TRAIL Inhibits Tumor Growth but Is Nontoxic to Human Hepatocytes in Chimeric Mice

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Abstract

Tumor necrosis factor (TNF) family ligand TNF-α and Fas ligand (FasL) can trigger apoptosis in solid tumors, but their clinical usage has been limited by hepatotoxicity. TNF-related apoptosis-inducing ligand (TRAIL) is a newly identified member of the TNF family, and its clinical application currently is under a similar debate. Here, we report a recombinant soluble form of human TRAIL (114 to 281 amino acids) that induces apoptosis in tumor cells but not human hepatocytes. We first isolated human hepatocytes from patients and showed that the human hepatocytes expressed Fas but no TRAIL death receptor DR4 and little DR5 on the cell surface. Antibody cross-linked FasL, but not TRAIL, triggered apoptosis of the human hepatocytes through cleavage of caspas. We then examined TRAIL hepatotoxicity in severe combined immunodeficient/Alb-uPA chimeric mice harboring human hepatocytes. Intravenous injection of FasL, but not TRAIL, caused apoptotic death of human hepatocytes within the chimeric liver, thus killing the mice. Finally, we showed that repeated intraperitoneal injections of TRAIL inhibited intraperitoneal and subcutaneous tumor growth without inducing apoptosis in human hepatocytes in these chimeric mice. The results indicate that the recombinant soluble human TRAIL has a profound apoptotic effect on tumor cells but is nontoxic to human hepatocytes in vitro and in vivo.

Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a recently identified tumor necrosis factor (TNF) family member (1, 2) and currently is under development as a potential chemotherapeutic agent because it kills many types of tumor cells but spares normal cells in cultures and experimental animals (3, 4). However, a polyhistidine-tagged human TRAIL was reported to kill isolated human hepatocytes (5), raising the same concern as noted with TNF-α and Fas ligand (FasL; ref. 6). Contradictory to this was a recent study reporting that nontagged human TRAIL (114 to 281 amino acids) was not toxic to isolated human hepatocytes and was well tolerated in nonhuman primates (7). In this study, we generated chimeric mice with human hepatocytes (8) and observed that recombinant nontagged form of human TRAIL (114 to 281 amino acids) inhibited tumor growth without inducing apoptosis of the human hepatocytes, whereas antibody cross-linked Flag-tagged human FasL triggered apoptotic death in the human hepatocytes and thus killed the chimeric mice.

Materials and Methods

Human Hepatocyte Isolation and Culture. For human hepatocyte isolation, segments of human liver tissue were obtained from specimens following surgical removal. The University of Alberta Faculty of Medicine and Dentistry Research Ethics Board approved this study. Hepatocytes were isolated by collagenase-based perfusion using 0.38 mg/mL Liberase CI (Boehringer, Ingelheim, Germany) in perfusate (DuPont, Wilmington, DE; ref. 8). Cells were plated in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and 1% antibiotics.

Cell Death and Flow Cytometry Assay. Isolated human hepatocytes and WM793 and H460 cell lines were seeded in 96-well plates at 3 × 105 cells/well and treated with the recombinant soluble human TRAIL (114 to 281 amino acids, M, 19,600; provided by PeproTech, Inc., Rocky Hill, NJ) or antibody cross-linked FasL (Alexis, San Diego, CA). Cell death was determined by the crystal violet assay, and the results were presented as the percentage cell death: 1−(absorbance of cells treated/absorbance at 550 nm of cells untreated) × 100 (9). Cellular apoptosis was examined under phase contrast light microscopy. Cell surface expression of DR4 and DR5 was analyzed by flow cytometry (9). In brief, 0.1 μg/mL of phycoerythrin-conjugated anti-human DR4 and DR5 (mouse IgG1; eBioscience, San Diego, CA) or mouse IgG1 (a negative control; eBioscience) were added to the 106 cells treated) in 200 μL of immuno-fluorescence buffer, and 10,000 cells were analyzed using a Becton Dickinson FACScan (Mountain View, CA), and the data were processed using CellQuest software (Becton Dickinson).

Generation and Treatment of Severe Combined Immunodeficient/Alb-uPA Chimeric Mice. The chimeric mice were generated through crossing hemizygous Alb-uPA transgenic mice (Jackson Laboratory, Bar Harbor, ME) with homozygous severe combined immunodeficient (SCID)/bg mice (strain C-b/h7GmnsTac-SCID/bgN7; Taconic Farms, Germantown, NY), and selective backcrosses bred the SCID trait to homozygosity (8). The litters of homozygous SCID mice carrying the homozygous Alb-uPA transgene were injected with freshly isolated human hepatocytes into inferior splenic pole. Successful engraftment of human hepatocytes was confirmed by serum levels of human α1-antitrypsin >100 μg/mL at 8 weeks after transplantation. The animals were housed in the Veterinary Animal Facility, following Canadian Council on Animal Care Guidelines. Two-month-old chimeric mice were treated with TRAIL or FasL before or after inoculation with tumor cells, as described in Results. Necropsy was performed to examine the mice for tumors. During the necropsy, approximately one third of each chimeric liver was snap frozen in liquid N2 and stored at −80°C for Western blot analysis. The remaining chimeric liver, other organs, and tumors were fixed in 10% formalin and embedded in paraffin. Sections were either stained with H&E or with mouse monoclonal antibody to human hepatocytes (OCHI15, 1:25; Dako, Carpinteria, CA) and rabbit antibody to cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) and visualized by Super Sensitive Immunodetection System (BioGenex, San Francisco, CA).

Human α1-Antitrypsin and Alanine Aminotransferase Assays. For antihuman α1-antitrypsin (hAAT) assay (8), 96-well plates (Corning Inc., Corning, NY) were coated with 100 μL of phenotype serum. After blocking with 5% nonfat dry milk, 100 μL of samples was added per well and incubated at 37°C for 1 h, followed by the addition of anti-human α1-antitrypsin antibody cross-linked to alkaline phosphatase. After washing, 100 μL of p-nitrophenyl phosphate substrate was added, and absorbance was measured at 405 nm. The absorbance was calculated as follows: A – (absorbance of samples/absorbance at 405 nm of wells treated) × 100 (8). The alanine aminotransferase (ALT) activity was measured using 96-well plates (Corning Inc., Corning, NY) and 100 μL of phenotype serum. After blocking with 5% nonfat dry milk, 100 μL of samples was added per well and incubated at 37°C for 1 h, followed by the addition of anti-human α1-antitrypsin antibody cross-linked to alkaline phosphatase. After washing, 100 μL of p-nitrophenyl phosphate substrate was added, and absorbance was measured at 405 nm. The absorbance was calculated as follows: A – (absorbance of samples/absorbance at 405 nm of wells treated) × 100 (8).
ing, NY) were coated overnight at 4°C with goat anti-hAAT antibody (DiaSorin, Saluggia, Italy), and 50 μL of chimeric serum dilution were added and incubated for 2 hours. Standard curves were generated from serial dilutions of human reference serum (Calibrator 4; DiaSorin). The plates were incubated with horseradish peroxidase–linked goat anti-hAAT antibody (Pierce, Rockford IL) for 2 hours, washed, and added with substrate solution (3,3′,5,5′-tetramethyl benzidine dihydrochloride; Sigma, St. Louis, MO). Horseradish peroxidase activity was measured spectrophotometrically, and hAAT levels were calculated with the Softmax software (Molecular Devices, Sunnyvale, CA). The alanine aminotransferase (ALT) in the chimeric serum was analyzed using the ALT Flex reagent cartridge (Dade Behring Inc., Newark, DE) and Dade Dimension chemistry analyzer (Dade Behring Inc.).

**Gel Electrophoresis and Western Blot Analysis.** The recombinant soluble human TRAIL was treated with 100 mmol/L DTT (Sigma) and subjected to SDS-PAGE and stained with Silver Stain Plus kit (Bio-Rad, Hercules, CA). For Western blot analysis, the chimeric liver tissue was homogenized in radioimmunoprecipitation assay buffer [10 mmol/L Tris (pH 8), 140 mmol/L NaCl, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 0.025% NaN3 with the addition of protease inhibitors], and cultured cells were lysed in lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol, 1% Triton X-100, 1% protease inhibitor mixture, and 1 mmol/L phenylmethylsulfonyl fluoride). Fifty micrograms of total proteins from each lysate were separated through 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4°C with mouse monoclonal antihuman caspase-8 (1:1000; Medical & Biological Laboratories, Nagoya, Japan), DNA fragmentation factor 45 (1:1000; StressGen, Victoria, British Columbia, Canada), rabbit antihuman caspase-3 (1:1000; Cell Signaling Technology), DR4, DR5 (1:500; ProSci, Inc., Poway, CA), and extracellular signal-regulated kinase 1/2 (1:1000; StressGen) and developed by chemiluminescence (Amersham Biosciences, Piscataway, NJ).

**Results and Discussion**

**TRAIL Is Nontoxic to Isolated Human Hepatocytes.** TRAIL primarily is expressed as a type II membrane protein with an extracellular COOH-terminal of 114 to 281 amino acids (1, 2) of the receptor binding site (10). The extracellular domain can be cleaved to yield a soluble and biologically active form of 114 to 281 amino acids.
(M, 19,000 to 20,000; ref. 11). TRAIL carries a zinc ion bound by a free cysteine residue (Cys230), which is an essential moiety of TRAIL capacity to induce apoptosis (12). The recently reported recombinant human TRAIL (114 to 281 amino acids) contained sufficient bound zinc ions and formed TRAIL homotrimers and killed tumor cells but was not toxic to isolated human and nonhuman primate hepatocytes (7). In contrast, recombinant polyhistidine-tagged human TRAIL (114 to 281 amino acids) implicated in human hepatotoxicity (5) showed poor zinc ion coordination and the formation of free disulfide-linked dimers (7). We previously have shown antitumor activity of a recombinant nontagged soluble human TRAIL of 114 to 281 amino acids (13). This human TRAIL was homogenous on nonreducing and reducing SDS-PAGE (Fig. 1A).

TRAIL-induced apoptosis occurs through binding of cell surface death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2; ref. 14). The receptors in turn recruit Fas-associated death domain and caspase-8 to form death-inducing signaling complex, in which caspase-8 is activated through autocatalytic cleavage (15, 16). Activated caspase-8 initiates apoptosis through a systemic cleavage of downstream caspase-3, a caspase-8 substrate, and DFF45, a caspase-3 substrate (17). We showed that the recombinant human TRAIL killed human melanoma cell line WM793, as shown by cell viability assay (Fig. 1B), through caspase cascade, as evident by the appearance of caspase-8 (p18), caspase-3 (p20, p17, and p10), and DFF45 (p25, p17, and p11) cleavage products on Western blot analysis (Fig. 1C). In contrast, Western blot analysis showed no cleavage of caspase-8, caspase-3, and DFF45 (Fig. 1C); a cell viability assay revealed no significant cell death (Fig. 1B); and phase contrast microscopy observed no cellular apoptosis (Fig. 1D) in human hepatocytes after exposure to TRAIL (300 ng/mL). Similar results were observed in human hepatocytes freshly prepared from 10 patients and maintained for 4 days either in DMEM or William's E medium.

Flag-tagged FasL cross-linked with anti-Flag antibody was reported to kill human hepatocytes in culture (18). We observed caspase-8, caspase-3, and DFF45 cleavage (Fig. 1C), significant cell death (Fig. 1B), and cellular apoptosis (Fig. 1D) in the human hepatocytes exposed to the antibody cross-linked Flag-FasL (50 ng/mL. FasL mixed with 2 μg/mL antibody). The results indicate that caspase-8–initiated apoptotic machinery exists in human hepatocytes and can be activated by FasL, but not by TRAIL.

DR4 and DR5 transcripts were reported in human liver tissue and isolated human hepatocytes (5). However, studies of DR4 and DR5 proteins have produced controversial results; one showed DR4 and DR5 on human hepatocyte surface (19), whereas others reported no DR5 (20). We compared DR4 and DR5 expression between isolated human hepatocytes and tumor cells. DR4 protein was not detected either by flow cytometry (Fig. 1E) or on Western blot analysis (Fig. 1F), whereas flow cytometry showed a little DR5 expression on human hepatocyte surface (Fig. 1E). DR5 proteins exist in three isoforms: one p60 intracellular form and two membrane p43 and p49 forms (15), and Western blot analysis detected a strong p60 band but weak p43 and p49 bands in human hepatocytes; the results confirmed that the hepatocytes express low levels of the membrane forms of DR5 proteins (Fig. 1F). In contrast, Fas was highly expressed on human hepatocytes (Fig. 1E), and DR4 and/or DR5 was highly expressed in human tumor lines WM793 and H460 (Fig. 1E and F). These studies indicate that the lack of DR4 and low levels of DR5 expression in human hepatocytes may contribute to the cell resistance to TRAIL.

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**Fig. 2.** TRAIL does not trigger apoptosis in human hepatocytes in the chimeric mice. A, serum tests for hAAT and ALT in the chimeric mice at 0 and 16 hours after intravenous injection of 500 μg TRAIL. B, H&E (top), antihuman hepatocyte (middle), and anticleaved caspase-3 antibody-stained sections (bottom) of the livers obtained from the chimeric mice injected intravenously with saline (left), 500 μg TRAIL (middle), or 30 μg FasL (right). The nodules of human hepatocytes (H) and surrounding mouse hepatocytes (M) were observed in the chimeric livers. C, Western blot detection of cleavage products of caspase-8 (Casp-8), caspase-3 (Casp-3), and DFF45 in the homogenized liver tissues from two chimeric mice intravenously injected with 500 μg TRAIL for 16 hours and two chimeric mice intravenously injected with 30 μg FasL for 1.5 hours. Extracellular signal-regulated kinase (ERK) 1/2 was used as a loading control.

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FasL but not TRAIL Induces Apoptosis in Human Hepatocytes in Chimeric Mice. Next we tested TRAIL and FasL hepatotoxicity in SCID/Alb-\textit{uPA} chimeric mice whose livers are largely composed of human hepatocytes (8). The chimeric mice were generated through crossing hemizygous Alb-\textit{uPA} transgenic mice with homozygous SCID/\textit{bg} mice as described in the Materials and Methods. Two-month-old chimeric mice were injected intravenously with 500 \mu g of TRAIL in 100 \mu L normal saline, 30 \mu g of the antibody cross-linked Flag-FasL (30 \mu g of Flag-FasL mixed with 12 mg antibody in 100 \mu L normal saline), or 100 \mu L normal saline. All of the chimeric mice that received cross-linked Flag-FasL injection succumbed within 90 minutes, whereas the chimeric mice injected with 500 \mu g TRAIL remained alive up to 4 and 16 hours when the mice were sacrificed for necropsy. Serum tests showed no significant difference in hAAT and ALT concentrations before and after TRAIL injection in chimeric mice (Fig. 2A). Histologic examination of the livers from the chimeric mice injected with FasL showed extensive necrosis, severe edema, and hemorrhage within the large nodules of human hepatocytes in the chimeric livers (Fig. 2B). Immunohistochemistry confirmed the human origin of the hepatocytes and revealed caspase-3 cleavage in the human hepatocytes (Fig. 2B). In contrast, the human hepatocytes in the chimeric livers obtained at 4 hours and 16 hours after TRAIL injection and 16 hours after normal saline injection were morphologically normal and free of caspase-3 cleavage (Fig. 2B). Western blot analysis detected caspase-8, caspase-3, and DFF45 cleavage in the homogenized liver tissues from the chimeric mice injected with FasL but not TRAIL (Fig. 2C). These results indicate that systemic administration of high doses of the human TRAIL caused no apoptotic injury to human hepatocytes \textit{in vivo}.

TRAIL Inhibits Tumor Growth but Causes No Injury to Human Hepatocytes in Chimeric Mice. To show TRAIL selective antitumor activity in the chimeric mice, we injected 2-month-old chimeric mice intraperitoneally with 8 \times 10^6 WM793 cells on the left side. On the next day, three chimeric mice received intraperitoneal injections of 100 \mu g TRAIL on the right side with 100 \mu L normal saline in the control group of three mice. Animals were treated twice daily for 10 days. All of the mice treated with TRAIL remained healthy, whereas in the control group, one mouse died on the 39th day, and the remaining two mice appeared sick. All of the remaining mice were sacrificed on the 40th day for necropsy. On histologic examination, the hepatocytes within nodules appeared normal and stained positively for a human hepatocyte marker but negatively for cleaved caspase-3 antibody in TRAIL- and saline-treated mice (Fig. 3A). Serum tests showed no significant difference in the hAAT concentrations before and after TRAIL injection in the chimeric mice (Fig. 3A). Necropsy showed large tumor masses limited to peritoneal space in the saline-treated control group, with an average tumor weight of 1.6 g (Fig. 3C). Of three TRAIL-treated mice, however, one remained free of tumor, whereas the other two mice harbored small tumor masses within the peritoneal space. The mean tumor weight of the TRAIL-treated mice was 0.25 g (Fig. 3C).

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Fig. 3. Intraperitoneal injection of TRAIL inhibits tumor growth in chimeric mice. A, H&E, antihuman hepatocyte, and anticleaved caspase-3 antibody-stained sections of the livers from chimeric mice that received intraperitoneal injections twice daily of 100 \mu g of TRAIL or 100 \mu L of normal saline for 10 days, followed by sacrifice on the 40th day. The tumors (T) invaded chimeric livers in a control mouse. B, serum test for hAAT concentrations in the chimeric mice on the day of tumor inoculation (week 0) and the following weeks up to the 4th week after tumor inoculation. C, mean intraperitoneal W793 tumor weights in the chimeric mice that received intraperitoneal injection of TRAIL or normal saline as described in A. All of the intraperitoneal tumor masses were grouped and weighed in each mouse for calculation of mean tumor weight. The data were compared using Student's \textit{t} test (\textit{P} < 0.01).

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injected with normal saline or TRAIL as described in saline or TRAIL as described in (C). Serum tests showed no significant difference in the con-

tumors were measured daily and showed significant growth in the (B). One day later, the treatment group of four chimeric mice received 100 µg TRAIL, whereas the control group of four chimeric mice received 100 µL normal saline, twice per day for 10 days. The sizes of subcutaneous tumors were measured daily and showed significant growth in the chimeric mice in the control group but not in the TRAIL-treated group (Fig. 3). Serum tests showed no significant difference in the concentrations of hAAT (Fig. 4A) and ALT (Fig. 4B) either before or after TRAIL injection or between the TRAIL-treated group and saline-treated group. Histologic examination revealed that the hepatocytes were normal morphologically and positive for a human hepa-
tocyte marker but negative for cleaved caspase-3 (data not shown). Caspase-8, caspase-3, and DFF45 cleavage products were not detected on Western blot analysis in the homogenized liver tissues from either TRAIL- or saline-treated chimeric mice (Fig. 4A). We conclude that the recombinant soluble human TRAIL (amino acids 114 to 281) has a profound apoptotic effect on tumors but is nontoxic to human hepatocytes in vitro and in vivo. This form of TRAIL may prove to be a safe and effective biological agent for cancer therapy in humans.

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References

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