Preclinical Differentiation between Apparently Safe and Potentially Hepatotoxic Applications of TRAIL Either Alone or in Combination with Chemotherapeutic Drugs

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Abstract

Purpose: Tumor necrosis factor-related apoptosis–inducing ligand (TRAIL/Apo2L) exhibits potent antitumor activity on systemic administration in nonhuman primates without deleterious side effects for normal tissue. However, there is a controversy about the potential toxicity of TRAIL on human hepatocytes. The use of different recombinant TRAIL forms only partially explains the contradicting reports on TRAIL sensitivity in primary human hepatocytes (PHH).

Experimental Design: To clarify this issue, we comprehensively tested four different recombinant forms of TRAIL for their apoptosis-inducing capacity on PHH obtained from a total of 55 human livers between day 1 and day 8 of in vitro culture.

Results: One day after single-cell isolation, all but one recombinant form of TRAIL [i.e., an untagged form of TRAIL (TRAIL.0)] induced apoptosis in PHH. Apoptosis induction by TRAIL in these cells could only be fully inhibited by concomitant blockade of TRAIL receptor 1 and TRAIL receptor 2. At day 4 of in vitro culture, when surrogate markers indicated optimal hepatocyte in vitro function, only high doses of cross-linked FLAG-TRAIL killed PHH whereas the other three recombinant TRAIL forms did not. Strikingly, cotreatment of day 4 PHH with cisplatin sensitized for TRAIL-induced apoptosis whereas 5-fluorouracil, etoposide, gemcitabine, irinotecan, or oxaliplatin, which are commonly used in the treatment of gastrointestinal cancers, did not.

Conclusion: Our data show that whereas TRAIL alone or together with selected chemotherapeutic drugs seems to be safe, the combination of TRAIL with cisplatin is toxic to PHH.

Tumor necrosis factor–related apoptosis–inducing ligand (TRAIL), also known as Apo2L, binds to four membrane bound receptors, TRAIL-R1 to TRAIL-R4, and one soluble receptor, osteoprotegerin (reviewed in ref. 1). Two of them, TRAIL-R1 and TRAIL-R2 (refs. 2 – 4; also known as DR4 and DR5), are capable of inducing apoptosis on activation, whereas TRAIL-R3 and TRAIL-R4 (refs. 5, 6; also known as DcR1 and DcR2) as well as osteoprotegerin (7) might serve as decoy receptors to block TRAIL-mediated apoptosis.

TRAIL induces apoptosis in a wide variety of human cancer cell lines (8), but not in most normal human cells (9 – 11). A non-tagged recombinant form of TRAIL (i.e., the Apo2L.0 form) is currently under development as a biotherapeutic agent (reviewed in refs. 12, 13). However, it was reported that normal human hepatocytes undergo apoptosis when incubated with a recombinant polyhistidine–tagged or a cross-linked FLAG–tagged soluble form of human TRAIL in vitro (14, 15). These findings raised the concern of potential liver toxicity of systemic TRAIL administration in humans (16). Thus far, the in vivo safety of TRAIL administration has mainly been shown in different animal models (9, 10, 17). Leucine-zipper (LZ)-tagged versions of human and murine TRAIL (LZ-TRAIL) did not show any adverse effects in normal tissue in mice (18). Apo2L.0 was not toxic to isolated human and cynomolgus hepatocytes and was well tolerated in vivo in cynomolgus monkeys and chimpanzees (17, 19). Thus far, adverse effects of TRAIL on hepatocytes have only been shown in isolated human hepatocytes. In addition, contradicting results have been reported on the TRAIL sensitivity of primary human hepatocytes (PHH), which could only partially be explained by the use of different forms of recombinant TRAIL. In preclinical studies, TRAIL has shown to be a promising antitumor agent, which, in combination with chemotherapeutic drugs, synergistically kills...
many tumor cells (reviewed in ref. 20). However, potential toxic effects of such a combined therapy on primary human liver cells have not been sufficiently investigated far.

In the present study, we comprehensively investigated the hepatotoxicity of four different recombinant TRAIL forms on PHH and continuously monitored the apoptosis-inducing potential of TRAIL between day 1 and day 8 of in vitro culture. A novel recombinant version of TRAIL, isoleucine-zipper-TRAIL (iz-TRAIL), was analyzed for its cytotoxic activity in hepatoma cell lines and PHH.

Furthermore, we tested a panel of chemotherapeutic drugs which are widely used in the clinic for their TRAIL-sensitizing potential on PHH. Of clinical relevance, we found that cisplatin sensitized PHH for TRAIL-induced apoptosis. It will be important to take this into account when the combined clinical use of TRAIL with chemotherapy is considered.

Materials and Methods

Cell lines. The human hepatoma cell line HepG2 was maintained in DMEM containing 10% FCS (Life Technologies, Inc., Karlsruhe, Germany).

Isolation and culture of PHH. PHH were isolated from healthy liver tissue obtained from patients undergoing partial liver resection with a two-step perfusion technique described by Berry and Friend (5) and modified by Schulze-Bergkamen et al. (21). The isolation procedure was approved by the Ethics Committee, Medical Faculty, University of Heidelberg. Cells were then seeded in maintenance medium supplemented with 10% FCS (Life Technologies) at a density of 1.0 × 10⁶ to 1.5 × 10⁸ viable cells/cm² on collagen-coated culture plates (collagen type I; Serva Biochemicals, Heidelberg, Germany). The maintenance medium was changed 2 hours after seeding for Williams’s medium E supplemented with 5% FCS, after another 12 hours and every day thereafter for serum-free Williams’s medium E. All experiments were done in FCS-free maintenance medium. Cells were incubated at 37°C and 10% CO₂.

Antibodies and reagents. The monoclonal anti-FLICE antibody C15 (22) and anti-APo-1 (23) were obtained from Alexis Corp. (San Diego, CA). Anti–poly(ADP-ribose) polymerase (clone C-2-10) was purchased from Biomol (Hamburg, Germany) and extracellular signal–regulated kinase 1 was from Transduction Laboratories (San Diego, CA). Anti–FLAG antibody was from Sigma (St. Louis, MO). Anti-CD95 antibody (clone 2F10) was from BD Biosciences PharMingen (San Diego, CA). Anti-human TRAIL-R1 to TRAIL-R4 antibodies, CD95, or control mIgG1, followed by biotinylated secondary goat anti-mIgG1 F(ab)₂ antibody fragments and streptavidin-phycoerythrin. Surface staining was determined on a FACS Calibur cytometer (Becton Dickinson, Heidelberg, Germany).

Western blot analysis. Preparation of cell lysates and immunoblotting were done as described before (26). For Western blot analysis, supernatants were denatured and 20 μg of protein were separated on 4% to 12% NuPage Bis-Tris gradient gels (Novex, San Diego, CA) in MOPS buffer. For stripping, blots were incubated in 50 mmol/L glycine-HCl (pH 2.3) for 15 minutes at room temperature. Subsequently, blots were washed, blocked, and reprobed again.

Reverse transcription-PCR. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Five micrograms of total RNA were transcribed into cDNA using the M-MuLV reverse transcriptase (AmpliChem, Darmstadt, Germany) according to the instructions of the manufacturer. PCR was done using 0.4 μmol/L of each primer (5′-actin, forward: GCGAGGAGGCCCCAGAGCA and reverse: CCCCCGCAAGCAGGGTGAGC; TRAIL-R1, forward: TGTCTGGTGTCACTCCGTCAAGGTGTTG and reverse: GAGGCGTTCCGTCCAGTTTTGTTG; TRAIL-R2, forward: GCGCCACAACAAAAGGGCTC and reverse: CAGCAGCTCGCTGCTGGGGTTTT), deoxynucleotide triphosphates (Sigma), Taq polymerase (Qiagen). PCR conditions were as follows: initial denaturation, 5 minutes 95°C; cycle, 1 minute 54°C, 1 minute 56°C, 1 minute 72°C; final elongation, 15 minutes 72°C.

Immunohistochemistry. Formalin-fixed, paraffin-embedded liver tissue samples were cut into 3-μm sections, dewaxed, and pretreated in 10 mmol/L citrate buffer. Unspecific antibody binding was blocked by incubation with 20 μg/mL bovine serum albumin (20 mg/mL; Serva) and human γ-globulin (Gamma-Venin, 1 mg/mL; Aventis-Behring, Marburg, Germany). Antibodies against TRAIL-R1 to TRAIL-R4 were incubated at 4°C overnight or, in the case of CD95, for 1 hour at room temperature. Sections were washed, incubated with blocking solution [20% normal goat serum (Dianova) in PBS], followed by the secondary antibody (biotinylated goat anti mouse, Dianova) at room temperature, rinsed twice and incubated with streptavidin-alkaline phosphatase (BioGenex, San Ramon, CA). After washing, color reaction was developed using Fast Red Substrate System (DAKO, Glostrup, Denmark). After counterstaining with hematoxylin (DAKO), the sections were mounted in glycerol (DAKO).

Hoechst 33342 stain. For the nuclear staining with Hoechst 33342, cells were seeded in chamber slides at a density of 100,000/cm². After treatment, cells were washed in PBS, fixed in 4% formalin, washed again, and incubated for 2 hours in Hoechst 33342 (1 μg/mL in PBS).

Results

Sensitivity of PHH for TRAIL-induced apoptosis does not only depend on the form of recombinant TRAIL but also on the duration of in vitro culture. Repeated administration of
LZ-TRAIL in mice (9) and Apo2L.0 in cynomolgus monkeys and chimpanzees (10, 19) induced no pathologic changes in these animals. However, contradictory results were obtained with respect to sensitivity of isolated PHH on incubation with polyhistidine-tagged TRAIL (14). Therefore, we investigated the influence of different culture conditions and different recombinant forms of TRAIL on the induction of apoptosis in isolated human hepatocytes from a total number of 55 different donors.

PHH were cultured for up to 8 days in vitro. Between in vitro day 1 and day 8, PHH were exposed to different concentrations of LZ-TRAIL for 24 hours and cell viability was measured (Fig. 1A). At day 1, up to 70% of the cells could be killed with LZ-TRAIL. However, at day 2 and day 3, increasingly less cells died on treatment with LZ-TRAIL. At day 4, PHH became TRAIL resistant even at high doses of LZ-TRAIL. This resistance was maintained at least until day 8 of in vitro culture (data not shown). In contrast, cells showed a high and sustained sensitivity for CD95L-induced apoptosis from day 1 until day 8. Thus, freshly isolated hepatocytes seem to be sensitive for TRAIL-induced cell death only directly after isolation.

To investigate how the induction of apoptosis depends on the form of TRAIL in PHH at day 1 and day 4 after isolation, we incubated these cells in the presence of four recombinant forms of TRAIL: TRAIL.0, iz-TRAIL, LZ-TRAIL, FLAG-TRAIL cross-linked with an anti-FLAG-antibody (M2), and, as a control, with LZ-CD95L (Fig. 1B and C). Of the four recombinant forms of TRAIL, cross-linked FLAG-TRAIL exhibited the highest potency to induce apoptosis in PHH, killing a substantial percentage of hepatocytes at day 1 and also at day 4. LZ-TRAIL and iz-TRAIL significantly reduced the viability of day 1 hepatocytes but could not induce cell death at day 4 of hepatocyte culture. TRAIL.0 was the only recombinant TRAIL form that neither induced apoptosis at day 1 nor at day 4 whereas LZ-CD95L efficiently induced hepatocyte cell death at both time points.

To test the apoptosis-inducing capacity in tumor cells, we also treated the hepatoma cell line HepG2 with the four recombinant forms of TRAIL. LZ-TRAIL showed the highest apoptosis-inducing capacity on HepG2 cells (Fig. 1D). At higher concentrations, iz-TRAIL and cross-linked FLAG-TRAIL killed nearly as efficiently. However, TRAIL.0, which did not
induce apoptosis in PHH at day 1 and day 4, was still able to induce substantial, albeit slightly reduced apoptosis in HepG2 cells in comparison with the other recombinant forms of TRAIL.

We next tested whether incubation of PHH with TRAIL or, as a control, with CD95L resulted in cleavage of caspase-8 and poly(ADP-ribose) polymerase and whether there was a difference at day 1 and day 4 of in vitro culture (Fig. 1E). Poly(ADP-ribose) polymerase, a substrate of executioner caspases, was cleaved on LZ-CD95L treatment of cultured hepatocytes at day 1 and day 4, but only in day 1 cultured hepatocytes when treated with LZ-TRAIL.

At day 1, caspase-8 processing was clearly visible after 4-hour treatment with LZ-CD95L. As expected, treatment of PHH at day 1 with LZ-TRAIL induced a significant caspase-8 cleavage. However, at day 4, even 8 hours of LZ-TRAIL treatment did not result in any detectable caspase-8 processing. Therefore, whereas PHH remained sensitive towards LZ-CD95L-induced apoptosis, they became resistant to LZ-TRAIL-mediated apoptosis.

**TRAIL-induced apoptosis of PHH at day 1 is mediated by TRAIL-R1 and TRAIL-R2.** Because TRAIL was capable of killing ~40% of PHH at day 1 but not at day 4, we tested whether TRAIL sensitivity is based on a differential expression of TRAIL receptors in native liver tissue and at day 1 and day 4 of in vitro culture. Transcripts of mRNA could be detected for all TRAIL receptors without any significant difference in the expression level between the primary liver tissue and liver cells of day 1 or day 4 culture (Fig. 2A). However, in none of 12 different preparations of PHH cell-surface expression of any of the TRAIL receptors could be detected at day 0, day 1, or day 4 by FACS analysis (Fig. 2B). Immunohistochemical staining of primary liver tissue showed a locally variable intensity for TRAIL-R1, which,
however, was restricted to a cytoplasmic pattern without any membrane-associated signal (Fig. 2C). Between faintly stained hepatocytes, some strongly TRAIL-R1-positive sinusoidal cells were visible, resembling the staining pattern of Kupffer cells as detected with a CD68 costaining (data not shown). Staining of hepatocytes for TRAIL-R2, TRAIL-R3, and TRAIL-R4 showed mostly a cytoplasmic pattern. In contrast, CD95 hepatocyte surface expression could be detected by both FACS stain and immunohistochemistry (Fig. 2C). These data suggest a very low level and rather a cytoplasmic, instead of a membrane-bound, TRAIL receptor expression, which is in contrast to numerous hepatoma cell lines where TRAIL receptors could be readily detected by either method (26–28).

As we have shown that PHH could be killed by certain recombinant forms of TRAIL at day 1, we subsequently investigated whether both death-inducing TRAIL-R1 and TRAIL-R2 were involved in this TRAIL-mediated apoptosis. Therefore, we preincubated the cells at day 1 with blocking antibodies, specific for either TRAIL-R1 or TRAIL-R2, or a combination thereof (Fig. 2D). Individual blockage of either TRAIL-R1 or TRAIL-R2 only partially protected PHH from apoptosis mediated by LZ-TRAIL. However, the combined blockage of both TRAIL-R1 and TRAIL-R2 completely abrogated TRAIL-induced cell death. Therefore, although the surface receptor level might be too low to be detected by FACS staining and immunohistochemistry, cell-surface expression of both TRAIL death receptors is responsible for TRAIL-mediated cell death in day 1 PHH.

Cisplatin sensitizes PHH for TRAIL-induced apoptosis. Many tumor cell lines and cells derived from primary human tumors have been shown to be resistant to TRAIL-induced apoptosis (29). Therefore, in a number of studies, irradiation (30, 31) or chemotherapeutic drugs, including 5-fluorouracil (26), etoposide (32), cisplatin (33), oxaliplatin (34), irinotecan (35, 36), and gemcitabine (36), have been used together with TRAIL to sensitize tumor cells for TRAIL-induced apoptosis. However, such cotreatments can only be of clinical benefit if they do not sensitize normal cells, especially normal human liver cells.

![Fig. 3. Various chemotherapeutic drugs do not sensitize PHH for TRAIL-induced apoptosis. A, PHH at day 4 were cotreated with LZ-TRAIL and chemotherapeutic drugs as indicated for 24 hours. Cell viability was measured in triplicates by MTT assay. Bars, SDs of three independent experiments. B, PHH at day 4 were treated overnight as indicated and nuclei were stained with Hoechst 33342.](image-url)
We therefore tested whether treatment with either of these drugs in combination with TRAIL exhibited any *in vitro* toxicity on PHH.

We exposed day 4 PHH to LZ-TRAIL in combination with increasing concentrations of 5-fluorouracil, irinotecan, gemcitabine, etoposide, oxaliplatin, and cisplatin, respectively, and determined cell viability after 24 hours (Fig. 3A). 5-Fluorouracil, irinotecan, gemcitabine, and etoposide neither showed a considerable toxicity when administered alone nor did they sensitize PHH for TRAIL-induced cell death. Although the highest concentrations of oxaliplatin were toxic to PHH, there was no sensitization for TRAIL-induced apoptosis. In contrast, cisplatin itself was not toxic to PHH but showed a synergistic effect for TRAIL-induced cell death at high concentrations.

The results of the treatment with different recombinant forms of TRAIL and the combinatorial treatment with iz-TRAIL and cisplatin were confirmed by nuclear staining of PHH with Hoechst 33342 after overnight treatment at day 4 of *in vitro* culture (Fig. 3B). The agonistic anti-CD95 monoclonal antibody anti-APO-1 was included as a positive control. Taken together, we could show that most of the chemotherapeutic drugs currently used for the therapy of gastrointestinal cancers, which have been reported to synergistically kill several cancer cell lines together with TRAIL, do not sensitize PHH for TRAIL-induced apoptosis. However, we also show that cisplatin was clearly capable of sensitizing PHH for TRAIL-induced apoptosis. This result may predict toxicity of selected combinations of TRAIL and chemotherapy, raising the concern about the safety of such combinations.

### Discussion

Controversial results have been published about the effect of recombinant soluble forms of TRAIL on isolated human hepatocytes (11, 14, 15, 17, 27). Polyhistidine-tagged (14) and FLAG-tagged (15) TRAIL induced substantial apoptosis in freshly isolated human hepatocytes, but not in nonhuman primates. An untagged form of TRAIL (Apo2L.0) seemed to be safe for normal human hepatocytes in primary culture (17) and for nonhuman primates on systemic administration (10, 19). Recently, Hao et al. (27) showed that untagged TRAIL neither induced apoptosis in isolated human hepatocytes nor in xenografted human hepatocytes in a mouse model. Here we studied and compared the potential toxicity of four different recombinant forms of TRAIL in a total of 55 human livers and analyzed the influence of the *in vitro* culture time after single-cell preparation of PHH.

We found that freshly isolated human hepatocytes had become partially sensitive to all but one form of TRAIL, but gradually reassumed resistance until day 5 and remained resistant until day 8 of *in vitro* culture (Fig. 1 and data not shown). After 4 days of *in vitro* culture, only high concentrations of cross-linked FLAG-TRAIL were still able to induce apoptosis (Fig. 1). At this time point, all other recombinant forms of TRAIL were unable to induce apoptosis in PHH. Only the untagged form of TRAIL (TRAIL.0) did not induce apoptosis in PHH at either time point.

Because we could not detect differences in the expression level of any one of the TRAIL receptors between TRAIL-sensitive PHH at day 1 and TRAIL-resistant PHH at day 4, we assume that intracellular mechanisms are responsible for the restored resistance of isolated human hepatocytes at day 4. Human hepatocytes (27) and liver cells from nonhuman primates (10, 19) have been shown to be TRAIL resistant *in vivo*. In accordance with our data, TRAIL-induced apoptosis has only been reported in freshly seeded hepatocytes (37, 38) but not after a 4-day period of *in vitro* culture (27). We hypothesize that the *in vitro* sensitivity of cultured human hepatocytes of day 1 could represent an *in vitro* artifact caused by the isolation procedure and the adaptation to the culture conditions. In this context, it has been shown that the albumin production rate, a surrogate marker of hepatocyte function, is dramatically reduced directly after single-cell isolation and reaches a maximum rate at about day 5 of *in vitro* culture (39, 40), indicating that freshly isolated human hepatocytes may not represent a suitable model in this respect.

On the other hand, cross-linked FLAG-TRAIL induces apoptosis in PHH not only at day 1 but also at day 4. In accordance with our data, Ichikawa et al. (15) reported an induction of apoptosis in PHH on treatment with a cross-linked FLAG-TRAIL. Yet interestingly, cross-linked FLAG-TRAIL was not the most potent inducer of apoptosis in cancer cells when compared with LZ-TRAIL or iz-TRAIL.

The surface expression of TRAIL receptors on isolated human hepatocytes is still a matter of debate (15, 27, 38). In contrast to Mori et al. (38), we and others (15, 27) could not detect a substantial surface expression of either TRAIL receptor on the surface of PHH. On the mRNA level, equal TRAIL receptor expression was detected at days 0, 1, and 4. The immunohistochemical analysis of paraffin-embedded liver tissue revealed cytoplasmic staining, but no membrane staining, for TRAIL-R1 and TRAIL-R2, which was also found by Spierings et al. (41). However, because blockade of either TRAIL-R1 or TRAIL-R2 substantially and equally inhibited TRAIL-induced apoptosis, both TRAIL receptors are mediators of TRAIL-mediated hepatocyte apoptosis at day 1 (Fig. 2D). Thus, these two receptors must both be present and functional on the surface of day 1 hepatocytes at levels which are below the detection limit of the sensitive surface stains shown in Fig. 2B. This is in accordance with the induction of apoptosis in freshly isolated human hepatocytes at day 1 by agonistic cross-linked TRAIL-R1 and TRAIL-R2 antibodies (38).

Many preclinical studies have shown that cotreatment with TRAIL and chemotherapeutic drugs can overcome resistance to chemotherapy in many cancer types (reviewed in refs. 12, 13). Therefore, the future clinical application of TRAIL might include the simultaneous or sequential cotreatment with different chemotherapeutic drugs. Treatment of PHH at day 4 with 5-fluorouracil, gemcitabine, etoposide, or irinotecan did not result in sensitization for TRAIL-induced apoptosis, indicative of nontoxicity of the combinatorial use of TRAIL with these chemotherapeutic agents on human liver tissue. These data are in accordance with a xenograft mouse model of hepatic metastases of a human colon cancer cell line (35). In contrast, in our experiments, cisplatin showed a substantial sensitization of PHH for TRAIL-mediated apoptosis, indicating that the combination of TRAIL with cisplatin seems to be unfavorable for future therapeutic strategies.

Taken together, we could show that TRAIL sensitivity of PHH depends not only on the specific recombinant form of TRAIL...
applied but also on the time point of the onset of TRAIL stimulation with respect to the start of hepatocyte in vitro culture. Recombinant iz-TRAIL, a novel stable form of recombinant soluble TRAIL, showed a potent antitumor activity but did not induce apoptosis in PHH at day 4 in vitro. Finally, we could show that chemotherapeutic agents differ substantially in their ability to sensitize TRAIL-resistant PHH for TRAIL-induced apoptosis. Our data show that the combination of TRAIL with certain, but not all, chemotherapeutic drugs might open the therapeutic window for more effective but still safe treatment of cancer.

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References

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