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

Irene Zinonos¹, Agatha Labrinidis¹, Michelle Lee¹, Vasilios Liapis¹, Shelley Hay¹, Vladimir Ponomarev³, Peter Diamond², Andrew C.W. Zannettino², David M. Findlay¹, and Andreas Evdokiou¹

¹Discipline of Orthopaedics and Trauma, Adelaide Cancer Research Institute, The University of Adelaide, Adelaide, South Australia, Australia

²Myeloma Research Laboratory, Bone and Cancer Laboratories, Division of Haematology, Hanson Institute, Adelaide, South Australia, Australia

³Department of Neurology, Memorial Sloan-Kettering Cancer Center, New York, New York

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.

Requests for reprints: Andreas Evdokiou, Discipline of Orthopaedics and Trauma Level 4, Bice Building, Royal Adelaide Hospital, North Terrace, Adelaide 5000, South Australia, Australia. Phone: 618-82223107; Fax: 618-8232-3065. andreas.evdokiou@adelaide.edu.au.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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 has now progressed to the next stage of its development with numerous early-phase clinical trials initiated (15).

As an alternative to soluble Apo2L/TRAIL, long-acting agonistic monoclonal antibodies, specific for cell death-inducing human 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 vivo*, 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 down-stream 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 Centre, 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 α MEM (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 μ g/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). Affinty Pure Goat Anti-Human IgG Fc γ 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, 1×10^4 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 Fc γ 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 $\pm$ 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 herpes simplex 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 1h. 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 acclimatized to the animal housing facility for a minimum period of 1wk 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 flasks with 2 mmol/L EDTA and resuspended in $1 \times \text{PBS}$ at 1×10^6 cells/10 μL and kept on ice in an Eppendorf tube. An equal volume of Matrigel-HC (BD Biosciences) 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 1×10^5 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 D-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 1s) 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 μ A, rotation step of 0.675, 0.5-mm Al filter, and scan resolution of 5.2 μ m/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 $\pm$ 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 without 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 ortho-topic 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 together with anti-Fc Ab induced morphologic changes characteristic of apoptosis, including chromatin condensation, DNA fragmentation (Fig. 2B), and a dose-dependent activation of caspase-3 (Fig. 2C). The presence of a pan-caspase inhibitor, zVAD-fmk, completely reversed the reduction in cell viability induced by Apomab plus anti-Fc, indicating that as with Apo2L/TRAIL, Apomab-mediated apoptosis is caspase dependent (Fig. 2C). Immunodetection showed that apoptosis induction by Apomab was associated with processing and activation of the initiator caspases-8 and caspase-10, leading to cleavage and activation of caspase-3, which was concomitant with cleavage of the apoptosis target protein poly ADP ribose polymerase. Engagement of the intrinsic apoptotic signaling pathway was also shown because apoptosis induction by Apomab was associated with caspase-8-mediated cleavage of the Bcl2 protein family member Bid and activation of caspase-9 (Fig. 2D).

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 cells 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 mm³ 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 was 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, starting on day 19 when the tumors were advanced and measured ~1,000 mm³. Animals were dosed weekly with Apomab at either 3 or 10 mg/kg, and tumor regression was monitored by BLI. A single dose of Apomab at either 3 or 10 mg/kg resulted in a strikingly rapid and complete regression of tumors, occurring within 3 days of treatment. With once weekly dosing, the animals remained free of tumors for the duration of the experiment (Fig. 3B). Tumor volume measured with callipers was well correlated with the mean photon counts in all animals, providing validation of our BLI analysis (Fig. 3C). Histologic examination of representative sections from the advanced mammary tumors 48 hours after treatment indicated that Apomab-induced apoptosis in a substantial proportion of the tumor mass, with intense terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive staining of tumor cells when compared with vehicle treated animals (Fig. 3D).

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). For ethical reasons, 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).

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 as shown by BLI was observed. Animals continued to be tumor free thereafter until the experiment was terminated on day 84 (Fig. 5A). TUNEL analysis of histologic sections from representative animals from each group confirmed the presence of apoptosis in the tibial tumor mass in the animals treated with Apomab for 48 hours but not in the vehicle-treated animals (Fig. 5B). Before Apomab treatment, all animals were restricted in their movement and the tumor-injected legs were no longer weight bearing due to high tumor load and bone destruction. However, following treatment with a single dose of Apomab, all animals were no longer hindered in their movement and animals continued to improve with weekly treatment. Representative animals were humanely killed at different times after Apomab treatment, and high-resolution *ex vivo* micro-CT analysis highlighted the progressive resolution of the bone lesions with continued 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 not 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 realtime *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 protocol inhibited both intraosseous and extraosseous tumor growth and completely prevented the development of breast cancer–induced osteolysis. In the delayed treatment protocol, Apomab treatment resulted in a strikingly rapid response with complete regression of tibial tumors as assessed by bioluminescence imaging. In addition, Apomab-induced apoptosis in bone *in vivo* was shown by TUNEL staining of decalcified bone tumor sections. Live micro-CT imaging performed in a subset of animals before Apomab treatment showed extensive osteolysis. At this time all animals were given pain relief and were no longer weight bearing due to tumor load and bone destruction. In contrast, Apomab treatment not only completely eradicated tibial tumors but also mediated progressive and profound remodeling of both trabecular and cortical bone, leading to remarkable resolution of the osteolytic lesions. The treated animals remained tumor free, no longer on pain relief, were weight bearing, and continued to improve with time. In the context of bone cancer therapy, many clinical observations and preclinical research has shown that chemotherapeutic drugs disturb bone tissue metabolism and bone remodeling and can cause osteopenia, largely as a result of the direct cytotoxic actions of these drugs on normal bone remodeling. An important attribute of Apomab is the apparent selectivity in toxicity for tumor cells over normal cells and our results clearly indicate that we can exploit this attribute for the treatment of cancer in bone without the detrimental side effects on normal bone metabolism.

In conclusion, our data suggest that patients diagnosed with primary breast cancer or with bone metastases could benefit from Apomab therapy and that tumor burden in the orthotopic site and in bone is remarkably reduced following treatment. These preclinical data provide strong support for the clinical development of Apo2L/TRAIL receptor agonistic antibody-based therapies in patients with advanced and metastatic breast cancer.

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Figure 1.

Activity of Apomab against breast cancer cells and normal cells *in vitro*. **A**, seven human breast cancer cell lines were seeded in 96-well plates at 1×10^4 cells per well and treated with increasing doses of Apomab plus anti-human IgG Fc, as indicated. Cell viability was assessed by crystal AlamarBlue staining 24 h after treatment. Apomab reduced cell viability in three of the seven cell lines tested, with the MDA-MB231-TXSA being the most sensitive. In contrast, the remaining four cell lines were relatively resistant to Apomab, even after cross-linking with anti-Fc. **B**, normal human bone cells (osteoblasts) from two independent donors NHB1 and NHB2, normal human foreskin fibroblasts (HFF), normal human gingival fibroblasts (HGF), embryonic fibroblasts (MRC-5), and normal human mammary epithelial cells (MCF-10A) were seeded into 96-well plates and treated with increasing doses of Apomab plus anti-Fc, as indicated. Cell viability was assessed by AlamarBlue assay or crystal violet staining 24 h after treatment. Data points show means of quadruplicate results from a representative experiment, repeated at least twice. *Points*, mean of quadruplicate wells and are expressed as a percentage of the number of control cells; *bars*, SD. **C**, cells were treated with 100 ng/mL of Apomab plus 100 ng/mL of anti-Fc for 24 h. The cells were lysed and subjected to gel electrophoresis and immunoblot analysis.

Figure 2.

Proapoptotic activity of Apomab against MDA-MB231-TXSA breast cancer cells *in vitro*.

A, MDA-MB-231-TXSA-TGL cells were treated with increasing doses of Apomab alone or Apomab plus equivalent doses of anti-Fc, as indicated. Cell viability was assessed by AlamarBlue assay 24 h after treatment and expressed as percentage of control. **B**, MDA-MB231-TXSA-TGL cells were seeded on chamber slides at 5×10^4 cells per chamber and were treated for 24 h with Apomab at 100 ng/mL plus anti-Fc. Cells were fixed with methanol and incubated with 4',6-diamidino-2-phenylindole before washing in PBS and mounting on PBS/glycerin. 4',6-Diamidino-2-phenylindole staining was visualized by fluorescence microscopy. **C**, *top*, MDA-MB231-TXSA cells were treated with Apomab plus anti-Fc for 1,3,6,9, and 12h. Celllysates were used to determine caspase-3-like activity, using the caspase-3-specific fluorogenic substrate, zDEVD-AFC, as described in the Materials and Methods. *Bottom*, MDA-MB231-TXSA cells were treated for 12 h with 100 ng/mL of Apomab plus anti-Fc alone or were coincubated with the broad specificity caspase inhibitors z-VAD-fmk (50 μ mol/L). To exclude possible toxic effects of the inhibitor, cells were also

treated with the inhibitor alone. Cell viability was determined using the AlamarBlue assay and expressed as percentage of control. *Columns*, means of quadruplicate results from a representative experiment repeated at least twice; *bars*, SD. **D** MDA-MB231 -TXSA-TGL cells were seeded at 2×10^6 per T25 flask and were either left untreated or treated with Apomab plus anti-Fc at a concentration of 100 ng/mL each. Cells were then lysed and protein was isolated at 1, 3, 6, 9, and 12 h after treatment. 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.

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 1×10^6 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 experiments 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 $\sim 1,000 \text{ mm}^3$ 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 $\pm$ 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.

Figure 4.

Apomab inhibits tumor growth in bone and protects against breast cancer-induced osteolysis. Early treatment protocol: MDA-MB231-TXSA-TGL breast cancer cells were injected directly into the tibial marrow cavity of 4-wk-old female athymic mice ($1 \times 10^5/10 \mu\text{L}$ injection), as described in the Materials and Methods. **A**, tibial tumors were allowed to establish for 14 d and mice were randomized into two groups ($n = 10$ per group), which received either vehicle or Apomab at 10 mg/kg once weekly. Representative whole body BLIs are shown, with the graph representing the average tumor signal over time, measured as mean photon counts per second. **B**, three-dimensional micro-CT images from representative animals taken at day 27 showed extensive osteolysis in the tibiae of the vehicle-treated animals, whereas Apomab treatment offered complete protection from cancer-induced osteolysis. The graph shows the average total bone volume in each group for both the tumor-bearing and nontumor-bearing tibiae; *, $P < 0.001$ with respect to the

nontumor-bearing control. Bone volume was measured from 750 micro-CT sections of the proximal tibia, starting from the growth plate and using the program CTan. Data shown in each case are the average bone volume from all animals in that group: *points*, means; *bars*, SEM.

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Figure 5.

Apomab treatment reduces tumor burden in bone and induces massive apoptosis of tumor cells in the bone marrow. Delayed treatment protocol: **A**, cells were injected intratibially and tumors were allowed to progress to an advanced stage before Apomab therapy so that by day 26, BLI and live micro-CT analysis showed high tumor load and extensive osteolysis. Animals ($n = 6$ per group) were treated with Apomab on day 26 at 10 mg/kg i.p. once weekly, and tumor regression was monitored by BLI at the indicated times. Shown are representative bioluminescence whole body images, demonstrating a striking reduction in tumor burden in the tibiae only 2 d after Apomab treatment, with all animals remaining tumor free thereafter with weekly treatment. **B**, histologic examination of representative sections from decalcified tibial sections 48 h after treatment indicated that Apomab induced apoptosis in a substantial proportion of the intraosseous and extraosseous tumor mass, with

intense TUNEL-positive staining of tumor cells when compared with vehicle-treated animals.

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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.