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 poly-histidine-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 (In Vitro, Carlsbad, CA) with 10% fetal bovine serum and 1% penicillin-streptomycin (In Vitro). Cells 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 (OCH1E5, 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., Corn-
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.
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 contract 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.

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.
FasL but not TRAIL Induces Apoptosis in Human Hepatocytes in Chimeric Mice. Next we tested TRAIL and FasL hepatotoxicity in SCID/Alb-uPA chimeric mice whose livers are largely composed of human hepatocytes (8). The chimeric mice were generated through crossing hemizygous Alb-uPA transgenic mice with homozygous SCID/bg mice as described in the Materials and Methods. Two-month-old chimeric mice were injected intravenously with 500 μg of TRAIL in 100 μL normal saline, 30 μg of the antibody cross-linked Flag-FasL (30 μg of Flag-FasL mixed with 12 mg antibody in 100 μL normal saline), or 100 μL 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 μg 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 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 × 10⁶ WM793 cells on the left side. On the next day, three chimeric mice received intraperitoneal injections of 100 μg TRAIL on the right side with 100 μL 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. 2A).

Histologic examination of the livers from the chimeric mice injected with the FasL showed extensive necrosis, severe edema, and hemorrhage within the large nodules of human hepatocytes in the chimeric livers (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 in vivo.
To further assess TRAIL antitumor activity without causing injury in human hepatocytes, we injected eight chimeric mice subcutaneously with $4 \times 10^6$ lung adenocarcinoma H460 cells that were sensitive to TRAIL, as evident by the cleavage of caspase-8, caspase-3, and DFF45 on Western blot analysis. Tumor viability and cell death were assessed by cell viability assay (Fig. 4B) and serum hAAT and ALT concentrations on the day of tumor inoculation (day 0) and day 10 in the TRAIL-treated group and control group but not in the TRAIL-treated group (Fig. 4C). Serum tests showed no significant difference in the concentrations of hAAT (Fig. 4D) and ALT (Fig. 4E) 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 hepatocyte 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 be used to determine a safe and effective biological agent for cancer therapy in humans.

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References

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