A Novel Anti-human DR5 Monoclonal Antibody with Tumoricidal Activity Induces Caspase-dependent and Caspase-independent Cell Death*

Received for publication, April 4, 2005, and in revised form, September 6, 2005 Published, JBC Papers in Press, October 18, 2005, DOI 10.1074/jbc.M503621200

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Like anti-Fas monoclonal antibodies, some monoclonal antibodies against tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors have tumoricidal activity too. In this article we report a novel mouse anti-human DR5 monoclonal antibody, AD5–10, that induces apoptosis of various tumor cell lines in the absence of second cross-linking in vitro and showed strong tumoricidal activity in vivo. AD5–10 does not compete with TRAIL for binding to DR5 and synergizes with TRAIL to induce apoptosis of tumor cells. AD5–10 induces both caspase-dependent and caspase-independent cell death in Jurkat cells, whereas TRAIL induces only caspase-dependent cell death. We show for the first time that DR5 can mediate caspase-independent cell death, and DR5 can mediate distinct cell signals when interacting with different extracellular proteins. Studies on AD5–10 help us to understand more on the functions of DR5 and may provide new ideas for cancer immunotherapy.

Tumor necrosis factor (TNF)²-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily with the ability to induce apoptosis in a wide variety of transformed cell lines of diverse origin (1). At least five receptors for TRAIL have been identified so far. Two of them, DR4 (TRAIL-R1) and DR5 (TRAIL-R2/TRICK2) (2–4), are capable of transducing an apoptosis signal, whereas the other three, decoy receptor (DcR) 1 (TRAIL-R3) (5), DcR2 (TRAIL-R4) (6), and osteoprotegerin (7), serve as decoy receptors to block TRAIL-mediated apoptosis. DR4 and DR5 share a common intracellular domain, called the death domain (DD), which is indispensable for initiation of the intracellular signaling cascade leading to cell death. The death domain motif is also found in the cytoplasmic adaptor proteins, such as Fas-associated protein with death domain (FADD) (8), TNF-R1-associated death domain protein (TRADD) (9), and receptor-interacting protein (RIP) (10), etc. These adaptor proteins are essential for the intracellular signals mediated by DR4 and DR5 (11). TRAIL triggers multiple cell signals, including the activation of apoptotic caspase cascade, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and NF-κB (11–13).

In contrast to TNFs and Fas ligand, TRAIL has been known to induce apoptosis in a variety of tumor cells and some virally infected cells but not in most normal cells. The potential and safety of soluble TRAIL (sTRAIL) as an anticancer therapeutic agent have been demonstrated in mice and non-human primates (14, 15). However, increasing experimental evidence on TRAIL-inducing apoptosis of normal cells (especially hepatocytes) were reported in recent studies (16, 17), arguing against the potential usefulness and safety of soluble TRAIL in cancer therapy. Meanwhile, there were also reports demonstrating that recombinant TRAIL without exogenous sequence tags was nontoxic to human hepatocytes both in vitro (18, 19) and in chimeric mice (20). In addition to sTRAIL, monoclonal antibodies (mAbs) against TRAIL receptors with tumoricidal activity are also potential candidates for cancer therapy. There are a number of agonistic mAbs against human DR4 or DR5 reported in previous studies (21–23), most of which need cross-linkers to ensure effective killing of tumor cells (21, 22). In 2001, Ichikawa et al. (23) reported a mouse anti-DR5 mAb, TRA-8, that showed strong tumoricidal activity in the absence of cross-linking and had no hepatocyte toxicity. TRA-8 competes with TRAIL for binding to DR5 and almost entirely mimics the apoptosis-inducing mechanism of TRAIL. The authors believed that DR5 was not sufficient to trigger apoptosis in normal hepatocytes (23). However, a recent study showed that at least some anti-DR5 and anti-DR4 mAbs did induce human hepatocytes apoptosis (24). Therefore, we cannot draw a definite conclusion on the hepatocyte toxicity of soluble TRAIL or mAbs against TRAIL receptors now. Studies on the mechanism of anti-DR5 mAbs will help us to understand the complicated signal pathways mediated by DR5.

In this article, we report studies on AD5–10, a novel monoclonal antibody against human DR5. AD5–10 induces apoptosis in various tumor cell lines in the absence of second cross-linking in vitro and exhibits a strong tumoricidal activity in vivo. AD5–10 does not induce cell death of human normal hepatocytes and primary peripheral blood lymphocytes, and the injection with high doses of AD5–10 in mice causes no toxic reaction in liver, spleen, and kidney. Unlike TRA-8, AD5–10 does not compete with TRAIL for binding to DR5, and there is a synergistic effect between TRAIL and AD5–10 on their tumoricidal activity. Downstream cell signals induced by AD5–10 and TRAIL were also compared. Both TRAIL and AD5–10 activate caspase cascade and induce a classical apoptosis in Jurkat cells. Interestingly, AD5–10 kills...
Jurkat cells in the presence of Z-VAD, whereas TRAIL does not, indicating a caspase-independent cell death promoted by AD5–10. Furthermore, we show that RIP is essential for this caspase-independent cell death. Z-VAD inhibits the activation of JNK/p38 by TRAIL but not AD5–10. Both TRAIL and AD5–10 are capable of activating of NF-κB, but there are differences between the regulation of NF-κB activity by TRAIL and that by AD5–10 in certain cell lines. These findings show that DR5 can mediate distinct cell signals when interacting with different extracellular proteins. The differences in the cell signals induced by AD5–10 and TRAIL are undoubtedly significant for further studies. Illustration of the mechanisms may lead to the development of new strategies for cancer immunotherapy.

MATERIALS AND METHODS

Reagents—Z-VAD-fmk (a pan-caspase inhibitor) was purchased from R&D Systems, Inc. (Minneapolis, MN). Phorbol 12-myristate 13-acetate (PMA) was purchased from Promega Co. (Madison, WI). pGL2 plasmid, the luciferase reporter vector, was from Promega. Caspase-8 dominant-negative plasmid was kindly provided by Dr. Shimin Hu (University of Pennsylvania). Recombinant soluble TRAIL (rsTRAIL, amino acids 95–281, nontagged) was prepared as previously described by Shi et al. (19). His-DR4 (histidine-tagged extracellular domain of human DR4; amino acids 110–240), His-DR5 (histidine-tagged extracellular domain of human DR5α; amino acids 52–183), and His-DR5-∆L2 (histidine-tagged extracellular domain of human DR5α; amino acids 52–183 with a deletion of amino acids 94–113) were expressed in Escherichia coli and purified using nickel nitrotriacetic acid His-Bind resin (Novagen, Inc., Milwaukee, WI). Anti-caspase-8 and anti-caspase-9 antibodies were purchased from Calbiochem. Anti-TRAIL, anti-caspase-3, anti-poly(ADP-ribose) polymerase (PARP), anti-actin, anti-phospho-JNK, anti-IκB, and anti-IκB antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-p38 antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Horseradish peroxidase-linked anti-mouse IgG, anti-rabbit IgG and anti-goat IgG were purchased from ZhongShan Co. (Beijing, China).

ELISA—ELISA plates (Costar) were coated with 5 µg/ml His-DR4, His-DR5, or His-DR5-∆L2 and blocked with 5% nonfat dry milk. Plates were then incubated with 100 µl/well of mAbs (or hybridoma culture supernatants) at the required dilutions. The bound mAbs were detected with 100 µl/well of horseradish peroxidase-anti-mouse IgG. For the binding of rsTRAIL, plates were incubated with 100 µl/well of rsTRAIL. After washing, 100 µl/well of rabbit anti-rat TRAIL antibody was added to wells. The specific binding was then detected with horseradish peroxidase-anti-rabbit IgG. The absorbency at 492 nm was measured on a microtiter reader (Thermo Labsystems). The results were analyzed using GraphPad Prism (GraphPad software, San Diego, CA).

Cell Culture and Cell Viability Assay—SMMC-7721 cells and U251 cells were purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Other tumor cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). HL-60-R cells were derived from HL-60 cells (ATCC). RIP-deficient Jurkat cells were kindly provided by Dr. Brian Seed (Department of Molecular Biology, Massachusetts General Hospital, Boston, MA). Normal human hepatocytes were provided by the Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China). Normal human primary peripheral blood lymphocytes cells were isolated from peripheral blood of healthy volunteers. Tumor cell lines and primary cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere of 5% CO₂. Hybridomas were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum and antibiotics as described above. Cell viability was determined using CellTiter 96 AQueous nonradioactive cell proliferation assay (MTS) according to the manufacturer’s instructions (Promega). Z-VAD-fmk or PMA was added 30 min before the addition of stimulants.

Generation of AD5–10—4-Week-old female BALB/c mice were immunized 4 times with 50 µg of DR5 extracellular domain protein at 2-week intervals. Three days after the final boost, lymphocytes from spleen were fused with SP2/0 myeloma cells (ATCC), and positive hybridomas were screened against DR5 and His-DR5 recombinant protein using ELISA. The selected mAbs were further tested for their tumoricidal activity.

Annexin-V and Propidium Iodide Staining—Cells after the indicated treatment were stained using the annexin-V kit according to the manufacturer’s instructions (BioSea Biotech Co., Beijing, China). Briefly, cells were washed once with phosphate-buffered saline and stained in 200 µl of binding buffer containing annexin-V-fluorescein isothiocyanate for 30 min on ice. Propidium iodide was then added into the buffer. 5 min later 300 µl of binding buffer was added. The samples were analyzed with a flow cytometer (FACScan, BD Biosciences). 10,000 cells were counted per sample.

Western Blot Analysis—After the required treatments, cells (2–3 ¥ 10⁶) were washed once with phosphate-buffered saline and lysed in the sample buffer (80–120 µl) for SDS-PAGE and immediately boiled for 5 min. Each sample was subjected to 8, 10, or 12% SDS-PAGE, and the proteins separated in the gel were subsequently electrotransferred onto a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was blocked with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl (pH 7.4), 8 g/liter NaCl, and 0.1% Tween 20) for 1–2 h at room temperature. The membrane was then incubated with the indicated primary antibodies in TBS-T containing 5% nonfat dry milk at 4 °C overnight. The membrane was washed 3 times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1.5–2 h. After washing four times with TBS-T, the protein was visualized using the ECL Plus Western blotting detection system according to the manufacturer’s instructions (Amersham Biosciences).

Analysis of Tumoricidal Activity in Vivo—6–8-Week-old female BALB/c nude mice were inoculated subcutaneously with SMMC-7721 cells (2 ¥ 10⁶). Three weeks later, after the indicated treatment, the mice were sacrificed, and the growth of tumor cells was determined by the weight of the tumor mass. 6–8-Week-old female BALB/c nude mice were inoculated subcutaneously with A549 cells (2 ¥ 10⁶). One month later, after the indicated treatment, the mice were sacrificed, and the growth of tumor cells was determined by the weight of the tumor mass.

Construction of Casp-8–DN Jurkat Cells—Jurkat cells were transfected with Casp-8–DN plasmid using the cell line Nucleofector kit V (Amaxa Biosystem) according to the manufacturer’s instructions and screened with G418 (Alexis Biochemicals, San Diego, CA).

Electron Microscopy—After the indicated treatment cells were fixed by dropwise addition of glutaraldehyde and analyzed according to standard procedures.

DNA Fragmentation Assay—After the required treatments, 2 × 10⁶ cells were collected and washed once with phosphate-buffered saline and lysed in 100 µl of buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100. Lysates were centrifuged at 14,000 ¥ g for 5 min at 4 °C, and supernatants were then subjected to digestion.
with ribonuclease A (0.2 mg/ml) for 1 h at 37 °C followed by incubation with proteinase K (0.2 mg/ml) for 1 h at 37 °C. DNA in the sample was precipitated by centrifugation at 14,000 × g for 15 min at 4 °C after treatment with 50% isopropyl alcohol and 0.5 M NaCl overnight at −20 °C. DNA was resuspended in 30 µl of Tris-EDTA buffer and analyzed by electrophoresis on a 2% agarose gel in the presence of 0.2 µg/ml ethidium bromide.

**NF-κB Activation Assay**—The NF-κB reporter plasmid was constructed as previously described (25). The two primers, p1 (5′-GC-GAGCTCGGGACTTCCGAGACTTTCCGGGACT-3′, containing SacI cut site (underlined)) and p2, 5′-CCGCTCGAGGAAAAGTC-CCGGAAGTCCCGGAAAG-3′, containing XhoI cut site (underlined)) were synthesized by BioAsia Co. (Shanghai, China). The overlapped sequences in the primers are indicated in italics. The primers were annealed to each other and amplified using PCR. The product corresponding to the three repeats of NF-κB DNA binding sequence was then cut with SacI and XhoI restriction endonucleases and inserted into the polyclonal sites of pGL2 plasmid (Promega). The recombinant plasmid was designated as pGL2-NF-κB. Cells were transfected with pGL2-NF-κB using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Equal count of transfected cells were treated, and luciferase activity in the cells was then determined using luciferase reporter assay system (Promega).

**Statistical Analysis**—Results were expressed as mean values ± S.D., and a Student’s t test was used for evaluating statistical significance. p values were considered to be statistically significant when less than 0.05.

**RESULTS**

**AD5–10 Binds to DR5 Specifically and Does Not Compete with TRAIL**—Human DR5 has two isoforms, designated as DR5a and DR5b, which is a result of pre-mRNA alternative splicing. DR5b has an insertion of 29 amino acids in the extracellular domain compared with DR5a (26). AD5–10 (IgG3-κ) was generated by immunizing BALB/c mice with a recombinant protein containing simply the
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FIGURE 2. AD5–10 and rsTRAIL induced tumor cells but not normal cell apoptosis in vitro. A and B, annexin-V and propidium iodide (PI) staining flow cytometry. Jurkat cells were treated with 100 ng/ml rsTRAIL or AD5–10 for 2 h (A) or 1 or 2 h (B). FITC, fluorescein isothiocyanate. C, Jurkat cells were treated with the indicated concentrations of rsTRAIL or AD5–10 for 8 h. Cell viability was determined by an MTS assay. D and E, tumor cell lines were treated with the indicated concentrations of rsTRAIL (D) or AD5–10 (E) for 24 h. F and G, human normal hepatocytes were treated with the indicated concentrations of rsTRAIL (F) or AD5–10 (G) in the presence or absence of 1 μg/ml CHX for 24 h. Cell viability was determined by an MTS assay. Results shown are the average of triplicate measurements and a representative of at least three independent experiments.
extracellular domain of DR5a (amino acids 52–183), which was expressed in E. coli and without any exogenous sequence tags. In previous studies on anti-TRAIL receptor mAbs, DR4-Fc and DR5-Fc fusion proteins were usually used as antigens for immunizing animals (21–23). We showed that the bacterially expressed DR4 and DR5 extracellular regions bound to their natural ligand, TRAIL, specifically (Fig. 1A), indicating that they are suitable for immunizing animals. There are two different TRAIL-binding sites on both DR4 and DR5 molecules (5, 6). In contrast, AD5–10 bound to DR5 via one binding site (K_d = 0.30 nM) and did not react with DR4 and Fas (Fig. 1B). Notably, competitive ELISA showed that there was no competition between AD5–10 and TRAIL for binding to DR5 (Fig. 1, C and D). There are 7 disulfide bonds on the extracellular domain of DR5. The loop (amino acids 94–113) formed by the second disulfide bond (from the N terminus) has been demonstrated essential for DR5 to interact with TRAIL (27). We expressed amino acids 94–113-deleted DR5 extracellular domain, designated as DR5-L2, in E. coli, for the binding assay. The result showed that TRAIL did not bind to DR5-L2, whereas AD5–10 bound to DR5-L2 specifically (Fig. 1E), indicating that AD5–10 and TRAIL bound to different sites on DR5, raising the possibility that AD5–10 and TRAIL may trigger different downstream cell signals.

**AD5–10 Induces Apoptosis in Multiple Tumor Cell Lines in Vitro and Is Nontoxic to Human Normal Cells**—Whether AD5–10 can kill tumor cells like TRAIL is what we are most interested in. Our results showed that AD5–10 and rsTRAIL strongly induced cell death in Jurkat cells. When treated with 100 ng/ml AD5–10, about 40% of Jurkat cells presented annexin-V positive after 1 h, and about 80% cells presented annexin-V positive after 2 h (Fig. 2A and B). Cell viability was also tested using MTS kit (Promega). The IC_{50} (inhibitory concentration 50%) of Jurkat cells treated with AD5–10 for 8 h was less than 10 ng/ml (Fig. 2C). Furthermore, various tumor cell lines, SMMC-7721 (human hepatocellular carcinoma cells), HeLa cells, MDA-MB-231 (human breast adenocarcinoma cells), U251 (human glioma cells), and HCT-116 (colorectal carcinoma cells) were killed by AD5–10 as well as
rsTRAIL in a concentration-dependent manner (Fig. 2, D and E). We noticed that Jurkat cells are more susceptible to AD5–10 than to rsTRAIL (Fig. 2, A–C), whereas apoptosis in HeLa cells were more easily induced by rsTRAIL (Fig. 2, D and E), which suggested that different mechanisms may be utilized by the two proteins. Nevertheless, these data demonstrated that both AD5–10 and rsTRAIL could efficiently induce cell death in various tumor cell lines in the absence of second cross-linking in vitro.

Some versions of soluble TRAIL and some mAbs against TRAIL receptors were reported toxic to human normal hepatocytes, whereas others were demonstrated nontoxic. To test if AD5–10 has normal cell toxicity, we treated human hepatocytes with various concentrations of rsTRAIL or AD5–10 for 24 h. No decrease of cell viability was detected, as determined by MTS assay (Fig. 2, F and G). We also tested if cycloheximide (CHX), a potent apoptosis enhancer, could sensitize hepatocytes to TRAIL or AD5–10-induced cell death. A 24-h co-incubation with CHX and 10 ng/ml rsTRAIL caused a 40% decrease of hepatocyte viability. In contrast, no decrease of cell viability was detected in cultures exposed to both CHX and various concentrations of AD5–10 (Fig. 2, F and G). Our results showed that both rsTRAIL and AD5–10 were nontoxic to human hepatocytes and suggested that AD5–10 was relatively safer to human hepatocytes than TRAIL. In addition, rsTRAIL, AD5–10, or the combination of the two agents was also nontoxic to human primary peripheral blood lymphocytes (data not shown).

**Tumoricidal Activity of AD5–10 in Vivo**—The tumoricidal activity of AD5–10 in vivo was tested using two tumor cell lines. BALB/c nude mice were inoculated subcutaneously with SMMC-7721, a human hepatocellular carcinoma cell line, or A549, a human lung carcinoma cell line. Both rsTRAIL and AD5–10 inhibited the SMMC-7721, forming solid tumors in early treatment or reduced tumor weight in late treatment significantly. The tumor weights were reduced 4–5-fold as compared with control treatments (Fig. 3 A and B). Tumor formation was completely invisible in 4 animals with AD5–10 early treatment (n = 10), and the tumors of 5 animals with AD5–10 late treatment completely disappeared (n = 10). An example from AD5–10-treated mice was shown along with one from control mice in Fig. 3 C. Similarly, AD5–10 inhibited the growth of A549 cells in BALB/c nude mice (Fig. 3 D). These results indicate that AD5–10 is a potent inhibitor of in vivo tumor cell growth.
growth. Injection with extremely high doses of rsTRAIL and AD5–10 (10-fold of the dose used in anti-tumor treatment experiments) to BALB/c mice caused no toxic reaction in liver, spleen, and kidney (data not shown).

AD5–10 and TRAIL Synergize to Induce Apoptosis of Tumor Cells—Because AD5–10 does not compete with TRAIL for binding to DR5, it is possible that AD5–10 synergizes with TRAIL to kill tumor cells. To test this hypothesis, Jurkat cells were co-incubated with AD5–10 and rsTRAIL. As expected, the killing activity of AD5–10 was significantly enhanced in the presence of 1 ng/ml rsTRAIL as determined by cell viability using MTS assay (Fig. 4A). The reciprocal experiment, where the cells were treated with fixed concentrations of AD5–10 and increasing amounts of rsTRAIL, produced a similar result (Fig. 4B). HL-60 cells can differentiate spontaneously into granulocytes or monocytes, which are termed HL-60-R for its resistance to some apoptotic stimuli including anticancer drugs or irradiation, although undifferentiated cells significantly respond to these stimuli (28). Our results showed that neither rsTRAIL nor AD5–10 alone was able to induce apoptosis in HL-60-R cells even at a concentration as high as 1000 ng/ml. However, co-incubation of the cells with both agents at a 10× lower concentration resulted in about a 50% decrease in cell viability (Fig. 4C). In Western blot analysis, PARP cleavage was only detectable in the cells exposed to both AD5–10 and rsTRAIL (Fig. 4D). These results support the
concept that AD5–10 and TRAIL synergize to induce apoptosis of tumor cells.

**AD5–10 Activates the Caspase Cascade**—It is known that TRAIL activates caspases in various cell lines. Naturally, we wondered if AD5–10 induces the same reactions. Western blot analysis showed that AD5–10 activated the caspase cascade in Jurkat cells like TRAIL (Fig. 5). Caspase-8, -9, and -3 were cleaved in a time-dependent manner within 1 h. The cleavage of PARP occurred with the cleavage of caspases. These results showed that AD5–10 could induce a classic apoptosis.

**AD5–10 Induces Caspase-independent Cell Death**—Because both TRAIL and AD5–10 activated the caspase cascade, we used a pan-caspase inhibitor, Z-VAD, to further investigate the signal pathways other than the caspase pathway. Fig. 6A showed that the cell death of Jurkat cells induced by TRAIL was completely inhibited by 5 μM
Z-VAD. Interestingly, Z-VAD failed to protect Jurkat cells from AD5–10-induced cell death (Fig. 6B), although the killing process was delayed (Fig. 6C). Moreover, AD5–10 killed Jurkat cells at all concentrations of Z-VAD, whereas this inhibitor blocked TRAIL-induced cell death at a lower concentration of 5 μM (Fig. 6D). In addition, Jurkat cells, which stably express a dominant-negative form of caspase-8 (Casp-8-DN) were resistant to TRAIL as previously reported (31, 32) but were susceptible to AD5–10 (Fig. 6E).

Annexin-V staining flow cytometry indicated that Jurkat cells incubated with AD5–10 in the presence of Z-VAD underwent a programmed cell death (Fig. 7A). However, the morphology of cells under this treatment was significantly different from that of the cells treated with TRAIL or AD5–10 alone. When analyzed by electron microscopy, Jurkat cells treated with TRAIL or AD5–10 displayed typical apoptotic changes including chromatin condensation. In contrast, cells treated with AD5–10 in the presence of Z-VAD showed no chromatin condensation (Fig. 7B). The DNA fragmentation assay showed that cells treated with TRAIL or AD5–10 alone formed a DNA ladder, which is a hallmark of apoptosis. But no DNA ladder was observed in AD5–10 and Z-VAD co-treated Jurkat cells (Fig. 7C). There was no cleavage of caspase-8 and PARP in cells treated with TRAIL or AD5–10 in the presence of various concentrations of Z-VAD, indicating that the caspase pathway was efficiently blocked by the inhibitor (Fig. 7D). These data showed that AD5–10 induced a caspase-dependent cell death pathway that was similar to the pathway induced by TRAIL; in addition, it could also induce a caspase-independent cell death pathway that proceeded more slowly, yet was efficient.

RIP Is Essential for the AD5–10-induced Caspase-independent Cell Death—Holler et al. (29) demonstrated in 2000 that the death domain protein RIP was required in Fas-mediated caspase-independent cell death in Jurkat cells. RIP is also a component of death-inducing signal complex triggered by TRAIL and is essential for TRAIL-induced activation of JNK and NF-κB (10). In our study we detected whether RIP contributes to AD5–10-induced caspase-independent cell death using RIP-deficient Jurkat cells described previously by Ting et al. (30). The results showed that both TRAIL and AD5–10 killed RIP-deficient Jurkat cells in a 24-h incubation, although the cells were not as susceptible as the wild type Jurkat cells. However, AD5–10 could not induce RIP-deficient Jurkat cells death in the presence of Z-VAD, indicating that RIP is essential for the AD5–10-induced caspase-independent cell death in Jurkat cells (Fig. 8, A and B).

AD5–10 Activates JNK/p38 via the Caspase-independent Pathway—Activation of JNK and p38 MAPK is an important event mediated by DR4 and DR5 (10, 12). JNK/p38 MAPK activation induced by rsTRAIL and AD5–10 was compared in our study. Western blot analysis showed that both rsTRAIL and AD5–10 activated JNK/p38 (Fig. 9A). AD5–10-induced JNK/p38 activation was also tested at various time points. The results showed that AD5–10 could activate JNK/p38 in the presence of Z-VAD as rapidly as in the absence of the inhibitor (Fig. 9B). These data demonstrated that the activation of JNK/p38 induced by TRAIL is caspase-dependent in Jurkat cells, whereas AD5–10 could activate JNK/p38 in a caspase-independent pathway.

FIGURE 8. RIP is essential to AD5–10-induced caspase-independent cell death. RIP-deficient (def.) Jurkat cells were incubated with indicated concentrations of rsTRAIL (A) and AD5–10 (B) in the absence or in the presence of various concentrations of Z-VAD-fmk for 24 h. Cell viability was determined by MTS assay.

FIGURE 9. Activation of JNK/p38 MAPK. A Western blot is shown. A, Jurkat cells were incubated with 200 ng/ml rsTRAIL or 200 ng/ml AD5–10 in the absence of Z-VAD for 1 h or in the presence of 5 μM Z-VAD-fmk for 4 h. JNK and p38 MAPK activation and PARP cleavage were tested using respective antibodies. B, Jurkat cells were incubated with 200 ng/ml AD5–10 in the absence or presence of 5 μM Z-VAD-fmk for indicated times. P-, phosphorylated.
FIGURE 10. Regulation of NF-κB activity by rsTRAIL and ADS-10. A, HL-60-R cells were transfected with pGL2-NF-κB reporter plasmid, then incubated with 200 ng/ml rsTRAIL or 200 ng/ml ADS-10 for the indicated times. Cells were lysed, and the luciferase activity of the lysate was analyzed. B, HL-60-R cells were incubated with 200 ng/ml rsTRAIL or 200 ng/ml ADS-10 for indicated times. The phosphorylation (P-) of IκB was tested using respective antibodies. C, Jurkat cells were transfected with pGL2-NF-κB reporter plasmid and then incubated with 10 ng/ml rsTRAIL or 10 ng/ml ADS-10 for the indicated times. Cells were lysed, and the luciferase activity of the lysate was analyzed. D, Jurkat cells were incubated with 10 ng/ml rsTRAIL or 10 ng/ml ADS-10 for the indicated times. The phosphorylation of IκB was tested using respective antibodies. E and F, Jurkat cells were incubated with indicated concentrations of rsTRAIL or ADS-10 in the absence or presence of 100 nM PMA for 8 h. Cell viability was determined by MTS assay. G, Jurkat cells were transfected with pGL2-NF-κB reporter plasmid, then incubated with 10 ng/ml rsTRAIL and 10 ng/ml ADS-10 in the absence or presence of 100 nM PMA or 5 μM Z-VAD-fmk for 8 h. The cells were lysed, and the luciferase activity of the lysate was analyzed. Cell viability was determined by MTS assay.
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Table One

| Differences in terms of biological and biochemical outcomes between TRAIL and AD5-10 |
|-----------------------------|--------------------------------------|-----------------------------|-----------------------------|
|                            | TRAIL (Z-VAD (-)) | TRAIL (Z-VAD (+)) | AD5-10 (Z-VAD (-)) | AD5-10 (Z-VAD (+)) |
| PARP cleavage               | +                     | -                     | +                     | -                     |
| Activation of JNK/p38       | +                     | -                     | +                     | +                     |
| Activity of NF-κB           | †                     | †                     | †                     | †                     |
| Phosphatidylyserine externalization | +                  | -                     | +                     | +                     |
| Chromatin condensation      | +                     | +                     | +                     | +                     |
| DNA ladder                  | +                     | +                     | +                     | +                     |
| Cell death                  | +                     | +                     | +                     | +                     |

Regulation of NF-κB Activity by AD5–10—Nuclear transcription factor NF-κB plays important roles in cell proliferation, apoptosis, and inflammatory reaction. Previous studies demonstrated that overexpressing DR4 or DR5 in certain cell lines, e.g. the human embryo kidney 293 cells activated NF-κB (4, 6). To investigate the regulation of NF-κB activity by AD5–10, we transfected cell lines with luciferase reporter vector pGL2 (Promega) containing the NF-κB regulation sequence (pGL2-NF-κB), then treated the cells with rsTRAIL or AD5–10. The activity of NF-κB was determined by the activity of luciferase in cell lysates using specific substrate. Both rsTRAIL and AD5–10 up-regulated NF-κB activity in HL-60-R cells in a time-dependent manner, which was similar to the effect of DR5 overexpression in human embryonic kidney 293 cells as previously reported (Fig. 10A). Western blot analysis of IκB phosphorylation confirmed this result (Fig. 10B). However, it was far more complicated in sensitive cell lines. Both TRAIL and AD5-10 inhibited the activity of NF-κB in a time-dependent manner in Jurkat cells, as determined by luciferase activity and IκB phosphorylation (Fig. 10, C and D). Notably, rsTRAIL, but not AD5–10, failed to induce cell death of Jurkat cells pretreated with PMA, an activator of NF-κB and protein kinase C (Fig. 10, E and F). Moreover, rsTRAIL, but not AD5–10, significantly activated NF-κB in PMA or Z-VAD-pre-treated cells (Fig. 10G). The coincidence of the NF-κB activity and cell viability indicated the relationship of NF-κB and cell death (Fig. 10, E–G). These results suggest that TRAIL and AD5–10 may regulate NF-κB activity via different mechanisms, which is also cell type-specific.

DISCUSSION

A novel monoclonal antibody against human DR5, AD5–10, binds to DR5 specifically and does not cross-react with DR4 and Fas. AD5–10 induces apoptosis of various tumor cell lines in the absence of cross-linking. AD5–10 recognizes an epitope out of the TRAIL-binding sites on DR5 and does not compete with TRAIL for binding to DR5, which indicates that domains other than TRAIL-binding sites on DR5 molecule interacting with certain proteins may also trigger cell death signals.

Receptor oligomerization was considered necessary to death signals mediated by death receptors. The fact that some anti-death receptor mAbs required exogenous cross-linking to ensure their tumoricidal activity in previous studies seemed to favor this argument. However, AD5–10 as well as another previously reported mAb TRA-8 (23) was able to kill tumor cells without cross-linking, raising questions to this hypothesis.

Both rsTRAIL and AD5–10 were nontoxic to human normal hepatocytes, and AD5–10 seemed relatively safer than rsTRAIL (Fig. 2, F and G). Considering the in vivo cases, as a cytokine with five receptors, TRAIL plays very broad roles, many of which are not known yet. Therefore, monoclonal antibodies against DR4 or DR5 may be better candidates as therapeutic agents than recombinant TRAIL for their specificity and relatively simple mechanism.

To investigate the similarities and differences of the cell signals triggered by TRAIL and AD5–10, Jurkat cells, which have been reported to dominantly express DR5 and few DR4 (31, 32), were chosen to minimize the involvement of DR4. We observed that AD5–10, similar to rsTRAIL, activated the conventional caspase cascade in Jurkat cells and caused a classical caspase-dependent cell death. The activation of the caspase cascade is the most important part of the cell signals triggered by TRAIL and is the basic function of DR5. Interestingly, Z-VAD failed to inhibit cell death induced by AD5–10, indicating that AD5–10 activated a caspase-independent signal pathway in addition to the classical caspase-dependent pathway. The morphological characteristics (Fig. 7B), DNA fragmentation assay (Fig. 7C), and Western blot analysis of PARP cleavage (Fig. 7D) further demonstrated a typical caspase-independent cell death in Jurkat cells treated with AD5–10 in the presence of Z-VAD. Although TRAIL cannot trigger caspase-independent cell death, DR5 does have the capability in mediating caspase-independent cell death. Thus, the cell signals mediated by DR5 interacting with TRAIL or a certain anti-DR5 mAB are not the total functions of the receptor. The process of caspase-independent cell death is much slower than the process of caspase-dependent cell death (Fig. 6C); therefore, only when the caspase-cascade was inhibited can we see the caspase-independent cell death induced by AD5–10.

Caspase-dependent cell death is the classical apoptosis and is well comprehended, whereas the caspase-independent programmed cell death remains largely uncharacterized, which is sometimes termed apoptosis-like or necrosis-like programmed cell death according to the degree of chromatin condensation (33). Caspase-independent cell death can be triggered by many factors, e.g. chemotherapeutic agents, ultraviolet radiation, or ischemia. Cytokines of the TNF superfamily are also important inducements for caspase-independent cell death. Previous studies have demonstrated that TNF-α and Fas ligand (29, 34–36) can induce caspase-independent cell death, but there was no solid evidence on TRAIL-induced caspase-independent cell death. Holler et al. (29) reported that TRAIL could induce caspase-independent cell death in Jurkat cells, and a similar observation in normal epithelial cells was reported by Thorburn et al. (37). However, in both studies a combination of TRAIL and CHX was used to treat cells. In fact, it is well known that TRAIL/TRAIL receptor can induce caspase-independent cell death when cooperating with various chemotherapeutic agents including Bis VIII, adriamycin, cisplatin (38, 39), and 8-chloroadenosine (25). TRAIL-induced caspase-independent cell death has also been mentioned in some papers on the roles of TNF-α or Fas ligand, but the results were not sufficient for a definite conclusion (29, 40). In contrast, many studies demonstrated that caspases were essential for the cell death induced by TRAIL/TRAIL receptor in various cell lines (31, 32, 41). We show for the first time that an anti-DR5 mAb, AD5–10, can induce a caspase-independent cell death in Jurkat cells and demonstrate that DR5 is capable of mediating caspase-independent cell death in certain cell lines.
The further investigation showed that the AD5–10-induced caspase-independent cell death in Jurkat cells requires RIP, which is similar to the Fas-mediated caspase-independent cell death in the same cell lines. Thus, RIP may be a general adaptor protein for death receptor-mediated caspase-independent cell death. Interestingly, RIP is also a component of death-inducing signal complex triggered by TRAIL and is necessary to the activation of JNK and NF-κB (10), but there is no caspase-independent cell death induced by TRAIL. This question needs further investigation.

JNK/p38 can be activated via caspase-dependent or caspase-independent pathways. Muhlenbeck et al. (12) reported in 1998 that TRAIL activated JNK via a caspase-dependent pathway in HeLa cells and a caspase-independent pathway in Kym-1 cells. MacFarlane et al. (42) reported in 2000 that TRAIL activated JNK/p38 via a caspase-dependent pathway, whereas chemical stimuli activated JNK/p38 via a caspase-independent pathway in Jurkat cells. However, our results showed that DR5 could induce JNK/p38 activation via both caspase-dependent and caspase-independent pathway in the same cell line.

Because AD5–10 induces caspase-independent activation of JNK/p38 and a caspase-independent cell death in Jurkat cells (Figs. 6 and 9A) and the activation of JNK/p38 is much earlier than the occurrence of cell death morphology (Fig. 9B), the JNK/p38 pathway may contribute to the caspase-independent cell death induced by AD5–10. However, SP600125 (an inhibitor for JNK) and SB202190 (an inhibitor for p38 MAPK) could not inhibit the caspase-independent cell death efficiently (data not shown), indicating that the JNK/p38 pathway was not the major cause for the caspase-independent cell death.

Although activated by most members of the TNF superfamily, JNK/p38 pathway has been considered relatively unimportant in cell death mediated by death receptors when compared with their critical roles in the cell death mediated by DNA damage and the mitochondrial pathway (43–46). Our results indicate that the JNK/p38 pathway may play a more important role in death receptor pathway than thought previously. Perhaps further studies on the cell signals induced by AD5–10 can give more explanations. The activation of JNK/p38 during AD5–10-induced caspase-independent cell death is somehow similar to the case when tumor cells are treated with chemotherapeutic agents. However, the toxicity of AD5–10 on normal cells is much less than that of chemotherapeutic agents.

Many members of TNF superfamily can activate NF-κB. Activated NF-κB functions as an anti-apoptotic factor by up-regulating caspase inhibitory proteins c-FLIP (caspase-8 inhibitory protein) and IAPs (inhibitors of apoptosis). Although TRAIL can activate NF-κB, the inhibition of NF-κB activity is necessary to TRAIL-induced apoptosis (47, 48). Our results showed that both TRAIL and AD5–10 activated NF-κB in resistant cells yet inhibited the factor in sensitive cells. However, in the presence of Z-VAD or PMA, AD5–10 and TRAIL performed differently in regulating NF-κB activity. These differences may be caused by the caspase-independent pathway induced by AD5–10.

Irrespective of the fact that AD5–10 and TRAIL interact with the same membrane receptor, DR5, the two proteins synergize to induce apoptosis of tumor cells. It is possible that there is a complementation between the cell signals triggered by TRAIL and AD5–10. The synergistic action of TRAIL and AD5–10 also suggests different cell signal mechanisms utilized by TRAIL and AD5–10. Moreover, because AD5–10 does not compete with TRAIL for binding to DR5, a receptor molecule can bind a TRAIL molecule and an AD5–10 molecule at the same time, thus triggering a much stronger death signal than the signal triggered by TRAIL or AD5–10 alone.

The differences in terms of biological and biochemical outcomes between TRAIL and AD5–10 are summarized in TABLE ONE. From this table one can clearly see that TRAIL can mediate distinct cell signals when interacting with different extracellular proteins. We hypothesize that because TRAIL and AD5–10 different changes in the conformation of DR5 molecule, the intracellular domains with different conformations may interact with different cytoplasmic adaptor proteins and trigger different cell signals.

The fact that different cell signals induced by TRAIL and AD5–10 and the synergistic action of TRAIL and AD5–10 in killing tumor cells evidently demonstrate the complexity of the cell signals mediated by DR5. The structure and function of DR5 are much more complicated than what have been considered. Our studies put forward DR5 a more attractive research target, and further exploration of the mechanisms of AD5–10 will add new knowledge at a good pace for the research on programmed cell death and cancer immunotherapy. The humanized version of AD5–10 may be a potential therapeutic agent in expectation in the future.

Acknowledgments—We are grateful to Dr. Brian Seed for the gift of RIP-deficient Jurkat cells. We thank Dr. Shimin Hu for the gift of caspase-8 dominant-negative plasmid.

REFERENCES

1. Wiley, S. R., Schooley, K., Smolak, P. J., Jin, W. S., Huang, C. F., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., and Goodwin, R. G. (1995) Immunity 3, 673–682
2. Pan, G., O’Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, K., Ni, J., and Dixit, V. M. (1997) Science 276, 111–113
3. Walczak, H., Degli-Esposti, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Boiani, N., Timour M. S., Gerhart, M. J., Schooley, K. A., Smith, C. A., Goodwin, R. G., and Rauch, C. T. (1997) EMBO J. 16, 5386–5397
4. Chaudhary, P. M., Eby, M., Jasmin, A., Bookwalter, A., Murray, J., and Hood, L. (1997) Immunity 7, 821–830
5. Degli-Esposti, M. A., Smolak, P. J., Walczak, H., Waugh, J., Huang, C. F., DuRose, R. F., Goodwin, R. G., and Smith, C. A. (1997) J. Exp. Med. 186, 1165–1170
6. Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., and Goodwin, R. G. (1997) Immunity 7, 813–820
7. Emery, J. G., McDonnell, P., Burke, M. B., Deen, K. C., Lynn, S., Silverman, C., Dul, E., Appelbaum, R. E., Eichman, C., DiPersio, R., Doods, R. A., James, I. E., Rosenberg, M., Lee, J. C., and Young, P. R. (1998) J. Biol. Chem. 273, 14363–14367
8. Chinnaiyan, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
9. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 495–504
10. Lin, Y., Devin, A., Cook, A., Keane, M. M., Kellihier, M., Lipkowitz, S., and Liu, Z. G. (2000) Mol. Cell Biol. 20, 6638–6645
11. Schneider, P., Thöne, M., Burns, K., Bodmer, J. L., Hofmann, K., Kataoka, T., Holler, N., and Tschope, J. (1997) Immunity 7, 831–836
12. Muhlenbeck, F., Haas, E., Schwenzer, R., Schubert, G., Grell, M., Smith, C., Scheurich, P., and Wajant, H. (1998) J. Biol. Chem. 273, 33901–33908
13. Jeremias, I., and Debatin, K. M. (1998) Eur. Cytokine Netw. 9, 687–688
14. Walczak, H., Miller, R. E., Arai, K., Gliick, B., Griffith, T. S., Kubin, M., Chinn, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schub, J. C., and Lynch, D. H. (1999) Nat. Med. 5, 157–163
15. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtry, A. E., Hebert, A., Deforge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrrokh, Z., and Schwab, R. H. (1999) J. Clin. Invest. 104, 155–162
16. Jo, M., Kim, T. H., Seol, D. W., Esplen, J. E., Dorko, K., Hilliar, T. R., and Strom, S. C. (2000) Nat. Med. 6, 564–567
17. Nitsch, R., Bechmann, I., Deiz, R. A., Haas, D., Lehmahn, T. N., Wendling, U., and Zipp, F. (2000) Lancet 356, 827–828
18. Lawrence, D., Shahrrokh, Z., Marsters, S., Achilles, K., Shih, D., Mounho, B., Hillan, K., Toptal, K., Deforge, L., Schow, P., Hooley, J., Sherwood, S., Pai, R., Leung, S., Khan, L., Gliick, B., Bussiere, J., Smith, C. A., Strom, S. S., Kelley, S., Fox, J. A., Thomas, D., and Ashkenazi, A. (2001) Nat. Med. 7, 383–385
19. Shi, J., Liu, Y., Li, Y., and Zheng, D. (2003) Chin. J. Bioeng. 23, 46–49
20. Hao, C., Song, J. H., Bii, B., Lewis, J., Song, D. K., Petrak, C. K., Tyrrell, D. L., and Kneteman, N. M. (2004) Cancer Res. 64, 8502–8506
21. Griffith, T. S., Rauch, C. T., Smolak, P. J., Waugh, J. Y., Boiani, N., Lynch, D. H., Smith, C. A., Goodwin, R. G., and Kubin, M. Z. (1999) J. Immunol. 162, 2597–2605
