RANKL Inhibition Blocks Osteolytic Lesions and Reduces Skeletal Tumor Burden in Models of Non–Small-Cell Lung Cancer Bone Metastases

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Introduction: Bone metastasis is a serious complication in patients with lung cancer, occurring in up to 40% of patients. Tumor cell–mediated osteolysis occurs ultimately through induction of RANK ligand (RANKL) within the bone stroma although this hypothesis has not been tested extensively in the setting of non–small-cell lung cancer (NSCLC). By using two novel NSCLC bone metastasis mouse models, we examined the effects of RANKL inhibition on osteolysis and tumor progression.

Methods: We treated mice bearing skeletal NSCLC tumors with osteoprotegerin-Fc (OPG-Fc) to assess whether osteoclast inhibition through RANKL inhibition would affect bone metastases at early or late stages of bone colonization. Progression of skeletal tumor was determined by radiography, longitudinal bioluminescent imaging, and histological analyses.

Results: OPG-Fc reduced development and progression of radiographically evident osteolytic lesions and also significantly reduced skeletal tumor progression in both NSCLC bone metastasis models. In the H1299 human NSCLC bone metastasis model, OPG-Fc plus docetaxel in combination resulted in significantly greater inhibition of skeletal tumor growth compared with either single agent alone. The observed ability of RANKL inhibition to reduce NSCLC osteolytic bone destruction or skeletal tumor burden was associated with decreases in tumor-associated osteoclasts.

Conclusions: These results demonstrate that RANKL is required for the development of tumor-induced osteolytic bone destruction caused by NSCLC cells in vivo. RANKL inhibition also reduced skeletal tumor burden, presumably through the indirect mechanism of blocking tumor-induced osteoclastogenesis and resultant production of growth factors and calcium from the bone microenvironment. RANKL inhibition also provided an additive benefit to docetaxel treatment by augmenting the reduction of tumor burden.

Key Words: RANK ligand inhibition, Osteoprotegerin, Bone metastasis, Osteolysis, Lung cancer.

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through the fully human anti-RANKL antibody denosumab, reduced the risk of skeletal adverse events and, moreover, was associated with improved overall survival when compared with zoledronic acid (ZA) in patients with lung cancer. It is therefore important to define the mechanisms by which RANKL inhibition may reduce skeletal tumor burden in lung cancer, specifically. In this study, we describe the development of two novel mouse models of non–small-cell lung cancer (NSCLC) bone metastasis, and evaluate the effects of the RANKL inhibitor human osteoprotegerin-Fc (OPG-Fc), either alone or in combination with the chemotherapeutic agent docetaxel, on experimental NSCLC-induced osteolytic lesions, skeletal tumor burden, and survival.

MATERIALS AND METHODS

Tumor Cell Lines

The human NSCLC cell lines H1975 (CRL-5908) and H1299 (CRL-5803; ATCC, Manassas, VA) were transduced to express Firefly luciferase (FLuc) and green fluorescent protein for bioluminescent imaging (BLI) as described. The ability of H1299 luciferase–expressing cells to seed and grow in bone was enhanced by one round of in vivo passage by isolating green fluorescent protein–luciferase-expressing cells from an osteolytic lesion after intracardiac (IC) challenge. In brief, after IC challenge with H1299 luciferase–expressing cells, a tumor site was identified by both BLI and the development of a radiographic lesion in the tibia of a challenged mouse. This location was excised and mechanically disrupted and placed in culture. The resultant isolate, which was named H1299Luc, resulted in 100% tumor take in the hind limbs of all IC-challenged mice, and was used for all subsequent experiments described. Cells were cultured in Roswell Park Memorial Institute (RPMI) media (Invitrogen, Grand Island, NY) with 10% fetal calf serum at 5% carbon dioxide, at 37°C.

Animals for In Vivo Studies

Athymic (R-Foxn1<nu>) nude female mice were obtained from Taconic Farms (Germantown, NY) and maintained under specific pathogen-free conditions. Purina Rodent Chow 5002 (Ralston Purina, St. Louis, MO) or Harlan Teklad Rodent Diet #8728C (Madison, WI) and tap water were provided ad libitum. The laboratory housing the cages provided a 12-hour light cycle and met all Association for Assessment and Accreditation of Laboratory Animal Care specifications. All experiments performed at Amgen Inc. (Thousand Oaks, CA) were approved and performed in accordance with guidelines set out by the Amgen Animal Use and Care Committee.

IC Model of Bone Metastasis

For the IC model of bone metastasis, 4- to 6-week-old mice were injected with 1 × 10^6 cells in a 100 μl volume into the left cardiac ventricle. In vivo BLI of mice was measured weekly beginning on the day of tumor challenge with an IVIS 200 imager (Xenogen, Alameda, CA) as described. For studies using H1975Luc, mice were pretreated with a low–molecular-weight heparin, enoxaparin (Lovenox; Sanofi Aventis, Bridgewater, NJ), to prevent thrombotic events. Enoxaparin sodium injection was diluted in 0.9% sterile saline to a concentration of 1 mg/ml and administered at 10 mg/kg intravenously into the tail vein 10 minutes before IC challenge. On the basis of a previously published study using intravenous enoxaparin in mice in a small-cell lung carcinoma mouse model of metastasis, we have chosen the enoxaparin dose that does not change the metastatic distribution of tumor cells. For both the H1299Luc and H1975Luc models, BLI confirmed that the majority of the tumor was distributed within the skeleton at hindlimbs during the entire study course. Some minimal additional tumor was also observed at other skeletal sites in the vertebrae and skull.

To examine the effects of OPG-Fc on osteoclast activity in NSCLC-induced bone destruction, mice bearing NSCLC tumors were randomly assigned to three treatment groups (n = 10 per group) after IC challenge with either H1299Luc or H1975Luc. The treatment groups consisted of (1) phosphate-buffered saline (PBS) thrice weekly subcutaneously (SC) beginning on day 1 after tumor challenge; (2) human OPG-Fc 3 mg/kg thrice weekly SC beginning on day 1 after tumor challenge (preventive protocol); and (3) human OPG-Fc 3 mg/kg thrice weekly SC beginning on day 7 after tumor challenge (established protocol). Mice were imaged twice weekly beginning on days 4 to 6 after tumor challenge, and images were gated for regions of interest on the hind limbs as described.

Animals were monitored weekly for progression of osteolytic lesion by digital radiograph (Faxitron, Wheeling, IL). Studies were terminated at day 19 and day 27 for H1299Luc and H1975Luc, respectively. Serum samples were collected at the time of necropsy and frozen at −80°C. Serum tartrate-resistant alkaline phosphatase 5b (sTRAP5b) levels were measured by enzyme-linked immunoassay by using MouseTRAP Assay (Immunodiagnostic Systems Limited, Bolton, United Kingdom). Histological measurements of tumor area were performed on hematoxylin-and-eosin–stained sections of tibias and femurs from each mouse and quantified using Osteo II software (Bioquant, Nashville, TN) in a blinded manner by a pathologist; intrabone tumor area was reported in square millimeters. Radiograph lesion areas were calculated using MetaMorph Imaging software (Molecular Devices Corporation, Downingtown, PA) on randomized blinded images.

To test the effects of combined OPG-Fc and chemotherapy on bone lesions and progression of skeletal tumor, the H1299Luc IC model was used for combination studies because of the greater size of skeletal tumors. Bioluminescent signals were detectable in hind limbs within 2 days post-tumor challenge, distributed into one of four possible treatment regimens (n = 10 per group) on the basis of rank randomization of day 5 BLI signals to normalize the distribution within each group. The control regimen comprised PBS (thrice weekly, SC) beginning on day 5, plus intraperitoneal (IP) saline on post-tumor challenge days 5 and 12. Active treatments included OPG-Fc (3 mg/kg thrice weekly SC) beginning on day 5 (plus IP saline on post-tumor challenge days 5 and 12); docetaxel only (35 mg/kg IP bolus injection on post-tumor challenge days 5 and 12) with PBS SC (thrice weekly); or docetaxel (35 mg/kg days 5 and 12) combined with OPG-Fc (3 mg/kg thrice weekly). Studies were terminated on...
day 21 post-tumor challenge and histological tumor volume, sTRAP5b, radiograph analysis, and histological examination of tumor-associated osteoclasts were each performed from samples obtained at this time point. For the survival study, the cohorts were treated as described above for the combination treatments; mice were continually treated according to that schedule and monitored until death or until they experienced hind limb paralysis or had 20% weight loss (operative definition of survival), at which point the mice were euthanized.

Subcutaneous Tumor Model

Twelve-week-old female athymic nu/nu mice were injected SC in the flanks with $2 \times 10^6$ human H1299Luc or H1975Luc cells suspended 1:1 in a mixture of Matrigel (BD Biosciences, Bedford, MA). Animals were randomized into the following treatment groups (10 mice in each treatment group for both H1299Luc and H1975Luc): (1) PBS thrice weekly SC beginning on the day of tumor challenge and (2) human OPG-Fc 3 mg/kg thrice weekly SC beginning on the day of tumor challenge. Tumor dimensions were measured by a digital caliper twice weekly. Tumor volumes were calculated as $[\text{length} \times (\text{width})^2]/2$.

Human RANK Transduction and Protein Expression

Cells were transduced with a retroviral LZRS-pBMNZ vector containing a full-length version of human RANK. Production of infectious retroviral vector particles was performed in 293-E Phoenix packaging cells as described. Cells were infected with varying amounts of the RANK retroviral construct in the presence of 5 μg/ml Polybrene. Forty-eight hours after infection, cells were lifted with Versene and analyzed by flow cytometry. Surface RANK expression was determined by flow cytometry after incubation with either 1 μg/ml mouse anti-RANK antibody (M331, NIH8, or N2B10; Amgen) or isotype control (anti-AGP3 mIgG1 clone 4D2; Amgen) in 2% fetal bovine serum followed by allophycocyanin-conjugated anti-mouse secondary antibody. Fluorescence was assessed by using a FAScan sorter (BD Biosciences, San Jose, CA).

Statistical Analyses

Statistical analyses were performed using JMP7 and SAS software (Cary, NC). BLI data were evaluated using a repeated-measures model which included the effects of treatment, day, and treatment-by-day interactions. Between-group BLI comparisons at individual time points were performed using an analysis of variance with a post hoc Dunnett’s test used for comparisons between the control and treatment groups. Comparisons of survival time were analyzed with the Wilcoxon test using JMP7 statistical software (SAS Institute, Inc.). A significance level of 0.05 was used for all comparisons.

RESULTS

Development of NSCLC Bone Metastasis Models

IC injection of human tumor cell lines into immune-compromised mice is an established model for studying tumor metastasis to bone. We combined IC challenge with BLI to develop new models to study the effects of inhibiting RANKL specifically on NSCLC-induced bone metastases. Initial experiments demonstrated that IC H1299 cells colonized approximately 50% of the hind limbs of challenged mice and produced osteolytic lesions (data not shown). This degree of penetrance was not sufficient for further experiments, and therefore we isolated and expanded H1299 cells from a bone metastasis to select a cell with more osteolytic bone metastatic characteristics. This isolate was termed H1299Luc and demonstrated greater penetrance, exhibiting skeletal metastasis in 100% of hind limbs of all challenged mice, which was suitable for pharmacology studies. Skeletal tumor colonization

**FIGURE 1.** Radiograph, bioluminescent imaging, and TRAP histochemical staining of H1299 and H1975 NSCLC bone metastases in vivo. Radiographs ($\times 2$ magnification) confirmed the presence of tumor-induced osteolysis shown by red arrows. In vivo monitoring of intracardiac-challenged mice was performed by BLI. Tumor bioluminescence is present in the long bones of the hind limbs and mandibular regions. Metastatic growth of both NSCLC models was limited to the skeleton as indicated by BLI, with no overt metastases to other tissues/organs. The color scale indicates the intensity of the BLI signal. TRAP5b-stained histological bone sections ($\times 40$ magnification) depict brown/purple TRAP-positive osteoclasts at the tumor-bone interface as indicated by arrows. In the histology image, T indicates tumor cells and B indicates bone tissue. Each of the above analyses performed on experimental day 19 for H1299Luc and day 27 for H1975Luc. BLI, bioluminescent imaging; TRAP, tartrate-resistant alkaline phosphatase; NSCLC, non–small-cell lung cancer.
and growth was particularly aggressive, such that tumor foci could be observed in hind limbs by BLI as early as 2 days post-tumor challenge. H1299Luc-challenged mice progressed rapidly to produce osteolytic lesions characterized by the presence of numerous osteoclasts at the tumor/bone interface (Fig. 1). Mice challenged with a second human NSCLC line, H1975Luc, also exhibited a high frequency of osteolytic hind limb bone metastasis (Fig. 1).

Metastatic growth of both NSCLC models was limited to the skeleton, with predominant occurrence in the long bones and jaw and no overt metastases to other tissues/organs (Fig. 1). Earlier detectable osteolytic lesions (data not shown) and a higher extent of osteolysis were observed in H1299Luc-challenged mice compared with H1975Luc (Fig. 1), indicating that these two NSCLC cell lines have distinct and overlapping bone colonization and growth characteristics.

**Inhibition of RANKL–Blocked NSCLC-Induced Osteolysis**

To examine the effect of osteoclast activity in the NSCLC-induced bone destruction and skeletal colonization or progression of metastases, mice bearing bone metastases from NSCLC were treated with the RANKL inhibitor OPG-Fc. Pilot BLI experiments indicated that NSCLC bone metastases were established 7 days after challenge with either H1299Luc or H1975Luc cells. To test the effect of RANKL inhibition on both the early stages of bone metastatic colonization and the progression of established bone metastasis, mice were treated with OPG-Fc on day 1 post-tumor challenge (preventive protocol) or on day 7 post-tumor challenge (established treatment protocol). Treatment of animals with OPG-Fc in both the highly osteolytic H1299Luc and the less osteolytic H1975Luc models eliminated all radiographic evidence of osteolytic lesions, irrespective of the protocol used (preventive or established treatment) (p < 0.00001 versus PBS treatment for all; Fig. 2A, B). The prevention of local, tumor-induced osteolysis by OPG-Fc was correlated with a significant reduction in systemic osteoclast function, as measured by sTRAP5b levels (p < 0.00001 for all versus PBS treatment; Fig. 2C, D).

Treatment with OPG-Fc either early (preventive protocol) or late (established treatment protocol) was also shown to significantly reduce the growth of H1299Luc tumors in bones as measured by both longitudinal BLI measurements and histological assessment at study end point. At the conclusion of the study (day 19 post-tumor challenge), reductions in the skeletal tumor growth rate measured by BLI were 77% and 67% in the preventive (day 1) and established (day 7) OPG-Fc–treatment groups, respectively (p < 0.001 for both; Fig. 3A). These results were confirmed by histological assessment: OPG-Fc treatment beginning 1 day after tumor challenge resulted in a 72%

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**FIGURE 2.** RANKL inhibition completely blocked non–small-cell lung cancer–induced osteolytic bone lesions. Radiograph osteolytic lesion area was quantified from femurs and tibia on day 19 post-tumor challenge (A, H1299Luc) and day 27 post-tumor challenge (B, H1975Luc). OPG-Fc treatment beginning on day 1 (preventive) or day 7 (established tumor treatment) post-tumor-challenge inhibited radiographically evident tumor-induced osteolysis as compared with PBS. The data represent the mean lesion area/mouse + SEM. Statistical analysis performed with JMP7 using Dunnett’s test (****p < 0.00001). RANKL inhibition reduces sTRAP5b, a systemic marker of osteoclasts, in H1299Luc (C) and H1975Luc (D) bone metastases models. Serum samples were collected at the time of necropsy and frozen at −80°C. Serum TRAP5b levels were measured by enzyme-linked immunoassay. The data represent the mean concentration ± SEM. Statistical analysis performed with JMP7 using Dunnett’s test (****p < 0.00001). PBS, phosphate-buffered saline; OPG-Fc, osteoprotegerin-Fc; RANKL, RANK ligand; sTRAP5b, serum tartrate-resistant alkaline phosphatase 5b; SEM, standard error of the mean.
reduction in skeletal tumor burden \( (p < 0.01) \), and the treatment of established skeletal tumors \( (\text{day } 7 \text{ initiation}) \) resulted in a 63% reduction \( (p < 0.01; \text{Fig. } 3B) \).

Although the overall skeletal tumor burden was lower in the less osteolytic H1975Luc model as compared with the H1299Luc model, similar responses were observed after RANKL inhibition in the two models. Among OPG-Fc–treated H1975Luc-bearing mice, skeletal growth rate reductions measured by BLI on day 27 were 68% \( (p < 0.05) \) and 63% \( (p = 0.053) \) with the preventive or established treatment protocols, respectively \( (\text{Fig. } 3C) \). Histological analyses confirmed the reductions in skeletal tumor burden with OPG-Fc–treated mice compared with controls. OPG-Fc treatment in the prevention protocol, beginning 1 day after tumor challenge, resulted in a 70% reduction in tumor burden \( (p < 0.05) \), and in the established treatment protocol \( (\text{initiated at day } 7) \) yielded a 75% reduction \( (p < 0.05; \text{Fig. } 3D) \).

Treatment with OPG-Fc had no effect on the growth of either H1299Luc tumors \( (p = 0.11) \) or H1975Luc tumors \( (p = 0.18) \) when tested in the nonosseous, subcutaneous setting (see Supplemental Figure 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A503, demonstrating that RANKL
inhibition had no effect on growth of H1299Luc or H1975Luc tumors), which supports the notion that the observed effect of RANKL inhibition on skeletal tumor growth is indirect and occurs through effects on the bone microenvironment.

Combination Effects of RANKL Inhibition with Docetaxel Treatment on Established NSCLC Bone Metastases

In this article, we examined the in vivo effects of the RANKL inhibitor, OPG-Fc, in combination with docetaxel on tumor-induced osteolysis, tumor burden, and survival in the H1299Luc mouse model of NSCLC metastasis in bone. Testing was performed in the H1299Luc model, as H1299 cells do not express functional RANK at the cell surface (see Supplemental Figure 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A504, showing lack of surface RANK expression on H1299 cells), allowing an exclusive testing of the indirect anti-tumor hypothesis. We used mice with established bone metastases to more closely mimic the clinical setting of a patient with existing bone metastases.

Compared with the vehicle control regimen, treatment of mice with established H1299Luc NSCLC bone metastases with all active agents resulted in a significant reduction ($p < 0.0001$ for all treatment groups versus vehicle control cohort) in skeletal tumor burden as assessed by BLI (Fig. 4A). Treatment with OPG-Fc as a single agent resulted in an 84.1% tumor growth inhibition compared with vehicle control at the termination of the study (day 21) (Fig. 4A). Docetaxel (35 mg/kg) as a single-agent treatment resulted in a significantly lower skeletal tumor burden as early as 3 days postinhibition of therapy, culminating in a 96.5% reduction in skeletal tumor by day 21 post-tumor challenge (Fig. 4A). The greatest reduction in skeletal tumor burden was observed with the combination treatment of OPG-Fc and docetaxel (35 mg/kg) at day 21 (99.7% tumor growth inhibition) compared with either docetaxel alone ($p < 0.001$) or OPG-Fc alone ($p < 0.001$) (Fig. 4A). Histological analysis of skeletal tumor burden area provided independent confirmation that all treatment groups experienced significant reductions compared with vehicle control in hind limb skeletal tumor area at day 21 ($p < 0.0001$ for each group; Fig. 4B). In contrast to BLI measurements, the limited sensitivity of histological analysis was perhaps insufficient to demonstrate differences between treatment groups.

RANKL inhibition by means of OPG-Fc completely prevented the development of osteolytic bone lesions as a single agent, consistent with the above results, and also when combined with docetaxel (Fig. 5A, B). The significant reduction in the systemic osteoclast marker sTRAP5b ($p < 0.0001$) (Fig. 5C) and in TRAP-positive osteoclasts localized at the tumor/bone interface (Fig. 5D) after OPG-Fc treatment was consistent with the marked effect on radiographically evident NSCLC-induced bone lesions. Docetaxel as a single agent also significantly reduced the size of osteolytic lesions ($p < 0.0001$) (Fig. 5A), but did not prevent their occurrence (Fig. 5A, B). Although the sTRAP5b levels in the docetaxel-treated group were significantly reduced compared with sTRAP5b levels in vehicle control group ($p < 0.001$), these levels remained significantly increased...
OPG-Fc and docetaxel treatments inhibited tumor-induced osteolysis of H1299Luc non–small-cell lung skeletal tumors through distinct mechanisms. OPG-Fc and/or docetaxel treatment began on day 5 post-tumor challenge. A, Radiograph lesion area was determined from femurs and tibia at day 21 post-tumor challenge. The data represent the mean lesion area/mouse + SEM. Statistical analysis was performed with JMP7 using Dunnett’s test. All treatment groups resulted in a significant reduction in osteolytic lesion area, \(* * * p < 0.0001\). B, Digital radiographs (×2 magnification) depicting tumor-induced osteolysis (red arrows). C, Serum samples were collected at the time of necropsy and sTRAP5b levels were measured by enzyme-linked immunosorbent assay. The data represent the mean concentration + SEM. Statistical analyses were conducted in SAS 9.3. Three separate analyses were conducted taking into account data normality and group variances homogeneity. For naïve versus vehicle, Wilcoxon rank-sum test was used. For Treatment versus vehicle analysis, an analysis of variance model allowing for heterogeneous group variance was used to model the data, and a Dunnett’s test was used for post hoc comparison between individual treatment groups to vehicle. The Treatment versus naïve analysis was done separately but in a similar manner. sTRAP5b levels were significantly increased relative to age-matched naïve in PBS/saline and 35 mg/kg docetaxel groups (p < 0.0001 and \(p < 0.05\), respectively. All treatments groups were significantly reduced relative to PBS/saline as indicated (****p < 0.0001, ***p < 0.001). D, Histological sections of the bone were stained with TRAP5b, showing osteoclasts (as indicated by arrows) at the tumor-bone interface in vehicle (PBS) and docetaxel groups, and no osteoclasts in the OPG-Fc and OPG-Fc/docetaxel treatment groups. The skeletal tumor mass is indicated by T. Histological images were captured from \(×20\) scans using an Aperio T2 Scanscope (Aperio Corporation, Vista, CA) with an Olympus \(×20/0.75\). DTX, docetaxel; OPG-Fc, osteoprotegerin-Fc; sTRAP5b, serum tartrate-resistant alkaline phosphatase; PBS, phosphate-buffered saline; SEM, standard error of the mean.

OPG-Fc Treatment Improved Survival of Mice with Established NSCLC Bone Metastases

Among mice with established bone metastases treated from day 5 until the end of the study, Kaplan–Meier survival curves demonstrated a significant improvement in survival upon treatment with OPG-Fc alone, docetaxel alone, and the combination of OPG-Fc with docetaxel (Fig. 6). Although the median survival time for mice bearing established H1299Luc NSCLC bone metastases in the vehicle control group was 26 days, OPG-Fc treatment led to a 15% increase in survival (p < 0.05 versus PBS) to a median of 30 days. Treatment of tumor-bearing mice with docetaxel increased survival to 31 days (p < 0.01 versus PBS). The combination of OPG-Fc with docetaxel prolonged survival to a median survival of 40 days, which was significantly greater compared with docetaxel treatment alone (p < 0.05 versus docetaxel alone) or OPG-Fc alone (p < 0.001 versus OPG-Fc alone).

DISCUSSION

Bone metastasis is observed in a high fraction (approximately 40%) in advanced NSCLC, and resulting skeletal complications may be related to a poor prognosis.\(^2\)\(^2\)\(^3\) In this study of two NSCLC models, we have demonstrated the critical operative effect of RANKL not only in osteoclast-mediated bone destruction but also in the development and progression of skeletal tumors and associated survival outcomes.

Tumor cells metastatic to the bone use a wide variety of signals to alter the tumor microenvironment, leading to an increased RANKL:OPG ratio and subsequent increases in osteoclast differentiation, survival, and activation and bone destruction.\(^2\)\(^5\) This diversity of signals reflects multiple
mechanisms, including unique signals from each different tumor type and heterogeneity within individual tumor types. Recent molecular characterizations demonstrated considerable complexity in NSCLC, even within well-defined histological subtypes.26 Considerable evidence has indicated the essential role of the RANKL pathway in osteoclast formation, function, and survival in physiological contexts and in tumor-associated bone diseases. However, it is important to understand whether this pathway is used in NSCLC-associated bone disease, and previous studies in lung cancer models have been limited. In one study using intratibial injection of A549 NSCLC cells, RANKL inhibition completely blocked tumor-associated osteoclasts and reduced the resulting mixed osteolytic/osteosclerotic lesion, whereas in a model using human lung squamous carcinoma (Hara) cells, neither OPG-Fc nor ZA treatment had any effect on H1299 and H1975 tumors grown in PBS, phosphate-buffered saline.

Potential mechanisms for improved survival might include direct and/or indirect effects on tumor cells. Indirect effects could occur through osteoclast inhibition resulting in blunting of the vicious cycle of feedback from the bone microenvironment to the tumor. In previous mouse studies of breast and prostate cancer bone metastases, RANKL inhibition not only reduced tumor-induced bone destruction but also slowed skeletal tumor progression,15,31 consistent with an indirect effect on tumor growth by means of the vicious cycle.

To address the potential of RANKL inhibition to affect skeletal tumor and survival responses exclusively through an indirect mechanism (targeting the bone microenvironment), we included experiments with an NSCLC cell line in the present study that does not express RANK (H1299). OPG-Fc, either alone or in combination with docetaxel, effectively reduced the burden of H1299Luc-induced tumors from this cell line, indicating an indirect anti-tumor mechanism by means of targeting the bone microenvironment and an interruption of the vicious cycle of feedback from the bone microenvironment to the tumor. Furthermore, RANKL inhibition was also associated with an increase in survival for mice with existing bone metastases, consistent with increase in survival for patients with NSCLC bone metastasis treated with the anti-RANKL antibody, denosumab, suggesting that the overall survival benefit may be partially owing to a decrease in skeletal tumor burden.17 It is important to note, however, that ethical guidelines for the mouse studies obligated an operational definition of survival as a composite of death with additional surrogates such as hind limb paralysis, excessive morbidity, or at least 20% weight loss, potentially impacting its translation to the human clinical study results. The observation that OPG-Fc treatment had no effect on H1299 and H1975 tumors grown in a nonosseous environment is also consistent with an indirect anti-tumor mechanism, through osteoclast suppression.

In addition to the well-established indirect anti-tumor benefit of RANKL inhibition in bone metastases, a functional role of the RANKL pathway in osteoclast formation, function, and survival has been well-established. However, it is important to understand whether RANKL inhibition would reduce NSCLC-associated bone destruction and associated skeletal tumor progression. Taken together, these preclinical studies show RANKL was clearly essential for tumor-associated osteoclastogenesis and subsequent osteolytic bone destruction, indicating that RANKL is a common signaling pathway for tumor-associated osteolysis induced by diverse lung cancer cells.

Recent clinical studies have tested the ability of denosumab, a fully human antibody against RANKL, in reducing skeletal-related events in patients with solid tumors and bone metastases, including NSCLC.16,29,30 Importantly, in an exploratory analysis among 702 patients in the NSCLC subgroup of patients with solid tumors other than breast or prostate, denosumab was associated with significantly improved overall survival compared with ZA (9.5 versus 8.0 months; hazard ratio, 0.78 [95% confidence interval, 0.65–0.94; \( p = 0.011 \)).

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role for the RANKL receptor, RANK, on tumor cells has been defined experimentally for breast and prostate cancer cells, leading to increased tumorigenesis or metastasis through direct action of RANK. RANKL promotes tumor formation and facilitates the migration and invasion of RANK-expressing tumor cells. This may be particularly relevant in metastasis to the bone, lymph nodes, or other distant sites, as the bone and lymph node stroma are a rich source of RANKL. Preliminary immunohistochemical analysis of RANK and RANKL expression on primary human NSCLC adenocarcinomas by our group has indicated that RANK and RANKL expression can be frequently observed (56% and 75%, respectively; Branstetter et al., manuscript in preparation). Although RANK expression is observed in approximately 50% of human lung cancer cell lines (Tometsko, Dougall data not shown) and expression of RANK on NSCLC has been reported to increase migration and expression of intercellular adhesion molecule-1 in a RANKL-dependent manner in vitro, we demonstrated here that RANK-negative (H1299) NSCLC will efficiently metastasize to the bone, indicating that RANK expression is not required for bone metastases. These data suggest that RANKL inhibition may affect NSCLC tumor growth in the skeleton potentially by indirect mechanisms (as defined in the present studies) and direct mechanisms. Ongoing research is exploring the role of functional expression of RANK on tumor cells in promoting tumor progression and/or metastatic activity, and whether RANKL inhibition will provide additional benefits in NSCLC specifically.

Because the reduction in skeletal tumor burden achieved with RANKL inhibition has been shown to be additive when combined with chemotherapy, hormonal therapy, or targeted therapies in models of breast and prostate cancer bone metastasis, we addressed whether similar combination effects could be obtained in NSCLC bone metastases. RANKL inhibition targeting the bone microenvironment through osteoclast reduction was combined with docetaxel, a standard of care treatment in NSCLC that is directly cytotoxic without any effects on systemic osteoclast activity. Given that docetaxel treatment had no apparent effect on osteoclasts localized at the tumor/bone interface but did significantly reduce skeletal tumor burden, the ability of docetaxel to reduce sTRAP5b measures and the size of radiographically evident NSCLC-induced bone lesions was likely a result of reduced tumor burden and not a direct effect on osteoclast activity. By using the sensitive tumor-imaging modality BLI, we showed that combination treatment with two agents having distinct mechanisms of action, OPG-Fc and docetaxel, reduced skeletal tumor burden to a significantly greater extent compared with either agent alone.

In conclusion, these preclinical studies define a critical role of RANKL pathway in NSCLC osteolytic bone metastasis. The present study also provides evidence supporting an anti-tumor benefit of RANKL inhibition in NSCLC at least through indirect mechanisms, through inhibition of pathologic bone resorption. These preclinical studies may also provide mechanistic insight into the clinical studies demonstrating the ability of denosumab to not only decrease skeletal-related events in NSCLC patients with bone metastases but also increase survival. We showed, in two novel mouse models of NSCLC-induced bone metastases, that RANKL inhibition significantly reduced destructive osteolysis and skeletal tumor burden, and was associated with a survival advantage. Furthermore, the observation that combining RANKL inhibitors with chemotherapy provided an even greater benefit compared with either agent alone demonstrates the potential of targeting the bone environment to enhance the benefit of cancer therapies and supports further clinical evaluation.

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