Selective targeting of death receptor 5 circumvents resistance of MG-63 osteosarcoma cells to TRAIL-induced apoptosis

Rachel M. Locklin,[1] Ermanno Federici,[1] Belen Espina,[1] Philippa A. Hulley,[1] R. Graham G. Russell,[1] and Claire M. Edwards[2]

[1]Institute of Musculoskeletal Sciences, Botnar Research Centre, Nuffield Department of Orthopaedic Surgery, University of Oxford, Oxford, United Kingdom and [2]Vanderbilt Center for Bone Biology, Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, Tennessee

Abstract

Tumor necrosis factor – related apoptosis-inducing ligand (TRAIL), a tumor necrosis factor superfamily member, targets death receptors and selectively kills malignant cells while leaving normal cells unaffected. However, unlike most cancers, many osteosarcomas are resistant to TRAIL. To investigate this resistance, we characterized the response of MG-63 osteosarcoma cells and hPOB-tert osteoblast-like cells to TRAIL and agonist antibodies to death receptor 4 (DR4) and death receptor 5 (DR5). We found that MG-63 osteosarcoma cells and hPOB-tert osteoblast-like cells show no or very little response to TRAIL or a DR4 agonist, but MG-63 cells undergo apoptosis in response to a DR5 agonist. Analysis of TRAIL receptor expression showed that normal osteoblastic and osteosarcoma cells express a variety of TRAIL receptors but this does not correlate to TRAIL responsiveness. Production of the soluble decoy receptor osteoprotegerin also could not explain TRAIL resistance. We show that TRAIL activates the canonical caspase-dependent pathway, whereas treatment with cycloheximide increases the sensitivity of MG-63 cells to TRAIL and anti-DR5 and can also sensitize hPOB-tert cells to both agents. Proapoptotic and antiapoptotic protein expression does not significantly differ between MG-63 and hPOB-tert cells or change following treatment with TRAIL or anti-DR5. However, sequencing the death domain of DR4 in several osteoblast-like cells showed that MG-63 osteosarcoma cells are heterozygous for a dominant-negative mutation, which can confer TRAIL resistance. These results suggest that although the dominant-negative form of the receptor may block TRAIL-induced death, an agonist antibody to the active death receptor can override cellular defenses and thus provide a tailored approach to treat resistant osteosarcomas. [Mol Cancer Ther 2007;6(12):3219 – 28]

Introduction

Osteosarcoma is the most common malignant bone cancer in youth, the third most common malignancy in children and adolescents, and accounts for 35% of all primary bone malignancies. Before 1970, osteosarcomas were treated with amputation and survival was poor, with 80% of patients dying from metastatic disease. With improvements in chemotherapy protocols, surgical techniques, and radiologic staging studies, long-term survival and cure rates have increased to 60-80% in patients with localized disease. However, major problems associated with chemotherapy still remain, particularly about the frequent acquisition of drug-resistant phenotypes; the associated cytotoxic effects of chemotherapy on normal tissues and organs also remain a serious drawback. Thus, there is a pressing need to develop alternative approaches to osteosarcoma treatment.

Tumor necrosis factor – related apoptosis-inducing ligand (TRAIL) is produced by activated T cells and is expressed as a type 2 transmembrane protein, which can be proteolytically cleaved from the cell surface (1, 2). TRAIL induces apoptosis of various transformed cells *in vitro* and has specific antitumor activity *in vivo*, and it has therefore been hailed as a promising new therapy for many cancers (3, 4). Previous reports have shown that at least 50% of all human cancer cell lines tested from a variety of tissues are sensitive to the apoptotic effects of TRAIL *in vitro*, whereas most normal cells, including osteoblasts (5), are resistant. However, many osteosarcoma cells respond poorly to the cytotoxic effects of TRAIL alone and induction of apoptosis requires additional treatment with other chemotherapeutic agents that induce various changes in the apoptotic pathway (6–9). The resistance of normal cells to TRAIL and the differential sensitivity of cell lines and tumor types is not presently understood. Furthermore, the resistance of osteosarcoma to cytotoxic agents is a serious problem in patient management.

TRAIL interacts with the cell surface through four distinct membrane-bound receptors, inducing apoptosis by acting through death receptor 4 (DR4) and death

doi:10.1158/1535-7163.MCT-07-0275

receptor 5 (DR5), which have cytoplasmic death domains. In cells that express both death receptors, heterocomplexes can be formed (10). Apoptosis can be triggered independently through either DR4 or DR5; DR4 and DR5 do not seem to have distinct functions and behave similarly, binding TRAIL with comparable binding affinities (11). TRAIL also binds to the decoy receptors DcR1, which lacks a death domain and is bound to the cell surface via a glycosyl-phosphatidylinositol anchor, and DcR2, which has a truncated death domain, as well as to another soluble member of the tumor necrosis factor–related superfamily expressed by osteoblastic cells, the decoy receptor osteoprotegerin (12, 13). Cells may therefore express different patterns of death-inducing and decoy receptors, and it has been suggested that expression of the decoy receptors may confer TRAIL resistance (5, 11, 14, 15).

TRAIL forms a homotrimer containing a zinc atom essential for stability and optimal activity (16). On binding to the death receptors, TRAIL initiates apoptosis by inducing receptor trimerization, clustering of the intracellular death domains, and the recruitment of the specific cytoplasmic protein Fas-associated death domain (FADD) (17, 18). FADD contains both a death domain and death effector domain, which allow it to recruit caspase-8 to DR4 and/or DR5 shortly after binding to form the death-inducing signaling complex (10, 17, 19, 20). Additional studies have shown that, in some cases, tumor necrosis factor receptor 1–associated death domain may also be recruited by both DR4 and DR5, resulting in both activation of nuclear factor-κB and induction of c-Jun NH$_2$-terminal kinase (JNK) activity (21–24). Overexpression of DR5 is also able to induce spontaneous cell death by clustering of the death domains (25).

One study has shown that osteogenic sarcoma cells in culture (BTK-143) gradually acquire TRAIL resistance due to progressive acquisition of the decoy receptor DcR2 (26). However, the pattern of receptor expression does not correlate to the TRAIL responsiveness of the cells concerned, and expression of decoy receptors is not sufficient to confer resistance (27, 28). Whereas the death receptors are also able to activate the antiapoptotic nuclear factor-κB pathway, the decoy receptors are believed to lack this property and in contrast are able to block nuclear factor-κB activation via the death receptors. Thus, loss of decoy receptor activity could in fact lead to a prosurvival signal.

There is some recent evidence that DNA-damaging chemotherapy up-regulates expression of DR4 and DR5, increasing the response to TRAIL (29), whereas irradiation may specifically up-regulate DR5 (30). These facts suggest that an agonist antibody to a TRAIL receptor may be a good candidate for anticancer therapy. Use of such TRAIL receptor-selective therapy could also avoid escape from the effects of TRAIL via decoy receptor–mediated antagonism, allowing the use of a lower therapeutic dose. Such an approach has not hitherto been used in the treatment of osteosarcoma and thus may provide an important new approach to treatment of this difficult disease.

Materials and Methods

Reagents

Recombinant human osteoprotegerin, anti-human osteoprotegerin neutralizing antibody, recombinant human TRAIL, and anti-human DR5 agonist antibody were purchased from R&D Systems. Anti-human DR4 agonist antibody, soluble DR5 protein, and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody were purchased from Abcam, and anti-human DR4 antagonist antibody and anti-FLIP monoclonal antibody were from Alexis. Polystyrene microspheres were from Polysciences, Inc. Fluorescence-activated cell sorting supplies were provided by Becton Dickinson UK Ltd. Caspase-3 inhibitor II (Z DEVD FMK), caspase-8 inhibitor II (Z IETD FMK), and caspase-9 inhibitor I (Z LEHD FMK) were all from Calbiochem/VWR. Anti-Bim polyclonal antibody and anti-cleaved caspase-3 antibody were purchased from Calbiochem. Anti-Bax polyclonal antibody was from Cell Signaling Technology (New England Biolabs). Anti-Bcl-2, anti-Bcl-X$_L$, and anti-Mcl-1 antibodies were from Santa Cruz Biotechnology. All other chemicals were from Sigma-Aldrich Ltd. unless otherwise stated.

Cell Culture

The human osteosarcoma cell line MG-63 was obtained from the American Type Culture Collection and the immortalized human osteoblast-like cell line hPOB-tert (31) was kindly provided by Nestec Ltd. (Nestlé Research Center). Primary human bone marrow cells were isolated from explants of trabecular bone marrow obtained, with informed consent and the approval of the hospital ethics committee, from patients undergoing routine hip replacement surgery. All were cultured as previously described (32, 33).

Treatment with TRAIL or TRAIL Receptor Agonist Antibodies

TRAIL and anti-DR5 were supplied lyophilized and reconstituted in PBS with 0.1% bovine serum albumin. Anti-DR4 was supplied as a solution in PBS. Both TRAIL and anti-DR5 were added in solution to culture medium, whereas anti-DR4 needed to be immobilized on plastic to exert its agonist effect. The antibody was allowed to bind to plastic, either the tissue culture plate or 1 μm polystyrene beads of the same surface area, at 37°C for 2 h, after which unbound antibody was washed off before the cells were added. Cells were treated with TRAIL or the receptor agonist antibodies for 72 h. This time was chosen because although there was a small response at 24 h, differences between treatment conditions were much more evident at 72 h.

Measurement of Osteoprotegerin Production

Osteoblast-like cells were plated at a density of 2.5 × 10^4/mL into 24-well plates (Costar) and allowed to adhere in their usual culture medium. Osteoprotegerin concentration was measured by ELISA using a commercially available kit (R&D Systems) as described previously (32).

Analysis of Receptor Expression by Flow Cytometry

Cells were trypsinized and then resuspended in tissue culture medium and incubated at 37°C, with gentle mixing,

for 3 to 4 h to allow regeneration of the receptors. They were then washed once in 1 mL wash buffer (PBS containing 1% FCS), resuspended in 100 μL wash buffer, and labeled with mouse anti-human DR4, mouse anti-human DR5 (Diaclone), mouse anti-human DcR1, or mouse anti-human DcR2 (R&D Systems) or an isotype control mouse IgG_1 (Diaclone) or IgG_{2B} (R&D Systems) for 30 min. All antibodies were directly conjugated to phycoerythrin. Cells were then washed twice in PBS and resuspended in 300 μL PBS for flow cytometric analysis. The level of phycoerythrin fluorescence was measured using a FACS-Calibur flow cytometer (Becton Dickinson).

Identification and Quantification of Apoptotic Cells

Cells were plated at a density of 2×10^4/mL into 24-well plates (Costar) and allowed to adhere for 24 h. Cells were then treated with either 50 ng/mL TRAIL or 1 μg/mL anti-DR5 or exposed to plastic previously coated with 100 μg/mL anti-DR4. The proportion of apoptotic cells was determined either by measurement of Alamar Blue fluorescence or by using a fluorescence *in situ* nick translation assay. In many cases, the measurement of apoptosis was conducted in parallel with cell counts. For the nick translation assay, the presence of apoptotic cells was detected by flow cytometric analysis after the incorporation of FITC-labeled dUTP into DNA strand breaks as described previously (34). The nick translation assay, originally proposed by Nose and Okamoto (35), is very sensitive, allows detection and quantification of both DNA damage and repair, and distinguishes between various types of induced damage, including apoptosis. Extensive DNA degradation is a characteristic event that occurs in the late stages of apoptosis. Cleavage of the DNA yields characteristic single-strand breaks (''nicks''), which can be detected by enzymatic labeling of the free 3′-OH termini with modified nucleotides.

Apoptosis was confirmed in separate experiments by visualization of nuclear morphology after 4′,6-diamidino-2-phenylindole staining (DAPI). Cells were fixed in 4% formaldehyde, stained with 1 μg/mL 4′,6-diamidino-2-phenylindole, and visualized as described previously (32).

Analysis of Protein Expression by Western blot

Following appropriate treatment, subconfluent cell cultures were collected, together with medium, and washed in buffer containing 1 mmol/L each of EDTA and phenylmethylsulfonyl fluoride and then lysed in buffer consisting of 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% NP40 and 0.1% SDS in TBS. The cell suspension was sonicated for 15 s and then ultracentrifuged at 12,000 rpm for 10 min at 4°C and protein content of the supernatant was measured by the detergent-compatible bicinchoninic acid protein assay (Pierce). Equal amounts of each extract were then added to 0.5 volumes 3× Laemmli sample buffer (Bio-Rad) and denatured at 95°C for 5 min.

Samples were subjected to SDS-PAGE on 10%, 12%, or 14% gels followed by transfer to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in TBS with 3-10% fat-free milk powder and 0.1% Tween 20 at room temperature before incubation with optimal dilutions of the appropriate primary antibody overnight at 4°C. Secondary antibodies conjugated to horseradish peroxidase [anti-rabbit IgG peroxidase-linked species-specific $F(ab')_2$ fragment from donkey (Amersham Biosciences) or immunopure goat anti-rabbit IgG H+L peroxidase conjugated (Pierce)] were then added. Antigen-antibody complexes were detected using either enhanced chemiluminescence reagents (Amersham Biosciences) or SuperSignal (Pierce) and light emission was captured using CL-XPosure film (Pierce) with an exposure time varying from 10 s to 60 min. Membranes were subsequently stripped and then reprobed for the loading control glyceraldehyde-3-phosphate dehydrogenase.

Treatment with Caspase Inhibitors

Caspase activity was blocked by treating cell cultures with inhibitors of caspase-3 (Z DEVD FMK), caspase-8 (Z IETD FMK), and caspase-9 (Z LEHD FMK). The inhibitors were dissolved in DMSO and added to cultures at a final concentration of 100 μmol/L at the same time as other treatments; 1% DMSO alone was used as control.

Caspase Assays

Activity of caspase-3/7 was measured in cultured cells using a Promega Apo-ONE Homogeneous Caspase-3/7 assay according to the manufacturer's instructions.

Sequence Analysis

To identify mutations in DR4, sections of exon 10 were amplified and sequenced. DNA was isolated from cells using the Qiagen FlexiGene kit following the manufacturer's instructions. Primers were designed to amplify the appropriate section based on the sequence of DR4 published by Kim et al (36). DNA (5 μL) was amplified by PCR in a total volume of 50 μL containing 2 mmol/L $MgCl_2$, 50 mmol/L KCl, 20 mmol/L Tris-HCl, 200 μmol/L deoxynucleotide triphosphates, 0.2 μmol/L primers (described below), and 1 unit Platinum Taq DNA polymerase (Invitrogen). Amplification was done in an MBS satellite thermal cycler (Thermo Hybaid). The first cycle was 94°C for 10 min followed by denaturation at 94°C, annealing at 65°C, and extension at 72°C, with a final cycle of 72°C for 10 min.

The primer sequences were as follows: 5′-TCATCTGGC-TGTCTCTGTGG-3′ (forward) and 5′-AACACCTAAGAG-GAAACCTCTGG-3′ (reverse).

PCR products were purified using Millipore MANU3050 filter plates, then 5 μL of the product were amplified in a 20 μL sequencing PCR containing 2 μL Ready Reaction Premix, 2 μL BigDye sequencing buffer, 1 μL 3.2 μmol/L either primer, and 10 μL water for 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 s. The PCR product was purified using Sephadex G50 gel and sequenced using an ABI Prism 3700 sequencer with SeqScape software.

Statistical Analysis

All of the experiments were done in quadruplicate and repeated on at least two separate occasions unless stated otherwise. Results are expressed as mean ± SE. Comparisons between control and treatment groups were done using Student's *t* test where only two groups were

compared. Comparisons between multiple groups were done using a one-way ANOVA with Tukey's or Dunnett's post hoc test. Significance was assumed at $P < 0.05$.

Results

Osteoblastic Cells Vary in Their Response to TRAIL or the Agonist Antibodies Anti-DR4 and Anti-DR5

Analysis of cell viability showed that none of the cell lines tested respond to TRAIL, except for a small response at very high concentrations in MG-63 cells after 72 h. In contrast, an agonist antibody to DR5 potently reduces the viable cell number of MG-63 cells but has no effect on other cells tested over the same period of exposure (Fig. 1A).

Nontumoral Osteoblastic Cells and Osteosarcoma Cells Express a Variety of TRAIL Receptors, But This Does Not Correlate with TRAIL Responsiveness

Analysis of cell surface receptor expression by fluorescence-activated cell sorting showed that MG-63 cells express both of the death receptors DR4 and DR5 but neither of the decoy receptors, whereas hPOB-tert cells express DR5 and DcR2 (Fig. 1B). Of other cell lines tested, Saka stromal cells, Cal72 osteosarcoma cells, and primary human osteosarcoma cells express DR4, DR5, and DcR2; primary human marrow stromal cells express DR5 and DcR2; and 143B osteosarcoma cells express DR4 and DR5, whereas SaOS-2 osteosarcoma cells do not appear to express any of the TRAIL receptors on the cell surface.

MG-63 Osteosarcoma Cells and hPOB-tert Osteoblast-like Cells Do Not Respond to TRAIL, But MG-63 Cells Undergo Apoptosis in Response to a DR5 Agonist

Dose-response analysis of MG-63 human osteosarcoma cells and hPOB-tert osteoblast-like cells in culture with TRAIL or the agonists anti-DR4 or anti-DR5 showed that hPOB-tert cells do not respond to TRAIL with a reduction in viability, whereas MG-63 cells show a small reduction in viability of 15% to 20% as measured by Alamar Blue fluorescence (Fig. 2A). However, MG-63 cells do respond at very high concentrations with a reduction in viability of ~90% at a concentration of 1,000 ng/mL. In contrast, anti-DR5 has a potent effect on MG-63 cells but no effect on hPOB-tert cells, and the reduction in viability is due to apoptosis as confirmed by nick translation assay (Fig. 2B). The agonist anti-DR4 also induces a small degree of apoptosis in MG-63 cells at very high concentrations; again, hPOB-tert cells are not affected (Fig. 2A). The presence of apoptotic nuclei in anti-DR5-treated cultures, and to a far lesser extent in anti-DR4-treated cultures, was confirmed by 4′,6-diamidino-2-phenylindole staining (Fig. 2C). The agonist antibodies were also confirmed to have no effect on normal human nonmalignant osteoblasts or bone marrow stromal cells (data not shown).

The Resistance of Osteoblastic Cells to TRAIL Is Not Due to Their Production of the Soluble Decoy Receptor Osteoprotegerin

Osteoprotegerin is known to bind TRAIL, and we have shown that recombinant human osteoprotegerin, or conditioned medium from osteoblast-like cells that contains high
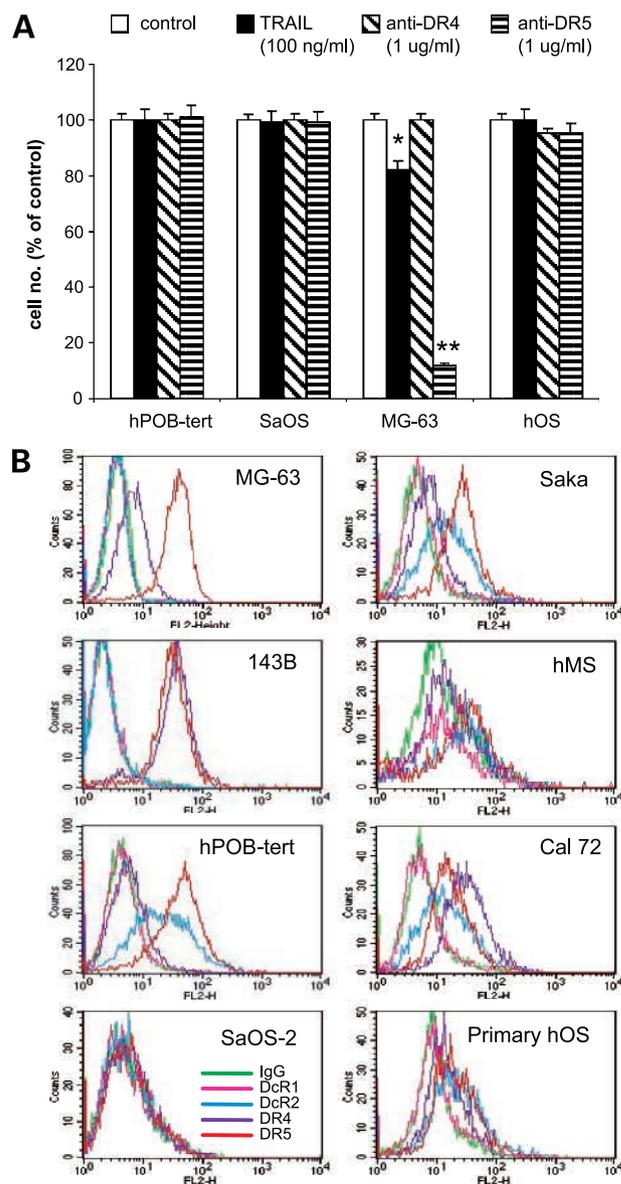

Figure 1. Osteoblastic cells showed variable responsiveness to TRAIL or the agonist antibodies anti-DR4 or anti-DR5. **A,** of a range of cell lines tested, only MG-63 cells showed any response to TRAIL or anti-DR5. *Columns,* mean of eight replicates from one of two experiments; *bars,* SE. **B,** analysis of cell surface TRAIL receptor expression by a variety of human osteoblast-like cell lines and primary cultures showed variable patterns of expression. 143B and MG-63 osteosarcoma cells express DR4 and DR5; Cal72 osteosarcoma cells, Saka stromal cells, and primary osteosarcoma cells express DcR2, DR4, and DR5; while the nontumoral osteoblastic line hPOB-tert and primary marrow stromal cells express DcR2 and DR5, whereas SaOS-2 cells do not appear to express any TRAIL receptors on the cell surface. The pattern of receptor expression was not altered by exposure to TRAIL (data not shown). *, $P < 0.05$; **, $P < 0.01$, compared with appropriate control.

levels of osteoprotegerin (measured by ELISA), can block the effects of TRAIL *in vitro* (32). It has been suggested that the large amounts of osteoprotegerin produced by MG-63 cells could be responsible for conferring their immunity to

TRAIL. However, treating the cells with TRAIL in the presence of excess of a neutralizing antibody to osteoprotegerin (20 μg/mL) did not render MG-63 cells more susceptible to TRAIL (Fig. 3A). The neutralizing antibody also had no effect on hPOB-tert cells, which produce levels of osteoprotegerin 1,000-fold lower that MG-63 cells and do not respond to TRAIL alone (Fig. 3A).

Apoptosis Induced by TRAIL Agonists Is due to Activation of the Canonical Caspase-Dependent Pathway

Measurement of caspase-3/7 activity in MG-63 cell cultures confirmed that whereas anti-DR5 induced a large increase in activity, anti-DR4 had no effect except at very high concentrations (Fig. 3B). To further confirm that TRAIL or the anti-DR5 agonist activates the classic
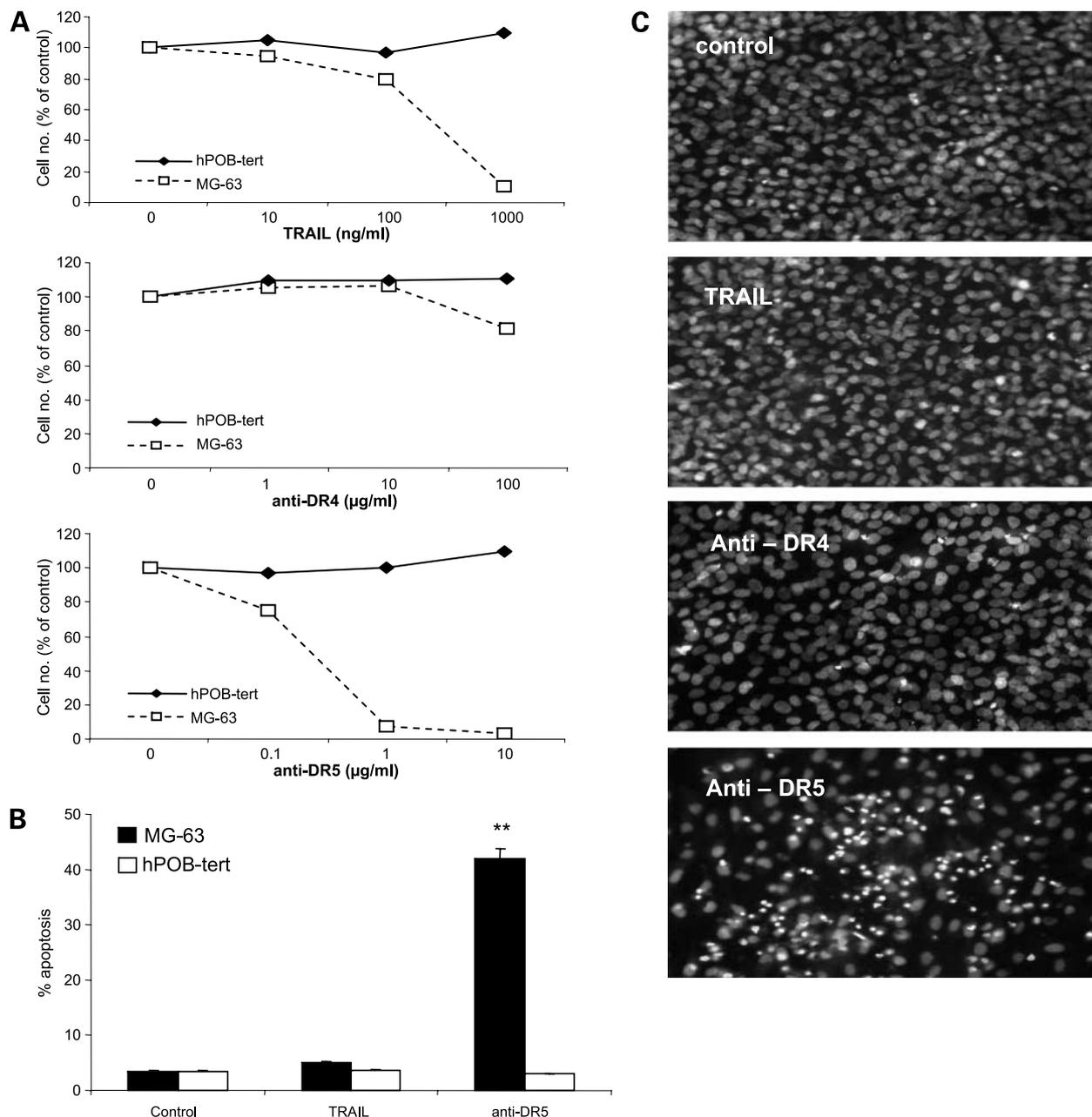
Figure 2. **A,** TRAIL and the agonist antibody anti-DR4 both caused a decrease in cell number of MG-63 cells at the highest concentrations tested, whereas anti-DR5 had a potent effect on MG-63 cells at all doses; in contrast, hPOB-tert cells were completely unaffected. **B,** nick translation assay confirmed that MG-63 cell death is due to apoptosis and showed that anti-DR5 (1 μg/mL) was able to increase apoptosis of MG-63 cells from a basal level of <4% to >40%. **C,** 4′,6-diamidino-2-phenylindole staining of MG-63 cell cultures showed extensive numbers of cells with the nuclear morphology typical of apoptotic cells in cultures treated with anti-DR5 (1 μg/mL) and to some extent in those treated with anti-DR4 (100 μg/mL) but not in those treated with TRAIL (50 ng/mL) after 72-h exposure. **,** $P < 0.01$, compared with control. Data show a typical result obtained in at least three experiments with four replicates.
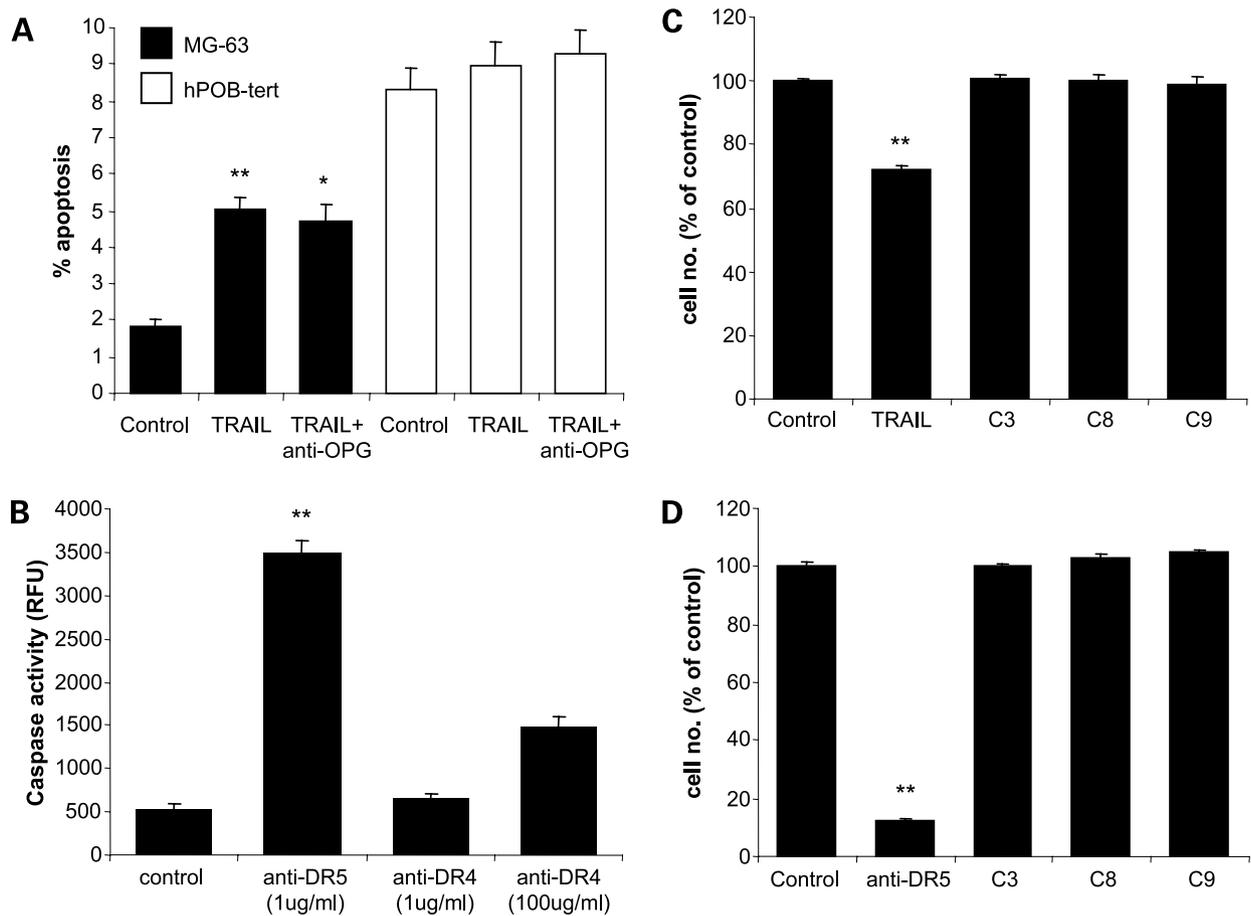
Figure 3. **A,** the addition of a neutralizing antibody to osteoprotegerin (*OPG*; 20 μg/mL) in combination with TRAIL (50 ng/mL) had no effect on levels of apoptosis in cultures of MG-63 or hPOB-tert cells, confirming that osteoprotegerin produced by these cells is not responsible for any protective effect. **B,** treatment of MG-63 cells with anti-DR4 (1 μg/mL) resulted in a significant increase in caspase-3 activity, whereas anti-DR4 had very little effect except at the highest concentration (100 μg/mL). *RFU,* relative fluorescence units. Treating MG-63 cells with TRAIL (50 ng/mL; **C**) or anti-DR5 (1 μg/mL; **D**) in combination with caspase inhibitors completely blocked the reduction in cell numbers, showing that cell death involves the caspase-dependent apoptosis pathway in both cases. *C3,* inhibitor of caspase-3; *C8,* inhibitor of caspase-8; *C9,* inhibitor of caspase-9. *, $P < 0.05$; **, $P < 0.01$, compared with appropriate control. Data show a typical result obtained in at least three experiments with four replicates.

apoptosis pathway, we treated MG-63 cells with TRAIL (Fig. 3C) or anti-DR5 (Fig. 3D) together with inhibitors of caspase-3, caspase-8, or caspase-9. Any one of the inhibitors was able to completely reverse the effect of the agonist.
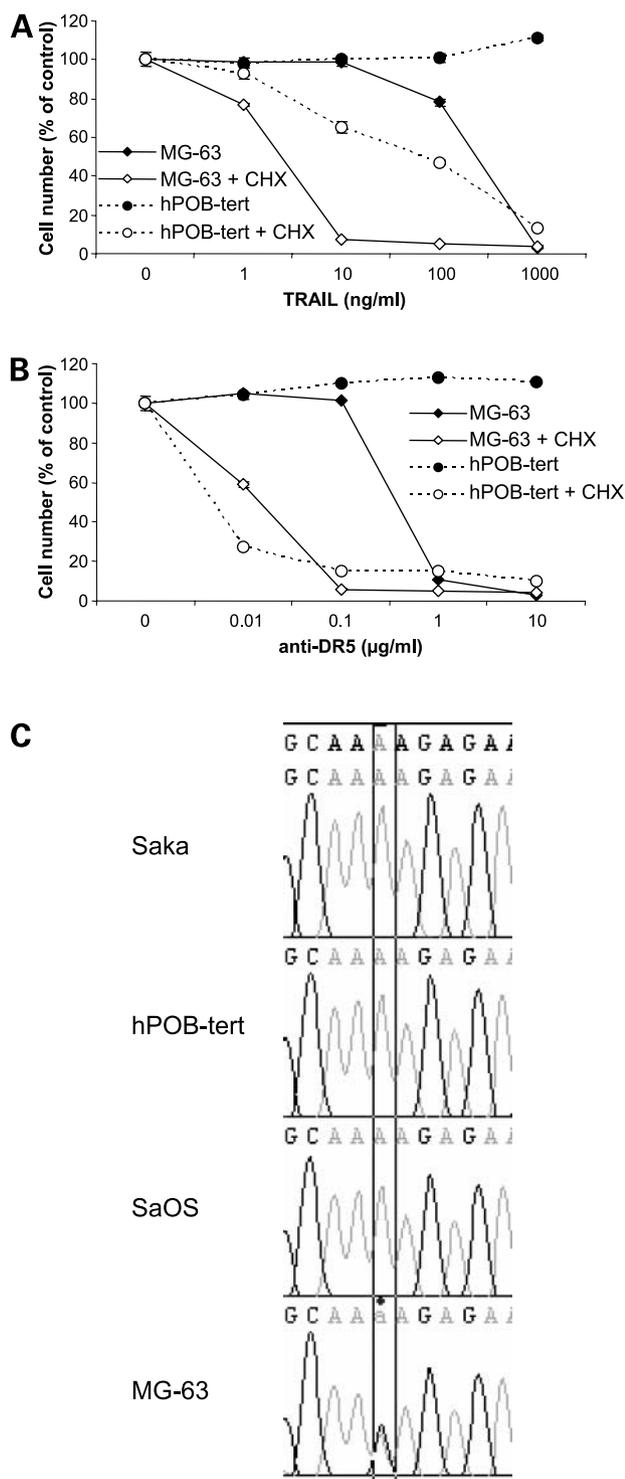
Treatment with Cycloheximide Increases the Sensitivity of MG-63 Cells to TRAIL and Anti-DR5 and Is Also Able to Sensitize hPOB-tert Cells to Both Agents

Cycloheximide inhibits protein translation and as such can shed light on whether new protein synthesis is involved in protection from apoptosis. Although MG-63 cells are largely unresponsive to TRAIL at normal concentrations, very high concentrations (1,000 ng/mL) do induce an effect, and this sensitivity can be increased by treatment with cycloheximide (1 μg/mL). In hPOB-tert cells, which are completely unresponsive to TRAIL at any of the concentrations tested, cotreatment with cycloheximide was also able to confer TRAIL sensitivity on these cells (Fig. 4A). A similar increase in sensitivity was observed

with anti-DR5 in combination with cycloheximide (Fig. 4B). This suggested that synthesis of antiapoptotic protein(s) could contribute to the defense of the cells against TRAIL-induced apoptosis.

Expression of Major Apoptotic and Antiapoptotic Proteins Does Not Differ Significantly between MG-63 and hPOB-tert Cells, and No Changes Are Apparent following Treatment with TRAIL or Anti-DR5

We analyzed expression of the antiapoptotic protein cFLIP, a key component of the extrinsic pathway, as well as Bcl-2, Bcl-X_L and Mcl-1, antiapoptotic proteins involved in the intrinsic pathway characterized by activation of caspase-9, which can augment the response to extrinsic signals. An increase in expression of any of these proteins could thus confer a protective effect. However, the difference in sensitivity to TRAIL between MG-63 and hPOB-tert cells could not be explained by any significant differences in the basal levels of cFLIP, Bcl-2, Bcl-X_L, or

Figure 4. **A,** treatment with cycloheximide (*CHX*; 1 mg/mL) in combination with TRAIL was able to greatly increase the cell death induced by TRAIL in MG-63 cells and also to sensitize otherwise insensitive hPOB-tert cells to TRAIL-induced cell death. **B,** a similar effect was observed with the agonist anti-DR5. *Points,* mean of four replicates from at least two experiments; *bars,* SE. **C,** sequencing exon 10 (encoding the death domain) of DR4 from several osteoblast-like cell lines revealed that only MG-63 cells were heterozygous for a single-base substitution, known to result in a dominant-negative effect of signaling through this receptor.

Mcl-1 proteins, as analyzed by Western blotting (data not shown). Analysis of the cells following treatment with TRAIL or anti-DR5 also showed no significant changes. Some other cells are reported to have developed TRAIL resistance as a result of loss of caspase expression (37); however, Western blotting also showed normal levels of expression of both caspase-3 and caspase-8.

MG-63 Osteosarcoma Cells Are Heterozygous for a Dominant-Negative Mutation in DR4

Sequencing of exon 10 containing the death domain of DR4 from several osteoblast-like cell lines revealed that MG-63 cells alone are heterozygous for a single-base substitution previously identified in other cell types (36) as a dominant-negative form of the TRAIL death receptor (Fig. 4C). This A to G substitution results in an arginine to lysine alteration at codon 441.

Discussion

In this study, we show that resistance of osteosarcoma cells to TRAIL can be overcome by the use of the appropriate receptor agonists, thus opening up new therapeutic possibilities for conditions that have been hitherto very difficult to treat.

Both MG-63 osteosarcoma cells and hPOB-tert human osteoblast-like cells are resistant to the proapoptotic effect of TRAIL. However, MG-63 cells, but not hPOB-tert cells, undergo apoptosis in response to the TRAIL receptor agonist antibody anti-DR5. Expression of the decoy receptors has been suggested to confer TRAIL resistance (5, 14, 26), and hPOB-tert cells express high levels of both DR5 and DcR2, suggesting expression of the decoy receptor as an obvious defense mechanism. However, hPOB-tert cells are also unaffected by the agonist anti-DR5, showing that expression of the decoy receptor is not responsible for conferring resistance. We also show that the resistance of MG-63 cells is not due to their innate or acquired expression of decoy receptors, as they respond to TRAIL at high concentrations and do not express the decoy receptors on the cell surface, either constitutively or following TRAIL exposure. Similarly, expression of the decoy receptor osteoprotegerin is a possible mechanism by which cells may protect themselves from TRAIL (32) and osteoprotegerin is known to act as a survival factor for other cancer cell types, including breast and prostate cancer (38, 39). We have previously shown that osteoprotegerin produced by osteoblastic cells can protect sensitive cells against TRAIL-induced apoptosis, suggesting that osteoprotegerin may function as a paracrine survival factor in the bone marrow microenvironment (32). However, we show that blocking the action of osteoprotegerin by the addition of an excess of a neutralizing antibody to osteoprotegerin does not render MG-63 cells sensitive to TRAIL.

In some cases, clustering of death receptors in the absence of binding can result in cell death, and some agonist antibodies have been shown to result in receptor clustering (40). Thus, clustering of DR5 when bound by the

agonist anti-DR5 is a possible mechanism underlying the apoptotic effect in MG-63 cells; however, as this effect is not observed in hPOB-tert cells, which also express high levels of DR5, it is unlikely to be responsible for the resistance.

We confirm that the action of TRAIL and anti-DR5 in inducing apoptosis involves the classic caspase pathway, as shown by increased expression and activity of caspase-3/7 and the blocking of apoptosis by caspase inhibitors. A recent study found that one mechanism of TRAIL resistance involved loss of procaspase-8 protein expression, likely due to a point mutation in the gene (41). However, Western blotting showed expression of caspase-3 and caspase-8 by MG-63 cells in our study (data not shown).

Other possible mechanisms of resistance include changes in expression of apoptotic or antiapoptotic proteins further down the signaling pathway, such as increased expression of cFLIP, the inhibitory caspase-8 analogue, which has been reported in several other cell types and cancer cell lines with varying TRAIL responses (36, 42). However, Western blot analysis has shown no significant difference in the basal levels of any proteins examined.

One recent study identified several homozygous mutations in DR4 in MG-63 cells (43). These mutations were absent in SaOS-2 cells that exhibited a dose-dependent sensitivity to TRAIL. A polymorphism in the death domain of DR4 has also been identified in a variety of cancer cell lines (36), leading to dominant-negative inhibition of cell signaling; this mutation in exon 10 was an A to G substitution resulting in an arginine to lysine alteration at codon 441 and was identical to one of those identified in MG-63 cells. Thus, changes to the receptors could affect signaling while leaving receptor expression unaffected.

We show that MG-63 cells are heterozygous for a mutation in DR4, which, in other cell types, can confer resistance to TRAIL. This polymorphism has been identified in DNA isolated from the blood of 20% of the normal population and inhibits DR4-mediated cell killing in a dominant-negative fashion (36). This would suggest that this mechanism could underlie the resistance of MG-63 cells to TRAIL in our study. This hypothesis is supported by another recent study showing that loss of DR4 expression is sufficient to confer TRAIL resistance in cells expressing both DR4 and DR5 (41). However, because MG-63 cells express both DR4 and DR5, with DR5 being present at far higher levels than DR4, there is still the possibility that TRAIL could act through DR5. In fact, there is some recent evidence that DR5 may make a greater contribution to apoptosis signaling than DR4 (16, 44), although this remains controversial and may depend on cell type (45). DR5 can also be up-regulated by irradiation or chemotherapy, leading to an increased response to TRAIL (44). These differences may be the result of differing responses to multimerization of TRAIL, with DR5 responding to higher-order multimerization as suggested by studies with cross-linked or non–cross-linked TRAIL (41, 46); again, this is controversial (16). Thus, it is possible that the DR5 agonist antibody may induce apoptosis by causing multimerization of the receptor to a greater extent

than TRAIL alone. However, the TRAIL receptor acts as a trimer and may form heterotrimers as well as homotrimers, although the way in which these receptors interact in a heterotrimer is not known (10). The dominant-negative DR4 could thus trimerize with either one or two normal DR5 species and block their function, in addition to forming inactive homotrimers. Thus, a dominant-negative mutation in DR4 in MG-63 cells seems a likely scenario for rendering the cells refractive to TRAIL treatment. This hypothesis is supported by the lack of effect of the agonist antibody anti-DR4 on MG-63 cells, except at very high concentrations when it is possible it may act through the normal allele; however, this would only occur in trimers of the normal form, which are likely to occur at a low rate compared with trimers of the mutant DR4 or trimers of this in combination with normal DR4 or DR5 species. This suggests that, in the presence of TRAIL that binds to both DR4 and DR5, the dominant-negative form of the receptor may block signaling except at concentrations that are high enough to saturate all available receptors and override the effect, whereas the agonist anti-DR5, acting through DR5 alone, induces apoptosis.

Treatment of both MG-63 and hPOB-tert cells with cycloheximide is able to sensitize both cell lines to TRAIL. This suggests that the resistance of hPOB-tert cells and of MG-63 cells to physiologic levels of TRAIL, is mediated by some other mechanism involving protein synthesis, which is blocked by cycloheximide treatment. As the hPOB-tert cells are a nontumoral cell line, it is likely that this mechanism reflects the resistance of most normal cells to TRAIL, whereas in MG-63 cells the process of transformation may have involved acquisition of an additional resistance mechanism to avoid the host immune response to cancerous cells. Exactly what proteins are involved in the resistance of nontransformed cells remains to be investigated.

A very recent publication (29) has shown that treatment of osteosarcoma cells, including MG-63 cells, with sulforaphane induces an increase in DR5 expression, which leads to an increased response to TRAIL. Although both sulforaphane and TRAIL were largely ineffective alone, when used in combination they were able to induce a significant increase in apoptosis. This raises the possibility that sulforaphane treatment in combination with a DR5 agonist would be even more effective in providing a targeted treatment for osteosarcoma as well as other cancers and would have the added benefit of being harmless to nonmalignant cells, unlike other antitumor agents such as etoposide or doxorubicin.

In conclusion, these findings show that an agonist antibody to an active TRAIL receptor provides a mechanism for overcoming the resistance of cells to the cytotoxic effects of TRAIL, which cannot be overridden by the intrinsic defense mechanisms of the cells. A detailed knowledge of the active receptors expressed by a particular tumor could thus enable treatment to be tailored specifically for that cell type, making treatment far more effective as well as reducing side effects and thus improving

patients' quality of life. Thus, the use of TRAIL receptor agonists could provide a novel therapeutic approach to cancers, such as osteosarcomas, which have hitherto proven extremely difficult to treat using existing therapies.

Acknowledgments

References

1. Martinez-Lorenzo MJ, Anel A, Gamen S, et al. Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles. J Immunol 1999;163:1274 – 81.

2. Mariani SM, Krammer PH. Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. Eur J Immunol 1998;28:1492 – 8.

3. Yagita H, Takeda K, Hayakawa Y, Smyth MJ, Okumura K. TRAIL and its receptors as targets for cancer therapy. Cancer Sci 2004;95:777 – 83.

4. Almasan A, Ashkenazi A. Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. Cytokine Growth Factor Rev 2003;14: 337 – 48.

5. Atkins GJ, Bouralexis S, Evdokiou A, et al. Human osteoblasts are resistant to Apo2L/TRAIL-mediated apoptosis. Bone 2002;31:448 – 56.

6. Hotta T, Suzuki H, Nagai S, et al. Chemotherapeutic agents sensitize sarcoma cell lines to tumor necrosis factor-related apoptosis-inducing ligand-induced caspase-8 activation, apoptosis and loss of mitochondrial membrane potential. J Orthop Res 2003;21:949 – 57.

7. Mirandola P, Sponzilli I, Gobbi G, et al. Anticancer agents sensitize osteosarcoma cells to TNF-related apoptosis-inducing ligand downmodulating IAP family proteins. Int J Oncol 2006;28:127 – 33.

8. Bu R, Borysenko CW, Li Y, Cao L, Sabokbar A, Blair HC. Expression and function of TNF-family proteins and receptors in human osteoblasts. Bone 2003;33:760 – 70.

9. Evdokiou A, Bouralexis S, Atkins GJ, et al. Chemotherapeutic agents sensitize osteogenic sarcoma cells, but not normal human bone cells, to Apo2L/TRAIL-induced apoptosis. Int J Cancer 2002;99:491 – 504.

10. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity 2000;12:611 – 20.

11. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG. The novel receptor TRAIL-R4 induces NF-κB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 1997;7:813 – 20.

12. Kimberley FC, Screaton GR. Following a TRAIL: update on a ligand and its five receptors. Cell Res 2004;14:359 – 72.

13. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ 2003;10:66 – 75.

14. Sheridan JP, Marsters SA, Pitti RM, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 1997; 277:818 – 21.

15. Drosopoulos KG, Roberts ML, Cermak L, et al. Transformation by oncogenic RAS sensitizes human colon cells to TRAIL-induced apoptosis by up-regulating death receptor 4 and death receptor 5 through a MEK-dependent pathway. J Biol Chem 2005;280:22856 – 67.

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

17. Kuang AA, Diehl GE, Zhang J, Winoto A. FADD is required for DR4- and DR5-mediated apoptosis: lack of trail-induced apoptosis in FADD-deficient mouse embryonic fibroblasts. J Biol Chem 2000;275: 25065 – 8.

18. Hymowitz SG, Christinger HW, Fuh G, et al. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. Mol Cells 1999;4:563 – 71.

19. Bodmer JL, Holler N, Reynard S, et al. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat Cell Biol 2000;2:241 – 3.

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

21. Chaudhary PM, Eby M, Jasmin A, Bookwalter A, Murray J, Hood L. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-κB pathway. Immunity 1997;7:821 – 30.

22. Schneider P, Thome M, Burns K, et al. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-κB. Immunity 1997;7:831 – 6.

23. Hu WH, Johnson H, Shu HB. Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-κB and JNK activation and apoptosis through distinct pathways. J Biol Chem 1999;274:30603 – 10.

24. MacFarlane M, Cohen GM, Dickens M. JNK (c-Jun N-terminal kinase) and p38 activation in receptor-mediated and chemically-induced apoptosis of T-cells: differential requirements for caspase activation. Biochem J 2000;348 Pt 1:93 – 101.

25. Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ, Bell JI. TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. Curr Biol 1997;7:693 – 6.

26. Bouralexis S, Findlay DM, Atkins GJ, Labrinidis A, Hay S, Evdokiou A. Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy. Br J Cancer 2003;89: 206 – 14.

27. Griffith TS, Lynch DH. TRAIL: a molecule with multiple receptors and control mechanisms. Curr Opin Immunol 1998;10:559 – 63.

28. Lincz LF, Yeh TX, Spencer A. TRAIL-induced eradication of primary tumour cells from multiple myeloma patient bone marrows is not related to TRAIL receptor expression or prior chemotherapy. Leukemia 2001;15: 1650 – 7.

29. Matsui TA, Sowa Y, Yoshida T, et al. Sulforaphane enhances TRAIL-induced apoptosis through the induction of DR5 expression in human osteosarcoma cells. Carcinogenesis 2006;27:1768 – 77.

30. Wen J, Ramadevi N, Nguyen D, Perkins C, Worthington E, Bhalla K. Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells. Blood 2000;96: 3900 – 6.

31. Darimont C, Avanti O, Tromvoukis Y, et al. SV40 T antigen and telomerase are required to obtain immortalized human adult bone cells without loss of the differentiated phenotype. Cell Growth Differ 2002;13: 59 – 67.

32. Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Cancer Res 2003;63:912 – 6.

33. Locklin RM, Croucher PI, Russell RG, Edwards CM. Agonists of TRAIL death receptors induce myeloma cell apoptosis that is not prevented by cells of the bone marrow microenvironment. Leukemia 2007;21:805 – 12.

34. Shipman CM, Rogers MJ, Apperley JF, Russell RG, Croucher PI. Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti-tumour activity. Br J Haematol 1997;98:665 – 72.

35. Nose K, Okamoto H. Detection of carcinogen-induced DNA breaks by nick translation in permeable cells. Biochem Biophys Res Commun 1983; 111:383 – 9.

36. Kim K, Fisher MJ, Xu SQ, el-Deiry WS. Molecular determinants of response to TRAIL in killing of normal and cancer cells. Clin Cancer Res 2000;6:335 – 46.

37. Chadderton A, Villeneuve DJ, Gluck S, et al. Role of specific apoptotic pathways in the restoration of paclitaxel-induced apoptosis by valspodar in doxorubicin-resistant MCF-7 breast cancer cells. Breast Cancer Res Treat 2000;59:231 – 44.

38. Holen I, Cross SS, Neville-Webbe HL, et al. Osteoprotegerin (OPG) expression by breast cancer cells *in vitro* and breast tumours *in vivo* —a role in tumour cell survival? Breast Cancer Res Treat 2005;92:207 – 15.

39. Holen I, Croucher PI, Hamdy FC, Eaton CL. Osteoprotegerin (OPG) is a survival factor for human prostate cancer cells. Cancer Res 2002;62: 1619 – 23.

40. Thomas LR, Johnson RL, Reed JC, Thorburn A. The C-terminal tails of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas receptors have opposing functions in Fas-associated death domain (FADD) recruitment and can regulate agonist-specific mechanisms of receptor activation. J Biol Chem 2004;279:52479 – 86.

41. Cheng J, Hylander BL, Baer MR, Chen X, Repasky EA. Multiple mechanisms underlie resistance of leukemia cells to Apo2 ligand/TRAIL. Mol Cancer Ther 2006;5:1844 – 53.

42. Leverkus M, Neumann M, Mengling T, et al. Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. Cancer Res 2000;60:553 – 9.

43. Dechant MJ, Fellenberg J, Scheuerpflug CG, Ewerbeck V, Debatin KM. Mutation analysis of the apoptotic ''death-receptors'' and the adaptors TRADD and FADD/MORT-1 in osteosarcoma tumor samples and osteosarcoma cell lines. Int J Cancer 2004;109:661 – 7.

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

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

46. Muhlenbeck F, Schneider P, Bodmer JL, et al. The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. J Biol Chem 2000;275: 32208 – 13.

AACR American Association for Cancer Research

Molecular Cancer Therapeutics

Selective targeting of death receptor 5 circumvents resistance of MG-63 osteosarcoma cells to TRAIL-induced apoptosis

Rachel M. Locklin, Ermanno Federici, Belen Espina, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-07-0275

Cited articles	This article cites 46 articles, 18 of which you can access for free at: http://mct.aacrjournals.org/content/6/12/3219.full#ref-list-1
Citing articles	This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/6/12/3219.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/6/12/3219. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.