A Functional, New Short Isoform of Death Receptor 4 in Ewing's Sarcoma Cell Lines May Be Involved in TRAIL Sensitivity/Resistance Mechanisms

Gaelle Picarda1,2, Sylvanie Surget3, Romain Guilo1,2, Stephanie Tetechea1,2, Martine Berreur1,2, Franck Tirode4,5, Catherine Pellat-Deceunynck6, Dominique Heymann1,2, Valerie Tricot1,2, and Francoise Redini1,2

Abstract

Ewing's sarcoma (ES) is a high-grade neoplasm arising in bones of children and adolescents. Survival rate decreases from greater than 50% to only 20% after 5 years for patients not responding to treatment or presenting metastases at diagnosis. TRAIL, which has strong antitumoral activity, is a promising therapeutic candidate. To assess TRAIL sensitivity, 7 human ES cell lines were used. Cell viability experiments showed that 4 of the 7 ES cell lines were resistant to TRAIL. Western blotting and flow cytometry analyses revealed that DR5 was uniformly expressed by all ES cell lines, whereas DR4 levels were higher in sensitive cell lines. In TRAIL-sensitive TC-71 cells, knockdown of TNFRSF10A/DR4 by short hairpin RNA (shRNA) was associated with a loss of sensitivity to TRAIL, in spite of DR5 presence. Interestingly, we identified a new transcript variant that results from an alternative splicing and encodes a 310-amino acid protein which corresponds to the 468 aa of DR4 original isoform but truncated of aa 11 to 168 within the extracellular TRAIL-binding domain. According to modeling studies, the contact of this new DR4 isoform (bDR4) with TRAIL seemed largely preserved. The overexpression of bDR4 in a TRAIL-resistant cell line restored TRAIL sensitivity. TRAIL resensitization was also observed after c-FLIP knockdown by shRNA in two TRAIL-resistant cell lines, as shown by XTT assay and caspase-3 assay. The results presented in this study showed that DR4, both as the complete form or as its new short isoform, is involved in TRAIL sensitivity in ES.

Introduction

Bone sarcomas are rare malignancies diagnosed in fewer than 700 individuals per year in the United States. Ewing’s sarcoma (ES) is the second most frequent primary bone tumors in children and adolescents. It is a high-grade neoplasm, representing 2% of childhood cancers with a peak incidence at age 15 (1–3), characterized by a rapid tumor growth and extensive bone destruction that can result in bone pain and pathologic fracture (4). Ewing’s tumors show a typical chromosomal translocation in more than 90% of cases linking the EWS gene on chromosome 22q12 to a member of the ETS transcription gene family, most commonly to FLI-1 on 11q24 (5,6), leading to an aberrant transcription factor that promotes tumorigenicity (7–9). Current treatment consists of several rounds of multidrug cytostatic therapy combined with surgery and radiotherapy for local control. With the use of alkylating agents, long-term survival can be achieved in more than 50% of patients with localized disease, whereas patients with clinically detectable metastases at diagnosis or who are not responding to therapy have a significantly poorer prognosis, with long-term survival decreasing to 15% to 25%, especially for patients with bone metastases (10). As the survival rates for metastatic and relapsed osteosarcoma or ES patients have not changed in the past 30 years, despite the use of intensive chemotherapy (11), new therapeutic approaches are therefore needed.

Among new strategies, TRAIL or APO-2L is a promising candidate. TRAIL is a member of the TNF superfamily that exerts a strong antitumor activity in a wide range of cancer cell lines, although being minimally cytotoxic to most normal cells and tissues (12). TRAIL binds 5 known receptors: 2 death receptors acting as activating receptors [DR4 (TRAIL-R1) and DR5 (TRAIL-R2)] and three decoy receptors DcR1 (TRAIL-R4), DcR2 (TRAIL-R5) and secreted osteoprotegerin (OPG), a member of the TNF
receptor superfamily: TNFRSF11B); ref. 13). The TRAIL decoy receptors are unable to trigger a death signal because they lack a functional death domain (DD; ref. 14). TRAIL activates the extrinsic apoptotic pathway by inducing Fas associated DD (FADD) protein recruitment to the DD, leading to caspase-8 activation and caspase-3 cleavage (15). In addition, it has been shown that TRAIL can induce the mitochondrial intrinsic apoptotic pathway through caspase-8 activation followed by Bid cleavage (16). Recombinant human (rh)TRAIL is being clinically evaluated for the treatment of both solid tumors and hematologic malignancies. A phase I trial with rhTRAIL is completed, showing no dose-limiting toxicities or clearly attributable toxicities to TRAIL (17). No anti-rhTRAIL antibodies have been detected. Clinical trials are also currently in progress testing fully humanized activating monoclonal antibodies (mAb) directed against DR4 or DR5. Several phase I and II studies targeting DR4 (Mapatumumab, TRM-1) or DR5 (Lextra- mumab) are carried out in patients with solid malignancies, associated or not to chemotherapy (18–22). Despite limited timespan, these studies show promising results on tumor progression. Kontny and colleagues reported that 80% of ES tumors express DR4 whereas all express DR5 (23). In another study, it has been shown that 72% of ES tumor samples express both DR4 and DR5 (of 32 samples), 25% express one receptor, and only 3% express none (24). These results suggest a high proportion of TRAIL sensitivity in ES tumors.

We have previously reported that TRAIL is indeed a good candidate for ES therapy, as it inhibited cell progression and increased animal survival in a xenograft model of ES induced by TRAIL-sensitive cell lines (25). However, half of the ES cell lines were resistant to TRAIL (4 of 7), suggesting that TRAIL or agonist antibodies cannot be proposed to all patients. Data from the literature have reported that TRAIL resistance occurs in numerous cancer cell lines, which can be attributed to dysfunctions in the TRAIL signaling pathway (14, 26). Among the major causes of TRAIL resistance, one is linked to the absence of activating DR4 and/or DR5 death receptors expressed on the surface of tumor cells, leading to an imbalance of the ratio death/decoy receptors (27). Initiation of death receptors DR4 and/or DR5 triggered apoptosis by formation of the death-inducing signaling complex (DISC) after recruitment of FADD, which binds procaspase-8 leading to caspase-8 activation. However, the presence of internal regulators of apoptotic machinery including cellular FLICE inhibitory protein (c-FLIP), a structural analog of procaspase-8 which inhibits caspase-8 activation, is another important determinant (28).

The aim of this study is to analyze the molecular mechanisms underlying TRAIL resistance in ES cell lines. For this purpose, 7 human cell lines were studied, all expressing the EWS-FLI-1 fusion gene but differing in their mutation status for p53, p16, p14, etc. The main finding of this study is the existence of a functional truncated form of DR4 in ES cells, restoring TRAIL sensitivity when reexpressed in resistant cell lines. The other main finding is the specific TRAIL sensitivity linked to DR4 expression (as both short and long forms) in ES, independent of the predominant DR5 expression. We also showed that the downregulation of c-FLIP expression in resistant ES cells represents another way to reestablish these cells to TRAIL.

Materials and Methods

ES cell lines

Seven human ES cell lines were used. A673, TC-32, SK-ES-1, SK-N-MC, and RD-ES cell lines were kindly provided by Dr. S. Burchill (Children’s Hospital, Leeds, United Kingdom) and the EW24 and TC-71 by Dr. O. Delattre (Institut National de la Santé et de la Recherche Medicale U830, Paris, France). The cell lines A673, TC-32, SK-ES-1, and RD-ES were cultured in Dulbecco’s modified Eagle’s medium (DMEM; BioWhittaker) with 10% FBS (HyClone) and SK-N-MC, EW24, and TC-71 cells were cultured in RPMI (BioWhittaker) with 10% FBS. In addition, TC-71 cells require type I collagen to grow.

Cells (2.93 HEK293) derived from human fetal kidney used in vitro cell transfection assays (29) were cultured in DMEM supplemented with 10% FBS and 2 mmol/L l-glutamine.

Mitochondrial activity

The mitochondrial activity was determined by a colorimetric assay using sodium 3’[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro-)benzene sulfonic acid hydrate (XTT; Roche Molecular Biochemicals). Two thousand cells per well were plated into 96-well plates and cultured for 72 hours in culture medium with 2 treatments with 0, 50, or 100 ng/mL of TRAIL (R&D systems). After the culture period, XTT reagent was added to each well and incubated for 5 hours at 37°C; absorbance was then read at 490 nm using a 96-multiwell microplate reader.

Caspase activity

Subconfluent ES cells cultured in 24-well plates were treated with 50 ng/mL TRAIL for 1 to 6 hours, washed, and lysed in radioimmunoprecipitation assay (RIPA) buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 1 mmol/L Na3VO4, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L NaF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin] for 30 minutes. The cells were then scraped off and the lysates were cleared of debris by centrifugation at 12,000 × g for 15 minutes, after which total protein was quantified using the bicinchoninic acid (BCA) + copper II sulfate assay (Pierce Chemical). Caspase-3 activity was assessed in 10 μL of cell lysate with the CaspACE assay kit (Promega) following the manufacturer’s recommendations. UV-treated cells were used as positive control for caspase activity.

Flow cytometry

Cell death was determined by Apo2.7-PE staining as described previously (30). Briefly, cells were harvested with PBS containing EDTA, washed and stained with anti-APO2.7-PE mAb (Beckman Coulter). Fluorescence was
analyzed on a FACSCalibur cytometer (BD Biosciences). To determine cell surface expression of DR4 or DR5, cells were stained with PE-conjugated anti-DR4 or DR5 (eBioscience) or PE-conjugated mouse as an isotype control (Beckman Coulter). A minimum of 10,000 cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cell viability was determined with PE-conjugated Apo2.7 labeling and analyzed by flow cytometry.

**Cell transfection**

Transfections with pcMV-SPORT6-DR4 (imaGenes GmbH) were done with polyethylenimine (PEI, kindly provided by Dr B. Pitard, UMR915, Nantes, France). PEI–DNA complexes were prepared by equipotometric mixing PEI (charge ratio, ±4) in water with plasmid DNA solution at the desired concentration (4 µg per well) in 150 mmol/L NaCl. Transfections were done at 70% to 80% of confluence in 0.5 mL of culture DMEM deprived of FBS. After 6 hours of incubation with DMEM containing 10% FBS and 1% penicillin/streptomycin, the transfection medium was replaced by 1 mL of DMEM containing 10% FBS and 1% penicillin/streptomycin (complete medium).

**RT-PCR analysis**

Forty-eight hours after transfection, total RNA was extracted from cells using TRIZol (Invitrogen) following the protocol suggested by the manufacturer. Reverse transcription PCR (RT-PCR) after total RNA extraction using TRIZol reagent was then done. First, RNA was reverse-transcribed using 400 units MMLV-RT (Invitrogen). Then, 2 µL RT reaction mixture was subjected to PCR using upstream (5′-CACCATGGGCCACCGACGC-TAGAGTACATCTA-3′) and downstream (5′-TTAACAGC-TCCTAACCTAAGGAGAACCTCTG-3′) primers (30 pmol each) of DR4 and Taq polymerase (1.25 units; Taq High Fidelity according to the manufacturer recommendation using the following DR4 primers: forward: GAGGCAGGGACAAGTTAC (start position 36, NM_003844.3) and downstream (30 pmol each) of DR4 and Taq polymerase (1.25 units; Eurobio).

**Quantitative RT-PCR analysis**

Short hairpin RNA (shRNA) gene silencing was assessed in subconfluent ES cells cultured in 6-well plates. c-FLIP and DR4 mRNA expression was determined by quantitative RT-PCR (qRT-PCR) after total RNA extraction using TRIZol reagent. Briefly, 10 ng of cDNA were amplified using the iQ SYBR Green Supermix (Bio-Rad) for the DR4, c-FLIP, and DR5 gene. Sense and antisense primers used are as follows (DR4: forward: GGAGGCAGGGACAAGTTAC and reverse: CAGCACCATTGTGCTGCTG; c-FLIP: forward: CAGCACCATTGTGCTGCTG and reverse: CTACACTACCTCTACCTCTCTACCT; DR5: forward: AGAGCCACAGGTGTCACACGT and reverse: CGCTCCTCCTCAGGACCC). Each sample was analyzed twice and quantified with the analysis software of the iCycler iQ Real-time PCR Detection System (Bio-Rad).

**Western blotting**

Cells were lysed with a lysis buffer (150 mmol/L NaCl, 5% Tris, pH 7.4, 1% Nonidet P-40, 0.25% Na deoxycholate, 1 mmol/L Na3VO4, 0.5 mmol/L PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin). After 30 minutes on ice, lysates were cleared by centrifugation at 10,000 × g for 10 minutes at 4℃. Total protein concentrations were determined using the BCA (Sigma) based method. Twenty micrograms of total cell lysate proteins was run on a SDS-PAGE and then electrophoretically transferred to Immobilon-P membrane (Millipore Corporation). The membrane was blotted with rabbit antibodies to human c-FLIP (Cell signaling), rabbit anti-human Caspase-8 (Santa Cruz biotechnology), goat anti-human DR4 (R&D systems), rabbit anti-human DR5 (Cell signaling), and rabbit anti-human actin was used as loading control (Sigma) in PBS, 0.05% Tween, and 3% bovine serum albumin. The membrane was washed and probed with the secondary antibody (goat and rabbit, respectively) coupled to horseradish peroxidase. Antibody binding was visualized with the Enhanced Chemiluminescence (ECL) system (SuperSignal West Dura kit; ThermoScientific). To visualize DR4, we changed the lysis buffer reagents (10 mmol/L Tris-HCL pH = 7.6, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L PMSF, 2 mg/mL aprotinin, 1% Triton X100). Then, after 40 minutes on ice, lysates were cleared by centrifugation at 10,000 × g for 30 minutes at 4℃. Eighty micrograms of cleared lysates were then separated by SDS-PAGE (10% acrylamide) and electrotransferred to Immobilon-P membrane (Millipore Corporation). Western blot analysis was done as before by standard techniques with a different ECL detection kit (Pierce Perbio Science France) this time.

**shRNA silencing**

Stably modified ES cells were obtained by lentiviral cell transduction essentially as described previously (31). Oligonucleotides were designed and cloned into pSUPER to produce the shRNA shDR4 and shc-FLIP directed, respectively, against human DR4 and c-FLIP genes. The targeted sequences were GGAGGCAGGGACAAGTTAC (start position 1055, NM_003879.4) for c-FLIP and GCCACGCTCCTCCTGAGAT (start position 36, NM_003844.3) for DR4. The cloned oligonucleotides were controlled by sequencing (Genome Express) before subcloning them with the upstream H1 promoter into the vector pFG12 (32). As a control, a vector pFG12 was developed to produce shRNA targeting LacZ gene as done by Qin and colleagues (2003). The resulting constructs allowed the expression of both green fluorescent protein (GFP) and target-specific shRNA. They were used for lentivirus production following the protocols provided with the ViraPower Lentiviral Expression System (Invitrogen). A multiplicity of infection of 10 was used to transduce ES cells. Seven days after transduction, GFP+ ES cells were sorted out using fluorescence-activated cell sorting (FACS).

**Short DR4 overexpression**

Total extract RNA from RD-ES ES cell line was reversed transcribed using MMLV-RT. Then, 0.5 µg of cDNA was amplified by PCR, with 1 Unit of Platinum Taq High Fidelity according to the manufacturer recommendation using the following DR4 primers: forward:
CACCATTGGCAGCCACCAGCTTAGGTACATCT-
TA; reverse: TTAATCTCTAACCCATAGGAACGACCTCTG.

Then 0.5 μL of the PCR mixture containing the truncated form of DR4 was cloned into the pLENTI TOPO plasmid according to the manufacturer protocol of the pLenti6/V5 Directional TOPO Cloning Kit. The SK-N-MC cell line stably expressing the DR4-truncated form was obtained by transduction with 20 to 150 μL lentiviral particles and selection with 3 μg/mL of blasticidin (Sigma).

Modeling DR4 isoforms and TRAIL interaction

The sequence corresponding to the extracellular domain of aDR4 (amino acids 120 to 236 of NP_003833.4) was aligned in Discovery Studio 2.5.5 with its corresponding DR5 domain sequence (69 to 185 of NP_003833.4) and adjusted manually to ensure that the cystein-rich domains (CRD) were correctly positioned (33). DR5 and aDR4 extracellular domains share 63% identity and 70% similarity. The crystal structure of the DR5 extracellular domains share 63% identity and 70% similarity.

The crystal structure of the DR5–TRAIL complex 1DV4 (34) was used to carry out the molecular modeling of aDR4 using the DR5 template. Of the 6 disulfide bridges present on DR5 extracellular domain, only 4 could be matched and modeled for aDR4. The final models were built using Modeller 9v5 (35). All resulting models were assessed for violations using the Protein Health module of Discovery Studio 2.5.5 (Accelrys Inc.) and corrected when necessary. The DR4–TRAIL complex was produced by molecular replacement of DR5 by the best model of aDR4 in the 1DV4 structure. The root mean square difference on main-chain atoms was 0.86 Å between the aDR4 model and the DR5 structure.

Caspase activity

Subconfluent ES cells cultured in 24-well plates were treated with 50 ng/mL TRAIL for 1 to 6 hours, washed, and lysed in RIPA buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 1 mmol/L Na3VO4, 0.5 mmol/L PMSF, 1 mmol/L NaF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin] for 30 minutes. The cells were then scraped off and the lysates were cleared of debris by centrifugation at 12,000 g for 15 minutes, after which the protein was quantified using the BCA + copper II sulfate assay (Pierce Chemical). Caspase-3 activity was assessed in 10 μL of cell lysate with the CaspACE assay kit (Promega) following the manufacturer’s recommendations. Cells treated one minute with UV were used as positive control for caspase activity.

Results

TRAIL resistance in ES cell lines is related to low DR4 levels despite high DR5 expression

DR4 and DR5 expression were first analyzed by qRT-PCR in 7 ES cell lines and presented in Fig. 1A. Protein levels of DR5 and DR4 were further detected by Western blotting and FACS analysis on the same ES cell lines (Fig. 1B and C, respectively). Levels of DR5 were similar in all cell lines, whereas DR4 levels were higher in RD-ES and TC-71 cells than in the 5 others. Complementary flow cytometry analyses were done to determine the presence of both DR4 and DR5 death receptors at the cell surface of 7 different ES cell lines (Fig. 1C). Levels of cell surface DR4 were about 3-fold higher than those detected for cell surface DR4 (range ratio 4.7–6.7 vs. 1.2–2.8, respectively). Confirming the different levels of DR4 detection by Western blot, flow cytometry analysis showed low cell surface DR4 levels for SK-N-MC, TC-32, SKES-1, and EW24 cells, intermediate for A673 and high for TC-71 and RDES (Fig. 1C). The same experiment was conducted on bone marrow–derived mesenchymal stem cells, and none of DR4 and DR5 was detected (data not shown).

Despite the fact that DR5 is the predominant TRAIL-activating receptor on ES cells, DR4 implication may be questioned. Indeed, 4 (SK-ES-1, SK-N-MC, EW24, and TC-32 cell lines) of 7 ES cell lines were resistant to TRAIL-induced cell death as shown by XTT cell viability assay (Fig. 1D) and low levels of DR4 were observed in these resistant cells (Fig. 1A–C). In contrast, RD-ES and TC-71 cell lines with high DR4 levels are TRAIL-sensitive as their viability is reduced to 17.6 ± 2.9% and 34.8 ± 2.9%, respectively, after TRAIL treatment (Fig. 1D). In the case of the A673 cell line, cell viability was reduced to 16.4 ± 0.7% in TRAIL-treated cells which showed intermediate DR4 levels. In addition, no correlation could be shown between decoy receptor (DcR1, DcR2, or OPG) and resistance/sensitivity in all ES cell lines studied (not shown).

Because all TRAIL-resistant cell lines tested expressed low levels of DR4, we wonder whether this death receptor may be involved in the regulation of TRAIL resistance in ES cells. To show this hypothesis, 2 strategies were envisaged: (i) to decrease DR4 expression in sensitive cells and (ii) to overexpress it in resistant cells.

DR4/TNFRSF10A knockdown in TRAIL-sensitive TC-71 cell line induced TRAIL resistance despite DR5 expression

Sensitive TC-71 cells were modified to stably produce either control shRNAs (shLacZ with no target in human cells) or targeting DR4 mRNA (shDR4) and were named shLacZ or shDR4TC-71, respectively. As shown by real-time PCR on reverse transcribed RNAs, DR4 expression was significantly reduced (2.6-fold compared with shLacZ-transduced TC-71 cells) in shDR4TC-71 cells as compared with TC-71 or shLacZ TC-71 (Fig. 2A, left panel). However, a concomitant 2.1-fold increase of DR5/TNFRSF10B level in shDR4TC-71 cells was noticed, although DR5 level was similar to shLacZTC-71 as compared with the parental TC-71 cell line (Fig. 2A, right panel). DR4 knockdown was associated with a 50% decrease of DR4 protein level in shDR4TC-71 cells as detected by Western blotting and at the membrane level by flow cytometry analyses (respectively Fig. 2B and C). Moreover, the DR4 reduction was associated with a loss of TRAIL sensitivity. As shown in Fig. 2D, 104 ± 1.9% viability was observed in shDR4TC-71 cells versus 39.7 ± 3.9% and 67.2 ± 7%, respectively, for the parental or shLacZTC-71 cell lines after 50 ng/mL
TRAIL treatment, and 102.2 ± 15.4% versus 19.5 ± 2.6% and 28 ± 3.7%, respectively, after 100 ng/mL TRAIL treatment. These results indicated that DR4 and DR5 functions may not be redundant. Indeed, even if the loss of DR4 seemed to be compensated by an augmentation of DR5 expression, it is not associated with restoration of TRAIL sensitivity.

Identification of a new DR4 splice transcript encoding a DR4 isoform truncated in the TRAIL-binding domain
To resensitize TRAIL-resistant cell lines to TRAIL by overexpressing DR4, we carried out DR4 cDNA amplification for cloning into lentivirus vector. As expected, using primers encompassing the start codon, and beyond the stop codon of the DR4 sequence (accession number NM_003844), a 1,400 bp fragment was obtained on reverse transcribed RNAs of TC-71 and RD-ES cells (Fig. 3A). Surprisingly, only a smaller fragment (900 bp) was obtained when these primers were used on reverse transcribed RNAs from 293FT cells transfected with a DR4-expressing plasmid (pCMV-sport6-DR4). We suspected that this unexpected isoform could result from an alternative splice site of DR4 transcript because (i) the expected 1,400 bp fragment was amplified with the same primers directly from pCMV-sport6-DR4 DNA plasmid and (ii) an additional fragment of 900 bp was also noticed within RT-PCR products of TC-71 and RD-ES RNAs (Fig. 3A). The 900 bp DR4 cDNA derived from RD-ES RNAs was cloned and sequenced. The
Comparison of both (900 and 1,400 bp) sequences showed a 473 bp deletion at the beginning of DR4 exon 1 to the end of exon 3 and then sequence identity beyond this deletion until the stop codon (Fig. 3B). This deletion including nucleotides 138 to 611 of NM_003844 DR4 sequence identifies an alternative intron as it shows typical intron feet [gt-ag].

The 900 bp DR4 cDNA was designed variant b (bDR4) as it could produce a protein shorter than the 1,400 bp one designed variant a (aDR4). The bDR4 transcript could potentially encode a 310–amino acid protein which corresponds to the 468–amino acid aDR4, but truncated of amino acids 11 to 168 within the extracellular TRAIL-binding domain. However, this truncated region shares 45% identity and 59% similarity with the corresponding DR5 region, whereas the remaining extracellular TRAIL-binding domain shares 76% identity and 84% similarity with the corresponding DR5 one (Fig. 3C).

On the basis of DR5-TRAIL crystal structure and DR5-DR4 similarities, the interactions of aDR4 or bDR4 to TRAIL were simulated. The DR5-TRAIL-binding interface is 1,398 Å² wide, whereas the predicted aDR4-TRAIL one is 1,196 Å². According to our modeling study, the contact of bDR4 with TRAIL seemed largely preserved with a relatively limited interface decrease (882 Å² for the bDR4-TRAIL-binding interface). However, the molecular dynamics simulation of the bDR4-TRAIL interface indicates that the bDR4-TRAIL binding is conserved even without these amino acids.

TRAIL sensitivity is induced by bDR4 overexpression in TRAIL-resistant SK-N-MC cell line

After bDR4 cDNA cloning into lentivirus vector, SK-N-MC cells were transduced with different quantities of virus containing supernatant (20, 50, 100, and 150 μL), then derived cell lines were designed bDR4-20, -50, -100, or -150 SK-N-MC, respectively. As shown in Fig. 4A (left panel), these derived cells produced various levels of bDR4 which were correlated to the volumes of virus containing supernatant. Using higher PAGE resolution, both aDR4 and overexpressed bDR4 isoforms were identified in bDR4-
150 SK-N-MC cells (Fig. 4A, right panel). In addition, bDR4 was detected as a minor isoform in TC-71 and A673 cells and also in the human multiple myeloma cell lines, as shown for an example with the KMM1 cell line. The proportion of bDR4 relative to aDR4 appeared more abundant in A673 cells than in KMM1 cells. SK-N-MC cells transduced with the truncated isoform bDR4 showed a gain of sensitivity to TRAIL-induced apoptosis. Indeed, around 95% of viability was observed after TRAIL treatment for parental, bDR4-20 or bDR4-50 SK-N-MC cells versus 78.9/C6 to 50.4/C6 for bDR4-100 and -150 SK-N-MC cells, respectively (Fig. 4B). Apoptosis assessed by APO2.7-PE staining in flow cytometry showed a 2-fold increase in TRAIL-induced apoptosis for bDR4-100 SK-N-MC cells as compared with parental cells (Fig. 4C).

These results indicated that TRAIL was able to induce apoptosis through the bDR4-truncated isoform, whereas DR5 was not able to transduce TRAIL signaling.

c-FLIP knockdown induces TRAIL sensitivity in originally resistant SK-N-MC cell line

TRAIL-induced apoptosis pathway can be regulated by the competitive recruitment of procaspase-8 and c-FLIP, as respective caspase initiator and negative regulator. As shown in Fig. 5A, procaspase-8 and c-FLIP levels are not correlated to sensitivity or resistance of ES cell lines. Indeed, procaspase-8 levels were higher in only 1 of the 3 TRAIL-sensitive ES cell lines (RD-ES vs. A673 and TC-71) and in 1 TRAIL-resistant cell line as compared with the others (TC-32 vs. EW24, SK-N-MC, SK-ES-1). In addition, similar levels of

Figure 3. New DR4 alternative transcript and its predicted bDR4 protein. A, PCR amplification of DR4 cDNA either after reverse transcription of RNAs of TC-71 and RD-ES ES cell lines, or in 293FT cells transfected with pCMV-sport6-DR4 (pDR4-293FT), or directly from 50 ng of pCMV-sport6-DR4 (pDR4). B, DR4 gene structure is displayed by boxes and triangles for exons and introns, respectively, whose sizes (bp) are indicated. Untranslated regions are shaded. aDR4 cDNA (1,400 bp; NM_003844) with numbered exons displayed by boxes is compared with bDR4 (900 bp; BankIt1442931 bDR4 JF729355) cDNA. The alternative intron containing part of exon 1, exon 2, and part of exon 3 is displayed by a broken line and the 2 nucleotides at both sides of this intron are indicated. C, the aDR4 and bDR4 sequences are aligned against the 69 to 185 amino acids of DR5, which correspond to the resolved DR5 crystal structure. Sequences were aligned using Jalview (41). Amino acids are highlighted using the Zappo color scheme (on the basis of their physicochemical properties), the consensus is displayed as a sequence logo. Disulfide bridges are represented by red lines. D, diagrams of predicted TRAIL (cyan) binding to aDR4 (red) or bDR4 (purple) are shown. The amino acids Ala163, Pro166, and Thr168 which are present in the aDR4–TRAIL–binding interface but absent of the bDR4–TRAIL one are indicated.
c-FLIP were detected in the 4 TRAIL-resistant cell lines (EW24, SK-N-MC, SK-ES-1, and TC-32) as compared with the 3 TRAIL-sensitive ones (RD-ES, A673, and TC-71; Fig. 5A). However, the implication of c-FLIP in DR4 expression in resistant/sensitive cell lines, c-FLIP was implicated in TRAIL resistance of ES cell lines.

In this study, we showed that all ES cell lines expressed DR5/DR4, rather than the presence of decoy receptors or c-FLIP expression. We share the same conclusion, except that, in this study, we showed the pivotal role of DR4 rather than DR5 by 2 complementary approaches: first, DR4 knockdown in TRAIL-sensitive TC-71 cell line induced TRAIL resistance despite DR5 expression and second, DR4 overexpression in resistant cells restored their sensitivity. Therefore, overexpression of DR4 may represent an alternative strategy to restore TRAIL sensitivity in ES cells and models.

During DR4 cloning procedure, we evidenced a splice transcript of DR4 corresponding to a new isoform truncated in the TRAIL-binding domain. Transcriptional modification by alternative splicing is known to be involved in the regulation of programmed cell death (36). Thus, alternative splicing has also been identified in genes of the TRAIL system, including DR4 and DR5; however, the isoform presented here has never been reported. For example, 2 isoforms of the DR5/TRICK2/TRAIL-R2/KILLER differing by a 29 amino acid extension in the extracellular domain are generated by alternative pre-mRNA splicing (37). The inserted sequence may represent a retained intron as it has flanking sequences that match consensus 5’ and 3’ splice sites. The authors hypothesized that the expression of these 2 isoforms of TRICK2/DR5, which have different spacing between the

Discussion

In this study, we showed that all ES cell lines expressed high levels of DR5, whereas DR4 was expressed only by 2 sensitive cell lines of 3, the third one presenting intermediate levels. None of the 4 resistant cell lines express significant levels of DR4. Furthermore, no correlation could be established with decoy receptor expression and TRAIL resistance (not shown). The correlation between TRAIL sensitivity and

![Figure 4](Image 259x693 to 334x723)

**Figure 4.** bDR4 overexpression in TRAIL-resistant SK-N-MC cell line. A, bDR4 isoform was detected by Western blotting in SK-N-MC cells transduced with bDR4-expressing virus contained in 50, 100, or 150 μL of supernatant (left). Both aDR4 and bDR4 were distinguished after high-resolution PAGE and Western blotting in KMM1, TC-71, A673, and bDR4-150 SK-N-MC cell lines (right). B, TRAIL sensitivity was determined by XTT assay for transduced and parental SK-N-MC and A673 cell lines after 72-hour treatment with 50 ng/mL TRAIL. C, percentage of cell death induced by a 48-hour treatment with 100 ng/mL TRAIL was determined by flow cytometry analysis after Apo2.7-PE staining.

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transmembrane domain and the TRAIL-binding domain, might regulate the response to TRAIL. In this study, bDR4 was detected as a minor form in TC-71 and A673 cells and also in the human multiple myeloma cell line KMM1. The proportion of bDR4 relative to aDR4 appeared more abundant in the ES cell line A673 than in KMM1 cells, suggesting that the new DR4 alternative splicing described here could be regulated by cell-specific factors and may not be an aberrant splicing which occurs proportionally to DR4 total expression. Therefore, it seems that DR4 splicing is not influenced by the initial amount of DR4 transcript in ES or in other tumor cells. In our models, the sensitivity of ES cells to TRAIL is not related to the aDR4/bDR4 ratio, but rather to the presence of DR4, expressed as truncated or complete forms, even if DR5 is predominant. This result seems to be specific to ES as it never had been reported in other cancer systems.

In our study, we showed that the truncated DR4 isoform is functional because its overexpression in TRAIL-resistant SK-

Figure 5. c-FLIP knockdown in TRAIL-resistant EW24 and SK-N-MC cell lines. A, c-FLIP and procaspase-8 were detected by Western blot of total cell lysates of 7 ES cell lines. Actin was used as loading control. B, c-FLIP expression was assessed by real-time PCR on reverse transcribed RNAs of EW24 and SK-N-MC cells unmodified or modified with lentivirus-expressing shRNAs which target either β-galactosidase or c-FLIP transcripts (shLacZ and shc-FLIP, respectively). C, c-FLIP protein expression was determined on whole-cell lysates of EW24 cells. D, TRAIL sensitivity was determined by XTT assay for transduced and parental EW24 or SK-N-MC cell lines after 72-hour treatment with 50 ng/mL TRAIL. E, caspase-3 activity was determined by enzymatic assay after 1 to 6 hours incubation of EW24 (left) and SK-N-MC (right) cells in the presence of 50 ng/mL TRAIL. UV exposition during 1 minute was used as positive control.
N-MC cells induced a gain of sensitivity to TRAIL-induced cell death. This result implies potential trimerization of bDR4 as homotrimerization or heterotrimerization with aDR4. At this stage, we have no indication about the trimeric nature of the short form in the absence of TRAIL. Because both the truncated and the complete DR4 isoforms are able to transduce TRAIL signaling, it indicates that the short DR4 isoform does not prevent the DR4 trimerization necessary for TRAIL binding. It is known that TRAIL binds with a higher specificity to DR5 via its loop AA' because the removal of this loop nearly abolishes DR5-TRAIL recognition (34). This TRAIL loop is in direct interaction with the CRD2 domain of DR5/DR4 but not with the CRD1 domain (34). Our modeling study indicates that the CRD2 domain involved in TRAIL binding is maintained in the bDR4 isoform. This suggests that most of the TRAIL–bDR4–binding specificity can be attributed to the CRD2 domain. This would explain why bDR4 is still able to sensitize cells to TRAIL activity.

Another interesting and novel result of this study relies to a second level of regulation of TRAIL resistance in ES, linked to c-FLIP downregulation. Indeed, even if no correlation could be evidenced between c-FLIP expression (or the ratio c-FLIP/caspase-8) and TRAIL sensitivity/resistance in ES cells, we showed that c-FLIP knockdown induced sensitivity in originally TRAIL-resistant SK-N-MC cell line. It is well known that TRAIL-induced apoptosis pathway can be regulated by the competitive recruitment of procaspase-8 and c-FLIP, respectively, as initiator or repressor of caspase-8 activity. Several publications have reported that inhibition of c-FLIP expression could represent an interesting strategy to sensitize TRAIL-resistant cells whatever the initial level of TRAIL expression (38–40). Therefore, using a RNA interference strategy, we showed that c-FLIP knockdown in TRAIL-resistant EW24 or SK-N-MC cells restored TRAIL sensitivity associated with an increased caspase-3 activity. We therefore hypothesize that the involvement of c-FLIP in TRAIL resistance in ES cells may be linked to its differential recruitment within the DISC. In RD-ES, A673, and TC-71 TRAIL-sensitive cell lines, cells express higher levels of DR4 (including both truncated and complete DR4 isoforms) than in resistant cells, but always less than DR5 levels. The intracellular domain of death receptors DR5 or DR4 binds FADD via the DD, then leading to the DISC formation and death signal transduction. The presence of c-FLIP interferes with procaspase-8 through the DISC domain, acting as a competitor and inhibiting the procaspase-8 cleavage and thus caspase-8 activation. In sensitive ES cells, DR4 is expressed together with DR5, inducing a high DISC formation with procaspase-8 recruitment that cannot be competed by c-FLIP. Because no significant difference have been observed in the procaspase-8/c-FLIP ratio between resistant and sensitive ES cells as analysed by Western blotting, other hypotheses could be proposed: (i) the relative availability of both antagonists (procaspase-8 or c-FLIP) is different in the cytoplasm near the DR4 or DR5/FADD/DED complex depending on the presence of lipid rafts, (ii) the relative affinity between FADD (linked to the intracellular domains of death receptors) to procaspase-8 or c-FLIP is different between DR4 and DR5, (iii) the procaspase-8 recruitment is more rapid or effective when DR4 is expressed than with DR5. These hypotheses have been partly confirmed when sensitive cells are treated with shDR4: the level of DR4 is decreased, so procaspase-8 is less recruited in DISC formation, and c-FLIP is therefore able to inhibit caspase-8 cleavage, then to induce TRAIL resistance.

TRAIL-resistant cell lines (TC-32, EW24, SK-ES-1, and SK-N-MC) express low (or no) levels of DR4, but still DR5. The addition of TRAIL induced the DISC formation but in a lesser extent than in DR4-expressing sensitive cells. c-FLIP is therefore able to inhibit caspase-8 cleavage in this complex. When resistant cells are treated with shc-FLIP, apoptosis is restored as the DISC complex could be activated. The other strategy developed to resensitize TRAIL-resistant ES cells is to overexpress DR4 (as truncated or complete isoform), restoring higher DISC formation by procaspase-8 binding to FADD, leading to apoptosis.

In conclusion, by using shDR4 in sensitive cells and overexpressing DR4 in TRAIL-resistant cells, the results presented in this study showed that DR4 is involved in TRAIL sensitivity in ES. We also evidenced a new isoform of DR4 as a consequence of an alternative transcript lacking exons 1 to 3, which is still functional and induced apoptosis through TRAIL binding. Finally, we hypothesized that the resistance/sensitivity in ES cells is linked to the DISC formation regulated by c-FLIP, but being also related to the presence of DR4 (in sensitive cells) or not (resistant cells).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Gaëlle Picarda, Sylvanie Surget, Romain Guiho, et al.


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