membrane-bound FasL-mediated apoptosis, we show that TR6 and TR6-Fc are equally efficacious in blocking the functional association between mouse cell-bound FasL and Fas (Fig. 4). This similar degree of FasL antagonism contrasts with results of studies with soluble FasL-killing of Jurkat and HT29 cells. The difference may depend upon the relative concentration of FasL in the assay system. Although an exact measurement has not been made, the level of membrane FasL is likely to be significantly lower than the levels of exogenous soluble FasL added to Jurkat cells in the killing assay shown in Fig. 3A. Since both TR6 and TR6-Fc bind FasL, they should show a similar level of efficacy in the presence of low levels of FasL, the difference in activity only becoming obvious as the concentration of FasL increases. Thus, in the L929 assay (Fig. 4), where membrane-bound FasL is likely to be present in only low concentrations, TR6 and TR6-Fc exhibit a similar degree of activity. In the Jurkat assay (Fig. 3A), however, FasL is titrated at ever higher and nonphysiological concentrations. Until addition of FasL at 100 ng/ml, the blocking activity of TR6 and TR6-Fc is identical. It is only at the very highest concentration of FasL that the antagonist activity of TR6 is overwhelmed.

In vivo, prophylactic administration of soluble decoy receptors blocked the hepatic apoptosis and mortality associated with the intravenous injection of cross-linked FasL into Fas−/− mice (Table 2, 3 and 4). TR6-Fc, TR6 and Fas-Fc all blocked FasL-induced mortality, which could have been predicted based on their high binding affinity for FasL, as measured by BLAcore analysis. It was impossible to determine whether the 4-fold difference in affinity between TR6 and TR6-Fc (Ka = 17.20 versus 4.61 nM) translated into reduced in vivo potency for TR6 compared with TR6-Fc, since a full in vivo dose-response curve was generated only for TR6-Fc. However, the in vivo data in the FasL mortality model does confirm the role of TR6-Fc as a decoy receptor for FasL, and supports a potential for the use of TR6-Fc in the modulation of diseases associated with Fas/FasL hyperactivity.

In addition to the model of liver injury described above, investigators have used transgenic and knockout mice to help determine the relevance of Fas/FasL involvement in a given disease (Nakamoto et al., 1997). In mouse models of graft-versus-host disease, the severity of disease is reduced when the graft cells are obtained from FasL−/− rather than positive stock (Baker et al., 1997). Likewise, in murine allergic encephalomyelitis, Fas−/− or FasL−/− mice have a greatly reduced pathology compared with control mice (Waldner et al., 1993; D'Souza et al., 1996).

Although the in vivo data in the FasL mortality model does confirm the role of TR6-Fc as a decoy receptor for FasL, and supports a potential for the use of TR6-Fc in the modulation of diseases associated with Fas/FasL hyperactivity, it is by no means definitive. To pick a clinical target, it is prudent to examine the association of FasL with human disease. FasL/Fas interactions have been implicated in several clinical conditions (DeMaria and Testi, 1998; Maggi, 1998). The liver, with its abundance of Fas+ hepatocytes, has often been cited as a target organ in FasL-associated diseases such as alcoholic cirrhosis (Nanji, 1998), viral hepatitis (Lau et al., 1998), and acute hepatic failure (Galle and Krammer, 1998). In common with hepatocytes, plaques from patients with multiple sclerosis have also been shown to express high levels of Fas and FasL (Suda et al., 1993; D’Souza et al., 1996).

FasL up-regulation has been noted in a number of autoimmune diseases. Patients with noninsulin-dependent diabetes have a superabundance of Fas+ T cells associated with their islet cells (Staissi et al., 1997), whereas patients with chronic renal failure over-express both FasL and Fas, leading to cellular fratricide (Schelng et al., 1997) of the renal tubule epithelium. In ulcerative colitis, Fas expression is observed on colonic epithelial cells, and FasL on lamina propria lymphocytes of patients with active lesions, but not those in remission (Ueyama et al., 1998). The neutrophil depletion seen in patients with large granular lymphocyte leukemia has been attributed to the high levels of circulating serum FasL (Tanaka et al., 1996). The possible involvement of FasL-induced apoptosis in these diseases has generated interest in the development of a FasL decoy receptor that could be used therapeutically to block FasL-mediated pathology.

Current work is focused on developing more relevant in vivo models of clinical disease. The soluble FasL model, where delivery of a 4-μg i.v. bolus injection of exogenous FasL results in 100% mortality within 2 h, is not a clinically relevant model of disease. It is not intended to be used to predict the effects of a therapeutic agent in a clinical setting with physiological levels of FasL. The model was chosen to establish the in vivo ability of TR6 to act as a decoy receptor and unambiguously block the lethal effects associated with FasL toxicity. This it clearly did. The issue is not whether TR6 will antagonize FasL activity in the clinic, but rather, in which disease is the presence of FasL most critical, and therefore most likely to be affected by TR6 treatment. That is a difficult question and one that may not be answered until TR6 is in clinical development.

Acknowledgments

We thank Nick Madery and his histology staff for preparation of tissue for histological evaluation.

References


Pages 33 and 34 of the document are not visible or readable in the image.