Nuclear Factor-κB–Dependent Mechanisms in Breast Cancer Cells Regulate Tumor Burden and Osteolysis in Bone

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Abstract

A central mediator of a wide host of target genes, the nuclear factor-κB (NF-κB) family of transcription factors, has emerged as a molecular target in cancer and diseases associated with bone destruction. To evaluate how NF-κB signaling in tumor cells regulates processes associated with osteolytic bone tumor burden, we stably infected the bone-seeking MDA-MB-231 breast cancer cell line with a dominant-negative mutant IκB that prevents phosphorylation of IκBα and associated nuclear translocation of NF-κB. Blockade of NF-κB signaling in MDA-MB-231 cells by the mutant IκB decreased in vitro cell proliferation, expression of the proinflammatory, bone-resorbing cytokine interleukin-6, and in vitro bone resorption by tumor/osteoclast cocultures while reciprocally up-regulating production of the proapoptotic enzyme caspase-3. Suppression of NF-κB transcription in these breast cancer cells also reduced incidence of in vivo tumor-mediated osteolysis after intratibial injection of tumor cells in female athymic nude mice. Immunohistochemistry showed that the cancerous lesions formed in bone by MDA-MB-231 cells express both interleukin-6 and the p65 subunit of NF-κB at the bone-tumor interface. NF-κB signaling in breast cancer cells therefore promotes tumor burden and tumor-mediated osteolysis through combined control of tumor proliferation, cell survival, and bone resorption. These findings imply that NF-κB and its associated genes may be relevant therapeutic targets in osteolytic tumor burden.

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Introduction

A coordinated balance exists between osteoclastic bone resorption and osteoblastic bone formation in normal bone remodeling (1). When metastatic breast cancer cells invade and seed themselves within the bone microenvironment, this balance becomes disrupted, usually leading to rapid bone loss. Uncontrolled production of an array of osteoclast-activating factors, including cytokines, growth factors, hormones, enzymes, adhesion molecules, and chemotactic factors, underlie progression of this disease state. Various pathologic conditions result, most notably bone fracture, bone pain, and hypercalcemia, which all contribute to increased morbidity among cancer patients with advanced bone tumors and metastases (2).

The clinical hallmark of disrupted bone remodeling in aggressively metastasizing breast cancers is extensive osteoclast-mediated bone destruction (3). Osteoclasts, which seem to be required for progression of osteolytic tumor burden, differentiate and activate in response to stimulation by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, all known to be secreted by breast cancer cells (4–6). Our data and those of other laboratories indicate that the cytokines produced by the cancer cells not only stimulate bone resorption by host osteoclasts but also induce other cells in the microenvironment to secrete high levels of the same cytokines (5, 7). This sustained release of catabolic cytokines drives a rapid loss of bone. Attempting to individually block the effect of any or all of these cytokines may not be the most practical approach to this clinical problem. Ideally, a distinct therapeutic target governing production of a spectrum of such bone-resorbing factors could be identified and exploited to more effectively reduce or prevent osteolytic bone destruction.

Many of the proinflammatory cytokines involved in stimulating osteoclastic activity, including TNF-α, IL-1, and IL-6, fall under the control of nuclear factor-κB (NF-κB) transcription (8). Targeting the NF-κB family of transcription factors holds significant promise in the treatment of various cancers and diseases associated with abnormal bone resorption (9–12). NF-κB controls progression of oncogenesis and associated disease states through suppression of apoptosis and cell differentiation as well as promotion of cell proliferation and migration. Many of the steps in the NF-κB pathway have been delineated. The dimeric form of the transcription factor resides in the cell cytoplasm bound to an inhibitor (of nuclear factor) of κB (IκB) protein. Various molecular stimuli can activate IκB kinases, which in turn phosphorylate IκB and target it for degradation in the 26S proteasome. After dissociating from IκB, the NF-κB dimer may then translocate to the nucleus and regulate expression of multiple genes potentially involved in cancer cell survival, growth, and osteolysis (9, 10). Blocking this single pathway therefore may inhibit diverse arrays of biological interactions that promote osteolytic tumor progression.

We suppressed NF-κB transcription and associated gene expression in the tumor cells by using a dominant-negative mutant IκBα (mIκBα) superrepressor of NF-κB signaling (13). This repressor was stably infected into the MDA-MB-231 cell line to determine the role of NF-κB signaling in cytokine production, apoptosis, proliferation, and bone resorption at a skeletal site.

Materials and Methods

Cell lines and culture conditions. The MDA-MB-231 breast cancer cell line was a gift from Dr. Theresa Guise (San Antonio, TX) and the 293GP viral packaging cell line was a gift from Dr. Inder Verma (Salk Institute, La Jolla, CA). MDA-MB-231 and 293GP cells were grown in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1%...
penicillin/streptomycin, and nonessential amino acids (Invitrogen). Cells were grown in a humidified 37°C atmosphere of 5% CO₂.

**Stable expression of lacZ and mIβ expression vectors.** MDA-MB-231 cells stably expressing a dominant-negative inhibitor of NF-κB, an IκBα with serine-to-alanine mutations at positions 32 and 36 (mIβ), were generated by infecting wild-type MDA-MB-231 cells with a recombinant retrovirus technique described previously and adapted for the MDA-MB-231 cell line (13, 14). Retrovirus was produced using 293 kidney fibroblasts stably expressing the Moloney gag and pol (293GP) under control of the cytomegalovirus promoter-enhancer. 293GP cells were plated in 10 cm culture dishes and grown to 50% confluency at 37°C in 10% DMEM/10% FBS/1% penicillin/streptomycin. Fresh serum-free DMEM was added before transfection. Vesicular stomatitis virus envelope (4 μg) downstream of the Rous sarcoma virus promoter-enhancer (to aid in assessing transfection efficiency of the mIβ) were transfected into 293GP cells using LipofectAMINE 2000 (Invitrogen). Transfected 293GP cells were incubated for 24 hours, after which retroviral supernatant was collected and treated with 10 μg/ml polybrene (Sigma, St. Louis, MO). Supernatants were filter sterilized and stored at −80°C until use. Transfection efficiency was evaluated by staining for β-galactosidase by fixing 293GP cells with 5 mL staining solution [5 mmol/L potassium ferricyanide, 5 mmol/L ferrocyanide, 2 mmol/L MgCl₂, PBS (pH 7.4), X-gal in DMSO (1 mg/mL)] at an I:40 dilution. Stained cells were incubated for 2 to 4 hours at 37°C. Whole cell or cytoplasmic protein extract on a 10% SDS-polyacrylamide gel using 200 μg/ml X-gal and 2 μmol/L β-nicotinamide adenine dinucleotide (NAD) is added to the binding reaction and incubated for 30 minutes at room temperature. NF-κB p65 subunit (Santa Cruz Biotechnology) for 20 minutes on ice.

**Western blot analysis.** Caspase-3 and IκBα protein expression was analyzed by Western blot as described previously by separating 20 μg whole cell or cytoplasmic protein extract on a 10% SDS-polyacrylamide gel using 200 μg/ml X-gal and 2 μmol/L β-nicotinamide adenine dinucleotide (NAD) is added to the binding reaction and incubated for 30 minutes at room temperature. NF-κB p65 subunit (Santa Cruz Biotechnology) for 20 minutes on ice.

**Electrophoretic mobility shift assay.** Electrophoretic mobility shift assay (EMSA) was used to assess NF-κB DNA binding as described previously (14). Nuclear protein extract (5 μg) was mixed with 2 μg poly(ddeoxyinosinic-deoxyctydilic acid) and DNA binding buffer [50 mmol/L NaCl, 5 mmol/L HEPES (pH 7.5), 5 mmol/L EDTA, 5 mmol/L EGTA, 30% glycerol, 1.25 μg bovine serum albumin] in a total volume of 10 μL and incubated on ice for 20 minutes. NF-κB and Oct-1 consensus oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA) were end labeled by the use of T4 polynucleotide kinase and [γ-32P]ATP (New England Nuclear, Boston, MA) and 20,000 counts per minute (cpm) of 32P-labeled oligonucleotide were added to the binding reaction and incubated for 30 minutes at room temperature. Complexes were then separated on 6% polyacrylamide gel under nondenaturing conditions at 125 V for 3 hours. Gels were dried on Whatman No. 3M paper and DNA-protein complexes were visualized by autoradiography. To identify the p65 NF-κB subunit of the heterodimeric complex, EMSA supershift was done by preincubating nuclear protein extracts with antibody specific for the active NF-κB p65 subunit (Santa Cruz Biotechnology) for 20 minutes on ice.

**Luciferase assay.** Our transfection reagents were 1.5 μg pNFκB-Luc (firefly luciferase driven by a TATA box with five NF-κB sites in the enhancer element) and 2 ng pRL-SV40 (Renilla luciferase driven by SV40) reporter constructs (Promega, Madison, WI) complexed to 3 μL LipofectAMINE 2000 transfection reagent in 100 μl serum-free culture medium. Cells (n = 300,000) seeded in six-well plates (Falcon, Franklin Lakes, NJ) in 3 ml culture medium were transfected by adding 100 μL LipofectAMINE 2000-plasmid complex. Luciferase activity was assayed after 24 hours using the Dual Luciferase Reporter Assay System (Promega). Reporter assays were performed on unstimulated cells after a 30-minute stimulation with 40 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) and 2 μmol/L ionomycin A23187 (Calbiochem, San Diego, CA), a common regimen used for activation of NF-κB.

**Figure 1.** Stability and functionality of MDA.mIβ cells. A, constitutive and induced NF-κB transcription is suppressed in MDA.mIβ cells. NF-κB luciferase reporter activity is observed to increase significantly after activation of MDA-MB-231 and MDA.lacZ control cells, but not MDA.mIβ cells, with PMA (40 ng/mL) and ionomycin (2 μmol/L) for 30 minutes *, P < 0.02 versus unstimulated and P < 0.01 versus PMA/ionomycin regimen. B, NF-κB p65 DNA binding activity is suppressed in MDA.mIβ cells. As analyzed by EMSA, constitutive NF-κB/DNA binding is seen in unstimulated (−) wild-type (WT) MDA-MB-231 nuclear extracts and after stimulation (+) with PMA (40 ng/mL) and ionomycin (2 μmol/L) for 30 minutes. To identify the active p65 NF-κB subunit of the heterodimeric complex, nuclear extracts from wild-type MDA-MB-231 cells were preincubated with antibody to human p65 for 30 minutes on ice.

Caspase-3 binding otherwise observed in wild-type MDA-MB-231 extracts is lost in MDA.mIβ cells. COLD, extracts treated with unlabeled, nonradioactive probe. C, IκBα is not degraded in MDA.mIβ clones. Western analysis shows that after stimulation with PMA (40 ng/mL) and ionomycin (2 μmol/L), IκBα levels decrease in MDA-MB-231 and MDA.lacZ control cells, whereas IκBα levels in MDA.mIβ cells remain the same. Endogenous IκBα is therefore replaced in MDA.mIβ cells by the mIβ, which cannot be degraded. D, an active form of the apoptosis marker caspase-3 (p20) is expressed in MDA.mIβ cells. Western analysis of whole cell lysates from tumor cells shows that after stimulation with TNF-α (10 ng/mL) for 0, 12, and 24 hours levels of the caspase-3 precursor CPP32 (−32 kDa) decrease and levels of an active subunit of caspase-3, p20 (−17 kDa), increase in MDA.mIβ cells but not in MDA-MB-231 and MDA.lacZ control cells.
ELISA detection and quantitation of cytokines. Cell lines were plated on six-well plates at a cell density of $10^5$/mL and grown in 2 mL DMEM/10% FBS/1% penicillin/streptomycin to near confluence. After incubation at 37°C for 16 hours, conditioned medium samples were collected and stored at −80°C until assayed for cytokine levels by ELISA. Protein standards, primary capture, and secondary biotin antibody pairs were obtained for human TNF-α, IL-1α, IL-6 (PharMingen, La Jolla, CA), IL-1β (Endogen, Woburn, MA), macrophage colony-stimulating factor (M-CSF), IL-11, and IL-17 (R&D Systems, Minneapolis, MN). Cytokine levels for 100 µL of each supernatant sample were evaluated using commercially available ELISA reagents (PharMingen).

**Real-time reverse transcription-PCR.** Total mRNA from MDA-MB-231 cells was reverse transcribed using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA) and PCR amplified using Platinum PCR Supermix (Invitrogen). PCR conditions for amplification were initial denaturation of 3 minutes at 94°C and subsequent denaturation for 25 seconds at 94°C, annealing for 25 seconds at 55°C, and extension for 50 seconds at 72°C. Quantitative PCR analysis was done using the Rotor-Gene 2000 Centrifugal Real-time PCR System (Corbett Research, Sydney, New South Wales, Australia). RNA samples were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal controls in each cell type and stimulus condition (17). IL-6 fragments (81 bp) were amplified with forward primer 5'-GGTACATCCTCGACGGCATCT-3' and reverse primer 5'-GTGCCCTCTTG-TGCTGTTTCAC-3' (Life Technologies, St. Paul, MN; ref. 18). Human parathyroid hormone-related protein (PTHrP) mRNA levels were evaluated using primers specific for a 285-bp PTHrP fragment (5'-GGCACGATTTCTCTTCACCC-3' and 5'-AGAAGTCTACACGCCAGGACG-3', corresponding to the sense and antisense strands of exon 4 of human PTHrP; ref. 19). GAPDH fragments (178 bp) were amplified with forward primer 5'-GACATCAAGAAGGTTGGTGAAC-3' and reverse primer 5'-TGTCATACAGGAATTGAGC-3' (20).

In vitro bone marrow/tumor cell cocultures. Transverse slices of femoral diaphyseal bovine cortical bone (4.0 × 4.0 × 0.3 mm) cut with a low-speed diamond saw (Buehler, Evanston, IL) were used. Long bones (tibias and femurs) were removed from 4- to 6-day-old euthanized rats, dissected free of adherent soft tissue, and minced with a ultrasonication in multiple changes of sterile water and stored at −20°C until used. Long bones (tibia and femurs) were removed from 4- to 6-day-old euthanized rats, dissected free of adherent soft tissue, and minced with a scalpel blade into 2 or 3 mL prewarmed medium 199 with Hank’s salts (Sigma; pH 7.0), buffered with 100 mM L HEPES containing 100 µg/mL penicillin/streptomycin. The resulting bone fragment and cell suspension was gently triturated 10 times with a transfer pipette. An aliquot (110 µL) of the bone cell suspension was then transferred to individual wells of 96-well plates, each well containing a single bone slice pre-wetted with 100 µL of the above medium. Following a settling period of 30 to 45 minutes at 37°C, medium was replaced with fresh 199 with Earle’s salts (Sigma), 10% heat-inactivated FBS, parathyroid hormone (10 mM/L), and 1.25(OH)$_2$ vitamin D$_3$ (10 mM/L) and adjusted to pH 6.8. Forty-eight hours after plating bone marrow cell suspensions onto bone slices, 10,000 tumor cells from each cell type (MDA-MB-231 and MDA.mI.B) were plated on each cortical bone slice. Tumor cells were cocultured with the bone marrow cell suspensions for 10 days on bone slices, changing medium every 48 hours. After 10 days, slices were removed, washed gently with 1× PBS, scraped free of cell debris, and stained with toluidine blue. Resorption area of each lacunae was quantified with Osteometrics software (Atlanta, GA). Digital image analysis generated a number expressing the percentage of the 16 mm² bone slice surface that was resorbed.
Intratibial injection model of tumor-mediated bone resorption.

Female athymic \( \text{nu/nu} \) mice (NIH/Charles River, Bethesda, MD) were given 60 mg/kg ketamine and 4 mg/kg xylazine i.p., providing 15 to 20 minutes of deep anesthesia before tumor cell injection. The injection site was sterilized with isopropyl alcohol, cleaned with Betadine, and allowed to dry. After making a small incision (5 mm) revealing the tibial tuberosity, a small hole was made with a 25 gauge needle. Then, a Hamilton syringe (10\( \mu \)L capacity, 26S gauge) was inserted in the proximal end of the tibia, and 10\(^5\) tumor cells suspended in 10\( \mu \)L PBS were injected into the intramedullary space. Radiographs were taken immediately after injection, while the mice remained anesthetized, to confirm correct insertion of the syringe without further fracture of surrounding bone. Radiographs were taken at 0, 14, and 28 days to track osteolytic lesion progression.

Radiographs and measurements of osteolytic lesion area. Animals were exposed for 6 seconds to X-ray in a prone position against XOMAT-AR film (Kodak, Rochester, NY) using a Faxitron system (Wheeling, IL) set to a tube voltage of 36 kVp. All radiographs were evaluated without knowledge of treatment groups. Radiographs were scanned into digital TIFF format, and quantitation of \textit{in vivo} osteolytic lesion area was done using image analysis software (NIH Scion Image, Frederick, MD and Adobe Systems Photoshop, San Jose, CA).

Histology/histomorphology. At sacrifice on day 28, tibias were dissected from all mice, fixed in 10% formalin, decalcified in EDTA, and embedded in paraffin. Sections were cut using a standard microtome, placed on poly-L-lysine-coated glass slides, and stained with orange G and H&E. Additional sections were stained for tartrate-resistant acid phosphatase (TRAP), human IL-6, and human p65 (Santa Cruz Biotechnology). Osteoclast number expressed per millimeter of tumor-bone interface was measured from TRAP-stained sections using digital imaging (NIH Scion Image and Adobe Systems Photoshop).

Tumor cell growth. To investigate whether tumor growth was altered in sites other than bone, tumor cell suspensions (5\( \times \)10\(^6\) cells/0.1 mL PBS) were inoculated s.c. into female athymic nude mice. Mice were sacrificed at day 28 and the tumors were excised and weighed. To confirm cell proliferation rates \textit{in vitro}, \( ^{3}\text{H}\)thymidine incorporation assays were done as described previously (22, 23). Briefly, cell cultures of 20,000 cells in 24-well plates (Becton Dickinson, Franklin Lakes, NJ) were labeled with 10 \( \mu \)Ci/mL \( ^{3}\text{H}\)thymidine (New England Nuclear) in the presence of 5 mmol/L unlabeled \( ^{3}\text{H}\)thymidine (to control for differences in radioactive \( ^{3}\text{H}\)thymidine pools) in culture medium. After 4 hours of incubation, the DNA was precipitated and centrifuged and the pellet was redissolved in NaOH. Radioactivity was determined by liquid scintillation spectrometry.

Results

Generation of MDA.mlkB and MDA.lacZ cells. To investigate the role of NF-\( \kappa \)B in bone tumor burden and associated tumor-mediated osteolysis, a recombinant retrovirus containing a mlkB expression cassette and a neomycin drug resistance marker were used to generate cells that stably express the mlkB. The mlkB cassette was mutated by replacing Ser\(^{32}\) and Ser\(^{36}\) with alamines. The mlkB binds NF-\( \kappa \)B, but because the mlkB cannot be

Figure 3. Establishment of osteolytic tumor lesions by intratibial injection. A, suppression of osteolytic tumor burden in mice implanted with MDA.mlkB cells. Tumor cells (10\(^5\)) were implanted in the proximal end of tibiae of female athymic nude mice. Arrows, approximate location and direction of tumor cell injection. Mice were X-rayed at days 0, 14, and 28 to track progression of osteolytic tumor lesions formed by wild-type MDA-MB-231, MDA.lacZ, and MDA.mlkB cells. B, suppression of osteolytic lesions in mice implanted with MDA.mlkB cells. On sacrifice at day 28, tibiae were harvested, fixed in formalin, and embedded in paraffin. Sections were stained with orange G and H&E. These images show the proximal end of representative tibiae where the tumor cells were injected. Note replacement of bone marrow with tumor cells in wild-type MDA-MB-231 and MDA.lacZ tissue sections. Magnification, \( \times 4\).
phosphorylated at two key sites NF-κB signal transduction is suppressed. The mutant therefore behaves as a dominant-negative inhibitor by blocking NF-κB gene expression. Using this technique, neomycin-resistant MDA.mI-B cells were isolated after stable infection of wild-type MDA-MB-231 cells. A lacZ retroviral vector was also stably infected into wild-type MDA-MB-231 cells to create neomycin-resistant MDA.lacZ control cells.

To examine stability and functionality of the newly isolated MDA.mI-B cells, functional activation of NF-κB transcription was examined by luciferase reporter assay. Suppression of functional NF-κB activation was exhibited by MDA.mI-B cells when compared with wild-type MDA-MB-231 and MDA.lacZ controls (Fig. 1A). EMSAs were done on isolated nuclear protein extracts to assess NF-κB/DNA binding activity. Wild-type MDA-MB-231 cells possessed constitutive NF-κB/DNA binding activity with or without stimulation by PMA/ionomycin for 30 minutes (Fig. 1B). The active p65 NF-κB subunit of the p50/p65 heterodimer was identified as a component of this shift, as evidenced by a supershift, after preincubating with antibody to human p65 for 30 minutes on ice (Fig. 1B). NF-κB/DNA binding associated with the p65 subunit, otherwise seen in nuclear extracts from MDA-MB-231 cells, was lost in MDA.mI-B cells (Fig. 1B). These experiments therefore show the effectiveness of the dominant-negative mI-B in reducing NF-κB activation in MDA.mI-B cells.

Further demonstration of the efficacy of the dominant-negative mI-B was shown by immunoblotting. Cytoplasmic extracts from wild-type MDA-MB-231, MDA.lacZ, and MDA.mI-B clones were probed for IκBα levels by Western blot analysis. Wild-type MDA-MB-231, MDA.lacZ, and MDA.mI-B cells produced similar amounts of IκBα before PMA/ionomycin treatment. However, 30 minutes after stimulation with PMA/ionomycin, IκBα levels in both wild-type MDA-MB-231 and MDA.lacZ cells decreased substantially, whereas IκBα levels in MDA.mI-B cells remained essentially unchanged. This supports the expectation that the predominant form of IκBα in the MDA.mI-B cells can no longer be degraded following stimulation (Fig. 1C).

Tumor cell survival is tightly regulated by NF-κB signaling, which protects against TNF-α-induced apoptosis (24). To assess TNF-α-induced apoptosis in MDA.mI-B cells, we employed caspase-3 as a biological marker. Caspase-3 whose activity is up-regulated in cells with reduced NF-κB signaling is required for DNA fragmentation and morphologic changes typical of cells undergoing apoptosis (25, 26). On induction of caspase-3 activity during apoptosis, the inactive 32-kDa proenzyme (CPP32) is cleaved at an aspartate residue, yielding the active subunit p20 (~17-21 kDa; ref. 27). Thus, expression of the p20 active form of caspase-3 serves as an indicator of apoptosis. After treatment with TNF-α for 12 and 24 hours, increased expression of active caspase-3 (p20) was observed in whole cell protein extracts of MDA.mI-B clones, whereas in wild-type MDA-MB-231 and MDA.lacZ extracts no expression of active caspase-3 was detected (Fig. 1D). These results support the notion that MDA-MB-231 cells are rendered susceptible to TNF-α-induced apoptosis after inhibition of NF-κB signaling.

**Screening of cytokine production in MDA-MB-231 cells.** Breast cancer cells promote in vivo osteolysis by inducing host osteoclast activity (4). To elucidate how tumor-derived NF-κB signaling increases bone-resorptive activity, MDA-MB-231 cells were screened for secretion of NF-κB-mediated factors with potential roles in bone resorption. Specifically, we examined levels of IL-1α, IL-1β, IL-6, IL-11, IL-17, M-CSF, and TNF-α, all of which are cytokines implicated in bone-resorptive processes and NF-κB signaling (6, 8, 28–34). MDA-MB-231 cells showed significantly higher constitutive levels of IL-11 in comparison with TNF-α, IL-1α, IL-1β, IL-6, IL-17, and M-CSF, and TNF-α for a prolonged period (16 hours), only IL-6 production increased significantly (data not shown).

MDA-MB-231 cells produce significant amounts of IL-6, a potent stimulator of osteoclast activity and formation whose gene promoter possesses NF-κB consensus motifs (28–30). These observations suggested that IL-6 secretion could be down-regulated after inhibition of NF-κB signaling in MDA-MB-231 cells. As measured by ELISA, MDA.mI-B cells secreted up to 99% less IL-6 than wild-type MDA-MB-231 cells after stimulation (n = 3; P < 0.01; Fig. 2A). This result was verified by real-time reverse transcription-PCR (RT-PCR), which shows suppression of IL-6 mRNA levels in MDA.mI-B cells treated with IL-1β (n = 3; P < 0.01; Fig. 2B). Secretion of IL-11, whose production was not further stimulated by IL-1β, was observed to be slightly up-regulated in MDA.mI-B clones (data not shown; n = 3; P < 0.02). MDA-MB-231 cells were also assayed for NF-κB-mediated PTHrP mRNA expression. PTHrP is a causal factor in tumor-mediated osteolysis whose fragments activate NF-κB signaling in osteoblasts (35, 36). Real-time RT-PCR did not show a statistically significant difference in PTHrP mRNA expression between wild-type MDA-MB-231 and MDA.mI-B cells (data not shown). NF-κB therefore does not seem to control PTHrP mRNA expression in MDA-MB-231 cells.

**Inhibition of nuclear factor-κB signaling in MDA-MB-231 cells down-regulates their capacity to stimulate bone resorption in vitro.** Next, we examined whether MDA.mI-B cells were altered in their ability to stimulate bone resorption in vitro. We cocultured wild-type MDA-MB-231 and MDA.mI-B cells with rat bone marrow cultures and quantified osteoclast lacunae formation in vitro on cortical (bovine) bone wafers using computerized imaging software. In comparison with bone marrow cell culture controls, addition of MDA-MB-231 cells significantly increased bone resorption lacunar area. Substitution of wild-type MDA-MB-231 cells with MDA.mI-B cells in this coculture, however, showed no measurable decrease in osteolysis whose fragments activate NF-κB signaling in osteoblasts (35, 36). Real-time RT-PCR did not show a statistically significant difference in PTHrP mRNA expression between wild-type MDA-MB-231 and MDA.mI-B cells (data not shown). NF-κB therefore does not seem to control PTHrP mRNA expression in MDA-MB-231 cells.

**Table 1. In vivo osteolytic tumor frequency and correlating immunohistochemistry**

<table>
<thead>
<tr>
<th></th>
<th>PBS (sham)</th>
<th>MDA-MB-231 (wild-type)</th>
<th>MDA.lacZ</th>
<th>MDA.mI-B</th>
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<td>Radiologic lesions</td>
<td>0/5</td>
<td>8/8</td>
<td>9/10</td>
<td>1/9*</td>
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<tr>
<td>Histologic lesions</td>
<td>0/5</td>
<td>8/8</td>
<td>9/10</td>
<td>3/9*</td>
</tr>
<tr>
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<td>3/9</td>
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<tr>
<td>IL-6 (+)</td>
<td>0/5</td>
<td>8/8</td>
<td>9/10</td>
<td>3/9</td>
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**NOTE:** Female athymic nude mice implanted with MDA-MB-231-derived tumor cells were monitored for osteolytic tumor progression until sacrifice (day 28). Osteolytic lesions were identified by radiography and histology. Presence of p65 and IL-6 was verified by immunohistochemistry. Fractions represent number of mice positive for tumor, NF-κB-p65, or IL-6/total number of mice in experiment. All experimental groups began as n = 10, except PBS sham (n = 5). Two mice in the wild-type MDA-MB-231 (n = 8) group and one mouse in the MDA.mI-B (n = 9) group died of complications unrelated to tumor injection.

* P < 0.01 versus wild-type MDA-MB-231 and P < 0.01 versus MDA.lacZ.
suppression of this osteolytic activity. Because inhibition of NF-κB signaling down-regulates IL-6 expression in MDA-MB-231 cells, wild-type MDA-MB-231/bone marrow cocultures were also treated with anti-human IL-6 antibody to ascertain any role tumor-derived IL-6 may have in in vitro bone resorption. A marked decrease in lacuna area was observed in wild-type MDA-MB-231 cocultures pretreated with anti-human IL-6 antibody (Fig. 2C). Thus, inhibition of NF-B signaling, as well as blockade of tumor cell-derived IL-6, down-regulates the ability of breast cancer cells to promote bone resorption.

Effect of the dominant-negative mlkB on in vivo osteolytic lesion formation by MDA-MB-231-derived tumor cell lines in female athymic nude mice. Our in vitro observations provided sufficient rationale to test whether inhibition of NF-κB signaling could suppress bone tumor growth and associated tumor-mediated bone resorption in vivo. Osteolytic lesion formation by MDA.mlkB cells in bone compared with MDA-MB-231 and MDA.lacZ controls was evaluated by intratibial injection in female athymic nude mice. After injecting the tumor cells (n = 10 per treatment group), radiographs were taken at days 0, 14, and 28. Radiography verified a high percentage of osteolytic lesions in MDA-MB-231 and MDA.lacZ treatment groups (Fig. 3A). At day 28, animals were sacrificed for histology.

Histologic specimens revealed replacement of bone marrow with tumor cells in the intratibial cavity of mice injected with either wild-type MDA-MB-231 or MDA.lacZ cells. In most mice injected with MDA.mlkB clones, however, the marrow remained intact (Fig. 3B). Tumor lesions formed by intratibial injection of MDA.mlkB cells were observed to be of significantly smaller magnitude than tumors formed by MDA-MB-231 and MDA.lacZ control cells. Frequency of osteolytic lesions (Table 1), osteolytic lesion area (Fig. 4A), and number of osteoclasts found at the tumor-bone interface (Fig. 4B) were significantly decreased in MDA.mlkB-bearing mice when compared with wild-type MDA-MB-231 and MDA.lacZ treatment groups. High numbers of TRAP-positive osteoclasts were seen at the tumor-bone interface (Fig. 4C), consistent with the pivotal role of osteoclasts in tumor-mediated bone destruction (4).

Immunohistochemistry of all tumors formed showed production of both NF-κB-p65 and IL-6 protein (Table 1; Fig. 5). The most intense IL-6 staining was found predominantly near areas where tumor cells interfaced with bone, consistent with the notion that IL-6 plays an important role in tumor-mediated bone resorption. These observations suggest that the presence of the NF-κB-p65 subunit and IL-6 correlates with formation of in vivo osteolytic lesions by MDA-MB-231 cells.

Effect of inhibition of nuclear factor-κB signaling in breast cancer cells on tumor growth. To determine if the effect of NF-κB signaling on bone tumor growth was indeed specific to bone, tumor cells (5 × 10^6/100 μL PBS) were injected s.c. into female athymic nude mice. S.c. tumor growth for each treatment group (n = 5) was monitored by palpation until sacrifice at day 28. On excision at day 28, tumors were weighed. Tumor mass (n = 5; P < 0.05 versus MDA-MB-231 and P < 0.01 versus MDA.lacZ; Fig. 6) differed significantly between both MDA-MB-231 or MDA.lacZ

Figure 4. Quantification and visualization of in vivo osteolysis. A, in vivo osteolytic lesion area quantified from radiographs of tibias on days 14 and 28. Osteolytic tumor lesion area was measured using computerized imaging analysis. Points, mean measurements from midsections of right tibias of mice injected with wild-type MDA-MB-231, MDA.lacZ, or MDA.mlkB cells (8-10 mice per group); bars, SE. *, P < 0.01 versus wild-type MDA-MB-231 and MDA.lacZ. B, inhibition of NF-κB signaling in MDA-MB-231 cells decreases osteoclast number per millimeter of bone in vivo. Histomorphometry of right tibias from female athymic nude mice injected with 10^6 tumor cells at day 0. Tibias were harvested at day 28 and stained with TRAP to count osteoclasts. Midsections of right mouse tibia. *, P < 0.01 versus MDA-MB-231 and MDA.lacZ. C, in vivo TRAP staining of osteoclasts at tumor-bone interface. After 28 days, tibias were harvested, decalcified, sectioned, and stained for TRAP. Implanted MDA.lacZ-positive control cells stimulating TRAP-positive osteoclasts (OC) at the tumor-bone interface. Similar sections were obtained for all mice implanted with tumor cells that developed osteolytic tumor lesions. Magnification, ×20.
controls and MDA.mIB cells. In vitro \(^{3}H\)thymidine incorporation assays confirmed MDA.mIB cells possessed a reduced capacity to proliferate compared with wild-type MDA-MB-231 cells (\(^{3}H\)thymidine DNA incorporation: wild-type MDA-MB-231 = 441.3 cpm and MDA.mIB = 252.7 cpm, \(n = 3; P < 0.01\)). Therefore, MDA.mIB cells show a decreased capacity to proliferate, independent of bone, as evidenced by their reduced s.c. tumor mass in vivo and decreased \(^{3}H\)thymidine incorporation in vitro.

**Discussion**

Patients presenting with bone cancers show significantly lower relative survival rates than patients with soft tissue cancers (37). Therefore, validating a therapeutic target, such as NF-\(\kappa\)B, governing production of numerous factors relevant to bone tumor and metastasis progression, remains of great interest. This study distinguished NF-\(\kappa\)B signaling as a key regulator of a tumor cell's capacity to survive in bone and form osteolytic tumors. Here, we show the essential role NF-\(\kappa\)B transcription may play in bone tumor cell survival, proliferation, bone resorption, and overall in vivo bone tumor burden. MDA-MB-231 breast cancer cells stably infected with a mI\(\kappa\)B, which suppressed NF-\(\kappa\)B transcription, displayed decreased in vivo bone resorption, decreased production of the bone-resorbing cytokine IL-6, and increased production of the active form of caspase-3, a known biological marker of apoptosis. Disruption of NF-\(\kappa\)B signaling in MDA-MB-231 cells also reduced in vivo tumor-mediated osteolysis, corresponding with suppressed in vivo production of IL-6 and the active p65 subunit of NF-\(\kappa\)B.

NF-\(\kappa\)B-mediated production of IL-6 by MDA-MB-231 cells may be of critical significance to the capacity of MDA-MB-231 cells to flourish in vivo, particularly in bone. Inhibition of NF-\(\kappa\)B signaling suppressed several biological phenomena (apoptosis, proliferation, cytokine production, and bone resorption) that combine to augment in vivo bone tumor growth and tumor-mediated osteolysis. IL-6 affects all of these processes and therefore warrants closer examination as a therapeutic target (38, 39). Production of IL-6 by breast cancer cells confers increased resistance to chemotherapeutic agents and plays a prominent role in several inflammatory disease states, including cancer-related anorexia and cachexia (40, 41).

Moreover, by increasing the pool of early osteoclast precursors available to mature into active, bone-resorbing osteoclasts, increased amounts of IL-6 secreted by tumor cells with active NF-\(\kappa\)B signaling could profoundly dysregulate normal bone remodeling and cause bone destruction. IL-6 likely works in concert with other bone-resorbing factors produced by breast cancer cells, such as PTHrP (35, 42). Augmented PTHrP expression can increase bone resorption by stimulating osteoblast RANKL mRNA expression, which in turn fosters bone-resorbing activity by the increased number of osteoclasts made available by IL-6 (43). PTHrP fragments also activate NF-\(\kappa\)B transcription in osteoblasts, leading to IL-6 gene induction and suggestive of an autocrine/paracrine role for PTHrP in NF-\(\kappa\)B-mediated osteolytic tumor burden (36, 44).

Other NF-\(\kappa\)B-related mechanisms, not directly dependent on RANKL activation of osteoclasts, exist in bone through which IL-6 may exert a bone-resorptive effect (45). These pathways could
include steroid hormone-regulated bone formation. Estrogen, whose loss is shown to result in IL-6 mediated stimulation of osteoclastogenesis, could protect bone via estrogen receptor interference with NF-κB binding (46, 47). Estrogen also induces production of the proapoptotic caspase-3 protein in association with preosteoclast apoptosis (48). This increase in caspase-3 can be explained by estrogen interference with NF-κB signaling. Production of caspase-3 and apoptosis of tumor cells also correlates with decreased bone tumor burden in the MDA-MB-231 bone metastasis model (49). Therefore, a strong rationale exists to further investigate the potential reciprocal relationship between estrogen and NF-κB signaling in cancer cells and how this affects osteolytic tumor burden. Because MDA-MB-231 cells are estrogen receptor negative and unable to suppress NF-κB signaling in this manner, this could explain why these cells significantly proliferate in bone (38).

Our observations here strengthen the notion that NF-κB-regulated IL-6 production by tumor cells causes dysregulation of bone remodeling, supportive of bone destructive processes, through multiple signaling pathways. An observed increase in IL-11 production by MDA-MB-231 cells, however, sharply contrasts with previous studies supporting its role as a stimulator of osteoclast formation and bone resorption and also as a predictor or promoter of osteolytic tumor burden or metastases (45, 50). IL-11 possesses anti-inflammatory properties and is reported to inhibit NF-κB activation by up-regulating production of IκBα (51). In this manner, IL-11 may override its known role as an osteoclast growth factor in favor of tumor suppression. Contrary to previous reports, therefore, IL-11 could function to inhibit as opposed to promote osteolysis in bone tumors with increased NF-κB activation.

Several therapeutic approaches targeting NF-κB signaling show promise in treating various cancers and inflammatory diseases and might be similarly applied to treatment of tumor-mediated osteolysis. Consistent with the results of our study, an NF-κB decoy oligonucleotide significantly stimulated apoptosis, upregulated caspase-3, and inhibited IL-6 expression in osteoclasts (52). This kind of decoy could decrease the numbers of osteoclasts available to induce osteolytic bone destruction and simultaneously suppress tumorigenic effects dependent on NF-κB activation (52, 53). Treating with proteasome inhibitors could prevent IκB degradation, otherwise required for NF-κB nuclear translocation. Velcade (PS-341), a proteasome inhibitor that suppresses degradation of IκB and abrogates IL-6 signaling, is currently used in patients with multiple myeloma (9, 54–56). Evaluating this agent in patients with metastatic or other native bone cancers would be a logical next step. Cytokine-induced NF-κB activation can be suppressed using a cell-permeable NF-κB essential modifier regulatory protein (NEMO)-binding domain peptide, which blocks association of NEMO with IκB kinase complexes upstream of NF-κB (57). Administration of the NEMO-binding domain peptide reduces NF-κB-mediated bone resorption in vivo and selectively blocks NF-κB activation induced by proinflammatory cytokines without disrupting basal NF-κB activity, an important consideration given the dual role of NF-κB activation in both proper immune system function and pathogenesis of disease (8, 57–59).

Collectively, our data support a potential role for therapeutics like these that could disrupt NF-κB signaling in cancers causing osteolysis. This investigation presents evidence on how NF-κB transcription may tightly control bone tumor cell survival and proliferation and associated bone resorption. Although there exist signaling pathways in tumor cells that promote osteolytic tumor burden but do not directly depend on NF-κB transcription, NF-κB remains a central target to consider when contemplating potential therapeutics in this regard.

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