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A novel agonistic antibody to human death receptor 4 induces apoptotic cell death in various tumor cells without cytotoxicity in hepatocytes

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Abstract

The proapoptotic tumor necrosis factor–related apoptosis inducing ligand (TRAIL) receptors death receptor (DR) 4 and DR5 are attractive targets to develop the receptor-specific agonistic monoclonal antibodies (mAb) as anticancer agents because of their tumor-selective cell death–inducing activity. Here, we report a novel agonistic mAb, AY4, raised against human DR4 in mice. ELISA analysis revealed that AY4 specifically bound to DR4 without competition with TRAIL for the binding. Despite distinct binding regions of AY4 on DR4 from those of TRAIL, AY4 as a single agent induced caspase-dependent apoptotic cell death of several tumor types through the extrinsic and/or intrinsic pathways without substantial cytotoxicity to normal human hepatocytes. Further, the AY4-sensitive cells followed the same cell death characteristics classified as type I and type II cells by the response to TRAIL, suggesting that the cell death profiles in responses to DR4 and/or DR5 stimulation are determined by the downstream signaling of the receptor rather than the kind of receptor. Noticeably, AY4 efficiently induced cell death of Jurkat cells, which have been reported to be resistant to other anti-DR4 agonistic mAbs, most likely due to the unique epitope property of AY4. *In vivo* administration of AY4 significantly inhibited tumor growth of human non–small cell lung carcinoma preestablished in athymic nude mice. Conclusively, our results provide further insight into the DR4-mediated cell death signaling and potential use of AY4 mAb as an anticancer therapeutic agent, particularly for DR4-responsive tumor types. [Mol Cancer Ther 2009;8 (8):2276–85]

Introduction

The proapoptotic tumor necrosis factor–related apoptosis inducing ligand (TRAIL) receptors death receptor (DR) 4 (TRAIL-R1) and DR5 (TRAIL-R2) are attractive targets for development of agonistic anticancer agents because they are widely expressed in many tumors and can selectively kill tumor cells with little cytotoxicity on normal cells *in vitro* and *in vivo* (1–3). The other cell surface–expressed TRAIL receptors, such as death decoy receptor (DcR) 1 (TRAIL-R3) and DcR2 (TRAIL-R4), are not able to transduce cell death signaling due to the lack of the functional cytoplasmic death domain (1–3).

Binding of trimeric or multimeric TRAIL to the proapoptotic receptors results in the receptor clustering to activate the intracellular death domain, promoting the formation of death-inducing signaling complex (DISC) by recruiting Fas-associated death domain (FADD) and then caspase-8, which can subsequently trigger apoptosis signaling through the extrinsic and/or intrinsic pathways (reviewed in refs. 1–4). Activated caspase-8 by the DISC formation triggers the extrinsic pathway by directly activating the downstream effector caspases, such as caspase-3, caspase-6, and caspase-7, which in turn cleave many cellular substrates to exert apoptosis (1–5). The extrinsic pathway can be amplified by cross-talk with the intrinsic pathway, the link of which is Bid cleavage by activated caspase-8 (1–5). Cleaved Bid (tBid) induces oligomerization of proapoptotic Bax and/or Bak, leading to the release of cytochrome *c* and Smac/Diablo from mitochondria into cytoplasm and activation of caspase-9 (1–5). The mitochondrial pathway eventually activates the effector caspases to execute apoptotic cell death. In some cells (designated type I cells), caspase-8 activation is sufficient to activate the effector caspases to execute apoptosis via the extrinsic pathway, whereas, in other cells (designated type II cells), amplification of the extrinsic pathway through the intrinsic (mitochondrial) pathway is needed to commit the cells to apoptotic cell death (1, 2, 6).

In addition to the cognate ligand of recombinant TRAIL, several agonistic monoclonal antibodies (mAb) have been developed by targeting DR4 (7–10) or DR5 (11–16), which also induce cell death in various types of tumors *in vitro* and *in vivo*. Some of them are now under various phases

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of clinical trials (1–3, 10). The advantages of agonistic mAbs compared with TRAIL include specificity of binding to their target receptor without cross-reactivity with the DcRs, much longer serum half-life, and the crystallizable fragment (Fc)–mediated effector functions exploiting human immune system, such as antibody-dependent cellular cytotoxicity, particularly for IgG1 isotype (1, 2). The cell death mechanism of most agonistic mAbs against DR4 or DR5 has been reported to be similar to that of TRAIL, inducing caspase-dependent apoptotic cell death through p53-independent apoptotic pathway (1, 2, 4). However, some malignant tumor cells even expressing DR4 and DR5 remain resistant to TRAIL and/or agonistic mAbs, the mechanism of which varies with the cellular context, including overexpression of cellular FLICE inhibitory protein (c-FLIP), absence of proper *O*-glycosylation in the receptors, and/or complex downstream regulation of the extrinsic and intrinsic apoptotic pathways (1–4, 17, 18).

Previous studies have shown that some cancer cells, despite their comparable expression of DR4 and DR5 on the cell surface, underwent apoptotic cell death by engaging only one receptor of the two proapoptotic DR4 and DR5, but not the other (2, 17, 19–21), suggesting that one of the receptors acts as a major mediator to the apoptotic signaling. For example, DR4 was the predominant apoptosis transducer for chronic lymphocytic leukemia and mantle cell lymphoma, which were resistant to anti-DR5 agonistic mAbs or DR5-selective TRAIL mutants (22–24). The preferential apoptotic cell death of certain tumor types through DR4 over DR5 represents the need to develop DR4-specific agonistic mAbs as anticancer agents, particularly for tumors selectively sensitive to DR4-mediated apoptosis. Further, different properties of mAbs, such as epitope, affinity, and isotype, can result in distinct apoptosis-inducing activity, providing further insight into the target receptor–mediated cell death signaling.

Here, we report a novel agonistic mAb, AY4, raised against human DR4 in mice, which did not compete for DR4 binding with TRAIL. The anti-DR4 agonistic AY4 mAb as a single agent induced caspase-dependent apoptotic cell death in various types of cancer cells without significant toxicity to normal human hepatocytes. AY4 reduced the growth of preestablished human lung tumors in a mouse xenograft model. Noticeably, AY4 efficiently induced cell death of Jurkat cells, which were only sensitive to TRAIL and anti-DR5 agonistic mAbs but resistant to DR4-selective TRAIL mutants and other anti-DR4 agonistic mAbs. The detailed molecular mechanism of AY4-mediated cell death was presented.

Materials and Methods

Generation and Characterization of AY4 mAb against Human DR4

Male BALB/c mice (6 wk old) were injected i.p. four times with 50 μg of the extracellular domain (ECD) of human DR4 (residues 1–215; PeproTech) at 2-wk intervals. Three days after the last injection, spleen cells from the immunized mice were fused with murine myeloma Sp2/O cells (American Type Culture Collection) in the presence of 50% (w/v) polyethylene glycol 1500 (Roche; ref. 25). From the screening of hybridoma supernatants against DR4-ECD using ELISA, 10 positive hybridomas were isolated (Supplementary Fig. S1A). Further, screening mAbs from each hybridoma for tumoricidal activity in model cells of HCT116 and H460 cells selected a mAb, dubbed as AY4, which exhibited the most effective cell death–inducing activity (Supplementary Fig. S1B). AY4 was purified from the cell supernatant by Protein A-Sepharose 4 Fast Flow (Pharmacia; ref. 25). Cloning of *AY4* gene from the hybridoma by reverse transcription-PCR and then sequence analysis revealed that the subclass of AY4 mAb was IgG1 for heavy chain and κ for light chain. The plasmids encoding heavy chain (deposit no. KCTC11392BP) and light chain (deposit no. KCTC11393BP) are available in the Korean Collection for Type Cultures. The construction of AY4 in a format of single-chain variable fragment, AY4-scFv, is described in Supplementary Materials and Methods. The experiments of size exclusion chromatography, ELISA, and surface plasmon resonance (SPR) of AY4 and AY4-scFv were done as described before (13, 26, 27). The detailed procedures are given in the figure legends and Supplementary Materials and Methods.

Reagents

All reagents were analytic grade unless specified otherwise. Z-VAD-fmk [benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone] and Z-LEHD-fmk [Z-Leu-Glu(OMe)-His-Asp(OMe)-fmk] were from Santa Cruz Biotechnology. Soluble TRAIL (with a COOH-terminal 6× His-tag; described as TRAIL hereafter), DR5-ECD, DR4-ECD, DcR1-ECD, and DcR2-ECD were prepared as previously described (26, 28). The ECD of CD95 was from Biovision. The gene encoding for mouse TRAIL receptor (mTR; ref. 29) was provided by Open Biosystems. The ECD (residues 53–169) of mTR was subcloned into pET21b (Novagen), expressed, and purified from *Escherichia coli* (26, 28). Details for materials and protein preparations are provided in Supplementary Materials and Methods.

Cell Viability Assays

Unless specified otherwise, human cell lines were purchased from the American Type Culture Collection and maintained as described previously (13, 30). Wild-type HCT116 and its Bax-deficient ($Bax^{-/-}$) and p53-deficient ($p53^{-/-}$) cell lines were obtained from the Cell Center of John's Hopkins University School of Medicine (30, 31). Jurkat E6-1 clone and Jurkat A3 clone cells were from the American Type Culture Collection and Korean Cell Line Bank, respectively (30). Jurkat A3 clone and its mutant cell lines deficient in receptor-interacting protein 1 (RIP1; RIP1 deficient), caspase-8 (Jurkat I9.2), and FADD (Jurkat I2.1) were kindly provided by Prof. John Blenis (Department of Cell Biology, Harvard Medical School, Boston, MA; refs. 30, 32). Normal human hepatocytes were purchased from BD Biosciences and cultured under the recommended conditions (13). Cell viability was analyzed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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(MTT)–based cell growth determination kit (Sigma), as described previously (13, 30). Then, the cell viability determined for at least triplicated experiments was presented as percentage of viable cells compared with untreated, control cells. These data were analyzed by a four-parameter sigmoidal curve fit on SigmaPlot software (SPSS, Inc.) to calculate EC_{50}, the concentration of proteins giving a 50% reduction in cell viability (13). For apoptosis detection and quantification, cells were stained with Annexin V and propidium iodide (PI; BD Pharmingen) and analyzed by flow cytometry (13).

Western Blotting Analysis

Cells (3×10^5 per well) were plated overnight in six-well plate and then treated under the conditions specified in the figure legends. The standard procedure for Western blotting was then done using rabbit antibodies as described previously (13, 30).

Analysis of Tumoricidal Activity *In vivo*

Animal experiments were approved by internal committee of Ajou University Medical School and done according to the guidelines established by the Institutional Animal Care and Use Committee. For the analysis of tumoricidal activity *in vivo*, female BALB/c athymic nude mice (Orient Bio) were inoculated s.c. with 5×10^6 cells of H460 cells in the right thigh. When the mean tumor size reached ~100 mm^3 (after 7-d growth), mice were randomized into two groups ($n = 6$ per group) and administered i.v. via tail vein in a dose/weight-matched fashion every 3 d (total, five times) with AY4 and isotype control (IC) antibody (mouse IgG1/κ; eBioscience), as specified in the figure legends. Details for the experiments are given in Supplementary Materials and Methods.

Results

AY4 Specifically Binds to DR4 without Competition with TRAIL for the Binding

Mouse mAb AY4 (IgG1, κ) was generated by immunizing BALB/c mice with human DR4-ECD. Reducing and nonreducing SDS-PAGE analyses confirmed that the purified AY4 from the hybridoma cells was correctly assembled (Supplementary Fig. S2). Further, size exclusion chromatography analysis revealed that AY4 existed in a monomeric, assembled form (~150 kDa) in solution at concentrations of up to 0.5 mg/mL (Fig. 1A). ELISA analysis revealed that AY4 bound only to DR4 but not to other homologous DRs of DR5, DcR1, DcR2, and CD95 (Fas; Fig. 1B). Further, AY4 did not cross-react with mTR (Fig. 1B), a mouse ortholog of human DR4 and DR5 (29). However, TRAIL bound to mTR, consistent with the previous result (29). Analysis of the kinetic binding interactions by SPR technique also confirmed that AY4 specifically bound to DR4, but not to DR5, DcR1, and DcR2, with association and dissociation rate constants of $6.8 \pm 0.08 \times 10^5$ $mol/L^{-1}s^{-1}$ and $4.8 \pm 0.14 \times 10^{-3}$ s^{-1}, respectively, resulting in a K_D value of 7.1 ± 0.6 nmol/L (Fig. 1C). These results showed that AY4 recognizes epitopes that are specific for DR4 but not shared by the other homologous TRAIL receptors.

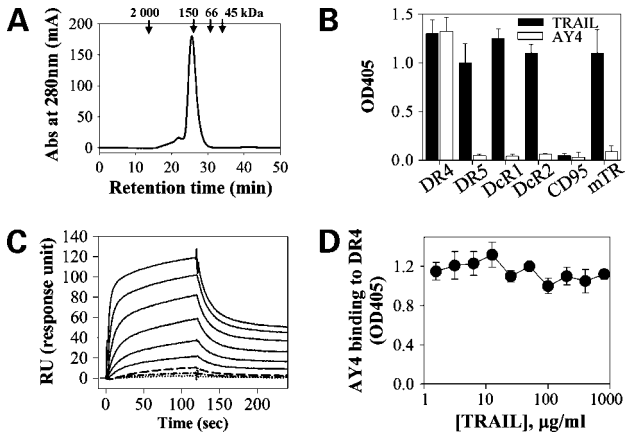

Figure 1. Biochemical characterization of mouse anti-DR4 AY4 mAb. **A,** size exclusion elution profile of AY4 injected at 0.5 mg/mL (≈3.3 μmol/L) and monitored at 280 nm. The arrows indicate the elution positions of molecular weight standards (alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa), including blue dextran 2000 (2,000 kDa) as a void volume marker. **B,** binding specificity of AY4 and TRAIL analyzed by ELISA. ELISA plates were coated with 10 μg/mL of DR4-ECD, DR5-ECD, DcR1-ECD, DcR2-ECD, mTR-ECD, or CD95-ECD and then incubated with 1 μg/mL TRAIL (*black columns*) or 1 μg/mL AY4 (*white columns*). **C,** kinetic interactions of AY4 with DR4-ECD determined by SPR analyses. SPR sensograms were obtained from injections of serially diluted DR4-ECD at 100, 50, 25, 12.5, 6.25, and 3.125 nmol/L (*solid lines* from *top* to *bottom*) over an AY4 immobilized surface at 2,400 response units. The injection of DR5-ECD (*long dashed line*), DcR1-ECD (*dotted line*), and DcR2-ECD (*dash-dotted line*) at 2 μmol/L exhibited negligible binding to AY4. **D,** competitive ELISA. Binding activity of AY4 (1 μg/mL) to DR4 in the presence of serially diluted TRAIL (from 800 to 1.56 μg/mL) was assessed in plates coated with DR4-ECD. **B** and **D,** *bars,* SD.

To determine whether AY4 recognized different regions of DR4 from those of TRAIL, competitive and sandwich ELISAs were carried out. Competitive ELISA showed that AY4 did not compete with TRAIL for the binding to DR4-ECD (Fig. 1D). Further, AY4 efficiently bound to DR4-ECD captured by surface-immobilized TRAIL on the sandwich ELISA (Supplementary Fig. S3). These results showed that AY4 recognizes epitopes that do not significantly overlap with TRAIL binding sites on DR4. For other anti-DR4 mAbs previously reported (7), mAb 4H6 (mouse IgG1) competed with TRAIL for binding to DR4, whereas mAb 4G7 (mouse IgG2a) did not.

AY4 Alone Induces Cell Death of Various Cancer Cells but Is Not Significantly Cytotoxic to Normal Human Hepatocytes

Cell death–inducing activity of AY4 was evaluated for various tumor type cells, which are known as TRAIL sensitive or resistant, as follows: H460 (non–small cell lung carcinoma), HCT116 (colon colorectal carcinoma), Ramos (Burkitt's lymphoma), Jurkat (acute T-cell leukemia), and HL60 (acute promyelocytic leukemia) cells for TRAIL-sensitive cells and U87MG (astrocyte glioma) and HepG2 (hepatocellular carcinoma) cells for TRAIL-resistant cells (13, 30, 33). The cells were treated with various concentrations of TRAIL (0.001–0.5 μg/mL) or AY4 (0.001–10 μg/mL) for 24 hours and cell viability was determined by MTT assay

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(13, 30). As expected, the TRAIL-sensitive cells, but not TRAIL-resistant cells, underwent cell death by TRAIL treatment in a concentration-dependent manner (Fig. 2A), displaying EC_{50} of 0.04 ± 0.02, 0.08 ± 0.02, 0.22 ± 0.03, 0.24 ± 0.02, and 0.95 ± 0.12 μg/mL for H460, HCT116, Ramos, HL60, and Jurkat cells, respectively. AY4 treatment as a single agent resulted in dose-dependent cell death for TRAIL-sensitive cells, such as H460 (EC_{50} ≈ 1.8 ± 0.2 μg/mL), Ramos (EC_{50} ≈ 1.6 ± 0.3 μg/mL), HCT116 (EC_{50} ≈ 7.2 ± 2.6 μg/mL), and Jurkat (EC_{50} ≈ 0.29 ± 0.06 μg/mL) cells, except for HL60 cells, which were completely resistant to AY4 even by the treatment of 20 μg/mL AY4 for 30 hours (Fig. 2A). The anchorage-dependent HCT116 and H460 cells dying by exposure to AY4 became rounded and were eventually detached from the culture plates to be floated (Supplementary Fig. S4A and B). AY4 did not show any significant cytotoxicity against TRAIL-resistant U87MG and HepG2 cells. The highest sensitivity of Jurkat cells to AY4 among the AY4-senstive cells was quite surprising because Jurkat cells have been reported to be only sensitive to anti-DR5 agonistic mAbs or DR5-selective TRAIL mutants but resistant to anti-DR4 agonistic mAb HGS-ETR1 or DR4-selective TRAIL mutants (23, 24, 34). When tested from other cell line bank (Korean Cell Line Bank), HL60 and Jurkat cells (E6-1 and A3 clones) exhibited the same response to AY4 and TRAIL as above.

We also generated monovalent AY4-scFv, which was expressed solubly in bacteria (Supplementary Fig. S2C). The bacterially purified AY4-scFv existed in the monomeric form in solution up to the concentration of 0.5 mg/mL (Supplementary Fig. S5A) and specifically bound to DR4 without cross-reactivity with other homologous receptors (Supplementary Fig. S5B). However, the monovalent AY4-scFv failed to induce the cell death of HCT116 and H460 cells (Supplementary Fig. S5C), which were sensitive to the bivalent AY4 mAb (Fig. 2A). This result suggested that the bivalency of AY4 mAb is required to activate DR4 for its proapoptotic activity. The bivalency of mAbs against DR5 has also been necessary for their apoptosis-inducing activity (11, 16, 23).

Some recombinant versions of TRAIL with exogenous tags and agonistic mAbs against DR4 or DR5 have been reported to be cytotoxic to normal human hepatocytes, raising the concern of their application as anticancer agents (12, 35). To determine whether AY4 induces hepatocellular toxicity *in vitro*, we treated normal human hepatocytes that have been reported to express DR5 and DR4 on the cell surface (12, 35) with various concentrations of AY4 and, for comparison, also with TRAIL. During 24-hour incubation, AY4 treatment exhibited very low levels of cytotoxicity with <10% of hepatocytes killed in the presence of 10 μg/mL AY4, which was similar to that of 0.5 μg/mL

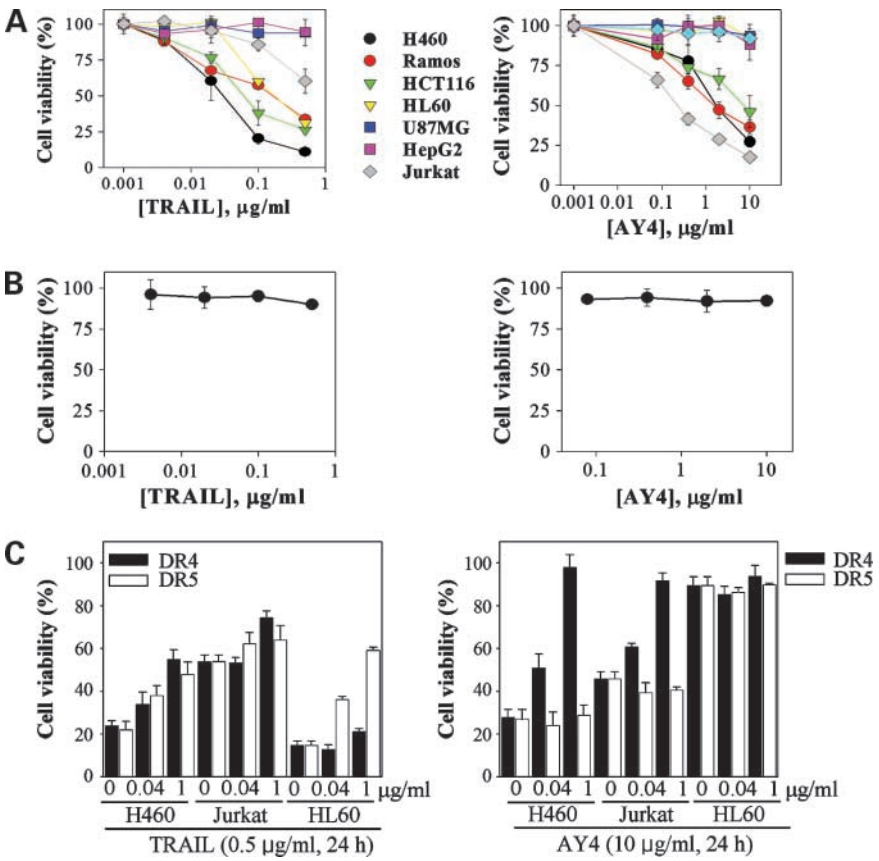

Figure 2. AY4 alone induces cell death of various human cancer cells through cell surface–expressed DR4 without significant cytotoxicity on normal human hepatocytes. **A** and **B,** cell killing activities of TRAIL (*left*) and anti-DR4 AY4 mAb (*right*) in various TRAIL-sensitive and TRAIL-resistant tumor cells (**A**) and in normal human hepatocytes (**B**). Cells were incubated with the indicated concentrations of TRAIL or AY4 for 24 h. **C,** inhibition of cell death induced by TRAIL (*left*) or AY4 (*right*) by the soluble competitor DR4-ECD (*black columns*) or DR5-ECD (*white columns*). Cells were treated with 0.5 μg/mL TRAIL (≈30 nmol/L) or 10 μg/mL AY4 (≈67 nmol/L) for 24 h in the presence of the indicated concentrations (0, 0.04, and 1 μg/mL) of DR4-ECD or DR5-ECD. *Bars,* SD.

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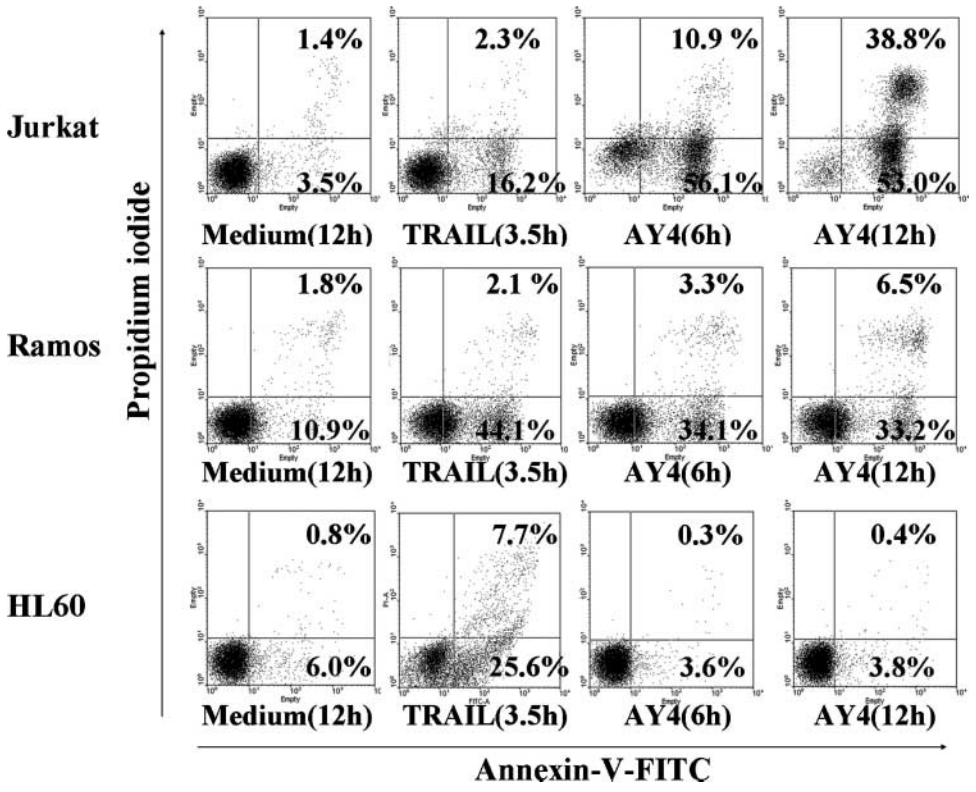

Figure 3. Dual staining of Annexin V-FITC and PI for Jurkat, Ramos, and HL60 cells treated with medium only for 12 h, 0.5 μg/mL TRAIL for 3.5 h, or 10 μg/mL AY4 for 6 and 12 h (as indicated in each panel). Samples were then analyzed by flow cytometry. In the dot plots, Annexin V-FITC$^+$/PI$^-$ cells (*bottom right quadrant*, % shown) and Annexin V-FITC$^+$/PI$^+$ cells (*top right quadrant*, % shown) are considered as "early apoptotic" and "dead" cells, respectively.

TRAIL treatment (~12% cell death; Fig. 2B; Supplementary Fig. S4C). Thus, AY4 was minimally cytotoxic on normal hepatocytes, like TRAIL.

To determine whether AY4 triggered cell death by specific binding to DR4 on the cell surface, soluble DR4-ECD was coincubated with AY4 in AY4-sensitive H460 and Jurkat cells using DR5-ECD as a control. As shown in Fig. 2C, the AY4-mediated cell death was blocked by the presence of DR4-ECD in a dose-dependent manner but not affected at all by DR5-ECD, showing that AY4 induced cell death signaling by specific binding to the cell surface–expressed DR4. Each soluble competitor partially, but not completely, suppressed the TRAIL-mediated cell death of H460 and Jurkat cells, indicating that TRAIL transduces apoptosis signaling through both DR4 and DR5 (Fig. 2C). In HL60 cells, however, DR5-ECD, but not DR4-ECD, effectively inhibited the TRAIL-mediated cell death, suggesting that DR5 is a preferential receptor for the TRAIL-mediated cell death of HL60 cells (36, 37). This result also partially explained why HL60 cells were resistant to anti-DR4 agonistic AY4.

To determine any correlations between DR4 expression level and AY4 sensitivity of cells, the cell surface expression levels of DR4 and DR5 were assessed by flow cytometry (Supplementary Fig. S6). AY4-sensitive H460 and HCT116 cells expressed much higher levels of DR4 on the cell surface compared with AY4-sensitive Ramos and Jurkat cells. TRAIL- and AY4-resistant U87MG and HepG2 cells expressed moderate and negligible levels of DR5 and DR4, respectively, compared with those of the other cells. Interestingly, AY4-resistant HL60 cells showed comparable DR4 expression level to that of AY4-sensitive Ramos and Jurkat cells. Thus, the cell surface expression level of DR4 was not strictly correlated with the susceptibility to AY4, suggesting that other factors are also involved in the DR-mediated apoptosis, as previously observed for TRAIL and anti-DR4 or anti-DR5 agonistic mAbs (2, 9, 11, 15).

AY4 Induces Caspase-Dependent Apoptotic Cell Death

The anchorage-dependent HCT116 and H460 cells dying by exposure to AY4 became rounded and broken into smaller pieces (Supplementary Fig. S4A and B), which are morphologic features of apoptotic cell death. To elucidate whether apoptosis is the principal mechanism of AY4-mediated cell death, cells exposed to AY4 were labeled by dual staining with Annexin V-FITC and PI and analyzed by flow cytometry. The AY4-sensitive Jurkat and Ramos cells treated with AY4 were significantly labeled as early apoptotic cells shown by Annexin V-FITC–positive staining, like the cells treated with TRAIL. The HL60 cells were positively labeled with Annexin V-FITC and PI only by TRAIL treatment, but negligibly by AY4, confirming that HL60 cells were TRAIL sensitive but AY4 resistant (Fig. 3).

The activations of intracellular apoptotic signaling molecules were further monitored by Western blotting in AY4-sensitive HCT116 and Jurkat cells and AY4-resistant HL60 cells treated with AY4, in comparison with TRAIL. In HCT116 and Jurkat cells, AY4 treatment for 24 hours significantly induced dose-dependent cleavages of procaspase-8,

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procaspase-3, procaspase-9, Bid, and poly(ADP-ribose) polymerase showing their activations, like the TRAIL-treated cells (Fig. 4A). Activation levels of the proapoptotic molecules were more intensive in Jurkat cells than HCT116 cells (Fig. 4A), consistent with the higher sensitivity of Jurkat cells to AY4 than HCT116 cells (Fig. 2A). Pretreatment of AY4-sensitive HCT116, Jurkat, H460, and Ramos cells with a pan-caspase inhibitor, Z-VAD-fmk (10 μmol/L, 0.5 hour), conferred the cells complete resistance to both TRAIL (0.5 μg/mL, 24 hours; Fig. 4B) and AY4 (10 μg/mL, 24 hours; Fig. 4C). For the TRAIL-sensitive but AY4-resistant HL60 cells, the proapoptotic molecules were only activated by TRAIL treatment but negligibly by AY4 (Fig. 4A). Resistance of HL60 cells to AY4 seemed to be upstream of caspase-8 activation, judged from the observation that little or no caspase-8 was processed in the AY4-treated cells. Taken together, these results showed that AY4 induces Z-VAD-fmk–inhibitable, or caspase-dependent, apoptotic cell death in various cancer cells primarily through DR4 stimulation.

FADD and Caspase-8 Are Essential for the AY4-Mediated Apoptotic Cell Death

Previously, DR4 stimulation by DR4-selective TRAIL mutant and wild-type TRAIL formed a DISC composed of DR4, FADD, and caspase-8 in tumor cells, the efficient formation of which determined the sensitivity (23, 32). To elucidate whether FADD and caspase-8 are also essential in the AY4-mediated cell death signaling through DR4, FADD-deficient, caspase-8–deficient, and RIP1-deficient Jurkat cells were treated with AY4 (0.001–10 μg/mL, 24 hours), in comparison with TRAIL (0.001–0.5 μg/mL, 24 hours). The cell surface expression levels of DR4 and DR5 in the mutant Jurkat cells have been reported to be almost identical to those of the wild-type cells (32). FADD-deficient and caspase-8–deficient Jurkat cells were completely resistant to both AY4 and TRAIL, whereas RIP1-deficient Jurkat cells were susceptible to both AY4 and TRAIL, showing comparable levels of cell death efficiency with those of wild-type Jurkat cells (Fig. 5A). The above results showed that FADD and caspase-8, but not RIP1, are essential for the AY4-mediated apoptotic cell death, suggesting that, like TRAIL, AY4-stimulated DR4 forms a DISC consisting of DR4, FADD, and caspase-8, although AY4 bound to different regions on DR4 from those of TRAIL.

AY4 Activates Both Extrinsic and Intrinsic Apoptotic Pathway

It is noteworthy that Bid and caspase-9 were activated by AY4 treatment in AY4-sensitive HCT116 and Jurkat cells (Fig. 4A), showing the extrinsic pathway triggered by AY4 binding to DR4 connected with the intrinsic pathway through Bid activation (1, 2, 4). As stated earlier, activated Bid exerts its proapoptotic function in mitochondria through its conformational activation of Bax and/or Bak (2, 4). Bax is required rather than Bak for TRAIL-mediated apoptotic cell death (38). The p53 tumor suppressor protein also facilitates apoptotic cell death by transcriptional up-regulation of many proapoptotic genes involved in both extrinsic and intrinsic pathways (38, 39). To determine the extent of involvement of the intrinsic pathway and p53 in the AY4-mediated apoptosis, HCT116 cells with two nonfunctional alleles of Bax ($Bax^{-/-}$) or p53 ($p53^{-/-}$) were treated with AY4, in comparison with TRAIL. $Bax^{-/-}$ HCT116 cells were almost completely resistant to both AY4 and TRAIL

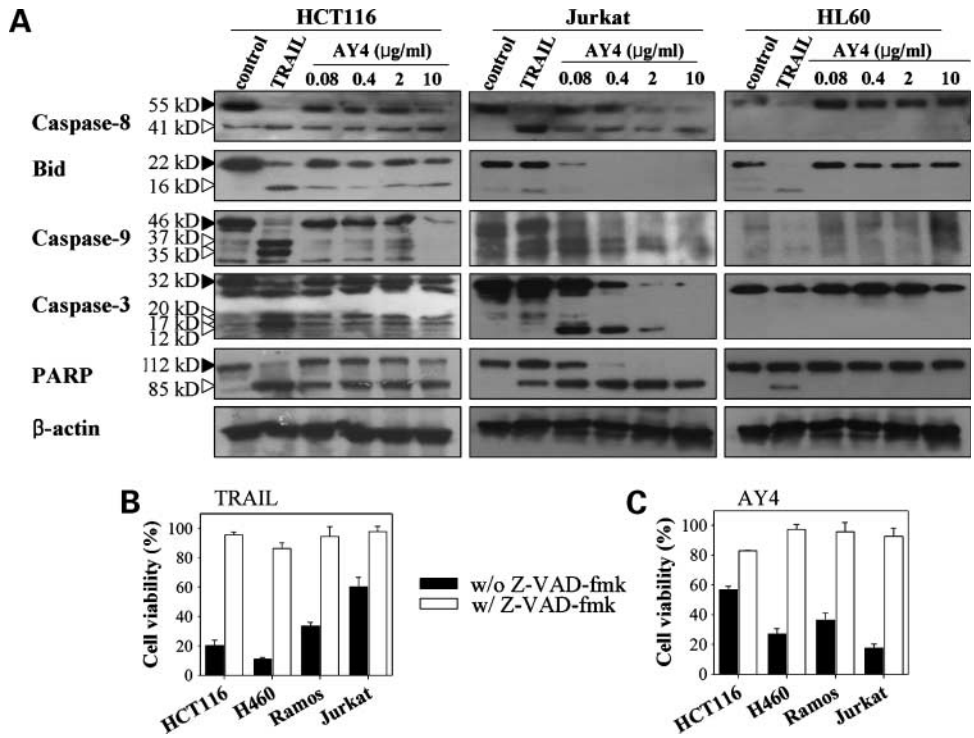

Figure 4. AY4 induces caspase-dependent apoptotic cell death of AY4-sensitive cells. **A,** detection of proteolytic processing of apoptotic molecules by Western blotting analyses in HCT116 (*left*), Jurkat (*middle*), and HL60 cells (*right*), which were untreated (control) or treated with either 0.5 μg/mL TRAIL for 3.5 h or the indicated concentrations of AY4 for 24 h. Proforms (*black arrowhead*) and cleaved forms (*white arrowhead*) of caspase-8, Bid, caspase-9, caspase-3, and poly(ADP-ribose) polymerase (*PARP*) are indicated. The β-actin protein levels are included as a control for protein loading. **B** and **C,** effects of a pan-caspase inhibitor, Z-VAD-fmk, on the cell viability of HCT116, H460, Ramos, and Jurkat cells treated with either TRAIL (**B**) or AY4 (**C**). Cells untreated (*black columns*) or pretreated (*white columns*) with Z-VAD-fmk (10 μmol/L) for 0.5 h were further incubated with either 0.5 μg/mL TRAIL or 10 μg/mL AY4 for 24 h. *Bars,* SD.

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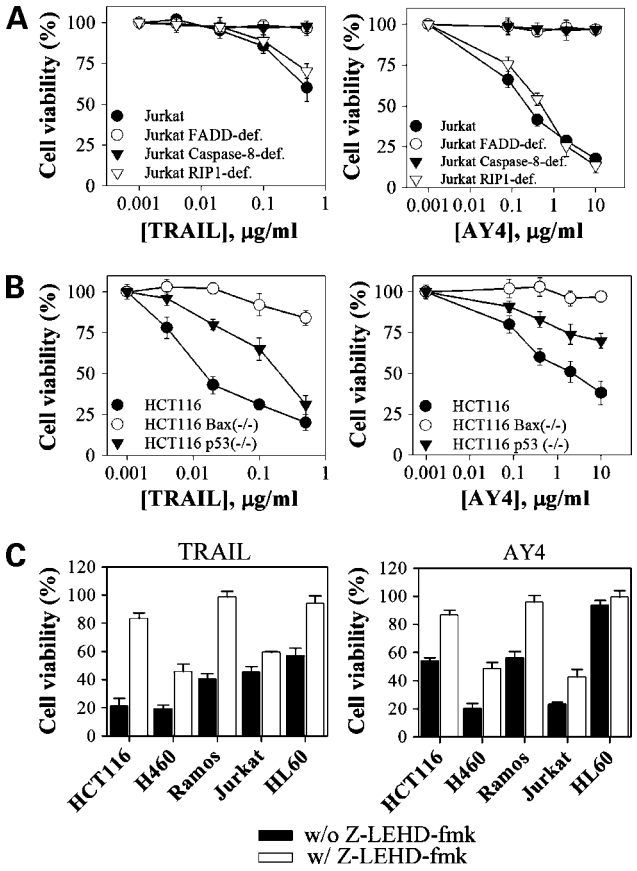

Figure 5. AY4-stimulated DR4 signaling requires FADD and caspase-8 and can transduce the extrinsic and/or intrinsic apoptotic pathways depending on the cell types. **A,** cell viability of Jurkat wild-type and its FADD-deficient (*FADD-def.*), caspase-8–deficient (*Caspase-8-def.*), or RIP1-deficient (*RIP1-def.*) cells, which were incubated with the indicated concentrations of TRAIL (*left*) or AY4 (*right*) for 24 h. **B,** HCT116 and its Bax-deficient ($Bax^{-/-}$) or p53-deficient ($p53^{-/-}$) cells were incubated with the indicated concentrations of TRAIL (*left*) or AY4 (*right*) for 24 h. **C,** effects of a caspase-9 inhibitor, Z-LEHD-fmk, on the cell viability of HCT116, H460, Ramos, Jurkat, and HL60 cells treated with either TRAIL (*left*) or AY4 (*right*). Cells untreated (*black columns*) or pretreated (*white columns*) for 0.5 h with Z-LEHD-fmk (20 μmol/L) were further incubated with either 0.5 μg/mL TRAIL or 10 μg/mL AY4 for 24 h. *Bars,* SD.

(Fig. 5B), showing that Bax is required for the apoptotic cell death of HCT116 cells induced by AY4 and TRAIL treatments. On the other hand, $p53^{-/-}$ HCT116 cells were still sensitive to AY4 in a concentration-dependent manner, but much less compared with the wild-type cells, like TRAIL, suggesting that p53 partially affects the AY4- and TRAIL-mediated apoptotic signaling in the upstream of Bax (38). The susceptibility of wild-type HCT116 cells but considerable resistance of $Bax^{-/-}$ HCT116 cells to AY4 treatment showed that HCT116 cells, defined as type II cells in response to TRAIL (5, 38), also behaved like type II cells in response to AY4.

To further determine whether AY4-mediated cell death requires the mitochondrial amplification signaling, the AY4-sensitive cells were pretreated with a caspase-9 inhibitor, Z-LEHD-fmk (20 μmol/L, 0.5 hour), followed by exposure to AY4 (10 μg/mL) or TRAIL (0.5 μg/mL) for 24 hours. The presence of Z-LEHD-fmk conferred HCT116 and Ramos cells substantial resistance to both AY4 and TRAIL but partially inhibited the AY4- and TRAIL-mediated cell death of H460 and Jurkat cells (Fig. 5C). These results indicated that HCT116 and Ramos cells behave like type II cells, whereas H460 and Jurkat cells are type I cells, in response to both AY4 and TRAIL, showing that the AY4-sensitive cells have the same cell death characteristics classified as type I and type II cells by the response to TRAIL. The AY4-resistant HL60 cells showed type II response to TRAIL treatment (Fig. 5C). The type I or type II behavior of the cells in response to TRAIL was consistent with the previous results (5, 6, 40). Taken altogether, AY4-stimulated DR4 activations can exert both the extrinsic and intrinsic pathways, at least for the AY4-sensitive cells.

AY4 Exhibits Antitumor Activity *In vivo*

The antitumor activity of AY4 *in vivo* was evaluated in a mouse xenograft model of non–small cell lung carcinoma, H460 cells. After H460 tumors were preestablished s.c. to a volume of ~100 mm^3, tumor-bearing mice were administered i.v. with AY4 or IC antibody at a single dosage of 5 mg/kg with five doses every 3-day interval. AY4 treatment showed strong antitumor activity from day 10, 3 days after the first injection of AY4, and resulted in ~2-fold reduction in tumor volume and weight at the end of treatment (22 day), compared with the IC antibody (Fig. 6A–C). Notably, no signs of systemic toxicity (e.g., no change in body weight, gross appearance, or behavior) were observed in the AY4-treated mice. These results suggested that AY4 is a potent antitumor agent of *in vivo* tumor growth.

Discussion

The cell death induced by anti-DR4 mAb AY4 was characterized by caspase-dependent apoptosis, which could be blocked by Z-VAD-fmk (Figs. 3 and 4). The specificity of AY4 to cell death signaling through DR4 was confirmed by the ability of DR4 as a soluble competitor to protect the AY4-sensitive cells from cell death but not by DR5 (Fig. 2C). The complete resistance of FADD-deficient or caspase-8–deficient Jurkat cells, but not RIP1-deficient Jurkat cells, to AY4-induced cell death (Fig. 5A) suggested that FADD and caspase-8 are essential components of DISC of DR4 formed by AY4 stimulation. Like the previously reported anti-DR4 HGS-ETR1 mAb (9), AY4-mediated DR4 stimulation activated the two apoptotic pathways of the extrinsic pathway, as evidenced by caspase-8 and caspase-3 activations, and the intrinsic pathway as shown by Bid and caspase-9 activations in the AY4-sensitive cells. HCT116 cells, which were defined as type II cells in response to TRAIL (38, 39), also exhibited the typical type II response to AY4 because the absence of Bax ($Bax^{-/-}$ HCT116 cells) and the presence of caspase-9 inhibitor significantly suppressed the AY4-mediated cell death (Fig. 5B and C). The $p53^{-/-}$ HCT116 cells were less susceptible to AY4 and TRAIL than the wild-type cells, suggesting that the tumor suppressor protein p53, which is a key signaling molecule

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connecting between DNA damage detection and apoptosis initiation, also partially implicated in the AY4- and TRAIL-mediated cell death most likely by transcriptional up-regulation of many proapoptotic genes involved in the extrinsic and/or intrinsic pathways, such as *DR4, DR5, Bax, Puma*, and *Noxa* (1, 4). However, the $p53^{-/-}$ HCT116 cells still exhibited significant cell death by AY4 exposure (Fig. 5B), suggesting that DR4 stimulation by AY4 can induce apoptotic cell death in p53-independent manner, like TRAIL (38). This result also indicated that AY4 can be used as an anticancer agent even for p53-mutated tumors, which are commonly resistant to conventional chemotherapy or radiotherapy (1, 2, 38). In addition to HCT116 cells, the other AY4-sensitive cells followed the same cell death characteristics classified as type I and type II cells by the response to TRAIL, suggesting that the cell death profiles in responses to TRAIL and anti-DR4/DR5 agonistic mAbs are determined by the downstream signaling of the receptor rather than the kind of receptor.

In contrast to previously reported anti-DR4 agonistic mAbs, 4H6 (7), 2E12 (8), and DR4-A (34), which required cross-linking agent to trigger potent apoptosis, the bivalent anti-DR4 AY4 alone was enough to activate DR4 to transduce cell death signaling for the TRAIL-sensitive cells, like anti-DR4 HGS-ETR1 mAb (9, 11). However, the monovalent AY4-scFv has no ability to induce cell death (Supplementary Fig. S5). Although how the bivalent AY4 mAb activates DR4 is not clear at this point, it is likely that AY4 binding to DR4 might directly induce or stabilize the receptor in an active conformation or induce clustering of the preligand assembled receptor, eventually promoting the formation of DISC for the intracellular death signaling (1, 9, 13, 28). Another distinctive property of AY4 from other anti-DR4 agonistic mAbs was its efficient killing activity of Jurkat cells (Fig. 2A), which, despite low-level DR4 expression on the cell surface (Supplementary Fig. S6), were only sensitive to the apoptotic cell death by TRAIL, DR5-selective TRAIL mutants, or anti-DR5 agonistic mAbs but neither to DR4-selective TRAIL mutants nor other anti-DR4 agonistic mAbs (12, 22, 23, 32, 34). AY4 induced apoptotic cell death of Jurkat cells dominantly through the extrinsic pathway, judged from the partial suppression by the caspase-9 inhibitor (Fig. 5C). All of these distinguished proapoptotic activities of AY4 from other anti-DR4 agonistic mAbs could be most likely attributed to its different epitopes on DR4 from those of other anti-DR4 mAbs (1), although the epitopes recognized by other anti-DR4 mAbs have not been reported yet. AY4 did not compete for DR4 binding with TRAIL (Fig. 1D), suggesting that binding regions of AY4 do not overlap with those of TRAIL on DR4. In another case, the anti-DR5 HW1 antibody that bound to different regions on DR5 from TRAIL also triggered cell death signaling through DR5 (13). These results indicated that agonistic mAb binding to the unique TRAIL binding regions on DR4 and DR5 is not a prerequisite for the activation of DR4 and DR5 to initiate cell death signaling. Accordingly, different profiles among anti-DR4 and anti-DR5 agonistic mAbs in terms of cross-linking requirement and cell death efficiency for various tumor cells might be most likely attributed to their distinct epitopes and/or affinity with their specific antigen. The differences in the epitopes and/or affinity among the mAbs might be reflected by their differential ability to activate DR4 and/or DR5, which in turn might determine the cell death efficiency of each agonistic mAb (1).

The TRAIL-sensitive H460 and HCT116 cells were also susceptible to either anti-DR5 or anti-DR4 agonistic mAbs (9, 13, 39). As stated above, however, Jurkat cells previously underwent cell death selectively through DR5 rather than DR4. Ramos cells, which were sensitive to anti-DR4 HGS-ETR1, but not to anti-DR5 HGS-ETR2 (23, 34), were also susceptible to AY4; nonetheless, their cell surface expression level of DR4 was much lower than DR5 (Supplementary Fig. S6). However, despite the similar expression level of DR4 to those of Jurkat and Ramos cells, the TRAIL-sensitive HL60 cells showed complete resistance to AY4. In HL60 cells, TRAIL triggered efficient activation of caspase-8 and Bid, but DR4-specific AY4 did not (Fig. 4A). These results are in line with the previous observation that HL60 cells were sensitive to TRAIL through DR5-mediated caspase-dependent apoptosis signaling, whereas they underwent monocytic differentiation by DR4 stimulation through nuclear factor-κB signaling pathways (37). These results, together with the selective responses of Jurkat and Ramos

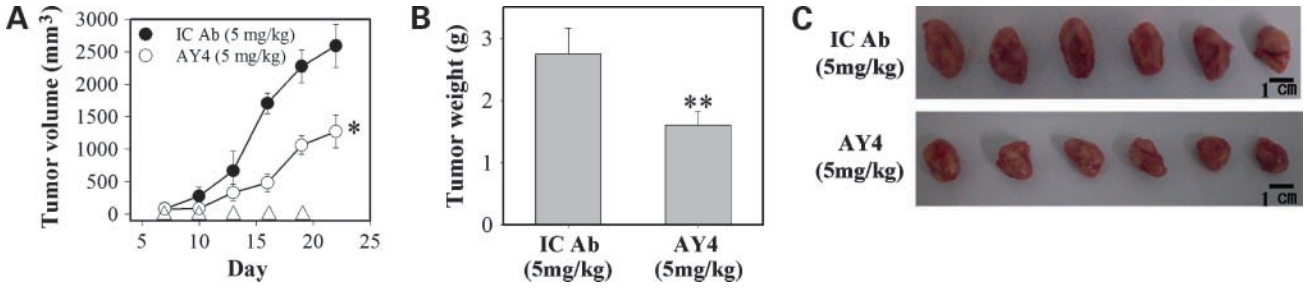

Figure 6. Antitumor effect of anti-DR4 AY4 on growth of preestablished H460 tumors in athymic nude mice. **A** to **C,** comparisons of tumor growth during the treatment (**A**) and isolated tumor weight (**B**) and tumor size (**C**) at the end of treatment (after 22 d of tumor inoculation) between mice treated with AY4 and IC antibody. After preestablishing of H460 cells with volume of 100 mm^3 (day 7), mice (n = 6 per group) were administered i.v. with five doses of AY4 or IC antibody at a single dosage of 5 mg/kg/injection (100 μg/injection) every 3 d from day 7 (as indicated by *white arrowheads*). *Bars,* SD. Significance was determined using the Student's t test versus control IC–treated group. *, $P < 0.0001$; **, $P < 0.01$.

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cells to anti-DR4 or anti-DR5 agonistic mAbs, indicated that, in some tumor cells, only one of the proapoptotic DR4 and DR5 is fully functional in terms of the ability to trigger apoptotic cell death signaling (12, 17, 21–23, 32, 34). In addition to the cell lines mentioned above, many tumor cells exhibited selective sensitivity to apoptotic cell death via one of DR4 and DR5, but not the other, regardless of their relative cell surface expression levels (2, 19, 20, 24). Accordingly, the comprehensive screenings of DR4- or DR5-specific agonistic mAbs in the cell death–inducing activity for a broad range of tumor types may be prerequisite for targeted treatment of particular tumors.

Finally, we have shown that the anti-DR4 mouse mAb AY4 as a single agent induced apoptotic cell death in various types of TRAIL-sensitive tumor cell lines but showed minimal cytotoxicity in normal hepatocytes *in vitro*. Although AY4 had distinct binding regions of DR4 from those of TRAIL, AY4 stimulation to DR4 could exert both the extrinsic and intrinsic apoptotic pathways for the AY4-sensitive cells, like TRAIL. For the preestablished solid tumors of H460 cells, AY4 significantly retarded the tumor growth in athymic nude mice, showing that AY4 treatment was pharmacodynamically effective. Conclusively, our results provide further insight into the DR4-mediated cell death signaling and potential use of AY4 mAb as an anticancer agent, particularly for AY4-sensitive tumor types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Ashkenazi A. Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. Nat Rev Drug Discov 2008;7:1001–12.

2. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 2008;8:782–98.

3. Bellail AC, Qi L, Mulligan P, Chhabra V, Hao C. TRAIL agonists on clinical trials for cancer therapy: the promises and the challenges. Rev Recent Clin Trials 2009;4:34–41.

4. Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. Targeting death-inducing receptors in cancer therapy. Oncogene 2007;26:3745–57.

5. Ozoren N, El-Deiry WS. Defining characteristics of types I and II apoptotic cells in response to TRAIL. Neoplasia 2002;4:551–7.

6. Rudner J, Jendrossek V, Lauber K, Daniel PT, Wesselborg S, Belka C. Type I and type II reactions in TRAIL-induced apoptosis—results from dose-response studies. Oncogene 2005;24:130–40.

7. Chuntharapai A, Dodge K, Grimmer K, et al. Isotype-dependent inhibition of tumor growth *in vivo* by monoclonal antibodies to death receptor 4. J Immunol 2001;166:4891–8.

8. Ohtsuka T, Buchsbaum D, Oliver P, Makhija S, Kimberly R, Zhou T. Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. Oncogene 2003;22:2034–44.

9. Pukac L, Kanakaraj P, Humphreys R, et al. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types *in vitro* and *in vivo*. Br J Cancer 2005;92:1430–41.

10. Tolcher AW, Mita M, Meropol NJ, et al. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. J Clin Oncol 2007;25:1390–5.

11. Georgakis GV, Li Y, Humphreys R, et al. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. Br J Haematol 2005;130:501–10.

12. Ichikawa K, Liu W, Zhao L, et al. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. Nat Med 2001;7:954–60.

13. Park KJ, Lee SH, Kim TI, et al. A human scFv antibody against TRAIL receptor 2 induces autophagic cell death in both TRAIL-sensitive and TRAIL-resistant cancer cells. Cancer Res 2007;67:7327–34.

14. Guo Y, Chen C, Zheng Y, et al. A novel anti-human DR5 monoclonal antibody with tumoricidal activity induces caspase-dependent and caspase-independent cell death. J Biol Chem 2005;280:41940–52.

15. Yada A, Yazawa M, Ishida S, et al. A novel humanized anti-human death receptor 5 antibody CS-1008 induces apoptosis in tumor cells without toxicity in hepatocytes. Ann Oncol 2008;19:1060–7.

16. Motoki K, Mori E, Matsumoto A, et al. Enhanced apoptosis and tumor regression induced by a direct agonist antibody to tumor necrosis factor-related apoptosis-inducing ligand receptor 2. Clin Cancer Res 2005;11:3126–35.

17. Locklin RM, Federici E, Espina B, Hulley PA, Russell RG, Edwards CM. Selective targeting of death receptor 5 circumvents resistance of MG-63 osteosarcoma cells to TRAIL-induced apoptosis. Mol Cancer Ther 2007;6:3219–28.

18. Wagner KW, Punnoose EA, Januario T, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med 2007;13:1070–7.

19. Kelley SK, Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. Curr Opin Pharmacol 2004;4:333–9.

20. van der Sloot AM, Tur V, Szegezdi E, et al. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. Proc Natl Acad Sci U S A 2006;103:8634–9.

21. Griffith TS, Rauch CT, Smolak PJ, et al. Functional analysis of TRAIL receptors using monoclonal antibodies. J Immunol 1999;162:2597–605.

22. MacFarlane M, Inoue S, Kohlhaas SL, et al. Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. Cell Death Differ 2005;12:773–82.

23. MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJ, Cohen GM. TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. Cancer Res 2005;65:11265–70.

24. Tur V, van der Sloot AM, Reis CR, et al. DR4-selective tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) variants obtained by structure-based design. J Biol Chem 2008;283:20560–8.

25. Song MY, Park SK, Kim CS, et al. Characterization of a novel anti-human TNF-α murine monoclonal antibody with high binding affinity and neutralizing activity. Exp Mol Med 2008;40:35–42.

26. Lee HW, Kim TI, Chan KH, et al. Inducing rigid local structure around the zinc-binding region by hydrophobic interactions enhances the homotrimerization and apoptotic activity of zinc-free TRAIL. Biochem Biophys Res Commun 2007;362:766–72.

27. Kim DS, Lee SH, Kim JS, Lee SC, Kwon MH, Kim YS. Generation of humanized anti-DNA hydrolyzing catalytic antibodies by complementarity determining region grafting. Biochem Biophys Res Commun 2009;379:314–8.

28. Lee HW, Lee SH, Lee HW, Ryu YW, Kwon MH, Kim YS. Homomeric and heteromeric interactions of the extracellular domains of death receptors and death decoy receptors. Biochem Biophys Res Commun 2005;330:1205–12.

29. Wu GS, Burns TF, Zhan Y, Alnemri ES, El-Deiry WS. Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. Cancer Res 1999;59:2770–5.

30. Park KJ, Lee SH, Lee CH, et al. Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death. Biochem Biophys Res Commun 2009;382:726–9.

31. Zhang L, Yu J, Park BH, Kinzler KW, Vogelstein B. Role of BAX in the apoptotic response to anticancer agents. Science 2000;290:989–92.

32. Sprick MR, Weigand MA, Rieser E, et al. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity 2000;12:599–609.

33. Kwon YS, Lee YR, Kim YS, Lee HW, Jang YJ. Effect of a novel fully human monovalent antigen-binding fragment on the survival of cancer cell lines. Oncol Rep 2007;18:513–7.

Published OnlineFirst July 28, 2009; DOI: 10.1158/1535-7163.MCT-09-0235

34. Natoni A, MacFarlane M, Inoue S, et al. TRAIL signals to apoptosis in chronic lymphocytic leukaemia cells primarily through TRAIL-R1 whereas cross-linked agonistic TRAIL-R2 antibodies facilitate signalling via TRAIL-R2. Br J Haematol 2007;139:568–77.

35. Mori E, Thomas M, Motoki K, et al. Human normal hepatocytes are susceptible to apoptosis signal mediated by both TRAIL-R1 and TRAIL-R2. Cell Death Differ 2004;11:203–7.

36. Kelley RF, Totpal K, Lindstrom SH, et al. Receptor-selective mutants of apoptosis-inducing ligand 2/tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling. J Biol Chem 2005;280:2205–12.

37. Secchiero P, Milani D, Gonelli A, et al. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and TNF-α promote the NF-κB-dependent maturation of normal and leukemic myeloid cells. J Leukoc Biol 2003;74:223–32.

38. Wang S, El-Deiry WS. Requirement of p53 targets in chemosensitization of colonic carcinoma to death ligand therapy. Proc Natl Acad Sci U S A 2003;100:15095–100.

39. LeBlanc H, Lawrence D, Varfolomeev E, et al. Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nat Med 2002;8:274–81.

40. Lamothe B, Aggarwal BB. Ectopic expression of Bcl-2 and Bcl-xL inhibits apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL) through suppression of caspases-8, 7, and 3 and BID cleavage in human acute myelogenous leukemia cell line HL-60. J Interferon Cytokine Res 2002;22:269–79.

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Molecular Cancer Therapeutics

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