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TAK1-TAB2 signaling contributes to bone destruction by breast carcinoma cells

Alfiya Safina¹, Paula Sotomayor¹, Michelle Limoge¹, Carl Morrison², and Andrei V. Bakin¹

¹Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, New York 14263

²Department of Pathology, Roswell Park Cancer Institute, Buffalo, New York 14263

Abstract

Advanced-stage breast cancers frequently metastasize to the bones and cause bone destruction, but the underlying mechanism is not fully understood. This study presents evidence that TGF- β -activated protein kinase 1 (TAK1) signaling in tumor cells promotes bone destruction by metastatic breast carcinoma cells, controlling expression of pro-metastatic factors, including MMP-9 and COX2. Suppression of TAK1 signaling by dominant-negative (dn) TAK1 in breast carcinoma MDA-MB-231 cells impairs bone colonization by carcinoma cells and bone osteolysis in the intra-cardiac injection model. Mechanistic studies showed that inhibition of TAK1 by dn-TAK1 or siRNA blocked expression of factors implicated in bone metastasis, such as MMP-9, COX2/PTGS2, PTHrP, and IL8, but did not affect activation of p38MAPK by TGF- β . TAK1 signaling is mediated by TAK1-binding partners TAB1, TAB2 and TAB3. Carcinoma cells express elevated mRNA levels of TAB2 and TAB3, whereas the TAB1 expression is noticeably low. Accordingly, depletion of TAB2 by siRNA reduced expression of MMP-9 and COX2. Together, these studies demonstrate that the TAK1-TAB2/TAB3 signaling axis is critical for carcinoma-induced bone lesions, mediating expression of pro-invasive and osteolytic factors. These findings identify the TAK1-TAB2 axis as a potential therapeutic target in bone metastasis.

Keywords

Invasion; metastasis; bone metastasis; TAK1; TGF-beta

INTRODUCTION

Patients with advanced-stage breast cancers frequently develop skeletal metastases that cause pain, bone fractures and other complications (1). Advanced breast cancers express elevated levels of transforming growth factor beta (TGF- β) cytokines that promote osteoclast-mediated bone destruction (2). Although, the TGF- β pathway is an attractive target for therapeutic intervention in the skeletal metastases, TGF- β is a potent inhibitor of cell growth and functions as a tumor suppressor in the early stages of cancer. In advanced cancers, TGF- β promotes tumor spreading by enhancing invasion and angiogenesis (3). Identifying the factors mediating tumorigenic activities of TGF- β is an important step in the development of effective therapeutics preventing tumor invasion and metastasis.

Cancer progression and bone metastasis have been linked to changes in tumor microenvironment and inflammation. Transforming growth factor beta (TGF- β) cytokines regulate the composition of extracellular matrix (ECM), matrix proteolysis and

inflammatory responses (4, 5). According to a current model, carcinoma cells respond to TGF- β with up-regulation of parathyroid hormone-related protein (PTHrP) that plays a key role in cancer-associated osteolysis (6). Within the bone microenvironment, PTHrP acts upon osteoblasts to stimulate production of RANKL (receptor activator of NF κ B ligand) that activates osteoclasts (7). Thus, TGF- β , both stored in the bone microenvironment and produced by carcinoma cells, promotes a “vicious cycle” within the bone, causing osteoclast-mediated bone destruction. In addition to the PTHrP-RANKL link, TGF- β may stimulate osteoclasts by regulating IL11, CTGF, COX2 and MMPs (7).

TGF- β cytokines signal through serine/threonine kinase transmembrane type I and type II receptors (8). TGF- β binding to the receptor complex triggers activation of the type I receptor, termed activin-like receptor kinase 5 (ALK5), which activates Smad transcription factors, mitogen-activated protein kinases (MAPKs) and PI3K-Akt signaling (9). ALK5 phosphorylates receptor-associated Smad2 and Smad3 (R-Smads) and induces translocation of R-Smads and Smad4 to the nucleus (8). Activation of MAPKs by TGF- β may involve TGF- β -activated kinase 1 (TAK1) or small GTPases (9), whereas TGF- β -mediated activation of PI3K-Akt signaling (10, 11) requires small Rho-like GTPases (10). MAPK and PI3K-Akt signaling cascades are activated independently of Smads (11, 12). All three cascades have been implicated in the regulation of tumor microenvironment and invasion, and potentially can contribute to the metastatic function of TGF- β (3).

TAK1, a mitogen-activated protein kinase kinase kinase (MAP3K7), was identified as a kinase that mediates TGF- β signaling to MAP kinases p38 and JNK (13). TAK1 is also involved in BMP signaling (14) as well as in inflammatory and innate immune responses (15). TAK1 is activated by association with TAK1-binding proteins TAB1, TAB2, TAB3 and BIRC4/XIAP (16). In addition to activating MAP kinases (ERK, JNK, and p38), TAK1 is an important activator of NF κ B transcription factor (17). Our recent studies have identified TAK1 as a critical regulator of matrix-metalloproteinase-9 (MMP-9) expression (18). MMP-9/gelatinase-B contributes to the invasive and metastatic potential of cancer cells (19–21), as well as to tumor angiogenesis, by promoting recruitment of endothelial cells (22) and pericytes (23). Indeed, breast tumor angiogenesis is significantly reduced by small-hairpin RNA (shRNA) to MMP-9 (21). TGF- β signaling, MMP-9 and NF κ B are implicated in breast and prostate cancer-induced bone lesions (7, 24–26). NF κ B transcription factor can also contribute to osteolytic bone lesions in breast carcinoma cells by regulating COX2/PTGS2, CSF2/GM-CSF and IL6 (26, 27). TGF- β -Smad signaling may negatively and positively interact with NF κ B signaling in various cell systems, but the role of TAK1 in this interaction is not fully understood (28–30).

The present study explored the role of TAK1 signaling in breast carcinoma-induced bone destruction using the intracardiac-injection model in SCID mice. Disruption of TAK1 signaling by forced expression of dominant-negative K63W mutant of TAK1 blocked the formation of osteolytic bone lesions by human breast cancer cell line MDA-MB-231. Mechanistic studies revealed the critical role of TAB2/TAB3 in the regulation of MMP-9 and COX2. These findings indicate that the TAK1-TAB2/TAB3 axis is important for TGF- β -mediated osteolytic bone lesions of breast carcinoma cells.

MATERIALS AND METHODS

Antibodies, plasmids and other reagents

TGF- β 1 was obtained from R&D Systems. Antibodies were obtained: TAK1 from Sigma (T7824), phospho-HSP27 (2401) and phospho-p38 MAPK (9211) from Cell Signaling Technology (Danvers, MA); GAPDH (sc25778) from Santa Cruz Biotechnology, Inc. Short interfering RNA (siRNA) to human TAK1 and p38MAPK were from Dharmacon, Inc

(Lafayette, CO). Scramble control RNA duplexes labeled with Rhodamine were from Qiagen (Chatsworth, CA). Kinase-inactive TAK1-K63W was a gift of Dr. Hiroaki Sakurai (31). Retroviral vector pBMN-EGFP and constructs encoding TAK1-K63W, Flag-tagged p38alpha-AF and HA-tagged ALK5-T204D are described in (18, 32). Plasmids encoding short hairpin RNA (shRNA) to human TAK1 in the pSHAG-MAGIC2 vector were obtained from the shRNA Core Resource at Roswell Park Cancer Institute (director Dr. Irwin Gelman; supported by the NCI Cancer Center Support Grant to the Roswell Park Cancer Institute, CA016056).

Cell culture

Human breast cell lines MDA-MB-231, MCF10A, MCF7, T47D, MDA-MB-468, MDA-MB-435, and prostate cancer cell line PC3 were obtained from the American Tissue Culture Collection (ATCC) and cultured as recommended by ATCC. MDA-MB-231-ALK5-T204D cells expressing constitutively active ALK5/TGF β R1 are described elsewhere (21). MDA-MB-231 cells expressing shRNA to TAK1 were cultured in the presence of 0.5 μ g/ml puromycin, while assays were performed in the absence of antibiotics.

Retroviral Infection

MDA-MB-231 cells were infected in the presence of 6 μ g/ml of polybrene with amphotropic retroviruses produced in Phoenix cells, as previously described (32). EGFP-positive cells were selected by FACS. MDA-MB-231 cells infected using retroviral vector pSHAG-MAGIC2 encoding shRNA to TAK1 and then were selected in the presence of 2 μ g/ml puromycin. Cells were routinely maintained in the presence of 0.5 μ g/ml puromycin.

Short Interference RNA

Cells were transfected with RNA duplexes using Oligofectamine reagents (Invitrogen) following the manufacturer's protocol. The media was replenished next day. The cells were grown for an additional 48h in the absence or presence of 2 ng/ml TGF- β 1 followed by gelatin zymography and immunoblotting.

Immunoblot analysis

Cells were incubated in medium containing 5% serum for 24 h prior to treatment with 2 ng/ml TGF- β 1. Kinase inhibitors were added 1h prior to treatment with TGF- β 1. Cells were lysed in buffer: 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 μ g/ml aprotinin, and 2 μ g/ml leupeptin. Immunoblot analyses of protein extracts were performed as described (32).

RT-PCR analysis

RT-PCR was performed as described (21). RNA extraction was performed using Versagene RNA kit (Centra, Minneapolis, MN). Amplification of transcripts was performed using 50 ng/ μ L of total RNA and one-step RT-PCR system from Invitrogen according to the manufacturing protocol. Primer sequences are presented in Table 1. The optimal number of the PCR cycles was determined for each primer set to ensure a linear range of amplification, typically 21–26 cycles.

Gelatin zymography

The assay was performed as described (21). Conditioned media samples were prepared from cells incubated in serum-free IMEM for 48h. SDS-PAGE gels were co-polymerized with gelatin at a final concentration of 1 mg/ml. After electrophoresis, the gels were renatured in 2.5% Triton X-100 and incubated at 37°C for 24h in 5 mM CaCl₂ and 50mM Tris-HCl buffer, pH 7.5, containing 0.05% NaN₃. The gels were stained with 0.5% Coomassie Blue

R-250 and destained in 10% methanol and 5% acetic acid in water. Gelatinolytic activities were detected as transparent bands on the blue background. The band densities were evaluated using NIH-Image software.

Intracardiac-injection model of bone metastasis with MDA-MB-231 cells in SCID mice

Female SCID/CB17 mice, 5 weeks of age, were obtained from a colony of SCID/CB17 mice that was bred and maintained at the Department of Laboratory Animal Resources (DLAR) facility at the Roswell Park Cancer Institute (RPCI). All animals were kept three to five mice per cage in microisolator units and provided with water and food *ad libitum* according to a protocol approved by the Institute Animal Care and Use Committee at RPCI. The facility has been certified by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulation and standards of the U.S. Department of Agriculture and the U.S. Department of Health and Human Services.

Exponentially growing breast cancer cells (200,000) in 0.1 ml of sterile PBS were injected using 27-G needle into the left heart ventricle of mice under anesthesia with isofluran according to the modification of the method described by (33). Formation of bone lesions was monitored bi-weekly using full-body X-ray imaging with a Faxitron MX-20 X-ray machine.

Histological analysis

Long bones (femur and tibia) were removed from the mice and fixed in 10% formalin for 24 hours and decalcified in 10% EDTA (pH 7.4) for 14 days, then embedded into paraffin blocks. Serial sections were prepared and stained with hematoxylin and eosin. Bone lesions observed by radiography were confirmed by histological examination. Rabbit polyclonal serum to GFP (NB600-308) from Novus Biological (Littleton, CO) was used for detection of tumor cells in histological bone sections.

TRAP staining for osteoclasts

Sections of the paraffin-embedded blocks of long bones (see above) were prepared. Tartrate Resistant Acid Phosphatase (TRAP) staining of the deparaffinized sections was carried out according to the manufacturer's protocol (387A-1KT; Sigma-Aldrich). Images were acquired using Olympus DP25 camera and DP2-BSW software (Olympus). TRAP-positive multinuclear cells were detected at the tumor-bone interface in diaphysis and metaphysis of tumor-carrying animals. The amount of TRAP-positive multinuclear cells were evaluated in four random fields within the same section that included normal bone marrow and tumor areas at 400 $\times$ magnification.

Statistical Analysis

Continuous data were compared using the Student's *t* test. Differences were considered significant when $P < 0.05$.

RESULTS

TAK1 is required for breast carcinoma-induced bone lesions

Both the TGF- β and NF κ B signaling pathways have been implicated in the formation of skeletal metastases (7, 24–26), although the underlying mechanisms are likely to be different, as these pathways have opposite effects in various cell systems (28–30). Our recent study has linked TAK1 to tumor invasion and angiogenesis via the TGF- β -TAK1-NF κ B-MMP-9 axis (18), while the role of TAK1 in skeletal metastases has not been addressed. The present study assessed the role of TAK1 in the bone metastatic process using

the human breast carcinoma MDA-MB-231 cell line in the mouse intracardiac-injection model (34–36). In this model, the inoculation of carcinoma cells into the left cardiac ventricle of immunodeficient mice results in osteolytic bone lesions, which closely match bone metastases observed in patients (37, 38). The breast carcinoma MDA-MB-231 cell line was established from the pleural effusions of a breast cancer patient with metastatic disease (39). Suppression of TAK1 signaling in MDA-MB-231 cells was achieved by expressing the dominant-negative TAK1-K63W mutant allele from a bi-cistronic EGFP-encoding retroviral vector (Figure 1A). The empty-vector control and dnTAK1 cells express EGFP from the internal-ribosome entry site (IRES) and have been characterized elsewhere (18). First, we optimized a number of injected cells, a time-frame of development of bone lesions, and a method for the de-calcification of bones. Formation of osteolytic bone lesions was monitored using full-body X-ray imaging. Radiographs showed that 80% of mice injected with the EGFP-control cells developed osteolytic lesions in limbs at four weeks after inoculation (Figure 1A). Histological analysis revealed that the control cells occupy bone marrow cavities in metaphysis (in the proximity of the growth plate) and diaphysis of tibia and femur bones (Figure 1B). With the exception of one case with a single metastatic site, mice from the dn-TAK1 group did not develop osteolytic bone lesions either radiographically or histologically at 4 weeks after inoculation (Figure 1B–D). Dn-TAK1 cells were absent from the bone marrow cavity in diaphysis (Figure 1D). These findings indicate that the dn-TAK1 cells exhibit a deficiency in the bone invasiveness compare to the control cells, and this is consistent with a reduced invasive and metastatic potential of MDA-MB-231-dnTAK1 cells (18). Dn-TAK1 did not affect the tumor cell growth in culture but markedly reduced tumor angiogenesis (18). Thus, the reduction in bone metastases by dn-TAK1 could not be explained by the growth defect, and it is likely associated with tumor-host interactions in the tumor angiogenesis and/or in the “vicious cycle” within the bone.

TAK1 contributes to osteoclastogenesis induced by breast carcinoma cells

In the intracardiac bone metastasis model, MDA-MB-231 cells induce the osteolytic bone lesions that are caused primarily by the disproportionate activity of osteoclasts (6, 7). The osteoclasts are derived from the pluripotent hematopoietic precursors in the bone marrow, and both TGF- β and NF κ B promote osteoclast differentiation and maturation (6, 26). To explore the effect of dn-TAK1 on osteoclasts, bone sections were stained for the enzymatic activity of tartrate resistant acid phosphatase (TRAP), a marker of osteoclasts. The activated osteoclasts were identified as TRAP-positive and multinucleated (>3 nuclei) cells (Figure 2). Microscopic inspection revealed high amounts of osteoclasts at the bone-tumor interface of the EGFP-control group compared to the bone-normal marrow interface in metaphysis and diaphysis (arrows), as well as within the tumor area (stars) vs. the normal bone marrow (Figure 2). The amount of osteoclasts in the dn-TAK1 tumor sections and tumor-bone boarder were significantly reduced compared to the EGFP-control areas (Figure 2). Thus, these findings show that carcinoma cells increase the amount of activated osteoclasts in the bone environment and suggest that dn-TAK1 affects either homing or tumor-bone interactions, reducing the osteoclast response.

TAK1 contributes to the activity of the NF κ B-MMP9 axis in breast carcinoma cells

Previous studies have suggested that tumor TGF- β signaling regulates expression of factors promoting the formation of osteoclasts (6, 40, 41). In MDA-MB-231 cells, TGF- β facilitates the bone osteolysis program by inducing IL11 and CTGF via Smads (40, 41), as well as up-regulating PTHrP via p38 MAPK (6). Previous studies have shown that dn-TAK1 does not inhibit Smad signaling (18) but rather reduces activity of NF κ B that regulates expression of MMP-9 and other osteolytic factors (18, 26, 27). To assess the effect of dn-TAK1 on p38MAPK signaling, we examined phosphorylation of p38 and its target HSP27 by immunoblotting. Dn-TAK1 did not block phosphorylation of p38 and HSP27 in response to

TGF- β in MDA-MB-231 cells (Figure 3A). Then, the effect of dn-TAK1 on MMP-9 expression in response to TGF- β and inflammatory cytokine IL1- β was explored using gelatin zymography. Dn-TAK1 abrogated up-regulation of MMP-9 by TGF- β or IL1- β alone, as well as by their combination (Figure 3B). To confirm this finding, TAK1 was suppressed by shRNA in MDA-MB-231 cells (Figure 3C). Gelatin zymography revealed a significant reduction of MMP-9 levels by depletion of TAK1 (Figure 3D), whereas suppression of TAK1 did not affect phosphorylation of HSP27, a target of p38 (Figure 3E). Together, these findings demonstrate that TAK1 contributes to TGF- β and IL1- β responses in breast carcinoma cells, and MMP-9 is a target of both pathways, whereas p38 signaling is not affected by the TAK1 suppression.

TAK1 and MMP-9 in breast carcinoma cell lines

Our findings demonstrate that TAK1 contributes to the invasive potential of tumor cells and tumor colonization of the bone marrow. TAK1 mediates expression of metalloproteinase MMP-9 in MDA-MB-231 cells (Figure 3), and MMP-9 is capable of degrading the bone matrix as well as it is critical for tumor angiogenesis (19–22). Thus, the TAK1-MMP-9 axis may represent an essential pathway in tumor-induced bone osteolysis. To assess whether this axis operates in other breast carcinoma cells, we first evaluated expression of TAK1 in a panel of human breast cancer cell lines. The immunoblot analysis showed that TAK1 is expressed in both triple-negative and ER-positive cells, whereas MDA-MB-435 and MDA-MB-468 cells have lower levels of TAK1 (Figure 4A). Gelatin zymography revealed that ER-positive MCF7 cells secrete MMP-9 and it is regulated by TGF- β , whereas MDA-MB-435 and MDA-MB-468 cells do not (Figure 4B). To investigate if production of MMP-9 requires TAK1 in MCF7 cells, TAK1 was suppressed using siRNA (Figure 4C). Zymography showed that basal and TGF- β -induced levels of MMP-9 were reduced by depletion of TAK1 (Figure 4D). These results demonstrate that TAK1 contributes to MMP-9 expression in ER-positive MCF7 and triple-negative MDA-MB-231, human breast carcinoma cell lines that are capable of bone metastasis in experimental animal models.

TAK1-TAB2 signaling in carcinoma cells

TAK1 signaling is controlled by TAK1-binding partners TAB1, TAB2 and TAB3 (16). TAB1 regulates TAK1 activation in some cell lines leading to phosphorylation of TAK1 at Thr187 (16, 42), whereas TAB2 and TAB3 link TAK1 to K63-ubiquitin-dependent signaling by TRAF6, TRAF2 and TRAF5 (43–46). To assess the contribution of TABs to the TAK1-MMP-9 axis, we first determined the expression levels of TAK1 and TABs in normal and carcinoma cell lines using RT-PCR with a range of cycles to ensure the proportionality of amplification. RT-PCR analysis showed that TAK1, TAB2, TAB3 and TRAF6 are expressed in all tested cell lines, whereas TAB1 levels were significantly lower (Figures 5A, B). TAB2 mRNA levels was more than 50-fold higher than TAB1. These results were confirmed by the Affymetrix microarrays (data not shown). The analysis also showed that TAK1 and TAB2 are expressed at elevated levels in tumor cell lines. To assess the contribution of TAB2 to the MMP-9 expression, we examined the effect of siRNA to TAB2 on MMP-9 in breast and prostate carcinoma cell lines. RT-PCR showed that TAB2 levels were efficiently depleted by siRNA to TAB2 in breast carcinoma MDA-MB-231 and MCF7 as well as in prostate carcinoma PC3 cells (Figure 5C). The specificity of TAB2 knockdown was confirmed in MDA-MB-231, where siRNA to TAB2 did not affect TAB1 and TAB3 levels (Figure 6C). Production of MMP-9 was evaluated by zymography. These assays revealed that depletion of TAB2 markedly reduced MMP-9 production in all tested cell lines (Figure 5D). Collectively, these findings demonstrate that TAB2 is required for MMP-9 production by breast and prostate carcinoma cells. It appears that TAB1 is expressed at low levels in human carcinoma cell lines.

Targets of TAK1-TAB2 signaling in carcinoma cell lines

To identify TAK1 effectors contributing to the bone metastatic potential of carcinoma cells, the mRNA levels of known TGF- β and IL1- β targets were assessed by semi-quantitative RT-PCR. Dn-TAK1 reduced basal mRNA levels of PTGS2/COX2, MMP-9, IL8 and PTHrP in MDA-MB-231 cells (Figure 6A). TGF- β -mediated induction of PTGS2, MMP-9 and IL8 was also inhibited by dn-TAK1, whereas up-regulation of PTHrP was not affected. As expected, the mRNA levels of IL11 and CTGF were not affected by dn-TAK1, as expression of these genes is controlled by TGF- β -Smad signaling (41). The comparison of PTGS2/COX2 and IL8 levels in tumor cells expressing dn-TAK1 and shRNA-TAK1 revealed that suppression of TAK1 by either dn-TAK1 or shRNA markedly reduced basal and TGF- β -induced levels of PTGS2, whereas primarily basal levels of IL8 were affected (Figure 6B). Finally, we evaluated the contribution of TAB1, TAB2, and TAB3 to PTGS2 expression using siRNA. The specificity of siRNA-mediated depletion was confirmed by RT-PCR (Figure 6C). The analysis revealed that PTGS2 expression was reduced by depletion of TAB2 and TAB3, whereas suppression of TAB1 had no effect (Figure 6C). These findings show that the TAK1-TAB2/TAB3 signaling module controls expression of bone metastatic factors PTGS2/COX2, PTHrP and IL8 by tumor cells, whereas the contribution of TAB1 is likely to be minimal.

DISCUSSION

The current study provides evidence that TGF- β -activated protein kinase 1 (TAK1) plays a critical role in the formation of osteolytic bone lesions by human breast carcinoma cells. Our findings indicate that TAK1 contributes to the bone-homing capacity of tumor cells as well as to the “vicious cycle” within the bone environment that regulates differentiation and activation of osteoclasts. Suppression of TAK1 signaling by dn-TAK1 in breast carcinoma cells impairs the bone invasive capacity of carcinoma cells and the bone osteolysis. Studies with dn-TAK1 and RNA interference in breast and prostate carcinoma cells reveal that TAK1 via signaling partners TAB2/TAB3 contributes to the expression of pro-invasive targets (COX2 and MMP9) as well as factors promoting osteoclast formation (PTHrP and IL8). Thus, the TAK1-TAB2/3 axis may facilitate bone metastasis by breast and prostate carcinoma, promoting the bone-invasive capacity of tumor cells and the osteolytic destruction of the bone.

Breast and prostate carcinomas frequently metastasize to the bone (47). Bone metastasis-capable tumors express factors that facilitate tumor homing to bone and pro-osteolytic factors that promote osteolysis by stimulating the formation and activation of osteoclasts. The current study demonstrates that TAK1 signaling is essential for tumor colonization of the bone cavity and the osteoclast response. TAK1 via TAB2/TAB3 signaling partners mediates expression of bone-invasive factors such as MMP-9 and COX2, as well as factors stimulating the osteoclast response, PTHrP and IL8 (Figure 7A). This TAK1-TAB2/TAB3 signaling axis can operate in both breast and prostate carcinoma cells. The link of TAK1 to MMP-9 and COX2 may explain a defect in bone colonization by dnTAK1-expressing tumor cells. MMP-9-deficient mice have abnormalities in vascularization and ossification of the skeletal growth plate (48). MMP-9 is also critical for matrix-degrading activity of tumor cells (21), and the release of growth factors in bone environment (22, 24). High levels of COX2/PTGS2 in primary tumor correlate with bone marrow micrometastases in breast cancer patients (49). Forced expression of COX2 enhances bone degradation in the bone metastasis model of breast carcinomas (50). The reduction of PTHrP and IL8 levels by dn-TAK1 may contribute to a decrease in the activated osteoclasts in the dn-TAK1 group. PTHrP is a major component of the “vicious cycle” within the bone, causing osteoclast-mediated bone destruction (Figure 7B). TAK1 is required primarily for the basal expression of PTHrP, but, apparently, it has limited input in the TGF- β -mediated up-regulation of

PTHrP that requires p38 MAPK (6). This is consistent with un-altered activation of p38 signaling in dn-TAK1 cells (Figure 3). The link of TAK1 to expression of IL8 could be of a particular importance. Chemokine IL8 activates macrophages and osteoclasts enhancing bone degradation and angiogenesis (47, 51, 52). Both PTHrP and IL8 may act directly on osteoclasts or up-regulate expression of RANKL, receptor activator of nuclear factor- κ B ligand, by osteoblast/stromal cells (47, 52). Breast carcinoma cells do not secrete RANKL and did not support osteoclastogenesis in *in vitro* experiments (our unpublished results). Hence, PTHrP and IL8 are likely to mediate tumor-induced osteoclastogenesis in the bone marrow environment. Together, these findings argue that the TAK1-TAB2/TAB3 axis represents an essential pathway for the bone metastatic potential of tumor cells, controlling expression of bone invasive factors MMP-9 and COX2 as well as osteolytic factors PTHrP and IL8 (Figure 7).

Human cancer cells that produce osteolytic lesions frequently express elevated levels of IL1- β and tumor necrosis factor alpha (TNF- α), which are involved in the stimulation of osteoclast differentiation and activation (52). The present study found that pro-inflammatory cytokines IL1- β and TNF- α cooperate with TGF- β in production of MMP-9 by breast carcinoma cells and TAK1 is required for this response. Elevated levels of MMP-9 are found in breast and prostate cancer patients with bone metastases (53) and in pediatric osteosarcomas (54). Inhibition of MMP-9 or MMP-2 reduces bone-degrading capacity of tumor cells *in vitro* and *in vivo* (55, 56). TGF- β is a potent anti-inflammatory cytokine (4, 5), and in normal epithelial cells TGF- β negatively regulates NF κ B transcriptional activity (57, 58), while pro-inflammatory cytokines repress Smad-dependent transcription (59, 60). The anti-inflammatory effect of TGF- β is likely mediated by Smads, as Smad3-null mice develop massive inflammation (5) and Smad3/Smad4 mediate suppression of NF κ B in renal inflammation (61, 62). In tumor cells, however, TGF- β can activate the NF κ B transcriptional response (28, 63). Breast carcinoma MDA-MB-231 cells express high levels of NF κ B activity (64) and constitutively active Ras-ERK signaling (65), which can inhibit Smad activity (66, 67). Thus, the attenuation of Smad signaling might contribute to a positive interaction of TGF- β and pro-inflammatory cytokines in tumor cells.

Our studies provide insights into the mechanism of TAK1-mediated up-regulation of COX2/PTGS2 and MMP-9. TAK1 can interact with the TAB1-XIAP complex (68, 69), regulating p38MAPK and NF κ B, and/or with the TAB2/TAB3-TRAF complexes, leading to the K63-ubiquitin-dependent activation of NF κ B and JNK (46). Disruption of TAK1 signaling by dn-TAK1 or by siRNA did not affect p38MAPK signaling in MDA-MB-231 cells. In agreement with this finding, breast and carcinoma cells express low levels of TAB1. These results argue that the interaction of TAK1 with the TAB1-XIAP complex is either transient or involves only a fraction of TAK1. In contrast, expression levels of the TAB2/TAB3/TRAF factors and TAK1 were comparable and suppression of TAB2 or TAB3 reduced expression of COX2/PTGS2 and MMP-9 in carcinoma cells. In support of these findings, recent studies have found that TAB1-null cells exhibit normal activation of TAK1-NF κ B signaling by IL1- β and TNF- α (70, 71).

High levels of TGF- β and pro-inflammatory cytokines in tumor microenvironment may facilitate the selection of tumor cells that are capable of utilizing the pro-tumorigenic functions of both pathways. In the natural history of the tumor, the TAK1-TAB2/3 signaling axis may emerge as a moderator of the two pathways favoring invasion and growth of tumor cells in the bone environment (Figure 7). This signaling axis may function in tumor cells independently of TGF- β -Smad and TGF- β -Src signaling events that regulate expression of IL11 and CTGF (41, 72, 73), as these factors are not affected by dn-TAK1. Thus, the TAK1-TAB2/3 axis may represent a novel target for anti-cancer therapy as well as a biomarker of bone-metastasis-capable cancers.

Abbreviations

TGF-β	transforming growth factor beta
TAK1	TGF-beta-activated protein kinase 1
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase

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Figure 1. TAK1 is required for breast carcinoma-induced bone lesions. **(A)** RT-PCR analysis of TAK1 mRNA levels in breast cancer MDA-MB-231 cells, EGFP-control cells and cells expressing dnTAK1. **(B)** MDA-MB-231 cells (EGFP-control and dn-TAK1) were injected into the left cardiac ventricle of SCID mice (10 mice in the control group, 14 mice in the dnTAK1 group). X-Ray radiographs were taken at 4 weeks after the injection. **(C)** The graph shows number of mice in the control and dn-TAK1 groups exhibiting bone lesions detected by radiography. Bone lesions were observed in 8 out of 10 animals in the control group, whereas no detectable bone lesions were found in the dn-TAK1 group. **(D)** Histological H&E-stained sections of long bones: panels (1, 4) show normal bone; panels (2, 5) show the femur bone of mouse inoculated with EGFP-control cells; (3, 6) - bones from the dnTAK1 group. Panels (2, 4) show the presence of tumor mass in metaphysis and diaphysis of bones in mice from the control group. Note the direct contact of tumor (TU) and bone marrow (BM) in diaphysis. Tumor mass was absent in the dnTAK1 group, except for a case presented in panels (3, 6). Magnification: panels (1–3) at 40x; (4–6) at 200x.

Figure 2. Detection of osteoclasts in histological sections of normal and tumor-carrying mouse bones. (A) Long bones were fixed in paraformaldehyde, decalcified and then sectioned after paraffin embedding. After deparaffinization, Tartrate Resistant Acid Phosphatase (TRAP) staining was carried out per manufacturer's protocol (Sigma-Aldrich). Images were acquired using Olympus DP25 camera and DP2-BSW software. Panels show TRAP staining in: (1) metaphysis area of mouse injected with control tumor cells; (2, 3) metaphysis of normal and tumor-containing bone; (4) Bone marrow in diaphysis of normal bone; (5) Tumor-bone interface in diaphysis of tumor-containing bone (the control-EGFP group); (6) Tumor-bone interface in diaphysis of the dn-TAK1 mouse. Images were acquired with 10× or 40× objective. (B) The graph shows the number of TRAP-positive and multinucleated cells (osteoclasts) per mm of the bone interface with normal bone marrow, the bone-EGFP-control tumor and dn-TAK1 tumor. Cells were counted from four random fields at 400× magnification. (C) Osteoclasts were counted from four random fields at 400× magnification

within the same section that included normal bone and tumor areas. BM, bone marrow; TU, tumor EGFP; TU-dnTAK1, tumor dn-TAK1; *, $P < 0.05$; ***, $P < 0.001$.

Figure 3.

TAK1 is required for up-regulation of MMP-9 in response to TGF- β and IL1- β . (A) Immunoblot analysis of whole-cell extracts from control and dn-TAK1-expressing MDA-MB-231 cells minus/plus treatment with 2ng/ml TGF- β 1. (B) Gelatin zymography of 48h-conditioned medium from control and dn-TAK1-expressing MDA-MB-231 cells treated with TGF- β 1 or/and IL1- β . MMP-9 secretion was significantly reduced by dn-TAK1. (C) Immunoblot of TAK1 and GAPDH (loading control) in whole-cell extracts from control MDA-MB-231 cells and MDA-MB-231 cells expressing shRNA to TAK1, minus/plus 2ng/ml TGF- β 1 for 24h. (D) Gelatin zymography of 48h-conditioned medium from control and shRNA-TAK1 MDA-MB-231 cells. (E) Immunoblotting of whole-cell extracts from MDA-MB-231 cells treated with TGF- β 1. Suppression of TAK1 by shRNA does not affect phosphorylation of HSP27 in response to TGF- β 1.

Figure 4.

TAK1 contributes to MMP-9 expression in MCF7 cells. (A) Immunoblot of whole-cell extracts from breast cancer cell lines using antibodies to TAK1 and GAPDH, a loading control. Cells (MDA-MB-231, MDA-MB-435, MCF7, MDA-MB-468, and T47D) were treated with 2ng/ml TGF-β1 for 24h. (B) Gelatin zymography of 48h-conditioned medium from breast cancer cell lines minus/plus treatment with 2ng/ml TGF-β1. (C, D) Gelatin zymography of 48h-conditioned medium and immunoblotting for GAPDH of whole-cell extracts from MCF7 cells transfected with siRNA to TAK1 or scramble-control siRNA, minus/plus treatment with 2ng/ml TGF-β1.

Figure 5.

The TAK1-TAB2 signaling axis is critical for MMP-9 regulation by TGF- β . (A) RT-PCR of TAK1, TAB1, TAB2, TAB3, TRAF6 and β -actin (ACTB) in MCF10A (1), MDA-MB-231 (2), MDA-MB-231-caALK5 (3), MCF7 (4) and PC3 (5) cell lines. (B) Relative expression of TAK1 partners evaluated by using the densitometry analysis of agarose gel in (A). (C) RT-PCR analysis of TAB2 to confirm the knockdown of TAB2 in MDA-MB-231, MCF7 and PC3 cell lines. (D) Gelatin zymography of 48h-conditioned medium from cell lines transfected with siRNA to TAB2.

Figure 6. Molecular targets of TAK1 in carcinoma cells that may contribute to bone metastasis. **(A)** RT-PCR analysis of TAK1, PTGS2, MMP-9, PTHrP, IL8, IL11, CTGF, and β -actin (ACTB) in control and dnTAK1 MDA-MB-231 cells; minus/plus treatment with 2ng/ml TGF- β 1 for 24h. **(B)** RT-PCR of PTGS2, IL8 and ACTB in MDA-MB-231 cells expressing dnTAK1 or shRNA to TAK1. **(C)** RT-PCR of PTGS2, TAB1, TAB2, TAB3 and ACTB in MDA-MB-231 cells transfected with siRNA to TAB1, TAB2, or TAB3.

Figure 7.

Model for the role of TAK1 signaling in bone metastasis. (A) TGF- β and pro-inflammatory cytokines can activate TAK1 in tumor cells. TAK1 is likely to interact with the TAB2/TAB3-TRAF complex leading to activation of NF κ B and up-regulation of MMP-9, COX2, IL8 and PTHrP. Expression of dnTAK1 (TAK1-K63W) hinders this signaling pathway in tumor cells. (B) In bone environment, the TAK1 signaling pathway in tumor cells promotes the “vicious cycle” leading to the disproportionate activity of osteoclasts and bone destruction.

TABLE 1

Primer sequences

Target Gene, Acc#	Forward Primer	Reverse Primer	Size, bp
ACTB, NM_007393	GCTGGTCGTCGACAACGGCTC	CAAACATGATCTGGGTCATCTTTTC	354
CTGF, NM_001901.2	ACTGTCCCGGAGACAATGAC	TGCTCCTAAAGCCACACCTT	339
GM-CSF, BC113999	ATGTGAATGCCATCCAGGAG	TTCCCAGTTCTTCCATCTGC	326
IL8, NM_000584	GTGCAGTTTTGCCAAGGAGT	CTCTGCACCCAGTTTTCTT	196
IL11, NM_000641.2	ACTGCTGCTGCTGAAGACTC	CCACCCCTGCTCCTGAAATA	218
MMP9, NM_004994	TTCATCTTCCAAGGCCAATC	CAGAAGCCCCACTTCTTGTC	290
PTGS2, NM_000963	TGAGCATCTACGGTTTGCTG	TGCTTGTCTGGAACAACCTGC	158
PTHrP, NM_198966.1	CAAGATTACGGCGACGATT	GGGCTTGCCTTCTTTTCT	239
TAB1, NM_006116.2	CCACCCTACCAACCAAAGC	GCTGTCACCACGCTCTGCTC	214
TAB2, NM_015093.3	CTCGAAGGCGCCTGAAAAGA	GGGTTTGGTGGCACAGGAC	218
TAB3, NM_152787.3	TGTACTCCATCACCATCTCCT	TGCTTTGCTAACCTCTCCAT	281
TAK1, AB009357	CAAGGAGATCGAGGTGGAAG	GGATTCAAGCAGGCTCCATA	224
TRAF6, NM_145803.1	CCATGCGGCCATAGGTTCTG	TGGCATTGGGGACAATCCAT	248