A Role for Endothelin-2 and Its Receptors in Breast Tumor Cell Invasion

Matthew J. Grimshaw, Thorsten Hagemann, Ayse Ayhan, Cheryl E. Gillett, Claudia Binder, and Frances R. Balkwill

1Cancer Research UK Translational Oncology Laboratory, Barts and The London, Queen Mary’s School of Medicine and Dentistry, London, United Kingdom; 2Department of Haematology and Oncology, University Göttingen, Göttingen, Germany; 3Department of Pathology, Faculty of Medicine, Hacettepe University, Ankara, Turkey; 4Cancer Research UK Breast Cancer Biology Group, Guy’s Hospital, and 5Hedley Atkins/Cancer Research UK Breast Pathology Laboratory, Guy’s Hospital, London, United Kingdom

ABSTRACT

We have studied the role of endothelins (ET-1, ET-2 and ET-3) and ET receptors (ET-RA and ET-RB) in the invasive capacity of breast tumor cells, which express ET-1 and ET-2 as well as ET-RA and ET-RB. Of five human breast tumor cell lines tested, all expressed mRNAs for ET-1, ET-2, and ET-RB. ET-RA mRNA was expressed by four of five tumor cell lines. Breast tumor cells migrated toward ET-1 and ET-2 but not toward ET-3. Chemotaxis involved signaling via both receptors, and a pertussis-toxin-sensitive p42/p44 mitogen-activated protein kinase (MAPK)-mediated pathway that could be inhibited by MAPK kinase (MEK)1/2 antagonists. Chemotaxis toward ETs did not involve p38 or stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) and was not inhibited by hypoxia. Incubation of tumor cells with ET-2 also increased chemotaxis toward the chemokines CXCL12 and CCL21. As well as inducing chemotaxis of tumor cells, ET-1 and ET-2 increased tumor cell invasion through Matrigel. Furthermore, stimulation of macrophage/tumor cell cocultures with ETs led to increased matrix metalloproteinase (MMP)-2 and -9 production by macrophages and a marked increase in invasion of tumor cells. Antagonism of either ET-RA or ET-RB decreased the invasion seen in ET-stimulated cocultures, as did a broad-spectrum MMP inhibitor. Immunohistochemical staining of human breast tumor sections showed increased ET and ET receptor protein expression by tumor cells in invasive ductal carcinoma compared with normal breast tissue or ductal carcinoma in situ. Furthermore, tumor cell ET and receptor expression was stronger at the invasive margin of invasive ductal carcinomas, in the lymphovascular space, and in lymph node metastases. ET expression often colocalized with ET-RB expression in all neoplastic tissue indicating a possible autocrine action of ETs. We suggest that expression of ETs and their receptors by human breast tumors, particularly in conjunction with a high macrophage infiltrate, may have a role in the progression of breast cancer and the invasion of tumor cells.

INTRODUCTION

Endothelins (ETs) are a group (ET-1, ET-2 and ET-3) of small (21-amino acid) vasoactive peptides with diverse paracrine/autocrine actions (1). ET-1 and ET-2 have similar structures, differing by only two amino acids, whereas ET-3 differs in structure at six amino acids (2). ETs bind at least two receptor subtypes, ET-A receptors (ET-RA) and ET-B receptors (ET-RB; Ref. 3), which are seven-transmembrane domain G protein-coupled receptors (4). ETs are expressed in a broad range of tumors, and there is a growing interest in ETs and cancer; most reports focus on the role of ET-1/ET-RA in tumor growth, progression, and angiogenesis (5). However, we recently proposed a role for ET-2/ET-RB in invasive ductal carcinoma (IDC) of the breast; binding of ET-2 to ET-RB protects tumor cells from hypoxia-associated apoptosis in an autocrine fashion (6). Both ET-2 and ET-RB are rarely detected in the nonneoplastic human breast, but breast carcinomas contain regions of hypoxia that increase production of ET-2 as well as ET-RA and ET-RB by tumor cells (6). Intratumoral injection of BQ-788, an ET-RB selective antagonist (7), causes increased necrosis and decreased tumor volume in a murine model of breast carcinoma (6); BQ-123, a selective ET-RA antagonist (8), also leads to decreased breast tumor volume but without a concomitant increase in necrosis.

High levels of macrophage infiltrate in breast tumors correlate with a poor prognosis (9) and hypoxic tumors have a higher invasive capacity and a poorer prognosis than well-oxygenated tumors (10). Hypoxia-associated ETs expression by tumor cells may also modulate tumor-associated macrophage distribution and activation in breast tumors; ET-1 and ET-2, but not ET-3, are chemoattractants for macrophages and lead to an activated phenotype in vitro (11). In vivo, ET expression in IDC not only correlates with hypoxia (6) but also with the presence of activated “foamy” tumor-associated macrophages (11).

ETs may also play a role in chemotaxis of several additional cell types: ET-1 and ET-3 are chemoattractants for pulmonary artery fibroblasts (12), and ET-2 is a chemoattractant for neutrophils (13). As well as being chemoattractant for several cell types, ET-1 may also modulate the invasive capacity of some tumor cells including ovarian carcinoma cells (14), Ewing’s sarcoma, and neuroblastoma cells (15). In ovarian carcinoma cells, ET-1 interacts with ET-RA to increase the expression and activation of metalloproteinases (MMPs) whereas decreasing the secretion of tissue inhibitor(s) of MMPs (TIMP; Ref. 14).

In view of the possible role of ETs in invasion of tumor cells, we investigated whether ETs are chemoattractants for breast carcinoma cells and, in conjunction with macrophages, whether they may modulate the invasive capacity of breast tumor cells. By examining human neoplastic breast tissues, we also analyzed whether ETs are associated with progression and invasion of tumor cells in ductal carcinoma in situ (DCIS), IDC, and lymph node metastases. Because of its regulation by hypoxia and increased expression in IDC compared with normal breast tissue (6), we focused on the potential role of ET-2/ET-RB.

MATERIALS AND METHODS

Peptides and Antagonists. ET peptides and receptor antagonists BQ-123 (ET-RA antagonist; Ref. 16) and BQ-788 (ET-RB antagonist; Ref. 7) were obtained from American Peptide Co., Sunnyvale, CA. MAPK kinase (MEK)1/2 inhibitors PD98059 and U0126 were purchased from Cell Signaling Technology Inc., Beverly, MA. Pertussis toxin (PTX; G-protein inhibitor) and FN439 (nonspecific MMP inhibitor) were supplied by Calbiochem-Novabiochem Corporation, San Diego, CA.

Cell Culture. HTH-K cells were cultured as described previously (17). Human cell lines (MCF-7, BT20, BT474, SKBR3, and MDAMB468) from the American Type Culture Collection (Manassas, VA) were cultured similarly.

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Requests for reprints: Matthew Grimshaw, Cancer Research United Kingdom, Breast Cancer Biology Group, 3rd Floor, Thomas Guy House, Guy’s Hospital, London SE1 9RT, United Kingdom. Phone: 00-44-20-7955-2335; Fax: 00-44-20-7955-2027; E-mail: matthew.grimshaw@cancer.org.uk.
then subjected to chemotaxis assays and counted as described previously (11). A 12-μm membrane, that had been treated with 6.5 μg/ml fibronectin solution at 37°C for 2 h and then dried was used. Migration was stopped after 5 h for each experiment.

**Western Blotting.** Protein extracts were prepared from cells that had been serum-starved for 24 h before stimulation with ETs. Western blotting for ET receptor was carried out as described previously (6) and for signaling pathways as described previously (11).

**Matrigel Invasion Assay.** In *vitro-*invasiveness was measured in a modified Boyden chamber as described in Hagemann et al. **M**-CF7 cells were seeded in gelatinase-free medium on polycarbonate membranes coated with Matrigel (ECM gel; Sigma-Aldrich Co. Ltd., Poole, United Kingdom). Macrophages were added at 2:1 (macrophages:tumor cells) in inserts that allow exchange of liquids but no direct cell-cell contact or invasion of macrophages. ETs (100 ng/ml) were added, and renewed every 24 h. After 96 h, the contents of the lower well were removed, and cells were collected by centrifugation. The supernatant was used for zymography. Cells were spun down onto coverslips and stained with 4′,6-diamino-2-phenylindol (DAPI, 200 ng/ml). Intact nuclei were counted in six high power fields using UV-microscopy (Axioskop; Zeiss, Jena, Germany).

**Zymography.** Protein content in the supernatant was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Twenty μg were separated on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin. After electrophoresis, gels were washed for 1 h in renaturation buffer (2.5% Triton X-100) and then were incubated for 36 h at 37°C in 50 mM Tris, 200 mM NaCl, and 5 mM CaCl₂ (pH 7.5). Gels were stained with 0.05% Coomassie Brilliant Blue, and MMPs were identified by size and comparison with the recombinant proteins (Calbiochem-Novabiochem Corporation).

**Immunohistochemistry of Human Breast Tumor Samples.** Intraductal and invasive human breast cancer archival samples and corresponding metastatic lymph node samples were supplied by the Hedley Atkins Breast Pathology Laboratory, Guy’s Hospital, London, United Kingdom. All of the specimens were formalin-fixed and paraffin-embedded. Protein expression of ETs and ET receptors were immunohistochemically examined as described previously (11). Sections from representative paraffin blocks of tumors were deparaffinized, rehydrated, and blocked for endogenous peroxidase activity using 3% H₂O₂. Macrophages were added at 2:1 (macrophages:tumor cells) in inserts that allow exchange of liquids but no direct cell-cell contact or invasion of macrophages. ETs (100 ng/ml) were added, and renewed every 24 h. After 96 h, the contents of the lower well were removed, and cells were collected by centrifugation.

**RESULTS**

**ET and ET Receptor mRNA Expression by Human Breast Tumor Cell Lines.** The murine breast carcinoma cell line HTH-K expresses ET-2, ET-RA, and ET-RB, and this expression is increased by exposure to hypoxia (6). We examined ET and ET receptor mRNA expression in five human breast tumor cell lines: SKBR3, MCF-7, BT20, BT474, and MDAMB468. Similar to the expression profile seen in IDC of the breast (6), all of the tumor cell lines expressed ET-1 and ET-2, but none expressed ET-3; all of the cell lines expressed ET-RB, whereas all but one, BT20, expressed ET-RA (Fig. 1A).

**Breast Tumor Cells Chemotax toward ET-2.** We used breast carcinoma cell lines to investigate chemotaxis of breast tumor cells toward ETs in *vitro*. MCF-7 cells migrated strongly toward 100 ng/ml ET-2 and less robustly toward ET-1 (Fig. 1B). Tumor cells did not

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Fig. 2. A, effect of endothelin (ET) receptor inhibition on tumor cell chemotaxis toward ET-2. MCF-7 cells were incubated with 100 ng/ml BQ-123 or BQ-788 (singly and in combination) for 30 min before and during the microchemotaxis assay toward 100 ng/ml ET-2. Both BQ-123 and BQ-788 could partially but significantly inhibit migration toward ET-2 (P < 0.001). 

B, chemotaxis of MCF-7 cells toward ET-2 under hypoxia. The microchemotaxis chamber was incubated at 0.1% oxygen for the duration of the assay. During hypoxia, MCF-7 cells were still able to migrate toward ET-2 (P < 0.05). C, inhibition of chemotaxis toward ET-2 by mitogen-activated protein kinase (MAPK) kinase (MEK)1/2 inhibitors. MCF-7 cells were incubated in 5 μM PD98059, 50 μM PD98059, or 10 μM U0126 for 30 min before and during the microchemotaxis assay toward 100 ng/ml ET-2. Migration was partially inhibited by 5 μM PD98059 (P < 0.001) and completely abolished by 50 μM PD98059 (P < 0.05) or 10 μM U0126 (P < 0.05). D, inhibition of chemotaxis toward ET-2 by inhibition of G proteins or by incubation in calcium-free medium (Ca-free). MCF-7 cells were incubated in 100 ng/ml pertussis toxin (PTX) or in Ca-free medium for 30 min before and during the microchemotaxis assay to 100 ng/ml ET-2. Migration was significantly inhibited by PTX (P < 0.001) or by incubation in Ca-free medium (P < 0.05). E, Western analysis of protein phosphorylation in MCF-7 cells on stimulation with ET-2. MCF-7 cells were serum-starved for 24 h before being stimulated with ET-2 for 0–24 h. ET-2 led to a rapid (15 min) phosphorylation of p42/p44 MAPK (Phospho-MAPK). p38, p46, and p54 were not phosphorylated in response to ET-2. Shown is one experiment of three. Phosphorylated and nonphosphorylated controls were either treated cell extracts [p38 (Phospho 38), stress-activated protein kinase/Jun N-terminal kinase (Phospho Sakp/JNK)] or control peptides (p42/44 MAPK) supplied by the antibody manufacturer. The bottom panel shows a graph of the phospho-MAPK:total MAPK ratio (normalized to the unstimulated control), as assessed by densitometry, from a single representative experiment (of three).

Fig. 3. Chemotaxis of MCF-7 cells toward 100 ng/ml CXCL12, CCL21, or CCL2 in the presence of 100 ng/ml endothelin (ET)-2. Chemotaxis of MCF-7 cells toward CXCL12 and CCL21 was significantly increased compared with control (P < 0.01), and this was further increased by the presence of ET-2 (P < 0.001). MCF-7 cells did not significantly migrate toward CCL2 with or without ET-2.
also inhibits ET-RB-mediated chemotaxis of macrophages toward ET-2 (11). Incubation of MCF-7 cells with PTX (100 ng/ml) led to decreased migration toward ET-2 (Fig. 2D). Incubation of MCF-7 cells with PTX (100 ng/ml) led to decreased migration toward ET-2 (Fig. 2D). Migratory assays in which 100 ng/ml ET was added to the upper chamber. ET-1 and ET-2 modestly but significantly ($P < 0.05$) increased invasion ET-3 had no significant effect. Migration of MCF-7 cells was also inhibited by incubating the cells in calcium-free medium before and during the chemotaxis assay (Fig. 2D).

Cell viability was not affected by any of the inhibitors used (data not shown).

To demonstrate that activation of the p42/p44 MAPK pathway was responsible for chemotaxis toward ETs, we analyzed the phosphorylation status of key molecules in several distinct signaling pathways. Incubation of serum-starved MCF-7 cells with 100 ng/ml ET-2 for 0–24 h led to a transient activation of p42 and p44 MAPK with maximal phosphorylation between 1 and 5 h (Fig. 2E). There was no activation of the stress-activated p38 or stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) pathways (Fig. 2E).

**ET-2 Increases Breast Carcinoma Cell Chemotaxis to Chemokines.** Because chemokines may play a role in breast tumor metastasis (22), we analyzed the ability of tumor cells exposed to ET-2 to chemotax toward CXCL12 and CCL21. Primary breast carcinoma cells and cell lines, including MCF-7, express the chemokine recep-
Peptides were added to the lower wells of the invasion chamber during whether ETs may induce invasion in a chemotactic manner, ET receptors at the cell surface (data not shown). Fluorescence-activated cell sorting analysis showed that there was no up-regulation of these receptors CXCR4 and CCR7 respectively. Chemokines CXCL12 and CCL21 was due to up-regulation of the chemokine receptors CXCR4 and CCR7 respectively. Furthermore, incubation of macrophages with ET-2 leads to increased activation of these cells (11). Therefore, we evaluated the effect of ETs on cocultured macrophages/tumor cells. The presence of macrophages led to increased MCF-7 tumor cell invasion through Matrigel compared with tumor cells cultured in isolation (Fig. 4C). Addition of ET-1 or ET-2 to the cocultured cells led to a further increase in invasion by MCF-7 cells, but ET-3 had no effect (Fig. 4C).

To test which ET receptor(s) was involved in the increased invasion of cocultured MCF-7 cells, we added ET-RA and ET-RB antagonists (singularly and in combination) to the invasion chambers. Unlike the chemotaxis assays, which showed partial inhibition of MCF-7 migration by both ET-RA and ET-RB antagonists and an additive effect when used in combination, both BQ-123 and BQ-788 could entirely abolish the effect of ET-2 on invasion of MCF-7 cells (Fig. 4D); the effects of BQ-123 and BQ-788 were not additive. Neither BQ-123 nor BQ-788 inhibited macrophage-induced invasion of MCF-7 cells when ETs were not added (data not shown).

ETs Increase MMP Production by Macrophages in Coculture. The increase in tumor cell invasion induced by ET-1 and ET-2 in coculture suggested that the macrophages were producing a soluble factor that aided tumor cell invasion. We analyzed MMPs-2 and -9 production by tumor cells and macrophages using zymography. MMP-7 cells produced little or no MMP-2 or -9 with or without stimulation by ETs (Fig. 5A). Cultured in isolation, macrophages produce MMP-2 and -9, but production was not altered by the addition of ETs (Fig. 5B). However, when cocultured tumor cells and macrophages were treated with ETs, all of the ETs caused an increase in MMP-2 production (Fig. 5C). The addition of 900 μM FN439, a nonspecific MMP inhibitor, to the coculture invasion assay abolished the increase in tumor cell invasion caused by ET-2 (Fig. 5D).

We analyzed MMP and TIMP (TIMP-1, TIMP-2, and TIMP-3) mRNA expression by macrophages in coculture by real-time PCR. The addition of ETs or receptor antagonists did not alter the macrophage mRNA expression of MMPs or TIMPs (data not shown).

ET and ET Receptor Expression Increases from DCIS to IDC to Lymph Node Metastases. In a preliminary study, we compared protein expression of ET and ET receptors in 9 cases of DCIS, 10 IDCs, and 10 lymph node metastases of primary breast carcinomas. ETs were expressed in the cytoplasm, with an increased level in tumor cell invasion by MCF-7 cells/macrophage. All of the ETs-stimulated release of active MMP-9 and -2 into the coculture medium. For all of the zymograms, cells were stimulated with 100 ng/ml ET for 96 h in gelatinase-free medium before the medium was collected. Shown is one representative experiment of three. D, inhibition of ET-induced invasion in MCF-7/macrophage coculture by a nonspecific MMP inhibitor, FN439 (900 μM) significantly reduced ET-2-stimulated invasion of MCF-7 cells in coculture (P < 0.05).
vivo and in view of the potential autocrine role(s) of ET-2 in breast neoplasia, we analyzed whether ET-2 induced ET receptor production by MCF-7 cells in vitro. Cells stimulated with 100 ng/ml ET-2 showed a rapid and sustained (1–24 h) increase in the production of ET-RB (Fig. 7); ET-RA production by MCF-7 cells exposed to ET-2 was increased by 24 h.

DISCUSSION

Many tumor-linked factors have been implicated in the development and progression of invasion and there has recently been interest in the role of ET-1/ET-RA, particularly in ovarian and prostate cancer metastasis (14, 23). Normal breast tissue expresses little ET or ET-RB, but intratumoral hypoxia (24, 25) induces expression of ET-2 and both ET receptors by tumor cells (6). We have now shown that ETs induce a chemotactic and invasive phenotype in breast tumor cells in vitro, particularly when tumor cells are cocultured with macrophages. Furthermore, data from the preliminary immunohistochemical study indicates that ET and ET receptor expression increases from normal breast to DCIS to IDC to metastatic deposits. This is the first time a possible role for ETs in breast cancer invasion has been identified and also the first time that ET-2/ET-RB has been implicated in invasion/progression.

It is interesting that tumor cells migrate strongly toward ET-2; however, it is difficult to understand whether this may have a broader role in metastasis. Physiologically, ET-2 expression is limited to the kidney and the intestine and is not released systemically in any great concentration (1). Thus, it is unlikely that chemotaxis toward ET-2 by...
breast tumor cells could explain organ-selective metastases, although breast cancers may metastasize to the kidney (27). It is also worth noting that ET-1 is expressed at several additional sites of breast cancer metastasis including bone (27).

It is conceivable, however, that migration toward ET-2 or ET-1 has an impact within the tumor, such that cells mass within hypoxic areas and become more aggressive because of exposure to ETs or by selective pressure of hypoxia. It is also possible that tumor cells may use ETs to migrate toward blood vessels, in which ET-1 is likely to be expressed (28), and thus can begin the process of intravasation. The high levels of ET and ET receptor expression by tumor cells within blood vessels and the high levels of gradient-independent invasion induced by ET-1 or ET-2 suggests that ET/ET receptor interactions have a role in intravasation and the preliminary steps of invasion and metastasis.

Systematically, it may be of more significance that treating tumor cells with ET-2 leads to increased chemotaxis toward the chemokines CXCL12 and CCL21, which are expressed by the lymph nodes and other sites of breast cancer metastasis. We were not able to detect an up-regulation of chemokine receptors on MCF-7 cells when stimulated by ET-2, and, thus, it may be that ET-2 impinges on the signaling pathways to amplify the response of tumor cells to chemokines.

Similar to macrophages (11), the preliminary steps in ET signaling in tumor cells is typical of chemokine signaling (29). Despite the action of ET-2 during cellular stress (6), ET-2 does not signal via the stress-activated p38 or SAPK/JNK pathways, but via the MEK1/2 and p42/p44 MAPK pathway. ET-2 signaling also involves activation of the G-protein-coupled ET receptors and an influx of extracellular calcium. Although ET-2 signaling in tumor cells has parallels with ET-2/ET-RB signaling in macrophages (11), it is clear that there are several differences. Macrophage chemotaxis toward ET-2 involves solely ET-RB, whereas chemotaxis and invasion of breast tumor cells involved both ET-RA and ET-RB. Chemotaxis toward ET-2 of both cell types can be inhibited by PD98059 or PTK, yet only tumor cells can migrate toward ET-2 under hypoxia. Both ET-2 and hypoxia increase ET receptor expression by tumor cells (6), and this may potentiate hypoxic migration. There may also be a role for ET-RA in hypoxic migration, which is expressed by tumor cells but not by macrophages (11). The chemotactic differences could also be due to the time of the chemotaxis assays; macrophage migration under hypoxia is inhibited rapidly by a short-term induction of MKP-1 (30), whereas tumor cells are allowed to migrate over a period of several hours.

Although the complete abolition of ET-induced chemotaxis required the inhibition of both receptors, ET-induced invasion of MCF-7 cells in coculture could be abolished by either receptor antagonist. This may be due to the complex processes and interactions that occur during invasion in macrophage/tumor cell coculture; individual elements of the invasion process may be mediated by one or other ET receptor allowing abolition of invasion by one receptor antagonist alone. The relative contributions of ET-RA and ET-RB signaling when tumor cells are stimulated by ET-2 are yet to be elucidated and may help us to understand the differences between macrophage and tumor cell signaling pathways.

Macrophages, which colocalize with ETs in breast cancer (11), increased MMP production in response to ETs, thus facilitating tumor cell invasion. Hagemann et al. (6) showed that the increase in MMP production in macrophage/tumor cell cocultures was due to the macrophages and not to the tumor cells. The inhibition of the MMPs was sufficient to prevent MCF-7 cell invasion, which shows the critical role of these enzymes in invasion. However, all of the ETs could increase production of MMP-2 and -9 by macrophages, yet ET-3 did not increase the invasion of tumor cells. This indicates that additional factors produced by cocultured cells and stimulated (or inhibited) by ETs, distinct from MMP-9 and MMP-2, must also play a role in tumor cell invasion. It is probable that there is a complex interaction between ET expression, hypoxia, and tumor-associated macrophages within the breast tumor such that invasion is increased. However, additional interactions may occur such as paracrine ET stimulation of tumor endothelial cells.

As well as the paracrine actions of ETs in breast tumors, those neoplasia that expressing ET in vivo also expressed the ET receptors, which may indicate an autocrine loop. We recently proposed an autocrine survival function for ET-2/ET-RB binding in breast carcinoma cells under hypoxic conditions (6). All of the human cell lines expressed ET-1 and ET-2 in vitro as well as one or more ET receptor; furthermore, production of the ET receptors by tumor cells in vitro was stimulated by ET-2. These data support an autocrine action of ETs to increase the invasiveness of tumor cells.

Examination of the expression of ETs and their receptors in a large number of breast carcinomas and metastatic deposits, and correlation with macrophase infiltrate, is now required to determine whether there is a correlation between ET/ET receptor expression and grade (or currently used conventional prognostic factors) and prognosis. Such studies may also indicate whether treatment of breast cancer and its metastases with ET receptor antagonists may have a therapeutic benefit. Because ETs stimulates not only breast cancer cell invasion but also chemotaxis toward chemokines, it may be that combined treatment with both ET receptor antagonists and chemokine receptor antagonists may potentially prevent breast cancer metastases better.
than do single treatments alone. Atrasentan, a selective ET-RA antagonist, is already in clinical trials for prostate cancer (31) and may be of therapeutic benefit for the treatment of IDC of the breast.

Up-regulation of ET-2 and its receptors by hypoxia (6), together with this data implying a role for ET-2 in invasion, may be one factor that explains why hypoxic tumors are more aggressive than nonhypoxic ones (10) and why hypoxia increases carcinoma cell invasion (32).

Not only is this the first time that a role for ET-2 or ET-RB in tumor cell invasion and metastasis has been proposed but it is also the first time the role of ET-stimulated macrophages in tumor cell invasion has been studied. The data showing ET-associated induction of MMP release by macrophages in coculture and increased chemotactic/invasive capacity of tumor cells together with increasing expression of ETs and ET receptor protein from DCIS to IDC to tumors in metastatic lymph nodes support a role for ET expression in progression and metastasis.

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