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Roles of Osteonectin in the Migration of Breast Cancer Cells Into Bone

Dianalee A. Campo McKnight,[1] Donna M. Sosnoski,[2] Jennifer E. Koblinski,[3] and Carol V. Gay[1,2]*

[1]The Pennsylvania State University, Intercollege Degree Program in Physiology, University Park, Pennsylvania
[2]The Pennsylvania State University, Department of Biochemistry and Molecular Biology, University Park, Pennsylvania
[3]National Institutes of Health, National Institute of Dental and Craniofacial Research, Craniofacial Developmental Biology and Regeneration Branch and Gene Therapy and Therapeutics Branch, Bethesda, Maryland

Abstract The focus of this study was to gain insight into the role(s) of osteonectin in the preferential metastasis of breast cancer cells to bone. Osteonectin was isolated from conditioned media of several cell lines including breast cancer (MDA-MB-435, MDA-MB-468), osteoblasts (hFOB1.19), non-neoplastic breast epithelial (hTERT-HME1), and vascular endothelial cells isolated from a bone biopsy (HBME-1). Chemical/physical properties of osteonectin from these five sources was analyzed to determine if unique configurations of osteonectin exist and therefore identify a chemotactic isoform. Osteonectin from all sources had a molecular weight of ~46 kDa, N-linked glycosylation, and undetectable phosphorylated serines, sialic acids and O-linked oligosaccharides. The cDNA for osteonectin from the breast cancer, osteoblast, and breast epithelial cell lines was identical, while the vascular endothelial cell cDNA contained point mutations that resulted in eight amino acid substitutions. Bone-derived osteonectin was then analyzed to assess its influence on breast cancer cell motility and migration. Although osteonectin increased undirected MDA-MB-231 cell motility, it did not chemoattract the same breast cancer cell line. However, the breast cancer cells did migrate toward the known chemoattractant vitronectin and to bone extracts derived from wild-type and osteonectin-null mice. Migration to vitronectin was enhanced when osteonectin was also present. We concluded that osteonectin was not a chemotactic factor. However, through its anti-adhesive properties, osteonectin induced undirected breast cancer cell motility, and may have enhanced chemoattraction to vitronectin. J. Cell. Biochem. 97: 288–302, 2006.

Key words: osteonectin; breast cancer; chemoattraction; bone matrix; vitronectin

Osteonectin (also called SPARC or BM-40) was originally discovered in bone by its ability to bind to type I collagen [Termine et al., 1981]. Later studies identified osteonectin in many other normal and neoplastic tissues [Porter et al., 1995]. This 32–46 kDa glycoprotein is characterized by three domains, each having a specific function. The acidic domain (aa 1–52) inhibits cell spreading and prevents chemotaxis [Motamed, 1999]. A second domain, the follistatin-like domain (aa 53–137), inhibits proliferation and disrupts focal adhesions. The extracellular Ca^{2+}-binding domain (aa 138–286) also inhibits cell spreading, proliferation, and focal adhesions, but in addition, binds cells and other matrix proteins in a calcium dependent manner [Brekken and Sage, 2000]. Although osteonectin binds to many matrix proteins, such as type I collagen, type IV collagen [Kato et al., 1998] and vitronectin [Rosenblatt et al., 1997], it is not responsible for the structural stability of extracellular matrix [Lane and Sage, 1994]. Instead, osteonectin has been characterized primarily as an anti-adhesive protein

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*Correspondence to: Carol V. Gay, Penn State University, 108 Althouse, University Park, PA 16802.
E-mail: cvg1@psu.edu

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[Murphy-Ullrich et al., 1995; Bradshaw and Sage, 2001] and may be important in cell motility. Murphy-Ullrich [2001] describes the ability of osteonectin to promote an "intermediate state of adhesion" which favors cell motility. Intermediate adhesion is characterized by the disassembly of stress fibers and focal adhesion complexes while maintaining integrin binding to a matrix.

The American Cancer Society reported that in 2002, cancer was the primary cause of death for American women [Jemal et al., 2005]. Breast cancer is the most common type of cancer in women and is the second deadliest next to lung cancer [Parker et al., 1997]. One in eight women will develop breast cancer in her lifetime [Parker et al., 1997] and many of these patients will suffer from bone metastasis. In fact, at autopsy, 75% of all women with breast cancer have bone metastases [Diel, 2001]. Despite the prevalence of breast cancer metastasis to bone, relatively little is understood about why bone is the preferred site of metastasis.

The relationship between osteonectin and cancer has gained some attention in recent years. Clinically, invasive breast carcinomas produce more osteonectin than normal breast tissue [Bellahcene and Castronovo, 1995]. This is also true for prostate cancer cells; osteonectin upregulation is correlated with bone metastases [Thomas et al., 2000]. It has been reported that osteonectin produced by bone cells is a chemoattractant for prostate cancer cells [Jacob et al., 1999; De et al., 2003]. Osteonectin as a chemoattractant for breast cancer cells has not been thoroughly examined; this glycoprotein could be a major contributing factor to their preferential metastasis to bone. However, because many metastatic breast cancer cells also produce osteonectin, it is counterintuitive to postulate that these same cells would be attracted to exogenous osteonectin. In order for a cell to migrate toward a chemotactic factor, a gradient must form so that a responsive cell can move toward greater concentrations. Because many metastatic breast cancer cells and bone cells produce osteonectin, a chemotactic gradient would not exist unless one of two conditions was present. The first condition is that the bone cells secrete a unique configuration of osteonectin, hence a chemotactic isoform. If tissue-dependent configurations of osteonectin exist, it is reasonable to hypothesize that bone-derived osteonectin could form a gradient and therefore attract breast cancer cells. The second condition is that the breast cancer cells that migrate to bone secrete little or no osteonectin and could thus respond to the bone-derived osteonectin. This second condition is plausible considering the Woelfle et al. [2003] report, which correlated a positive diagnosis of bone micrometastases to primary tumors with reduced expression of osteonectin.

In this study, we characterized osteonectin derived from several cell lines including breast cancer, skeletal-derived vascular endothelial, non-neoplastic breast epithelial, and osteoblasts to identify a chemotactic isoform. Osteonectin from these sources was analyzed in terms of molecular weight, phosphorylation, glycosylation, and cDNA sequence. We also examined the ability of exogenous osteonectin to influence cell motility and migration of a breast cancer cell line that secretes low levels of osteonectin.

MATERIALS AND METHODS

Cell Lines

The human breast cancer cell lines used were the MDA-MB-231 [Cailleau et al., 1974], MDA-MB-468, and MDA-MB-435 [Cailleau et al., 1978]. These breast cancer cell lines and the human bone marrow endothelial cell line, HBME-1 [Lehr and Pienta, 1998] which was derived from iliac crest biopsies, were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) and supplemented with 5% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Herndon, VA). The human mammary epithelial cell line, hTERT-HME1 (BD Biosciences-Clontech, Palo Alto, CA), was grown using the MEGM® BulletKit® media system (Cambrex, Walkersville, MD). A human fetal osteoblast cell line, hFOB1.19 (hFOB) [Harris et al., 1995], was grown in DMEM/F-12 (Mediatech) with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All cell lines were cultured at 37°C in a 5% CO_2 humidified incubator.

Protein Isolation: Osteonectin Immunopurified From Cell Lines

Osteonectin was immunopurified from conditioned media collected from the MDA-MB-435, MDA-MB-468, hTERT-HME1, hFOB, and HBME-1 cell lines. Briefly, cells were grown to confluency in their respective media, rinsed

three times in phosphate buffered saline (PBS, pH 7.2), and then exposed to DMEM/F-12 with Serum Replacement 3 (Sigma-Aldrich), 100 IU/ml penicillin and 100 μg/ml streptomycin for 24 h; normal growth media was used for the hTERT-HME1 cell line. Serum replacement was used because osteonectin is present in FBS; the hTERT-HME1 growth media does not contain osteonectin and was, therefore, suitable for conditioned media. Osteonectin produced by the cultured cells was isolated using anti-human osteonectin mouse IgG (Haematologic Technologies, Essex Junction, VT) linked to an AminoLink® Plus Immobilization kit (Pierce, Rockford, IL). The monoclonal antibody (1 μg) was coupled to the AminoLink Plus Gel using the "pH 7.2 coupling buffer" procedure for ligand immobilization as described by the manufacturer. Approximately 250 ml of conditioned media was mixed with the antibody-coupled gel at 4°C overnight while rotating at 160 rpm on a rotary platform. Samples were eluted from the gel with 0.1 M glycine, dialyzed against PBS, and stored at −80°C. Concentrations of osteonectin were determined by an ELISA (Haematologic Technologies). Purity of samples was confirmed with a Coomassie blue stained SDS–PAGE gel.

Protein Isolation: Mouse Bone Extracts

The femurs and tibias of 7–9 week old 129SV × C57BL/6 osteonectin knockout, heterozygous and wild-type mice were a gift from Dr. Hynda Kleinman (National Institutes of Health, National Institute of Dental and Craniofacial Research) [Norose et al., 1998]. Mice were euthanized by CO_2 inhalation; their hind limbs were removed and freed of muscle, tendon, and bone marrow. The cleaned bones were then rinsed in PBS, flash frozen in liquid nitrogen, and stored at −80°C.

A modified method of Termine et al. [1980] was used to extract protein; the conditions used are known to release native osteonectin that binds to collagen and hydroxyapatite [Termine et al., 1981]. Frozen bones were crushed into a fine powder under liquid nitrogen using a mortar and pestle. One gram of bone powder was diluted into 50 ml of extraction buffer (4.0 M guanidine HCl, 0.05 M Tris, 0.1 M 6-aminohexanic acid, 5 mM benzamidine HCl, and 1 mM phenylmethanesulfonyl fluoride (PMSF) pH 7.2) and mixed on a rotary platform at 160 rpm for 24 h in 4°C. Samples were centrifuged at 1,800*g* for 6 min, the supernatant was removed and kept for further analysis; it was found to be devoid of osteonectin by immunoblotting. A 10 ml volume of extraction buffer containing 0.5 M EDTA was added to the remaining residue. The samples were incubated at 4°C for 72 h and mixed on a rotary platform at 160 rpm. The samples were then centrifuged at 1,800*g* for 6 min and the supernatant collected. Bone extracts were dialyzed twice against dH_2O with protease inhibitors (0.1 M 6-aminohexanic acid, 5 mM benzamidine HCl, and 1 mM PMSF) at 4°C for a total of 48 h. The samples were lyophilized and reconstituted in dH_2O with protease inhibitors and stored at −80°C. Protein concentrations were determined by using the BioRad Protein Assay system (BioRad, Hercules, CA). Samples were separated on a 12% SDS–PAGE gel, stained with 7.5% SYPRO® orange in acetic acid (Molecular Probes, Eugene, OR) and imaged by a Typhoon™ Variable Mode Imager (Amersham Biosciences, Piscataway, NJ).

Immunoblotting

Immunopurified osteonectin samples (10 ng) derived from cultured media were diluted with Laemelli loading buffer, reduced with 100 mM DTT and boiled for 5 min. Following separation on a 15% SDS–PAGE gel, proteins were transferred to nitrocellulose membranes (BioRad). The membranes were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Membranes were subsequently exposed to primary antibody (anti-human osteonectin mouse IgG, Haematologic Technologies) at a dilution of 1:15,000 in blocking solution and incubated overnight at room temperature. A secondary antibody, sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP, Amersham Biosciences), was used at a dilution of 1:3,750 in PBS and incubated for 1 h at room temperature. Immunolabeled bands were detected using an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

The detection of osteonectin from mouse bone extracts was accomplished by the same immunoblotting method described with the following modifications. Blots were blocked with 5% nonfat dry milk and 1% BSA in PBS. The primary antibody, anti-mouse osteonectin rabbit IgG, was diluted to 1:1,000 in blocking solution [Wewer et al., 1988]. A horse anti-rabbit IgG

conjugated to HRP (Amersham Biosciences) was diluted to 1:5,000 in PBS.

Deglycosylation

Analysis of osteonectin glycosylation was accomplished with an enzymatic protein deglycosylation kit (Sigma-Aldrich). Briefly, 50 ng of immunopurified osteonectin was denatured and mixed with either (a) reaction buffer alone (control), (b) PNGase F, (c) PNGase F with α-2 neuraminidase, (d) *O*-glycosidase, (e) *O*-glycosidase with α-2 neuraminidase, (f) *O*-glycosidase with α-2 neuraminidase and β-galactosidase, or (g) *O*-glycosidase with α-2 neuraminidase and β-*N*-acetylglucosaminidase. Samples were enzymatically deglycosylated at 37°C for 3 h. The deglycosylated samples were then analyzed for shifts in migration by immunoblotting for immunopurified osteonectin.

Phosphoserine Detection

Detection of phosphoserines was accomplished by immunoblotting the immunopurified osteonectin samples collected from MDA-MB-435, MDA-MB-468, hTERT-HME1, hFOB, and HBME-1 cell lines. The samples (10 ng) were separated on a 12% SDS–PAGE gel and transferred to nitrocellulose. The nitrocellulose membranes were blocked in 3% BSA in PBS with 0.2% Tween-20 and phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride). The primary antibody was an anti-phosphoserine mouse IgG (Sigma-Aldrich) diluted to 1:5,000 in blocking solution. The secondary antibody was diluted to 1:3,750 in PBS with 0.2% Tween-20 and phosphatase inhibitors; all rinses were done in the presence of phosphatase inhibitors. Rat brain extracts (Biomol, Plymouth Meeting, PA) were used as the positive control.

cDNA Sequencing

Cell cultures of MDA-MB-435, MDA-MB-468, hFOB, hTERT-HME1, and HBME-1 were rinsed in PBS and lysed using the QIAshredder™ kit (Qiagen, Valencia, CA). RNA was isolated with an RNeasy® kit (Qiagen) and stored at −80°C. Full length cDNA transcripts were generated using the reverse transcriptase Retroscript kit (Ambion, Austin, TX). The MDA-MB-435, MDA-MB-468, hFOB, and hTERT-HME1 osteonectin cDNA coding region (911 bp) was amplified by PCR with the following primers: forward CCT-GCC-TGC-CAC-TGA-GG and reverse TAA-ACA-TTG-GGG-GAA-ACA-CG (GenBank accession no. NM003118, MWG-Biotech, High Point, NC). The HBME-1 cDNA was amplified using the same forward primer with the reverse primer GCA-GAA-CAA-CAA-ACC-ATC-CA (MWG-Biotech). PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide; bands were excised from of the gel and DNA was extracted with the QIAquick® gel extraction kit (Qiagen). Internal primers were designed approximately 400 base pairs apart to obtain overlapping sequence verification. Sense and anti-sense strands of the PCR products were then sequenced on an Applied Biosystem Hitachi 3100 Genetic Analyzer (Foster City, CA).

The amino acid sequence was translated from the resulting cDNA sequence using the ExPASy web-based program (us.expasy.org/tools/dna.html).

Cell Motility Assay

MDA-MB-231 cells were grown to confluency on 4-well permanox chamber slides (Nalge Nunc, International, Naperville, IL). A cross-shaped "wound" was created in each well by scraping the cell layer with a pipette tip (diameter ∼0.6 mm). Detached cells were removed. The cross-shaped wound provided a point of reference for images collected at two different time points; images were collected at the same distance from the center of the cross-shaped wound at 0 and 6 h in each chamber. DMEM/F-12 with Serum Replacement 3 and either vehicle (PBS only) or 500 ng of osteonectin immunopurified from MDA-MB-468, hFOB, or HBME-1 cells were added to each well. The chamber slides were incubated at 37°C in a 5% CO_2 humidified incubator. Wounds were imaged using a 10× objective and phase contrast microscopy. Wound closure was calculated as percent change in the distance between the borders of cell growth.

Transwell Migration Assays: Addition of Chemoattractants

A modified chemoattraction assay, previously described by Byzova et al. [2000] was employed. Briefly, the chemoattractant was diluted as described below and air-dried to the lower surface of the membrane of Falcon® FluoroBlock™ transwell chamber inserts (Becton Dickinson, Franklin Lakes, NJ). The transwell

inserts were in the 24-well format and were opaque with 8 μm pores. The chemoattractants used were (A) osteonectin derived from cell secretions of five cell lines, (B) bovine bone osteonectin (Haematologic Technologies), (C) bovine bone osteonectin and human vitronectin (Chemicon, Temecula, CA), or (D) protein extracts from mouse femurs and tibias. In experiment A, osteonectin secreted from MDA-MB-435, MDA-MB-468, hFOB, hTERT-HME1, or HBME-1 was diluted in PBS; 0 ng (vehicle control), 25 or 50 ng of protein in a 33 μl volume were air-dried to the lower surface of each well. In experiment B, 0 ng (vehicle control), 50 ng, 100 ng, 200 ng, or 1 μg of bovine bone osteonectin in a 10 μl volume of PBS was air-dried to the lower surface of each well. Experiment C utilized human vitronectin (200 ng), bovine bone osteonectin (200 ng), and a mixture of vitronectin and osteonectin (200 ng total protein) in a 10 μl volume of dH_2O with protease inhibitors (0.1 M 6-aminohexanic acid, 5 mM benzamidine HCl, and 1 mM PMSF) air-dried to the lower surface of transwell membranes. Finally, in experiment D, bone extracts were diluted in dH_2O with protease inhibitors (0.1 M 6-aminohexanic acid, 5 mM benzamidine HCl, and 1 mM PMSF). The lower surface of the wells were coated with either 0 ng (vehicle control), 50, 100, or 200 ng of extracts in a total volume of 10 μl of dH_20 with protease inhibitors.

Transwell Migration Assays: Addition of Cells

MDA-MB-231 cells were stained with Vibrant® DiI (Molecular Probes) and seeded to the upper surface of the chamber at 5×10^4 cells per well in the presence of 300 μl DMEM/F-12 with Serum Replacement 3, 100 IU/ml penicillin and 100 μg/ml streptomycin. In experiment C, GRGESP (RGE) and GRGDSP (RGD) blocking peptides (200 μg of peptides, Gibco, Rockville, MD) were diluted into 1 ml of DMEM/F-12 with Serum Replacement 3 and Vibrant DiI stained MDA-MB-231 cells. The cell and blocking peptide mixture (300 μl) was then added to the upper chamber of each well. DMEM/F-12 (800 μl) with Serum Replacement 3, 100 IU/ml penicillin and 100 μg/ml streptomycin was added to the lower chamber. The cells were allowed to migrate in a 37°C, 5% CO_2 humidified incubator for either 6 or 48 h. The membranes were then rinsed with PBS, fixed with 4% paraformaldahyde, and mounted to a microscope slide with Fluoromount-G (Southern Biotech, Birmingham, AL). Migrated cells found on the lower surface of the membrane were visualized and counted with the use of 549 nm excitation and 565 nm emission wavelengths at 100× magnification. Each experiment was done in triplicate (total of $n = 9$ independent replicates) and a Student's *t*-test was used for statistical analysis.

RESULTS

Immunoblotting of Osteonectin Secreted by Various Cell Types

Osteonectin is secreted by many normal and neoplastic cells in the body; we selected an array of cell lines that represent many of the cell types involved in breast cancer metastasis to bone. We used a non-neoplastic breast epithelial (hTERT-HME1) cell line and three human breast cancer cell lines (MDA-MB-435, MDA-MB-468, and MDA-MB-231); these cells provide a useful model of both normal and neoplastic breast tissue. The MDA-MB-435 and MDA-MB-231 cells lines are considered highly metastatic and will metastasize to bone in mice [Yoneda et al., 2001; Harms and Welch, 2003]. In contrast, the MDA-MB-468 cell line is substantially less metastatic compared to the MDA-MB-435 and MDA-MB-231 cell lines [Zhang et al., 1991]. To represent the bone microenvironment, we utilized a human bone marrow vascular endothelial cell line (HBME-1) derived from iliac crest biopsies and a human osteoblast cell line (hFOB).

We first determined which of these cell lines secreted osteonectin. The MDA-MB-435, MDA-MB-468, hFOB, HBME-1, and hTERT-HME1 cell lines all secreted detectable levels of osteonectin by immunoblotting (Fig. 1). The MDA-MB-231 cell line did not secrete measurable levels of osteonectin; however, low levels of osteonectin mRNA were detected by RT-PCR (data not shown and [Dhanesuan et al., 2002]. Furthermore, the MDA-MB-435, MDA-MB-468, hFOB, HBME-1, and hTERT-HME1 cell lines all secreted osteonectin with the same molecular weight (~46 kDa). Although osteonectin from the various cell types had similar molecular weights, a chemotactic gradient could exist if one of the secreted forms had unique post-translational modifications. Therefore, we further analyzed osteonectin from these cell sources to identify specific post-translational modifications.

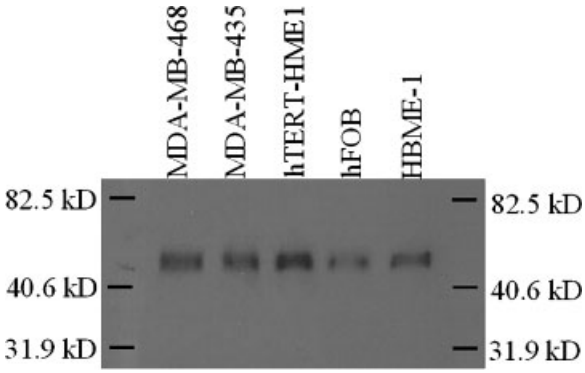

Fig. 1. Immunoblotting of osteonectin. Osteonectin (10 ng) immunopurified from conditioned media was reduced and separated on a 15% SDS–PAGE gel. Osteonectin from the MDA-MB-468, MDA-MB-435, hTERT-HME1, hFOB, and HBME-1 cell lines was detected at ~46 kDa.

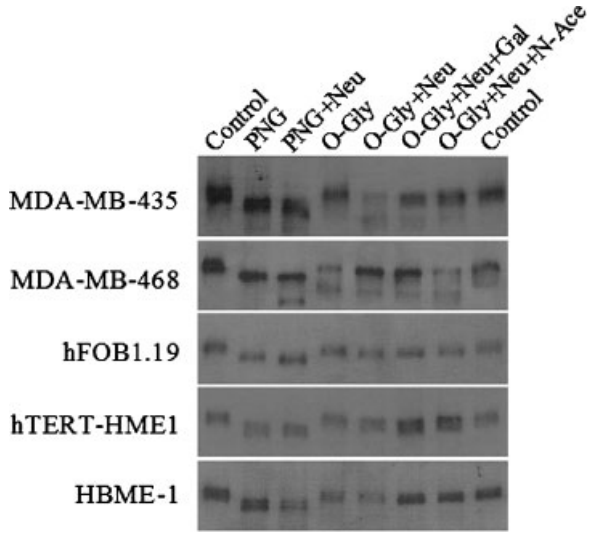

Fig. 2. Deglycosylation of osteonectin from different cell lines. Osteonectin was deglycosylated with a series of enzymes: PNGase F (PNG), neuraminidase (Neu), *O*-glycosidase (*O*-Gly), β-galactosidase (Gal), and *N*-acetylglucosaminidase (*N*-Ace). Samples were then separated on a 15% SDS–PAGE gel and immunoblotted. Compared to the control (no enzyme), the MDA-MB-435, MDA-MB-468, hFOB1.19, hTERT-HME1, and HBME-1 derived osteonectin displayed a molecular weight shift in response to PNGase F only indicating that N-linked glycosylation was present.

Identification of Post-Translational Modifications of Osteonectin

Two common post-translational modifications that could contribute to the formation of a unique configuration of osteonectin are glycosylation and phosphorylation. The glycosylation pattern of osteonectin secreted by the MDA-MB-435, MDA-MB-468, hFOB, HBME-1, and hTERT-HME1 cell lines was generated by using a series of deglycosylation enzymes (Fig. 2). All of the sources of osteonectin exhibited a marked gel shift when exposed to PNGase F. This pattern of enzymatic deglycosylation indicated that the MDA-MB-435, MDA-MB-468, hFOB, HBME-1, and hTERT-HME1 cell lines secreted osteonectin with N-linked oligosaccharides. There was no detectable gel shift in response to treatment with neuraminidase, *O*-glycosidase, β-galactosidase, or *N*-acetylglucosaminidase which remove sialic acid, O-link oligosaccharides, galactose, and β-linked *N*-acetylglucosamine residues, respectively. These data demonstrated that osteonectin from the cell lines examined had the same pattern of glycosylation.

Osteonectin has been described as a phosphoglycoprotein [Triffitt, 1987; Sage et al., 1989] with multiple serine residues as possible phosphorylation sites [Fisher et al., 1987]. We analyzed osteonectin from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1, and hTERT-HME1 cell lines for the presence of phosphoserines and determined that none of the tested osteonectin samples contained phosphorylated serines (Fig. 3).

Osteonectin cDNA Sequencing and Amino Acid Analysis

Because breast, bone, and vascular cells secreted osteonectin with the same post-trans-

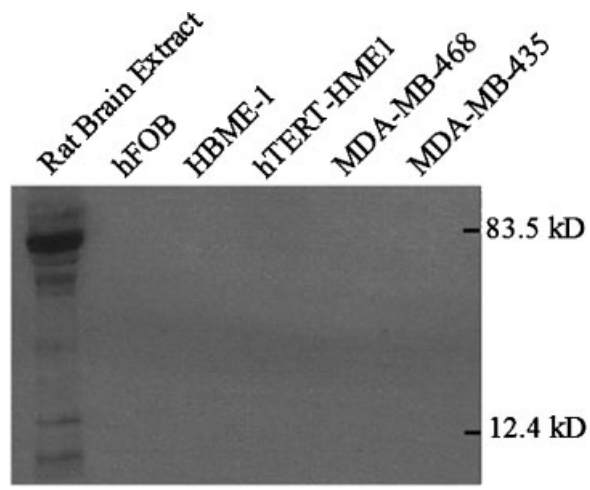

Fig. 3. Detection of phosphorlyated serines of osteonectin from different cell lines. Immunopurified osteonectin (10 ng) from the hFOB, HBME-1, hTERT-HME1, MDA-MB-468, and MDA-MB-435 cell lines was detected for the presence of phosphorylated serines. The osteonectin was reduced and separated on a 12% SDS–PAGE gel. Rat brain extract was used as the positive control. None of the examined cell lines secreted osteonectin with phosphorylated serines.

lational modifications, we compared the amino acid sequence for translational differences. Osteonectin cDNA was sequenced from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1, and hTERT-HME1 cell lines. The resulting nucleic acid sequences were then translated into amino acid sequences (Fig. 4). We found the MDA-MB-435, MDA-MB-468, hFOB, and hTERT-HME1 samples had identical cDNA sequences and therefore were assumed to have identical amino acid sequences to each other and to the published human osteonectin sequence (GenBank accession no. NM003118). The HBME-1 cDNA sequence had a number of nucleic acid point mutation that resulted in eight amino acids that differed from the published sequence. The published osteonectin amino acid sequence, with the eight substitutions found in the HBME-1 sample, is illustrated in Figure 4. The substitutions resulted in (1) threonine to alanine, (2) valine to glycine, (3) serine to proline, (4) aspartic acid to glutamic acid, (5) alanine to glycine, (6) glutamic acid to aspartic acid, (7) glutamine to glutamic acid, and (8) lysine to glutamine. We concluded that the amino acid sequence of breast cancer cells (MDA-MB-435 and MDA-MB-468), osteoblasts (hFOB), and non-neoplastic breast epithelial cells (hTERT-HME1) appeared to be identical. However, the human bone marrow vascular endothelial cell line (HBME-1) produced osteonectin with eight substituted amino acids.

Osteonectin-Induced Motility and Migration

To test the ability of osteonectin to increase cell motility, we utilized the MDA-MB-231 cell line. This cell line secretes undetectable amounts of osteonectin and can, therefore, be used to study its response to osteonectin from another cell type [Dhanesuan et al., 2002]. In "wound-healing" assays, MDA-MB-231 cells were treated with either vehicle (PBS) or osteonectin immunopurified from MDA-MB-468, HBME-1, or hFOB cell-conditioned media. The MDA-MB-231 cells displayed greater cell outgrowth in the presence of osteonectin compared to the vehicle control (Fig. 5). After 6 h, there was no wound closure in the control treatment. However, the hFOB-derived osteonectin induced a 23% increase in wound closure. There was also a 42% and 46% increase in wound closure by the HBME-1 and MDA-MB-468 derived osteonectin, respectively. These data support the literature that exogenous osteonectin enhances cell motility [Greenwood and Murphy-Ullrich, 1998; Murphy-Ullrich, 2001].

Even though osteonectin from the cancer cells (MDA-MB-435 and MDA-MB-468) and bone cells (hFOB) appeared to be identical, it is possible that a chemotactic gradient could exist if the bone cells secrete higher levels of osteonectin than the breast cancer cells. We conducted migration assays to test this possibility. The migration of MDA-MB-231 cells, which do not secrete detectable levels of osteonectin, toward osteonectin isolated from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1, and hTERT-HME1 cell lines was investigated (Fig. 6). After 6 h, there was no significant migration toward either 25 or 50 ng of osteonectin from the MDA-MB-435, MDA-MB-468, and hFOB cell lines. Cells did migrate toward the highest dose of hTERT-HME1 derived osteonectin (fivefold increase). However, the actual number of cells that migrated to the

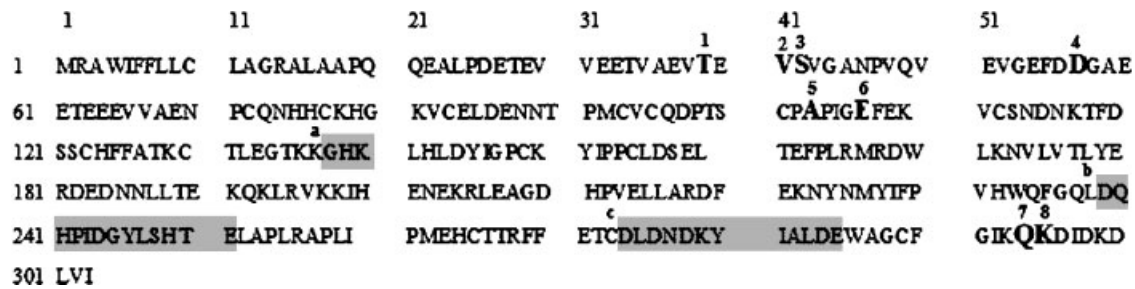

Fig. 4. The published amino acid sequence of human osteonectin with the location of the substitutions found in the HBME-1 osteonectin. MDA-MB-468, MDA-MB-435, hTERT-HME1, and hFOB osteonectin amino acid sequence was identical to the published sequence. There were eight substitutions identified in the amino acid translation of the HBME-1 sample, these substitutions were positioned at numbers 1–8. The substitutions were (1) threonine to alanine, (2) valine to glycine, (3) serine to proline, (4) aspartic acid to glutamic acid, (5) alanine to glycine, (6) glutamic acid to aspartic acid, (7) glutamine to glutamic acid, and (8) lysine to glutamine. The ''GHK'' (**a**), EF-hand 1 (**b**), and EF-hand 2 (**c**) peptides are highlighted.

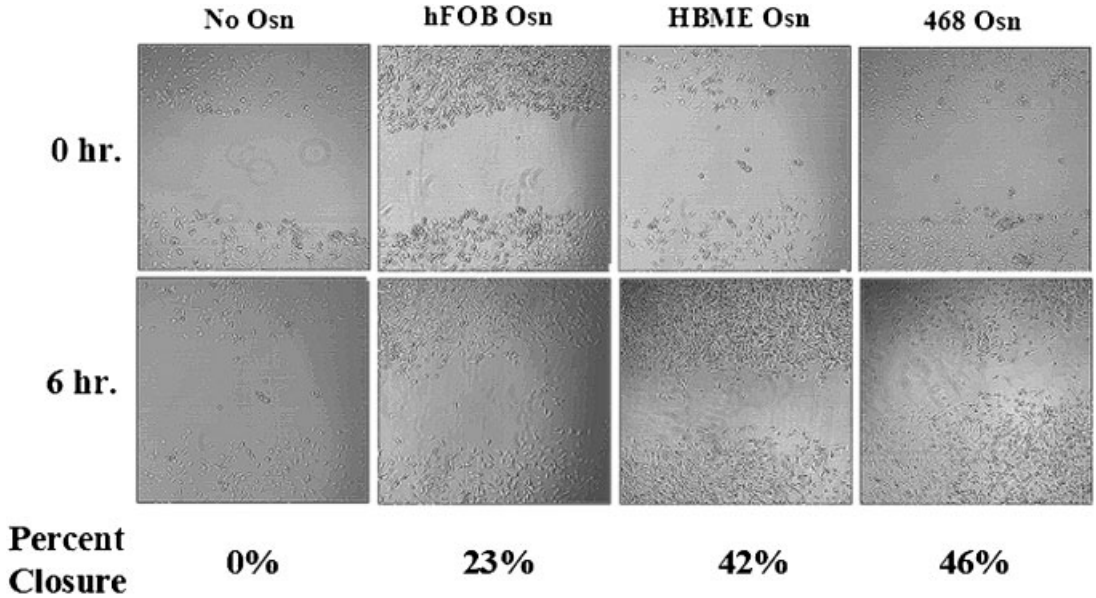

Fig. 5. Cell motility induced by osteonectin. MDA-MB-231 cells were grown to confluence on permanox chamber slides. A cross-shaped ''wound'' was then cut into the monolayer and the remaining attached cells were exposed to control (PBS only) or 500 ng of osteonectin from MDA-MB-468 (468), hFOB, and HBME-1 (HBME) cells for 6 h. Compared to the control, the hFOB derived osteonectin induced a 23% wound closure while the HBME-1 and MDA-MB-468 osteonectin induced 42% and 46% closure, respectively. These images have been 30× magnified and represent typical results from duplicate experiments.

hTERT-HME1-derived osteonectin was very low, only about 10 cells migrated for every square millimeter. There was also some migration to the HBME-1 derived osteonectin; we observed a twofold and threefold increase in migration toward the 25 and 50 ng doses, respectively. The number of migrated cells toward the HBME-1 derived osteonectin was also low. We observed about four cells in every mm^2 in the 25 ng dose while the number of migrated cells toward the 50 ng dose increased to about six cells per mm^2.

Chemoattraction of breast cancer cells to purified bovine bone-derived osteonectin was also tested. We extended the migration time to 48 h and increased the doses of osteonectin to 50, 100, 200 ng, and 1 μg of protein per well. The metastatic breast cancer cells did not display

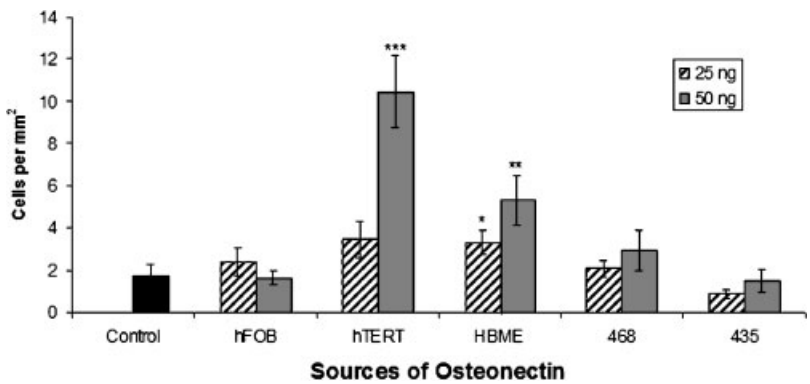

Fig. 6. MDA-MB-231 cell migration toward osteonectin. Either 0 (control), 25, or 50 ng of osteonectin isolated from hFOB, hTERT-HME1, HBME, MDA-MB-468 (468), and MDA-MB-435 (435) cell lines was air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells (5×10^4 cells per well) were added to the upper chamber. After 6 h, membranes were fixed and migrated cells were counted. Data represents three replicate experiments. There was migration to 50 ng of hTERT-HME1 derived osteonectin (fivefold increase). A twofold and threefold increase in migration was observed to 25 and 50 ng of HBME-1 derived osteonectin, respectively. No significant migration was observed toward the osteonectin isolated from the MDA-MB-435, MDA-MB-468, or hFOB cell lines. ($N = 9$ membranes per peptide dose, mean ± SEM, *, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$ compared to control).

any increase in migration to bovine bone osteonectin (data not shown).

To confirm that osteonectin is not chemotactic to breast cancer cells, we compared the migration of MDA-MB-231 cells to osteonectin and the known chemoattractant vitronectin [Bartsch et al., 2003]. Vitronectin has been shown to chemoattract breast cancer cells through its RGD domain [Meyer et al., 1998]. We conducted migration assays with 200 ng osteonectin, 200 ng vitronectin, or 100 ng of both vitronectin and osteonectin (vitronectin/osteonectin mixture, 200 ng of total protein) as the chemoattractant. The MDA-MB-231 breast cancer cells were pretreated with either GRGESP (RGE) or GRGDSP (RGD) blocking peptides and exposed to the chemoattractants for 6 h (Fig. 7). There was no significant migration of the breast cancer cells to osteonectin in the presence of RGE or RGD peptides. In contrast, there was substantial migration to vitronectin in the presence of RGE peptides. When the vitronectin/osteonectin mixture was used as a single chemoattractant in the presence of RGE peptides, breast cancer cell migration was also significantly increased. The migration of breast cancer cells to either vitronectin or the vitronectin/osteonectin mixture was significantly inhibited with RGD peptides. In this assay, osteonectin did not appear to chemoattract breast cancer cells. However, the breast cancer cells were chemoattracted to vitronectin though its RGD domain. Interestingly, the levels of migration to vitronectin (200 ng) compared to the vitronectin/osteonectin mixture were statistically equivalent despite the reduced dose of vitronectin in the mixture (100 ng).

Migration of MDA-MB-231 Cells Toward Bone Extracts

To further assess the chemotactic potential of osteonectin, migration assays utilizing protein extracts collected from the femurs and tibias of wild-type, heterozygous, and osteonectin-null mice were performed. We first assayed the bone extracts for the presence of osteonectin. In a SYPRO stained SDS–PAGE gel, the wild-type sample had a single band that was noticeably absent in the knockout sample and reduced in the heterozygous sample (Fig. 8A). There was very little effect on the presence of other proteins as indicated by the number and density of protein bands between the samples. An immunoblot confirmed the absence of osteonectin in the knockout sample (Fig. 8B).

We then used wild-type and osteonectin-null bone extracts in a transwell chamber migration assay; De et al. [2003] used a similar method to demonstrate that osteonectin is the main chemotactic factor in bone extracts for prostate cancer cells. In Figure 8C, the migration of MDA-MB-231 cells toward either vehicle control (dH_2O with protease inhibitors) or 50, 100, and 200 ng of wild-type or osteonectin-null extracts is represented. The cancer cells migrated to all doses of wild-type or osteonectin-

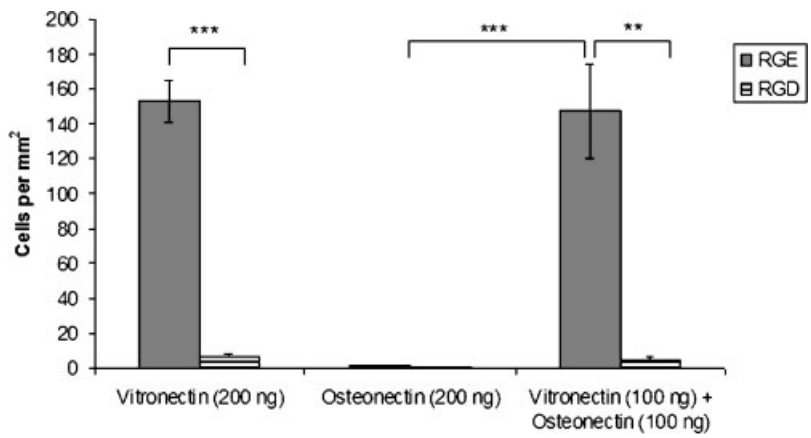

Fig. 7. Migration of MDA-MB-231 cells toward osteonectin and vitronectin. Either 200 ng osteonectin, 200 ng vitronectin or 100 ng of both osteonectin and vitronectin (vitronectin/osteonectin mixture, 200 ng total protein) was air-dried to the lower surface of a transwell membrane. Vibrant DiI stained MDA-MB-231 cells (5×10^4 cells per membrane) were combined with 200 μg/ml of RGE or RGD peptides. Experiments were done in duplicate. After 6 h, there was no migration to osteonectin in the presence of RGE or RGD blocking peptides. There was migration to vitronectin and the vitronectin/osteonectin mixture; this migration was significantly inhibited with RGD blocking peptides. ($N = 6$ membranes per peptide dose, mean ± SEM, **, $P \leq 0.01$, ***, $P \leq 0.001$).

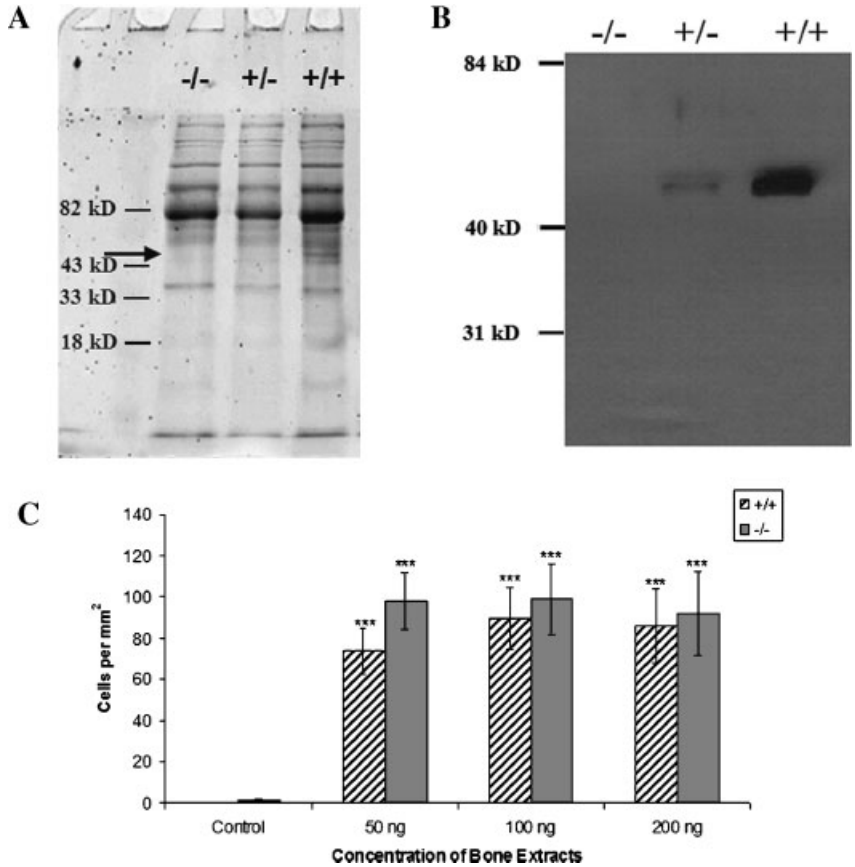

Fig. 8. Migration of MDA-MB-231 cells toward bone extracts. **A**: Bone extracts from the femurs and tibias of wild-type or osteonectin-null mice were separated by SDS–PAGE and stained with SYPRO®. There was a single band absent (denoted with an arrow) in the knockout extract (−/−) and reduced in the heterozygous (+/−) sample. **B**: Bone extracts were analyzed by immunoblotting and osteonectin was detected in the wild-type (+/+) and heterozygous extract but absent in the knockout sample. **C**: 0 ng, 50, 100, or 200 ng bone extracts were air-dried to the lower surface of a transwell membrane. Vibrant DiI stained MDA-MB-231 cells were seeded (5×10^4 cells per well) and allowed to migrate for 6 h before being fixed. Data represents three replicate experiments. There was increased migration to all bone extracts (38- to 50-fold increase). No difference in migration toward the wild-type or osteonectin-null extracts of equal concentrations was detected. ($N = 9$ membranes per extract dose, mean ± SEM, ***, $P \leq 0.001$ compared to control).

null bone extracts. There was no difference in migration toward the wild-type or osteonectin-null extracts when compared at equal doses. This experiment indicates that the metastatic breast cancer cells were attracted to bone extracts but that the presence of osteonectin was not required.

DISCUSSION

It is well-established that breast cancer cells have a high propensity to metastasize to bone [Diel, 2001]. We investigated the role that osteonectin plays in this phenomenon. Osteonectin has previously been described as a chemoattractant for prostate cancer cells [Jacob et al., 1999; De et al., 2003]. The possibility that osteonectin has the same effect on breast cancer cells has not been fully examined; such an effect could explain why bone is a preferred site of metastasis. Osteonectin has been shown to increase MDA-MB-231 invasiveness through Matrigel™ [Jacob et al., 1999]. Also, further evidence supporting a role for osteonectin in the development of skeletal malignancy is the observation that breast cancer cells have a high propensity to metastasize to trabecular bone where osteonectin is found at its greatest concentration within the bone [Ninomiya et al., 1990].

In order for a substance to act as a chemoattractant, a diffusible gradient must form, thus attracting a responsive cell toward higher concentrations. A paradox exists in that osteoblasts and metastatic breast cancer cells both secrete osteonectin. This dual production of

osteonectin could negate the formation of a gradient to which breast cancer cells respond, assuming that both forms of osteonectin are identical and secreted at similar concentrations. We analyzed osteonectin to determine if tissue dependent configurations and hence if a chemotactic isoform exists.

Our results indicated that metastatic breast cancer cells (MDA-MB-435 and MDA-MB-468), osteoblasts (hFOB), non-neoplastic breast epithelial cells (hTERT-HME1), and vascular endothelial cells (HBME-1) secreted osteonectin with a similar molecular weight (~46 kDa). We also found that osteonectin from these cells had similar post-translational modifications. All forms of osteonectin tested in this study had the same glycosylation pattern, namely, all had N-linked oligosaccharides and undetectable sialic acids and O-linked oligosaccharides. N-linked glycosylation is important because it has been correlated to the binding affinity of osteonectin to collagen [Kelm and Mann, 1991; Kaufmann et al., 2004]. However, osteonectin from these cell lines appeared to have no variation in N-linked glycosylation. Osteonectin from these cell types also had undetectable phosphorylated serines.

While the breast cancer, osteoblast, and normal breast epithelial cell lines generated an identical osteonectin cDNA sequence, the vascular endothelial cells produced a distinctly different osteonectin cDNA. However, at the amino acid level, these differences appeared minor. Substitutions numbers 1, 3, 4, 5, 6, 7, and 8 were common evolutionary changes; these substitutions resulted in amino acids with similar polarity and mass and therefore most likely did not affect the function of the protein [Dayhoff and National Biomedical Research Foundation, 1979]. There were no changes in the number or position of cysteines, which indicated that disulfide bonding was unaffected. There was a loss of a serine (substitution 3) and a threonine (substitution 1), which could have affected phosphorylation. The EF-hands, which sequester Ca^{2+} and bind to other matrix proteins, were not affected by the substitutions [Busch et al., 2000]. In addition, the GHK peptide within the follistatin-like domain that is responsible for stimulating angiogenesis and proliferation, was also unaffected by the substitutions found in the HBME-1 osteonectin. [Brekken and Sage, 2000]. Furthermore, the slight difference in the osteonectin cDNA sequence could also be a result of variations in the genetic background of the different cell lines. We concluded that there were no significant differences in the cDNA sequences, and therefore amino acid sequences, generated from the breast cancer, osteoblast, vascular endothelial and non-neoplastic breast epithelial cell lines.

Because our investigations did not reveal notable differences between the osteonectin secreted by breast cancer cells and osteoblasts, we concluded that it was unlikely that a chemotactic gradient, based on a unique configuration of osteonectin, could exist. However, one can argue that a gradient of osteonectin can form due to variable secretion levels as opposed to the presence of a tissue-dependent configuration. If cancer cells secrete reduced levels of osteonectin, while osteoblasts secrete high concentrations, a chemotactic gradient could still exist. This concept is supported by the clinical observation that breast cancer patients with primary tumors that express low levels of osteonectin have a greater frequency of developing bone micrometastases [Woelfle et al., 2003]. To test this possibility in vitro, we analyzed the ability of osteonectin to increase cell motility and migration on a breast cancer cell line (MDA-MB-231), which does not secrete detectable levels of osteonectin.

Osteonectin has been described as a matricellular protein with de-adhesive qualities [Murphy-Ullrich, 2001]. Soluble osteonectin induces loss of focal adhesion plaques in endothelial cells while vinculin and F-actin are redistributed within the cytoplasm [Greenwood and Murphy-Ullrich, 1998]. Cells remain attached to an extracellular matrix by surface integrins while intracellular focal adhesion plaques are lost. Such cells appear to have a normal morphology, namely, they remain spread over a matrix [Murphy-Ullrich et al., 1995]. When a cell maintains integrin binding to a matrix and loses focal adhesion plaques, it is described as being in a state of "intermediate adhesion" which is considered ideal for cell motility. In contrast, strong attachment is characterized by the presence of many focal adhesion plaques that render the cell immobile. A weakly adherent cell has little or no integrin binding and is, therefore, unable to develop the contractile force needed for movement [Murphy-Ullrich, 2001]. Soluble osteonectin is a potent stimulator of intermediate

attachment and therefore enhances cell motility [Greenwood and Murphy-Ullrich, 1998].

We tested the ability of osteonectin to induce cell motility in a "wound-healing" assay. Osteonectin from osteoblasts, breast cancer cells, and vascular endothelial cells increased MDA-MB-231 cell motility, as determined by the ability of osteonectin to enhance cell outgrowth into the wound. Notably, osteonectin from cancer and vascular endothelial cells induced high levels of motility; nearly half of the wound was covered with mobile cells after 6 h. Osteonectin from the osteoblasts had less of an impact on breast cancer cell motility but still increased greater cell motility compared to the vehicle control. These results support the current literature that exogenous osteonectin enhances cell motility [Greenwood and Murphy-Ullrich, 1998; Murphy-Ullrich, 2001]. However, the ability of a single concentration of soluble osteonectin to increase cell motility is distinctly different from chemoattraction, a process which is dependent on a gradient. To test for chemoattraction (or directed cell migration), we needed to measure cell movement toward a distant source of osteonectin.

Osteonectin from several cell lines was tested for its ability to chemoattract MDA-MB-231 cells utilizing transwell chamber assays. The hTERT-HME1 derived osteonectin induced a fivefold increase in migration. Although the migration toward the normal breast epithelial osteonectin was statistically significant, the actual number of migrated cells was very low ($\sim$10 cells per mm^2). It is reasonable to assume that the increase in migration toward hTERT-HME1 derived osteonectin could be attributed to increased cell motility and not true chemoattraction. This event could have resulted from the anti-adhesive properties of osteonectin. Nevertheless, if we assume there was true migration and not just increased motility, we were still unable to explain why breast cancer cells preferentially migrate to bone. We did, however, address the role osteonectin may have on cancer cells in the primary tumor. The epithelial-derived osteonectin could assist in the de-adhesion of cancer cells from the original tumor. Osteonectin upregulates matrix metalloproteinase-2 activation which is important for proteolysis of the extracellular matrix and hence a contributor of tumor invasion [Gilles et al., 1998; Jacob et al., 1999]. In either case, epithelial-derived osteonectin would not direct breast cancer migration toward bone.

Osteonectin from the endothelial (HBME-1) cells also increased migration (up to threefold); this could again be a result of its anti-adhesive properties. We found that the HBME-1 derived osteonectin increased cell motility in wound healing assays, thus the migration to this source of osteonectin could also be a result of increased cell motility.

Some migration to osteonectin from normal breast epithelial cells and vascular endothelial cells was observed, however, there was no migration to breast cancer or osteoblast-derived osteonectin in transwell chamber assays. Furthermore, the MDA-MB-231 cells did not respond to the bovine bone-derived osteonectin up to a dose of 1 μg and a migration time of 48 h. We concluded that bovine bone osteonectin did not attract breast cancer cells.

The lack of breast cancer cell chemoattraction toward osteonectin was further demonstrated by the comparison of migration toward osteonectin and vitronectin. Vitronectin is known to chemoattract MDA-MB-231 cells through the binding of $\alpha_v\beta_3$ on the breast cancer cell surface to the RGD domain of vitronectin [Meyer et al., 1998; Bartsch et al., 2003]. This is in contrast to osteonectin, which does not contain the RGD domain. RGD and RGE blocking peptides were used to demonstrate the RGD-dependence of breast cancer cell migration to vitronectin; RGE peptides provided a negative control. There was no migration of the breast cancer cells to bone-derived osteonectin; this was consistent with our previous findings that MDA-MB-231 cells did not migrate to high doses of bovine bone osteonectin after 48 h. There was marked breast cancer cell migration toward vitronectin in the presence of RGE peptides. RGD peptides significantly inhibited MDA-MB-231 migration toward vitronectin as expected [Meyer et al., 1998; Bartsch et al., 2003]. We also observed increased breast cancer cell migration toward the vitronectin/osteonectin mixture. In fact, the number of migrated cancer cells toward the vitronectin/osteonectin mixture was equal to the migration levels induced by vitronectin alone. These findings were surprising considering there was half the amount of vitronectin (100 ng) in the vitronectin/osteonectin mixture compared to when vitronectin was used alone (200 ng). In other experiments, MDA-MB-231 cell migration was shown to exhibit a dose

response to increasing amounts of vitronectin (data not shown). Migration toward the vitronectin/osteonectin mixture was also inhibited with RGD blocking peptides and was, therefore, deemed RGD-dependent.

The migration of the MDA-MB-231 cells toward the vitronectin/osteonectin mixture was mainly induced by the presence of vitronectin as demonstrated by blocking RGD-dependent migration. However, there appeared to have been a synergistic effect induced by the presence of osteonectin; the number of migrated cells toward the vitronectin/osteonectin mixture (100 ng vitronectin) was equal to the migration levels produced by vitronectin (200 ng). This observation supports the concept that osteonectin enhanced cell motility through its anti-adhesive properties. Murphy-Ulrich describes that intermediate adhesion can only exist when a cell is bound to a matrix through integrins. In this experiment, the MDA-MB-231 cells bound to the RGD-domain of vitronectin through integrins. Osteonectin was then able to generate an intermediate state of adhesion, which fostered a greater cellular response to the chemotactic vitronectin. Although this is speculation based upon an observation and not a tested hypothesis, it is important to consider the indirect effects osteonectin may have on breast cancer cell metastasis to bone.

Despite the lack of migration to purified osteonectin, physiologically, osteonectin is in the presence of many other matrix proteins in the bone microenvironment. The attractive quality of osteonectin may be dependent on its proximity to these other matrix proteins. This concept was supported by our observation that osteonectin increased breast cancer cell migration toward vitronectin. As stated earlier, osteonectin increases cell motility when the cells are bound to a matrix through integrins. The ability of osteonectin to enhance chemoattraction toward other matrix proteins, in addition to vitronectin, may be based on its de-adhesive properties.

Bone extracts from wild-type and osteonectin-null mice were utilized to assess the chemotactic potential of osteonectin in the presence of other matrix proteins. We detected increased migration toward the wild-type and osteonectin-null bone extracts. Furthermore, at equal protein doses, the metastatic breast cancer cells migrated to the wild-type and osteonectin-null bone extracts at the same rate. This experiment provided evidence that metastatic breast cancer cells were attracted to the bone extracts and therefore attracted to unknown factors in the demineralized portion of the bone matrix. However, it also demonstrated that osteonectin was not a relevant contributor to the chemotaxis of breast cancer cells to bone. Other studies have reported osteonectin to be the primary attractive force in bone extracts for prostate cancer cells [De et al., 2003]. We utilized a similar chemoattraction assay as De et al. [2003] but were unable to reproduce their results for breast cancer cells. The inability of osteonectin to increase breast cancer cell migration has been previously reported by Dhanesuan et al. [2002]. In this study, MDA-MB-231 cells were transfected to express increased levels of osteonectin. These cells did not display an increase in cellular migration as a result of increased osteonectin expression [Dhanesuan et al., 2002]. Here, we report that osteonectin by itself was also not chemoattractive for these breast cancer cells.

There are other factors within the mineralized matrix of bone, possibly RGD-containing proteins such as vitronectin, bone sialoprotein and thrombospondin that may be responsible for the attraction of breast cancer cells. Bone sialoprotein has been shown to increase MDA-MB-231 cell migration in an RGD-dependent manner [Sung et al., 1998]. Another study has demonstrated the chemoattraction of MDA-MB-435 cells toward thrombospondin-1 through the $\alpha_v\beta_1$ integrin [Chandrasekaran et al., 1999].

This study presents data that showed osteonectin derived from several sources stimulated breast cancer cell motility. Osteonectin may also enhance the chemoattraction of breast cancer cells toward vitronectin. However, bone-derived osteonectin alone was not chemoattractive for breast cancer cells and most likely does not direct cancer cell migration into the bone microenvironment.

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REFERENCES

Bartsch JE, Staren ED, Appert HE. 2003. Adhesion and migration of extracellular matrix-stimulated breast cancer. J Surg Res 110:287–294.

Bellahcene A, Castronovo V. 1995. Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. Am J Pathol 146:95–100.

Bradshaw AD, Sage EH. 2001. SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. J Clin Invest 107:1049–1054.

Brekken RA, Sage EH. 2000. SPARC, a matricellular protein: At the crossroads of cell-matrix. Matrix Biol 19: 569–580.

Busch E, Hohenester E, Timpl R, Paulsson M, Maurer P. 2000. Calcium affinity, cooperativity, and domain interactions of extracellular EF-hands present in BM-40. J Biol Chem 275:25508–25515.

Byzova TV, Kim W, Midura RJ, Plow EF. 2000. Activation of integrin alpha(V)beta(3) regulates cell adhesion and migration to bone sialoprotein. Exp Cell Res 254:299–308.

Cailleau R, Young R, Olive M, Reeves WJ, Jr. 1974. Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 53:661–674.

Cailleau R, Olive M, Cruciger QV. 1978. Long-term human breast carcinoma cell lines of metastatic origin: Preliminary characterization. In Vitro 14:911–915.

Chandrasekaran S, Guo NH, Rodrigues RG, Kaiser J, Roberts DD. 1999. Pro-adhesive and chemotactic activities of thrombospondin-1 for breast carcinoma cells are mediated by alpha3beta1 integrin and regulated by insulin-like growth factor-1 and CD98. J Biol Chem 274:11408–11416.

Dayhoff MO, National Biomedical Research Foundation. 1979. Atlas of protein sequence and structure. Silver Spring, MD: National Biomedical Research Foundation.

De S, Chen J, Narizhneva NV, Heston W, Brainard J, Sage EH, Byzova TV. 2003. Molecular pathway for cancer metastasis to bone. J Biol Chem 278:39044–39050.

Dhanesuan N, Sharp JA, Blick T, Price JT, Thompson EW. 2002. Doxycycline-inducible expression of SPARC/osteonectin/BM40 in MDA-MB-231 human breast cancer cells results in growth inhibition. Breast Cancer Res Treat 75: 73–85.

Diel IJ. 2001. Prognostic factors for skeletal relapse in breast cancer. Cancer Treat Rev 27:153–157; discussion 159–164.

Fisher LW, Hawkins GR, Tuross N, Termine JD. 1987. Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J Biol Chem 262:9702–9708.

Gilles C, Bassuk JA, Pulyaeva H, Sage EH, Foidart JM, Thompson EW. 1998. SPARC/osteonectin induces matrix metalloproteinase 2 activation in human breast cancer cell lines. Cancer Res 58:5529–5536.

Greenwood JA, Murphy-Ullrich JE. 1998. Signaling of de-adhesion in cellular regulation and motility. Microsc Res Tech 43:420–432.

Harms JE, Welch DR. 2003. MDA-MB-435 human breast carcinoma metastasis to bone. Clin Exp Metastasis 20: 327–334.

Harris SA, Enger RJ, Riggs BL, Spelsberg TC. 1995. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Miner Res 10:178–186.

Jacob K, Webber M, Benayahu D, Kleinman HK. 1999. Osteonectin promotes prostate cancer cell migration and invasion: A possible mechanism for metastasis to bone. Cancer Res 59:4453–4457.

Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. 2005. Cancer statistics. CA Cancer J Clin 55:10–30.

Kato Y, Sakai N, Baba M, Kaneko S, Kondo K, Kubota Y, Yao M, Shuin T, Saito S, Koshika S, Kawase T, Miyagi Y, Aoki I, Nagashima Y. 1998. Stimulation of motility of human renal cell carcinoma by SPARC/osteonectin/BM-40 associated with type IV collagen. Invasion Metastasis 18:105–114.

Kaufmann B, Muller S, Hanisch FG, Hartmann U, Paulsson M, Maurer P, Zaucke F. 2004. Structural variability of BM-40/SPARC/osteonectin glycosylation: Implications for collagen affinity. Glycobiology 14:609–619.

Kelm RJ, Jr., Mann KG. 1991. The collagen binding specificity of bone and platelet osteonectin is related to differences in glycosylation. J Biol Chem 266:9632–9639.

Lane TF, Sage EH. 1994. The biology of SPARC: A protein that modulates cell–matrix interactions. Faseb J 8:163–173.

Lehr JE, Pienta KJ. 1998. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. J Natl Cancer Inst 90:118–123.

Meyer T, Marshall JF, Hart IR. 1998. Expression of alphav integrins and vitronectin receptor identity in breast cancer cells. Br J Cancer 77:530–536.

Motamed K. 1999. SPARC (osteonectin/BM-40). Int J Biochem Cell Biol 31:1363–1366.

Murphy-Ullrich JE. 2001. The de-adhesive activity of matricellular proteins: Is intermediate cell adhesion an adaptive state? J Clin Invest 107:785–790.

Murphy-Ullrich JE, Lane TF, Pallero MA, Sage EH. 1995. SPARC mediates focal adhesion disassembly in endothelial cells through a follistatin-like region and the Ca(2+)-binding EF-hand. J Cell Biochem 57:341–350.

Ninomiya JT, Tracy RP, Calore JD, Gendreau MA, Kelm RJ, Mann KG. 1990. Heterogeneity of human bone. J Bone Miner Res 5:933–938.

Norose K, Clark JI, Syed NA, Basu A, Heber-Katz E, Sage EH, Howe CC. 1998. SPARC deficiency leads to early-onset cataractogenesis. Invest Ophthalmol Vis Sci 39: 2674–2680.

Parker SL, Tong T, Bolden S, Wingo PA. 1997. Cancer statistics. CA Cancer J Clin 47:5–27.

Porter PL, Sage EH, Lane TF, Funk SE, Gown AM. 1995. Distribution of SPARC in normal and neoplastic human tissue. J Histochem Cytochem 43:791–800.

Rosenblatt S, Bassuk JA, Alpers CE, Sage EH, Timpl R, Preissner KT. 1997. Differential modulation of cell adhesion by interaction between adhesive and counter-adhesive proteins: Characterization of the binding of vitronectin to osteonectin (BM40, SPARC). Biochem J 324(Pt 1):311–319.

Sage H, Vernon RB, Funk SE, Everitt EA, Angello J. 1989. SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading in vitro and exhibits

Ca^{2+}-dependent binding to the extracellular matrix. J Cell Biol 109:341–356.

Sung V, Stubbs JT III, Fisher L, Aaron AD, Thompson EW. 1998. Bone sialoprotein supports breast cancer cell adhesion proliferation and migration through differential usage of the alpha(v)beta3 and alpha(v)beta5 integrins. J Cell Physiol 176:482–494.

Termine JD, Belcourt AB, Christner PJ, Conn KM, Nylen MU. 1980. Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel. J Biol Chem 255:9760–9768.

Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML, Martin GR. 1981. Osteonectin, a bone-specific protein-linking mineral to collagen. Cell 26:99–105.

Thomas R, True LD, Bassuk JA, Lange PH, Vessella RL. 2000. Differential expression of osteonectin/SPARC during human prostate cancer progression. Clin Cancer Res 6:1140–1149.

Triffitt JT. 1987. The special proteins of bone tissue. Clin Sci (London) 72:399–408.

Wewer UM, Albrechtsen R, Fisher LW, Young MF, Termine JD. 1988. Osteonectin/SPARC/BM-40 in human decidua and carcinoma, tissues characterized by de novo formation of basement membrane. Am J Pathol 132:345–355.

Woelfle U, Cloos J, Sauter G, Riethdorf L, Janicke F, van Diest P, Brakenhoff R, Pantel K. 2003. Molecular signature associated with bone marrow micrometastasis in human breast cancer. Cancer Res 63:5679–5684.

Yoneda T, Williams PJ, Hiraga T, Niewolna M, Nishimura R. 2001. A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. J Bone Miner Res 16:1486–1495.

Zhang RD, Fidler IJ, Price JE. 1991. Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis. Invasion Metastasis 11:204–215.