

Endothelins Induce CCR7 Expression by Breast Tumor Cells via Endothelin Receptor A and Hypoxia-Inducible Factor-1

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Abstract

Endothelin expression is increased in breast tumors and is associated with invasion and metastasis, whereas CCR7 expression by breast tumor cells may have a role in the organ specificity of breast cancer spread. In this article, we have analyzed whether endothelins influence breast tumor cell expression of the chemokine receptor CCR7. Stimulation of human breast tumor cell lines with endothelins increased cell surface expression of CCR7 via endothelin receptor A. The iron chelators desferrioxamine and cobalt chloride, which induce hypoxia-inducible factor (HIF)-mediated transcription, also increased CCR7 expression; transfection of a dominant-negative version of the HIF regulatory subunit, HIF-1 α , into MCF-7 cells abolished CCR7 induction by endothelins, indicating that increased expression is due to HIF-1 stabilization. Endothelin stimulation promoted invasion toward the CCR7 ligands CCL19 and CCL21. Endothelin-mediated chemokine-independent invasion itself is dependent on CCR7 activity and could be abolished using a CCR7-neutralizing monoclonal antibody. In human breast carcinomas, mRNA expression of endothelins correlated with the level of CCR7 expression, both of which were associated with the presence of lymph node metastases. Expression of the CCR7 ligands CCL19 and CCL21 was also higher in breast cancer patients with lymph node involvement compared with those without, but expression of these chemokines did not correlate with endothelin expression. These data show that CCR7 may be regulated by the breast tumor microenvironment and further support the use of endothelin receptor antagonists in the treatment of invasive and metastatic breast cancer. (Cancer Res 2006; 66(24): 11802-7)

Introduction

There is increased endothelin (ET-1, ET-2, and ET-3) and endothelin receptor (ET-RA and ET-RB) expression in breast tumors compared with nonneoplastic tissue (1, 2), and "endothelin axis" expression is associated with invasion and metastasis (3–5). We showed recently that endothelin expression by breast tumor cells, which express both ET-RA and ET-RB, leads to the tumor cells becoming more invasive (reviewed in ref. 6). The mechanism by which endothelins induce an invasive phenotype is complex and not fully understood but involves stimulation of both tumor and stromal cells (3) and modulation of matrix metalloproteinases and cytokines (6).

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Chemokine receptors, such as CCR7 and CXCR4, are expressed by many immune cells and are involved with trafficking to the lymph nodes. Cells expressing CCR7 respond to two ligands [i.e., CCL19 (MIP-3 β) and CCL21 (6Ckine)], although the ligand for CXCR4 is CXCL12 (SDF-1 α). As on leukocytes, chemokine receptor expression by tumor cells may control migration and survival. Expression of CCR7 and CXCR4 by breast tumor cells modulates the invasion and organ specificity of breast cancer metastases; the chemokine ligands for these receptors, CCL21 and CXCL12, respectively, are released by the proximal lymph nodes (7).

CCR7 is highly expressed in human breast cancer cells, malignant breast tumors, and metastases, and ligand binding to CCR7 induces chemotactic and invasive responses, including actin polymerization and pseudopodia formation (7). Several other tumor types express CCR7, and expression correlates with lymph node metastases and poor prognosis in esophageal cancer (8), non-small cell lung cancer (9), and squamous cell carcinoma of the head and neck (10). Melanoma cells express CCR7 and migrate to CCL21 *in vitro* (11). Although the importance of CCR7 has been shown in breast cancer metastasis (7), there have been few reports describing the mechanism by which CCR7 expression is up-regulated in tumor cells. There is epigenetic up-regulation of CCR7 in melanoma cells; treatment with 5-aza up-regulates CCR7 mRNA and protein (12).

In contrast to CCR7, CXCR4 regulation in tumor cells has been extensively studied. CXCR4 expression can be up-regulated by hypoxia in ovarian cancer cells (13) or by vascular endothelial growth factor (VEGF) in breast carcinoma cells (14). CXCR4 induction by hypoxia is dependent on stabilization of the regulatory hypoxia-inducible factor (HIF) subunit HIF-1 α (13). In ovarian carcinoma cells, ET-1 stabilizes HIF-1 α , leading to increased expression of VEGF (15).

As HIF-1 α stabilization may influence chemokine receptor expression by tumor cells, we have examined whether endothelins modulate CCR7 expression by breast tumor cells and may have a role in the organ specificity of breast cancer metastasis. We report here that endothelins modulate CCR7 expression in breast cancer cell lines via a mechanism involving ET-RA and HIF-1 and that expression of ET-1 and CCR7 correlates in human breast carcinomas.

Materials and Methods

Peptides and antagonists. Endothelin peptides, ET-RA antagonist BQ-123 (16, 17), and ET-RB antagonist BQ-788 (18) were obtained from American Peptide Co. (Sunnyvale, CA).

Cell culture. Breast tumor cell lines MCF-7, SKBR3, and MDAMB231 were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 25 μ g/mL insulin. Endothelins or endothelin receptor antagonists were added to the cell culture medium to give a final concentration of 100 ng/mL, and cells were incubated in the presence of peptides or antagonists for 24 hours unless otherwise stated.

Figure 1. CCR7 expression by tumor cells is induced by ET-1. *A*, comparison of CCR7 expression by MCF-7, SKBR3, and MDAMB231 cells using quantitative real-time PCR. Chemokine receptor mRNA (relative to β -actin mRNA) is normalized to untreated cells. One representative experiment. *Columns*, mean ($n = 3$); *bars*, SD. ET-1 (100 ng/mL, 24 hours) significantly increased expression of CCR7 by tumor cells. *, $P < 0.05$. *B*, flow cytometry analysis of cell surface chemokine receptor expression by tumor cells. *Dashed line*, unstimulated cells; *filled line*, endothelin-treated cells; *gray line*, matched isotype control. Cell surface CCR7 could be detected on all cell lines. Stimulation with ET-1 increased CCR7 expression by all cell lines compared with untreated cells and this could be reversed by BQ-123; BQ-788 had no effect.

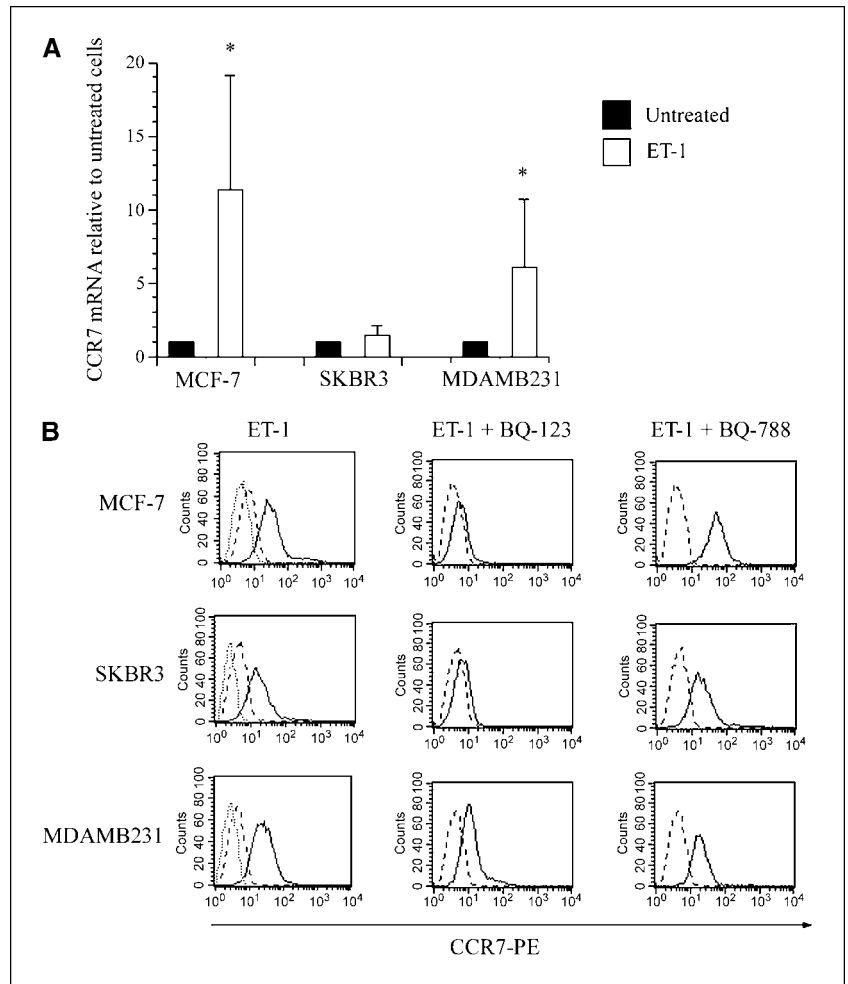
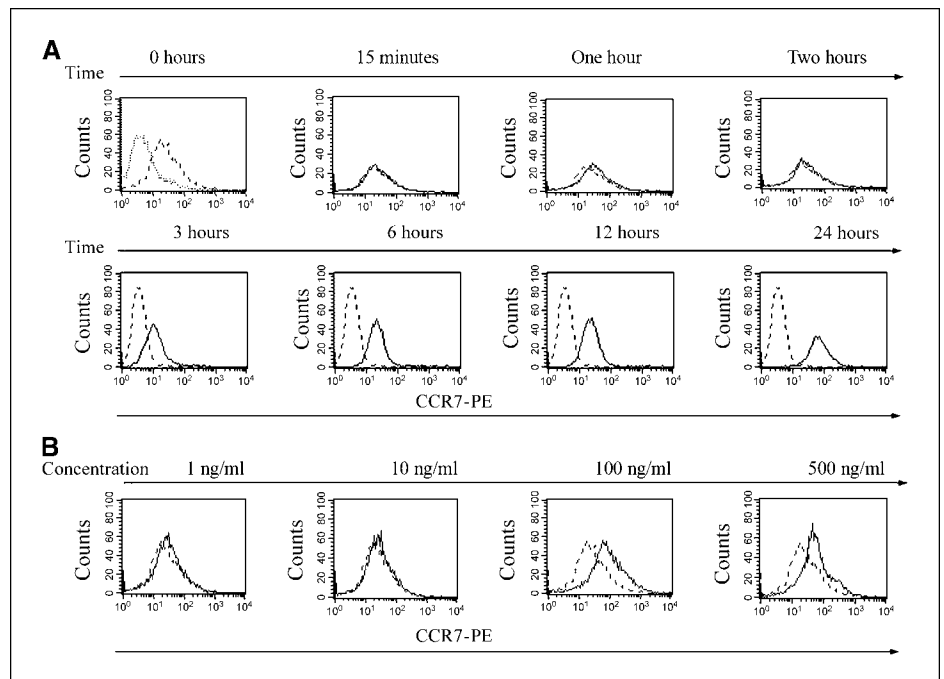


Figure 2. Kinetics of endothelin-mediated CCR7 induction. *A*, MCF-7 cells were stimulated with 100 ng/mL ET-1 for 0 to 24 hours and their cell surface CCR7 was analyzed by flow cytometry. *Dashed line*, unstimulated cells; *filled line*, endothelin-treated cells; *gray line*, matched isotype control. CCR7 was increased by 3 hours and remained increased at 24 hours. *B*, MCF-7 cells were stimulated with 0 to 500 ng/mL ET-1 for 24 hours. Cell surface CCR7 was not markedly increased at 1 or 10 ng/mL ET-1, but 100 ng/mL ET-1 produced a robust increase in CCR7. Higher concentrations of ET-1 did not induce CCR7 further.



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RNA extraction, reverse transcription, and quantitative real-time PCR. Total RNA was extracted from cells using TriReagent (Sigma-Aldrich Co. Ltd., Poole, United Kingdom). DNase-treated RNA (2 μ g) was reverse transcribed using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen Ltd., Paisley, United Kingdom). Primers for human chemokine receptors (and β -actin control) and their product sizes were as follows: β -actin, 5'-CCCTCCATCGTCCACCGCAAATGCTTC and 3'-CGACTGTGTACCTTCACCGTTCCAG (204 bp) and CCR7, 5'-GAAAG-TCCAGAACTGTTCCACCTGC and 3'-TTCCTACCAAGCCAAGAGTCT-CCCC (202 bp).

Quantitative real-time PCRs were done in triplicate using the Opticon Real-time PCR Analysis System (MJ Research, Inc., Waltham, MA) and a hot-start PCR that contained SYBR Green (Sigma-Aldrich) and 2 μ M/L forward and reverse primers. After an initial 10 minutes at 95°C, 40 cycles were done: 15 seconds at 94°C, 30 seconds at 60°C, 30 seconds at 72°C, and 10 seconds fluorescence detection at 78°C. Each sample was normalized to β -actin by removing the cycle threshold (C_t) value of β -actin from the C_t value of the gene under investigation (ΔC_t). The fold difference was calculated by subtracting the average ΔC_t of the test sample from the average ΔC_t control sample to give $\Delta\Delta C_t$, and then fold difference = $2^{-\Delta\Delta C_t}$.

HIF-1 α dominant-negative vector. HIF-1 α dominant-negative vector was a kind gift from Dr. D.E. Richard (Centre de Recherche de L'Hotel-Dieu de Québec, Quebec City, Quebec, Canada). Tumor cells were transfected as described in ref. 5. Stable transfectants were generated using the selectable marker Neo, and HIF-1 activity was analyzed using a HIF-1 activity ELISA (data not shown).

Monoclonal antibodies and flow cytometry. Phycoerythrin-labeled monoclonal antibodies (mAb; R&D Systems, Abingdon, United Kingdom) against chemokine receptors and isotype-matched labeled controls were used to characterize cell surface phenotypes by flow cytometry. For staining, cells were washed and resuspended in PBS supplemented with 1% heat-inactivated FBS and 0.01% NaN_3 . Antibodies were diluted in this buffer and used at a final concentration of between 2 and 20 μ g/mL. Incubations with antibodies were carried out for 30 minutes on ice. Following the final washing step, labeled cells were fixed with 1% formaldehyde solution and 10,000 cells were analyzed by flow cytometry on a FACScan flow cytometer using CellQuest software (Becton Dickinson, Oxford, United Kingdom). The CCR7-neutralizing mAb was also obtained from R&D Systems.

Invasion assay. Invasion was measured by assessment of the cell migration rate through an 8- μ m membrane coated with an artificial basement membrane (BD Matrigel from BD Biosciences Europe, Erembodegem, Belgium) in a modified Boyden chamber as described in ref. 3. Briefly, tumor cells (10^5 per well in 2 mL DMEM) were seeded into the upper well of the chamber, whereas the lower well contained medium supplemented with chemoattractant. All invasion assays were carried out for 72 hours unless otherwise stated. After 72 hours, cells were collected from both the lower side of the membrane and lower well, then spun down onto coverslips and then stained with 4',6-diamidino-2-phenylindole. The number of intact nuclei per high power field was counted by UV microscopy (Axioskop, Zeiss, Jena, Germany). If no cells could be detected in the lower well, cells were stained and counted directly on the lower side of the membrane.

Patients. The study population consisted of female breast cancer patients participating in a standard postsurgical follow-up program at the University Hospital Göttingen (Göttingen, Germany). The study was approved by the local ethics committee. Patients were prospectively enrolled between 1990 and 1995, when they consented to scientific analysis of their clinical and laboratory data and to storage of tumor samples for further evaluation. Tumor samples were taken at the time of surgery of the primary tumor and before chemotherapy. Frozen tissue was homogenized in a mill under liquid nitrogen (Glen Creston, Stanmore, United Kingdom) before RNA extraction.

Statistical analysis. All experiments were done in triplicate, and representative data are shown. Linear regression and statistical significance (ANOVA or Student's *t* test) were tested using InStat version 3.0 software.

Results

Endothelins induce breast tumor cell CCR7 expression via ET-RA. Using quantitative real-time PCR, we analyzed CCR7 mRNA expression by breast cancer cell lines (MCF-7, SKBR3, and MDAMB231) that express both ET-RA and ET-RB (5). CCR7 was expressed by all cell lines, and stimulating cells with ET-1 (100 ng/mL for 24 hours) led to increased CCR7 mRNA (Fig. 1A). SKBR3, which expresses lower ET-RA mRNA than either MCF-7 or MDAMB231 cells (5), increased CCR7 mRNA in response to ET-1 stimulation less robustly (<1.5- to 2-fold) compared with the other cell lines.

We next used flow cytometry to analyze cell surface chemokine receptor expression in response to endothelin stimulation in the breast tumor cell lines (Fig. 1B). In all cell lines, cell surface CCR7 protein was induced by ET-1 and this could be reversed by the ET-RA antagonist BQ-123; the ET-RB antagonist, BQ-788, had no effect.

Cell surface CCR7 is increased within 3 hours of endothelin stimulation. We next studied the time course of induction of CCR7 protein at the cell surface by ET-1 in MCF-7 cells. Cell surface expression of CCR7 remained unchanged between 15 minutes and 2 hours of endothelin stimulation, but increased CCR7 could be seen by 3 hours of ET-1 stimulation and remained elevated at 6, 12, and 24 hours (Fig. 2A) and also at 48 and 72 hours (data not shown).

A concentration curve of endothelin stimulation of MCF-7 cells (0, 1, 10, 100, and 500 ng/mL ET-1 for 24 hours) showed that CCR7

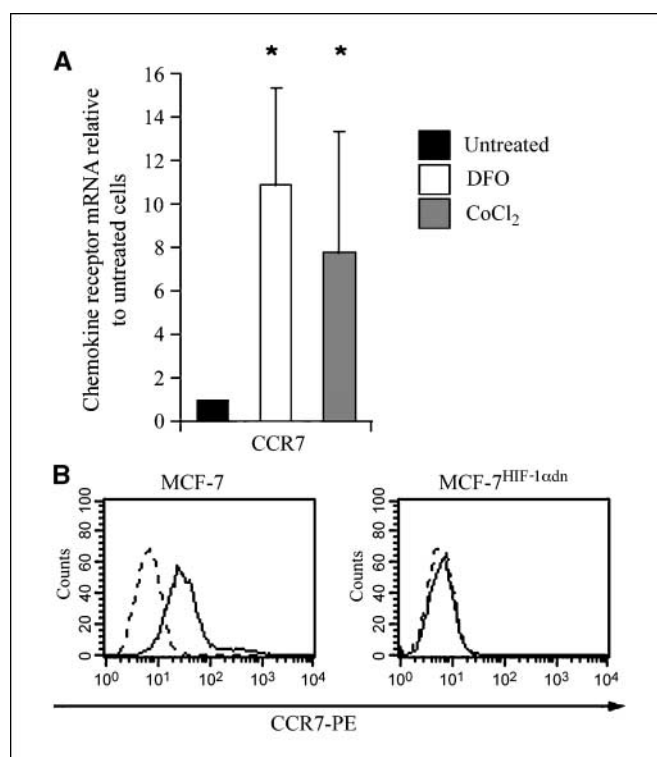


Figure 3. Endothelin stimulation of CCR7 involves HIF-1. **A**, MCF-7 cells were stimulated with the HIF-1 α stabilizers, 100 μ M/L desferrioxamine (DFO) or 50 μ M/L CoCl_2 for 24 hours, and CCR7 expression was analyzed by quantitative real-time PCR. Both desferrioxamine and CoCl_2 significantly increased CCR7 mRNA abundance compared with untreated cells. *, $P < 0.05$. **B**, mock-transfected MCF-7 cells and MCF-7 cells expressing dominant-negative HIF-1 α (MCF-7^{HIF-1 α dn}) were stimulated with ET-1, and cell surface expression of CCR7 was analyzed by flow cytometry. Mock transfectants treated with ET-1 (filled line) had increased CCR7 compared with untreated cells (dashed line); however, no such increase was seen in MCF-7^{HIF-1 α dn} cells.

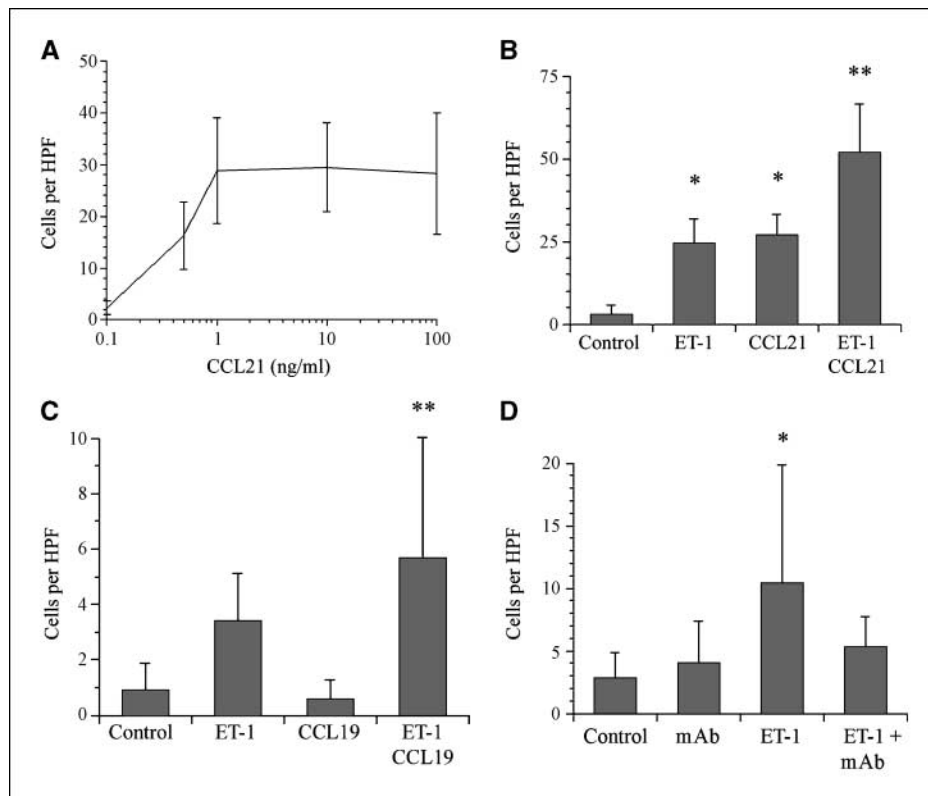


Figure 4. Stimulation of invasion by ET-1 and CCR7 ligands. *A*, MCF-7 cells were used in invasion assays with increasing concentrations (0–100 ng/mL) of CCL21 in the lower chamber of the invasion assay. The maximum rate of invasion was achieved at 1 ng/mL. *B*, MCF-7 cells were stimulated with 100 ng/mL ET-1 for 24 hours and then washed before being used in invasion assays with CCL21 in the lower chamber of the assay. ET-1 stimulation before the assay induced chemokine-independent invasion significantly greater than unstimulated control cells (*, $P < 0.05$), whereas CCL21 in the lower chamber induced chemokine-dependent invasion (*, $P < 0.05$). ET-1 stimulation together with CCL21 in the lower chamber had an additive effect and produced a level of invasion significantly higher than either endothelin or CCL21 alone. **, $P < 0.05$. One representative experiment of three. *Columns*, mean ($n = 10$ fields); *bars*, SD. *C*, ET-1-stimulated MCF-7 cells were used in invasion assays with 100 ng/mL CCL19 in the lower chamber. In these experiments (Fig. 5C and D), cells were counted directly on the membrane and hence the number of cells per high power field is lower than that in Fig. 5B. CCL19 alone did not stimulate invasion, but ET-1 stimulation together with CCL19 in the lower chamber significantly increased invasion compared with ET-1 alone. **, $P < 0.05$. *D*, MCF-7 cells in chemokine-independent invasion assays were treated with 5 μ g/mL CCR7 mAb. Whereas ET-1 stimulated invasion compared with unstimulated control cells (**, $P < 0.05$), endothelin-mediated invasion was abolished by the addition of the CCR7-neutralizing antibody. An isotype control mAb had no effect on invasion (data not shown).

was not markedly or reproducibly increased at low (1–10 ng/mL) concentrations but that CCR7 was strikingly increased at 100 ng/mL ET-1 (Fig. 2B). Higher doses (≥ 500 ng/mL ET-1) did not induce CCR7 further.

Endothelin-mediated chemokine receptor production is HIF-1 dependent. Endothelins may stabilize HIF-1 α in ovarian carcinoma cells (15), and the induction of CXCR4 expression during hypoxia is HIF-1 dependent (13). We incubated MCF-7 cells with the iron chelators desferrioxamine or cobalt chloride (CoCl₂), which induce HIF-1-dependent transcription (19). Cells stimulated for 24 hours with 100 μ mol/L desferrioxamine or 50 μ mol/L CoCl₂ increased CCR7 mRNA expression compared with untreated cells (Fig. 3A).

To confirm that endothelin-mediated chemokine receptor induction involved the activation of HIF-1, we transfected MCF-7 cells with a vector encoding a dominant-negative HIF-1 α . Stable transfectants showing decreased HIF-1 activity (MCF-7^{HIF-1 α dn}) were stimulated with ET-1, and cell surface CCR7 was compared with mock transfectants. Expression of the HIF-1 α dominant-negative vector by MCF-7 cells led to the complete abolition of the induction of cell surface CCR7 by ET-1 (Fig. 3B).

ET-1 and CCR7 ligands induce MCF-7 cell invasion. Endothelins induce invasion of MCF-7 cells through Matrigel independent of the presence of a chemokine (6), which complicates

any assay to measure chemotactic invasion of endothelin-stimulated cells. Nevertheless, it is important to show that endothelin-stimulated cells can invade toward a chemokine to a greater extent than unstimulated cells. MCF-7 cells were stimulated with ET-1 for 24 hours and then washed before being used in invasion assays with either CCL19 or CCL21 as a chemoattractant in the lower chamber of the assay. Unstimulated MCF-7 cells reached their maximum rate of invasion toward CCL21 at 1 ng/mL (Fig. 4A); higher concentrations of the chemokine did not induce further invasion. ET-1 induced invasion of MCF-7 cells through Matrigel similar to the maximum level of invasion induced by CCL21 (Fig. 4B). However, when MCF-7 cells were treated with ET-1 before the invasion assay, a level of invasion that was approximately additive (ET-1 stimulated plus CCL21 stimulated) was achieved.

In contrast, MCF-7 cells did not significantly increase invasion when 100 ng/mL CCL19 was added to the lower chamber of the invasion assay; cells treated with ET-1 however, invaded strongly toward CCL19 (Fig. 4C).

Next, to determine whether CCR7 is involved in endothelin-mediated invasion, we blocked CCR7 function with a neutralizing mAb. Although ET-1 stimulates (chemokine independent) invasion through Matrigel, this was entirely blocked by CCR7 inhibition (Fig. 4D).

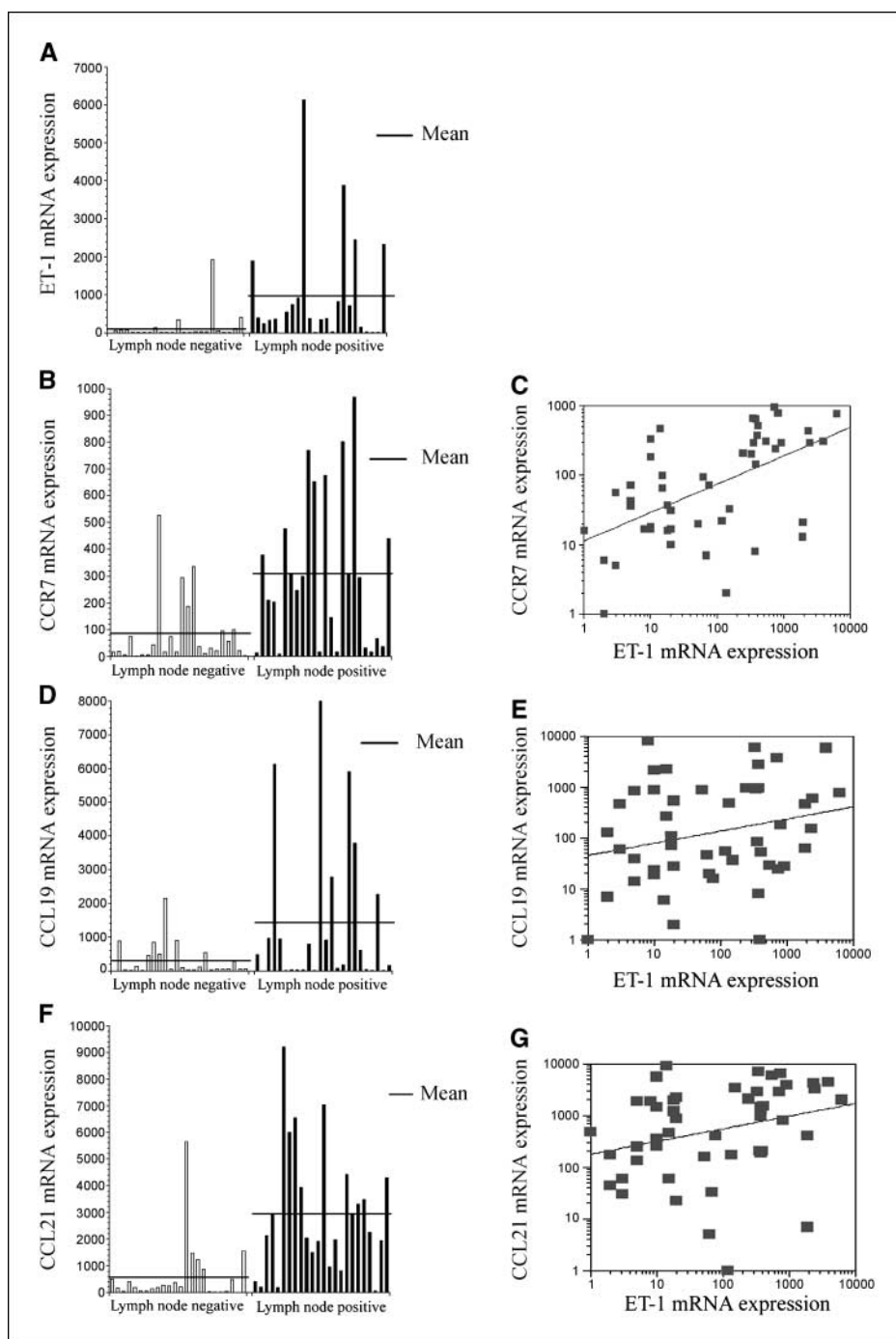


Figure 5. Analysis of expression of ET-1, CCR7, CCL19, and CCL21 mRNA expression by breast tumors with and without lymph node involvement. Total RNA was extracted from breast carcinomas without lymph node involvement ($n = 24$) and from carcinomas with lymph node metastases ($n = 24$). Expression of ET-1, CCR7, CCL19, and CCL21 was analyzed by quantitative real-time PCR. For each gene, samples were normalized to the sample with the highest ΔC_t value (i.e., the lowest expression). **A**, ET-1 expression (mean, $550.5 \pm 1,140$) is significantly higher in tumors with lymph node involvement than those without ($P < 0.0005$). **B**, expression of CCR7 (mean, 195.6 ± 247.7) was significantly higher in lymph node-positive tumors ($P < 0.005$). **C**, ET-1 expression was plotted against CCR7 expression. Linear regression analysis showed that ET-1 expression significantly correlated with CCR7 expression [$P = 0.0022$; correlation coefficient (r) = 0.43; $r^2 = 0.19$; SD of residuals (Sy.x) = 226.0; multiple comparisons correction (MCC) = 10.0%]. **D** and **E**, CCL19 expression (mean, $862.7 \pm 1,723$) was higher in lymph node-positive tumors ($P < 0.05$), but ET-1 and CCL19 mRNA expression did not correlate ($P = 0.27$; $r = 0.16$, $r^2 = 0.027$; Sy.x = 1,718.3; MCC = 100%). **F** and **G**, CCL21 expression (mean, $1,762.2 \pm 2,199.5$) was higher in lymph node-positive tumors ($P < 0.0001$), but ET-1 and CCL21 mRNA expression did not correlate ($P = 0.18$; $r = 0.20$; $r^2 = 0.04$; Sy.x = 2,179.9; MCC = 99.99%).

ET-1 expression correlates with CCR7 expression in invasive breast carcinoma and is associated with lymph node-positive breast tumors. Using quantitative real-time PCR, we assessed the level of expression of ET-1 and CCR7 in breast tumors from patients with lymph node metastases (24 patients) compared with patients with breast cancer with no lymph node involvement (24 patients). There was significantly higher mRNA expression of both ET-1 and CCR7 in tumors from patients with lymph node metastases than from those without (Fig. 5A and B). Linear regression analysis of ET-1 expression versus expression of CCR7 showed that the level of tumor ET-1 expression positively correlated with the level of CCR7 mRNA expression (Fig. 5C).

Expression of the CCR7 ligands CCL19 and CCL21 was also significantly higher in lymph node-positive tumors compared with breast tumors with no lymph node involvement (Fig. 5D and F). There was, however, no statistically significant correlation between the level of ET-1 expression and the level of CCL19 or CCL21 expression (Fig. 5E and G).

Both ET-RA and ET-RB mRNAs can be detected in the majority of invasive ductal carcinomas (IDC; ref. 3), although moderate/strong immunostaining is observed in approximately half of IDCs (ET-RA, 45.3%; ET-RB, 55.7%; ref. 4). Where available, patient cDNA was tested for ET-RA and ET-RB expression. All tumors tested (24 of 24) expressed ET-RA, but level of expression did not

correlate with expression of CCR7 (data not shown). ET-RB could be detected in 17 of 24 tumors, but CCR7 expression did not correlate with ET-RB expression (data not shown).

Discussion

The endothelin axis is overexpressed in carcinomas of the breast, and expression is associated with invasion and lymph node metastasis (3, 4). The mechanism by which endothelins increase invasion includes autocrine and paracrine actions resulting in increased chemotaxis of tumor cells and the production of proteases. The chemokine receptors CCR7 and CXCR4 have a critical role in breast cancer invasion and the organ specificity of metastasis. In this article, we show that expression of CCR7 is modulated by endothelin stimulation in established breast cancer cell lines and is associated with endothelin expression in human breast cancer tissue biopsies.

CXCR4 is induced by hypoxia via HIF-1 in several cell types (13), and stabilization of the regulatory subunit HIF-1 α by endothelins has previously been shown in ovarian carcinoma cells (15); in this system, endothelin stimulation via ET-RA leads to transcription of VEGF. It was these data that led us to analyze regulation of CCR7 by endothelins in breast tumors. The mechanism for endothelin induction and up-regulation of CCR7 in breast cancer involves signaling via ET-RA and requires the HIF-1 transcription factor, suggesting a similar pathway to that shown in ovarian carcinoma cells. Epigenetic mechanisms may also influence CCR7 expression on tumor cells at least *in vitro* (12). However, although the HIF-1 transcription factor is involved in CCR7 up-regulation, the increase in CCR7 mRNA expression on endothelin stimulation is relatively modest (particularly in SKBR3 cells) compared with the striking increase at the cell surface, suggesting that further posttranscriptional/translational controls are also involved.

Our patient data show that, in breast cancer, ET-1 expression correlates with CCR7 expression and that this combination is

associated with lymph node involvement. Hence, it seems that coexpression of these two molecules results in a more invasive phenotype facilitating the development of distant metastasis. Indeed, the maximum rate of breast cancer cell line invasion *in vitro* was observed when cells were stimulated with both ET-1 and the CCR7 ligand CCL21. Endothelin stimulation also potentiated invasion toward the second CCR7 ligand, CCL19.

Recent prognostic studies also strengthen our conclusions relating to CCR7 expression and tumor spread in breast cancer (20), colorectal cancer (21), and oral and oropharyngeal cancer (22).

As well as endothelin stimulation inducing CCR7-dependent chemotactic invasion, it was also found that CCR7 signaling is involved in endothelin-mediated chemokine-independent invasion. As noted previously, the exact mechanism by which endothelins stimulate invasion is not fully understood and may involve several disparate actions, and this is the first indication that CCR7 is involved in endothelin-induced invasion.

The importance of endothelin expression (5), chemokine receptor expression (7), and HIF-1 activity (23) has all been shown previously in invasive breast cancer. We have now shown that endothelins induce CCR7 expression in breast cancer cells via a mechanism involving ET-RA and HIF-1 and that ET-1 and CCR7 expressions correlate in breast cancer biopsies. Targeting either ET-RA or CCR7 may be therapeutically beneficial in the treatment of invasive or metastatic breast cancer.

Acknowledgments

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