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Osteoblast-Conditioned Media Influence the Expression of E-Selectin on Bone-Derived Vascular Endothelial Cells

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Abstract Breast cancer cells frequently metastasize to the ends of long bones, ribs and vertebrae, structures which contain a rich microvasculature that is closely juxtaposed to metabolically active trabecular bone surfaces. This study focuses on the effects of osteoblast secretions on the surface presentation of adhesive proteins on skeletal vascular endothelial cells. Vascular endothelial cells were isolated from trabecular bone regions of the long bones of 7-week-old Swiss Webster mice and also from the central marrow cavity where trabecular bone is absent. Both types of endothelial cells were placed in culture for 7 days, then exposed 24 h to conditioned media from MC3T3-E1 osteoblasts. Conditioned medium (CM) from two different stages of osteoblast development were tested: (1) from immature MC3T3-E1 cells cultured for 5–7 days and (2) from mature MC3T3-E1 cells cultured for 28–30 days. The immature osteoblasts were in a stage of rapid proliferation; the mature osteoblasts formed a matrix that mineralized. Following exposure to the conditioned media, the vascular cells were exposed to anti-P-selectin, anti-E-selectin, anti-ICAM-1, and anti-VCAM-1 to detect the corresponding adhesive proteins on their surfaces. Breast cancer cells are known to bind to these adhesive proteins. Of the four proteins evaluated, E-selectin was consistently found on more cell surfaces (~30%) of bone-derived vascular endothelial cells (BVECs) when exposed to the immature CM whereas vascular endothelial cells from marrow (MVECs) did not show this response to either immature CM or mature CM. These studies suggest that the BVEC blood vessels near immature bone cells express more surface adhesive protein that could enhance entrapment and extravasation of breast cancer cells. Once cancer cells have undergone extravasation into marrow adjacent to bone, they could be readily attracted to nearby bone surfaces. J. Cell. Biochem. 98: 1221–1229, 2006. © 2006 Wiley-Liss, Inc.

Key words: vascular endothelial cells; bone vasculature; surface adhesion molecules; E-selectin

Breast cancer is the second leading cause of cancer deaths among women in the United States. Breast carcinomas that do not spread to other tissues are cured at a rate of 90% [Welch et al., 2000]; however, when metastasis to skeletal sites has occurred, the survival rate decreases dramatically [Kurschat and Guise, 2003]. For breast cancer metastasis, the preferred secondary sites are within the highly vascularized regions of bone such as the vertebrae, ribs, ends of long bones, and skull [Mundy and Guise, 2000]. As a consequence, the cancer cells are in a protected site which makes treatment and/or ablation difficult. An understanding of the biological basis of metastasis of breast cancer cells is an area of critical importance for reducing the morbidity of this disease.

Metastasis occurs when a small subset of cells within a primary tumor acquires the ability to complete all steps in the metastasis cascade: detachment from the primary tumor, migration, intravasation, dissemination throughout the body, extravasation, and proliferation at a secondary site [Welch, 1997; Welch et al., 2000; Gaßmann et al., 2004]. Breast cancer cells arriving in the vasculature of a given tissue encounter a layer of endothelial cells which form the luminal surface of the blood vessel. One role of the vascular endothelium is to modulate the passage of specific types of cells across the endothelial layer, such as leukocytes and cancer

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cells. This selectivity is fostered by a unique array of adhesion molecules present on the endothelial cell surfaces that interact with ligands on the surface of circulating cells.

The transendothelial migration of leukocytes during an inflammatory response and the molecular interactions involved provide a model for the process of breast cancer cell extravasation [Chambers et al., 2000; Gaßmann et al., 2004]. In the case of leukocytes, recruitment to areas of inflammation is largely orchestrated by inflammatory cell cytokines and adhesion molecules (CAMs); the latter are present on both vascular endothelial cells and leukocytes. Soluble inflammatory molecules, such as TNFα and IL-1 activate the endothelial cells which results in the presentation or increased presentation of several surface tethering and adhesion molecules; these include P-selectin, E-selectin, ICAM-1, and VCAM-1 [Ulbrich et al., 2003]. The surface presentation of P- and E-selectin allows activated endothelial cells to transiently tether leukocytes traveling in the vessel lumen. E-selectin interacts with E-selectin ligand-1 (ESL-1) whereas P-selectin binds to P-selectin glycoprotein ligand-1 (PSGL-1) [González-Amaro et al., 1998]. After initial tethering, leukocytes are activated by both intracellular signals induced by the binding of selectins and by chemokines. The activation of leukocytes causes an increased avidity of leukocyte integrins, which are receptors for ICAM-1 and VCAM-1 located on the surface of activated endothelial cells [Feng et al., 1998]. Integrin-mediated adhesion provides an approximately 10-fold stronger binding force than selectin-mediated interactions [Gaßmann et al., 2004]. Once leukocytes have firmly adhered to the endothelial layer, they are in a position to migrate between the endothelial cells in response to the chemotactic gradient generated by cells at the site of inflammation [Muller, 2001].

A similar process may occur for breast cancer cells. Breast cancer cells have been shown to express the PSLG-1 and ESL-1 as well as a variety of integrins [Price et al., 1995; Renkonen et al., 1997; Lundström et al., 1998]; hence, it is possible that extravasation of breast cancer cells may occur in the same fashion as described for leukocytes as suggested by Chambers et al. [2000] and Gaßmann et al. [2004]. Upon lodging in the secondary site, metastatic cells are then able to proliferate and form a new carcinoma.

Osseous metastasis is most often found in areas rich in trabecular (spongy) bone, as opposed to cortical bone [Chambers et al., 2000; Welch et al., 2000; Gaßmann et al., 2004]. Trabecular bone, a loosely organized meshwork of calcified extracellular matrix, is present in ribs, vertebrae, and the metaphyses of long bones. In adults, the metaphyses contain a rich blood supply, metabolically active trabecular bone, and hematopoietic tissue [Aubin and Triffitt, 2002]. The trabecular bone meshwork is covered mainly by bone lining cells and quiescent osteoblasts.

One route of entry of breast cancer cells into the bone tissue space is through the vascular endothelium; hence, the specialized features of the vascular network in trabecular bone are of particular interest. Blood vessels that course through the trabecular bone meshwork have two characteristic features which make them well-suited as extravasation sites. Instead of having a network of capillaries, the vasculature in trabecular bone regions are comprised of sinusoids which have exceptionally large lumens. As a consequence, there is a reduction in the blood flow rate of $\sim$30-fold as compared to that found in capillaries [Mazo et al., 1998; Mazo and von Andrian, 1999]. Secondly, the endothelial cells forming the sinusoids constitutively and simultaneously express an array of tethering and adhesive proteins, including P-selectin, E-selectin, ICAM-1, and VCAM-1 [Frenette et al., 1998; Mazo et al., 1998; Mazo and von Andrian, 1999]. This is in contrast to the microvasculature in other tissues, where adhesion proteins are intermittently expressed when stimulated by inflammatory cytokines [Frenette et al., 1998]. The corresponding ligands and receptors for the four adhesion molecules of interest have been shown to be present on the surface of breast cancer cells [Price et al., 1995; Renkonen et al., 1997; Lundström et al., 1998].

Because of the close proximity of the vasculature to osteoblasts and bone lining cells along the trabecular surfaces, we hypothesized that, as a response to bone cell secretions, a unique combination of surface proteins are presented on the vascular endothelial cells which would then serve to transiently tether slowly moving metastatic breast cancer cells. This would allow time for the breast cancer cells to migrate toward chemotactic factors emanating from osteoblasts [Campo McKnight et al., 2005]. In

order to investigate this hypothesis, we isolated two types of vascular endothelial cells from 7- to 9-week-old mice, that is, trabecular bone-derived vascular endothelial cells (BVECs) and marrow-derived vascular endothelial cells (MVECs). The two types of endothelial cells were exposed to three treatments: immature osteoblast-conditioned media, mature osteoblast-conditioned media, or unaltered growth media. Immature and mature osteoblast conditioned media were obtained from cultures of the murine osteoblast cell line, MC3T3-E1.

The purpose of this study was first to determine if expression of tethering and surface adhesion molecules, namely, P-selectin, E-selectin, ICAM-1, and VCAM-1, known to bind breast cancer cells, would be greater on the BVECs than on MVECs. The second aim of this study was to investigate the effects of osteoblast secretions on the expression of surface adhesion molecules of both types of vascular endothelial cells isolated from bone. The information obtained could partially explain the high frequency of breast cancer metastasis into regions of highly vascularized bone.

MATERIALS AND METHODS

Isolation of Primary Mouse Vascular Endothelial Cells

All animals were handled in accordance with an approved protocol (IACUC #18823). For each experiment, six 7- to 9-week-old mice were euthanized by CO_2 inhalation. Femurs and tibias were removed and cleaned of muscle and connective tissue and split longitudinally.

Marrow vascular endothelial cells were isolated by from the central third of the bone shafts. BVECs were isolated from trabecular bone fragments obtained by scraping the metaphyses with a No. 1 curette. Marrow from the bone shafts and metaphyseal scrapings were treated with 0.1% collagenase (Sigma, St. Louis, MO) at 37°C for 30 min to release a crude cell isolate that included endothelial cells as well as other types of cells. Following the incubation with collagenase, MVECs and BVECs were cultured on gelatin-coated chamber slides in Medium 199 (Mediatech, Herndon, VA) supplemented with 20% fetal bovine serum (Sigma), 1% penicillin/streptomycin (Mediatech), and 1% endothelial cell growth factor (Sigma) for 7 days at 37°C in a 5% CO_2 humidified incubator. The medium was changed every other day.

Acetylated-Low Density Lipoprotein (LDL) Uptake Assay

Since endothelial cells endocytose and metabolize acetylated-LDL at a higher rate than other cell types [Voyta et al., 1984], the acetylated-LDL uptake assay provided a means of distinguishing the endothelial cells from other cell types present in the culture. After 7 days in culture, the media was removed from both types of endothelial cells. Medium 199 supplemented with 20% fetal bovine serum and 5 μg/ml DiI acetylated-LDL [excitation 549 nm/emission 565 nm] (Biomedical Technologies, Stoughton, MA) was placed on the cultured cells. Cultures were covered with aluminum foil and incubated for 4 h at 37°C in a 5% CO_2 humidified incubator to allow endothelial cells to take up the acetylated-LDL. After incubation, the media containing DiI acetylated-LDL was removed. Cells were rinsed with phosphate buffered saline (PBS) and treated with either osteoblast-conditioned media or with non-conditioned control media.

Identification of Vascular Endothelial Cells

In addition to acetylated-LDL uptake, the vascular endothelial cells were stained for platelet endothelial cell adhesion molecule (PECAM) and von Willebrand factor, two additional vascular endothelial cell markers. For PECAM, cells were grown on gelatin-coated chamber slides for 1 week. Slides were rinsed in PBS, fixed with cold methanol and blocked with 10% normal donkey serum (Sigma) for 1 h at room temperature. Primary antibody to murine PECAM (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:10 in 1.5% normal donkey serum and applied to the slides for 1 h at room temperature. Slides were rinsed (3×10 min) in PBS and exposed to donkey anti-goat IgG conjugated with the fluorophore Alexa 488 [excitation 488 nm/emission 519 nm] (Molecular Probes, Eugene, OR) for 1 h at room temperature. All antibodies were diluted in PBS. Slides were washed in PBS (3×10 min) then mounted with Fluoromount-G (Southern Biotech, Birmingham, AL). For von Willebrand factor detection, cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After rinsing in PBS, cells were permeabilized with 0.5% Triton X-100 in PBS (5 min), then blocked for 30 min in PBS with 10% normal rabbit serum. The primary antibody, sheep

anti-rat von Willebrand Factor (Cedarlane Laboratories, Hornby, Ontario, Canada) was diluted 1:1,250 in PBS 1% normal rabbit serum and applied to the cells for 1 h. Cells were rinsed in PBS plus 1% normal rabbit serum (3 × 20 min), then incubated for 1 h in a 1:200 dilution of rabbit anti-sheep IgG conjugated to a Cy3 fluorophore [excitation 550 nm/emission 570 nm] (Chemicon, Temecula, CA) in PBS (3 × 20 min) and mounted with Fluoromount-G.

Collection of Osteoblast-Conditioned Media

The mouse osteoblastic cell line MC3T3-E1 (a gift from Dr. Norman Karin, University of Delaware), was cultured in Alpha Modified Eagle's Medium (αMEM, Mediatech) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 50 μg/ml ascorbic acid (Aldrich, Milwaukee, WI), and 10 mM β-glycerophosphate (Sigma). The cells were grown at 37°C in a 5% CO_2 humidified incubator. Culture medium was replaced every 3 days. MC3T3-E1 cells cultured for 5–7 days were considered to be immature as they had not yet begun to express alkaline phosphatase by histochemical staining. MC3T3-E1 cells cultured for 29–31 days were shown to be making a mineralized matrix and therefore considered to be mature osteoblasts. Following the designated culture period, the cells were rinsed twice in PBS and the medium was replaced with αMEM supplemented with serum replacement 3 (SR3, Sigma) which contains albumin, transferin, and insulin. After 24 h, media from the cultures were then collected and replaced with fresh αMEM and SR3 (αMEM/SR3) for another 24 h. This procedure was repeated once more. All conditioned media were filtered and stored at −20°C.

Treatment of Endothelial Cells

Three separate isolations of both MVECs and BVECs were each treated with three types of media: immature osteoblast-conditioned media, mature osteoblast-conditioned media, and αMEM/SR3 as the control media. The endothelial cells were exposed to the three types of media for 24 h at 37°C in a 5% CO_2 humidified incubator.

Immunocytochemical Staining

Following treatment with immature osteoblast-conditioned media, mature osteoblast-conditioned media, or control media, the endothelial cell cultures were rinsed two times with PBS. Live cells were blocked with 3% bovine serum albumin (BSA, Sigma) in PBS (30 min, room temperature). Goat anti-mouse P-selectin, E-selectin, ICAM-1, or VCAM-1 (Santa Cruz Biotechnology) primary antibodies were applied at a 1:10 dilution in 0.01M PBS with 3% BSA. The cells were incubated for 1 h at 4°C to prevent endocytosis of the antibodies. Following the incubation, the cells were rinsed (3 × 5 min) in blocking solution. The secondary antibody, donkey anti-goat IgG conjugated to the fluorophore Alexa 488, was applied at a 1:200 dilution in PBS plus 3% BSA. Cells were incubated for 1 h at 4°C. Any unbound secondary antibody was removed with three 5-min rinses in blocking solution. The cells were then fixed in 4% paraformaldehyde (10 min, room temperature) and subsequently rinsed two times in PBS. Cover slips were mounted onto chamber slides using Fluoromount-G for viewing by confocal microscopy.

Data Collection and Analysis

Three identical experiments were performed. For each experiment, the two types of endothelial cells (BVECs and MVECs) were treated with three types of media. The acetylated-LDL uptake assay and immunocytochemical staining for four cellular adhesion molecules was performed for each cell type and treatment. For each cell type, treatment, and adhesion molecule, five images were captured on a Bio-Rad MRC 1024 confocal laser microscope (Bio-Rad, Hercules, CA) using a 40× objective lens. Between 15 and 25 optical sections, each having a thickness of 1 μm, were captured for each field of view. The images were collected sequentially for the 488 nm and 568 nm excitation wavelengths. A phase contrast image was also collected.

The number of cells that displayed acetylated-LDL uptake and surface staining for each adhesion molecule was determined for each experimental parameter. The percentage of endothelial cells exhibiting surface staining for each adhesion molecule was subsequently calculated. A total of 5,663 BVECs and 5,551 MVECs were counted.

Statistical comparison of adhesion molecule expression in untreated BVECs and MVECs was analyzed using a one-way student *t*-test. Statistical analysis of the data from treated cell cultures was performed using a three-way factorial analysis (ANOVA). Each data set

was evaluated and appropriately adjusted for normal distribution of means and residuals. The Tukey method was used for pairwise comparisons of the data to determine significant differences between means. The discriminator for significance was set at a threshold *P*-value of 0.05.

RESULTS

Figure 1 shows typical confocal and phase contrast images of the same field of cells. Virtually all of the cells had taken up DiI acetylated-LDL (panel A), a marker for endothelial cells, indicating that the cell preparation was mainly comprised of vascular endothelial cells.

The initial comparison of surface adhesion molecule expression between untreated bone-derived endothelial cells and marrow-derived endothelial cells yielded no significant differences between the two cells types (Fig. 2). This result was contrary to our hypothesis that BVECs and MVECs differentially express cell surface adhesion molecules. However, these cells were cultured for 1 week and thus were removed from the influences of bone cell secretions for that period of time.

In our next series of experiments, the endothelial cells were treated with conditioned media from immature and mature cultured murine osteoblasts. Of the four surface proteins examined, only the surface presentation of E-selectin was found to vary with treatment. Figure 3 is a representative image of the surface expression of E-selectin by BVECs when treated with either immature or mature osteoblast-conditioned media as compared to control media. Images similar to those shown in Figure 3 were obtained for surface expression of the other three surface proteins, P-selectin, ICAM-1, and VCAM-1, on BVECs as well as for all four surface proteins on MVECs (images not shown). Numbers of cells that had taken up

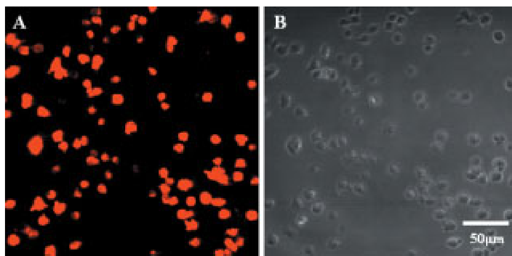

Fig. 1. **A**: A view of a typical culture of BVECs which have taken up DiI actylated-LDL, a marker of vascular endothelial cells. **B**: Phase contrast image of the cells shown in panel A.

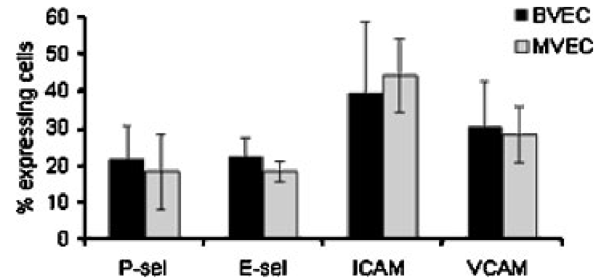

Fig. 2. Comparison of expression levels of surface proteins in 7-day cultures of BVECs and MVECs. Percentage of cells expressing P-selectin, E-selectin, ICAM-1, and VCAM-1 is shown. No difference between cell types was found.

acetylated-LDL and stained for adhesion molecules were counted from images such as these.

The means of the numbers of BVECs expressing each surface protein in response to treatment compared to control are shown in Figure 4. BVECs treated with immature osteoblast-conditioned media consistently exhibit an ~30% increase in surface presentation of E-selectin ($P < 0.05$). BVECs treated with mature osteoblast-conditioned media show a 53% reduction in surface presentation of E-selectin ($P < 0.001$). MVECs treated with immature or mature osteoblast-conditioned media did not exhibit enhanced surface presentation of E-selectin that was significantly different from that of MVECs treated with control media (Fig. 5). Treatment of either type of endothelial cell with immature osteoblast- or mature osteoblast-conditioned media did not result in significantly different surface presentation of P-selectin, ICAM-1, or VCAM-1 compared to the control media treatment (Figs. 4 and 5).

DISCUSSION

At autopsy, 75–80% of patients with metastatic breast cancer are found to have metastases in bone tissue [Diels, 2001]. The tumor cells that migrate to bone most often metastasize to regions of trabecular bone, which are highly vascularized. In previous studies, a marked increase has been observed in adhesion of several prostate cancer cell lines and one breast cancer cell line, MCF-7, to a vascular endothelial cell line (HBME-1) derived from the trabecular bone when compared to human umbilical vein endothelial cells [Lehr and Pienta, 1998]. The HBME-1 endothelial cells are immortalized cells that are similar to the BVECs used in the experiments described in the

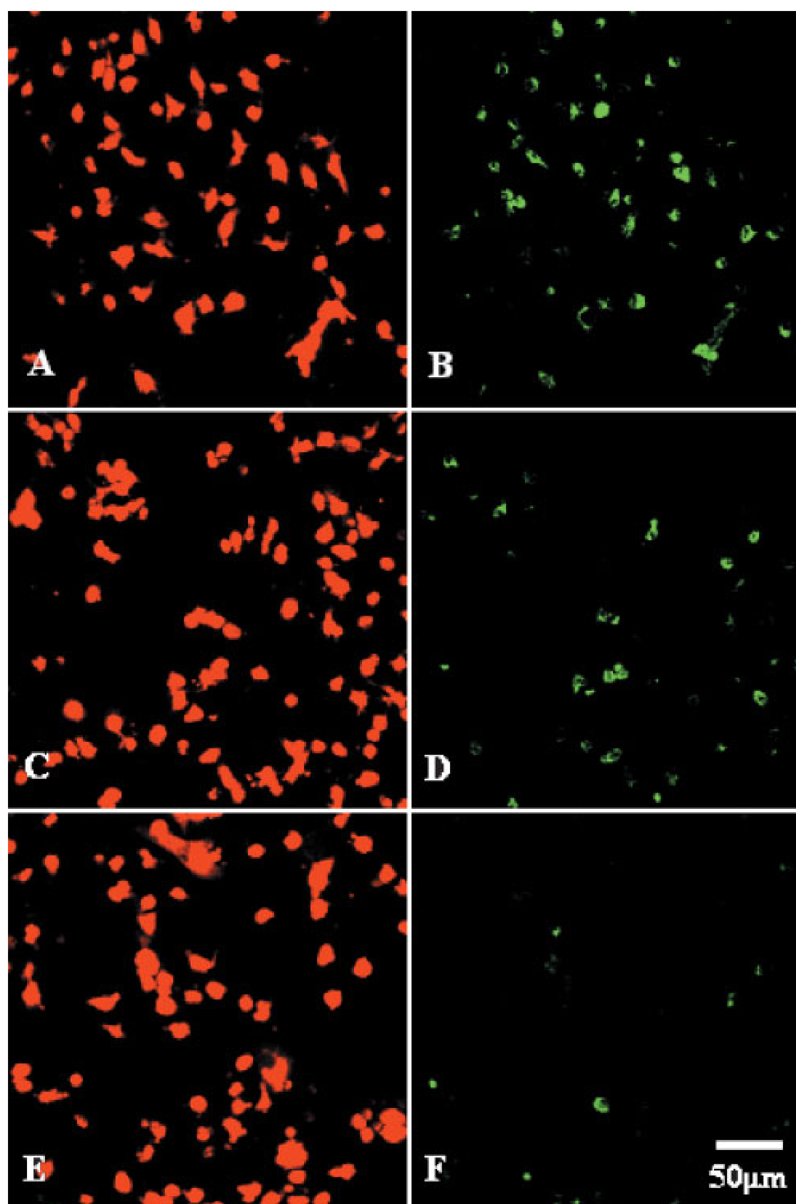

Fig. 3. Typical images of BVECs treated with (**A** and **B**) immature osteoblast conditioned medium, (**C** and **D**) control medium, or (**E** and **F**) mature osteoblast conditioned medium. DiI acetylated-LDL cells are shown on the left and surface expression of E-selectin are on the right.

present study in that they were derived from an aspirate of iliac crest, a region that is rich in trabecular bone. Additionally, the HBME-1 cells were found to present P-selectin, E-selectin, ICAM-1, and VCAM-1 on their surfaces. The specific receptor/ligand interactions between tumor cells and vascular endothelial cells have also been demonstrated in other studies. Blocking antibodies to E-selectin and VCAM-1 have been shown to reduce the adhesion of metastatic breast cancer cells to vascular endothelial cells [Moss et al., 2000]. The addition of peptides containing arginine-glycine-aspartic acid (RGD) sequences decreases the adhesion of prostate cancer cells to human bone marrow endothelial cells by 70%, implicating a major role for integrin interactions between tumor and endothelial cells [Lehr and Pienta, 1998]. We have postulated that, as a response to secretions of osteoblasts, a unique combination of the surface proteins develops on bone vascular endothelium which can tether or bind

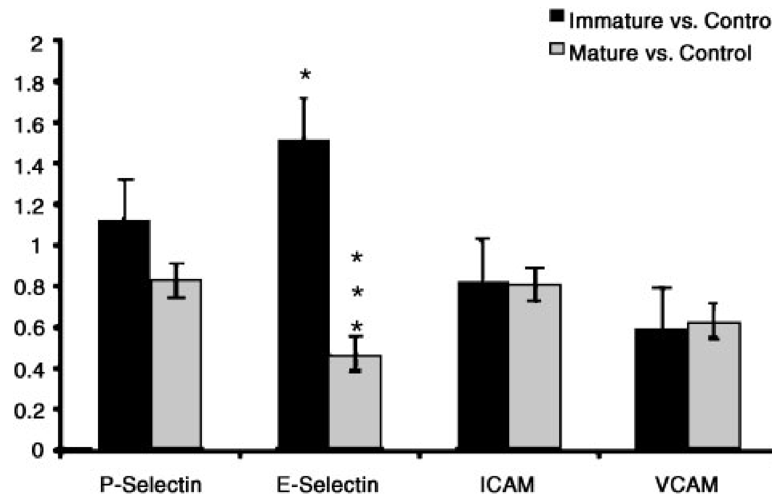

Fig. 4. BVEC expression of surface proteins when treated with either immature or mature osteoblast conditioned medium as compared with control values. E-selectin was found to vary; the other three surface proteins were unaffected.

metastatic breast cancer cells. The occurrence of this phenomenon in a region of sluggish blood flow [Mazo et al., 1998; Mazo and von Andrian, 1999] creates a situation in which tethered cancer cells can more easily respond to a gradient of bone-derived chemotactic factors and eventually extravasate into the surrounding bone tissue.

In our study, two types of vascular endothelial cells were isolated from the tibias and femurs of 7- to 9-week-old mice. BVECs were isolated from the blood sinusoids that reside within the meshwork of trabecular bone in the metaphyses of long bones. MVECs were isolated from the blood vasculature in the central marrow cavity of long bones, which is devoid of trabecular bone.

The results of this study show that BVECs treated with immature osteoblast-conditioned media exhibit a significant increase in surface presentation of E-selectin. Most bone surfaces are believed to be covered with immature osteoblasts and/or bone lining cells. Conversely, when treated with mature osteoblast-conditioned media, BVECs show a significant reduction in surface presentation of E-selectin. Treatment of MVECs with either type of media did not result in significantly different presentation of any of the four CAMs examined. These data suggest that vascular endothelial cells which are in close proximity to numerous immature osteoblasts on trabecular bone surfaces may be upregulated for E-selectin surface expression. This increases the likelihood that

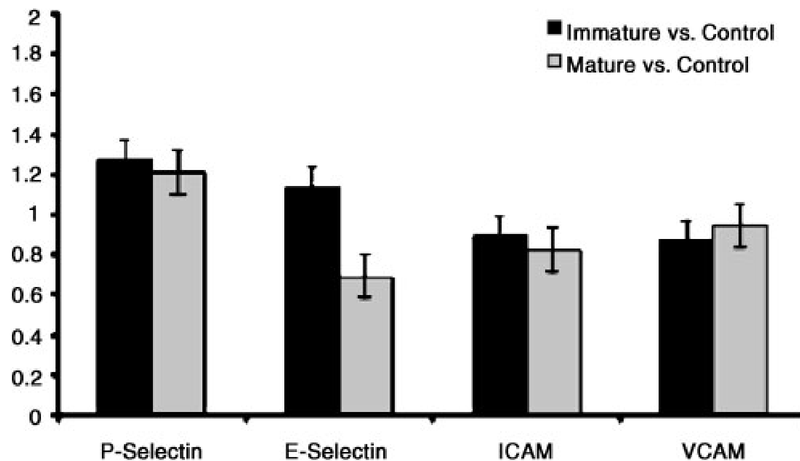

Fig. 5. MVEC expression of surface proteins when treated as described in Fig. 4. No variations were found.

breast cancer cells, which express a ligand for E-selectin [Price et al., 1995; Renkonen et al., 1997], will become attached to the endothelium at these sites.

Metastatic breast cancer cells must undergo extravasation in order to complete the metastasis cascade. Our data indicate that the secretions of immature osteoblasts have a stimulatory effect on BVECs, causing them to present more E-selectin than normal. Hence, components of the immature osteoblast-conditioned media that could be responsible for eliciting the stimulatory response are of interest.

During development, osteoblasts secrete several cytokines and growth factors which could be responsible for the upregulation of E-selectin. Primary osteoblasts of both human and murine origin have been shown to produce IL-1, IL-6, and VEGF [Taichman and Emerson, 1998]. All three of these proteins have demonstrated the ability to upregulate E-selectin in vascular endothelial cells as well as to increase adhesion of leukocytes to an endothelial cell layer [Barkalow et al., 1996; Watson et al., 1996; Kim et al., 2001]. Our results have revealed that bone-derived endothelial cells react differentially to the secretions of osteoblasts, suggesting that there are intrinsic differences between the endothelial cells of the bone metaphysis and those of the central marrow cavity.

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