Apomab, a fully human agonistic antibody to DR5, exhibits potent antitumor activity against primary and metastatic breast cancer

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Abstract
Apomab, a fully human agonistic DR5 monoclonal antibody, triggers apoptosis through activation of the extrinsic apoptotic signaling pathway. In this study, we assessed the cytotoxic effect of Apomab in vitro and evaluated its antitumor activity in murine models of breast cancer development and progression. MDA-MB-231-TXSA breast cancer cells were transplanted into the mammary fat pad or directly into the tibial marrow cavity of nude mice. Apomab was administered early, postcancer cell transplantation, or after tumors progressed to an advanced stage. Tumor burden was monitored progressively using bioluminescence imaging, and the development of breast cancer–induced osteolysis was measured using micro-computed tomography. In vitro, Apomab treatment induced apoptosis in a panel of breast cancer cell lines but was without effect on normal human primary osteoblasts, fibroblasts, or mammary epithelial cells. In vivo, Apomab exerted remarkable tumor suppressive activity leading to complete regression of well-advanced mammary tumors. All animals transplanted with breast cancer cells directly into their tibiae developed large osteolytic lesions that eroded the cortical bone. In contrast, treatment with Apomab following an early treatment protocol inhibited both intraosseous and extraosseous tumor growth and prevented breast cancer–induced osteolysis. In the delayed treatment protocol, Apomab treatment resulted in the complete regression of advanced tibial tumors with progressive restoration of both trabecular and cortical bone leading to full resolution of osteolytic lesions. Apomab represents a potent immunotherapeutic agent with strong activity against the development and progression of breast cancer and should be evaluated in patients with primary and metastatic disease. [Mol Cancer Ther 2009;8 (10):2969–80]

Introduction
Breast cancer is one of the leading causes of cancer death among women, with a worldwide incidence of around one in eight women. Bone metastasis occurs in >75% of patients with breast cancer and is associated with extensive bone destruction, leading to bone pain, pathologic fractures, spinal cord compressions, and hypercalcemia (1). Despite a significant increase in patient survival attributable to improvements in the treatment of the primary tumor, patients with metastatic or recurring disease, continue to have a poor prognosis. Although metastatic breast cancer is initially responsive to hormonal therapy and some forms of chemotherapy, including taxanes, many patients relapse, underscoring the urgent need for novel therapeutic approaches for advanced disease.

Recombinant soluble Apo2 ligand (Apo2L)/tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is emerging as a promising new cancer therapeutic for the treatment of solid and hematological malignancies. Apo2L/TRAIL induces apoptosis in a wide variety of cancer cell lines but not in most normal cells (2, 3). Induction of apoptosis by Apo2L/TRAIL is mediated by interactions with its death domain–containing cell surface receptors DR4 and DR5 to activate caspases that carry out the cell death program (reviewed in ref. 4). Three homologous human decoy receptors for Apo2L/TRAIL have also been identified, including DcR1, DcR2, and osteoprotegerin (OPG; ref. 4). Unlike DR4 and DR5, DcR1 and DcR2 lack functional death domains and cannot mediate apoptosis. OPG is a widely expressed soluble member of the tumor necrosis factor receptor family that is capable of binding to Apo2L/TRAIL, and although it has lower affinity for Apo2L/TRAIL at normal physiologic temperatures, it can block Apo2L/TRAIL–induced apoptosis in vitro (5–8). The potential of soluble Apo2L/TRAIL as an anticancer therapeutic agent has been well demonstrated in mouse xenograft models of human soft tissue cancers, including colorectal (9), breast (3), lung (10), multiple myeloma (11), and glioma (12). Apo2L/TRAIL
was active alone and exhibited synergistic activity with certain chemotherapeutic agents or radiotherapy, causing marked regression or complete remission of tumors, with no evidence of toxicity to normal tissues and organs of the animals (13, 14). Recombinant soluble Apo2L/TRAIL receptors DR4 and DR5, have been developed and are in early-phase clinical development (16–18). These have shown potent antitumor activity against tumor xenografts in preclinical models, which is enhanced by combination chemotherapy treatment (19–21). Apomab is a fully human agonistic antibody that is designed to specifically bind to and activate the human Apo2L/TRAIL death receptor DR5. In vitro, Apomab can induce apoptosis in various human cancer cell lines while sparing normal hepatocytes. In vitro, Apomab showed tumor suppressive activity in various xenograft models of cancer, including colorectal cancer, non–small cell lung carcinoma, pancreatic cancer, and lung (22, 23). Data from phase I studies examining the safety, pharmacokinetic profile, and antitumor efficacy in a cohort of patients with solid and hematological malignancies has shown that Apomab is well tolerated, and capable of inducing prolonged stable disease (16). Phase II clinical trials evaluating the efficacy of Apomab as a single agent, and in combination with chemotherapy, in a variety of malignancies are ongoing. However, to date, the efficacy of agonistic antibodies to DR5 against primary breast cancer growing in an orthotopic site in the mammary tissue, or the effect of treatment against metastatic breast cancer growing in bone and cancer-induced bone destruction, have not been reported.

In this study, we assessed the cytotoxic effect and downstream signaling consequences of Apomab treatment of breast cancer cells in vitro and evaluated its antitumor activity in murine models of breast cancer development and progression in both the orthotopic mammary tissue and in bone. Apomab exerted remarkable tumor suppressive activity as a single agent, leading to complete regression of well-advanced tumors within the mammary tissue, with the animals showing no evidence of recurrence. Importantly, Apomab reduced tumor burden within the bone marrow cavity and completely protected the bone from breast cancer-induced osteolysis, thus highlighting the need to clinically evaluate Apomab in patients with primary and metastatic breast cancer.

Materials and Methods

Cells
The MDA-MB231, MDA-MB453, MDA-MB468, ZR-75, MCF-7, MCF10A, MRC-5, and T47D human breast cancer cell lines were obtained from American Type Culture Collection. The MDA-MB231 derivative cell line, MDA-MB231-TXSA, was kindly provided by Dr. Toshiyuki Yoneda (University of Texas Health Sciences Center, San Antonio, Texas). Normal human foreskin fibroblasts and normal human gingival fibroblasts were provided by A/Prof. Stan Gronthos (Institute of Medical and Veterinary Science, Adelaide, Australia) and were cultured as previously described (24). Normal human osteoblasts (NHB) were grown from needle aspirates from the iliac crest of normal healthy donors and grown in αMEM (SAFC Biosciences) containing 10% fetal bovine serum (Biosciences) and ascorbic acid 2-phosphate (NovaChem). MCF10A cells were cultured in membrane epithelial basal media (Lonza) and all other cells were cultured in DMEM, supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 160 μg/mL gentamicin, and 10% fetal bovine serum (Biosciences) in a 5% CO2-containing humidified atmosphere.

Reagents
Apomab and the vehicle control [0.5 mol/L arginine succinate, 20 mmol/L Tris, 0.02% Tween 20 (pH 7.2)] reagents were a kind gift from Dr. Avi Ashkenazi (Genentech, Inc., South San Francisco, CA). Affinity Pure Goat Anti-Human IgG Fcγ Fragment was purchased from Jackson Immunoresearch Laboratories, Inc. and ZVAD-fmk (Caspase Inhibitor-1) from Calbiochem, Inc.

Cell Viability Assay
To determine the cytotoxic effects of Apomab on cell growth, 1 × 10⁴ cells per well were seeded in 96-well microtiter plates and allowed to adhere overnight. Cells were treated with increasing concentrations of Apomab (25–200 ng/mL) for 24 h. For all in vitro experiments, Apomab was cross-linked with anti-human IgG Fcγ antibody (Jackson Immunoresearch Laboratories, Inc.) for 30 min at 4°C before use. Cell viability was assessed using the AlamarBlue Cell Viability Assay (Promega) as well as Crystal Violet staining, and absorbance was measured at 570-nm wavelength. Experiments were done in triplicate and repeated a minimum of three times. Results of representative experiments are presented as the mean ± SD. Apoptosis assays were done as previously described (25).

Retroviral Infection of MDA-MB-231-TXSA Cells with the Triple Reporter Gene Construct SFG-NES-TGL
Luciferase-expressing MDA-MB231-TXSA cells were generated using the retroviral expression vector SFG-NES-TGL, which gives rise to a single fusion protein encoding herpessimplex virus thymidine kinase, green fluorescent protein (GFP), and firefly luciferase. Virus particle–containing supernatants were generated and filtered to remove any cellular debris and then used to infect cells, as described previously (26, 27). The retrovirally transduced cells were grown as bulk cultures for 48 h and subsequently sorted for positive GFP expression, using fluorescence-activated sorting (Aria BD Biosciences). The cells were allowed to proliferate, and the 10% of cells expressing GFP most strongly were obtained by fluorescence-activated sorting to generate the subline MDA-MB231-TXSA-TGL.

Western Blot Analysis
MDA-MB231-TXSA cells were treated with Apomab plus anti-Fc at 100 ng/mL in a time-dependent manner (0, 3, 6, 9, 12 h) and lysed in buffer containing 10 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, 0.1% sodium...
dodecylsulfate, 2 mmol/L sodium vanadate, and a protease inhibitor cocktail (Roche Diagnostics) and stored at -70°C until ready to use. Anti–caspase-8 and pAb anti–caspase-9 were purchased from Cell Signaling Technology, monoclonal antibody anti–caspase-10 from MBL, monoclonal antibody anti–caspase-3 from Transduction Laboratories, pAb anti-bid from Chemicon International, and pAb anti–poly ADP ribose polymerase from Roche Diagnostics. Anti-actin monoclonal antibody (SIGMA) was used as a loading control. Membranes were then rinsed several times with PBS containing 0.1% Tween 20 and incubated with 1:5,000 dilution of anti-mouse, anti-goat, or anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Pierce) for 1 h. Visualization and quantification of protein bands was done using the ECF substrate reagent kit (GE Healthcare) on a FluorImager (Molecular Dynamics, Inc.).

**Animals**

Female athymic nude mice ages 4 and 8 wk (Institute of Medical and Veterinary Services Division) were acclimated to the animal housing facility for a minimum period of 1 wk before the commencement of experimentation. The general physical well-being and weight of animals were monitored continuously throughout the experiments. All mice were housed under pathogen-free conditions, and all 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 Committees of the University of Adelaide and the Institute of Medical and Veterinary Science, Adelaide, SA, Australia.

**Mammary Fat Pad Injections of Breast Cancer Cells**

MDA-MB231-TXSA-TGL human breast cancer cells were cultured as described above until they reached 70% to 80% confluency. Adherent cells were removed from dishes with 2 mmol/L EDTA and resuspended in 1 × PBS at 1 × 10⁶ cells/10 μL and kept on ice in an Eppendorf tube. An equal volume of Matrigel-HC (BD Biocosciences) was added to the cells and resuspended. The 8-wk-old mice were anaesthetized by isoflurane (Faulding Pharmaceuticals), the mammary fat pad area of the mice was wiped with ethanol, and the skin was lifted over the left outermost nipple. Finally, a 25 G needle was inserted and 20 μL of cells were injected into the mammary fat pad. Mice were allowed to recover under the heat lamp before being transferred into cages.

**Intratibial Injections of Breast Cancer Cells**

Cells were prepared for injection as described above. Four-week-old female BALB/c Nu/Nu mice were housed under pathogen-free conditions, in accordance with the guidelines approved by the Institute of Medical and Veterinary Science animal ethics research committee. The mice were anaesthetized by isoflurane (Faulding Pharmaceuticals), the left tibia was wiped with 70% ethanol, and a 27-gauge needle coupled to a Hamilton syringe was inserted through the tibial plateau with the knee flexed, and 1 × 10⁵ MDA-MB231-TXSA-TGL cells resuspended in 10 μL of PBS were injected into the marrow space. All animals were injected with PBS in the contralateral tibia, as a control. Mice were randomly assigned into two groups of 10 animals each, and Apomab, starting 2 wk after cancer cell implantation, was given as a weekly dose of 10 mg/kg body weight.

**In vivo Bioluminescent Imaging**

Noninvasive, whole body imaging to monitor luciferase-expressing MDA-MB231-TXSA cells in mice was done weekly using the IVIS 100 Imaging system (Xenogen). Mice were injected i.p. with 100 μL of the δ-luciferin solution at final dose of 3 mg/20 grams mouse body weight (Xenogen) and then gas anaesthetized with isoflurane (Faulding Pharmaceuticals). Images were acquired for 0.5 to 30 s (images are shown at 1 s) from the front angle, and the photon emission transmitted from mice was captured and quantitated in photons/s/cm²/sr using Xenogen Living image (Igor Pro version 2.5) software.

**Microcomputed Tomography Analysis**

**In vivo Live Microcomputed Tomography Imaging.** Computed tomography (CT) images were obtained using a SkyScan-1076 in vivo micro-CT scanner (Skyscan) while the animals were anaesthetized. The micro-CT Scanner was operated at 80 kV, 120 μA, rotation step of 0.5, 0.5-mm Al filter, scan resolution of 17.4 μm/pixel, and imaging time of 15 min. The cross-sections were reconstructed using a cone-beam algorithm (software Cone_rec, Skyscan). Files were then imported into CTAn software (Skyscan) for three-dimensional analysis and three-dimensional image generation. All images are viewed and edited using ANT visualization software.

**Ex vivo Micro-CT Imaging.** Limbs for micro-CT analysis were surgically resected and scanned using the SkyScan-1072 high-resolution micro-CT Scanner (Skyscan). The Scanner was operated at 80 kV, 120 μA, rotation step of 0.675, 0.5-mm Al filter, and scan resolution of 5.2 μm/pixel. The cross-sections were reconstructed using a cone-beam algorithm (software Cone_rec, Skyscan). Using the two-dimensional images obtained from the micro-CT scan, the growth plate was identified and 750 sections were selected starting from the growth plate/tibial interface and moving down the tibia. For quantification, three-dimensional evaluation was done on all data sets acquired by selecting two separate regions of interest, one for total bone and another containing the trabecular spongiosa of the proximal tibia only, to determine three-dimensional bone morphometric parameters (software CTAn, Skyscan).

**Histology**

Tibiae were fixed in 10% (v/v) buffered formalin (24 h at 4°C), followed by 2 wk of decalcification in 0.5 mol/L EDTA/0.5% paraformaldehyde in PBS (pH 8.0) at 4°C. Complete decalcification was confirmed by radiography and tibiae were then paraffin embedded. Five-micrometer longitudinal sections were prepared and stained with H&E. Analysis was done on an Olympus CX41 microscope.

**Data Analysis and Statistics**

Experiments were done in triplicate, and data were presented as mean ± SEM. All statistical analysis was done using SigmaStat for Windows version 3.0 (Systat Software, Inc.) using the unpaired Student’s t test. Measures of association between two variables were assessed using the
Spearman Rank correlation coefficient. Comparisons between groups were assessed using a one-way ANOVA test. In all cases, \( P \) value of <0.05 was considered statistically significant.

Results

Effect of Apomab on Breast Cancer Cells and on Normal Primary Cell Cultures

A panel of human breast cancer cell lines was examined for their sensitivity to the cytotoxic effects of Apomab. As shown in Fig. 1A, Fc cross-linked Apomab induced a dose-dependent increase in apoptosis in MDA-MB-231-TXSA, MDA-MB-231, and MDA-MB-468 after 24 hours of treatment, reaching a maximum of 40% to 90% cell death at the highest dose of 200 ng/mL of Apomab (Fig. 1A). The remaining breast cancer cell lines were refractory to the effects of Apomab even upon anti-Fc cross-linking. An important attribute of Apomab is the apparent selectivity in toxicity for tumor cells over normal cells. Apomab showed no cell death induction in primary cultures derived from normal human foreskin fibroblasts, normal human gingival fibroblasts, or NHBs. In addition, Apomab was with out effect on human embryonic fibroblasts (MRC-5) or on mammary epithelial cells (MCF10A), even upon anti-Fc cross-linking (Fig. 1B). The expression of the four Apo2L/TRAIL receptors and other downstream proapoptotic and antiapoptotic factors known to be involved in death receptor signaling was analyzed and was compared with Apomab sensitivity (Fig. 1C). Predictably, the Apomab-sensitive MB-231, MB-231-TXSA, and MB-468 cell lines expressed relatively higher levels of DR5 when compared with the resistant cell lines. This was also the case with the other death receptor DR4. Of note, sensitive cell lines exhibited high expression of the initiator caspases-8 and caspase-10, whereas FADD expression was comparable in all cell lines tested. The expression profile of additional downstream proapoptotic and antiapoptotic factors including FLIP, BAX, BID, BCL2, XIAP, AIF, and CIAP could not be correlated with sensitivity or resistance to Apomab-induced apoptosis (Fig. 1C).

In light of these preliminary in vitro findings and as a lead up to the present in vivo study for testing the activity of Apomab in breast cancer development and progression, we selected the human MDA-MB-231-TXSA breast cancer cell line for a detailed analysis. This cell line forms aggressive, rapidly growing tumors when injected into the orthotopic site of the mammary fat pad of nude mice, and stimulate the formation of osteolytic lesions when injected into the tibial marrow cavity (28). In addition, our previous...
studies show that these cells are highly sensitive to the apoptotic effects of Apo2L/TRAIL in vitro and when tested in vivo (28). Consistent with the hypothesis that receptor multimerisation and aggregation augments death receptor signaling, cross-linking with an anti-Fc Ab dramatically enhanced Apomab-mediated apoptosis in vitro, shifting the concentration response curve such that a 50-fold lower concentrations was effective (Fig. 2A). Treatment of the sensitive MDA-MB-231-TXSA cells with Apomab plus anti-Fc for 1, 3, 6, 9, and 12 h. Cell lysates were analyzed by PAGE and transferred to polyvinylidene difluoride membranes for immunodetection. The caspase-8, caspase-9, caspase-10, caspase-3, poly ADP ribose polymerase (PARP), and Bid antibodies detect both full-length and processed forms of the antigen.

Effect of Apomab as a Single Agent on the Growth of Orthotopic Breast Cancer Xenografts

To investigate the activity of Apomab against MDA-MB-231-TXSA tumors growing in an orthotopic site, we used a model in which cells were injected directly into the mammary fat pad of athymic female nude mice. For noninvasive bioluminescence imaging (BLI) of tumor growth, the parental cell were retrovirally infected with a triple-fusion protein reporter construct encoding herpes simplex virus thymidine kinase, GFP, and firefly luciferase (29). After infection,
MDA-MB-231-TXSA cells were enriched for high level expression of GFP by two rounds of fluorescence-activated cell sorting. The subline-designated MDA-MB-231-TXSA-TGL exhibited a 1,000-fold induction of luciferase activity when analyzed in vitro. When compared with the parental noninfected cells, MDA-MB-231-TXSA-TGL cells were equally responsive to Apomab, with both lines showing high sensitivity to the apoptotic effects of the antibody and with similar kinetics of caspase-3 activity (data not shown). We conducted two studies in this model.

In the first series of experiments, treatment was initiated as early as 7 days after cancer cell transplantation to investigate the effect of Apomab on early tumor establishment and low tumor burden. Animals treated with vehicle exhibited an exponential increase of mean photon emission that was directly associated with an increase in tumor burden, which was clearly evident from day 7 onwards reaching a maximum signal at day 21, at which point tumor volume exceeded 1,000 mm$^3$ as measured by calipers, requiring the animals to be humanely killed. In contrast, all animals treated i.p. with 10 mg/kg of Apomab once weekly showed complete responses and remained tumor free for 90 days, at which time the experiment was terminated (Fig. 3A). In a separate cohort of animals, treatment with Apomab was terminated on day 14, after two weekly doses, and tumor burden monitored weekly for tumor recurrence. These animals remained tumor free, and showed no evidence of recurrence for up to 90 days (Fig. 3A).

In the second study, we tested the efficacy of Apomab against well advanced tumors in a “delayed treatment protocol” (Fig. 3B). In this case, orthotopic mammary tumors were allowed to progress for a longer period before initiation of Apomab therapy. In the first experiment, treatment was initiated on day 14 after cancer cell transplantation following confirmation that breast cancer cells had established growth in the bone marrow cavity but had no evidence of osteolysis. All vehicle-treated animals showed an exponential increase of mean photon emission associated with an increase in tumor burden, which was clearly evident from day 14 onwards. By day 27, all animals developed large intratibial tumors that penetrated the cortical bone with the tumor mass invading the surrounding soft tissue. In contrast, all animals in the Apomab-treated group showed progressive reduction of bioluminescence signal and complete regression of tibial tumors by day 21, only 1 week after treatment commenced. These animals continued to show a complete response with weekly treatment until the experiment was terminated on day 90 (Fig. 4A). Qualitative and quantitative three-dimensional micro-CT analysis showed extensive osteolysis in the vehicle-treated group, with the amount of bone loss exceeding 50% in the tumor-bearing tibiae when compared with the contralateral nontumor-bearing tibiae (Fig. 4B).

In the second experiment, we allowed the intratibial tumors to grow for a longer period before initiation of Apomab therapy. Live micro-CT analysis of these advanced tumors confirmed the presence of extensive osteolysis. Apomab was administered on day 26, and within 2 days of Apomab therapy, a striking regression of tibial tumors was observed. In contrast, all vehicle-treated animals were humanely killed on day 27 due to high tumor load and extensive osteolysis. In contrast, treatment with Apomab resulted in a remarkable conservation of the tibiae, showing complete protection from cancer-induced osteolysis when compared with vehicle treatment (Fig. 4B).

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Effect of Apomab on Breast Cancer–Induced Bone Destruction

To evaluate the efficacy of Apomab against tumor growth within bone and its effects on cancer-induced bone destruction, we used a xenogeneic tumor model in which the MDA-MB-231-TXSA-TGL cells were transplanted directly into the tibial marrow cavity of athymic nude mice. A limitation in measuring tumor burden in bone is that it is not possible to accurately assess the progression of tumor growth by palpation, as in soft tissue tumors, before they break through the cortical bone. However, noninvasive bioluminescence imaging provided sensitive real-time in vivo assessment of breast cancer growth in bone. Progressive development of breast cancer–induced bone destruction was qualitatively and quantitatively assessed using high-resolution live micro-CT analysis. We conducted two experiments in this model, using an early and a delayed treatment protocol. In the first experiment, we initiated treatment on day 14 after cancer cell transplantation following confirmation that breast cancer cells had established growth in the bone marrow cavity but had no evidence of osteolysis. All vehicle-treated animals showed an exponential increase of mean photon emission associated with an increase in tumor burden, which was clearly evident from day 14 onwards. By day 27, all animals developed large intratibial tumors that penetrated the cortical bone with the tumor mass invading the surrounding soft tissue. In contrast, all animals in the Apomab-treated group showed progressive reduction of bioluminescence signal and complete regression of tibial tumors by day 21, only 1 week after treatment commenced. These animals continued to show a complete response with weekly treatment until the experiment was terminated on day 90 (Fig. 4A). Qualitative and quantitative three-dimensional micro-CT analysis showed extensive osteolysis in the vehicle-treated group, with the amount of bone loss exceeding 50% in the tumor-bearing tibiae when compared with the contralateral nontumor-bearing tibiae (Fig. 4B).

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Figure 3. Antitumor activity of Apomab as a single agent against orthotopic mammary tumors. A, early treatment protocol: 8-wk-old female nude mice were injected with $1 \times 10^6$ MDA-MB-231-TXSA-TGL cells into the mammary fat pad, as described in the Materials and Methods. Seven days after cancer cell transplantation, mice were randomized into two groups of six mice per group, which received either vehicle or Apomab at 10 mg/kg i.p. once weekly until termination of the experiment. Mice were imaged weekly using the Xenogen IVIS 100 bioluminescence imaging system. Representative whole body images of vehicle and Apomab-treated animals during the course of the experiment are shown. All vehicle-treated animals were humanely killed on day 19 due to high tumor load, whereas all Apomab-treated animals exhibited complete responses and remained free of tumor with weekly treatment until day 90 when the experiment was terminated. In a separate cohort of animals ($n = 4$), where Apomab treatment was discontinued on day 21, animals also remained free of mammary tumors, with no evidence of recurrence. B, delayed treatment protocol. Mammary tumors were allowed to progress to an advanced stage of $\sim 1,000$ mm$^3$ and mice were randomized into two groups of six mice per group. Apomab was administered on day 19 at either 3.0 or 10 mg/kg i.p. once weekly until termination of the experiment. Representative whole body images and quantification of bioluminescence signal demonstrating complete regression of tumors in mice treated with Apomab at both doses are shown. C, tumor burden as measured by BLI is well correlated with tumor volume of palpable tumors. BLI measurements are expressed as the sum of integrated photon counts per second and data are expressed as mean ± SEM (solid line). Tumor dimensions were measured by digital caliper, and tumor volumes were calculated as $[\text{Length} \times \{\text{Width}\}^2]/2$ measured by calipers and expressed as mm$^3$ (dotted line). D, histologic examination of representative sections from the mammary tumors 48 h after treatment indicated that Apomab induced apoptosis in a substantial proportion of the tumor cells, with intense TUNEL-positive staining of tumor cells when compared with vehicle-treated animals.
treatment (Fig. 6). By 2 weeks after Apomab treatment, the osteolytic lesions were resolved as a result of the bone remodeling that occurred in these young animals. Two-dimensional and three-dimensional micro-CT analysis, together with histology of bone sections, showed extensive bone remodeling of the trabecular network that was prominent under the growth plate and extended down the shaft of the tibia where the tumor mass resided (Fig. 6A). This extensive trabecular bone remodeling was associated with a significant increase in the number of TRAP-positive multinucleated osteoclasts lining the newly formed trabecular bone surface. Remarkably, the cortical bone, although initially porous, was progressively regenerated (Fig. 6A). Representative animals were also analyzed at 5 and 9 weeks after Apomab treatment. In these animals, the cortical bone was fully mineralized and had a near normal appearance with a clearly defined thickness and density (Fig. 6B and C). There was a concomitant decrease in the trabecular bone network in response to this remodeling process, and such as the contralateral nontumor-bearing tibiae, osteoclasts were now only found directly under the growth plate. Collectively, these data clearly suggest that Apomab can inhibit tumor growth in bone and can protect the bone from breast cancer–induced osteolysis. In the delayed treatment setting, in which osteolysis was prominent, Apomab therapy led to complete regression of advanced tumor, highlighting the potential for this therapy to allow for repair of osteolytic lesions.

Discussion
The activity of soluble Apo2L/TRAIL may be abrogated in the bone marrow microenvironment, where high levels of...
OPG produced by bone cells and endothelial cells (30) may antagonize the apoptotic actions of Apo2L/TRAIL, perhaps limiting its effectiveness against cancer growth in bone. Therefore, alternative approaches that target the Apo2L/TRAIL/DR4/DR5 system, and preferably agonistic antibodies that specifically activate DR4 and/or DR5 without competing for OPG binding, may have increased efficacy against cancer growth in bone. Additional advantages of targeting the Apo2L/TRAIL pathway using agonistic receptor antibodies are the longer half-life in the circulation of antibodies compared with recombinant Apo2L/TRAIL and the higher specificity of these reagents (31). In humans, the serum half-life of agonistic antibodies can range from 15 to 20 days, whereas the half-life of recombinant soluble Apo2L/TRAIL in serum is only 20 to 30 minutes (9, 15, 16, 32), requiring frequent administration to maintain efficacy and making the therapeutic development and clinical application of Apo2L/TRAIL protein a challenge.

The preclinical efficacy of various DR4 or DR5 agonistic antibodies in vitro and on tumor growth in vivo in s.c. xenograft models has been reported (20, 21, 33–38). Recently, the inhibitory effect of Apomab, a fully human DR5 agonistic antibody against lung tumor xenografts growing s.c. or in the orthotopic tissue has been shown. In these studies, Apomab was active alone and cooperated with Taxol or carboplatin, to inhibit tumor growth in lungs and improve survival (22, 23). To date, however, the activity of any agonistic antibodies to DR5 or DR4 against breast cancer growing in the orthotopic tissue of the mammary gland or in the distant metastatic site of bone has not been reported.
Apomab binds specifically to Apo2L/TRAIL death receptor DR5 and triggers apoptosis through activation of the extrinsic apoptotic signaling pathway (22). To test the activity of Apomab in a preclinical model of breast cancer development and progression, we selected the human MDA-MB-231-TXSA breast cancer cell line, which we have shown in vitro to be highly sensitive to Apomab treatment. This is a subline of the parental MDA-MB-231, which was generated by sequential passages in nude mice and in vitro selection for cells metastatic to bone (39). We have shown that these cells form rapidly growing tumors in the mammary gland and, when injected into bone, grow and reproducibly produce osteolytic lesions in the area of injection (28). A limitation in measuring tumor burden in bone is that it is not possible to accurately assess the progression of tumor growth by palpation, as in soft tissue tumors, before they break through the cortical bone. However, our noninvasive bioluminescence imaging approach enabled sensitive real-time in vivo tracking of breast cancer growth in the orthotopic site and in bone and allowed us to group animals with equivalent tumor burden before treatment commenced. In addition, high-resolution in vivo micro-CT imaging provided a powerful means for the simultaneous and progressive assessment of tumor burden and cancer-induced bone destruction before and after treatment. Our in vivo studies clearly showed the potent antitumor activity of Apomab against the development and progression of breast cancer at the orthotopic site. Although Apomab completely inhibited tumor growth in the early treatment protocol, of particular significance was the striking and rapid response to Apomab of well-advanced mammary tumors. Importantly, all animals treated with Apomab either early or when tumors were allowed to progress advanced remained tumor free and showed no evidence of recurrence even when treatment ceased after the second dose, thus highlighting the remarkable in vivo efficacy of Apomab in this model.

In the intratibial injection model, treatment with Apomab 7 days after cancer cell transplantation in the early treatment

Figure 6. Apomab treatment reduces tumor burden and promotes healing of osteolytic lesions. Animals were treated as described in Fig. 6. BLI and live micro-CT imaging were done on all animals before treatment on day 26 and the effect of Apomab therapy at 2 wk (A), 5 wk (B), and 9 wk (C) after Apomab treatment was assessed in the same animals. BLI showed complete tumor regression, whereas comparison of longitudinal and cross-sectional two-dimensional and three-dimensional live micro-CT images before and after treatment showed marked resolution of osteolytic lesions. Shown also are the corresponding histologic sections from decalcified tibiae of Apomab-treated animals stained with H&E or TRAP for the detection of new bone formation and the presence of osteoclasts on the bone surface.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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