

Death Induction by Recombinant Native TRAIL and Its Prevention by a Caspase 9 Inhibitor in Primary Human Esophageal Epithelial Cells*

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The cytotoxic death ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a tumor-specific agent under development as a novel anticancer therapeutic agent. However, some reports have demonstrated toxicity of certain TRAIL preparations toward human hepatocytes and keratinocytes through a caspase-dependent mechanism that involves activation of the extrinsic death pathway and Type II signaling through the mitochondria. We have isolated and purified both His-tagged protein and three versions of native recombinant human TRAIL protein from *Escherichia coli*. We found that 5 mM dithiothreitol in the purification process enhanced oligomerization of TRAIL and resulted in the formation of hyper-oligomerized TRAILs, including hexamers and nonomers with an extremely high potency in apoptosis induction. Although death-inducing signaling complex formation was much more efficient in cells treated with hyper-oligomerized TRAILs, this did not convert TRAIL-sensitive Type II HCT116 colon tumor cells to a Type I death pattern as judged by their continued sensitivity to a caspase 9 inhibitor. Moreover, TRAIL-resistant Type II Bax-null colon carcinoma cells were not converted to a TRAIL-sensitive Type I state by hyper-oligomerized TRAIL. Primary human esophageal epithelial 2 cells were found to be sensitive to all TRAIL preparations used, including trimer TRAIL. TRAIL-induced death in esophageal epithelial 2 cells was prevented by caspase 9 inhibition for up to 4 h after TRAIL exposure. This result suggests a possible therapeutic application of caspase 9 inhibition as a strategy to reverse TRAIL toxicity. Hyper-oligomerized TRAIL may be considered as an alternative agent for testing in clinical trials.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)¹ is a Type II transmembrane protein that binds to one of four receptors that have been identified in humans, including TRAIL-R1/DR4 (1), TRAIL-R2/KILLER/DR5 (2–4), TRAIL-R3/DcR1/TRID (5, 6), and TRAIL-R4/DcR2/TRUND (7). Both DR4 and DR5 are pro-apoptotic receptors, which contain a cytoplasmic death domain and mediate apoptosis on binding to TRAIL. By contrast, TRID and TRUND do not contain a cytoplasmic death domain and block the function of TRAIL by competing with TRAIL-R1 and TRAIL-R2 for binding of TRAIL (8).

Apoptosis is an essential process for the regulation of homeostasis and development. The apoptotic process can be induced by two different pathways (9). The intrinsic pathway, also called the mitochondrial pathway, is induced by intracellular signals such as oncogene activation, DNA-damaging agents, and growth factor deprivation. Mitochondrial release of cytochrome *c* into the cytosol in response to death signals allows assembly of a multiprotein caspase activating complex called the apoptosome (10, 11). The extrinsic pathway is activated by cell surface death receptors, and activation of the death receptors by death ligand engagement induces the formation of a death-inducing signaling complex (DISC), which consists of the receptor FADD as an adaptor and caspase 8 as an initiator caspase (12). Once the DISC is formed, the caspase 8 is auto-processed and activated by induced proximity (13, 14). The TRAIL death ligand induces apoptosis via the extrinsic pathway (15).

TRAIL is a promising agent for development as a cancer-specific therapeutic agent because it induces apoptotic cell death in a wide variety of transformed cell lines and tumors in animal models but not in most normal cells or tissues (15–17). Even in tumor cell lines that show resistance to TRAIL, combined treatment with chemotherapeutic drugs or ionizing radiation has revealed induction of tumor cell death (18–24). However, some reports have demonstrated a toxicity of certain TRAIL preparations against primary human hepatocytes through a caspase-dependent mechanism that involves activa-

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¹ The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DISC, death-inducing signaling complex; hTR, His₆-tagged TRAIL; Ni-NTA, nickel-nitrilotriacetic acid; FADD, Fas-associating death domain-containing protein; nTR, native TRAIL preparation; GFP, green fluorescent protein; DTT, dithiothreitol; c-FLIP, cellular FLICE (FADD-like interleukin-1 β -converting enzyme)-inhibitory protein; Bax, BCL-2-associated X protein.

TABLE I
Conditions for generation of recombinant TRAILs

	Tag	Amino acids residues	Growth medium	Purification buffer
hTR	His ₆	95–281, 23 kDa	LB	Phosphate buffer with 10 mM BME ^a
nTR1	—	114–281, 19 kDa	LB	Phosphate buffer with 10 mM BME ^a
nTR2	—	114–281, 19 kDa	LB	Phosphate buffer with 5 mM DTT ^b
nTR3	—	114–281, 19 kDa	LB with 100 μM ZnSO ₄	Phosphate buffer with 5 mM DTT ^b

^a BME, β-mercaptoethanol.

^b DTT, dithiothreitol.

tion of the extrinsic death pathway (25, 26). Further studies suggested that this toxicity could be avoided by use of native TRAIL but not tagged TRAIL (27). In this study, we generated His-tagged and native TRAIL using several biochemical purification methods. In the case of native TRAIL produced in the presence of dithiothreitol, we observed and purified hyperoligomerized forms that were found to be highly potent in death induction assays. We investigated the toxicity of the various recombinant TRAIL preparations toward normal human esophageal epithelial cells and found evidence of significant toxicity. We further developed a strategy for using caspase 9 blockade to inhibit toxicity and now report that administration of a caspase 9 inhibitor is effective for a period of up to 4 h following TRAIL exposure. Our studies have implications for the use of TRAIL as an anticancer agent during its utilization in clinical trials.

EXPERIMENTAL PROCEDURES

Expression Plasmids for Tagged and Untagged Soluble Human TRAIL—The extracellular portion (aa 95–281) of the human TRAIL cDNA was amplified and cloned into pQE80L (Qiagen) after digestion with BamHI and HindIII to generate the His₆-tagged TRAIL expression plasmid (pQE-hTR). The primer sequences used were 5'-AAGGCGGATCCACCTGAGGAAACCATTTTC-3' (hTR-S) and 5'-CCCAAGCTTTAGCCAATAAAAAGGCCCGA-3' (hTR-AS). To make a non-tagged native human TRAIL expression plasmid, the His₆ tag and amino acids 95–113 were removed from pQE-hTR by a two-step PCR-mediated ligation. The first PCR step was performed to amplify the vector and TRAIL sequences using primers for the vector sequence 5'-CGTATCAGGAGGCCCTTTCGTC-3' (Head-S) and 5'-TTTCTCTCACCATAGTTAATTTCTCTCTTAA (Head-AS)-3' and for TRAIL aa 114–281, 5'-GAAATTAACATGGTGAGAGAAAGAGGTCCTCA-3' (TR114) and 5'-CCCAAGCTTTAGCCAATAAAAAGGCCCGA-3' (hTR-AS), respectively. *Pfu* Turbo DNA polymerase was used to prevent 3'-nucleotide addition. The amplified products from the first step were mixed and reamplified with primers Head-S and hTR-AS. The amplified product was cloned into pQE80L after digestion with EcoRI and HindIII to generate pQE-nTR.

Purification of Soluble Recombinant Human TRAIL—Recombinant TRAILs were expressed in DH10B bacteria as the expression host. The overnight seed culture was diluted 100-fold into 1.0 liter of LB broth and incubated for 3 h at 37 °C. Isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) was added to induce recombinant protein expression, and bacterial cells were incubated overnight at 30 °C. Bacteria were harvested and homogenized in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 10 mM β-mercaptoethanol or 5 mM dithiothreitol). Recombinant TRAILs were isolated from the soluble fraction using Ni-NTA agarose beads (Qiagen) after washing with lysis buffer containing 20 mM imidazole. Isolated proteins were dialyzed against phosphate-buffered saline with 10 mM β-mercaptoethanol. The purity of the recombinant TRAIL proteins was confirmed by SDS-PAGE and Coomassie Blue gel staining.

Separation of Oligomerized Forms of Soluble Recombinant Human TRAIL—The recombinant human TRAIL purified from the Ni-NTA-agarose column application was subjected to gel filtration chromatography to isolate oligomerized forms of TRAIL. The HiLoad 60/120 Superdex 200 prep grade column was used in the AKTA fast protein liquid chromatography system (Amersham Biosciences).

Cell Lines and Culture—Human primary esophageal epithelial cells (EPC2) were described previously (28). EPC2 cells were cultured in keratinocyte-SFM (Invitrogen) containing bovine pituitary extract (40 μg/ml) and epidermal growth factor (1 ng/ml). Human foreskin fibroblasts were described previously (29) and grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics. The

recombinant human soluble DR5 was produced using the *Pichia pastoris* system as we described previously (30). The purified soluble DR5 contains 1–2 ng of lipopolysaccharide/mg of protein as determined by *Limulus* ameocyte lysate assay. This is comparable with human serum albumin purchased from Sigma, which contains 1–4 ng of lipopolysaccharide/mg of protein.

Assessment of TRAIL-mediated Apoptosis—For detecting apoptosis mediated by TRAIL, the active caspase 3 assay was performed using a Cytofix/Cytoperm kit (BD Biosciences) as described previously (31). Briefly, 5×10^5 cells were seeded into a 6-well plate. After 24 h, the cells were treated with TRAIL. After treatment, the cells were harvested, fixed, and incubated with 0.125 μg/μl rabbit anti-active caspase 3 antibody (clone C92-605, Pharmingen) for 20 min. After washing, the cells were probed with 0.125 μg/μl phycoerythrin-conjugated goat anti-rabbit secondary antibody (CALTAG Laboratories) for 20 min. The intensity of phycoerythrin was analyzed by flow cytometry using a Beckman Coulter Epics Elite analyzer. For the analysis of the sub-G₁ fraction, cells were fixed in 70% ethanol, stained with 50 μg/ml propidium iodide and RNase A for 30 min at room temperature, and then analyzed by flow cytometry.

DISC Immunoprecipitation—H460 cells (4×10^7) were harvested and suspended in 2 ml of Dulbecco's modified Eagle's medium with 100 ng/ml trimer or hexamer TRAIL. After incubation with TRAIL preparations for 5, 10, or 20 min, cells were collected and lysed in 1 ml of DISC immunoprecipitation buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Triton X-100) with protease inhibitor mixture. Cell lysates (400 μl) were incubated overnight with 5 μl of rabbit anti-FADD antibody (Cell Signaling) at 4 °C. Complexes were precipitated by protein A-agarose (Invitrogen) and suspended in 50 μl of SDS sample buffer after three washes with DISC immunoprecipitation buffer. Immunoprecipitates were subjected to SDS-PAGE and Western blotting.

RESULTS

Generation of Recombinant TRAILs and Their Toxicity against Human Cancer Cell Lines—hTR, which has an N-terminal histidine tag, was purified from the DH10B *Escherichia coli* strain after overnight incubation in 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for protein induction. We used three different expression and purification schemes for the generation of native TRAILs (Table I). All TRAILs were purified using an Ni-NTA-agarose column application, and TRAIL protein purity was confirmed by SDS-PAGE and Coomassie Blue staining (Fig. 1A). Although the native TRAIL preparations (nTRs) did not contain a His₆ tag in their sequence, they still bound to the Ni-NTA-agarose column, but the interaction was relatively weak, allowing separation from the beads at 40 mM imidazole, whereas the His-tagged hTR required 250 mM imidazole to be released (data not shown).

The death-inducing activity of each TRAIL was tested in H460, a human lung adenocarcinoma cell line, which is sensitive to TRAIL. All the purified recombinant TRAIL proteins could induce apoptosis in H460 cells (Fig. 1B). The hTR and nTR1 proteins induced less than 15% cell death at 10 ng/ml. The hTR protein induced more than 40% cell death at 100 ng/ml, whereas nTR1 was only slightly less toxic than hTR, resulting in 30% cell death at 100 ng/ml. Incubation of TRAIL proteins in the presence of soluble DR5 protein resulted in a blockade of apoptosis, thereby confirming the specificity of hTR and nTR1 for the TRAIL-mediated death receptor pathway. We also used a decoy receptor-expressing SW480 human colorectal carcinoma cell line to further confirm the specificity of hTR and

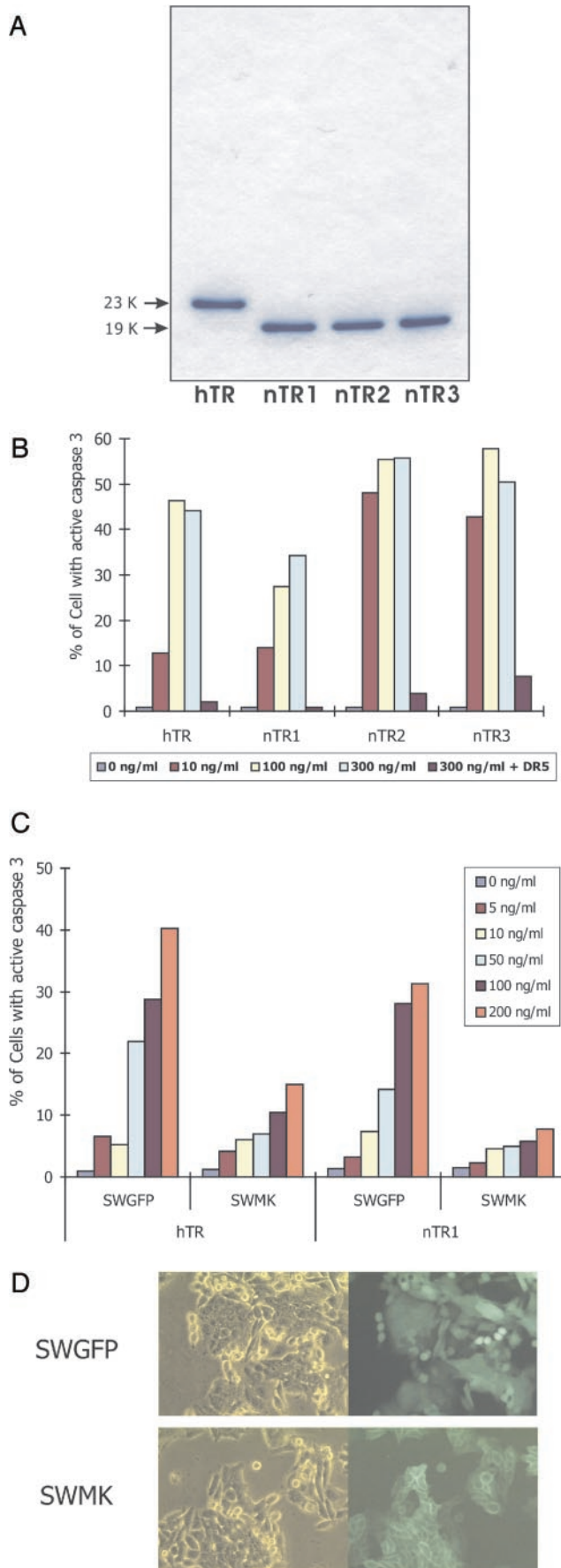


FIG. 1. Generation of recombinant TRAILs and their cytotoxicity. *A*, each TRAIL preparation was purified using a Ni-NTA-agarose column after overnight induction as described under “Experimental

nTR1 for TRAIL receptor-mediated apoptosis (Fig. 1, *C* and *D*). We introduced a cytosolic death domain-deleted mouse *KILLER/DR5* gene, which was fused with GFP into the SW480 cells (SWMK), and found that this artificial decoy receptor could protect from cell death induced by hTR or nTR1. The death-inducing activity of hTR was comparable with that of commercially available preparations (32, 33). Interestingly, the death-inducing activity of nTR2 and nTR3 appeared to be higher than that observed with nTR1, although all three were expressed from the same expression plasmid and host. Death induction following exposure to nTR2 and nTR3 resulted in a cell death rate greater than 40%, even at 10 ng/ml TRAILs, and death occurred rapidly by 1 h after treatment with higher doses of TRAIL. This hyperactivity of nTR2 and nTR3 was mediated specifically by the cell surface TRAIL death receptors because soluble DR5 protein almost completely blocked their cytotoxicity. These results suggested that nTR2 and nTR3 were somehow different from hTR and nTR1, although they were composed of a homogeneous TRAIL component. These results prompted us to examine the multimeric state of the TRAIL molecules.

Hyper-oligomerization Correlates with High Cytotoxicity of nTR2 and nTR3—Gel filtration chromatography revealed that, depending on the method of purification, TRAIL molecules were heterogeneous in their oligomeric state (Fig. 2). In the case of hTR, one peak corresponding to the trimer was observed, whereas nTR1 appeared to be a mixture of monomer, dimer, and trimer molecules. The existence of an N-terminal His tag appeared to facilitate the trimerization of TRAIL molecules. Surprisingly, nTR2 and nTR3, which were purified in a 5 mM DTT-containing buffer, appeared to consist of trimer, hexamer, and a small amount of nonomer. Each peak corresponding to a trimer, hexamer, or nonomer was composed of TRAIL molecules as verified by reducing SDS-PAGE. There was no apparent difference in the molecular composition of nTR2 versus nTR3, which suggested that the addition of zinc ions in the culture medium did not influence the oligomerization state of TRAIL molecules. However, there was a highly significant difference in the molecular conformation in nTR1 versus nTR2, although the same procedure was used in the overexpression and purification of the TRAIL molecules, except that the reducing agent was changed from 10 mM β -mercaptoethanol to 5 mM DTT. The use of DTT during purification greatly enhanced the oligomerization of native TRAIL molecules, which resulted in the formation of hexamers and nonomers. DTT also led to the disappearance of monomers and dimers observed with nTR1.

We next investigated the apoptosis-inducing capacity of various TRAIL preparations and found that hexamers and nonomers were the highly cytotoxic component of nTR2 and nTR3. Hexamer and nonomer TRAIL proteins could kill the majority of H460 cells within 1 h of treatment at 100 ng/ml, and no cells survived after 4 h of treatment. In contrast, trimer TRAIL

Procedures.” The purity of the purified recombinant TRAIL proteins was checked by SDS-PAGE and Coomassie Blue staining of the protein gel. *B*, the cytotoxicity of each TRAIL preparation was investigated in H460 cells. Each TRAIL preparation was added for 4 h, and cells were harvested and stained for the active form of caspase 3 and analyzed by fluorescence-activated cell sorter. nTR2 and nTR3 showed high potency in inducing apoptosis. *C*, SW480 cells expressing either GFP (SWGFP) or the cytosolic domain-truncated mouse KILLER/DR5-GFP fusion protein (SWMK) were subjected to the treatment by the indicated concentration of TRAIL proteins for 4 h, and apoptosis was checked by the active caspase 3 assay. *D*, bright field (left panels) and fluorescent (right panels) microscopic images of SWGFP and SWMK. The decoy receptor expression was identified as the membrane expression of GFP fusion protein.

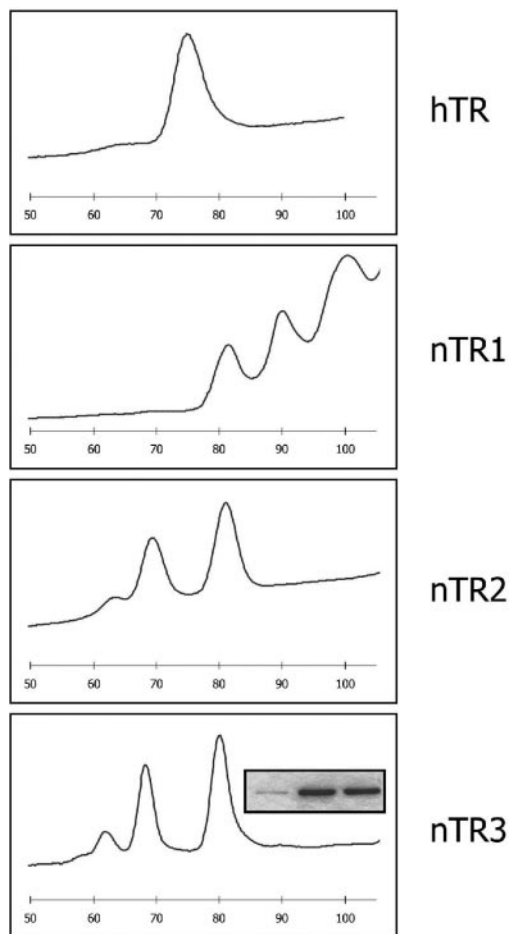


FIG. 2. Hyper-oligomerization of TRAIL was observed in nTR2 and nTR3. The oligomeric state of TRAIL proteins was analyzed by gel filtration chromatography. The hTR was composed of trimer TRAIL and showed one peak corresponding to 65 kDa. nTR1 gel filtration showed a mixture of three forms, which consist of monomer, dimer, and trimer. Trimer, hexamer, and nonomer forms of TRAIL were identified in nTR2 and nTR3. Each peak in nTR3 was collected and subjected to SDS-PAGE for the verification of the TRAIL molecule. Gel filtration samples for hTR, nTR1, and nTR2 were run at a flow rate of 1.0 ml/min, but nTR3 was run at 0.5 ml/min velocity, which resulted in sharper peaks and a good separation between the hexamer and the nonomer peaks. *x* axis represents the elution volume.

exposure resulted in more modest cell killing under the same conditions. Cell death induced by hyper-oligomerized TRAIL proteins was blocked by the soluble DR5 protein (Fig. 3A). We also checked the cleavage of caspases following TRAIL exposure. Trimer TRAIL treatment resulted in cleavage of pro-caspases 8, 9, and 3, but significant amounts of pro-caspases remained after 4 h of treatment. In the case of hexamer TRAIL, we observed cleavage of most of the cellular caspases 8 and 3 within 1 h, accompanied by complete disappearance of the pro-form of caspases 8 and 3 within 2 h. Significant amounts of pro-caspase 9 remained at 1 h but disappeared by 2 h on treatment with hexamer TRAIL. The extent of cleavage of the caspases was proportional to the potency of the TRAILs (Fig. 3B).

Hyper-oligomerized TRAIL Induced More Efficient DISC Formation—Because hexamer TRAIL induced apoptosis more efficiently than trimer TRAIL, we wondered whether DISC formation was more efficient following binding of hexamer *versus* trimer TRAIL. We performed DISC immunoprecipitation using anti-FADD antibody to examine the recruitment and cleavage of DISC components following exposure of H460 cells to either trimer or hexamer TRAIL. Using 100 ng/ml trimer or

hexamer TRAIL, we observed a time-dependent increase in the recruitment and cleavage of caspase 8 and c-FLIP to the DISC (Fig. 3C). We also observed increased recruitment of DR4 to the DISC. This increase was much greater in hexamer TRAIL-treated cells showing a significantly greater amount of recruitment and cleavage even at a 5-min exposure, which was comparable with the amount observed following 20 min of exposure to the trimer TRAIL. We also investigated whether the more efficient DISC formation and death induction by hexamer TRAIL might influence whether cell death occurred by a Type I *versus* a Type II mechanism. To determine whether cell death became less sensitive to inhibition by the caspase 9 inhibitor LEHD-fluoromethyl ketone (34), we treated either H460 (Type I) or HCT116 (Type II) cells with trimer or hexamer TRAIL and examined cell death induction at 6 h (Fig. 4A). H460 cells showed marginal death protection by the caspase 9 inhibitor (C9I) treatment, whereas complete death protection was conferred by the caspase 8 inhibitor (C8I), consistent with a Type I cell death protection pattern. There was no difference in the degree of observed death protection by the caspase inhibitors whether trimer or hexamer TRAIL was used to treat the H460 cells. In HCT116 cells, the C9I completely blocked cell death induced by either trimer or hexamer TRAIL. Despite more efficient formation of DISC by hexamer TRAIL, this did not alleviate the need to amplify the death signal through mitochondrial activation of caspase 9 as occurs in the Type II cell death pattern. We further confirmed these observations by examining the sub-G₁ fraction and colony viability (Fig. 4, B and C, respectively) of Bax-null HCT116 cells, which are TRAIL-resistant because the Bax deficiency prevents their death by a Type II mechanism (33, 35). Bax-null cells remained resistant regardless of the oligomeric state of TRAIL used.

Native TRAIL, Including Trimer TRAIL, Induced Death of Human Primary Esophageal Epithelial Cells—TRAIL is believed to selectively induce cell death of tumor cells but not most normal cells. We tested our TRAIL preparations for potential cytotoxic effects toward normal human cell lines. Human foreskin fibroblasts appeared completely refractory to TRAIL-induced death regardless of the method of TRAIL preparation (Fig. 5A). In contrast, EPC2 cells displayed significant sensitivity to the various TRAIL preparations (Fig. 5B). We recently demonstrated that these cells are sensitive to His-tagged recombinant TRAIL (36). We treated the EPC2 cells with 50 ng/ml of each TRAIL preparation and, using the active caspase 3 fluorescence-activated cell sorter assay, found that EPC2 were sensitive to all four TRAIL preparations (Fig. 5B); however, the hyper-oligomerized TRAIL preparations were clearly more toxic toward the EPC2 cells.

There have been some reports that His-tagged or leucine zipper TRAIL preparations induced cell death in human primary keratinocytes or hepatocytes (25, 27, 37). There was also a report that optimally trimerized soluble TRAIL did not induce apoptosis in human hepatocytes (27). When we compared the dose dependence of cell death induced by trimer TRAIL in EPC2 and H460 cells, EPC2 cells showed a sensitivity to TRAIL similar to that of the H460 lung cancer cells (Fig. 5C), although our trimer TRAIL has optimally trimerized conformation according to our gel filtration results (Fig. 2).

TRAIL-induced Cell Death in Human Primary Esophageal Epithelial Cells Was Prevented by Treatment with a Caspase 9 Inhibitor Even after Initiation of Apoptosis—We previously reported that human primary hepatocytes were sensitive to His-tagged TRAIL treatment, but this death was prevented by co-treatment with the C9I (34). We also showed that the C9I could not prevent apoptosis induced by TRAIL in H460 and SW480 cell lines because these tumor cell lines were killed by

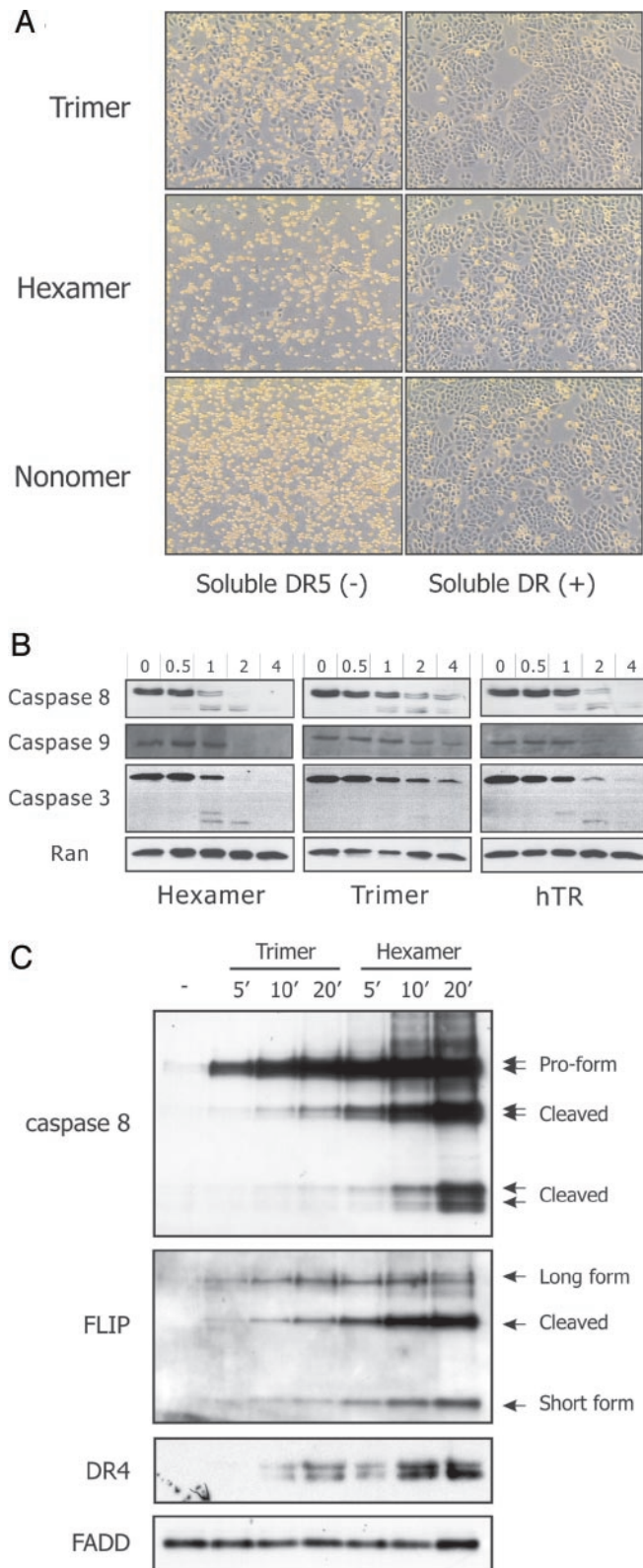


FIG. 3. Hyper-oligomerization of TRAIL correlated with the high potency of nTR2 and nTR3 in cytotoxicity assays. *A*, H460 cells were subjected to treatment with 100 ng of each TRAIL preparation/ml for 4 h, and cell death was checked by phase-contrast microscopy examination. Hyper-oligomerized TRAIL preparations (hexamer and nonomer) induced total cell death, and their activity was blocked by 1.5 μ g/ml soluble DR5 protein treatment. *B*, H460 cells were exposed to 100 ng of each TRAIL preparation/ml and harvested for the Western blot at the indicated time points. Cleavage of caspases and death substrates was proportional to the activity of the TRAIL preparations. *C*, DISC immunoprecipitation of H460 cells was carried out at 5, 10, or

a Type I mechanism that was not sensitive to blockade of caspase 9 activated by the mitochondrial signaling pathway. In the present study, we evaluated the death-protective effect of C9I especially *after* the death signal had been initiated by TRAIL. First, we added the C9I or the soluble DR5 protein at 0-, 1-, 2-, 4-, and 6-h time points during a 6-h culture of EPC2 cells exposed to 100 ng/ml trimer TRAIL at time 0 (Fig. 6A). At the zero time point, when each of the death blockers, C9I or soluble DR5, was added with trimer TRAIL, the death was prevented completely. At the 1-h time point, in the case when the death blockers were added at 1 h after trimer TRAIL, the C9I prevented the death completely, whereas the soluble DR5 only protected by ~50% from death induced by the 6-h treatment using trimer TRAIL. Even at the 2-h time point, we found a potent protective effect of the C9I. Remarkably, the death-protective effect of C9I addition could still be demonstrated as a 50% protection from cell death even after 4 h of trimer TRAIL exposure, whereas soluble DR5 showed no protection from death induced by the total 6-h treatment of trimer TRAIL (when the inhibitors were added similarly at 4 h). Next, we extended the cell culture time with trimer TRAIL to 24 h and investigated whether this death protection was transient (Fig. 6B). These results confirmed that even if EPC2 cells were exposed for 24 h to trimer TRAIL, the addition of C9I at 4 h after TRAIL exposure resulted in 50% protection from cell death, observed at 24 h.

We also tested the death-protective effect of C9I on death induced by hexamer TRAIL, which induced a more potent and rapid apoptosis than that observed with trimer TRAIL. As shown in Fig. 6C, we observed more than 50% cell death protection by C9I at the 2-h time point when 2 or 5 ng/ml hexamer TRAIL was used. The dose that showed similar death inducing activity was 100 ng/ml for trimer TRAIL, whereas 10 or 20 ng/ml hexamer TRAIL induced massive apoptosis such that less than 50% cell death protection was observed at the 2-h time point of C9I treatment. Next, we checked whether using the 2-h time point for caspase 9 blockade could rescue the death of HCT116, which shows a Type II cell death pattern, and found significant death protection by the 2-h time point C9I treatment in either hexamer and trimer TRAIL-treated cells (Fig. 6D).

DISCUSSION

In the present studies, we generated several forms of native TRAIL as well as His-tagged TRAIL and examined their toxicity toward human cancer cell lines and human primary cells. We found that native TRAIL could adopt different oligomeric states depending on the purification conditions, particularly in regard to the use of DTT during purification. Moreover, the cytotoxicity of various TRAIL preparations varied greatly, and this appeared to correlate with their oligomeric state.

Soluble TRAIL was identified as a stable trimer, which contains a zinc ion in the x-ray structural analysis (38). We found that addition of 5 mM DTT during purification significantly increased the oligomerization of TRAIL, which resulted in higher order molecules, including hexamers and a small amount of nonomers in addition to trimer TRAIL. We observed no difference in the oligomeric composition of nTR2 (purified with DTT without additional zinc) *versus* nTR3 (purified with

20 min with 100 ng of TRAIL trimer or hexamer/ml and analyzed for recruitment of caspase 8, DR4, and c-FLIP. Immunoprecipitation was performed using 5 μ l of polyclonal anti-FADD antibody (Cell Signaling) and protein A-agarose for 24 h. Immunoblotting was performed for caspase 8 (mouse monoclonal, clone 3-1-9, BD Biosciences), DR4 (goat polyclonal, R&D Systems), c-FLIP (mouse monoclonal, clone NF6, Alexis), and FADD (mouse monoclonal, BD Biosciences).

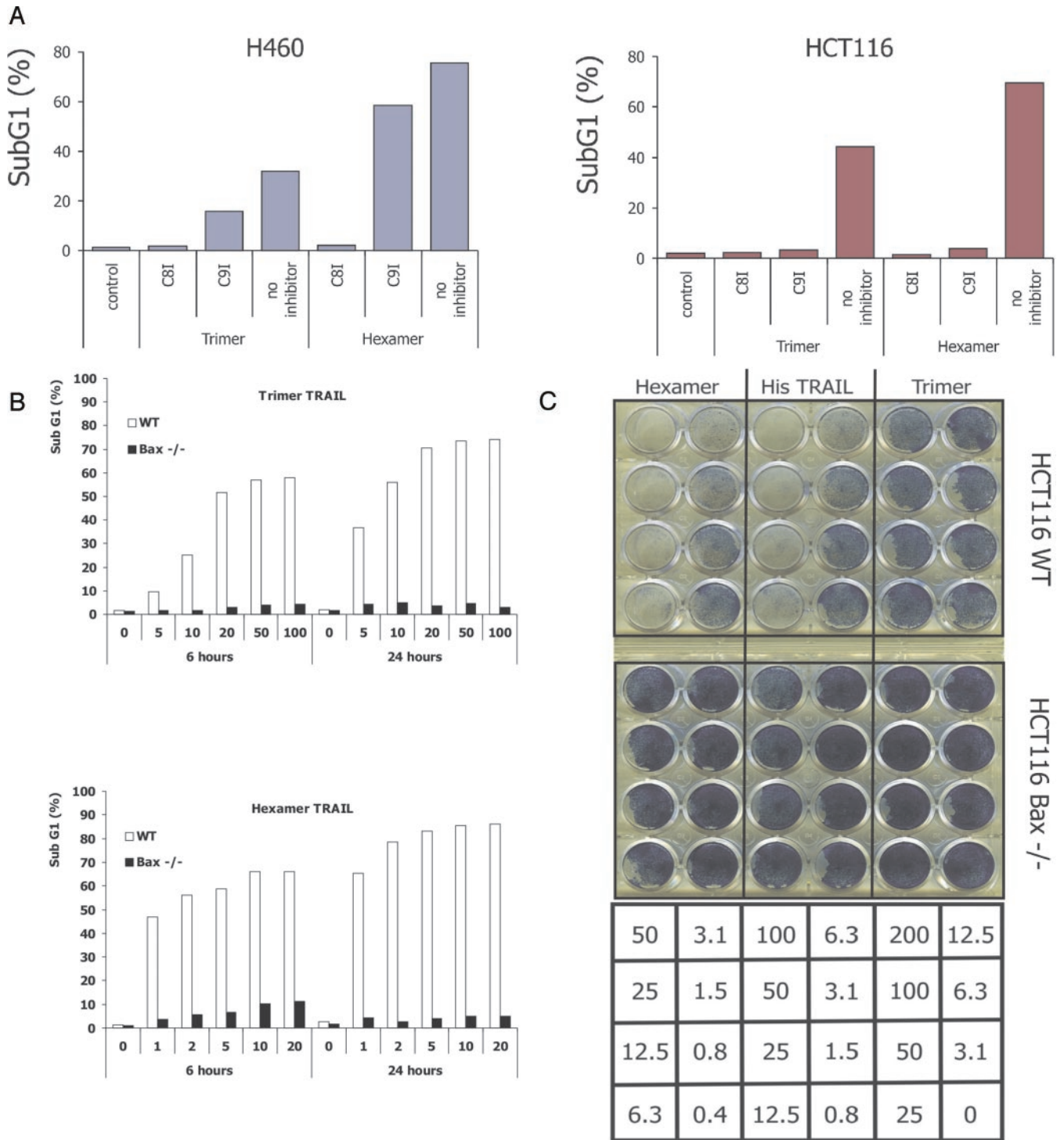


FIG. 4. Type II cell death mechanism was not changed by highly potent hexamer TRAIL. *A*, H460 cells were exposed to trimer (100 ng/ml) or hexamer (10 ng/ml) TRAIL for 6 h. 50 μ M caspase 8 or 9 inhibitor was added at the same time with TRAIL. Cell death was measured by propidium iodide staining and sub-G₁ fraction. In the case of HCT116, which is much more sensitive to TRAIL, 20 ng/ml trimer or 2 ng/ml hexamer TRAIL was used. *B*, dose-dependent cell death was measured in HCT116 wild-type (WT) and Bax-null cells. Cell death was checked at the 6- and 24-h time points. *C*, death-inducing activity of each TRAIL preparation was compared using HCT116 wild-type and Bax-null cells. The upper plate contains wild-type HCT116 cells, whereas the lower plate contains Bax-null HCT116 cells. The final dose of TRAIL preparations (ng/ml) used is indicated in the grid. 1×10^5 cells were plated in each well, cells were exposed to TRAIL for 48 h, and then plates were stained with Coomassie Blue.

DTT plus additional zinc), and there was no difference in their oligomeric profile as seen in Fig. 2. This suggests that additional ZnSO₄ in the LB medium did not influence the oligomeric state of TRAIL molecules. According to Lawrence *et al.* (27) and Hymowitz *et al.* (38), the zinc ion concentration in the culture medium may have an effect on the proper oligomerization of the TRAIL molecule. These studies used M9 minimal

medium for the culture of *E. coli*, which is reported to contain trace metal ions, whereas we used LB medium, which may contain metal ions. This is possibly one reason for the lack of observed differences in the activity and conformation of our nTR2 and nTR3 preparations.

Hyper-oligomerized hexamer and nonomer TRAIL showed a much higher cytotoxicity compared with trimer TRAIL and

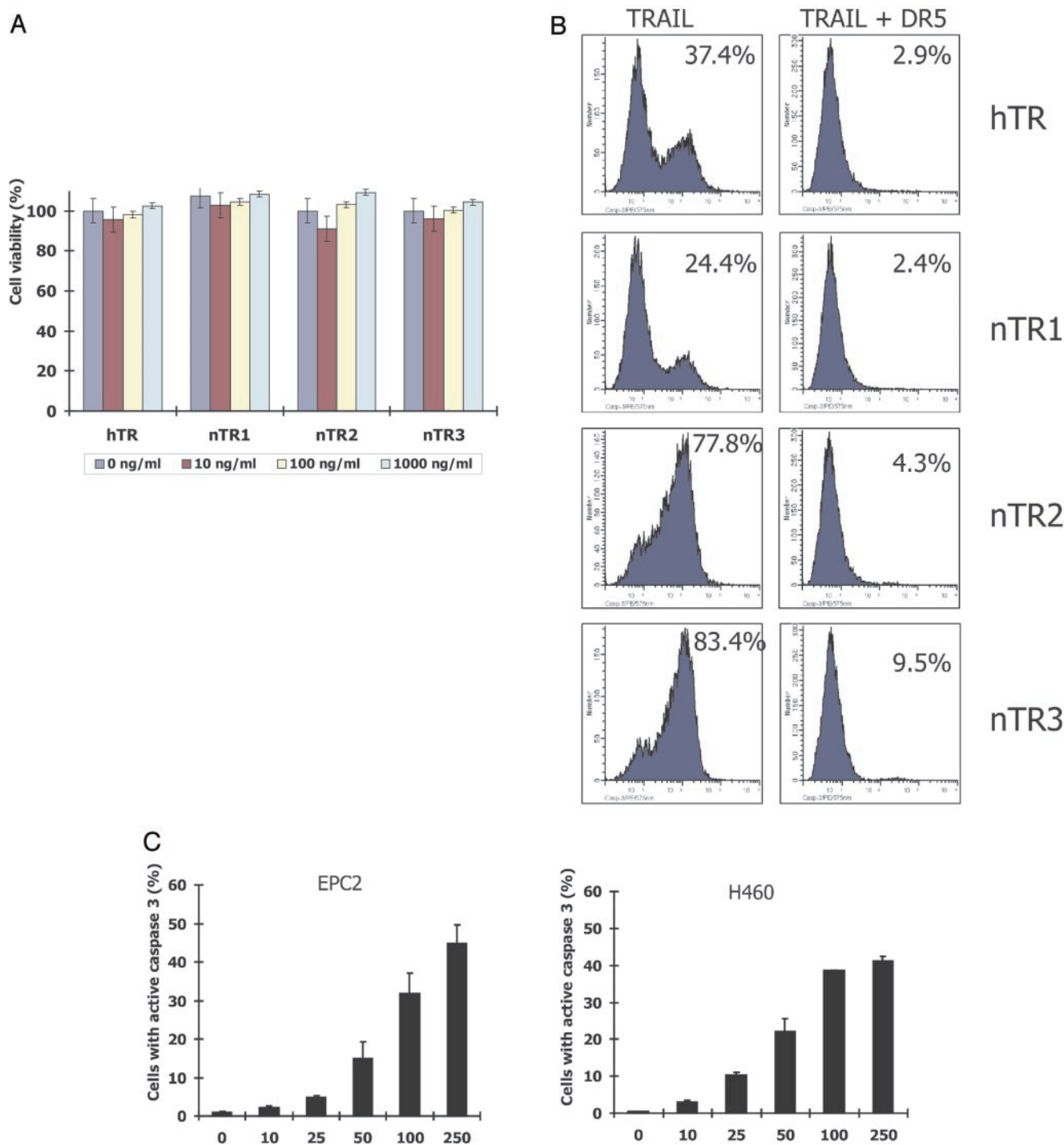


FIG. 5. Sensitivity of primary human cells to TRAIL. *A*, human foreskin fibroblasts were tested for sensitivity to TRAIL and appeared totally refractory to all TRAIL preparations used, up to 1 μ g/ml. Cell survival was checked by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 24 h of treatment with TRAIL. *B*, EPC2 cells were exposed to 100 ng/ml TRAIL preparations with or without soluble DR5 receptor for 4 h, and cell death was measured by staining for the active form of caspase 3 and analysis by flow cytometry. Although this is a normal cell line, significant death was induced by all TRAIL preparations used. The percentage of active caspase 3-positive cells is shown for treatment with only TRAIL (*left panels*) and TRAIL with soluble DR5 receptor cells (*right panels*). *C*, EPC2 and H460 cells were exposed to trimer TRAIL at the indicated concentrations (ng/ml) for 4 h. Sensitivity to trimer TRAIL of EPC2 cells was almost the same as that of H460 lung cancer cells.

induced more rapid apoptosis. This hyperactivity might be explained by the facilitation of receptor clustering that was found in a Fas-signaling pathway (39). Hyper-oligomerized TRAIL could potentially aggregate more than three receptor molecules, thereby inducing rapid receptor clustering, which results in rapid and strong intracellular signal transmission. We found that hexamer TRAIL was more efficient at recruitment of the DISC components (Fig. 3C) but was not capable of

promoting death of Type II cells in the presence of caspase 9 blockade (Fig. 4, *A* and *B*). Treatment with hyper-oligomerized TRAIL also did not result in killing of TRAIL-resistant Bax-null HCT116 cells (Fig. 4C) that die by a Type II mechanism in response to TRAIL. This is in contrast to the effect of the combination of TRAIL plus CPT11 that we recently showed was capable of killing the Bax-null HCT116 cells through an apparent conversion to the Type I mechanism, which in this

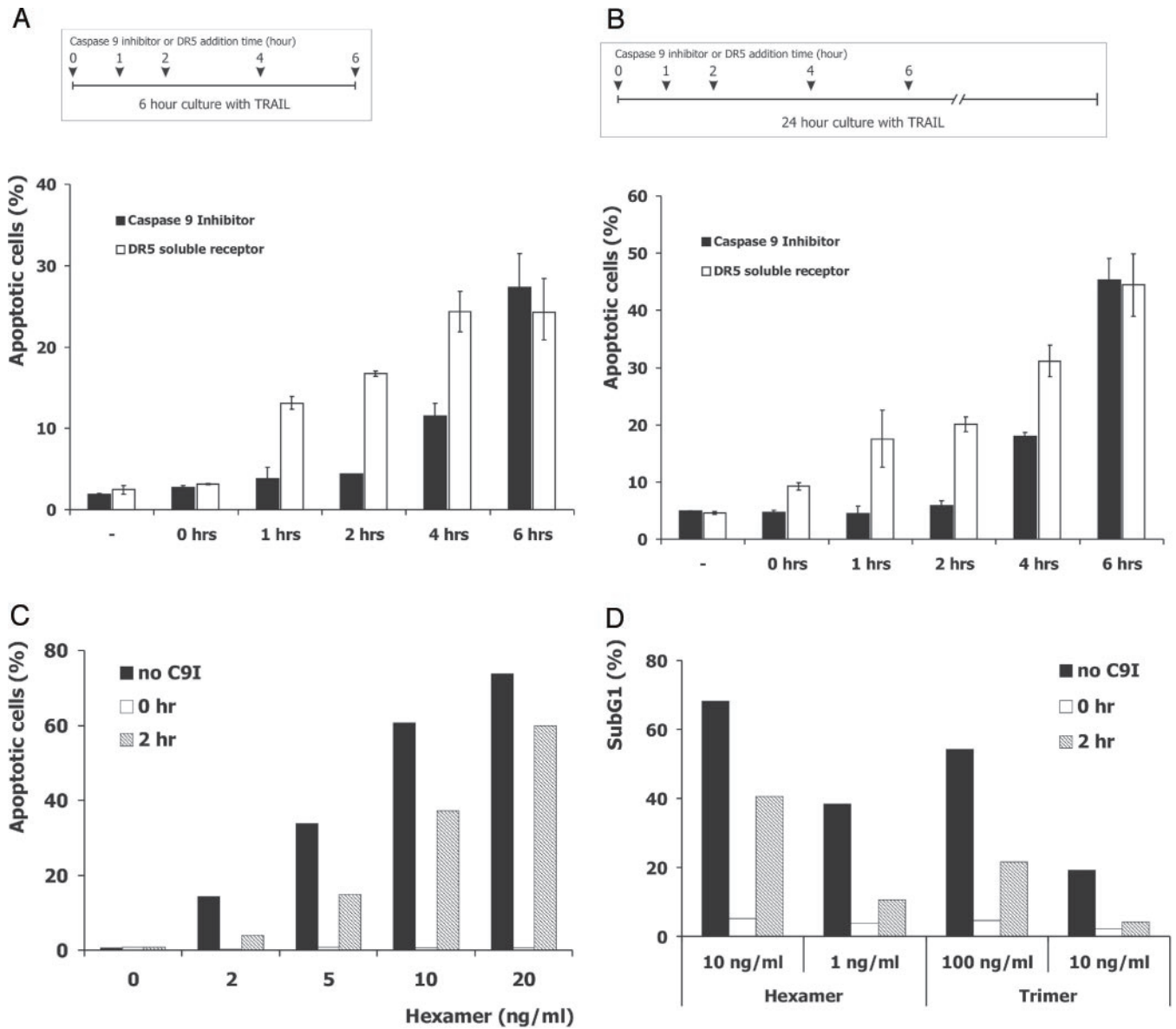


FIG. 6. Toxicity of TRAIL toward EPC2 cells could be reversed by use of a caspase 9 inhibitor treatment even after initiation of TRAIL-induced cell death. A, the 6-h time point for the protection from death by death inhibitors (caspase 9 inhibitor (filled bars) and soluble DR5 (open bars)) is shown. During 6 h of culture of EPC2 cells with 100 ng/ml trimer TRAIL, C9I or soluble DR5 was added to the medium at the indicated time points. At 6 h, cells were harvested for the active caspase 3 assay. B, the 24-h time point experiment for the protection from death by death blockers. All conditions were the same as in B except the EPC2 cell culture time with trimer TRAIL, which was continued for 24 h. C, EPC2 cells were exposed to hexamer TRAIL for 6 h, and death protection by caspase 9 inhibitor was measured at 0- (open bars) and 2-h (hatched bars) time points. Filled bars, no C9I. D, HCT116, which shows Type II cell death, was exposed to hexamer or trimer TRAIL for 6 h, and death prevention by caspase 9 inhibitor was measured at 0- (open bars) and 2-h (hatched bars) time points. Filled bars, no C9I.

case involved, at least in part, p53-dependent up-regulation of KILLER/DR5 (35). Thus, hyper-oligomerized TRAIL does not promote Type I-mediated killing of Type II cells.

Recombinant human soluble TRAIL is a candidate tumoricidal agent, which can induce apoptosis in a variety of human cancer cell lines but not in normal cells. Although some cancer cell lines are not sensitive, TRAIL could induce apoptosis in resistant cell lines when combined with chemotherapeutic drugs, ionizing radiation, or cytokines (18–24). It has been shown that TRAIL could induce apoptosis in several animal tumor xenograft models (16, 17). One potential obstacle for clinical trials lies in the fact that TRAIL might be toxic to certain human cells, especially hepatocytes because His-tagged TRAIL induced a strong apoptotic response in cultured human primary hepatocytes (25). Some studies, however, have suggested that native TRAIL (unlike tagged TRAIL) was less toxic

toward normal hepatocytes and keratinocytes in culture. There has been no published explanation as to why tagged TRAIL acts differently than native TRAIL only with respect to killing of normal human primary cells because both TRAIL preparations presumably use the same signaling pathway, and their effect can be blocked by a caspase-9 inhibitor (34).

The toxicity of TRAIL to human primary cells could represent a hindrance to the use of TRAIL to treat cancer patients. In this study, we demonstrated that human primary esophageal epithelial cells were sensitive to trimer and hyper-oligomerized TRAIL preparations. Importantly, we showed that this toxicity could be avoided by caspase 9 inhibitor treatment. We evaluated the death-protective effect of C9I or soluble DR5. The soluble DR5 protein treatment represents the clearing of TRAIL molecules in the culture environment and could not block the death pathway already initiated by TRAIL prior to

clearance of the TRAIL. On the other hand, C9I showed an excellent death-protective effect, and nearly no cell death was noticed even when administered 2 h after trimer TRAIL exposure. Furthermore, C9I prevented nearly 50% of cell death at 4 h of trimer TRAIL exposure, and this was observed even with a 24-h total duration of trimer TRAIL exposure. We believe this is a significant finding that provides a safety window for the monitoring of TRAIL toxicity, which might occur during clinical use of recombinant human TRAIL. After administration of TRAIL, any sign of cell death in major organs in 2–4 h may possibly be reversed by caspase 9 inhibitor treatment. Although it was not as strong as trimer TRAIL-treated cells, the C9I treatment showed significant cell death protection when EPC2 cells were exposed to hexamer TRAILs. The hyper-oligomerized TRAIL preparations, which show high potency and also high toxicity to EPC2 cells, could be considered a therapeutic agent for tumor-specific therapy.

In conclusion, we generated several recombinant soluble TRAIL preparations and found that the oligomerization status of TRAIL determines the potency of TRAIL in killing cells. We also found that human primary esophageal epithelial cells are sensitive to various TRAIL preparations and that the death induced by TRAIL could be reversed by caspase 9 inhibitor treatment even after the death-signaling pathway was initiated. The death protection by caspase 9 inhibitor treatment is a specific phenomenon to the cells with the Type II cell death mechanism. This is not unique to normal cell lines because we could observe similar death protection by caspase 9 inhibition in the HCT116 human colon cancer cell line. But fortunately, many tumor cells acquire mesenchymal characteristics during tumor progression and tend to die by a Type I pathway, whereas normal epithelial cells tend to die by a Type II mechanism. This phenomenon has been recently demonstrated for Fas signaling (40). This evolution during tumor progression may provide an Achilles' heel for many tumor cells that may be sensitive to Type I TRAIL-mediated cell death (not inhibitable by caspase 9 blockade), although normal epithelial cells can be protected. The protective effect of the caspase 9 inhibitor may be useful in the clinic in combination with recombinant TRAIL.

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