J Cancer Res Clin Oncol (2000) 126:631–640

ORIGINAL PAPER

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Human breast cancer cells induce angiogenesis, recruitment, and activation of osteoclasts in osteolytic metastasis

Received: 25 January 2000 / Accepted: 11 April 2000

Abstract ***Purpose:*** The purpose of this study was to elucidate the potential of human breast cancer cells (BCC) to induce matrix degradation and neo-vascularization, essential for continued tumor growth, in osteolytic lesions. ***Methods:*** BCC were inoculated into the left cardiac ventricle of female athymic mice and osteolytic lesions were radiologically visualized within 4 weeks from inoculation. ***Results:*** Histomorphometric analysis of bone sections revealed a significant increase in the number and maturity of osteoclasts (OCl) lining the bone surfaces next to tumor tissue when compared to corresponding bone surfaces in healthy mice. In addition, a large number of newly formed blood vessels could be visualized by immunohistochemistry at the periphery of and within tumor tissue. When bone marrow (BM) cells were cultured in the presence of BCC the OCl formation was increased threefold. These OCl were also found to be more mature and to have greater resorptive activity. Moreover, BCC were found to stimulate proliferation, migration, and differentiation of BM-derived endothelial cells. ***Conclusions:*** Matrix destruction and neo-vascularization are accomplished by BCC arrested in the BM cavity by increasing recruitment and activity of OCl and by induction of angiogenesis within or in proximity to the tumor tissue.

Key words Bone metastasis · Neo-vascularization · Osteoclast recruitment · Osteolysis · MDA-231 cells

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Introduction

Bone is one of the three most favored sites of solid tumor metastasis. In fact, 90% of the patients who die of breast carcinoma have bone metastases (Woodhouse et al. 1997). Breast cancer metastasis to bone often presents with severe symptoms due to osteolytic lesions and may cause multiple complications for the patient. These include pain, impaired mobility, hypercalcemia, pathologic fracture, spinal cord or nerve root compression, and bone marrow infiltration (Coleman 1997; Sasaki et al. 1995). Most importantly, once cancer cells become housed in the skeleton, the cancer is essentially noncurable with current treatments. Agents such as the bisphosphonates, which inhibit osteoclastic bone resorption, have proven to decrease the complications associated with bone metastases (Kanis et al. 1996; Diel et al. 1998).

Bone has been described as a storehouse for a variety of cytokines and growth factors, which provides an extremely fertile environment for breast cancer cells (Yoneda et al. 1994). However, because bone is mainly comprised of a hard, mineralized tissue, it is more resistant to destruction than other tissues. Thus, for cancer cells to grow in bone, they must be capable of causing bone destruction (Guise 1997). This is believed to be accomplished by the potential of breast cancer cells to recruit and activate osteoclasts (OCls) (Yoneda et al. 1994; Clohisy et al. 1996; Pederson et al. 1999).

So far, only a very few breast cancer cell-derived factors, which may be involved in cancer-induced osteolysis in vivo, have been identified. Yoneda and colleagues have shown that parathyroid hormone-related protein (PTHrP) might play an important role for cancer-induced osteolysis in vivo. They have found that mice inoculated with human breast cancer cells (MDA-231) receiving neutralizing anti-human PTHrP antibodies developed fewer and smaller osteolytic metastases than mice receiving nonspecific antibodies or vehicle as control (Guise et al. 1996). The presence of PTHrP at

the mRNA and protein levels in bone metastasis still remains to be demonstrated.

The generation of blood vessels (angiogenesis) is an essential step for the primary tumor and metastasis to grow (Woodhouse et al. 1997; Hanahan and Folkman 1996). Smaller tumors may receive all nutrients by diffusion, but further growth depends on an adequate blood supply, accomplished through cancer-induced angiogenesis (Fidler and Ellis 1994). Therefore, after the initial arrest and growth in the bone environment, the induction of angiogenesis becomes crucial for the continued secondary tumor growth.

The purpose of the present study was to investigate the influence of human breast cancer cells on OCl recruitment, maturation, and resorptive activity, and on endothelial cell proliferation, migration, and differentiation. We used bone sections from athymic mice with or without bone metastasis for immunochemical quantification of possible changes in OCl numbers and newly formed vessels within or in proximity to the tumor tissue as compared to normal bone marrow. Moreover, several bone marrow/breast cancer cell co-culture assays were used to highlight the relative importance of cancer-derived factors on each single step in OCl and endothelial cell activity.

Materials and methods

Animals and drugs

Four-week-old female Balb/C athymic (nude) mice were obtained from M&B, Denmark. Culture media, penicillin, and streptomycin were purchased from Life Technologies, Denmark. Rabbit anti-human pan-cytokeratin polyclonal antibodies (Code No. A-0575), control antibodies (Code No. X-0903), and EnVision+ were purchased from DAKO, Glostrup, Denmark. The rat anti-mouse CD31 antibody (MEC 13.3) and rat immunoglobulin isotype controls were purchased from PharMingen (San Diego, Calif., USA). Rat anti-mouse CD34 antibody (MEC 14.7) was a kind gift from Dr. Cecilia Garlanda (Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy). Biotinylated rabbit anti-rat antibodies, DAB, ExtrAvidin Peroxidase Staining kit, and Mitomycin C were obtained from Sigma Chemical, St. Louis, Mo., USA.

Cell lines

The parental human estrogen receptor-negative breast cancer cell line MDA-231/P was kindly supplied by Professor Nils Brünner, Finsen Laboratory, Denmark. An in vivo selected bone-seeking subclone, called MDA-231/B, of the MDA-231 cell line was generated by us and maintained as the parental cell line for six passages. The murine bone marrow-derived stromal cell line MBA-2.1 (endothelial-like cells) (Zipori et al. 1987) was kindly supplied by Professor Dov Zipori, Weizman Institute of Science, Israel.

In vivo studies

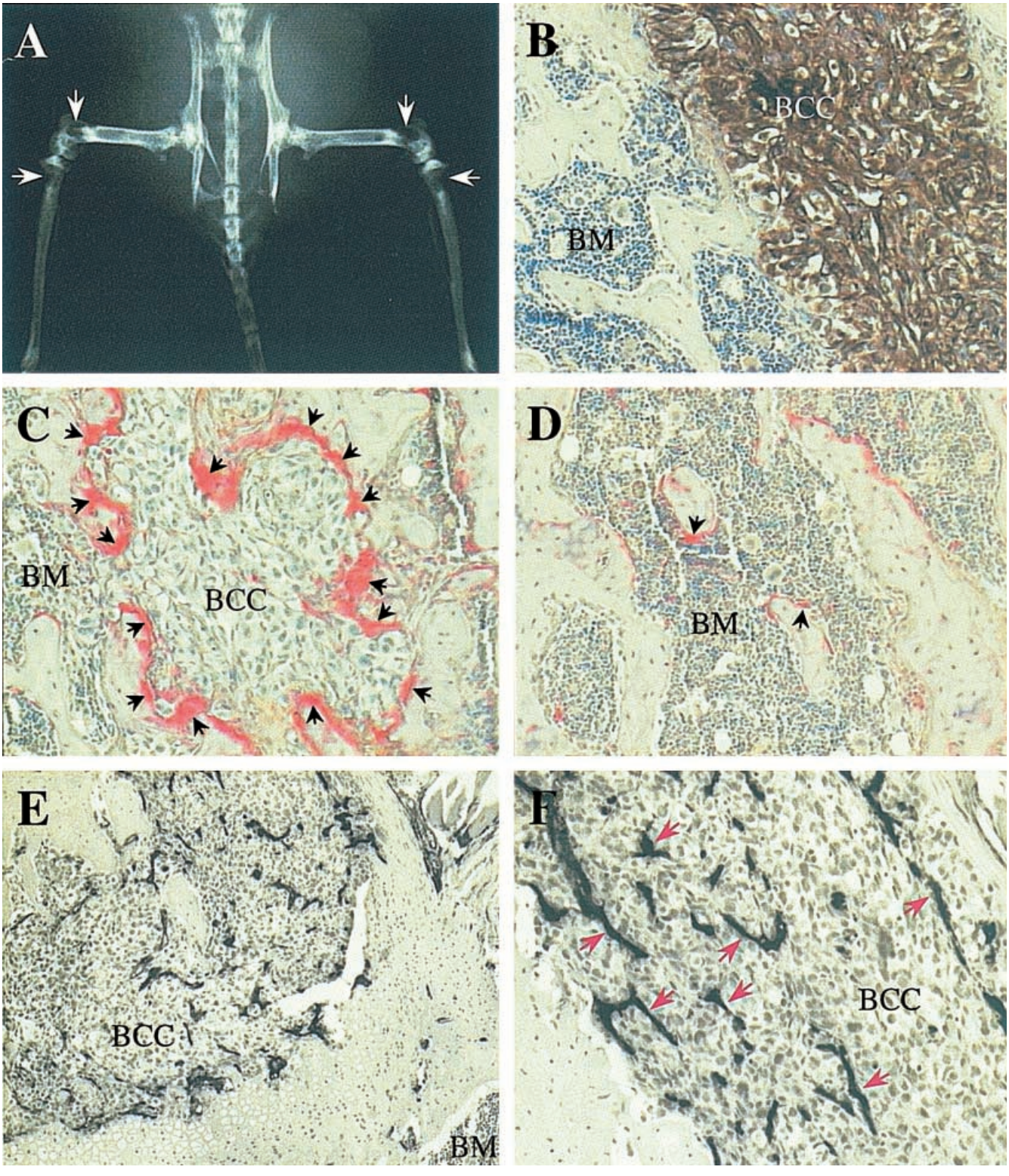
MDA-231/B cells were cultured in 175 cm^2 flasks (NUNC, Denmark) to confluence. The cells were released by trypsin/EDTA and after repeated washing in phosphate-buffered saline (PBS) resuspended in E-MEM without additives at a cell density of 10^6 cells/ml. The cancer cell suspension (0.1 ml) was slowly inoculated into the left cardiac ventricle of anesthetized 4-week-old mice. Less than 10% of animals died from the inoculation procedure. Four weeks after cancer cell inoculations animals were anesthetized and placed in prone position against a single coated HCM film (Imation Medical Imaging, Denmark), and exposed to X-rays at 30 kVp, 5 mA, for 18 s using a Biotron radiographic inspection unit (Renberg and Jensen, Denmark). Films were developed with the use of a 3 M Model XP-2000 (Imation Medical Imaging, Denmark). Osteolytic metastases (Fig. 1A) were detected in approximately 90% of the cancer-inoculated animals.

Fig. 1A–F Radiography and histology of osteolytic bone metastasis. **A** A representative radiography of a mouse with osteolytic lesions in the hind limbs (*arrows*). Human breast cancer cells (BCC) in single cell suspension had been inoculated into the left cardiac ventricle of 4-week-old female Balb/C athymic mice 4 weeks prior to the X-ray. **B** Using a rabbit polyclonal antibody raised against the human epithelial cell marker, cytokeratin, a strong and specific immunoreaction to human BCC was observed, without non-specific staining of bones or normal bone marrow (BM). **C** When a corresponding bone section was stained for tartrate-resistant acid phosphatases (TRAP; red color reaction), a rim of large TRAP+ cells (*arrows*) was found covering the bone surfaces next to the tumor tissue. **D** In contrast, at bone surfaces distant from tumor tissue, only a low number of small TRAP+ cells (*arrows*) was identified. **E** The MEC14.7 antibody, raised against mouse CD34, formed immunoreactions with endothelial cells lining smaller blood vessels near the cartilage growth plate in the BM and strong immunoreaction with small blood vessels and capillaries at the periphery and within tumor tissue (BCC). **F** At higher magnification the vessel-like structures (*red arrows*) of immunoreactive cells can clearly be seen. Replacing MEC14.7 antibodies with an isotypic control antibody as primary antibody confirmed the specificity of the immunoreactions, as no signal was observed in bone sections from healthy or metastatic mice (data not shown)

Histology

Bones were isolated from animals immediately after being killed and immersed in 4% formaldehyde and kept at 4 °C for 48 h. The bones were decalcified in 15% EDTA-solution (Tritriplex III, Merck), pH 7.4, for 3 weeks at 4 °C. The decalcification buffer was changed twice a week. Bones were subsequently dehydrated in increasing concentrations of ethanol before embedding in paraffin. Five-micrometer sections of tibias or femurs placed on Superfrost PLUS slides were air-dried overnight at 37 °C. Slides were deparaffinized in two changes of toluene and rehydrated in decreasing concentrations of ethanol for the tartrate-resistant acid phosphatases (TRAP)-staining procedure or dehydrated in two changes of 99% ethanol before immunohistochemistry (IHC). Following dehydration, endogenous peroxidase activity in tissue sections for IHC was blocked with 0.45% hydrogen peroxide in 99% ethanol.

For the demonstration of tumor cells in bone sections we used rabbit anti-human pancytokeratin antibodies (A 0575, DAKO, Denmark). Rabbit non-specific polyclonal antibodies were used in the same dilution as the primary antibody in adjacent sections to verify the specificity of the observed signals. Sections were washed in TBS and rinsed in running tap water before slides were treated with 0.05% protease (P 5147, Sigma) for 5 min at 37 °C to reveal antigenic sites. To reduce background staining, slides were incubated in a TBS blocking solution containing 0.5% (w/v) casein (C5890, Sigma) for 20 min. The blocking solution was used as diluent for primary/control antibodies, secondary antibody, and as washing solutions in between. Sections were incubated in primary or control antibodies (1:500) for 2 h at room temperature, washed, and subsequently incubated with Envision+, peroxidase, anti-rabbit assay (DAKO, Denmark) for 30 min at room temperature. Immunoreactive sites were visualized by submerging slides in DAB^+ (0.05% DAB (D 5637, Sigma) in TRIS/Imidazole buffer pH 7.6, to which 0.01% hydrogen peroxide had been added (just before



use) for 4 min. Sections were counterstained with Mayers hematoxylin, rinsed in running tap water, and finally slides were coverslipped with DPX Mountant (Fluka, Neu-Ulm, Switzerland). We did not observe any non-specific background staining on slides treated with control antibodies.

For the immunohistochemical demonstration of endothelium of blood vessels and of newly formed capillaries we used the two rat monoclonal antibodies MEC 13.3 and MEC 14.7 raised against mouse CD31 (PECAM-1) and mouse CD34, respectively. The appropriate rat immunoglobulin isotype control was used in the same dilution as primary antibodies in adjacent sections to verify the specificity of the observed signals. Sections were washed in TBS and rinsed in running tap water before slides were treated with 0.1% trypsin for 20 min at 37 °C to reveal antigenic sites. To reduce background staining, slides were incubated in a TBS blocking solution containing 0.5% (w/v) casein for 20 min. The blocking

solution was used as diluent for primary/control antibodies, secondary antibody, and ExtrAvidin, and as washing solutions in between. Sections were incubated in primary or control antibodies (1:100) for 2 h at room temperature, washed, and subsequently incubated with the secondary antibody (biotinylated rabbit anti-rat immunoglobulin, Sigma) for 30 min at room temperature. Subsequently, slides were incubated twice with ExtrAvidin for 30 min. Immunoreactive sites were visualized by submerging slides in DAB^+ for 4 min. Sections were counterstained with Mayers hematoxylin and rinsed in running tap water, and finally the slides were coverslipped with Aquamount (BDH Laboratories Supplies, UK). We did not observe any non-specific background staining on slides treated with control antibodies.

Bone histomorphometry

Serial sections (5 μm in thickness) of tibias of three mice with radiographically verified osteolytic lesions and four healthy control mice were cut longitudinally and examined using a semi-automatic system (Image-Pro Plus, Media Cybernetics, Silver Spring, Md., USA). The demonstration of TRAP-positive cells (=OCls) in bone sections was performed as previously described (Scheven et al. 1986). Histomorphometric analyses were performed 0.5–1.0 mm to the growth plate cartilage in three non-consecutive sections from each tibia at a magnification of 200. The parameters for osteoclast number and maturity included: number of osteoclasts per millimeter of bone surface (N.Oc/mm BS); osteoclast surface per bone surface (Oc.S/BS, %); osteoclast surface per osteoclast (Oc.S/Oc, μm); and number of nuclei per osteoclasts (N.Nu/Oc).

In vitro studies

Osteoclast assays

The OCl formation assay was performed as previously described (Takahashi et al. 1988). Briefly, primary bone marrow cells were harvested from tibias and femurs isolated from 5-week-old mice. The collected bone marrow cells were seeded on devitalized bovine cortical bone slices (28 mm^2) placed in 96-well plates or seeded in culture-treated 24-well plates (Falcon, Becton Dickinson) at cell densities of 5×10^5 and 1.5×10^6 cells/well, respectively. The cells were cultured in α-MEM containing 5% heat-inactivated fetal calf serum (HI-FCS) for the indicated number of days (normally 8 days) with conditioned medium (CM) being collected and replaced with fresh culture medium every second day. At the end of the culture period adherent cells were washed twice in PBS (pH 7.4), fixed in 5% glutaraldehyde–formaldehyde and stained for detection of TRAP+ cells according to the manufacturer's instructions (Sigma Chemical). The OCl escavation pits formed on the bovine cortical bone slices were stained and the area measured as previously described (Foged et al. 1996; Winding et al. 1997). The CM was analyzed for TRAP activity and CrossLaps (i.e., a carboxy-terminal telopeptide proteolytic degradation product of the α_1 chains of type I collagen) as previously described (Foged et al. 1996; Pederson et al. 1997). To study the influence of breast cancer cells on the number, maturity, activity, and survival of OCls formed in this assay, cancer cells at increasing seeding densities of proliferating (10^2–10^4 cells/well) or non-proliferating (pre-treated with 40 μg/ml Mitomycin C for 45 min) (10^3–10^5 cells/well) cells were added to the bone marrow cultures with direct cell–cell contact or separated by a membrane filter insert (pore size 0.45 μm, Falcon).

Endothelial cell assays

The influence of cancer cell-derived factors on migration, proliferation, and differentiation of bone marrow-derived endothelial cells were studied in co-cultures. For the proliferation and migration studies breast cancer cells and endothelial cells were physically separated by a membrane insert, which allowed the exchange of soluble factors but prevented direct cell–cell contact. Cancer cells were plated onto 12-well plates with increasing cell density. Cell cultures were allowed to adjust for 24 h in a culture medium containing 0.1% albumax.

In the proliferation assay, endothelial cells (10^4) were plated onto type I collagen precoated membrane inserts with a pore size of 0.45 μm and allowed to adjust for 24 h. Subsequently, inserts were transferred to 12-well plates with breast cancer cells and co-cultured for 48 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromid (MTT, Boehringer Mannheim, Germany) was added to the cell cultures for the last 3 h.

In the migration assay endothelial cells (3×10^4) were plated onto type I collagen gel-precoated membrane inserts with a pore size of 12 μm and allowed to form a monolayer on the inserts. Subsequently, the inserts were transferred to 12-well plates with breast cancer cells and co-cultured for 14 h before fixation of the endothelial cells in 5% glutaraldehyde and staining with 0.5% toluene blue in 2.5% $NaCO_3$. The number of migrating endothelial cells was measured semi-automatically by counting in hundreds randomly, but evenly distributed computer-generated counting fields for each insert.

In the microtubule formation assay MDA-231/B cells were plated onto 24-well plates at increasing seeding densities and cultured for 24 h. Cells were washed three times in serum-free media before addition of 300 μl Matrigel D-MEM (2:1) to each well. Cultures were subsequently incubated at 37 °C for 30 min, causing the Matrigel to jellify. Endothelial cells (3×10^4) were plated on top of the Matrigel and the cells were co-cultured for 48 h. Cells were fixed in 5% glutaraldehyde for 30 min and washed three times with PBS containing 50 mM glycine at 30 min intervals. The area of tubules formed in co-cultures was measured semi-automatically by counting the number of hits in 24 randomly distributed computer-generated grids for each well.

Cytokine levels

The levels of mouse-derived cytokines, i.e., interleukin-6 (IL-6), interleukin-1beta (IL-1β), and tumor necrotic factor-alpha (TNF-α), secreted into the conditioned media during cultivation of either mouse bone marrow alone or in co-culture with breast cancer cells were measured by ELISA (R&D Systems Europe, UK). The levels of human-derived cytokines were determined in conditioned media obtained from confluent MDA-231/B cell cultures. Briefly, MDA-231/B cells were plated onto 10-cm petri dishes and cultured to confluence in α-MEM containing 5% HI-FCS. Conditioned medium was harvested after culturing the cells in fresh medium for 48 h. The human cytokines, vascular endothelial growth factor (VEGF), macrophage colony stimulating factor (M-CSF), IL-6, interleukin-1alpha (IL-1α), IL-1β, and TNF-α, were measured by ELISA (R&D Systems Europe). Parathyroid hormone-related protein (PTHrP) was measured by a radioimmunoassay (Peninsula Laboratories Europe, Merseyside, UK). The background levels in conditioned media from petri dishes without cells were found to be zero for all tested cytokines.

Detection of cytokine transcripts

MDA-231 cells were cultured to confluence as described above. Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform as previously described (Chomczynski and Sacchi 1987). Expression of M-CSF, IL-1α, IL-1β, PTHrP, TNF-α, and VEGF mRNA was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) using GeneAmp Thermostable rTth kit (Perkin Elmer, USA) according to the manufacturer's recommendations. Briefly, first strand cDNA was synthesized from 250 ng total RNA with a final concentration of the downstream primer at 0.75 mM. Upstream primer and $MgCl_2$ were added to the cDNA mixture in final concentrations of 0.15 mM and 1.5 mM, respectively. PCR was performed in a Perkin Elmer 9600 PCR machine

with 1 min denaturation at 95 °C, 35 cycles of 10 s at 95 °C, and 15 s at 60 °C and a final extension step for 7 min at 60 °C.

For analysis of IL-6 mRNA expression cDNA was synthesized with the First-Strand cDNA Synthesis Kit (Pharmacia-Biotech, USA) according to the manufacturer's recommendations. In brief, 5 μg total RNA was reversibly transcribed with the supplied Not I-d(T)18 primer in a final reaction volume of 33 μl. The PCR reaction mixture contained 5 μl cDNA, 2.5 mM $MgCl_2$, 0.2 mM dATP, dCTP, dGTP, dTTP (Pharmacia-Biotech, USA), 30 pmol of each primer, 1 × PCR buffer, 2.5 U AmpliTaq Gold (Perkin Elmer, USA). The PCR was performed for 11 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 64 °C, and 1 min at 72 °C, and for 7 min at 72 °C as a final extension step in a Perkin-Elmer 9600 PCR machine. Control PCR was performed with MilliQ; in none of these samples were PCR products detected.

Primers used for RT-PCR were as previously reported (Brown et al. 1996); details are available on request. The oligonucleotides were obtained from DNA Technology, Denmark. The sizes of the PCR products were analyzed on a 2% wt/vol agarose gel and the specificity was confirmed either by sequencing with the 70770 Sequenase version 2.0 kit (Amersham, USA) or for VEGF by Southern blot analysis.

For the Southern blot analysis the agarose gel was pre-treated by washing: 10 min in 10 mM HCl, 2 min in MilliQ, 2 × 30 min in 0.5 M NaOH, 1.5 M NaCl, 2 min in MilliQ, and 2 × 30 min in 0.5 M Tris, 3.0 M NaCl. The products were blotted onto a nitrocellulose membrane (Schleicher and Shuell, Keene, N.H., USA) by capillary transfer o.n. using 2 × SSC. Prehybridization was performed at 58 °C in 5 × SSC, 2.5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA for 1.5 h. A oligonucleotide 5′-CATCACCATGCAGATTATGCGGATCAAA CC-3′ (Brown et al. 1996), which hybridizes to all VEGF splice-variants, was labeled with γP32-ATP (Amersham Pharmacia Biotech, USA) using T4 polynucleotide kinase (Promega, Madison, Wis., USA) and purified by ProbeQuant G-50 micro column (Amersham Pharmacia Biotech, USA). Hybridization was performed at 58 °C o.n. 10 pmol labeled oligonucleotide and post-hybridization washes were: 3 × 20 min in 2 × SSC, 0.1% SDS at r.t., 2 × 20 min in 0.1 × SSC, 0.1% SDS at r.t., and 30 min in 0.1 × SSC, 0.1% SDS at 65 °C. Specifically bound oligonucleotide was visualized with a PhophorImager (Molecular Dynamics, Sunnyvale, Calif., USA).

Results

Osteolysis

Human breast cancer cells or vehicle were injected into the left cardiac ventricle of 4-week-old female athymic mice. In mice injected with cancer cells osteolytic lesions developed within 4 weeks from inoculation with a preference for the distal femurs and proximal tibias (Fig. 1A). The human origin of the tumor tissue was confirmed by species-specific immunohistochemical detection of the human epithelial cell marker, cytokeratin, in the breast cancer cells (Fig. 1B). Histomorphometric analyses of tibias and femurs showed a significant increase in the number of OCls lining bone surfaces facing nests of tumor cells as compared to bone surfaces distant from tumor cells in the same bone sample or in bones from healthy control mice (Fig. 1C, D). Furthermore, the OCls lining bone surfaces near tumor tissue were significantly larger and with a higher number of nuclei per sectioned OCl than OCls lining corresponding surfaces of long bones in healthy mice (Table 1).

Table 1 Histomorphometric analysis on bones isolated from mice with osteolytic metastases or healthy mice. The sample area was from 0.50–1.0 mm to the growth plate cartilage. *N.Oc/mm BS* number of osteoclasts per millimeter of bone surface, *Oc.S/BS*, %, osteoclast surface per bone surface, *Oc.S/Oc*, μm, osteoclast surface per osteoclast, *N.Nu/Oc*, number of nuclei per osteoclasts. Data are expressed as mean ± SEM

	Diseased mice (n = 3)	Healthy mice (n = 4)	P value
N.Oc/mm BS	14.6 ± 2.6	8.6 ± 0.7	< 0.05
Oc.S/BS, %	72 ± 9	22 ± 3	< 0.005
Oc.S/Oc, μm	52 ± 8.7	25 ± 1.1	< 0.05
N.Nu/Oc	2.5 ± 0.3	1.2 ± 0.2	< 0.05

MDA-231/B cells were also found to increase the number of OCls formed in bone marrow cultures. A nearly threefold increase in the number of TRAP + multinucleated cells was observed in both direct and indirect co-cultures of bone marrow and non-proliferating breast cancer cells when compared to bone marrow cultures alone (Fig. 2A, B). In contrast to the pure bone marrow cultures, the number of OCls remained high after day 8 in culture in bone marrow/breast cancer cell co-cultures, suggesting that breast cancer cell-derived factor(s) may prolong OCls survival. Moreover, the maturity and resorptive activity of OCls formed in bone marrow/breast cancer cell co-cultures, measured as the number of nuclei per OCls and the area of pits formed on slices of devitalized cortical bovine bone, respectively, were significantly increased when compared to control bone marrow cultures (Fig. 3A, B). Furthermore, the level of type I collagen degradation products (CrossLaps) released into the conditioned medium in bone marrow/breast cancer cell co-cultures was significantly elevated when compared to cultures of bone marrow alone (Fig. 3B). The breast cancer cells alone were unable to degrade bone and to release collagen fragments from the bone slices (Fig. 3B). The level of TRAP activity measured in the conditioned media was increased tenfold ($P < 0.0001$, $n = 12$) in bone marrow/breast cancer cell co-cultures when compared to bone marrow cultures. Conditioned medium from breast cancer cell cultures did not contain detectable levels of TRAP activity.

Cytokines affecting formation and activity of OCls

The data from non-contact co-cultures of bone marrow and breast cancer cells strongly suggest that soluble breast cancer cell-derived factors can increase the OCl number and resorptive activity. The effect may be mediated solely by breast cancer cell-derived factors or may be due to a concurrent action of breast cancer cell and autocrine and/or paracrine bone marrow-derived factors.

As summarized in Table 2, confluent breast cancer cell layers expressed high levels of M-CSF, moderate levels of IL-6, IL-1α, and LIF, and undetectable levels of

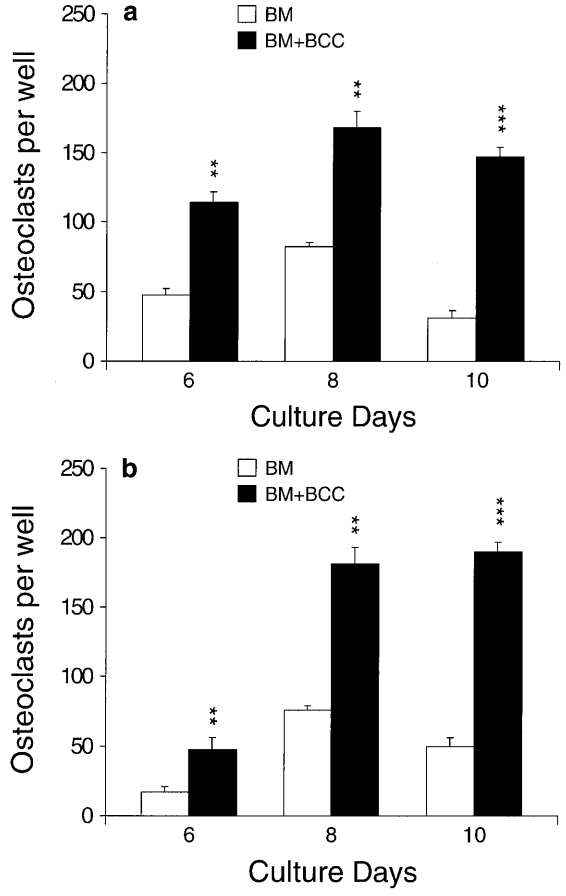


Fig. 2a, b Osteoclast recruitment in bone marrow/breast cancer co-cultures. Bone marrow cells (BM) were cultured for 6, 8 or 10 days in the absence (*white bars*) or in the presence (*gray bars*) of non-proliferating MDA-231/B breast cancer cells (BCC). Co-cultures were performed with either direct cell–cell contact (**a**) or when cells were separated by a membrane insert (**b**). At the indicated days cultures were rinsed, fixed, and stained for TRAP (*red*). TRAP+ cells with three or more nuclei were counted as mature OCls. The results are expressed as mean (+SEM) number of OCls ($n = 4$). The experiment was repeated three times with similar results. Significance levels, $^{**}P < 0.01$, $^{***}P < 0.001$

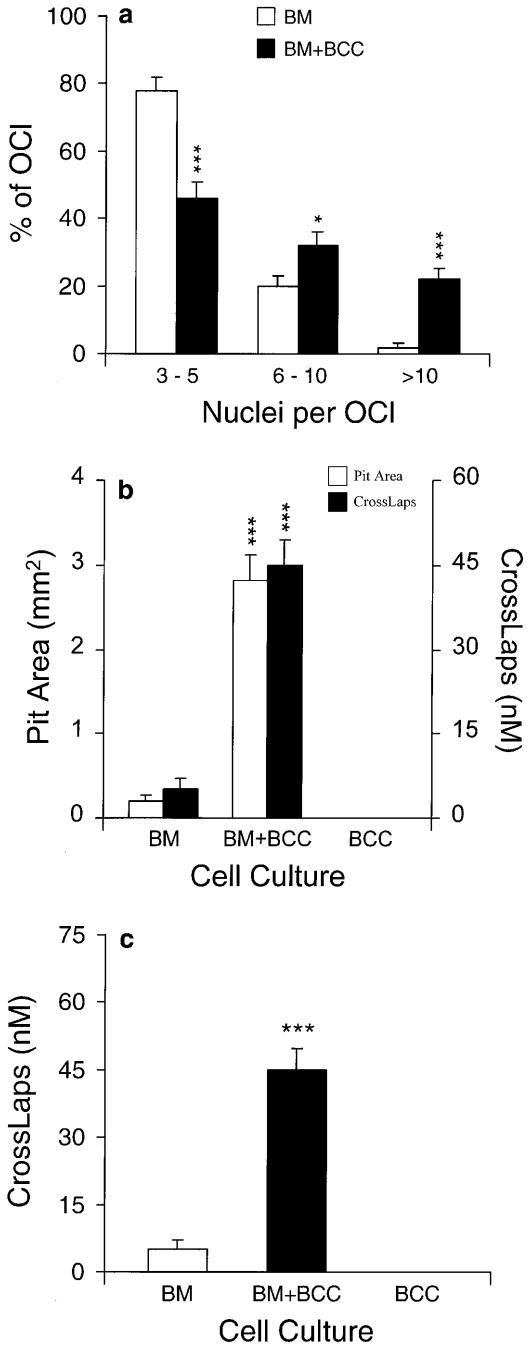


Fig. 3a–c Osteoclast maturity and resorptive activity in co-culture with breast cancer cells. **a** The osteoclast (OCl) maturity, measured as the number of nuclei per OCl, was determined in 8-day-old bone marrow cultures (BM) when cultured on plastic in the absence (*open bars*) or in the presence (*gray bars*) of 3×10^4 non-proliferating MDA-231/B breast cancer cells (BCC). **b** The resorptive activity of OCls formed in 8-day-old BM with or without BCC was measured as the plan surface area of excavation pits formed on the bovine cortical bone slices (*white bars*) or as the release of type I collagen fragments (CrossLaps) into the conditioned medium (*gray bars*). The results are expressed as mean (+SEM) nuclei per OCl, pit area, or CrossLaps concentration, respectively. The experiments were repeated three times with similar results. Significance levels, $^{*}P < 0.05$, $^{***}P < 0.001$

PTHrP, IL-1β, and TNF-α, both at mRNA and protein levels measured by RT-PCR and ELISA or RIA, respectively.

The levels of three selected mouse-derived cytokines were analyzed in CM collected every second day over 8 days from cultures of bone marrow alone and when co-cultured with breast cancer cells (Fig. 4). The secretion of bone marrow-derived IL-6 was found to be significantly increased when bone marrow was co-cultured with breast cancer cells. The secretion of mouse IL-1β in bone marrow cultures decreased during cultivation and was independent of the presence or absence of breast cancer cells. Finally, mouse TNF-α was found to be fairly constantly secreted during cultivation and just slightly affected by the presence of breast cancer cells (Fig. 4). CM harvested from cultures of breast cancer cells alone, as expected, did not contain antigenic constituents according to the ELISAs for mouse IL-6, IL-1β or TNF-α.

Table 2 Levels of human cytokines in MDA-231 cell culture. Human breast cancer cells (MDA-231) were cultured to confluence in 10-cm petri dishes. Conditioned medium was harvested after 48 h and the level of cytokines was measured by ELISA or by RIA for PTHrP (*). Cellular RNA extracts were used for determination of the expression of cytokines by reverse transcriptase-polymerase chain reaction (RT-PCR)[1)] . (–) no signal, √ clear signal, *n.p.* not performed

	ELISA (pg/mg cellular protein)	RT-PCR[1)]
IL-1α	13 ± 3	√
IL-1β	< 3.9	(–)
IL-6	171 ± 20	√
M-CSF	7462 ± 460	√
PTHrP*	< 2.0	(–)
TNF-α	< 15.6	(–)
LIF	41 ± 4	n.p.
VEGF	6224 ± 120	√

Angiogenesis

Many large and small blood vessels were found in the bone marrow and within the tumor tissue (Fig. 1E, F). The vessels were visualized by immunohistochemical detection of mouse CD31 and/or CD34 positive endothelial cells with the two rat monoclonal antibodies MEC 13.3 (Vecchi et al. 1994) and MEC 14.7 (Garlanda et al. 1997), respectively. High levels of immunoreactivity for CD31 were observed in endothelial cells lining larger blood vessels in the bone marrow and moderate immunoreactivity was observed in some smaller blood vessels in both bone marrow and at the periphery of and within tumors. Immunoreactivity for CD34 was clearly restricted to small blood vessel endothelial cells. In the bone marrow and in the tumor tissue only newly formed blood vessels and capillaries appeared highly CD34 positive. The blood vessels seemed to be infiltrating the tumor tissue from the periphery.

Fig. 4A–C The levels of three mouse bone marrow-derived cytokines. Interleukin-6 (IL-6) (**A**), interleukin-1 beta (IL-1β) (**B**), and tumor necrosis factor-alpha (TNF-α) (**C**), were measured in the conditioned medium harvested every second day from co-cultures or single cultures of bone marrow (BM) and breast cancer cells (BCC). Results are expressed as mean ± SEM ($n = 4$). The experiments were repeated twice with similar results. Significance levels, $*P < 0.05$, $***P < 0.001$

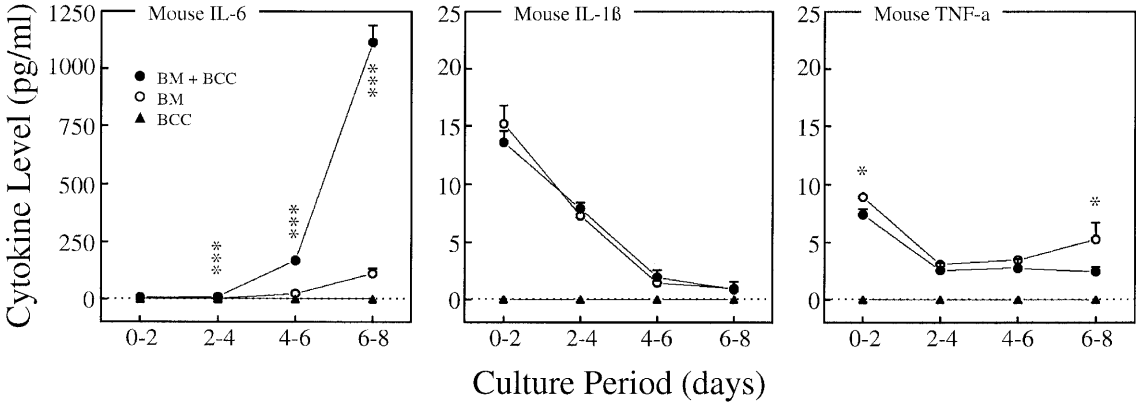
Membrane-separated co-cultures showed that MDA-231-derived factors were able to stimulate the growth of the murine stromal endothelial cell line MBA-2.1 (Fig. 5A). Under indirect co-culture conditions, breast cancer cells also induced a 16-fold increase in the number of endothelial cells migrating through a thin layer of type I collagen ($P < 0.001$, $n = 8$) (Fig. 5B). Finally, similar conditions showed that breast cancer cells dose – dependently stimulated the differentiation of endothelial cells, since the total area of tubule-like structures formed in a three-dimensional Matrigel was increased nearly 230-fold in the presence of the highest cancer cell numbers when compared to cells cultured in the absence of cancer cells ($P < 0.001$, $n = 4$) (Fig. 5C).

Confluent breast cancer cell cultures expressed high levels of VEGF mRNA and protein (Table 2). As revealed by RT-PCR, and confirmed by Southern blotting, the two lower-molecular-weight isoforms of VEGF mRNA, $VEGF_{121}$, and $VEGF_{165}$, were expressed by the breast cancer cells, whereas the three other identified isoforms $VEGF_{145}$, $VEGF_{189}$, and $VEGF_{206}$, were not detectable (Fig. 6A, B).

Discussion

The in vivo bone metastasis model in nude mice was first described by Arguello et al. 1988, and later modified by Yoneda et al. 1994 for the study of bone metastasis of human breast cancer. One of the characteristic histological findings in sections of osteolytic tumor lesions has been the dramatic increase in the number of multinucleated OCls lining the bone surfaces near the tumor tissue. By use of the in vivo bone metastasis model, we were able to quantify the significantly increased size and maturity of OCls lining bone surfaces in the proximity of tumor cells compared to OCls lining normal bone (Table 1). Furthermore, the in vivo findings were found to be closely mimicked in the bone marrow OCl formation assay, where the number (Fig. 2), maturity (Fig. 3A), and activity (Fig. 3B) of OCls were increased in co-cultures of bone marrow and breast cancer cells as compared to bone marrow alone.

Cultures of breast cancer cells were found to secrete high levels of M-CSF (Table 2), which is an indispensable



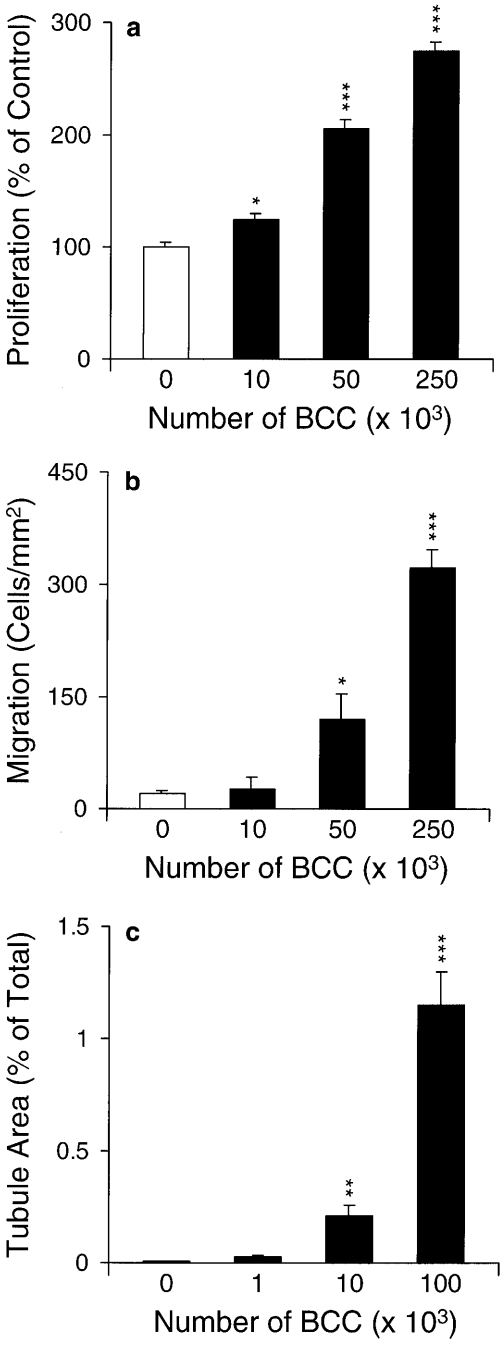


Fig. 5a–c Co-cultures of breast cancer and endothelial cells. **a** The diagram shows the proliferation of endothelial cells, measured as radiolabeled thymidine incorporation into DNA, in response to indirect co-culture with breast cancer cells (BCC). The proliferation is expressed as mean level ± SEM of thymidine incorporation after normalization to control values. The experiments were repeated three times with similar results. **b** The number of endothelial cells, migrating through a thin layer of type I collagen gel on a membrane insert, was significantly and dose dependently increased by indirect co-culture with BCC. **c** Likewise, the area of endothelial tubule-like structures, formed in a three-dimensional artificial basal-membrane (Matrigel), was significantly and dose dependently increased when plated onto increasing numbers of BCC. Results are expressed as mean ± SEM. Significance levels, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

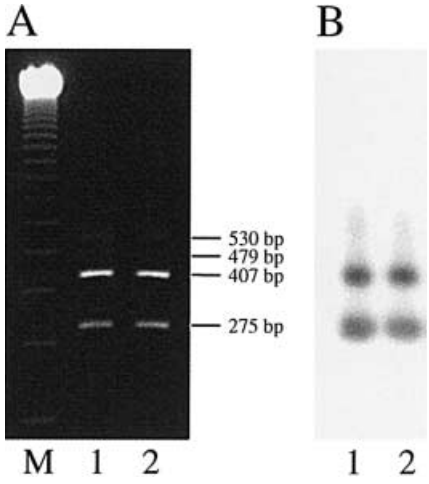


Fig. 6A, B Expression of VEGF isoforms in the human breast cancer cell line MDA-231. **A** RT-PCR demonstrating the expression of the two VEGF isoforms, $VEGF_{121}$ and $VEGF_{165}$, of the expected product size of 275 and 407 base pairs, respectively. No clear RT-PCR products were observed for the two higher isoforms of VEGF, $VEGF_{189}$, and $VEGF_{206}$, of expected product size of 479 and 530 base pairs, respectively. *Lane M*: 123 bp DNA marker; *lane 1*: MDA-231/P RNA; *lane 2*: MDA-231/B RNA. **B**. Southern blotting confirming the identity of $VEGF_{121}$ and $VEGF_{165}$

cytokine in normal OCl development (Tanaka et al. 1993). This has been highlighted by the fact that mice lacking the gene for M-CSF become osteopetrotic due to a very low number of matured OCls in the skeleton (Felix et al. 1990a,b). It is, therefore, likely that breast cancer cell-derived M-CSF participate as a paracrine factor in the recruitment and maturation of OCls from the bone marrow. This has been supported by recent data from our laboratories, where monoclonal antibodies raised against human M-CSF were found to decrease the breast cancer cell-induced recruitment of OCls by 40% (Misander et al. 1999).

IL-6 was found to be expressed by breast cancer cells in culture at moderate levels. IL-6 is involved in the early stages of OCl development, while its role in the regulation of the activity of mature OCls is controversial and the results depend on the system used for evaluation (de la Mata et al. 1995; Kurihara et al. 1990; Lowik et al. 1989; Ohsaki et al. 1992; Roodman 1996). Moreover, IL-6 has been implicated in several diseases involving increased bone resorption, such as osteoporosis, Paget's disease, myelomatosis, and Gorham-Stout disease (Roodman 1996; Jilka et al. 1992). In co-culture with breast cancer cells, the bone marrow was found to secrete tenfold higher levels of mouse IL-6 than bone marrow cultures alone (Fig. 4). The results suggest that breast cancer cells induce an up-regulation of bone marrow expression of IL-6, which subsequently can act as a co-factor in OCl recruitment.

Other potent stimulators of osteoclastogenesis and OCl activity in vivo and in vitro, i.e., IL-1β, and TNF-α, were expressed at low or undetectable levels by breast cancer cells and by bone marrow in co-cultures or when

cultured alone. PTHrP, which has been suggested to be important for breast cancer cell-induced osteolysis in vivo (Guise 1997; Guise et al. 1996), was not secreted in detectable levels by the MDA-231/B cells in culture nor could it be detected by RT-PCR. It is possible that cytokines other than those selected for analysis in this study, such as transforming growth factor-beta (TGF-β), IGF-II, and IL-11 (Pederson et al. 1999; Lacroix et al. 1998; Morinaga et al. 1997), may also participate in the observed breast cancer cell-induced increase in OCl number and activity.

Cancer cell-induced angiogenesis is essential for sustained tumor growth as it allows tumor oxygenation and nutrient perfusion and the removal of tumor waste products. Recent studies have shown that angiogenesis in breast cancer assessed by microvessel count is a significant prognostic factor (Bosari et al. 1992; Horak et al. 1992; Toi et al. 1995). In line with the human studies (Horak et al. 1992; Shibusa et al. 1998), antibodies directed against mouse CD31 and mouse CD34 can be used to assess the amount of neo-vascularization in tumors in the in vivo bone metastasis model. It has previously been suggested that the MEC 14.7 antibody, recognizing mouse CD34, can be used to discriminate between larger blood vessels and smaller blood vessels including newly formed capillaries in tumors in mice (Garlanda et al. 1997). Our observations in nude mice with bone metastasis support this finding. Indeed, the distinct reaction of the MEC 14.7 antibody with small, newly formed capillaries in both the peripheral and central part of osteolytic tumors implies that the cancer cells – not only in vitro, as shown clearly by our co-culture experiments, but also in vivo – have the capacity to induce endothelial cell proliferation, migration, and differentiation. The induction of an endothelial cell response may be mediated solely by cancer cell derived cytokines and growth factors or may be concerted in action by growth factors, such as TGF-β1 and insulin-like growth factors, released from the bones during osteoclastic resorption (Oursler 1994). TGF-β has been implicated as being a very potent co-factor to VEGF in the induction of angiogenesis in cancer (Tsujii et al. 1998; Nakanishi et al. 1997; Donovan et al. 1997; Benefield et al. 1996). The high levels of VEGF expression in the human breast cancer cell line MDA-231/B (Fig. 6, Table 2) suggest that this growth factor may be involved in the paracrine communication between breast cancer cells and endothelial cells as has been shown for colon cancer cell-induced angiogenesis (Tsujii et al. 1998). This hypothesis is currently being studied in our laboratory.

The feasibility of targeted drug delivery to tumor vasculature has previously been elegantly demonstrated by Ruoslahti and co-workers, taking advantage of the selective expression of several proteins in endothelial cells in the angiogenic vessels within solid tumors as compared to endothelial cells located in established blood vessels (Arap et al. 1998; Pasqualini et al. 1997; Pasqualini and Ruoslahti 1996). Although skepticism regarding the success of this approach has recently been called for by Schnitzer (1998), similar results have been found by several other investigators (Hammes et al. 1996; Strawn et al. 1996; Spragg et al. 1997; Molema et al. 1997). Using targeted drug delivery to bone metastasis vasculature, inhibition of OCl activity, tumor growth, and/or angiogenesis could be achieved with improved efficacy and tolerability and with reduced systemic toxicity and side effects.

Matrix destruction and neo-vascularization are necessary for all solid tumors to expand. This study has shown that breast cancer cells arrested in the bone marrow accomplish this by two events. First, the hard calcified bone matrix is removed by osteoclasts increased in number and activity by cancer-derived factors and, second, new blood vessel formation is enhanced due to a cancer-stimulated increase in endothelial cell proliferation, invasion, and differentiation.

Acknowledgements The continuous help from Professor Toshiyuki Yoneda and Paul Williams, Texas University, San Antonio, is highly appreciated.

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