Article

Characterization of a Novel Anti-DR5 Monoclonal Antibody WD1 with the Potential to Induce Tumor Cell Apoptosis

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TNF-related apoptosis-inducing ligand (TRAIL) is a TNF family member capable of inducing apoptosis. Death receptor 5 (DR 5) is a key receptor of TRAIL and plays an important role in TRAIL-induced apoptosis. To prepare monoclonal antibodies (mAbs) against DR5, cDNA encoding soluble DR5 (sDR5) was firstly amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) with specific primers, and then inserted into a prokaryotic expression vector pET-30a. The recombinant plasmid was expressed in *Escherichia coli* strain BL21 (DE3), and sDR5 was purified by nickel affinity chromatography. As an antigen, sDR5 was used to immunize mice. Hybridomas secreting antibodies against sDR5 were identified. One positive clone was selected to produce antibody, WD1. ELISA and immunofluorescence demonstrated that WD1 could bind recombinant sDR5 and membrane-bound DR5 (mDR5) on Jurkat and Molt-4 cells. ATPLite assays showed that Jurkat and Molt-4 cells were sensitive to the antibody in a dose dependent manner. The Annexin V/PI assays and Giemsa's staining both showed that WD1 could induce Jurkat cell apoptosis efficiently. Transient transfection of 293T cells and indirect immunofluorescence assay demonstrated that mAb (WD1) couldn't cross-react with DR4. Our findings indicated that the novel antibody, WD1 could act as a direct agonist, bind DR5 characteristically, and initiate efficient apoptotic signaling and tumor regression. Thus, WD1 would be a leading candidate for potential cancer therapeutics. *Cellular & Molecular Immunology*. 2008;5(1):55-60.

Key Words: TRAIL, death receptor 5, apoptosis, monoclonal antibody, ATPLite

Introduction

TNF-related apoptosis-inducing ligand (TRAIL) is a TNF family member capable of inducing apoptosis (1). TRAIL interacts with five distinct receptors: TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, TRAIL-R4/DcR2, and osteoprotegerin/OPG. DcR1 is a GPI-anchored membrane protein without a death domain, and DcR2 contains a truncated death domain. Both DcR1 and DcR2 are unable to recruit FADD or signal apoptosis, and inhibit apoptosis (2). OPG is a soluble receptor that may have a more prominent role in bone and myeloid (2).

DR4 and DR5 are able to transduce apoptosis signals, which induce formation of the death inducing signaling complex (DISC) and activation of the caspase-8 and mitochondrial pathway (3, 4). As a type I transmembrane protein, DR5 contains a cytoplasmic death domain and mediates apoptosis upon ligation to TRAIL (5). Recent studies demonstrated that DR5 might contribute more than DR4 to TRAIL-induced apoptosis in cancer cells that express both death receptors. So DR5 is a key receptor of TRAIL and plays an important role in TRAIL-induced apoptosis. TRAIL has been tested on a number of different tumor cell lines, and the results show that TRAIL induces apoptosis in various tumor cell lines, with no cytotoxicity to many normal cell types (6), which indicates it as a promising reagent for cancer therapy. Since TRAIL may be toxic to human liver cells, TRAIL agonists can be alternative reagents to kill tumor cells. So it is meaningful to obtain anti-DR5 monoclonal antibodies that can activate apoptosis signal upon binding to DR5 and are used as potential anti-tumor drugs.

In this study, sDR5 was expressed in bacteria and purified to immunize BALB/c mice. A new anti-sDR5 monoclonal antibody, WD1 was obtained and characterized *in vitro* biochemically and biologically.

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Received Dec 18, 2007. Accepted Jan 20, 2008.

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Materials and Methods

Materials

Recombinant expressing plasmid pET30a/sDR5 was prepared previously (7). Midipreps DNA purification kit was purchased from Promega. Anti-human DR5 mAb YM366 and negative control antibody P218 were prepared in our laboratory. Anti-subclass antibody was purchased from Sigma Corp. ECL Western blot detection reagents were purchased from Amersham Life Science Corp. Fluorescein isothiocyanate (FITC) labeled goat anti-mouse antibody was purchased from Sigma-Aldrich (St. Louis, MO). ATPLite kit was purchased from PerkineElmer Corp. Annexin V and Propidium iodide (PI) kit was purchased from BD Corp. Human lymphoma cell lines (Jurkat and Molt-4), HEL (human erythroleukemia) and 293T cells were stored in our laboratory. ExGen 500 was purchased from MBI Fermentas Corp. Anti-human DR4 mAb was purchase from eBioscience Corp. Recombinant expressing plasmid pcDNA3.1/DR4 was prepared in our laboratory.

Expression and purification of sDR5

The right expression plasmids for sDR5 were transformed into BL21 (DE3) using a standard calcium chloride method. A single colony from a Luria-Bertani broth (LB) plate supplemented with kanamycin was picked up, inoculated into LB plus kanamycin, and incubated at 37°C overnight. Cell culture was transferred to 2 L of LB plus kanamycin and incubated at 37°C until the mid-exponential phase (A600 was about 0.6), when isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The cells were allowed to continue to grow at 30°C for 4 hours and harvested by spinning the culture at 9,000 rpm, 4°C, for 15 min. After washing with phosphate-buffered saline (PBS; 10 mM PB, 150 mM NaCl [pH 7.4]) once, the bacteria pellet was resuspended in PBS and sonicated on ice. After centrifugation at 12,000 rpm, 4°C, for 15 min, the soluble fraction was separated from insoluble pellet. To purify sDR5, the supernatant of cell lysate was loaded on a nickel-chelated affinity column preequilibrated with PBS. Then the column was washed with 50 mM imidazole and 200 mM imidazole in PBS, respectively. Finally, the bound proteins were eluted by 500 mM imidazole, dialyzed against PBS overnight, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis

Protein samples were resolved by 12% SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk in PBS at room temperature for 1 h, the membrane was incubated with 10 μg/ml anti-DR5 mAb YM366 (8) at 4°C overnight. The membrane was washed with PBST (PBS supplemented with 0.05% Tween) three times at room temperature, and incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody for 1 h at room temperature. Then the membrane was rinsed with TBST three times at room temperature. Bound antibodies were visualized by an enhanced chemiluminescence (ECL) method using ECL Western blot detection reagents.

Production and purification of mAb

Immunization and production of mAbs were carried out using standard protocols (9). Three 5-week-old female BALB/c mice were subcutaneously immunized with 100 μg of purified recombinant sDR5 in complete Freund's adjuvant per animal. The animals were boosted twice at 3-week intervals using 100 μg of antigen in incomplete Freund's adjuvant. Three days after the final booster, one mouse was sacrificed, and its splenocytes were fused with NS-1 at a 5:1 ratio, and 200 μl of cells was plated in each well on five 96-well plates. Hybrids were selected in a Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal calf serum and 5×10^{-3} M hypoxanthine, 2×10^{-5} M aminopterin, and 8×10^{-4} M thymidine (HAT). After 10 days, cell clones secreting antibodies against sDR5 were screened by enzyme-linked immunosorbent assay (ELISA), some positive cell clones were selected as secreting antibodies against sDR5 and further subcloned to the stable hybridoma. To produce the mAb in large quantity, 5×10^6 hybridoma cells were injected into the peritoneal cavity of BALB/c mice. After 14 days, ascites were withdrawn and centrifuged at 1,500 rpm for 5 min at 4°C. The supernatant was collected and applied to a column of protein A-Sepharose 4B, which was pre-equilibrated with PBS. The bound mAb was eluted with pH 4.0 citric acid buffer and dialyzed against PBS overnight. The purified proteins were analyzed by SDS-PAGE.

Subclass of a monoclonal antibody WD1

Double immunodiffusion method was carried out to identify the subclass of mAb WD1. Briefly, 1% agarose solution was prepared in PBS containing 0.05% sodium azide and melted. Three milliliters of the solution was poured onto a glass slide. When the agar set, the wells were punched, and each well was 1 cm apart. Then 10 μl concentrated culture media of the hybrid was added to the middle ring and 10 μl of anti-subclass antibody (Sigma-Aldrich) was added to the outer well. The glass slides were placed in a humidified chamber and incubated at 37°C. After 48 h, the gel was stained with Coomassie Blue.

ELISA analysis

Binding of mAb WD1 to sDR5 was analyzed by ELISA. ELISA plates (Nunc, Roskilde, Denmark) were coated with 2 μg/ml of sDR5 in 0.1 M sodium carbonate buffer (pH 9.6) and incubated at 4°C overnight. Then the plate was blocked with 5% milk powder in PBS for 1 h at 37°C. After washing the plates three times with PBST, different concentrations of purified mAb were added and incubated for 1 h at 37°C. Human IgG was used as a control. The plate was washed three times with PBST. Then 0.1 ml of 1:4,000 diluted goat-anti-mouse IgG antibody labeled with HRP was added and incubated for 1 h at 37°C. After three washes with PBST, the peroxidase reaction was developed with color development

solution containing 5.5 mM OPD and 8.5 mM H_2O_2. The light absorbance was measured at 492 nm with an ELISA reader.

Flow cytometric analysis

Culture cells (1×10^6) were collected by centrifugation at 4°C for 5 min at 1,000 rpm. After washing once, cells were resuspended in FACS buffer (PBS containing 2% FCS and 0.01% sodium azide) and incubated with 10 μg/ml of purified antibody WD1 for 1 h at 4°C. Mouse IgG1 was set as negative control. After washing twice with FACS buffer, the cells were incubated with 50 μl of 1:100 diluted FITC-conjugated goat anti-mouse IgG1 for 30 min at 4°C with gentle shaking, then washed twice with FACS buffer and fixed in 300 μl of 1% paraformaldehyde. Samples were analyzed on a FACSCalibur (Becton-Dickinson) using the software program CellQuest.

ATPLite assays

A total of 1×10^5 cells per well were cultured in flat-bottom 96-well plate in triplicates in 10% FCS/RPMI 1640 medium with the indicated concentration of the purified mAb WD1 or antibody P218 as a negative control. Cell viability was determined using an ATPLite kit according to the manufacturer's instructions.

Analysis of apoptosis

Annexin V staining and PI uptake were analyzed for apoptosis evaluation. Jurkat cells were incubated with 10 μg/ml or 1 μg/ml of WD1 for 30 min at 37°C. Cells were collected and double stained with FITC-conjugated Annexin V and PI. Annexin V and PI emissions were detected in FL-1 (530/30 nm) and FL-2 (585/40 nm) channels, respectively. For each sample, data from approximately 10,000 cells were recorded list mode on logarithmic scales. Data analysis was performed with CellQuest software (Becton-Dickinson).

Giemsa's staining

Jurkat cells were incubated with 10 μg/ml of WD1 for 30 min at 37°C. Cells were collected by centrifugation at 4°C for 5 min at 1,000 rpm, then given a quick wash in 0.01 mol of sterile phosphate buffer (Flow, USA, pH 7.4). Cells were sprayed on glass slides with a Cytospin (Shandon, Astmoor, England), air dried and fixed with methanol for 1 min, stained with 10% Giemsa's stain for 15 min, washed with distilled water, and finally air dried. The samples were examined in light microscopy.

Transient transfection and indirect immunofluorescence analysis of mAb specificity

293T cells (2×10^5) were transfected with 3 μg of pcDNA3.1/DR4 expression plasmid in a 6-well plate using 6 μl of ExGen 500 in DMEM supplemented with 10% fetal calf serum. Following the manufacturer's instructions, 293T cells without transfection were set as negative controls. After incubated for 36 hours at 37°C, 293T and transfected 293T cells were collected by centrifugation at 4°C for 5 min at 1,000 rpm, resuspended in FACS buffer and incubated with 10 μg/ml of purified antibody WD1 and 2 μg/ml anti-human DR4 mAb for 1 h at 4°C. Mouse IgG1 was set as negative control, and 293T cells transfected with pcDNA3.1/DR4 expression plasmid (293T/DR4) were set as positive control. After washed twice with FACS buffer, the cells were incubated with 50 μl of 1:100 diluted FITC-conjugated goat anti-mouse IgG1 for 30 min at 4°C with gentle shaking. Then cells were washed twice with FACS buffer and fixed in 300 μl of 1% paraformaldehyde. Samples were analyzed by FACS.

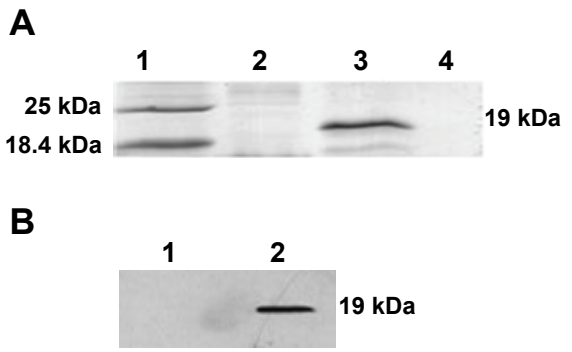

Figure 1. Purification and binding assay of sDR5. Soluble fraction of cell lysate was loaded on a Ni^+ affinity column. The bound proteins were eluted by 200 mM imidazole and resolved on 12% SDS-PAGE, transferred on a nitrocellular membrane and immunoblotted with mAb YM366. (A) SDS-PAGE analysis. Lane 1, protein marker; Lane 2, pET30a transformed into BL21 (DE3); Lane 3, pET30a/sDR5 transformed into BL21 (DE3); Lane 4, BL21 (DE3) control; (B) Western blot. Lane 1, pET30a transformed BL21 (DE3); Lane 2, pET30a/sDR5 transformed BL21 (DE3).

Statistical analysis

Experimental conditions were compared by using Student's *t* test for single measurements or ANOVA for repeated measurements. A *p* value less than 0.05 was considered statistically significant.

Results

Expression and purification of sDR5

pET30a/sDR5 plasmid was used for preparation of sDR5. The expression of sDR5 was induced by 0.1 mM IPTG in *Escherichia coli*. Analysis of protein samples by 12% SDS-PAGE indicated that sDR5 could express only in induced cell lysate, not in non-induced cell lysate. Most of the recombinant proteins existed in soluble fraction. SDS-PAGE analysis showed that, compared with the negative control, there was one more specific band at predicting 19 kDa in recombinant bacterial group, which was considered as sDR5 (Figure 1A). To test whether purified sDR5 could be specifically recognized by anti-DR5 monoclonal antibody, sDR5 was resolved on 12% SDS-PAGE and transferred to a nitrocellular membrane. Western

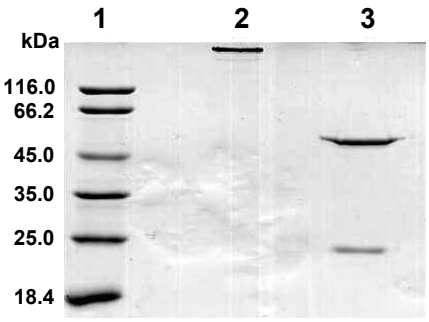

Figure 2. SDS-PAGE analysis of the purified monoclonal antibody WD1. Lane 1, protein marker; Lane 2, under a non-reduced condition; Lane 3, under a reduced condition.

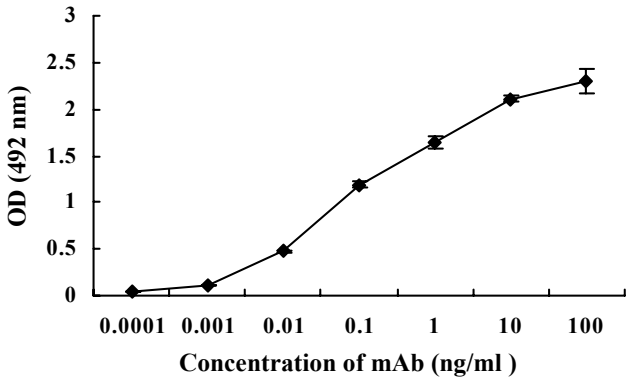

Figure 4. ELISA analysis of the mAb WD1. ELISA plates were coated with sDR5 (2 μg/ml), and blocked with 2% BSA. Then different amounts of purified WD1 were added and incubated at 37°C for 1 hour. The bound WD1 was detected with an anti-mouse antibody labeled with HRP.

blot analysis showed that 10 μg/ml anti-DR5 mAb YM366 could recognize the antigen, while no specific band was detected in negative control group (Figure 1B).

Identification of anti-sDR5 mAb

To obtain anti-sDR5 mAbs, we used sDR5 to immunize BALB/c mice and fused their splenocytes with NS-1 cells to establish hybridoma cell lines. WD1 was produced by one of them. After purification, mAb was analyzed under reduced or nonreduced conditions on SDS-PAGE. As shown in Figure 2, under a reduced condition, two bands of about 25 and 50 kDa were generated; meanwhile only a single band of about 150 kDa was shown under a nonreduced condition. To identify the subclass of WD1, we carried out double immuno-diffusion and found that WD1 could interact specifically with anti-IgG1 antibody and formed a visible line, suggesting that mAb WD1 was IgG1 (Figure 3). To analyze binding property of WD1 with sDR5, various amounts of purified mAb were used. When sDR5 was fixed at 2 μg/ml, the mAb could bind with sDR5 in a dose-dependent manner (Figure 4). To test if the mAb WD1 could bind membrane-bound DR5, we used Jurkat and Molt-4, which are human T cell leukemia cell lines, expressing DR5 on their membrane (5, 6). As shown in Figure 5, mAb WD1 bound to Jurkat cells (54.40%) and Molt-4 (20.59%) specifically, compared with the negative control mouse IgG1 (0.63%). To test whether WD1 could cross-react with DR4, we used HEL cell line, which expresses DR4 on cell membrane (10). The FACS results showed that WD1 couldn't bind to the cell, compared with DR4 mAb control. We also used the 293T cells transfected with pcDNA3.1/DR4 expression plasmid to prove the specificity of the antibody WD1. The result also showed that WD1 could not bind to 293T cells transfected with pcDNA3.1/DR4 (2%) (Figure 6). All these results demonstrated that WD1 couldn't cross-react with DR4 and bound only human DR5.

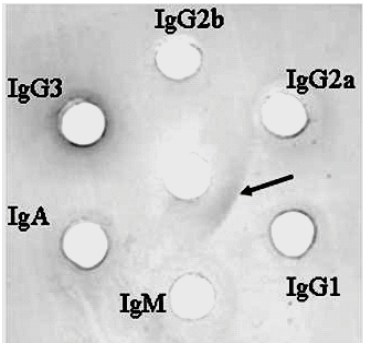

Figure 3. Subclass of mAb WD1. Agarose solution at the concentration of 1% was prepared in PBS containing 0.05% sodium azide and melted, poured onto a glass slide, and the agar set, wells were punched, 10 μl concentrated culture media of the hybrid was added to the middle ring, and 10 μl of anti-subclass antibody was added to the outer well. After incubated at 37°C for 48 h, the gel was stained.

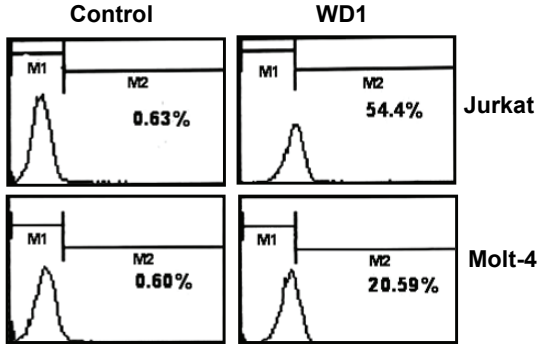

Figure 5. Flow cytometric analysis. Cells were collected by centrifugation and washed once, and resuspended in FACS buffer at 4°C. Cells were incubated with purified antibody WD1 (10 μg/ml) or an isotype-sepcific IgG1 antibody as negative control for 1 h at 4°C. Then after washing, cells were incubated with 1:100 diluted FITC-conjugated goat anti-mouse IgG1 antibody. Finally, cells were fixed in 1% paraformaldehyde for FACS analysis.

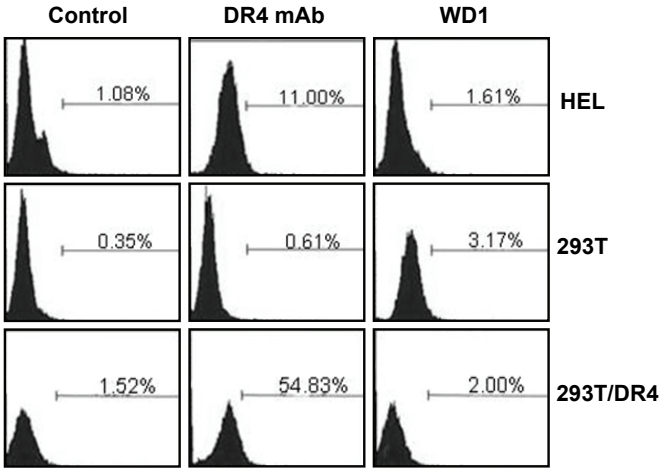

Figure 6. Transient transfection and indirect immunofluorescence analysis of mAb (WD1) specificity. Cells (HEL, 293T and 293T transfected with pcDNA3.1/DR4 expression plasmid) were incubated with 10 μg/ml of purified antibody WD1 and 2 μg/ml anti-human DR4 mAb for 1 h at 4°C. Mouse IgG1 was set as negative control. Then the cells were incubated with FITC-conjugated goat anti-mouse IgG1 polyclonal antibody. After fixed, the cells were assayed by flow cytometry.

Functional analysis of WD1

To test if the mAb WD1 was functional, we examined the cytotoxic effect of WD1 antibody against Jurkat or Molt-4 cells *in vitro*. Jurkat and Molt-4 cells were treated with increased amounts of WD1 and the cytotoxicities were measured after 24 hours. Both Jurkat cells and Molt-4 cells died in the presence of WD1 in a dose-dependent manner. The survival ratio of Molt-4 cells could decrease to 52% when cultured with 10 μg/ml WD1 for 24 h (IC_{50} = 10 μg/ml), meanwhile Jurkat cells were more sensitive to WD1 (IC_{50} = 0.1 μg/ml) (Figure 7). Then we hypothesized that WD1 could inhibit survival of the cell lines by inducing cell apoptosis. To test this hypothesis, we performed the Annexin V/PI assay and Giemsa's staining experiment. The FACS results showed that compared with negative control, WD1 could induce Jurkat cell apoptosis efficiently. The ratio of total cell apoptosis was about 67.48% in the presence of 1 μg/ml WD1, and the ratio of early apoptotic cells could reach 59.81% within 30 minutes; meanwhile, the ratio of total cell apoptosis and early apoptosis were 60.04% and 48.39% in the presence of 10 μg/ml WD1, respectively (Figure 8). Moreover, Giemsa's staining (Figure 9) also showed that after incubated with 10 μg/ml of WD1, Jurkat cells acquired typical features of apoptosis, including cell shrinkage, membrane blebbing and nuclear pyknosis.

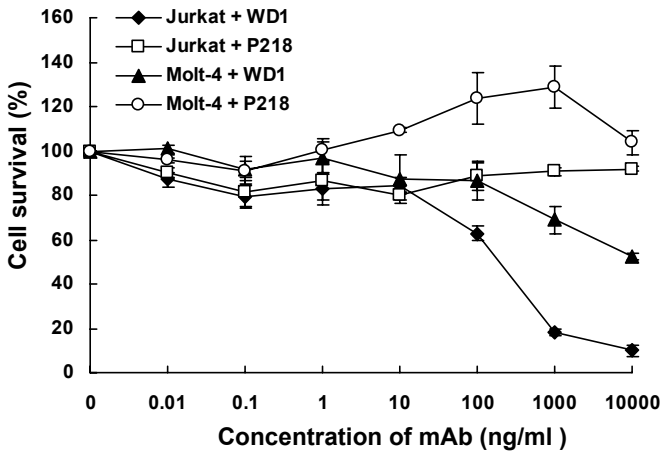

Figure 7. ATPLite assay. Jurkat and Molt-4 cells were cultured in 96-well flat-bottom plate in triplicates in 10% FCS/RPMI 1640 medium with the indicated concentration of the purified mAb WD1 or P218 as a negative control. Cell viability was determined using the ATPLite kit. The data are representative of three independent assays.

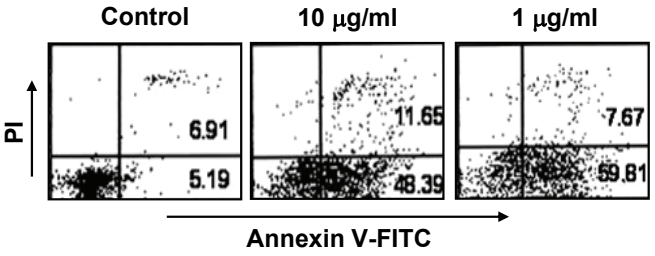

Figure 8. Flow cytometric analysis of apoptosis. Jurkat cells were incubated with 10 μg/ml and 1 μg/ml of WD1 for 30 min at 37°C. Cells were collected and double stained with FITC-conjugated Annexin V and PI.

Discussion

Apoptosis is an essential mechanism for maintaining normal development and homeostasis. TRAIL, as a member of the TNF family, can bind DR5/DR4 and induce apoptotic cell

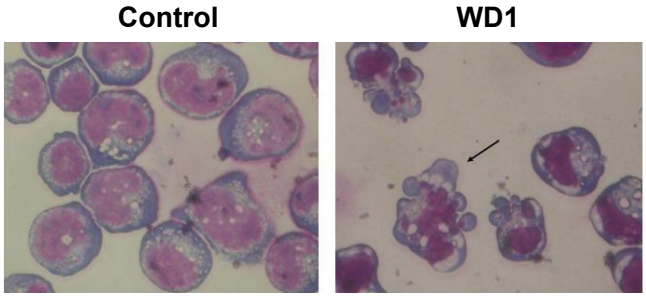

Figure 9. Giemsa's staining. Jurkat cells were incubated with 10 μg/ml of WD1 for 30 min at 37°C. Cells were collected by centrifugation at 4°C and then were sprayed on glass slides with a Cytospin, air dried and fixed with methanol for 1 min, stained with 10% Giemsa's stain for 15 min. The samples were examined in light microscopy at 40× magnification.

death in a wide variety of tumor cell lines *in vitro*; besides, it plays an important role in T cell- and natural killer cell-mediated tumor surveillance (11). Therefore, administration of rTRAIL (soluble recombinant TRAIL), apoptosis-inducing anti-DR5/DR4 antibodies, or an agent that increases endogenous TRAIL expression are promising strategies to improve conventional therapies for cancer (12, 13). However, abundant experiments showed the capability of soluble human TRAIL inducing apoptosis of normal human hepatocytes *in vitro,* so the agonists focusing on the receptors (DR4 and DR5) are becoming the new research hotspots.

So far, several clinical trials are ongoing with different TRAIL receptor agonists alone or in combination with other anti-cancer drugs (14). The potential for disease-specific targeting and low toxicity profiles have made monoclonal antibodies attractive therapeutic drug candidates. Moreover, some antibodies to DR4 and DR5 have been reported to exhibit anti-tumor effects without cross-linking, and an enhanced anti-tumor effect was observed in the presence of a cross-linking reagent. For example, anti-immunoglobulin antibodies can be used for crossing-linking under *in vitro* conditions, but *in vivo* the cross-linking reagent is limited to the complement component C1q and Fc receptor (FcR) present on most effector cells (15). So direct agonists to TRAIL receptors that trigger apoptosis independent of cross-linking may be more desirable for antibody-based cancer therapy.

In this study, we developed an anti-DR5 mAb, WD1, which could recognize sDR5 and membrane DR5 on the surface of Jurkat and Molt-4 cells, without cross-react with DR4. Furthermore, WD1 exhibited transparent activity to efficiently induce Jurkat and Molt-4 cell apoptosis without cross-linking. In fact, the novel specified agonist antibody WD1 might directly initiate efficient apoptotic signaling and tumor regression independent of host effector function. Therefore, WD1 could be a leading candidate for cancer therapeutics, with recent advances in genetic engineering to generate chimeric, humanized, or fully human antibodies to decrease antibody immunogenicity and increase antibody half-life (16). In the near future, WD1 may be a potential therapeutic agent in clinical application in expectation.

Acknowledgements

We thank Xiaoling Lang for her excellent technical assistance and Dr. Jian Sun for English corrections. This study was supported by National “863” Fund of China (No. 2006AA02A254) and National “863” Fund of China (No. 2006AA020503).

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