Apo2l/Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Prevents Breast Cancer–Induced Bone Destruction in a Mouse Model

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Abstract
Breast cancer is the most common carcinoma that metastasizes to bone. To examine the efficacy of recombinant soluble Apo2 ligand (Apo2L)/tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) against breast cancer growth in bone, we established a mouse model in which MDA-MB-231 human breast cancer cells were transplanted directly into the marrow cavity of the tibiae of athymic nude mice producing osteolytic lesions in the area of injection. All vehicle-treated control animals developed large lesions that established in the marrow cavity, eroded the cortical bone, and invaded the surrounding soft tissue, as assessed by radiography, micro-computed tomography, and histology. In contrast, animals treated with recombinant soluble Apo2L/TRAIL showed significant conservation of the tibiae, with 85% reduction in osteolysis, 90% reduction in tumor burden, and no detectable soft tissue invasion. Tumor cells explanted from Apo2L/TRAIL–treated animals were significantly more resistant to the effects of Apo2L/TRAIL when compared with the cells explanted from the vehicle-treated control animals, suggesting that prolonged treatment with Apo2/TRAIL in vivo selects for a resistant phenotype. However, such resistance was readily reversed when Apo2L/TRAIL was used in combination with clinically relevant chemotherapeutic drugs, including taxol, etoposide, doxorubicin, cisplatin, or the histone deacetylase inhibitor suberoylanilide hydroxamic acid. These studies show for the first time that Apo2L/TRAIL can prevent breast cancer–induced bone destruction and highlight the potential of this ligand for the treatment of metastatic breast cancer in bone. (Cancer Res 2006; 66(10): 5363-70)

Introduction
Breast cancer is the major cancer affecting females in the western world with an overall incidence of about 1 in 11, and in this group, it constitutes the major cause of cancer mortality. In the United States alone, currently around 40,000 female and 1,500 male breast cancer deaths occur per annum (1). Mortality almost uniformly results from metastatic spread to organs, principally to the bones, lungs, and liver. Bone metastasis occurs in 44% to 71% of autopsy cases in fatal breast cancer cases, with associated significant clinical preterminal debilitation arising from extensive bony destruction, bone pain, pathologic fractures, hypercalcemia, and spinal cord compression. In real terms, there have been only minimal improvements in therapy for destructive bony metastases; therefore, new and safe approaches to this clinical problem are urgently required. One approach, not previously explored with regard to therapy of breast cancer bone metastases, is the potential use of apoptosis inducing agents to delay, slow, or eliminate growth of breast cancer cells within bone. Previous in vitro work has shown a cooperative synergistic effect between an agent Apo2 ligand (Apo2L)/tumor necrosis factor (TNF)–related apoptosis-inducing ligand, which is essentially nontoxic for normal cells, and chemotherapeutic agents for inducing death of cells from primary bone tumors and soft tissue sarcomas (2, 3).

Several members of the TNF superfamily of cytokines have been considered as potential treatments for cancer because of their ability to induce cell death through their cognate death receptors (4). Apo2L/TRAIL induces apoptosis in a variety of cancer cell types, which is mediated by interactions with its death domain–containing receptors DR4 and DR5 (5–12). Although other apoptosis-inducing members of the TNF family, such as TNFα and Fas ligand, initially carried great promise as anticancer agents, their severe toxicity towards normal tissues precluded their clinical use (4). In contrast, Apo2L/TRAIL seems to be selectively toxic to cancer cells and exhibits no toxicity to most normal cells in vitro (3, 13, 14). The therapeutic potential of Apo2L/TRAIL was realized when a recombinant soluble human Apo2L/TRAIL was produced. Repeated administration of this molecule to nonhuman primates (cynomolgus monkeys and chimpanzees) was shown to be safe and nonimmunogenic (15, 16). Administration of Apo2L/TRAIL alone or in combinations with other therapies resulted in marked regression or complete remission of tumors with no evidence of toxicity to normal tissues and organs in several murine xenograft models of human cancer (13, 14, 17–23). However, its in vivo efficacy against breast cancer present in bone has not been investigated.

The aim of these studies was to investigate the effect of recombinant soluble Apo2L/TRAIL on the growth of transplanted breast cancer cells in a murine model and the potential for a combined synergistic action between Apo2L/TRAIL and a number of chemotherapeutic agents for treatment of resistant cell clones that sometimes arise. We used a well-established animal model, in which the highly osteolytic breast cancer subline MDA-MB231-TXSA was transplanted directly into the tibial marrow cavity of athymic mice. As previously described for tumors in soft tissues, Apo2L/TRAIL exerted a potent anticancer activity on breast cancer cells in bone and significantly protected the bone from cancer–induced bone destruction. However, treatment with Apo2L/TRAIL in vivo failed to eradicate tumor cells from the bone, which was found to be due to the emergence of Apo2L/TRAIL–resistant cells.
Such resistance could be reversed in vitro when explanted tumor cells were treated with Apo2L/TRAIL in combination with clinically relevant chemotherapeutic drugs. These results provide proof of principal for the efficacy of Apo2L/TRAIL against breast cancer growth with in the bone microenvironment and have provided important information that is likely to advance current treatment strategies for breast cancer growth in bone.

Materials and Methods

Cells and reagents. The MDA-MB231 derivative cell line MDA-MB231-TXSA was kindly provided by Dr. Toshiyuki Yoneeda (University of Texas Health Sciences Center, San Antonio, TX). These cells have been selected for their increased propensity to metastasize to bone and for their increased osteolytic activity (24). All cells were cultured in DMEM, supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 160 μg/mL gentamicin, and 10% fetal bovine serum (Biosciences, Sydney, Australia) in a 5% CO2-containing humidified atmosphere. Recombinant soluble Apo2L/TRAIL was a gift from Dr. Avi Ashkenazi (Genentech, Inc., South San Francisco, CA; refs. 13, 15). Suberoylanilide hydroxamic acid (SAHA) was generously provided by Aton Pharma, Inc. (Tarrytown, NY). Doxorubicin, etoposide, and cisplatin were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia), and Taxol (paclitaxel) was purchased from Calbiochem (Merck Pty Ltd., Victoria, Australia).

Generation of green fluorescent protein–expressing cells. To generate cells stably expressing green fluorescent proteins (GFP) and the G418-resistant gene, MDA-MB231-TXSA cells were seeded at a density of 1 × 105 per 25-cm2 flasks and transfected with 1.0 μg of PEGFP-N (G418R) vector using the Fugene-6 reagent (Boehringer Mannheim, Castle Hill, New South Wales, Australia) according to the manufacturer’s instructions. GFP-expressing cells were selected with neomycin (Sigma Chemical Co., St. Louis, MO) for ~3 weeks.

Animals. Female athymic nude mice at 4 to 6 weeks old (Institute of Medical and Veterinary Services Division, Gilles Plains, South Australia, Australia) were acclimatized to the animal housing facility for a minimum period of 1 week before the commencement of experimentation. The general physical well-being and weight of animals were monitored continuously throughout the experiments. All of the experimental procedures on animals were carried out with strict adherence to the rules and guidelines for the ethical use of animals in research and were approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science, Adelaide (South Australia, Australia).

Intratibial implantation of breast cancer cells. Breast cancer cells were inoculated intratibially as described previously (25). Briefly, mice were anesthetized with ketamine/xylazine (Parnell Laboratories Ltd., New South Wales, Australia) and Taxol (paclitaxel) was purchased from Calbiochem (Merck Pty Ltd., Victoria, Australia). The left tumor-bearing tibia and the right contralateral non–tumor-bearing control tibia of individual mice were scanned simultaneously. Three-dimensional images were generated using Cone-Beam Reconstruction and 3D Realistic Visualisation software (SkyScan). Bone volume analysis was conducted using CTAn software (SkyScan). Bone volume calculations made comparisons between the left tumor-bearing and right non–tumor-bearing tibia of individual mice at selected longitudinal sections beginning at the growth plate and extending 250 μCT cross-sections downwards each at a thickness of 17 μm.

Histology and measurement of tumor area. Tumor area was fixed in 10% formalin followed by a slow decalcification process using 10% EDTA w/v for 2 weeks. Step sections were done, and 10-μm-thick sections representative of 10 levels of the block were stained with H&E for routine histologic evaluation. For detection of GFP, tissue sections were incubated overnight at 4°C in the presence of 2 μg/mL of rabbit anti-GFP antibody (Molecular Probes, Victoria, Australia) in 5% goat serum/PBS. This was done subsequent to inactivation of endogenous peroxidase activity by incubation of sections with 3% hydrogen peroxide/PBS and blocking with 5% goat serum/PBS for 1 hour at 4°C. Slides were then washed with 0.05% Tween 20/PBS followed by incubation with a biotinylated secondary anti-rabbit antibody and stained using streptavidin-peroxidase conjugate commercial reagents according to the manufacturer’s recommendation (Vectors Laboratories, Inc., Burlingame, CA). Hematoxylin (DakoCytomation, New South Wales, Australia) was used as a counterstain. Normal rabbit IgG (H+L; R&D Systems, Minneapolis, MN) was used as the negative control antibody.

Measurement of cell viability. For cytotoxic assays, 1 × 104 cells per well in 96-well microtiter plates and allowed to adhere for 24 hours. Cells were then treated as indicated. Treatment time was for 24 hours unless otherwise stated. Cell viability was assessed using crystal violet staining and measurement of absorbance at 570 nm wavelength (λ570 nm). Experiments were done in triplicates and repeated at least thrice. Results of representative experiments are presented as the mean ± SD.

Measurement of DEVD-caspase activity. Measurement of caspase-3 activity was done as previously described (3). Briefly, the assay involves the cleavage of zDEVD-afc (z-asp-gluf-val-asg-4-trifluoro-methyl-coumarin), a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly(ADP-ribose) polymerase (PARP). Cells were seeded at 5 × 104 per well in 48-well plates and were treated as indicated for 24 hours. Cells were washed once in HBSS and lysed in 100 μL of NP40 lysis buffer containing 5 mmol/L Tris-HCl, 5 mmol/L EDTA, and 0.5% NP40 (at pH 7.5) for 15 minutes on ice. Insoluble material was pelleted at 15,000 × g, and an aliquot of the lysate was analyzed for caspase-3 activity. For each 20 μL of cell lysate, 8 μmol/L of the fluorogenic substrate (zDEVD-afc) was added in 1 mL of protease buffer [50 mmol/L HEPES, 10 % sucrose, 10 mmol/L DTT, and 1% CHAPS (pH 7.4)] and incubated for 4 hours at room temperature in darkness. Fluorescence activity was quantified using a Perkin-Elmer LS50 spectrofluorometer at 400 nm excitation and 500 nm emission wavelengths. Results were expressed relative to the protein concentration of the sample, determined.
using a commercial bicinchoninic acid (BCA) protein assay reagent from Pierce (Rockford, IL). Experiments were conducted in triplicates and repeated at least thrice. Representative experiments are presented as the mean ± SD.

**Western blot analysis.** Cells were treated as indicated for 24 hours and lysed in buffer containing 10 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 2 mmol/L sodium vanadate, and a protease inhibitor cocktail (Boehringer Mannheim) and stored at −70°C until ready to use. The amount of protein in each sample was quantified using the BCA protein assay reagent (Pierce) according to the manufacturer's instructions. Before loading, protein extracts were mixed with an equal volume of running buffer containing 12 mmol/L Tris HCl (pH 6.8), 6% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.03% bromophenol blue. Protein samples were then heated at 70°C for 10 minutes and loaded into 4% to 20% polyacrylamide gels for electrophoresis under reducing conditions. Separated proteins were electrophoretically transferred to polyvinylidene difluoride transfer membranes (Novex, San Diego, CA) and blocked in PBS containing 5% blocking reagent (Amersham) for 1 hour at room temperature. Immunodetection was done overnight at 4°C in PBS/blocking reagent containing 0.1% Tween 20, using the following primary antibodies at the dilutions suggested by the manufacturer. Primary antibodies were purchased from Chemicon International [Temecula, CA: polyclonal antibody (pAb) anti-bid], PharMingen International (San Diego, CA; pAb anti-caspase-8), Cell Signaling Technology (Beverly, MA pAb anti-caspase-9), Transduction Laboratories (Lexington, KY; monoclonal antibody anti-caspase-3), and Roche Diagnostics (Mannheim, Germany; pAb anti-PARP). Anti-actin pAb (Santa Cruz, CA) was used to normalize for protein concentration. Membranes were then rinsed several times with PBS containing 0.1% Tween 20 and incubated with 1:5,000 dilution of anti-mouse or anti-rabbit alkaline phosphatase–conjugated secondary antibodies (Amersham) for 1 hour. Visualization and quantification of protein bands was done using the Vistra ECF substrate reagent kit (Amersham) on a FluorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Generation of Apo2L/TRAIL–resistant MDA-MB231-TXSA cells in vitro.** MDA-MB231-TXSA-GFP cells were seeded in a T75 flask until 75% confluence. Cells were exposed to Apo2L/TRAIL at 25 ng/mL for the first 2 weeks, at which time the concentration of Apo2L/TRAIL was increased to 50 ng/mL for a further 2 weeks followed by treatment in 100 ng/mL for a further 4 weeks. During the selection period, cell debris was removed every 3 days, and cells were incubated with fresh media containing Apo2L/TRAIL at 75 ng/mL. By week 8 in the continual presence of Apo2L/TRAIL, these cells were almost completely resistant to Apo2L/TRAIL. Thereafter, Apo2L/TRAIL was maintained for a further 4 weeks. During the selection period, cell debris was removed every 3 days, and cells were incubated with fresh media containing Apo2L/TRAIL at 75 ng/mL. By week 8 in the continual presence of Apo2L/TRAIL, these cells were almost completely resistant to Apo2L/TRAIL. Thereafter, Apo2L/TRAIL–resistant cells, denoted MDA-MB231-TXSA-R, were cultured in Apo2L/TRAIL–free medium and were found to remain resistant.

**Statistics.** Student's *t* test was used for analysis with *P* < 0.05 accepted as significant. Results are expressed as mean ± SD.

**Results**

**Apoptotic activity of Apo2L/TRAIL on parental and GFP-expressing MDA-MB231-TXSA cells.** To test the activity of Apo2L/TRAIL in a preclinical model of breast cancer growth in bone, we have selected the human MDA-MB-231-TXSA breast cancer cells, a subline of the parental MDA-MB-231 generated by sequential passages in nude mice and *in vitro* selection for cells metastatic to bone (24). These tumor cells when injected into bone, grow, and reproducibly produce osteolytic lesions in the area of injection. To enable imaging, and for selection of tumor cells in bone marrow explants, we have generated a subline of MDA-MB-231-TXSA stably expressing GFP. GFP-expressing cells were morphologically identical and had a similar growth rate to their nontransfected counterparts (data not shown). These cell lines were similar in response to Apo2L/TRAIL, with both lines showing high sensitivity to the apoptotic effects of Apo2L/TRAIL. Treatment caused a dose-dependent loss of cell viability with only 20% of cells viable after treatment for 24 hours at 25 ng/mL of Apo2L/TRAIL (Fig. 1A). Apo2L/TRAIL–induced morphologic changes characteristic of apoptosis, including chromatin condensation and DNA fragmentation as assessed by 4',6-diamidino-2-phenylindole staining of nuclei concomitant with processing and activation of caspase-8, caspase-9, and caspase-3 and the cleavage of PARP (Fig. 1B and C).

**Effect of Apo2L/TRAIL on the growth of breast cancer cells in bone and the development of cancer-induced osteolysis.** To examine the antitumor activity of Apo2L/TRAIL, *in vivo* in the context of breast cancer in bone, we used a mouse model in which MDA-MB-231-TXSA-GFP human breast cancer cells were transplanted into the tibiae of athymic mice. Of the 10 animals transplanted with MDA-MB-231-TXSA-GFP breast cancer cells and treated with PBS vehicle only, all developed large tumors that invaded the marrow cavity and eroded the cortical bone with

![Image](https://example.com/image1.png)

**Figure 1.** Apo2L/TRAIL–mediated induction of apoptosis in MDA-MB-231-TxSA breast cancer cells *in vivo*. A, comparison of the effect of Apo2L/TRAIL on GFP-expressing MDA-MB-231-TXSA cells and their untransfected counterpart cell line. Cells were left untreated or treated for 24 hours with increasing Apo2L/TRAIL concentrations of 10, 25, 50, and 100 ng/mL, and cell viability was analyzed using crystal violet. B, cells were seeded on chambers slide at 5 × 10⁴ per chamber and were treated with Apo2L/TRAIL at 25 ng/mL for 24 hours. Cells were fixed with methanol and incubated with 4',6-diamidino-2-phenylindole staining of nuclei concomitant with processing and activation of caspase-8, caspase-9, and caspase-3 and the cleavage of PARP. C, cells were seeded at 2 × 10⁴ per T75 flask and were either left untreated or treated with Apo2L/TRAIL at a concentration of 25 ng/mL. Cells were then lysed, and total cell lysates analyzed by polyclonal antibody gel electrophoresis and transferred to polyvinylidene difluoride membranes for immunodetection. The caspase-8, caspase-3, caspase-9, and PARP antibodies detect both full-length and processed forms of the antigen.
progressive invasion into the surrounding soft tissue, as assessed by radiography, μCT, and histology (Fig. 2A and C). In contrast, animals treated i.p. with 30 mg/kg/dose of Apo2L/TRAIL for five consecutive days followed by once weekly injection for 4 weeks showed remarkable conservation of the tibiae with no evidence of soft tissue invasion (Fig. 2B and D). The bioavailability of Apo2L/TRAIL from the peritoneal cavity is ~30%, and the peak plasma levels expected with 30 mg/kg i.p. dose given over 5 days is ~7 μg/ml previously shown to have efficacy in vivo (13). Reconstructed three-dimensional images of μCT scans revealed the complete absence of tumor-induced osteolysis in eight of the 10 Apo2L/TRAIL–treated animals, with small osteolytic lesions detected in the remaining two animals (Fig. 2B). Histologic analysis revealed the presence of cancer cells within the marrow space in most of the Apo2L/TRAIL–treated animals, with small osteolytic lesions detected in the remaining two animals (Fig. 2B). Although tumor cells persisted in the marrow cavity of Apo2L/TRAIL–treated mice, these tumors were significantly smaller and were confined within the marrow space and only at the site of transplantation (Fig. 2C). These smaller tumors produced bone lesions that could not be detected by standard X-ray radiography but were readily detectable by μCT analysis (Fig. 2B).

The effect of Apo2L/TRAIL on osteolysis was quantified using μCT analysis. We compared the left tumor-bearing tibiae with the contralateral right non–tumor-bearing tibiae at a selected region beginning at the growth plate and extending downwards 250 μCT bone slices each at a thickness of 17 μm. This region included most of the tibial length containing osteolytic lesions. Treatment with Apo2L/TRAIL reduced the volume of bone lost by 85% ($P < 0.05$, $n = 10$) compared with vehicle control ($n = 10$; Fig. 3A). The tumor burden was assessed from histologic sections and took into account the tumor mass confined within the marrow cavity. Treatment with Apo2L/TRAIL reduced the mean tumor area by 95% ($P < 0.05$, $n = 10$) compared with vehicle control ($n = 10$; Fig. 3B). These data provide evidence that Apo2L/TRAIL is a potent anticancer agent that can inhibit the growth of breast cancer cells within the bone microenvironment and protect the skeleton from tumor-induced osteolysis.

Development of acquired resistance after prolonged treatment with Apo2L/TRAIL in vivo. The persistence of tumor cells in the Apo2L/TRAIL–treated animals may be an indication that the therapy and dosing was insufficient, or that Apo2L/TRAIL treatment resulted in the selection of Apo2L/TRAIL–resistant clones in vivo. We tested this latter hypothesis by isolating tumor cells from the bones of Apo2L/TRAIL–treated animals to assess their resistance to Apo2L/TRAIL in vitro. Tumor explants were isolated from Apo2L/TRAIL–treated and vehicle-treated animals.
at time of sacrifice. Marrow cells were cultured in the presence of G418 to select for GFP-expressing MDA-MB231-TxSA cells, which were tested for Apo2L/TRAIL sensitivity. Cancer cells explanted from Apo2L/TRAIL–treated animals were significantly more resistant to the apoptotic effects of Apo2L/TRAIL. \( EC_{50} = 50 \text{ ng/mL} \) compared with cells explanted from the vehicle-treated control animals showing much reduced cell viability \( EC_{50} = 20 \text{ ng/mL} \). (Fig. 4A). The effect of Apo2L/TRAIL on cancer cells explanted from the vehicle-treated control animals was no different from that observed in the parental nontransplanted cells growing in culture, suggesting that acquired Apo2L/TRAIL resistance was not due to growth \textit{in vivo} but rather due to selection pressure imposed by prolonged treatment of animals with Apo2L/TRAIL. (Fig. 4A).

To test the hypothesis that resistance to Apo2L/TRAIL could be produced by treatment of cells with this agent, parental Apo2L/TRAIL–sensitive MDA-MB-231-TxSA cells were cultured continuously \textit{in vitro} for 8 weeks in medium containing Apo2L/TRAIL, as described in the Materials and Methods. These \textit{in vitro} generated resistant cells (denoted "parental \textit{in vitro} resistant") were strongly resistant to Apo2L/TRAIL, showing almost 100% viability even when cultured in a 20-fold higher concentration of Apo2L/TRAIL. (Fig. 4A). These data show the potential for prolonged exposure of MDA-MB-231-TxSA cells to Apo2L/TRAIL to result in cells resistant to this agent.

Acquired Apo2L/TRAIL resistance is reversible by combination with chemotherapeutic drugs. The cells that acquired resistance to Apo2L/TRAIL were not cross-resistant to a number of clinically relevant chemotherapeutic drugs, including taxol, doxorubicin, etoposide, cisplatin, and a novel histone deacetylase inhibitor SAHA, suggesting that the \textit{intrinsic} apoptotic signaling pathway was intact (data not shown). When used in combination with Apo2L/TRAIL, suboptimal doses of these agents augmented the apoptotic effect of Apo2L/TRAIL in this cell population to a level comparable with that seen when the sensitive cells were treated with Apo2L/TRAIL as a single agent (Fig. 4B). Similar levels of sensitization to Apo2L/TRAIL by these agents were also shown in the highly resistant population of MDA-MB-231-TxSA cells that were selected after prolonged treatment with Apo2L/TRAIL \textit{in vitro} (data not shown).

Consistent with chemotherapy-mediated augmentation of Apo2L/TRAIL–induced apoptosis, immunoblot analysis revealed that combined treatment with Apo2L/TRAIL and chemotherapeutic drugs, such as taxol or doxorubicin, resulted in enhanced processing of caspase-8, caspase-9, with a concomitant increase in Bid and PARP cleavage and amplification of the activity of the executioner caspase-3 (Fig. 5A and B). Taken together, our data clearly indicate that combination of chemotherapy and Apo2L/TRAIL resulted in the amplification of the caspase cascade that ultimately leads to increased cell death compared with that achieved by each agent alone.
Discussion

Breast cancer metastasis to bone is a serious debilitating clinical problem often associated with metastases to other organs. Unfortunately, metastasis to bone is unpredictable and prevention of this is difficult. Bone metastases can lead to extensive bone destruction, with bone pain, hypercalcemia, pathologic fractures, and spinal cord compression (26). To date, there are no totally efficacious treatment modalities for breast cancer bone metastasis. Apo2L/TRAIL is a member of the TNF family of ligands that induces apoptosis of a variety of cancer cell types but has limited or no toxicity to most normal cells (3, 13, 14). The antitumor activity of Apo2L/TRAIL has been well shown in several mouse xenograft models of human soft tissue cancers, including colorectal (13, 17, 18, 27), prostate (22), glioma (14, 21), multiple myeloma (19), and lung (20). Apo2L/TRAIL was active either alone or exhibited synergistic activity with certain chemotherapeutic agents or radiotherapy, causing marked regression of tumors with significant survival benefit and no evidence of toxicity to normal tissues and organs of the animals (13, 20). Previous in vitro studies have shown that Apo2L/TRAIL is capable of inducing apoptosis of breast cancer cell lines, although the sensitivity varied considerably, with the majority of cell lines being relatively resistant (23, 28). However, these resistant breast cancer cells could be sensitized to the effects of Apo2L/TRAIL by combination with chemotherapeutic drugs, often resulting in synergistic or additive induction of cell death (23, 28, 29). In vivo, the efficacy of Apo2L/TRAIL against breast cancer was shown in tumors growing in a s.c. setting (18, 23, 30), and no studies on its effects on cancer growth within bone have been described. In this study, we show that Apo2L/TRAIL as a single agent prevents breast cancer–induced osteolysis by inhibiting breast cancer growth within the bone microenvironment. In addition, we provide evidence that prolonged exposure to Apo2L/TRAIL in vivo results in the selection of Apo2L/TRAIL resistance, which ultimately would lead to tumor recurrence. However, such resistance is readily reversed by combination with clinically relevant chemotherapeutic drugs.

Animal models of breast cancer growth and metastasis have been useful for the development of novel therapies. Many human breast cancer–derived cell lines are tumorigenic in nude mice and selected cell variants, which have increased incidence of metastasis to bone, have been generated. We used the estrogen-independent MDA-MB231 cell line derivative MDA-MB231-TxSA, which was selected in vivo for its preferential bone metastasis properties and enhanced osteolytic activity (24). When transplanted directly in bone, these tumor cells grow and produce osteolytic lesions in the area of injection. We used the intratibial injection animal model for this study because of the following. First, there is a need to establish whether the pharmacodistribution and pharmacokinetics of Apo2L/TRAIL ensures its efficient accumulation in the bone microenvironment and ability to exert anticancer activity. Second, this model results in localized tumor growth at a single bony site that produces a consistent measurable outcome, in which the effect of Apo2L/TRAIL treatment could be assessed. With the current advances in screening methods, primary breast cancer is now increasingly diagnosed at an earlier stage before the appearance of bone metastases or when tumor in bone is at the initial stage of being established. For this reason, we have chosen to treat animals 2 days after cancer cell transplantation to recapitulate the human disease so that we best assess the efficacy of Apo2L/TRAIL in the early establishment of breast cancer cell growth in the bone microenvironment when tumor load is low.

In vitro, these cells were highly sensitive to the apoptotic effects of Apo2L/TRAIL, showing >80% cell death when exposed to 25 ng/mL ligand for 24 hours. Consistent with apoptosis induction, Apo2L/TRAIL treatment resulted in activation of caspase-8, caspase-9, and caspase-3 and cleavage of Bid and PARP. In vivo, 10 of 10 animals transplanted with MDA-MB-231-TxSA breast cancer cells and treated with vehicle alone, developed large lesions that established in the marrow cavity, eroded the cortical bone, and invaded the surrounding soft tissue. In contrast, animals treated with recombinant Apo2L/TRAIL showed dramatic conservation of the tibiae with 83% reduction in osteolysis, 90% reduction in tumor burden, and no detectable soft tissue invasion. Although tumor cells persisted in the marrow cavity of Apo2L/TRAIL–treated mice, these tumors were significantly smaller and were only confined to the site of transplantation. The persistent growth of tumor cells within the marrow cavity of the Apo2L/TRAIL–treated mice suggested that either the therapy and dosing was insufficient, or that prolonged treatment with Apo2L/TRAIL resulted in the selection of Apo2L/TRAIL–resistant clones in vivo. These cells when explanted from Apo2L/TRAIL–treated animals and tested in vitro...
for Apo2L/TRAIL sensitivity were found to be significantly more resistant to the effects of the ligand when compared with cells explanted from the vehicle-treated control animals. These data suggest that prolonged treatment in vivo with Apo2L/TRAIL selects for tumor cells that are resistant to Apo2L/TRAIL, ultimately resulting in tumor recurrence. However, these Apo2L/TRAIL–resistant cells were not cross-resistant to a number of clinically relevant chemotherapeutic drugs, including, taxol, doxorubicin, etoposide, cisplatin, and a novel histone deacetylase inhibitor SAHA, suggesting that the intrinsic apoptotic signaling pathway was intact. When used in combination with Apo2L/TRAIL these agents resensitized these in vivo generated partially resistant cells to the apoptotic effects of the ligand to a level comparable with that seen when the sensitive cells were treated with Apo2L/TRAIL as a single agent.

Apo2L/TRAIL induces apoptosis by engaging the cell-extrinsic apoptotic signaling pathway (4, 31). Activation of death receptors by Apo2L/TRAIL results in the rapid assembly of a death-inducing signaling complex and activation of the caspase cascade. The basis for Apo2L/TRAIL resistance is not well understood and multiple mechanisms have been proposed. These include, differential expression of death and decoy receptors for Apo2L/TRAIL (28, 32–36) and modulation of intracellular inhibitory proteins, such as FLIP (37) or intracellular inhibitor of apoptosis molecules (38, 39). In addition, mutation of the BCL2 family member BAX can confer resistance to Apo2L/TRAIL–induced apoptosis (40). Resistance to Apo2L/TRAIL–induced apoptosis is likely to be a major factor dictating the efficacy of Apo2L/TRAIL in cancer therapy. Consistent with the observed increase in apoptosis induction by Apo2L/TRAIL in the presence of chemotherapeutic, the resistant cells displayed enhanced processing and activation of caspase-8 and PARP cleavage. In particular, there was enhanced cleavage of the proapoptotic BCL2 family member Bid, resulting in enhanced activation of caspase-9 with a consequential overall increase in activity of the executioner caspase-3. These results highlight the importance of a multifaceted combinatorial approach to cancer therapy using Apo2L/TRAIL and suggest that cooperation between the cell extrinsic and intrinsic apoptotic signaling pathways results in an increased and more efficient apoptosis induction.

Taken together these studies show for the first time that recombinant Apo2L/TRAIL as a single agent prevents breast cancer–induced bone destruction and highlights the potential of this ligand for the treatment of bone metastases from breast cancer. However, a more comprehensive study(ies) will be required to optimize treatment schedules with Apo2L/TRAIL, either as a single agent or in combination with other cytotoxics to achieve complete cures. Our findings have important implications for the future planning of additional preclinical studies assessing the efficacy of Apo2L/TRAIL on cancers that metastasize in the unique microenvironment of bone.

Acknowledgments

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