A Secreted Isoform of ErbB3 Promotes Osteonectin Expression in Bone and Enhances the Invasiveness of Prostate Cancer Cells

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Abstract

The propensity for prostate cancer to metastasize to bone led us and others to propose that bidirectional interactions between prostate cancer cells and bone are critical for the preferential metastasis of prostate cancer to bone. We identified previously a secreted isoform of ErbB3 (p45-sErbB3) in bone marrow supernatant samples from men with prostate cancer and bone metastasis and showed by immunohistochemical analysis of human tissue specimens that p45-sErbB3 was highly expressed in metastatic prostate cancer cells in bone. Here, we show that p45-sErbB3 stimulated mouse calvaria to secrete factors that increased the invasiveness of prostate cancer cells in a Boyden chamber invasion assay. Using gene array analysis to identify p45-sErbB3–responsive genes, we found that p45-sErbB3 up-regulated the expression of osteonectin/SPARC, biglycan, and type I collagen in calvaria. We further show that recombinant osteonectin increased the invasiveness of PC-3 cells, whereas osteonectin-neutralizing antibodies blocked this p45-sErbB3–induced invasiveness. These results indicate that p45-sErbB3 enhances the invasiveness of PC-3 cells in part by stimulating the secretion of osteonectin by bone. Thus, p45-sErbB3 may mediate the bidirectional interactions between prostate cancer cells and bone via osteonectin. [Cancer Res 2007; 67(14):6544–8]

Introduction

Prostate cancer is the most common malignancy among men in the United States. Men with advanced prostate cancer often have symptoms attributable to bone metastases, including pain, spinal cord compression, nerve root impingement, and bone marrow failure (1). Autopsy studies of men who died of prostate cancer indicate that 83% of them had evidence of bone metastasis (2). The proclivity of prostate cancer to metastasize to bone is not fully understood, but interactions between the disseminated cancer cells and the bone microenvironment seem to be critical for the process (3, 4).

One explanation for the preferential growth of prostate cancer in bone is the presence of bidirectional interactions in which prostate cancer cells secrete paracrine factors that affect bone and bone in turn releases factors that modulate prostate cancer cell activity (5, 6). In support of this concept are findings that injection of prostate cancer cells in combination with bone stromal cells increased tumorigenicity in vivo (3) and that coculturing prostate cancer cells with osteoblasts in vitro led to increased proliferation of both the osteoblasts and the cancer cells (7), suggesting that bone stromal cells provide factors that enhance prostate cancer progression in bone. Identification of the molecular determinants that mediate such interactions will provide the basis for developing strategies to prevent or treat bone metastases.

Our previous investigation of proteins that participate in prostate cancer bone metastases led to the identification of a soluble form of ErbB3, with a molecular mass of 45 kDa (p45-sErbB3, previously named MDA-BF-1), in pooled bone marrow supernatant samples from men with prostate cancer that had metastasized to bone (8). Subsequent examination of this soluble p45-sErbB3 during prostate cancer progression showed it to be expressed in metastatic prostate cancer cells in lymph nodes and bone but not in prostate cancer cells in the prostate (8). The p45-sErbB3 protein exhibited specific binding to plasma membranes prepared from cells of osteoblastic lineage but not to plasma membranes from PC-3 or HEK293 cells, suggesting that p45-sErbB3 might have specific interactions with osteoblasts (9). Indeed, functional studies showed that p45-sErbB3 promoted the growth and differentiation of osteoblasts both in vitro and in vivo.5 These observations support the hypothesis that p45-sErbB3 is a prostate cancer cell–derived paracrine factor that affects bone homeostasis.

In this study, we found that p45-sErbB3 stimulated calvaria to secrete several extracellular matrix proteins including osteonectin, which in turn enhanced the invasiveness of the prostate cancer cell lines PC-3 and C4-2B. These observations suggest that p45-sErbB3 may be involved in the bidirectional interactions between prostate cancer cells and bone by stimulating the bone to release factors that subsequently facilitate the metastasis of prostate cancer to bone.

Materials and Methods

Cell cultures. The prostate cancer cell lines LNCaP and PC-3 were obtained from American Type Culture Collection. C4-2B was kindly provided by Dr. L.W. Chung (Emory University School of Medicine, Atlanta, GA). These cells were propagated at 37°C with 5% CO2 in complete RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum.

Generation of recombinant p45-sErbB3 and osteonectin. Recombinant adenovirus containing p45-sErbB3 cDNA (GenBank accession no. U88588) with a histidine–tag at the COOH terminus was generated and used to infect PC-3 cells. The histidine-tagged p45-sErbB3 was secreted into the

Note: N. Chen and X.C. Ye contributed equally to this work.

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5 S.-H. Lin et al., submitted for publication.
Factors secreted by osteoblasts exposed to p45-sErbB3 enhance the invasiveness of prostate cancer cells. A, experimental plan for examining the effect of p45-sErbB3 on mouse calvaria, both in organ culture assays and in conditioned medium (CM). B, histologic analysis indicates that treatment of calvaria with p45-sErbB3 increased calvarial bone formation. C, in Transwell Boyden chambers, conditioned medium from p45-sErbB3–treated calvaria increased the invasiveness of PC-3 cells. Representative microscopic photographs show reassembled images of the entire Transwell membrane, on which cells that migrated to the outside of the upper chamber were stained and counted. D, quantification of the invasiveness of PC-3, LNCaP, and C4-2B cells in response to conditioned medium from p45-sErbB3–treated calvaria in a Transwell assay. *, P < 0.05; ***, P < 0.001.

Organ culture of mouse calvaria and preparation of conditioned medium. Calvaria of 4-day-old CD1 mice were excised and cut in half along the midline for paired comparisons: one half was cultured in a BGJb medium (Invitrogen) containing 0.1% bovine serum albumin (BSA) and with 100 ng/mL of recombinant p45-sErbB3. After 4 days of culture, calvaria were processed for staining with H&E or for RNA extraction. Conditioned medium was concentrated by using a Centriplus YM-10 (Millipore) and used as a control, and the other half was cultured in the same medium but treated or not treated with p45-sErbB3. 18S rRNA was used as internal control for calibrating the RNA loading. The experiment was repeated thrice, with similar data obtained each time. Right, fold changes in osteonectin, biglycan, and type I collagen production induced by p45-sErbB3. *, P < 0.05; ***, P < 0.001. C, left, Western blotting of osteonectin in conditioned medium from p45-sErbB3–treated calvaria. BSA was stained to show that equal aliquots of medium were loaded. Result was representative of three similar experiments. *, P < 0.05.

cell culture medium, from which it was purified to homogeneity by metal affinity chromatography. Recombinant osteonectin was expressed in insect cells by using baculoviral vector and purified as described previously (10).

Western blotting. Proteins from conditioned medium were denatured and separated on 4% to 12% acrylamide gels (Invitrogen) by SDS-PAGE and transferred onto nitrocellulose membranes. Polyclonal anti-osteonectin antibody (H-90, Santa Cruz Biotechnology) was used for immunoblotting.

Invasion assay. The ability of prostate cancer cells to penetrate a synthetic basement membrane was assessed in a Matrigel-Boyden chamber invasion assay (BD Biosciences). In this assay, concentrated conditioned medium or purified recombinant osteonectin was reconstituted in RPMI 1640 and added to the bottom wells. For the antibody neutralization experiments, the conditioned medium in the bottom wells had been preincubated with 1 μg/mL affinity-purified rabbit polyclonal anti-osteonectin antibody, mouse monoclonal anti-osteonectin antibody 293 (11), or normal rabbit or mouse IgG, for 2 h at room temperature. LNCaP, C4-2B, or PC-3 cells in RPMI 1640 were seeded in the upper chambers and incubated at 37°C for 17 to 20 h. After the incubation, cells that remained in the chambers were wiped off with cotton swabs, and cells that had reached the other side of the filter membrane were fixed and stained with toluidine blue.

**Osteogenesis GEarrays containing skeletal-related genes (SuperArray Bioscience). Raw data were normalized by the GEarray Analyzer software.**

RNA preparation, gene array analysis, and Northern blotting. Total RNA was extracted with Trizol (Invitrogen) and was further purified by the use of a Mini RNA extraction kit (Stratagene). RNAs were used to probe Osteogenesis GEarrays containing skeletal-related genes (SuperArray Bioscience). Raw data were normalized by the GEarray Analyzer software. For Northern blotting, hybridization probes for mouse osteonectin, type I collagen, and biglycan were obtained by reverse transcription-PCR. Primers for osteonectin were 5'-GATCAGACCCCATGGTGA-3' and 5'-TAAGCACAGAGTCTGGTTGA-3'; primers for type I collagen were 5'-TCTGAAGTCCAGGTTGTTGT-3' and 5'-ACTGCCGATGAGACCCCTGG-3'; and primers for biglycan were 5'-CCTGAGTTTCTGCTACC-3' and 5'-AGGGAGTCTCTAGTGGGACA-3'. A probe for hybridization of mouse 18S ribosome RNA, obtained from Ambion, was used for calibrating the RNA sample on the hybridization membrane.
Each membrane was subjected to serial photomicroscopy over the entire membrane. Cells were counted with the aid of the NIH Image J program.

**Statistical analysis.** All data are expressed as means with SDs. The statistical significance of differences between two groups was analyzed by Student’s t tests. Differences with P values of <0.05 were considered statistically significant.

**Results**

**Conditioned medium from p45-sErbB3–treated calvaria increases the invasiveness of prostate cancer cells.** We propose that the osteoblastic character of prostate cancer bone metastases is initiated by osteoblast regulatory factors that increase bone formation; the newly formed bone in turn provides factors that enhance the invasion, survival, or proliferation of the prostate cancer cells in the bone (6). To examine the effect of p45-sErbB3 on bone growth, we used a bone organ culture assay in which paired halves of newborn mouse calvaria were treated or not treated with 100 ng/mL of purified recombinant p45-sErbB3 for 4 days. Calvaria were subsequently processed for histologic analysis and the conditioned medium was collected for protein functional studies (Fig. 1A). We found that the calvaria treated with p45-sErbB3 were thicker than the untreated controls (Fig. 1B). This confirmed our previous observation that p45-sErbB3 leads to increased bone formation. 5

To investigate the possibility that p45-sErbB3 stimulates the newly formed bone to secrete factors that promote the metastasis or growth of prostate cancer in bone, we examined the effect of conditioned medium from the p45-sErbB3–treated calvaria on the proliferation or invasiveness of LNCaP, C4-2B, and PC-3 cells. The LNCaP cell line was derived from a lymph node metastasis; C4-2B is a LNCaP subline derived from a xenograft bone metastasis; whereas PC-3 was derived from a prostate cancer bone metastasis. The conditioned medium from the p45-sErbB3–treated calvaria had no effect on the proliferation of any of the three cell lines compared with medium from control (untreated) calvaria (data not shown). However, exposure to the same conditioned medium significantly enhanced the invasiveness of the bone-derived PC-3 cells (Fig. 1C and D) and C4-2B cells (Fig. 1D) in a Boyden chamber Transwell assay. Treatment with recombinant p45-sErbB3 alone did not have an effect on the invasiveness of PC-3 or C4-2B cells (data not shown), suggesting that the invasion-stimulatory factor(s) is from p45-sErbB3 conditioned medium. Invasiveness of LNCaP cells in this assay was low to undetectable and was not enhanced by treatment with p45-sErbB3 conditioned medium (Fig. 1D).

**Identification of p45-sErbB3–induced factors from mouse calvaria.** Conditioned medium from calvaria cultures contain many secretory proteins, some of which may be enhanced by...
treatment with p45-sErbB3 and act to stimulate prostate cancer cell invasiveness. To identify such factors, we purified total RNA from the calvaria treated or not treated with p45-sErbB3 and used microarray analysis to profile the expression of skeletal-related genes in response to the p45-sErbB3 treatment. Results showed that the expression of osteonectin (also known as SPARC, the secreted protein acidic and rich in cysteine), biglycan, and type I collagen was increased by exposure to p45-sErbB3 (Fig. 2A). To validate these findings, we used Northern blotting with RNA prepared from calvaria treated or not treated with p45-sErbB3. We found that p45-sErbB3 treatment led to a 1.6-fold (1.6 ± 0.2; n = 3) increase in osteonectin mRNA, a 2.4-fold (2.4 ± 0.5) increase in type I collagen, and a 1.9-fold (1.9 ± 0.2) increase in biglycan (Fig. 2B).

Of the three calvarial proteins increased by p45-sErbB3, osteonectin is known to promote prostate cancer cell migration and invasion (12, 13). Thus, the enhanced invasiveness of prostate cancer cells by p45-sErbB3 may be mediated, at least in part, by osteonectin produced by bone. To evaluate osteonectin protein levels in the conditioned medium of calvarial cultures, we used Western blotting and confirmed the elevated expression of osteonectin by calvaria treated with p45-sErbB3 (compared with control calvaria) at day 4 after treatment (Fig. 2C).

Osteonectin increases prostate cancer cell invasiveness. Next, we examined whether osteonectin has a direct effect on prostate cancer cell invasiveness by measuring the effect of recombinant osteonectin on LNCaP, C4-2B, and PC-3 cell invasion. Purified osteonectin protein promoted the invasiveness of PC-3 cells and that of C4-2B cells, but not that of LNCaP cells, in a concentration-dependent manner in the Transwell assay, suggesting that osteonectin alone is sufficient to stimulate the invasiveness of PC-3 cells (Fig. 3A). However, osteonectin did not affect the proliferation of any of the three cell lines (data not shown).

Neutralization of osteonectin results in inhibition of p45-sErbB3-induced prostate cancer cell invasiveness. To further examine our observation that the conditioned medium of calvaria treated with p45-sErbB3 enhances the invasiveness of PC-3 and C4-2B cells is due to osteonectin, we used two different neutralizing antibodies against osteonectin to block its function in the invasion assay. These antibodies, a polyclonal and a monoclonal IgG, but not a control antibody, significantly reduced the invasion of PC-3 cells exposed to p45-sErbB3-conditioned medium (Fig. 3B), suggesting that p45-sErbB3 enhances the invasiveness of these cells via osteonectin.

Collectively, we have shown that p45-sErbB3 has an osteoblastic effect on bone and stimulates the expression of osteonectin, which enhances prostate cancer cell invasiveness (Fig. 3C). In addition to osteonectin, other factors (such as biglycan or type I collagen) are probably also involved in this process; nevertheless, the increase in osteonectin might be one of the pathophysiologic responses to p45-sErbB3 that contribute to prostate cancer bone metastasis.

Discussion

Advanced prostate carcinoma is often associated with bone metastases. The mechanisms responsible for the organ-specific metastasis of this form of cancer are not known. In our quest to identify factors that mediate the bidirectional interactions between prostate cancer cells and the bone microenvironment, we show here that p45-sErbB3 stimulates bone to secrete osteonectin, which subsequently enhances the invasiveness of bone-tropic prostate cancer cells. Although mouse calvaria were used in this study, we consider that our observations are likely to be translatable to human bone. Mouse model has been widely used in addressing both developmental and disease-related aspects of human bone biology and most of the known human mutations that cause abnormal bone density, remodeling, and mineralization have resulted in similar phenotypes in mice. Both mouse and human osteoblasts originate from the mesenchyme and respond similarly to various stimuli in vitro. In addition, human osteonectin peptide sequence is 92% identical to mouse osteonectin. Thus, our study warrants further investigation of the correlation of p45-sErbB3 and osteonectin in clinical specimens of the bone metastasis of prostate cancer.

Osteonectin is a major noncollagenous glycoprotein originally identified in bovine bone (14, 15). Osteonectin is important in bone formation; osteonectin-null mice exhibit profound osteopenia due to decreases in the numbers and surface areas of osteoblasts and osteoclasts (16). Osteonectin may also be involved in the progression of prostate cancer to bone metastatic disease (17). Jacob et al. (12) showed that osteonectin promoted the invasiveness of bone-metastasizing cancer cells including PC-3. Similarly, De et al. (13) reported that osteonectin increases prostate cancer cell invasiveness by interacting with the cell surface receptors αvβ3 and αvβ5. The functional activity of αvβ3 and αvβ5 integrins is higher on the bone-metastasizing C4-2B cells than on the lymph node–metastasizing LNCaP cells despite the similar expression levels of these two integrins in C4-2B and LNCaP. In the study reported here, we showed that p45-sErbB3 stimulates osteonectin secretion from bone, which increased the invasiveness of prostate cancer cells PC-3 and C4-2B. These observations are consistent with the conclusion that osteonectin is an osteoblastic factor that could selectively interact with the bone-tropic cancer cells and enhance their invasiveness.

The mechanism by which osteonectin increased the invasiveness of PC-3 cells and the C4-2B cells in our study is not clear. Jacob et al. (12) showed that treating PC-3 cells with osteonectin increased the activity of matrix metalloproteinase (MMP)-2 in PC-3 cells and that this increased activity may lead to increased tumor cell invasiveness. MMPs are a group of proteinases that degrade collagens, elastin, and other components of the extracellular matrix. To see whether p45-sErbB3 would increase the expression of MMPs by PC-3 cells, we examined MMP-2 and MMP-9 activity in medium from PC-3 cells that were treated or not treated with conditioned medium from calvaria treated with p45-sErbB3. In contrast to the report by Jacob et al. (12), we did not detect significant differences in MMP-2 or MMP-9 activity in these PC-3 cells by zymography (data not shown). Whether other MMPs or matrix-degrading enzymes are involved in the invasion activity seen in our study is not known.

Other bone extracellular matrix proteins, such as osteopontin and bone sialoprotein, have also been reported to contribute to the survival and growth of prostate cancer cells in bone. Osteopontin assists in cell adhesion, migration, and survival (18). Overexpression of osteopontin in LNCaP and PC-3 cells has been shown to increase the invasion of those prostate cancer cells (19). Bone sialoprotein facilitates the attachment of cancer cells to the bone and enhances the metastatic potential of cancer cells (20). In our study, we found that osteonectin stimulated the
invasiveness of C4-2B cells, but neutralizing antibodies against osteonectin could not inhibit the invasiveness of C4-2B cells in response to conditioned medium from p45-sErbB3–treated calvaria (data not shown), suggesting that other invasion-enhancing factors are present in the conditioned medium. Whether biglycan, type I collagen, or both are responsible for differences in prostate cancer cell invasiveness awaits further investigation.

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