Vascular Endothelial Growth Factor Promotes Breast Carcinoma Invasion in an Autocrine Manner by Regulating the Chemokine Receptor CXCR4

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Abstract

We report that vascular endothelial growth factor (VEGF), a major angiogenic factor, is also a requisite autocrine factor for breast carcinoma invasion in vitro and that the VEGF receptor Neuropilin-1 but not Flt-1 is essential for this function. VEGF regulates expression of the chemokine receptor CXCR4, and this VEGF target is needed for invasion but not for cell survival. CXCR4 mediates migration of breast carcinoma cells toward stromal-derived factor-1, and this migration is dependent on autocrine VEGF. Of interest, a CXCR4-inhibitory peptide that is currently in HIV clinical trials suppressed invasion. Our findings indicate that a VEGF autocrine pathway induces chemokine receptor expression in breast carcinoma cells, thus promoting their directed migration toward specific chemokines.

Introduction

Tumor cells often acquire the ability to support autocrine signaling pathways by expressing growth factors and their cognate surface receptors. In this direction, we reported recently that VEGF, a major angiogenic factor, is also an essential autocrine factor for the survival of metastatic breast carcinoma cells in vitro (1, 2). Key issues, however, are establishing the extent to which such autocrine pathways facilitate tumor invasion and defining the mechanisms involved. Although previous studies have indicated the ability of exogenous VEGF to promote tumor cell migration, they did not establish an autocrine function for this factor (3–5). We report here that VEGF produced by breast carcinoma cells is critical for their invasion, and that the chemokine receptor CXCR4, which mediates the migration of these cells toward SDF-1, is an important target of this autocrine pathway.

Materials and Methods

Reagents. The MDA-MB-231 breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository (Georgetown University). The generation of MDA-MB-435 breast carcinoma cells that stably express the α6β4 integrin has been described previously (6). Human dermal fibroblasts were provided by Dr. Donald Seng (Beth Israel Deaconess Medical Center). Reagents used in this study, and their respective sources, are as follows: ZVAD-FMK (Promega), Annexin V-FTTC (Biosource), pertussis toxin (Calbiochem), cholera toxin (Calbiochem), recombinant VEGF165 (Biological Resources Branch, National Cancer Institute), recombinant human SDF-1 (Calbiochem), and ALX40–4C (N-acetyl-nona-t-arginine amide; American Peptide Company). The Abs used in this study were obtained from the following sources: rabbit IgG, mouse IgG1, mouse IgG, mouse IgG2b, and rabbit anti-actin (Sigma); Flt-1 polyclonal Ab (Santa Cruz); SDF-1-neutralizing Ab (R&D Systems); and horseradish peroxidase-conjugated goat antimouse and goat antirabbit IgG (Jackson Immunoresearch). A polyclonal Ab directed against rat NP-1 amino acids 813–827 and a NP-1-specific mAb were purchased from Oncogene Sciences and Santa Cruz, respectively. CXCR4-specific Abs (mAb 12G5, IgG2a, 44717.111, IgG 2b and 44716.111, IgG 2b ) were provided by the NIH AIDS Research and Reference Reagent Program. A rabbit polyclonal Ab directed against human VEGF was provided by Dr. Donald Seng.

Determination of Protein Expression. Proteins were extracted from cells directly in their wells with radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0)]. VEGF expression was assessed by immunoblotting, as described previously (2). Similarly, CXCR4 was detected by immunoblotting extracted proteins with a CXCR4-specific mAb (12G5). To control for protein loading, these blots were also probed with an actin-specific Ab.

To assess Flt-1 and NP-1 cell surface expression, cells were incubated with the following Abs at 2 μg/ml: mouse IgG, rabbit IgG, NP-1-specific mAb, and Flt-1 polyclonal Ab, followed by the appropriate phycoerythrin-conjugated secondary Ab; the cells were then analyzed by flow cytometry. To determine cell surface CXCR4 expression, cells were fixed with 4% paraformaldehyde and were incubated on ice for 20 min with the CXCR4-specific mAb 12G5 (5 μg/ml) or a mouse IgG2A (5 μg/ml). These cells were then washed with PBS, incubated on ice for 20 min with a phycoerythrin-conjugated goat antimouse IgG, and analyzed by flow cytometry.

Invasion and Migration Assays. Matrigel invasion assays, using NIH3T3-conditioned medium as a chemoattractant, were performed as described previously (6). Migration toward SDF-1 (100 nM in 0.1% BSA/DMEM) was assessed using collagen (Cohesion; 15 μg/ml)-coated Transwell chambers. The effects of inhibitory Abs, drugs, or peptides on invasion were determined by preincubating cells with the indicated inhibitory reagent for 30 min on ice. The ability of these cells to invade Matrigel was then assessed, as described above, in the presence of the indicated reagent.

RT-PCR. cDNA was isolated using the Qiagen RNAeasy kit. This RNA was added to one-step RT-PCR reactions (Qiagen), using either human Flt-1 specific primers (7) or NP-1-specific primers (8). RT was performed at 50°C for 30 min, followed by a 95°C 15-min heat inactivation step. This cDNA was then subjected to 30 cycles of amplification using the following parameters: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. A final extension step was then performed for 10 min at 72°C.

Results and Discussion

Autocrine VEGF Is Required for the Invasion of Breast Carcinoma Cells. We assessed the impact of reducing VEGF expression in invasive breast carcinoma cells on their ability to invade Matrigel using antisense phosphorothioate oligodeoxynucleotides (oligos). These antisense (AS) oligos reduced VEGF expression in each of two breast carcinoma cell lines by ~70% compared with a control sense oligo, as determined by immunoblotting (Fig. 1, A and B). To prevent the apoptosis that occurs in these cells after a reduction in VEGF expression (1, 2), the cells were maintained in the continual presence of the caspase inhibitor, ZVAD-FMK. As shown in Fig. 1A, this reagent inhibited the apoptosis that we had previously observed in VEGF AS-transfected MDA-MB-231 cells (96% effective). Expression of AS VEGF oligos resulted in a significant reduction (65%) in Matrigel invasion of MDA-MB-231 cells (Fig. 1B). This effect was a specific consequence of reduced VEGF expression because the addi-
tion of recombinant VEGF restored their ability to invade Matrigel (Fig. 1B). In addition, AS VEGF oligos inhibited the Matrigel invasion of MDA-MB-435/β4 cells, a highly invasive breast carcinoma cell line that stably expresses the α6β4 integrin (Fig. 1B; Ref. 6). We conclude from these data that VEGF produced by breast carcinoma cells is necessary for their invasion in vitro.

The VEGF Receptor NP-1 Supports VEGF Autocrine Invasive Function in Breast Carcinoma Cells. If VEGF autocrine signaling is an important component of invasion, then invasive cells must express specific receptors that mediate this signaling. The results from previous studies indicated that MDA-MB-231 cells lack the classical VEGF receptor tyrosine kinases Flt-1 and KDR (9) but express NP-1 (1, 10). On the basis of these results, it has been assumed that NP-1, a receptor that lacks classical consensus signaling sites, must cooperate with a signaling-competent coreceptor to deliver VEGF signals in MDA-MB-231 cells. Interestingly, we detected both NP-1 and Flt-1 in both MDA-MB-435/β4 and MDA-MB-231 cells by RT-PCR (Fig. 1C). To confirm these findings at the protein level, we also detected NP-1 and Flt-1 expression on the surface of MDA-MB-231 (Fig. 1C) and MDA-MB-435/β4 (data not shown) cells by flow cytometry. To examine the involvement of Flt-1 in invasion, MDA-MB-231 cells were incubated with either a rabbit immunoglobulin or Flt-1 polyclonal Ab that inhibits monocyte migration by blocking VEGF binding to Flt-1 (11). Surprisingly, invasion was not decreased by this Flt-1 Ab (Fig. 1D), even at 100 μg/ml (data not shown). In contrast, a NP-1-specific Ab inhibited invasion significantly, compared with a control IgG in the presence of ZVAD-FMK (Fig. 1D), but it did not influence survival under these conditions (data not shown). This NP-1-specific Ab also inhibited the invasion of MDA-MB-435/β4-expressing cells (data not shown). These data establish that endogenously expressed NP-1, but not Flt-1, contributes to breast carcinoma invasion.

Heterotrimeric G Protein Activity Is Important for Breast Carcinoma Invasion but not Survival. Given that our studies indicate the importance of autocrine VEGF in both breast carcinoma survival (1, 2) and invasion, we postulated that different downstream targets of VEGF signaling mediate these distinct VEGF functions. The fact that GPCRs are important for cell migration and invasion (12–14) provided a logical focal point to examine this possibility. As shown in Fig. 2A, pertussis toxin, a specific inhibitor of Go proteins,
inhibited invasion completely but cholera toxin, an inhibitor of Go proteins, had no effect. Surprisingly, however, neither drug influenced the survival of these cells (Fig. 2A). These findings indicate a requirement for Go signaling in invasion but not in survival.

The Chemokine Receptor CXCR4 Is a Target of VEGF Autocrine Signaling That Promotes Breast Carcinoma Invasion but not Survival. The dependence of invasion on Go proteins suggested that VEGF might promote invasion by regulating a GPCR. For this reason, we were intrigued by the observation that the Go coupled receptor CXCR4 is expressed in breast carcinoma cells and that it significantly impacts their metastasis (12). On the basis of this report, we postulated that CXCR4 is a target of VEGF autocrine signaling and that CXCR4 links VEGF to invasion. To test this hypothesis, we examined the effect of inhibiting VEGF on the expression of CXCR4. Indeed, we detected significantly reduced levels of CXCR4 protein in VEGF AS- relative to VEGF sense-transfected MDA-MB-231 and MDA-MB-435/Δ4 cells, and the addition of recombinant VEGF to AS-transfected cells restored CXCR4 expression (Fig. 2B). Also, a significant reduction in CXCR4 surface expression (36%) was observed in VEGF AS-transfected relative to sense-transfected MDA-MB-231 cells (Fig. 2C). In contrast, we detected similar surface expression of uPAR in VEGF sense-transfected and VEGF AS-transfected cells (Fig. 2C), demonstrating that the expression of all cell surface proteins is not influenced by VEGF. Together, these data establish that endogenous VEGF is an important determinant of CXCR4 expression in breast carcinoma cells.

VEGF Promotes the Migration and Invasion of MDA-MB-231 Breast Carcinoma Cells toward Exogenous Sources of SDF-1. We next sought to identify the source of the CXCR4 ligand, SDF-1, that stimulates CXCR4 signaling in breast carcinoma cells. The invasion of MDA-MB-231 cells toward conditioned NIH3T3 medium was inhibited significantly (40%) by an SDF-1-neutralizing Ab (Fig. 3A). This finding supports the importance of SDF-1 in breast carcinoma invasion, but it does not exclude a potential contribution of SDF-1 produced by the tumor cells themselves. However, we were unable to detect either SDF-1α or -1β mRNA in MDA-MB-231 cells (Fig. 3B). Furthermore, we did not detect SDF-1α or -1β protein in the culture medium of these cells by ELISA (data not shown). These data indicate that the ability of MDA-MB-231 cells to invade Matrigel is dependent on an exogenous source of SDF-1.

The above data imply that CXCR4-dependent migration and invasion require VEGF. A definitive test of this hypothesis would be to demonstrate that a reduction in VEGF expression abrogates migration toward SDF-1, a ligand for CXCR4 and not for other GPCRs (15, 16). As expected from previous studies (12), MDA-MB-231 cells migrate robustly toward SDF-1 (Fig. 3C). This migration, however, was reduced substantially by expression of the VEGF AS oligonucleotide (Fig. 3D). Although these data do not exclude a role for VEGF in chemokinesis, they indicate that endogenous VEGF regulates breast carcinoma invasion by inducing CXCR4 expression, which drives their directed migration toward SDF-1.

Finally, to establish a definitive role for CXCR4 in invasion, the effect of a CXCR4-neutralizing peptide (ALX40–4C) was tested. This peptide blocks the binding of SDF-1 to CXCR4 without influencing the binding of other chemokines to their respective GPCRs (17, 18). As shown in Fig. 4A, ALX40–4C inhibited Matrigel invasion by ~75%, but it did not decrease cell viability (data not shown). The invasion but not the survival of MDA-MB-231 cells was also inhibited by a CXCR4-specific Ab (Fig. 4B), substantiating the peptide results. These data demonstrate a distinct contribution of CXCR4 to the invasion but not to the survival of breast carcinoma cells. On the basis of these data, we suggest that ALX40–4C may be an effective therapeutic in preventing breast cancer spread. Of note, Phase I/II clinical trials in HIV-infected individuals have demonstrated that the
References


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