

# A Human scFv Antibody against TRAIL Receptor 2 Induces Autophagic Cell Death in Both TRAIL-Sensitive and TRAIL-Resistant Cancer Cells

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## Abstract

**Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induces apoptotic cell death in a variety of tumor cells without significant cytotoxicity on normal cells. However, many cancer cells with apoptotic defects are resistant to treatment with TRAIL alone, limiting its potential as an anticancer therapeutic. Here, we report on the tumoricidal activity of a human single-chain fragment variable, HW1, which specifically binds to TRAIL receptor 2 (TR2) without competing with TRAIL for the binding. HW1 treatment as a single agent induces autophagic cell death in a variety of both TRAIL-sensitive and TRAIL-resistant cancer cells, but exhibits much less cytotoxicity on normal cells. The HW1-induced autophagic cell death was inhibited by an autophagy inhibitor, 3-methyladenine, or by RNA interference knockdown of *Beclin-1* and *Atg7*. We also show that the HW1-mediated autophagic cell death occurs predominantly via the c-Jun NH<sub>2</sub>-terminal kinase pathway in a caspase-independent manner. Analysis of the death-inducing signaling complex induced by HW1 binding to TR2 exhibits the recruitment of TNF receptor-associated death domain and TNF receptor-associated factor 2, but not Fas-associated death domain, caspase-8, or receptor-interacting protein, which is distinct from that induced by TRAIL. Our results reveal a novel TR2-mediated signaling pathway triggering autophagic cell death and provides a new strategy for the elimination of cancer cells, including TRAIL-resistant tumors, through nonapoptotic cell death.** [Cancer Res 2007;67(15):7327–34]

## Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and its associated receptors (TRAIL-R/TR) are attractive targets for cancer therapy as they can kill tumor cells with little cytotoxicity on normal cells *in vitro* and *in vivo* through the p53-independent extrinsic apoptotic pathway (1–3). TR1 (TRAIL-R1/DR4) and TR2 (TRAIL-R2/DR5) contain a cytoplasmic death domain that triggers apoptosis upon TRAIL binding, whereas TR3

(TRAIL-R3/DcR1) and TR4 (TRAIL-R4/DcR2) lack such a functional death domain (reviewed in ref. 2). TRAIL binding to TR1 or TR2 results in conformational changes of the receptor that promote the formation of death-inducing signaling complexes (DISC), which consists of receptors, Fas-associated death domains (FADD), and activated caspase-8 and/or caspase-10 to trigger apoptosis signaling (2). However, many highly malignant tumor cells (>50%) even expressing TR1 and/or TR2 remain resistant to TRAIL-induced apoptosis, the underlying mechanism of which has been poorly understood and varies with the cellular context (4, 5). TRAIL-resistant cells become sensitized to apoptotic cell death via a combination of TRAIL with either chemotherapeutic agents or irradiation (6–10). A number of agonistic monoclonal antibodies (mAb) that specifically target TR1 or TR2 have been isolated to induce apoptotic cell death in multiple tumor cell types, and some of these also exhibit minimal cytotoxicity on normal cells (11–16). However, all of the TR1- or TR2-specific mAbs reported thus far have shown tumoricidal activities as a single agent in most TRAIL-sensitive cells, but not in TRAIL-resistant cells (12, 13). Thus, the isolation of agonistic mAbs that are cytotoxic to TRAIL-resistant cells but not to normal cells is highly desirable for developing novel cancer therapeutics.

When stimulated by either TRAIL or agonistic mAbs, TR1 and/or TR2 can mediate apoptotic cell death in most TRAIL-sensitive cancer cells predominantly through the sequential activation of caspases (2, 12, 15). Depending on the external stimulus and specific cell types, TR1 and/or TR2 can also transduce multiple cellular signaling pathways in a caspase-independent manner, including c-Jun NH<sub>2</sub>-terminal kinase (JNK) and p38, although the components of which remain largely undefined (4, 7, 8, 14, 17). Furthermore, TRAIL can also induce caspase-independent autophagic cell death in normal epithelial cells (18) and in the breast cancer cell line MCF-10A (19), implying that TR1 and/or TR2 are involved in autophagic cell death. However, the specific roles of TR1 and/or TR2 in autophagic cell death have not yet been defined. Autophagy is an intracellular process in which double membrane-bound vesicles encapsulate cytoplasmic proteins and organelles to degrade by fusion with lysosomes in response to external stresses (reviewed in refs. 20, 21). Recent studies have suggested that autophagy is another cell death pathway that is morphologically distinct from apoptosis (22, 23).

Here, we have characterized the cell death-inducing properties of an agonistic anti-TR2 single-chain fragment variable (scFv), HW1, in multiple TRAIL-sensitive and TRAIL-resistant cancer cell types. Unlikely with TRAIL and agonistic TR2-specific mAbs previously reported, HW1 induced autophagic cell death as a single agent in both TRAIL-sensitive and TRAIL-resistant cancer cells exhibiting much less cytotoxicity on normal cells. We also found that HW1-mediated autophagic cell death occurs predominantly via

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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caspase-independent JNK activation. DISC analysis showed that HW1 binding to TR2 induced the recruitment of TNF receptor (TNFR)-associated death domain (TRADD) and TNFR-associated factor 2 (TRAF2), but not FADD, caspase-8, or receptor-interacting protein (RIP), showing that the components of HW1-induced DISC are distinct from those of TRAIL-induced DISC. These results provide a potential therapeutic strategy to kill not only TRAIL-sensitive but also TRAIL-resistant cancer cells through TR2-mediated autophagic cell death, which is distinct from TRAIL-mediated apoptotic pathway.

## Materials and Methods

**Cell lines and reagents.** Adherent human cancer cell lines, HCT116, MCF-7, HepG2, DU-145, ZR75-1, and U87MG were purchased from American Type Culture Collection and grown in DMEM medium supplemented with 10% (v/v) FCS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Life Technologies Invitrogen). Nonadherent human cancer cell lines, Molt-4 and HL-60, were from American Type Culture Collection and grown in RPMI 1640 supplemented with 10% FCS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. Normal human hepatocytes and mammary epithelial cells were purchased from Cambrex BioScience and were cultured under the recommended conditions. Normal human astrocytes were prepared and subcultured as described previously (9). Recombinant human TRAIL (residues 114–281) without any tags were from KOMA Biotech. Soluble TR2-Fc, TNFR1, and TNFR2 were from R&D Systems, and CD95 was from Biovision. AlexaFluor633 and AlexaFluor488 labeling kits were from Molecular Probes. Mouse anti-His<sub>6</sub> IgG was from Sigma. The following rabbit antibodies were used for Western blotting: anti-caspase-3 and anti-caspase-8 were from Stressgen, Inc.; anti-poly(ADP-ribose) polymerase (PARP) and anti-FADD were from Upstate Biotechnology; anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-TRADD, anti-TRAF2, and anti-β-actin were from Cell Signaling Technology; anti-TR2 was from Koma Biotech; and anti-RIP was from BD Bioscience.

**Biochemical characterizations.** Previously, we isolated human scFv HW1<sup>4</sup> (281 amino acids, 30.9 kDa) against the extracellular domain of TR2 (TR2-ECD) from a yeast surface-displayed pseudoimmune scFv library (24). The detailed experimental methods of expression and purification of HW1 (with COOH-terminal FLAG and 6× His tags), TRAIL-His (residues 114–281 with a COOH-terminal 6× His-tag), TRAIL-Flag (residues 114–281 with a COOH-terminal Flag tag), TR2-ECD, TR1-ECD, TR3-ECD, and TR4-ECD (25, 26), competitive ELISA (25), size exclusion chromatography (25), and surface plasmon resonance (SPR; refs. 25, 26) are described in the Supplementary Data.

**Specific binding of HW1 to cell surface-expressed TR2.** Plasmids of T010 and T30 encoding TR2ΔCD-YFP fusion protein (the cytosolic domain deleted TR2 fused to yellow fluorescent proteins) and TR4ΔCD-YFP (the cytosolic domain deleted TR4 fused to yellow fluorescent proteins), respectively, were kindly provided by Prof. F.K. Chan (University of Massachusetts). HCT116 cells were transfected with the respective plasmid by electroporation (1,000 V and 1,500 µF) using a 0.1-cm diameter capillary (Digital Biotech, Korea), seeded at a density of  $5 \times 10^4$  cells/well in 24-well plate over glass coverslips and grown for 30 h. The cells were then washed once with PBS containing 2% FCS and then stained with either Alexa633-labeled HW1 (5 µg/mL) or TRAIL-His (5 µg/mL) for 30 min at 4°C. The cells were then washed thrice with 2% FCS and fixed using 4% paraformaldehyde in PBS for 1 h at 4°C. Images were obtained using a LSM510 model laser scanning confocal fluorescence microscope (LSM510; Carl Zeiss).

**Cell viability assays.** Cells seeded at a density of  $1 \times 10^4$  cells/well in 96-well plates were cultured overnight and treated with HW1 or TRAIL in the absence and presence of the following agents, as specified in the figure legends: Z-VAD [benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl

ketone; Santa Cruz Biotechnology], SP600125 (Calbiochem), SB203580 (Sigma), and 3-methyladenine (3-MA; Sigma). Cell viability was analyzed using a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based cell growth determination kit (Sigma; refs. 6, 26).

**Transmission electron microscopy.** Cells treated with TRAIL-His or HW1, as specified in the figure legends, were prefixed in Karnovsky's solution (2% glutaraldehyde, 1% paraformaldehyde in 100 mmol/L of sodium cacodylate buffer; pH 7.4) at room temperature for 2 h. The samples were rinsed with cacodylate buffer, postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h, dehydrated through a graded series of ethanols (50–100%), and then embedded in Poly Bed 812 resin (Pelco). Ultrathin sections were cut on a Reichert Ultracut E microtome, double-stained with uranyl acetate and then lead citrate, and viewed under an electron microscope (EM 902A; Carl Zeiss).

**Light and fluorescence microscopy.** For the analysis of green fluorescent protein-fused LC3 (GFP-LC3) localization, U87MG cells were transfected with a plasmid encoding GFP-LC3 (27, 28) kindly provided by Prof. T. Yoshimori (Osaka University) and stably expressing cells were selected with changes of media containing 500 µg/mL of G418. After treatment of the stable cell lines expressing GFP-LC3 with HW1 (25 µg/mL, 24 h), images were obtained under a live fluorescence microscope (Axiovert 200M; Carl Zeiss). The nucleus was stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories). The images were obtained using a LSM510 model laser scanning confocal fluorescence microscope (LSM510; Carl Zeiss). The fluorescence images taken and processed using identical settings are representative of experiments done at least in duplicate.

**Western blotting.** Cells ( $3 \times 10^5$  cells/well) were seeded in six-well plates, grown overnight, and then treated under the conditions specified in the figure legends. The standard procedure for Western blotting was then done as described previously (6, 9). The secondary anti-rabbit IgG conjugated to horseradish peroxidase (Zymed Laboratories) was used for developing by chemiluminescence (Amersham Pharmacia Biotech).

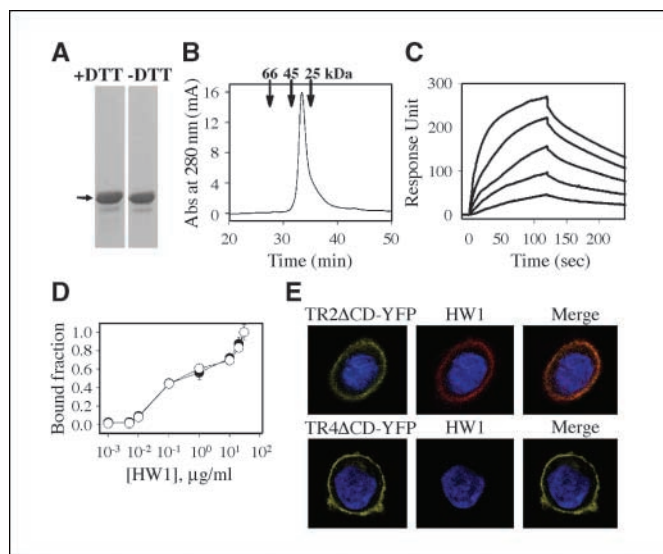
**RNA interference.** Small interfering RNA (siRNA) oligonucleotides were synthesized at Bioneer Co. The mRNA sequences targeted by *Beclin-1* and *Atg7* siRNAs were 5'-CAGUUUGGCACAAUCAUA-3' and 5'-GGAGUCA-CAGCUCUUCUU-3', respectively (22). An unrelated siRNA with a sequence of 5'-AGACACACGCACUCGUCUC-3' was employed as a control. HCT116 or U87MG cells were transfected with 1 µmol/L of siRNA by electroporation in a 0.1-cm diameter capillary (Digital Biotech). Transfected cells were incubated for 48 h at a density of  $3 \times 10^5$  cells/well in a six-well plate for Western blotting using anti-Beclin 1 (Novus Biologicals) or anti-Atg7 antibody (Novus Biologicals). Twenty-four hours posttransfection, the cells ( $1 \times 10^4$  cells/well in 96-well plate) were treated with 200 ng/mL of TRAIL for 2 h or 25 µg/mL of HW1 for 30 h prior to MTT assay.

**Immunoprecipitations.** Cells ( $1 \times 10^7$  cells/time point) were left untreated or treated with 2 µg/mL of Flag-tagged TRAIL (29) or 25 µg/mL of Flag-tagged HW1 for the indicated time periods (see figure legends). The cells were collected and lysed for 30 min on ice in 1 mL of a lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 2 mmol/L EDTA] with protease inhibitor cocktail (Roche). After removing cell debris by centrifuging at  $15,000 \times g$  for 15 min at 4°C, the cell lysates (800 µL, 15 mg protein) were precipitated with 80 µL of EZview Red anti-Flag M2 Affinity Gel (Sigma) overnight at 4°C. The complexes were subsequently washed five times with TBS buffer [50 mmol/L Tris, 150 mmol/L NaCl (pH 7.4)], suspended in 20 µL of 2× SDS sample buffer [125 mmol/L (pH 6.8), 10% (w/v) SDS, 20% (v/v) glycerol, 14.4 mmol/L 2-ME, and 0.0002% (w/v) BPP], and subjected to SDS-PAGE and Western blotting.

## Results

**HW1 specifically binds to TR2 without competition with TRAIL for the binding.** Human scFv HW1 recently isolated against TR2-ECD from a pseudoimmune scFv library (24) was solubly expressed and purified without intermolecular disulfide bonds from bacterial supernatants with >98% purity (Fig. 1A). HW1 existed in a monomeric form (~30.9 kDa) in solution at concentrations of up to 500 µg/mL (~16 µmol/L), as determined

<sup>4</sup> The HW1 sequence data has been deposited into the Genbank under accession no. EF088679.



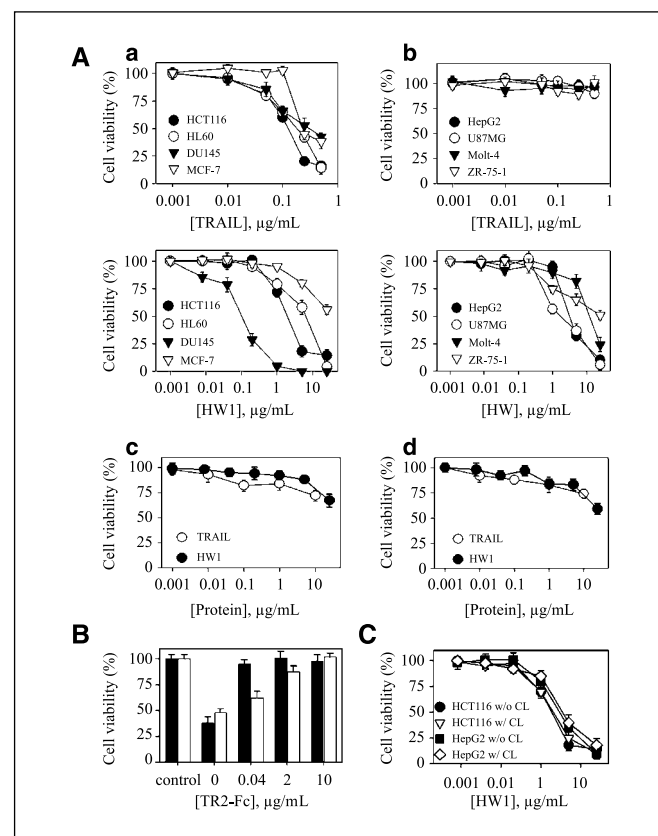
**Figure 1.** Biochemical characterizations of human scFv HW1. *A*, reducing (+DTT) and nonreducing (–DTT) SDS-PAGE analyses of the purified HW1. *B*, size exclusion elution profile of HW1 injected at 500  $\mu\text{g/mL}$  ( $\sim 16 \mu\text{mol/L}$ ) and monitored at 280 nm. *C*, kinetic interactions of HW1 with TR2-ECD determined by SPR analyses. SPR sensograms were obtained from injections of serially diluted HW1 at 1.5, 0.75, 0.38, 0.19, and 0.09  $\mu\text{mol/L}$  (from top to bottom) over a TR2-ECD immobilized surface. *D*, no competition between HW1 and TRAIL-His for the binding to TR2. ELISA plates coated with TR2-ECD were incubated with the indicated concentrations of HW1 in the absence or presence of 20  $\mu\text{g/mL}$  of TRAIL-His. Points, relative bound fractions of HW1 to TR2-ECD from experiments done in triplicate; bars, SD. *E*, specific binding activity of HW1 for cell surface-expressed TR2. Alexa633-labeled HW1 (5  $\mu\text{g/mL}$ ) was incubated for 30 min at 4°C with HCT116 cells transiently transfected with TR2 $\Delta$ CD-YFP (*top*) and TR4 $\Delta$ CD-YFP (*bottom*), respectively, and then visualized by confocal fluorescence microscopy. Nuclei were costained with DAPI. Magnification,  $\times 630$ .

by size exclusion chromatography (Fig. 1*B*). SPR analyses showed that HW1 bound to TR2-ECD with association and dissociation rate constants of  $2.33 \pm 0.02 \times 10^4 \text{ (mol/L)}^{-1} \text{ s}^{-1}$  and  $4.71 \pm 0.05 \times 10^{-3} \text{ s}^{-1}$ , respectively, resulting in a  $K_D$  value of  $202 \pm 6 \text{ nmol/L}$  (Fig. 1*C*). However, HW1 bound to neither the homologous ECDs of TR1, TR3, and TR4, nor the TNF family death receptors of TNFR1, TNFR2, and CD95 (Supplementary Fig. S1*A*), which was further confirmed by ELISA (data not shown). Thus, HW1 specifically binds to TR2 without cross-reactivity with the other death receptors. Notably, competitive ELISA showed that HW1 did not compete with TRAIL-His for the binding to TR2-ECD (Fig. 1*D*), suggesting that it recognizes epitopes which do not overlap with the TRAIL-binding sites on TR2-ECD.

The specific binding activity of HW1 for cell surface-expressed TR2 was also analyzed by confocal fluorescence microscopy in HCT116 cells transiently transfected with a plasmid encoding TR2 $\Delta$ CD-YFP using TR4 $\Delta$ CD-YFP as a control (Fig. 1*E*; ref. 30). The staining of cells expressing TR2 $\Delta$ CD-YFP with Alexa633-labeled HW1 at 4°C revealed a discrete colocalization pattern of HW1 and TR2 along the plasma membrane, similar to the staining with Alexa633-labeled TRAIL-His. However, HW1 did not bind to cells expressing TR4 $\Delta$ CD-YFP (Fig. 1*E*), which colocalized with Alexa633-labeled TRAIL-His on the plasma membrane (Supplementary Fig. S1*B*). Upon incubation at 37°C for 5 min, the colocalized fluorescence signal from the HW1-TR2 complexes exhibited strong punctuated foci (Supplementary Fig. S1*C*). These observations indicate that HW1 specifically binds to TR2 to form oligomeric HW1-TR2 complexes on the cell surface.

Flow cytometry analysis of Alexa488-labeled HW1 binding to endogenous TR2 on HCT116 cells, which overexpressed TR2 on the cell membrane following pretreatment with sulforaphane (6), showed a much more enhanced binding activity in the sulforaphane-treated cells compared with untreated cells, further indicative of the specific binding activity of HW1 for TR2 (Supplementary Fig. S2*A*).

**HW1 induces cell death in multiple TRAIL-sensitive and TRAIL-resistant tumor cells with much less cytotoxicity on normal cells.** Tumoricidal activities of HW1 were evaluated using multiple human cancer cells with different sensitivities to TRAIL. These included HCT116 (colorectal carcinoma), HL-60 (monocyte leukemia), Du145 (prostate cancer), and MCF-7 (breast cancer) for TRAIL-sensitive cells and HepG2 (hepatoma), U87MG (glioma), Molt-4 (T cell leukemia), and ZR-75-1 (breast cancer) for TRAIL-resistant cells (4). The cells were incubated with various concentrations of TRAIL-His (0.008–0.5  $\mu\text{g/mL}$ ) and HW1



**Figure 2.** HW1 alone induces cell death in both TRAIL-sensitive and TRAIL-resistant tumor cells with much less cytotoxicity on normal cells. *A*, cell killing activities of TRAIL-His (*a* and *b*, top) and HW1 (*a* and *b*, bottom) in various TRAIL-sensitive (*a*) and TRAIL-resistant tumor cells (*b*). Cells were incubated with the indicated concentrations of TRAIL-His for 8 h (*a*) or 30 h (*b*) and HW1 for 30 h. *c* and *d*, cytotoxicity assay of non-tagged TRAIL and HW1 in normal human hepatocytes (*c*) and mammary epithelial cells (*d*), which were incubated with the indicated concentrations of non-tagged TRAIL or HW1 for 30 h. *B*, inhibition of cell death induced by TRAIL-His (filled columns) or HW1 (open columns) by the soluble competitor TR2-Fc. HCT116 cells were treated with 0.1  $\mu\text{g/mL}$  of TRAIL-His for 8 h or 5  $\mu\text{g/mL}$  of HW1 for 30 h in the presence of the indicated concentrations of TR2-Fc. *C*, no effects of cross-linked HW1 on the cell death activity in HCT116 and HepG2 cells. Cells were incubated for 30 h with the indicated concentrations of HW1, which were untreated or cross-linked by preincubation (4°C for 1 h) with a secondary cross-linking agent of mouse anti-His<sub>6</sub> IgG (10  $\mu\text{g/mL}$ ). *A* to *C*, cell viability was determined by MTT assay. Points, percentages of viable cells compared with untreated control cells from experiments done at least in triplicate; bars, SD.

(0.008–25  $\mu\text{g}/\text{mL}$ ) for the indicated periods specified in the figure legends. Cell viability was determined by MTT assay (6, 26). All of the known TRAIL-sensitive cells were susceptible to both TRAIL-His- and HW1-mediated cell deaths in a dose-dependent manner (Fig. 2A, a). DU145 cells were most susceptible to HW1, exhibiting an  $\text{EC}_{50}$  (50% effective concentration) of  $\sim 100$  ng/mL. As expected, the known TRAIL-resistant cells did not show significant cell death (<12%) by TRAIL-His treatments of up to 0.5  $\mu\text{g}/\text{mL}$  for 30 h (Fig. 2A, b). Strikingly, however, HW1 exhibited cell-killing activity for all of these TRAIL-resistant cells in proportion to the concentration starting from 12 h at  $>1$   $\mu\text{g}/\text{mL}$  (Fig. 2A, b). Cells dying because of HW1 treatment became rounded and were detached from the culture plates. The sensitivity to HW1-induced cell death varied among the cells, with U87MG displaying the highest susceptibility ( $\text{EC}_{50} \approx 1.5$   $\mu\text{g}/\text{mL}$ ).

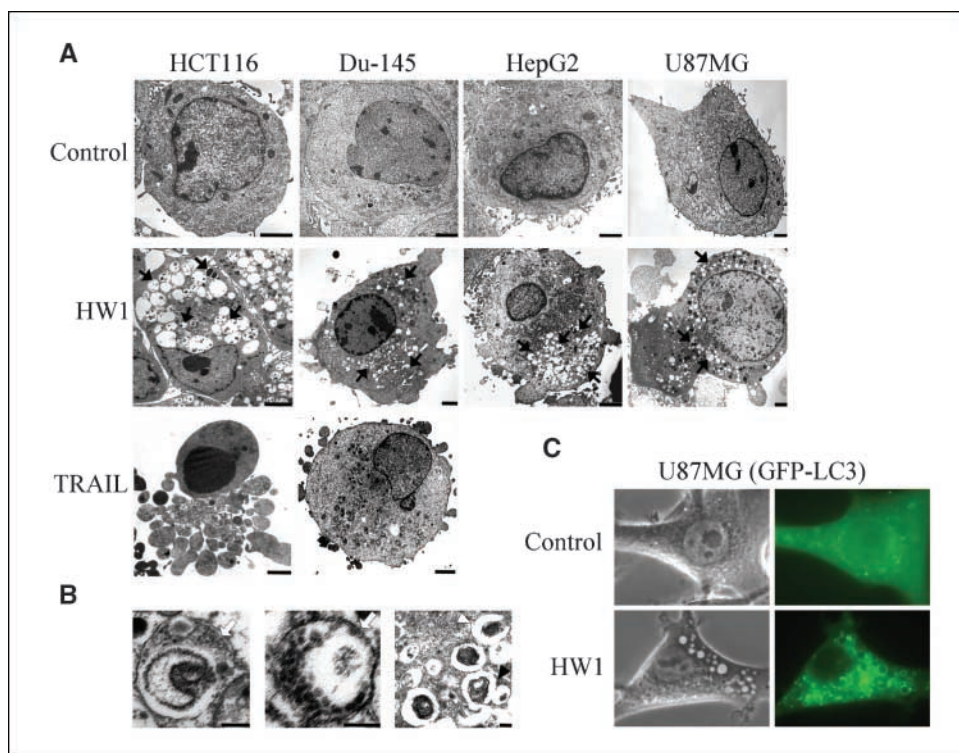
Next, we determined the cytotoxicity of HW1 on normal cells using human hepatocytes, mammary epithelial cells, and astrocytes in comparison with non-tagged TRAIL (residues 114–281). Tagged TRAIL with  $\text{NH}_2$ -terminal  $6 \times$  His exogenous sequences have previously exhibited cytotoxicity on normal human hepatocytes (31), whereas non-tagged TRAIL and some anti-TR2 mAbs have not (3, 14, 15). HW1 treatment exhibited very low levels of cytotoxicity ( $\leq 20\%$ ) at doses  $<5$   $\mu\text{g}/\text{mL}$  for 30 h incubation in normal human hepatocytes and mammary epithelial cells, like the non-tagged TRAIL (Fig. 2A, c and d). Treatment with 25  $\mu\text{g}/\text{mL}$  of HW1 for 30 h induced  $\sim 40\%$  cell death in both normal human cells (Fig. 2A, c and d). However, the cytotoxicity of HW1 for the normal cells showed far lower levels than for the cancer cells tested under the same conditions (Fig. 2A). For normal human astrocytes, HW1 did not show any cytotoxicity even up to 40  $\mu\text{g}/\text{mL}$  for 48 h incubation (data not shown). Thus, HW1 exhibited much less cytotoxicity on normal human cells compared with multiple cancer cells.

Whether HW1 induced cell death through specific binding to TR2 was assessed using a soluble competitor of TR2-Fc-conjugated

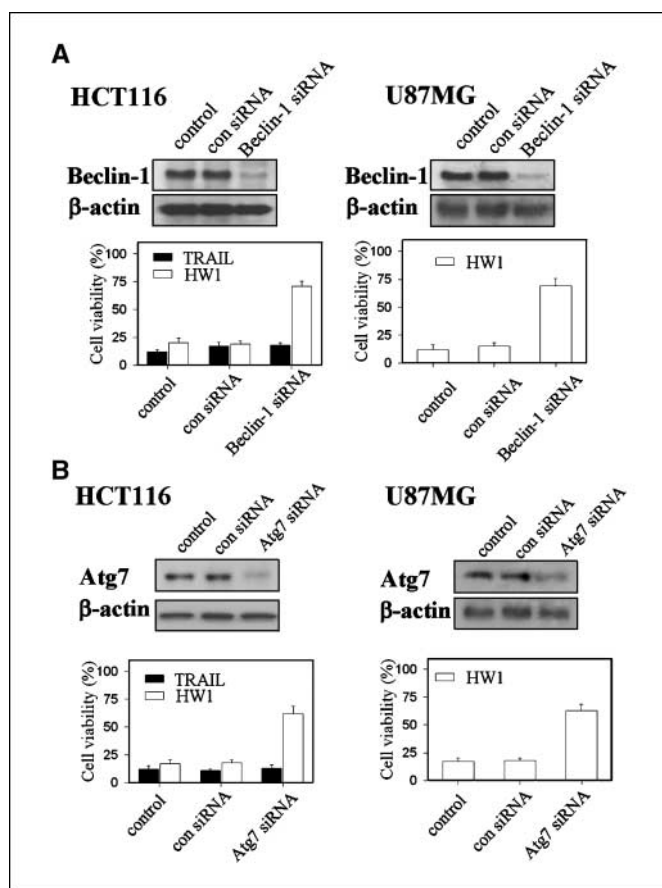
protein (TR2-ECD fused to the constant region of the immunoglobulin) for cell surface-expressed TR2. Cell death in HCT116 cells mediated by either HW1 or TRAIL-His was gradually inhibited with increased concentrations of TR2-Fc (Fig. 2B). Furthermore, HW1 showed enhanced cell death activity for HCT116 cells with TR2 overexpressed by pretreatment with sulforaphane, compared with the untreated control cells (Supplementary Fig. S2B). These results showed that HW1 specifically transduces cell death signaling through TR2.

Some bivalent TR2-specific IgG mAbs induced apoptotic cell death as a single agent (11, 14, 15), but their respective monovalent Fab forms could not (16, 32), indicating that the oligomerization of TR2 is required to recruit and activate subsequent molecules involved in cell death, like other TNFRs (2). However, monovalent scFv HW1 induced cell death in the absence of secondary cross-linking agents. Even cross-linking of  $6 \times$  His-tagged-HW1 using mouse anti-His<sub>6</sub> IgG did not augment the cell death activity in HCT116 and HepG2 cells examined (Fig. 2C). In contrast, the cross-linking of TRAIL-His under the same conditions showed significantly enhanced killing activity in HCT116 cells (data not shown), consistent with previous results (1, 32).

**HW1 induces autophagy in both TRAIL-sensitive and TRAIL-resistant tumor cells.** The tumoricidal activity of HW1, even for TRAIL-resistant cells, prompted us to characterize the associated cell death morphology by transmission electron microscopy. Compared with untreated cells exhibiting normal nuclear and cytoplasmic morphology (Fig. 3A, top), TRAIL-sensitive HCT116 and Du145 cells treated with TRAIL-His showed typical characteristics of apoptotic cell deaths, such as nuclear shrinkage, chromatin condensation, and membrane blebbing (Fig. 3A, bottom; refs. 20, 21). However, HW1-treated cells exhibited numerous membrane-bound vesicles occupying the major cytoplasmic space ( $>90\%$ ), which frequently contained electron-dense materials of cytoplasmic fragments and organelles, such as entire or damaged



**Figure 3.** HW1 induces autophagy in both TRAIL-sensitive and TRAIL-resistant cancer cells. **A**, representative transmission electron microscopy images of TRAIL-sensitive HCT116 and DU145 cells and TRAIL-resistant U87MG and HepG2 cells, which were left untreated as controls (top), or treated with either 25  $\mu\text{g}/\text{mL}$  of HW1 for 20 h (middle) or 0.2  $\mu\text{g}/\text{mL}$  of TRAIL-His for 5 h (bottom). Autophagic vesicles (black arrows); bars, 2  $\mu\text{m}$ . **B**, representative high-magnification images of multiple- (left) or double membrane-bound (middle) autophagosomes (white arrows), and autophagosome induction stage (white arrowhead) and autophagosome fusion with autophagic vacuoles (black arrowhead; right); bars, 0.2  $\mu\text{m}$ . **C**, HW1-induced LC3 accumulation in autophagic vacuoles. Phase contrast (left) and fluorescence images (right) of U87MG cells stably expressing GFP-LC3, which were left untreated (top) or treated with 25  $\mu\text{g}/\text{mL}$  of HW1 for 24 h (bottom). Magnification,  $\times 800$ .



**Figure 4.** Inhibition of the HW1-mediated autophagic cell death by siRNAs against *Beclin-1* (A) and *Atg7* (B) in TRAIL-sensitive HCT116 cells (left) and TRAIL-resistant U87MG cells (right). After transfection with *Beclin-1*, *Atg7*, or unrelated (con) siRNA, the cells were cultured for 48 h and then subjected to Western blotting using anti-*Beclin-1* or anti-*Atg7* antibodies. Cell viability was determined by MTT assay for siRNA-transfected cells cultured for 24 h and then further incubated with 200 ng/mL of TRAIL-His for 2 h or 25  $\mu$ g/mL of HW1 for 30 h. Untransfected cells (Control) were also treated with TRAIL or HW1 under the same conditions. Columns, percentages of viable cells compared with untreated control cells from experiments done in triplicate; bars, SD.

mitochondria, in both TRAIL-sensitive and TRAIL-resistant cells (Fig. 3A, middle). These are typical autophagic vesicle structures associated with autophagy (20, 21). Higher magnification of images clearly revealed multiple or double membrane-bound autophagosomes with fragmented cellular organelles and debris inside (Fig. 3B, left and middle; refs. 19–21). Simultaneously, autophagosome induction stages where membrane-bound vacuoles surround mitochondria and autophagosome fusion with autophagic vacuoles were also observed (Fig. 3B, right). The dying cells by HW1 treatment also exhibited common characteristics of apoptosis and autophagy, such as shrunk nuclei, partial chromatin condensation, and membrane blebbing (Fig. 3A, middle; refs. 20, 21).

Another distinctive hallmark of autophagy is the translocation of LC3 from the cytosol to the autophagic vacuoles (27, 28). When TRAIL-resistant U87MG cells stably expressing GFP-LC3 were incubated with HW1, the accumulation of GFP-LC3 was observed both around and inside the autophagic vacuoles, with strong discrete dots as shown by confocal fluorescence microscopy, compared with untreated cells (Fig. 3C). Furthermore, when the HW1-induced dying cells of HCT116 and U87MG were stained with lysotracker red, which

accumulates in acidic vesicles (20, 21), they exhibited numerous punctuate staining patterns, compared with untreated cells exhibiting only very weak diffuse staining (Supplementary Fig. S3A and B). This supports the cytosolic accumulation of autophagic vacuoles. To determine whether autophagy was responsible for the HW1-induced cell death, the effect of 3-MA, an autophagy inhibitor (21), on the HW1-induced cell death was evaluated. Pretreatment of HCT116 and U87MG cells with 3-MA (50  $\mu$ mol/L) significantly abolished the cytosolic accumulation of autophagic vacuoles monitored by lysotracker red staining (Supplementary Fig. S3A and B) and completely suppressed the HW1-induced cell death at 125  $\mu$ mol/L (Fig. 5B), such as the TRAIL-mediated autophagic cell death of epithelial cancer cells (19). Furthermore, long-term clonogenic assays showed that inhibiting the HW1-induced autophagic cell death of HCT116 cells by 3-MA rescued the colony-forming ability of the cells (Supplementary Fig. S4).

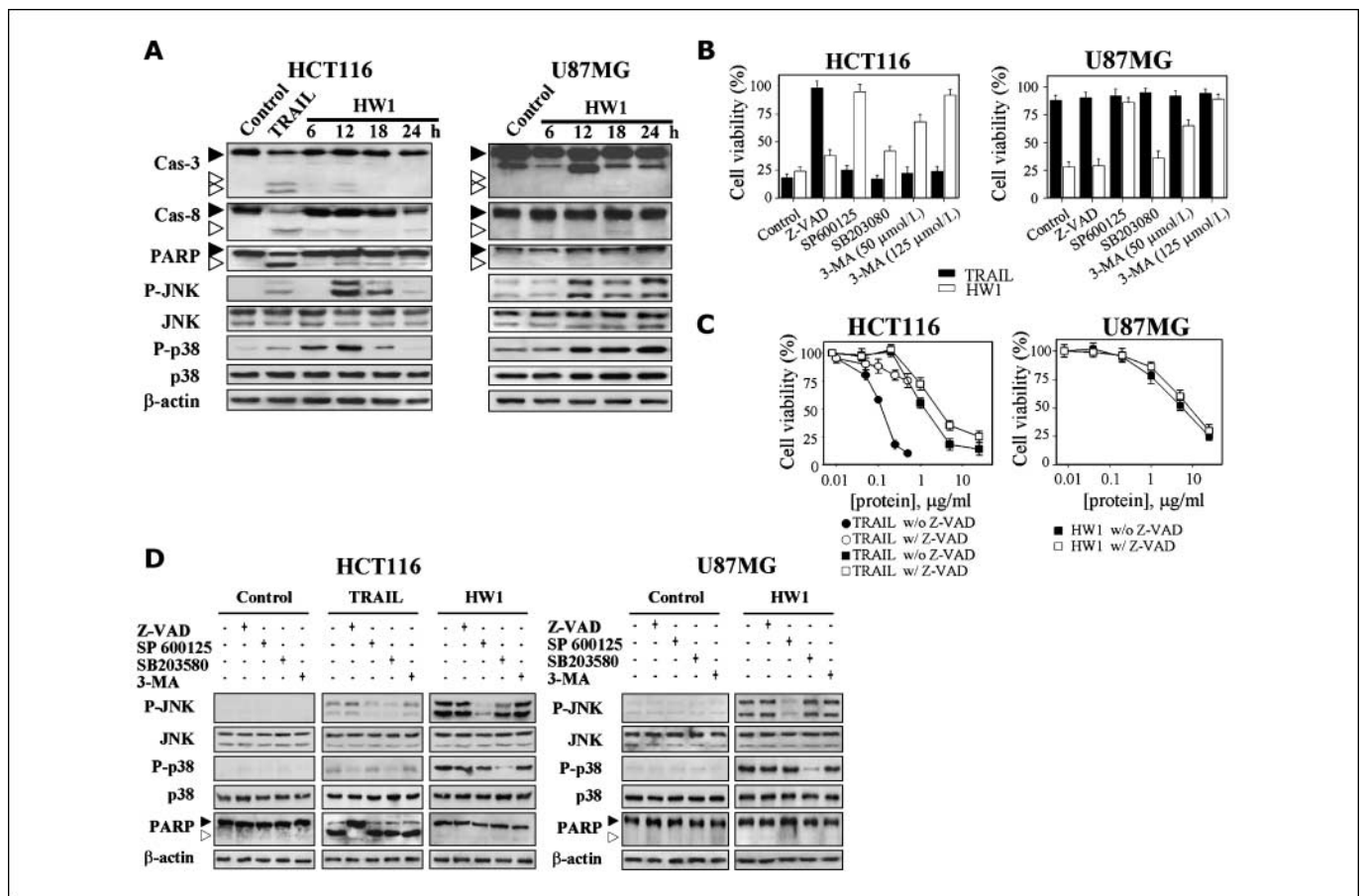
Several autophagy-related (*Atg*) genes, including *Beclin-1* and *Atg7*, have been known to be associated with the autophagic pathway (20–22). To determine whether *Beclin-1* and/or *Atg7* were involved in HW1-induced cell death, we reduced the expression of *Beclin-1* and *Atg7* by RNA interference in TRAIL-sensitive HCT116 and TRAIL-resistant U87MG cells before HW1 treatment (Fig. 4). The significantly suppressed expression of the two respective genes by siRNA caused a dramatic inhibition of the HW1-induced cell death in both HCT116 and U87MG cells, but exhibited negligible effects on TRAIL-induced cytotoxicity in HCT116 cells, compared with cells untreated or treated with the unrelated siRNA (Fig. 4). This result suggested that *Atg7* and *Beclin-1* are closely associated with the HW1-induced autophagic cell death, consistent with typical autophagic cell death (20–22, 33). Taken together, we concluded that HW1 induces autophagic cell death not only in TRAIL-sensitive cancer cells, but also in TRAIL-resistant cancer cells.

**HW1-induced autophagic cell death occurs mainly through the JNK pathway.** To elucidate the mechanisms underlying HW1-induced cell death, activation of some participants involved in cell death were monitored by Western blotting in TRAIL-sensitive HCT116 and TRAIL-resistant U87MG cells. Caspase-3, caspase-8, and PARP were significantly cleaved from their pro-forms in HCT116 cells treated with TRAIL-His for 2 h, but was very slightly cleaved in both HCT116 and U87MG cells treated with HW1 for the various time periods (Fig. 5A). Instead, compared with TRAIL-His-treated or untreated control cells, HW1-treated cells exhibited significant activations of both JNK and p38 by at least 3-fold starting from 12 h, which closely paralleled the time course of cell death in both cells (Fig. 5A). We then examined further whether caspases, JNK, or p38 plays a critical role in HW1-induced autophagic cell death, using their specific inhibitors. The pan-caspase inhibitor Z-VAD (10  $\mu$ mol/L) completely blocked the cleavage of PARP by caspase-3 and cell death in HCT116 cells treated with TRAIL-His (Fig. 5B–D). However, for HW1-treated HCT116 and U87MG cells, Z-VAD did not attenuate JNK activations at all and only slightly reduced the cell death (Fig. 5B–D), indicating that HW1-mediated JNK activation and cell death occur independently of caspase activations. The p38 inhibitor SB203580 (10  $\mu$ mol/L) efficiently inhibited HW1-mediated p38 activation, but not JNK activation, resulting in only a slight reduction in the HW1-induced cell death (Fig. 5B and D). Hence, even though p38 was significantly activated in HW1-treated cells (Fig. 5A), its activation was not essential for HW1-induced cell death. Strikingly, the JNK inhibitor SP600125 (10  $\mu$ mol/L), which

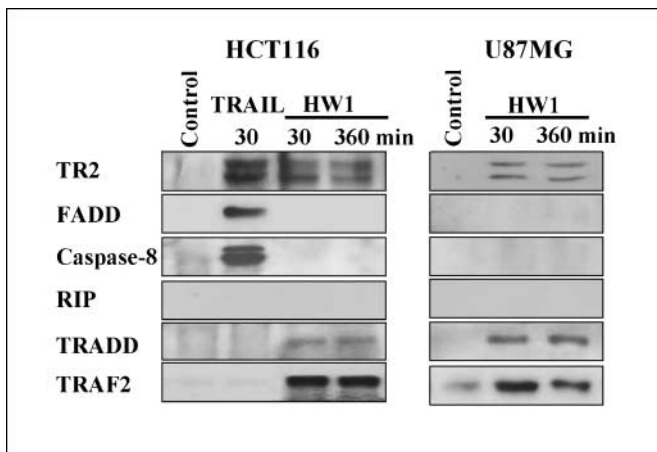
efficiently inhibited JNK activations, completely suppressed HW1-mediated cell death in both TRAIL-sensitive and TRAIL-resistant cells (Fig. 5B and D), indicating an essential requirement of JNK activation in HW1-mediated cell death. Furthermore, blocking the cell death of HCT116 cells induced by HW1 with the JNK inhibitor SP600125 efficiently rescued the colony-forming capability of the cells (Supplementary Fig. S4). 3-MA (50 μmol/L) did not inhibit JNK activations, but completely blocked the HW1-stimulated cell death at 125 μmol/L (Fig. 5B and D), suggesting that it attenuates autophagic cell death by operating downstream of JNK activation. The HCT116 and U87MG cells dying because of HW1 in the presence of Z-VAD or SB203580, but not with SP600125 or 3-MA, exhibited numerous punctuate lysotracker red stainings (Supplementary Fig. S3), indicative of the accumulation of autophagic vacuoles, and thus, autophagic cell death. The inhibitors against JNK, p38, and autophagy (3-MA) did not affect the TRAIL-induced cell death at all in HCT116 cells (Fig. 5B), whereas Z-VAD almost blocked it (Fig. 5B and C). Taken together, the results showed that

the specific inhibitors of JNK and autophagy (3-MA), but not of caspases and p38, effectively protected both TRAIL-sensitive and TRAIL-resistant tumor cells from the HW1-induced autophagic cell death, consistent with the Z-VAD-mediated autophagic cell death of L929 fibroblastic cells (22).

To further understand the role of JNK in HW1-induced cell death, we transiently transfected HCT116 and U87MG cells with wild-type JNK2 (wtJNK2) or dominant-negative JNK2 mutant (dnJNK2) to modulate JNK activity. The expression of these two respective proteins in both cells was confirmed by Western blotting using anti-JNK antibody (Supplementary Fig. S5). The ectopic expression of dnJNK2 significantly inhibited the HW1-induced cell death in both cells, whereas that of wtJNK2 showed the opposite effect of slightly enhancing the HW1-mediated cell death, compared with control cells transfected with an empty vector and then treated with HW1 (Supplementary Fig. S5). This result, together with that of the JNK inhibitor, indicated that JNK activation is required for the HW1-induced autophagic cell death.



**Figure 5.** HW1-induced autophagic cell death occurs predominantly via JNK activation in a caspase-independent manner in both TRAIL-sensitive HCT116 and TRAIL-resistant U87MG cells. **A**, HW1 treatment slightly activates caspases, but significantly activates JNK and p38 in both HCT116 and U87MG cells. Cells were left untreated (*Control*), or treated with either 0.2 μg/mL of TRAIL-His for 2 h or with 25 μg/mL of HW1 for the indicated periods, and then whole cell lysates were prepared and subjected to Western blotting. **B**, effects of various inhibitors on the cell death of HCT116 and U87MG cells treated with either HW1 or TRAIL-His. Cells pretreated for 1 h with Z-VAD (10 μmol/L), SB203580 (10 μmol/L), SP600125 (10 μmol/L), or 3-MA (50 and 125 μmol/L) were further incubated with either 0.2 μg/mL of TRAIL-His in HCT116 for 5 h and U87MG cells for 30 h or 25 μg/mL of HW1 in both cells for 30 h. **C**, no significant effects of Z-VAD on the cell death of HCT116 cells and U87MG cells induced by varying concentrations of HW1. Cells pretreated with 10 μmol/L of Z-VAD for 1 h were further incubated with the indicated concentrations of TRAIL-His (8 h) or HW1 (30 h) in HCT116 cells and HW1 (30 h) in U87MG cells. Cell viability was determined by MTT assay; percentages of viable cells compared with untreated control cells (*columns, B*; and *points, C*) from experiments done at least in triplicate; *bars, SD*. **D**, effects of various inhibitors on the activations of JNK and p38, and cleavage of PARP in HCT116 treated with TRAIL or HW1 and U87MG cells treated with HW1. Cells pretreated for 1 h with either Z-VAD (10 μmol/L), SB203580 (10 μmol/L), SP600125 (10 μmol/L), or 3-MA (50 μmol/L) were further incubated with 25 μg/mL of HW1 for 30 h or 0.2 μg/mL of TRAIL-His for 5 h. **A** and **D**, the phosphorylated forms of JNK (*P-JNK*) and p38 (*P-p38*), and pro-forms (*black arrowhead*) and cleaved forms (*white arrowhead*) of caspase-8, caspase-3, and PARP. The β-actin protein levels were included as a control for protein loading.



**Figure 6.** HW1 induces a different DISC from that of TRAIL in TRAIL-sensitive HCT116 (*left*) and TRAIL-resistant U87MG (*right*) cells. Cells were treated with 2  $\mu\text{g}/\text{mL}$  of Flag-tagged TRAIL for 30 min (only for HCT116 cells) or 25  $\mu\text{g}/\text{mL}$  of Flag-tagged HW1 for 30 or 360 min, and lysed. The TR2-associated DISC was coimmunoprecipitated from the lysates using anti-Flag antibody and analyzed by Western blotting for the presence of TR2, FADD, caspase-8, TRADD, RIP, and/or TRAF2. The addition of beads alone to unstimulated cell lysates (*Control*) was used to control for nonspecific interactions.

**TRADD and TRAF2, but not FADD, caspase-8, or RIP, are recruited to the HW1-TR2 signaling complex.** To elucidate the molecular components involved in HW1 signaling through TR2, we analyzed DISC associated with HW1-TR2 complex by immunoprecipitation after Flag-tagged HW1 treatment of HCT116 and U87MG cells, compared with Flag-tagged TRAIL treatment (Fig. 6). The TR2-associated DISC was coimmunoprecipitated from the cell lysates using anti-Flag antibody and analyzed by Western blotting. TRAIL stimulated a DISC assembly containing TR2, FADD, and caspase-8, but not RIP, TRADD, or TRAF2 in HCT116 cells, consistent with the previous results (29). However, HW1 induced a DISC composed of TR2, TRADD, and TRAF2, whereas FADD, caspase-8, and RIP were not detected in the TR2-associated complex in both HCT116 and U87MG cells (Fig. 6). This result suggested that HW1 binding may lead to the recruitment of TRADD into the intracellular death domain of TR2, and subsequent association of TRADD with TRAF2, which seems to trigger subsequent signaling for the JNK activation (34). Taken together, our data suggested that HW1 triggers autophagic cell death by forming a distinct DISC from that of TRAIL.

## Discussion

TRAIL or agonistic anti-TR2 mAbs induce apoptotic cell death in various cancer cells, but was not sufficient as a single agent to kill TRAIL-resistant tumor cells, which can be sensitized to cell death by combined therapy with chemotherapeutics (6–12). We showed here that HW1 alone can induce autophagic cell death in both TRAIL-sensitive and TRAIL-resistant cancer cells *in vitro* with much less cytotoxicity to normal cells. The tumoricidal activity of TR2-specific HW1 was blocked by the soluble TR2-Fc and augmented by TR2 overexpression on the cell surface, suggesting that TR2-mediated extrinsic signaling is sufficient to kill various tumors, including TRAIL-resistant cells, through the distinct cell death pathway of autophagy from apoptosis. This is indirectly supported by the TRAIL-mediated autophagic cell death of normal epithelial cells (18, 19). Similar to typical autophagy (20–22), two key autophagy genes, *Beclin-1* and *Atg7*,

seem to be involved in HW1-induced autophagic cell death. Although TRAIL induced apoptotic cell death in TRAIL-sensitive cells via the caspase-dependent pathway, HW1 triggered autophagic cell death in both TRAIL-sensitive and TRAIL-resistant tumors predominantly through the JNK pathway in a caspase-independent manner. Caspase-independent JNK activation has been reported to contribute to autophagic cell death induced by TNF- $\alpha$  (33) and chemicals (22, 35), which, together with our results, indicates a molecular link between JNK activation and autophagic cell death. DISC analysis revealed that TRADD and TRAF2 were recruited to the HW1-TR2 complex, but not FADD and caspase-8, which are essential for TRAIL-induced apoptotic cell death. Thus, it is most likely that TRAF2 mediated HW1-induced JNK activation, eventually triggering autophagy and subsequent cell death (22, 33). Even though the detailed mechanism of how HW1-triggered JNK activation mediates autophagic cell death is yet to be determined, our study suggests that induction of autophagic cell death through the JNK pathway might be one of the strategies to overcome TRAIL-resistance of tumor cells.

In contrast to previous reports showing that at least the bivalency of TR2-specific mAbs is required to trigger cell death (12, 14, 16, 32), monovalent HW1 alone induced cell death without enhanced tumoricidal activity through cross-linking, raising the question of how it directly activates TR2 to transmit the cell death signal. Similar to TNFRs (2), preligand assembled oligomeric complexes of TR2 through lateral interactions of TR2-ECD have been observed on the cell surface (26, 30). The observed punctuate colocalization signals upon HW1 binding to TR2 (Fig. 1E; Supplementary Fig. S1C) indicates the formation of oligomeric HW1-TR2 complexes on the cell membrane. It is not clear, however, whether HW1 binds to preassembled homomeric TR2 complexes or to monomeric TR2, and then immediately induces TR2 clustering. The latter might be feasible due to the different binding sites of HW1 from those of TRAIL on TR2. In either case, DISC analysis indicated that HW1 binding to TR2 may induce different conformations of the TR2 intracellular death domain, which recruits distinct adaptor molecules of TRADD and TRAF2 from those induced by TRAIL. This distinct DISC assembly seems to explain the differences in the eventual outcome between HW1 and TRAIL upon binding to the same target TR2. For example, in contrast with HW1-induced autophagic cell death, TRAIL-resistant U87MG cells were sensitized to apoptotic cell death by the combination of TRAIL and the chemotherapeutic agent rottlerin, which was mediated by caspase activations without any activations of JNK and p38 (9).

The specific role of autophagy in programmed cell death, whether it is a survival mechanism or another cell death pathway, has been controversial. However, accumulating evidence suggests that autophagy represents another cell death pathway in certain circumstances, which occurs in the absence or presence of apoptosis (20–23). Most TRAIL-resistant cancer cells have apoptotic defects. For example, antiapoptotic molecules of FLICE-like inhibitory proteins and Bcl-2 family are overexpressed in TRAIL-resistant cells (5). Thus, similar to HW1-induced autophagic cell death, targeting nonapoptotic cell death might be an alternative strategy to apoptotic cancer therapy for various cancers, particularly for TRAIL-resistant tumors (21, 23). Although we need to further characterize the tumoricidal activity of HW1 *in vivo*, our study shows that TR2-specific HW1 can induce autophagic cell death in various cancer cell types, including TRAIL-resistant

tumors, providing a novel strategy of cancer therapy through nonapoptotic cell death.

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