

Enhanced Apoptosis and Tumor Regression Induced by a Direct Agonist Antibody to Tumor Necrosis Factor – Related Apoptosis-Inducing Ligand Receptor 2

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Abstract Purpose: Substantial evidence indicates that supraoligomerization of the death receptors for Fas ligand and tumor necrosis factor – related apoptosis-inducing ligand (TRAIL) is necessary for efficient activation of the apoptotic pathway. Bivalent IgG antibodies can induce the efficient apoptosis by mimicking the natural ligands but only after these antibodies are further oligomerized by cross-linking. In this study, we generated a novel agonist antibody to TRAIL receptor 2 (TRAIL-R2) capable of inducing apoptosis without cross-linking and elucidated its mode of action and efficacy. **Experimental Design:** A fully human antibody to TRAIL-R2, KMTR2, was generated from KM Mouse immunized with TRAIL-R2 ectodomain. Apoptosis-inducing activities of unfractionated or purified monomeric IgG of KMTR2 was evaluated in the presence or absence of cross-linkers, secondary antibodies or Fc receptor – expressing effector cells, against human colorectal adenocarcinoma Colo205. Oligomerization of TRAIL-R2 was analyzed by size exclusion chromatography and confocal microscopy, and *in vivo* efficacy was examined in Colo205 xenograft model. **Results:** KMTR2 specifically recognized TRAIL-R2 and induced apoptosis with or without cross-linking. Size exclusion chromatography showed that the apoptosis activity coeluted with monomeric IgG and was effective independent of secondary antibody or Fc receptor – expressing effector cells. The antibody formed supracomplexes with soluble recombinant and membrane-anchored TRAIL-R2 and enhanced clustering of TRAIL-R2 on cell surface without cross-linking. KMTR2 was dramatically efficacious in reducing established human tumor. **Conclusion:** Our findings indicate that novel agonist antibody KMTR2 can direct antibody-dependent oligomerization of TRAIL-R2 and initiates efficient apoptotic signaling and tumor regression independent of host effector function. Thus, the direct agonist would be a lead candidate for cancer therapeutics.

Oligomerization of death receptors via their specific trimeric, membrane-anchored ligands can activate tumor cell apoptosis (1). Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily initially defined by its ability to selectively induce apoptosis of tumor cells (2–4). Recombinant soluble TRAIL (sTRAIL) activates apoptosis selectively on a broad variety of human tumor cells, but not most normal cells (5–7), and therefore has garnered significant interest as a therapeutic agent for the treatment of

cancer. Recombinant sTRAIL showed an absence of detectable apoptosis activity on normal primary cells from lung, bone, liver, breast, brain, and kidney (6, 8). Importantly, short-term treatment of mouse, monkey, and chimpanzee with recombinant sTRAIL has shown a reasonable safety profile for this ligand (6, 9). Alternative mechanisms of activating the TRAIL pathway include the use of specific agonist antibodies (10) that exploit the receptor discriminating specificity and prolonged bioavailability of IgG.

The TRAIL receptors are members of the TNF receptor superfamily defined by a cysteine-rich extracellular domain. Five distinct receptors and binding proteins for TRAIL have been identified to date (1, 11). TRAIL-R1 (TNFRSF10A, DR4; ref. 12) and TRAIL-R2 (TNFRSF10B, DR5; refs. 13–15) contain a cytoplasmic death domain (DD) capable of transducing an apoptotic signal on ligation with the ligand TRAIL, whereas the other three TRAIL receptors, TRAIL-R3 (TNFRSF10C, DcR1; ref. 16), TRAIL-R4 (TNFRSF10D; DcR2; ref. 17), and osteoprotegerin (TNFRSF11B; OPG; ref. 18), lack a DD and do not activate apoptosis and may function as decoy receptors (19, 20). Assembly and trimerization of TRAIL-R1 and TRAIL-R2 are

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requisite for transducing the death signal (1, 11). Some antibodies to TRAIL-R1 and TRAIL-R2 have been reported to exhibit weak or marginal antitumor effects without cross-linking but an enhanced antitumor effect in the presence of a cross-linking reagent (21, 22). Under *in vitro* conditions, a variety of exogenous reagents, including anti-immunoglobulin antibodies, protein A, and chemical reagents, can be used for cross-linking, but *in vivo* effector molecules and/or cells involved in cross-linking are presumably limited to the complement component C1q and Fc receptors (FcR) present on most immune effector cells (22, 23). Because the effector function of such endogenous cross-linkers may be variable in individual patients due to immunosuppressive therapies (24) and polymorphism of FcR (25, 26), a direct agonist to TRAIL receptor that triggers apoptosis independent of cross-linking may be more desirable for antibody-based cancer therapy.

We reported previously the generation of several human monoclonal antibodies (mAb) specific for either TRAIL-R1 or TRAIL-R2 that were derived from transchromosomal mice expressing human immunoglobulin locus (KM Mouse; ref. 27). Antibodies specific to either TRAIL-R1 or TRAIL-R2, such as B12 or H48, were effective at inducing apoptosis; however, they required cross-linking to exert apoptotic activity (27). Here, we identified a novel anti-TRAIL-R2 mAb (KMTR2) that acts as a direct agonist possessing the ability to induce apoptosis without secondary cross-linking reagents. This agonist mAb can oligomerize sTRAIL-R2 and clusters membrane TRAIL-R2 on cell surface without cross-linking, inducing death of human tumor cells *in vitro* and established tumors *in vivo*. The results support the concept that a direct agonist antibody is more efficacious than indirect agonist antibodies and further support its evaluation as an immunotherapeutic for cancer.

Materials and Methods

Reagents. Anti-human IgG polyclonal antibody was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan) and used as cross-linking reagent for *in vitro* assay. The antibody cocktail to FcR was prepared by mixing anti-CD16 mAb (BD Biosciences PharMingen, San Jose, CA), anti-CD32 mAb (Serotec, Inc., Raleigh, NC), and anti-CD64 mAb (Caltag Laboratories, Burlingame, CA) at same concentration. Human IgG labeling kit was purchased from Molecular Probes (Eugene, OR) and used for confocal microscopy analysis. Control human antibody to human serum albumin (HSA) or 2,4-dinitrophenol (DNP) was prepared in our laboratory from Chinese hamster ovary cells transfected with variable region of the hybridoma-derived antibody and the framework of either IgG4 or IgG1 according to the method described below. Whole human IgG was purchased from Sigma-Aldrich Japan K.K. and used as control IgG for *in vivo* xenograft model. sTRAIL-R2:Fc fusion protein was prepared in our laboratory from stably transfected Chinese hamster ovary cells with a vector that contains cDNA encoding the extracellular domain of TRAIL-R2 fused to Fc portion of human IgG1. sTRAIL-R1, sTRAIL-R3, and sTRAIL-R4 were purchased from Alexis (Läufelfingen, Switzerland).

Cell lines. Colo205 human colorectal adenocarcinoma was obtained from American Type Culture Collection (Rockville, MD). Colo205 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and streptomycin. L929/DR5 Δ DD and L929/DR4 Δ DD transfectants were produced in our laboratory from parent cells L929 (American Type Culture Collection) by transfecting DD-deleted TRAIL-R2 (amino acids 1-348) or TRAIL-R1 (amino acids 1-351) as reported previously (27). Both transfectants were maintained in DMEM supplemented with 10% fetal bovine serum and streptomycin.

Generation of fully human agonistic monoclonal antibodies to tumor necrosis factor-related apoptosis-inducing ligand receptor 2. KM/H2-D mice generated by crossing the original KM Mouse (28) with a BALB/c background were i.p. immunized with L929/DR5 Δ DD transfectants (1×10^6 : 5×10^6 cells per head per shot) weekly or biweekly for 2 months. A mouse exhibiting high titer specific to TRAIL-R2 was selected for final boost with TRAIL-R2:Fc fusion protein (20 μ g/head i.v.) and interleukin-6 (5 μ g/head i.v.). Spleen cells were prepared 3 days after the final boost and fused with SP2/0 cells (American Type Culture Collection). Antibody-secreting hybridomas were initially screened by a proliferation assay without cross-linking as described below and cloned by limiting dilution. Isotypes of mAbs were determined with an isotyping ELISA (The Binding Site, Birmingham, United Kingdom), and the subclass of KMTR2 produced from original hybridoma was found to be IgG4, different from other IgG1 mAbs to TRAIL-R2, including H48 obtained previously in our laboratory (27).

Preparation of recombinant human antibody. The heavy and light chain mRNAs were cloned from hybridoma of KMTR2, H48, and control antibodies (anti-HSA mAb and anti-DNP mAb) in pGEM-T Easy Vector (Promega Corp., Madison, WI). Variable regions of the antibodies were genetically spliced into N5KG1-Val Lark vector (Biogen IDEC, Inc., Cambridge, MA) or N5KG4-Val Lark (Biogen IDEC) with puromycin resistance gene, respectively. Antibodies were expressed in Chinese hamster ovary cells and purified with protein A (Amersham Biosciences Corp., Piscataway, NJ) by standard methods from culture supernatant of Chinese hamster ovary cells. These recombinant antibodies were used for the following experiments.

Separation of monomeric antibodies. Protein A purified antibody was applied to Superdex 200HR column (Amersham Biosciences Corp.), equilibrated with PBS, and eluted with PBS at a flow rate of 0.25 mL/min. Eluted fractions were collected and filtered through a membrane filter (0.22 μ m Ultrafree-MC Sterile, Millipore, Billerica, MA). The absorbance of each fraction was measured at 280 nm and the antibody concentration of each fraction was calculated using 1.4 absorbance = 1 mg/mL. Fractions containing sufficient amount of antibody were applied to Colo205 proliferation assay at final concentration of 1,000 ng/mL, but fractions below detectable levels at A280 were tested without dilution. IgG in the monomeric fractions was subjected to re-chromatography for determining the purity of monomers, and highly purified monomer fractions (purity of >99.8%) were used for confocal microscopic analysis, immune complex analysis, and proliferation assay.

Specific binding of KMTR2 to tumor necrosis factor-related apoptosis-inducing ligand receptors. L929/DR5 Δ DD or L929/DR4 Δ DD cells were plated in 96-well round-bottomed plates (BD Biosciences PharMingen) at 1×10^5 cells per well. Antibodies were added into each well at indicated concentration and incubated at 4°C for 1 hour. Plate was centrifuged at 2,000 rpm for 2 minutes and washed thrice with PBS containing 2% FCS. Horseradish peroxidase-conjugated goat anti-human IgG(Fc) (IBL, Gunma, Japan) was added into each well and plate was incubated at 4°C for 30 minutes. After washing thrice with PBS, TMB substrate (DAKO Cytomation Japan, Kyoto, Japan) was added into each well. Enzyme reaction was stopped by 0.5 mol/L sulfonic acid. The absorbance at 450 nm (reference wavelength at 570 nm) was measured by microplate reader.

Proliferation assay. Colo205 cells were seeded in 96-well flat-bottomed plate at 0.5×10^4 to 1×10^4 cells per well and cultured overnight at 37°C under 5% CO₂. Anti-TRAIL-R2 mAbs were added to each well at various concentrations (1-1,000 ng/mL), and cells were cultured for an additional 2 days. When anti-TRAIL-R2 mAbs were cross-linked, goat anti-human IgG was added at a concentration of 10 μ g/mL ~0.5 to 1 hour after the addition of anti-TRAIL-R2 mAbs. In competition assay, human control IgG1 (anti-HSA mAb) was added just before the addition of cross-linking reagent. After culture for 2 days, each well was gently washed once with PBS to remove cell debris and immediately filled with fresh medium. Then, cell viability was determined by MTS dye reduction assay (CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay kit, Promega Corp.) and

calculated by following formula: cell viability (%) = (absorbance of sample well – absorbance of blank well) / (absorbance of medium-treated well – absorbance of blank well) × 100.

Cross-linking anti-tumor necrosis factor-related apoptosis-inducing ligand receptor 2 monoclonal antibody with human peripheral blood mononuclear cells. Human peripheral blood was obtained from health volunteers according to the protocol approved by the internal committee at our laboratory, and signed informed consent was obtained from all volunteers. Peripheral blood mononuclear cells (PBMC) were prepared from peripheral blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences Corp.) and freeze-stocked until assay. Colo205 cells (0.5×10^4 – 1×10^4 per well) were seeded and anti-TRAIL-R2 mAbs were added as described in proliferation assay. Cryopreserved human PBMCs were gently thawed in water bath at 37°C and suspended with fresh medium. Viable PBMCs were counted and adjusted at appropriate cell density. After irradiated with 50 Gy of X-ray, PBMCs were added as cross-linker at effector (PBMC)/target (Colo205 cells) ratio (E/T ratio) of 12.5, 25, and 50, and the plate was cultured for additional 2 days. In competition assay or FcR-blocking assay, control human IgG1 (anti-HSA mAb) was added before the addition of PBMCs, or PBMCs pretreated with anti-FcR antibody cocktails were added to target cells. After culture for 2 days, each well was gently washed once with PBS to remove cell debris and immediately filled with fresh medium. Then, cell viability was determined and calculated as described in proliferation assay.

Caspase activity. Colo205 cells were seeded in 96-well flat-bottomed plate (Corning Life Sciences, Corning, NY) at 1×10^4 cells per well and cultured overnight at 37°C under 5% CO₂. Anti-TRAIL-R2 mAbs were added to each well at 1,000 ng/mL and subsequently incubated for ~0.5 to 1 hour. Cells were added with or without goat anti-human IgG at a concentration of 10 µg/mL. Two hours after the addition of anti-TRAIL-R2 mAbs, caspase-3/7 activity was measured by Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega Corp.).

Detection of apoptosis. Apoptosis was evaluated by detecting cytoplasmic histone-associated DNA fragments (mononucleosome and oligonucleosomes). Colo205 cells were seeded in 96-well flat-bottomed plate (Corning Life Sciences) at 1×10^4 cells per well and cultured overnight at 37°C under 5% CO₂. Anti-TRAIL-R2 mAbs were added to each well at 1,000 ng/mL and subsequently incubated for ~0.5 to 1 hour followed by the addition of goat anti-human IgG. Two hours after the addition of anti-TRAIL-R2 mAbs, DNA fragments resulting from apoptotic cell death were measured with Cell Death Detection ELISA^{PLUS} (Roche Diagnostics, Penzberg, Germany).

Immune complex analysis of soluble receptor and antibody. Anti-TRAIL-R2 antibody was mixed with sTRAIL-R2 extracellular domain or TRAIL-R2:Fc fusion protein in PBS at the molar ratio of 2:1 or 1:1, respectively. The mixture was incubated for 30 minutes at 37°C and then loaded to Superdex 200HR gel filtration column equilibrated with 20 mmol/L phosphate buffer (pH 7.0) containing 200 mmol/L NaCl. The protein complexes of each fraction were detected by UV absorbance at 280 nm and molecular weight of complexes was determined by light scattering analysis.

Analysis of cell surface receptor-antibody immune complex. Colo205 cells (1×10^5 per mL) were incubated with anti-TRAIL-R2 mAbs at a concentration of 100 ng/mL for 30 minutes at 37°C. After washing the cells to remove free antibodies, cell suspensions were mixed with 2 mmol/L of chemical cross-linker [3,3'-dithiobis(sulfosuccinimidyl-propionate), Pierce Biotechnology, Inc., Rockford, IL] and incubated on ice for 2 hours. Tris-HCl buffer (pH 7.5) was added at a final concentration of 50 mmol/L to inactivate the excessive amount of chemical cross-linker for 15 minutes. Cells were then collected by centrifugation and lysed in PBS containing 0.1% Triton X-100 (Nakarai Tesque, Inc., Kyoto, Japan). The suspension was incubated on ice for 30 minutes followed by the centrifugation for 30 minutes to remove insoluble material. Cell lysate (100 µL) was loaded onto gel filtration column (Superose 6, 1×30 cm, Amersham Biosciences Corp.) and eluted with PBS containing 0.1% CHAPS (Dojindo Laboratories,

Kumamoto, Japan) at a flow rate of 0.5 mL/min (high-performance liquid chromatography system, L-6000 series, Hitachi High-Technologies Corp., Kyoto, Japan). After the eluted protein fraction was collected by gel filtration, each fraction (100 µL) was assayed for human IgG ELISA. The human IgG ELISA was done by using rabbit anti-human IgG polyclonal antibody (DAKOCytomation Japan) as a capture antibody and horseradish peroxidase-labeled rabbit anti-human IgG polyclonal F(ab')₂ antibody (DAKOCytomation Japan) as a detection antibody.

Confocal microscopic analysis. Colo205 cells were seeded in collagen I two-well CultureSlides (BD Biosciences Pharmingen) at 2×10^5 cells per well and cultured overnight at 37°C under 5% CO₂. Cells were incubated with gel filtration fractionated monomeric KMTR2 or H48 at a concentration of 100 ng/mL for 1 hour at 37°C under 5% CO₂ and subsequently incubated with or without cross-linker, mouse monoclonal anti-human IgG (HyTest Ltd., Turku, Finland). After washing by ice-cold PBS, Alexa 488-labeled Fab fragment specifically recognizing human IgG (Zenon Human IgG Labeling kits, Molecular Probes) was added for 30 minutes at 4°C. For nuclear staining, cells were fixed with 70% ethanol and stained with propidium iodide (Sigma Aldrich Japan K.K.). Cells were washed and mounted in Fluoromount-G (Southern-Biotech, Birmingham, AL) and visualized with confocal microscope (Carl Zeiss, Oberkochen, Germany), ×40 objective lens.

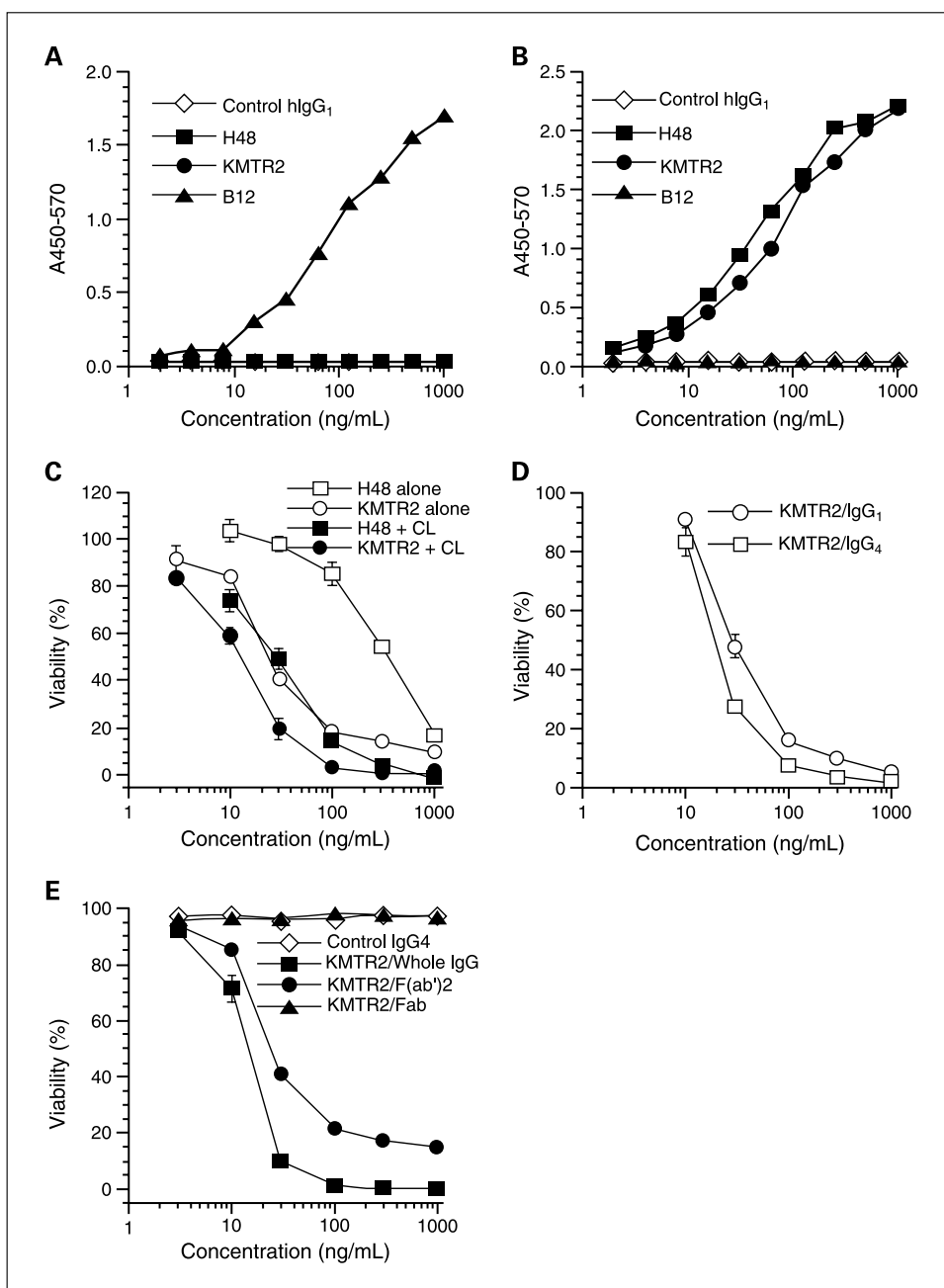
In vivo tumor model. Antitumor activity of KMTR2 and H48 was evaluated in Colo205 xenograft model (29). Experimental procedures for xenograft model were approved by internal committee of our laboratory, and mice were maintained and treated according to the institutional guidelines. Female BALB/c athymic nude mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and allowed to acclimatize to local condition of our animal facility for >1 week before the initiation of experiment.

Colo205 colon cancer cells were s.c. inoculated into right flank of nude mice at 4×10^6 or 5×10^6 cells per mice. Seven or 8 days after implantation, nude mice bearing palpable tumor at volumes ranging from 60 to 110 mm³ were selected and assigned into groups to make the average volume of 75 or 100 mm³. In a large tumor model, nude mice bearing Colo205 at tumor size of 230 to 530 mm³ were assigned into groups to make average volume ~350 mm³. Each treatment regimen was started on the same day when mice were assigned into groups. Vehicle and antibodies were i.p. given into mice every other or every 3 days (total three times) at dosages of 4, 20, and 100 µg/head/injection. Length, width, and height of each tumor mass were measured by calipers twice or thrice weekly, and tumor volume was calculated as: tumor volume (mm³) = (length × width × height) / 2.

Results

Direct agonist antibody specific for tumor necrosis factor-related apoptosis-inducing ligand receptor 2. Hybridoma supernatants from KM mice immunized with TRAIL-R2 ectodomain were screened for death-inducing activity on Colo205 cells in the absence of secondary cross-linking reagents. One cloned hybridoma line producing an IgG4 isotype was identified, designated KMTR2, and biological activities of recombinant IgG1 or IgG4 of KMTR2 were evaluated. Specificity of the antibody was shown by specific binding to mouse L cells transfected with human TRAIL-R2 but not TRAIL-R1 (Fig. 1A and B; ref. 27). In addition, KMTR2 bound to soluble ectodomain of TRAIL-R2 as a chimeric protein fused to Fc of human IgG1 but not to other soluble Fc fusion proteins containing TRAIL-R1, TRAIL-R3, or TRAIL-R4 (data not shown). The concentration-response curves for Colo205 cell death were identical for KMTR2 (IC₅₀, 10-40 ng/mL) with or without cross-linking reagent (goat anti-human IgG), whereas cross-linking the anti-TRAIL-R2 antibody H48 (IgG1) dramatically enhanced the death-inducing

Fig. 1. Binding specificity and activity of the anti-TRAIL-R2 human IgG, KMTR2. *A* and *B*, L929/DR4ΔDD (*A*) and L929/DR5ΔDD (*B*) transfectants in microtiter wells were incubated with KMTR2/IgG₁, H48/IgG₁, specific antibody to TRAIL-R1 (B12/IgG₁), or control IgG₁ (anti-DNP mAb) at indicated concentrations for 1 hour and binding was assessed by ELISA specific for human IgG (Fc). *C*, Colo205 cells were plated at 1×10^4 per well and cultured with anti-TRAIL-R antibody preparations at the indicated concentrations for 2 days at 37°C in the presence or absence of cross-linker (CL), goat anti-human IgG (10 μg/mL), and then cell viability was determined by MTS dye reduction assay. *D*, recombinant expressed KMTR2 containing either IgG₁ or IgG₄ heavy chain subclass was tested for proliferation assay as described above. *E*, Colo205 cells were incubated with whole antibody, F(ab')₂ or Fab fragment of IgG₄ isotype of KMTR2, or control IgG₄ (anti-DNP mAb) without the secondary antibody and cell viability was determined by MTS dye reduction assay.



activity (Fig. 1C). Effector caspase activation and DNA degradation was correlated with cells death induced by anti-TRAIL-R2 mAbs (data not shown), indicating the cell death was triggered by an apoptotic signal. Reengineering the KMTR2 heavy chain from a γ_4 to γ_1 did not alter apoptosis concentration-response curve (Fig. 1D). Moreover, bivalent F(ab')₂ of KMTR2 was nearly as effective as intact IgG (Fig. 1E), although monovalent Fab fragments were inactive, indicating that multivalency of KMTR2 is necessary for apoptosis-inducing activity.

Direct agonist KMTR2 induces apoptotic cell death regardless of cross-linking. Aggregates of IgG often contaminate purified antibody preparations (30), which mimic the effects of a cross-linked antibody, accounting for the direct apoptotic activity of anti-TRAIL-R2 antibody. To examine this possi-

bility, we purified anti-TRAIL-R2 antibody by size exclusion gel filtration chromatography to determine whether the apoptosis-inducing activity of KMTR2 was associated with IgG monomers or oligomers. KMTR2 exerted apoptotic activity against Colo205 in fractions corresponding to IgG monomers (fractions 27-32) regardless of the presence of cross-linking reagent (Fig. 2A). Highly purified IgG monomer of KMTR2 with or without cross-linking reagent showed similar concentration-response curves (Fig. 2C) on the death of Colo205 cells. In addition, IgG monomer of KMTR2 induced marked caspase-3/7 activation (Fig. 2E) and DNA fragmentation (Fig. 2F) at a concentration of 1,000 ng/mL. By contrast, some apoptotic activity was detected in the oligomeric fractions (23-25) of H48 antibody but not in fractions where monomeric IgG eluted (fractions 27-32),

unless the cross-linking reagent was added (Fig. 2B). Similarly, caspase-3/7 activation, DNA fragmentation, and resultant apoptotic cell death were observed only when the purified monomer IgG of H48 was cross-linked (Fig. 2D-F), clearly different from the case of unfractionated H48 (Fig. 1C).

The monomer fractions of purified anti-TRAIL-R2 antibody H48 induced apoptosis of Colo205 cells only when cross-linked with goat anti-human IgG. However, apoptotic activity of H48 was blocked in a concentration-dependent fashion by the addition of competing human IgG1. By contrast, the apoptotic activity of KMTR2 was unaffected by human IgG1 (Fig. 3A). PBMCs expressing FcR γ can be used as a cross-linking system to aggregate immune complexes. The apoptosis activity of H48 was induced by addition of PBMC (50:1 ratio of PBMC/Colo205 cells) but was blocked by the addition of human IgG1 (Fig. 3B). By contrast, the addition of PBMC to KMTR2-treated Colo205 cells did not alter the degree of apoptosis and the activity of KMTR2 was not inhibited by addition of human IgG1 (Fig. 3B). Furthermore, the apoptosis-

inducing activity of H48 in the presence of PBMC was proportional to the E/T ratio, indicating dependence on cellular FcR γ (Fig. 3C and D). The PBMC-dependent apoptotic activity was substantially blocked by excessive amount of competing IgG1 (100-fold excess of H48) and by the pretreatment of PBMC with a mixture of anti-FcR γ antibodies, although both blocking reagents failed to abolish the activity of KMTR2 in the presence of PBMC (Fig. 3C and D). The results are consistent with the idea that KMTR2 acts as a direct agonist to TRAIL-R2.

KMTR2 antibody enhances the oligomerization of soluble receptors and membrane-bound receptors. The idea that KMTR2 acts as a direct agonist suggests that antibody binding induces aggregation of TRAIL-R2. To test this hypothesis, the molecular mass of the immune complex formed between KMTR2 and TRAIL-R2 ectodomain was estimated by size exclusion chromatography. The extracellular domain of TRAIL-R2 was eluted with a molecular mass of 43 kDa with large deviation, suggesting that the TRAIL-R2 ectodomain was equilibrated between monomer and trimer (Fig. 4A and B);

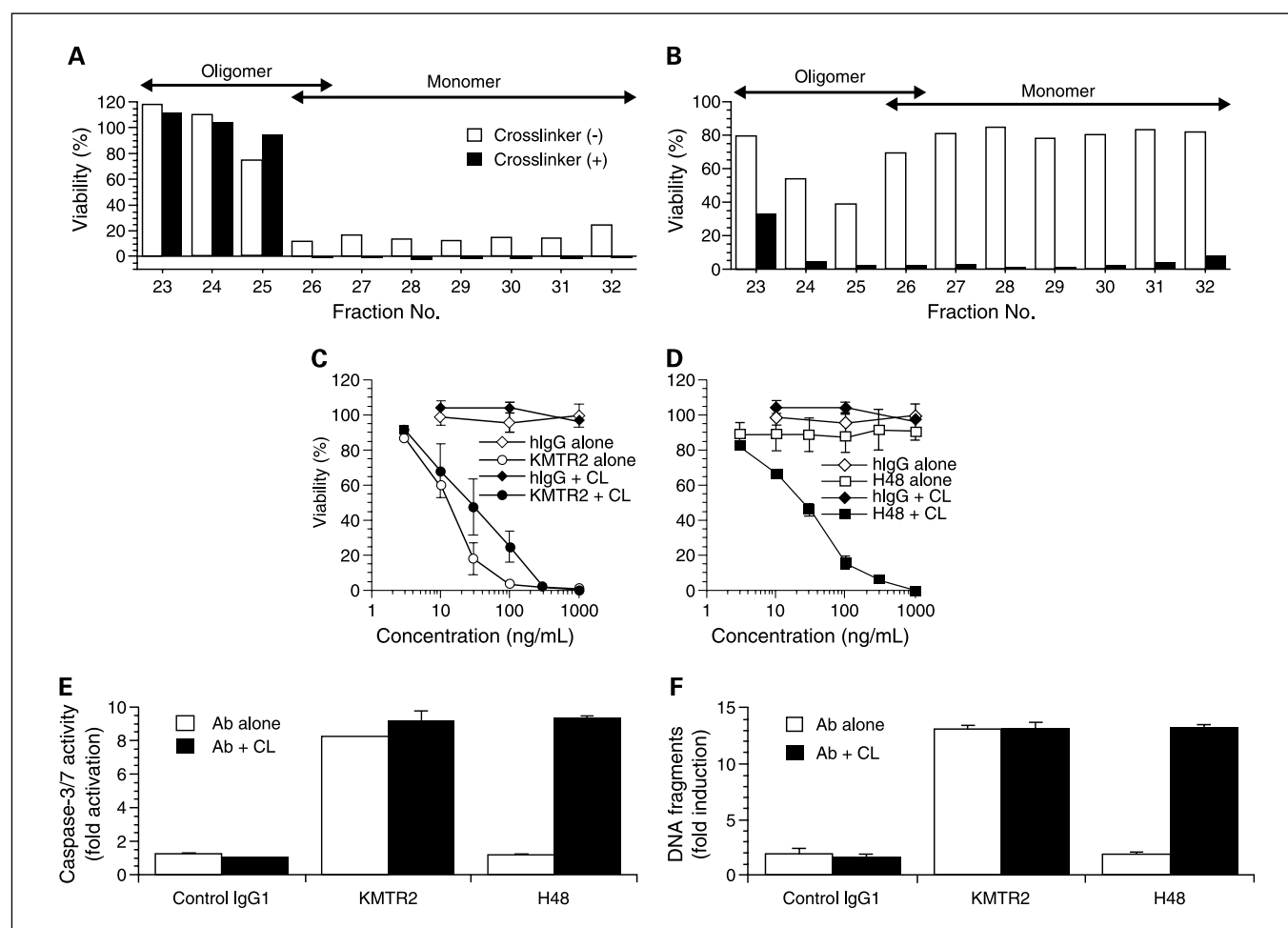
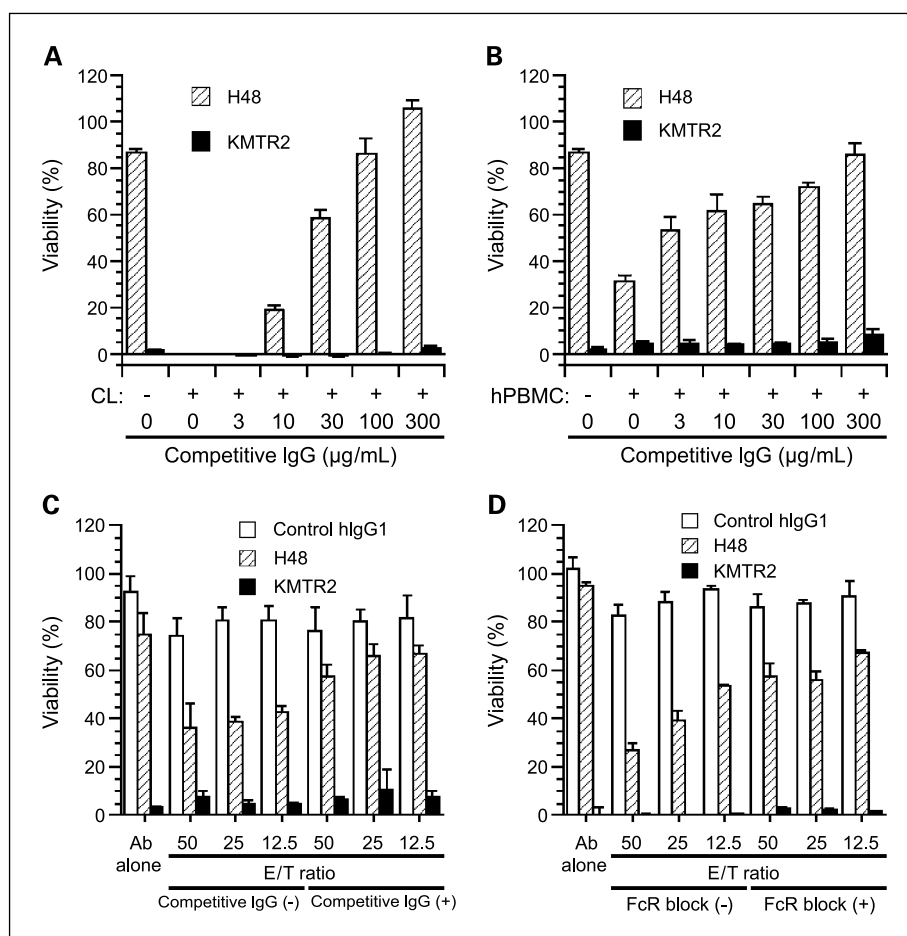


Fig. 2. Apoptosis-inducing activity of gel filtration purified KMTR2. *A* and *B*, purified preparations of KMTR2 or H48 were subjected to gel filtration chromatography and the effect of each fraction on the viability of Colo205 cells (1×10^4 per well) was assessed by MTS reduction assay. KMTR2/IgG1 (*A*) or H48/IgG1 (*B*) was added at $\leq 1 \mu\text{g/mL}$ in the presence (*black columns*) or absence (*white columns*) of cross-linker, goat anti-human IgG ($10 \mu\text{g/mL}$). *C* and *D*, concentration response of the apoptosis-inducing activity of purified monomer fraction of KMTR2/IgG1 (*C*) or H48/IgG1 (*D*) and unfractionated control IgG1 (anti-DNP mAb) was evaluated in the presence or absence of the cross-linker goat anti-human IgG. *E*, effector caspase activity of Colo205 was assessed 2 hours after the treatment with purified monomer fraction of KMTR2/IgG1 or H48/IgG1 or unfractionated control IgG1 (anti-DNP mAb). *F*, apoptosis of Colo205 was estimated by DNA fragmentation using Cell Death Detection ELISA^{PLUS} 2 hours after the treatment with purified monomer fraction of KMTR2/IgG1 or H48/IgG1 or unfractionated control IgG1 (anti-DNP mAb).

Fig. 3. Effect of cross-linking by anti-IgG or human PBMCs on the apoptosis-inducing activity of anti-TRAIL-R2 antibody. Colo205 cells were plated at 0.5×10^4 (C and D) or 1×10^4 (A and B) per well and cultured with KMTR2/IgG1 or H48/IgG1 from monomer fractions (1 $\mu\text{g}/\text{mL}$) with or without cross-linker. A and B, anti-TRAIL-R2 mAbs KMTR2 or H48 were cross-linked by 10 $\mu\text{g}/\text{mL}$ anti-human IgG (A) or with mononuclear cells (5×10^5 per well; B) with or without indicated concentrations of competitive human IgG1 (anti-HSA mAb). C, anti-TRAIL-R2 mAbs or control human IgG1 (anti-DNP mAb) were cross-linked by 50 Gy-irradiated human PBMCs (0.625, 1.25, or 2.5×10^5 per well) in the presence or absence of competitive human IgG1 (anti-HSA mAb, 100 $\mu\text{g}/\text{mL}$). D, anti-TRAIL-R2 mAbs or control human IgG1 (anti-DNP mAb) were cross-linked by 50 Gy-irradiated human PBMCs (0.625, 1.25, or 2.5×10^5 per well) preincubated with anti-human FcR mAb cocktails (anti-CD16/CD32/CD64 mAb) each at concentration of 10 $\mu\text{g}/\text{mL}$. Cell viability was determined by MTS dye reduction assay 2 days after culture at 37°C .



KMTR2 and H48 IgG both eluted at 157-158 kDa (Fig. 4A and B). When immune complexes were formed at a molar ratio of 2:1 (receptor to antibody) and then subjected to size exclusion chromatography, both KMTR2 and H48 were detected in the ~ 180 -kDa fraction (Fig. 4A and B), consistent with the idea that each bivalent antibody bound two TRAIL-R2 ectodomains. However, when KMTR2 was mixed at equimolar ratio with the dimeric TRAIL-R2:Fc (91 kDa), the immune complex eluted in the void volume fraction at $>3,000$ to $5,000$ kDa as the major peak (Fig. 4C). By contrast, the immune complex formed with H48 antibody and TRAIL-R2:Fc eluted within the inclusion volume at 250 kDa (Fig. 4D). Light scattering analysis revealed that the molecular mass of the KMTR2/TRAILR2:Fc immune complex was 5,130 kDa, which is consistent with the idea that the KMTR2 antibody forms a relatively stable oligomeric complex. Moreover, large complexes were not detected when monomeric Fab fragments of KMTR2 were mixed with TRAIL-R2:Fc, indicating that oligomerization is dependent on multivalency of the antibody (data not shown). These results suggest that KMTR2 antibody can directly induce receptor oligomerization.

To determine if KMTR2 clustered TRAIL-R2 on the cell surface, immune complexes were formed by incubating Colo205 cells with either KMTR2 or H48 for 30 minutes at 37°C and the cells were then treated with a bifunctional chemical cross-linker with a short cross-linking arm (1.1 nm) to

stabilize complexes. Nonionic detergent was used to solubilize the immune complexes followed by gel filtration chromatography to determine the relative molecular mass. Immune complexes from KMTR2-treated Colo205 cells were detected in fractions corresponding at high molecular mass (Fig. 5A) compared with immune complexes from H48-treated cells formed under identical conditions. Indeed, H48 complexes eluted primarily at ~ 200 kDa, suggesting that H48 is unable to directly induce high molecular mass complexes on the cell surface.

The formation of immune complexes on the surface of Colo205 cells was visualized by confocal microscopy. Consistent with the idea that KMTR2 induces receptor clustering, Colo205 cells incubated with KMTR2 at 37°C (Fig. 5B and C), but not 4°C , formed specific patches (Fig. 5E). In addition, inhibiting lateral diffusion of membrane proteins of Colo205 cells by 4% paraformaldehyde before incubation with KMTR2 also prevented patches from forming (Fig. 5F), suggesting that TRAIL-R2 is initially dispersed on the cell surface. By contrast, the H48 antibody incubated at 37°C showed dispersed staining on Colo205 cells (Fig. 5G), but clustering was apparent if secondary antibody was included at 37°C (Fig. 5H).

Direct agonist KMTR2 elicits tumor regression. These results suggest that KMTR2 mimics the natural ligand and might prove to be more efficacious in halting tumor cell growth compared with indirect agonist antibodies. To examine this

possibility, human Colo205 tumors were established s.c. in the flanks of immunodeficient nude mice, and after 7 days (mean tumor volume, 75 mm³), the mice were assigned to groups and treated with KMTR2/IgG4, H48, or control human IgG1 (anti-HSA human IgG1) and tumor growth was assessed over 2 weeks. Tumor growth was significantly retarded in H48-treated mice with maximal response at 20 µg/injection, indicating that indirect agonist antibody is effective at slowing tumor growth (Fig. 6A); however, KMTR2 was drastically effective at inducing rapid tumor regression at 20 µg/injection (Fig. 6A). Similar effects were observed when nude mice bearing Colo205 tumors (mean tumor volume, 100 mm³) were treated with KMTR2/IgG1, and the established tumor regressed after i.p. treatment with KMTR2/IgG1 at a lowest dose of 4 µg/injection (Fig. 6B). Maximal efficacy

was achieved by highest dose (100 µg/injection) and tumor did not regrow at 57 days postinitiation of treatment (Fig. 6B). Moreover, a large tumor burden (~350 mm³) regressed after highest dose treatment of KMTR2/IgG1 (Fig. 6C). Thus, KMTR2 exhibits enhanced efficacy in the treatment of tumor in xenotransplantation model.

Discussion

Some mAbs to TRAIL-R have been reported to exhibit agonistic activity without cross-linking, but this activity was drastically enhanced by cross-linking. We describe here a novel anti-TRAIL-R2 mAb KMTR2 with direct agonistic activity. The KMTR2 mAb is more efficacious in the induction of tumor

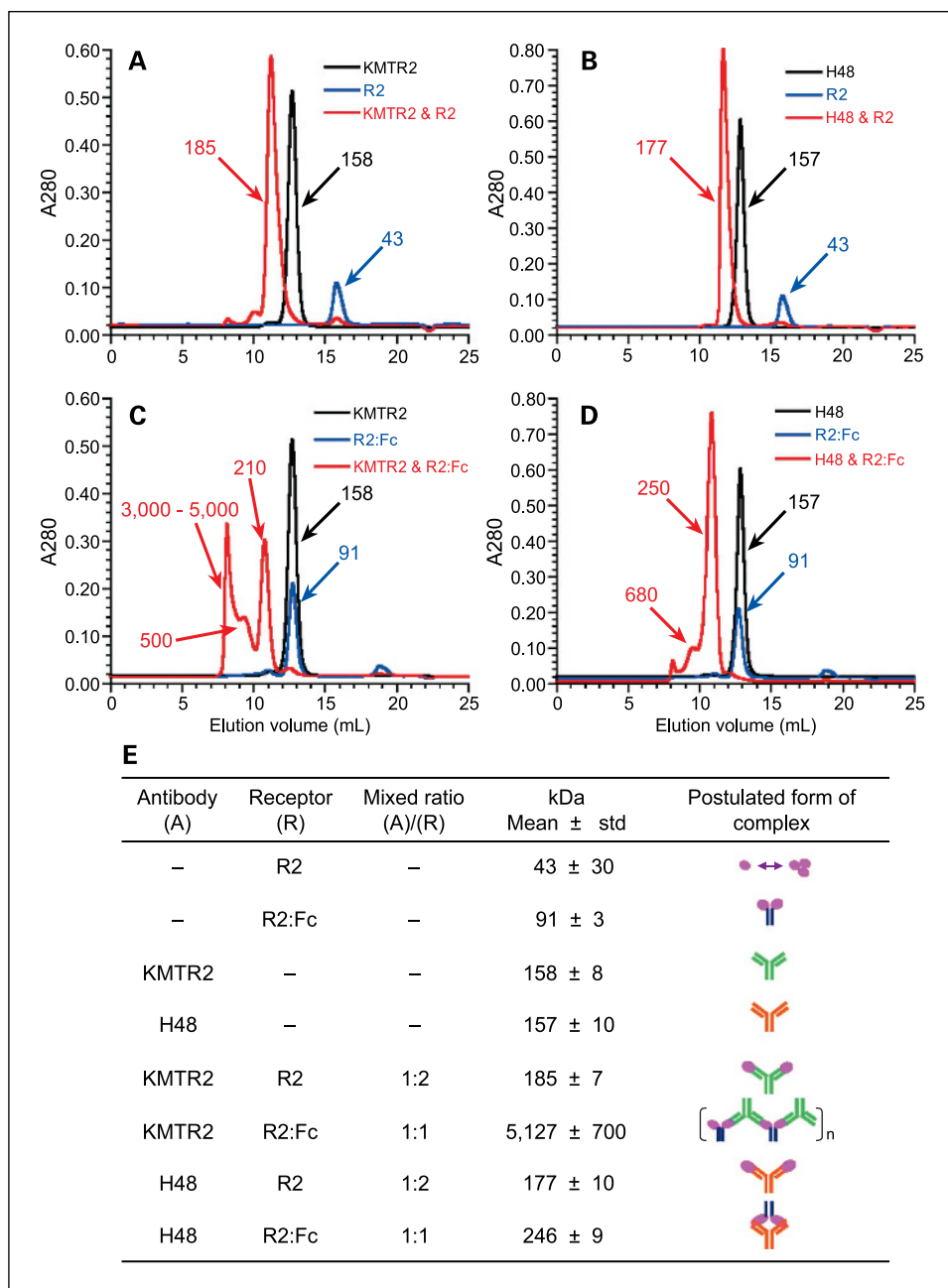


Fig. 4. Immune complex analysis of soluble receptor and antibody. KMTR2/IgG4 (A and C) or H48/IgG1 (B and D) was mixed with extracellular domain of sTRAIL-R2 (R2) or TRAIL-R2:Fc fusion protein (R2:Fc) in PBS at the molar ratio of 1:2 (A and B) or 1:1 (C and D); total amount; 90 µg in A and B and 120 µg in C and D). The mixture was incubated for 30 minutes at 37 °C and then loaded to Superdex 200HR gel filtration column equilibrated with 200 mmol/L NaCl, 20 mmol/L phosphate buffer (pH 7.0). The protein complexes of each fraction were detected by UV absorbance at 280 nm. Each figure is overlaid by control chromatogram of antibody or receptor alone. E, properties of the immune complexes formed and their theoretical stoichiometry are speculated by molecular mass determined by light scattering analysis. (kDa represents kilodaltons).

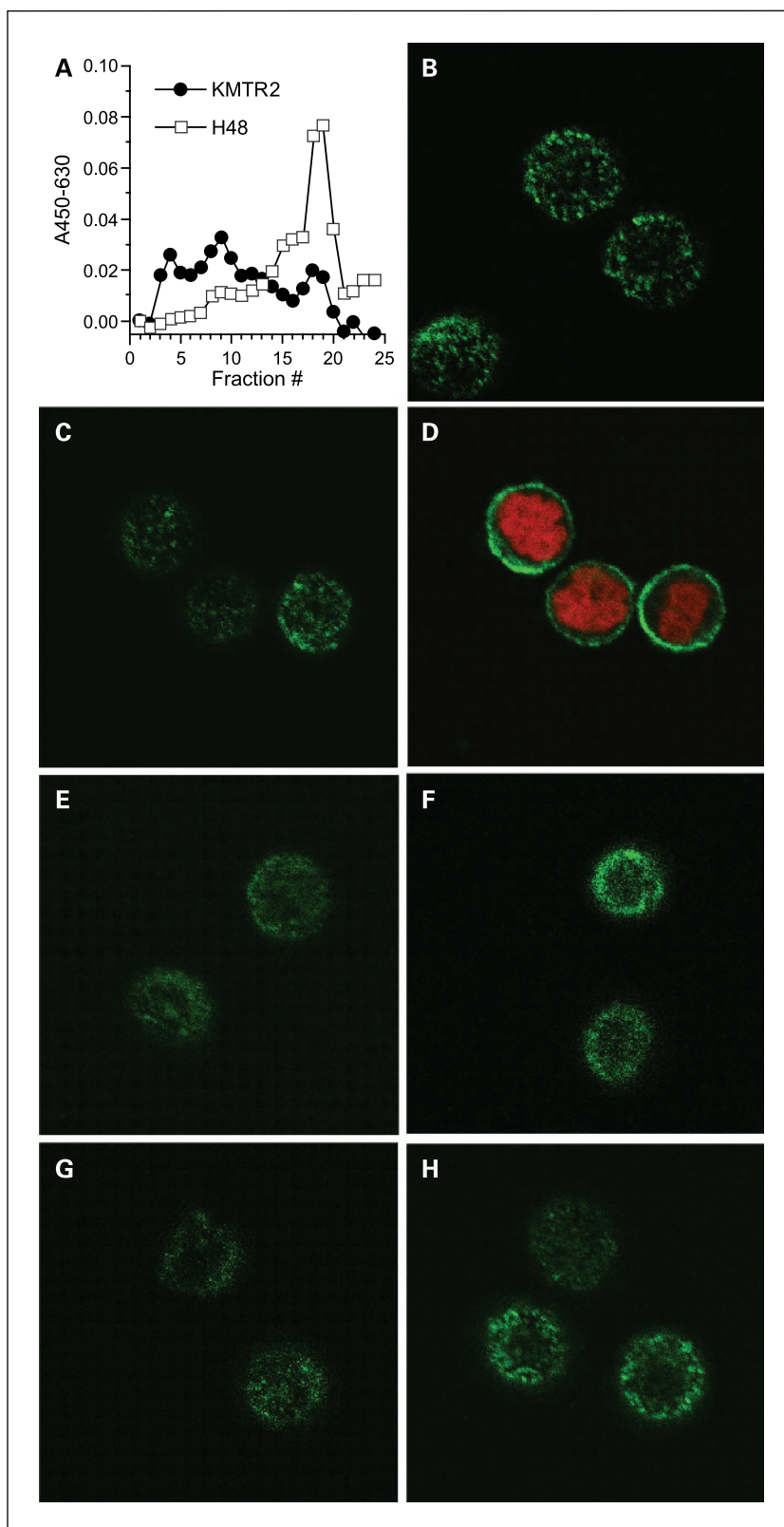


Fig. 5. Analysis of TRAIL-R2 immune complexes formed on cell membranes. *A*, Colo205 cells (1×10^5 per mL) were preincubated with 100 ng/mL of either KMTR2/IgG1 or H48/IgG1 for 30 minutes at 37°C and subsequently treated with 2 mmol/L chemical cross-linker [3,3'-dithiobis(sulfosuccinimidylpropionate)] on ice for 2 hours. Soluble cell lysate was prepared by the treatment of cells in PBS containing 0.1% Triton X-100. Cell lysate solution (100 μ L) was loaded onto gel filtration column (Superose 6) and eluted with PBS containing 0.1% CHAPS at a flow rate of 0.5 mL/min. Human IgG was detected in each fraction by ELISA. *B-H*, cell surface TRAIL-R2 immune complexes on Colo205 cells visualized by confocal microscopy. Cells were incubated with monomeric KMTR2/IgG1 at 37°C for 1 hour and then labeled with Alexa488 and examined by 2microscopy (*B-D*). Nuclei were costained with propidium iodide (*D*). Membrane protein diffusion was restricted by incubation of cells at 4°C with KMTR2 (*E*) or treatment with 4% paraformaldehyde at 4°C before addition of KMTR2 followed by incubation at 37°C for 1 hour (*F*). Colo205 cells were incubated with H48/IgG1 at 37°C for 1 hour alone (*G*) or together with mouse anti-human IgG (*H*). Magnification, $\times 40$.

regression *in vivo* than an anti-TRAIL-R2 antibody that acts indirectly and requires secondary cross-linking reagents. Some reports describe mAbs to TRAIL-R2 exerting similar agonistic activities without cross-linking (21, 22). However, there is the

possibility that this activity was not exclusively due to the monomeric form of the antibody (1), and as shown here, the presence of aggregated IgG in unfractionated preparations can account for apparent "direct" agonist function of some

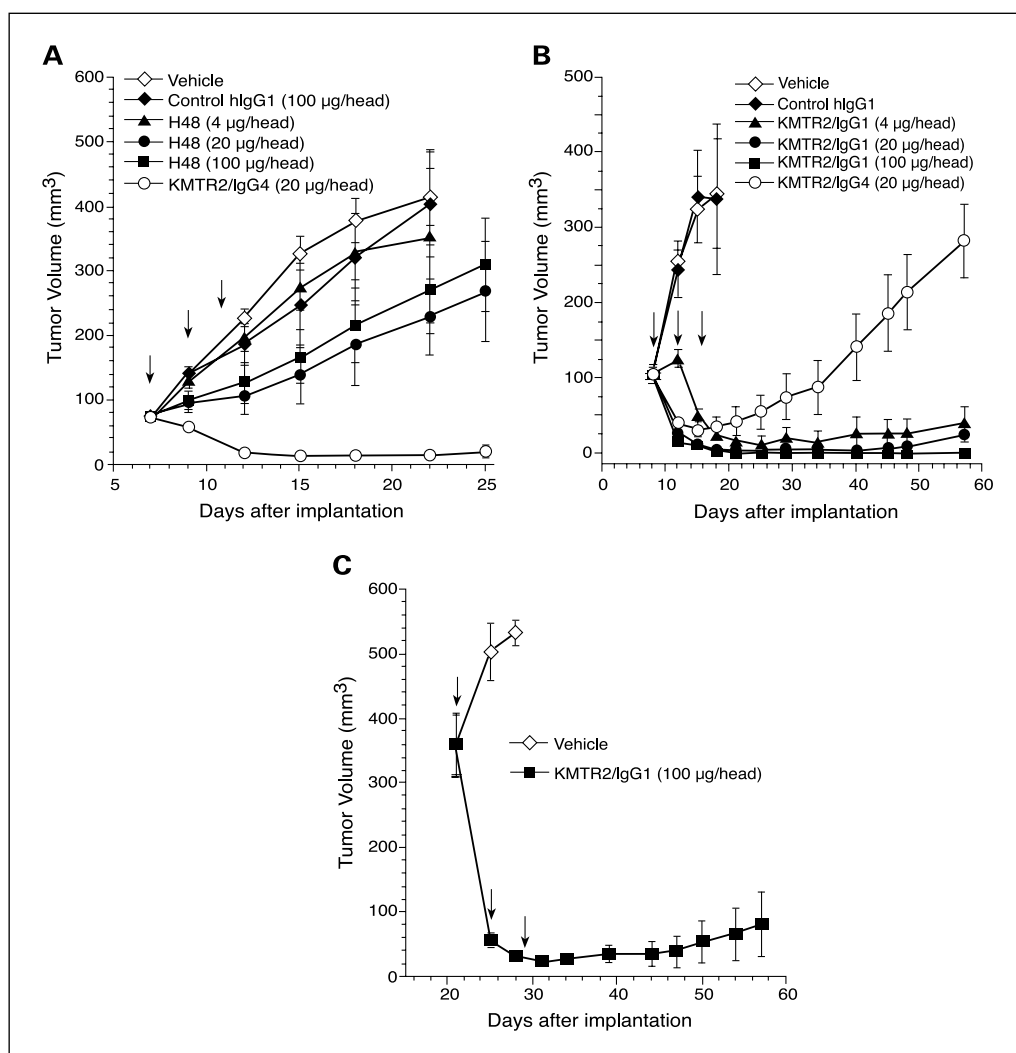


Fig. 6. Antitumor effects of anti-TRAIL-R2 antibodies on human colon tumor xenografts. Colo205 human cells, 5×10^6 (A) and 4×10^6 (B and C), were subcutaneously inoculated at the right flank of female nude mice. After 7 (A), 8 (B), or 22 (C) days, mice ($n = 5$) were assigned to the indicated groups controlling for average tumor volume. Mice were i.p. injected thrice (arrows, the treatment days) with vehicle (PBS containing 1% nude mouse serum) or the indicated dose of H48/IgG1, KMTR2/IgG1, KMTR2/IgG4, or control antibody [IgG1 isotype of anti-HSA mAb (A) or whole human IgG (B)]. Length, width, and height of each tumor mass were measured by calipers, and tumor volume was calculated as: tumor volume (mm^3) = (length \times width \times height) / 2. Points, average tumor volume; bars, SE.

mAb. A similar result was also reported for an anti-CD40 mAb (31), indicating that the monomer fraction is essential for the confirmation of direct agonist activity.

Alternative methods to generate direct agonist/oligomeric TNF receptor or other cell surface molecules have been described (32, 33). Chemically coupled dimeric forms of IgG to CD19, CD20, CD21, CD22, and Her-2 exhibited more profound activities in comparison with the monomeric form of IgG (32). A series of multivalent human IgG antibody, including tetravalent IgG, tetravalent $F(ab')_2$, and linear Fab multimer to DR5 (TRAIL-R2) induced apoptosis without cross-linking (33). These chemically or genetically modified antibodies were efficacious but may be impractical for development as therapeutic agents. The multivalent antibodies showed a short half-life (33), suggesting that *in vivo* efficacy could be lower than expected. In addition, artificially constructed mAbs, even if humanized, would be highly immunogenic in humans or proinflammatory.

The direct agonist KMTR2 was able to oligomerize the soluble dimeric form of TRAIL-R2 and to form large clusters with membrane-anchored TRAIL-R2. However, KMTR2 failed to oligomerize the soluble monomeric form of TRAIL-R2, and both cold treatment and prefixation with paraformal-

dehyde abolished large cluster formation on cell surface. It has not been precisely elucidated why the direct agonist provokes supraoligomerization of TRAIL-R2, yet two scenarios could be envisioned in which the direct bivalent mAb mediates supraoligomerization of TRAIL-R2. Cell surface receptors from the TNF receptor superfamily, including TRAIL-R1 and TNF receptor (34), are present in preassembled complexes formed independently of ligand (nonsignaling). This provides the possibility that the TRAIL-R2 exists as a multimeric complex (minimally bivalent) and can be efficiently cross-linked by a bivalent mAb. Alternatively, the cytoplasmic domains could promote aggregation between similar receptors or bivalent complexes could be recruited into specialized membrane domains, such as lipid rafts where Fas (35), CD20 (36), and TNF (37) are known to localize.

The greater efficacy of KMTR2 may be due to its ability to directly activate apoptosis independent of whether host effector functions are operative. This direct activity could be advantageous as an anticancer agent. Chemotherapy or radiotherapy frequently impairs the effector functions of FcR-expressing cells (24) and polymorphisms of $Fc\gamma RIIIa$ can also influence clinical outcome (25, 26). In addition, circulating endogenous immunoglobulin might interfere with the necessary interaction

between antibodies that require Fc function and FcR, although the antibody engaged with the corresponding receptor has higher affinity to FcR than free antibody. Indeed, in experiments presented here, human IgG1 blocked the apoptosis-inducing activities of an indirect agonist antibody cross-linked by secondary antibody or effector PBMCs. Furthermore, we found that the direct agonist KMTR2 exerted a marked antitumor effect

in xenograft models with many human tumor lines.⁴ The data indicate that a direct agonist antibody can provide a highly efficacious antitumor activity in these experimental models and therefore emerges as a lead candidate for cancer therapeutics.

⁴ In preparation.

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