

Vascular Endothelial Growth Factor Is an Autocrine Survival Factor for Neuropilin-expressing Breast Carcinoma Cells¹

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Abstract

We identify a novel function for the vascular endothelial growth factor (VEGF) in its ability to stimulate an autocrine signaling pathway in metastatic breast carcinoma cells that is essential for their survival. Suppression of VEGF expression in metastatic cells *in vitro* induced their apoptosis, in addition to inhibiting the constitutively elevated phosphatidylinositol 3'-kinase activity that is characteristic of these cells and important for their survival. Hypoxia enhanced the survival of metastatic cells by increasing VEGF expression. The importance of the VEGF receptor neuropilin was indicated by the ability of a neuropilin-binding VEGF isoform to enhance breast carcinoma survival. Moreover, the expression of neuropilin in neuropilin-deficient breast carcinoma cells protected them from apoptosis. The identification of this VEGF autocrine signaling pathway has important implications for tumor metastasis and therapeutic intervention.

Introduction

Breast epithelial cells are able to survive and differentiate because of the tissue architecture and growth factor milieu present in the mammary gland (1). This rich environment, however, is progressively lost during transformation, especially as malignant cells become invasive and metastatic. One of the most formidable barriers to tumor survival is hypoxia (2). Although hypoxia kills most normal cells and some tumor cells (3–5), it also provides a strong selective pressure for the survival of the most aggressive and metastatic cells (6). These considerations substantiate the importance of defining the molecular characteristics of tumor cells that enable their survival. One survival strategy utilized by breast carcinomas and other tumors is the secretion of proteins that elicit an angiogenic response. For example, vascular permeability factor or VEGF⁵ appears to be an essential factor for breast carcinoma progression (7–9). It is widely assumed that the function of VEGF produced by breast carcinoma and tumor stromal cells is to stimulate angiogenesis by acting in a paracrine fashion on vicinal endothelium (8, 10). Surprisingly, however, the possibility that VEGF functions in an autocrine fashion on breast carcinoma cells to stimulate signaling pathways that maintain their survival has not been considered. This autocrine activity of VEGF could be important for survival in hypoxic, poorly vascularized areas

of solid tumors. Our results define a novel signaling pathway in breast carcinoma cells involving VEGF, the VEGF receptor neuropilin, and PI3-kinase that is likely to play an important role in breast carcinoma progression.

Materials and Methods

Cells. MDA-MB-231, MDA-MB-435, and MDA-MB-453 cells were obtained from the Lombardi Breast Cancer Depository (Georgetown University). Primary endothelial cells were provided by Dr. Donald Senger (Beth Israel Deaconess Medical Center). For hypoxia experiments, cells were cultured in low-serum culture medium (DMEM/0.5% FBS) and maintained in either normoxic (5% CO₂, 20% O₂, 75% N₂) or hypoxic (5% CO₂, 3% O₂, 94.5% N₂) conditions for the indicated amounts of time.

VEGF Antisense Strategy. Cells (2 × 10⁵ cells/well of a 12-well plate; 50% confluence) were transfected with either a VEGF antisense 2'-O-methyl phosphorothioate oligodeoxynucleotide (5'-CACCCAAGACAGCAGAAG-3') or a VEGF sense 2'-O-methyl phosphorothioate oligodeoxynucleotide (5'-CTTCTGCTGTCTTGGGTG) at a concentration of 0.3 μM in the presence of Lipofectin reagent (Life Technologies, Inc.; 2 μg/ml). These oligonucleotides contain 2'-O-methyl modifications at the last five nucleotides (3'). The design of these oligonucleotides has been described previously (11). After 4 h, the cells were washed with PBS and allowed to recover in DMEM/10% FBS. These conditions were determined to be optimal for inhibiting VEGF expression in these cell lines as determined by quantitative RT-PCR (data not shown).

Quantifying VEGF mRNA. mRNA was isolated from cellular extracts using the RNeasy kit (Qiagen). cDNA was synthesized from this RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and quantified by RT-PCR. Primers and probes were synthesized by Oligo Therapeutics (Wilsonville, OR) and Perkin Elmer (Foster City, CA), respectively. Primer and probe sequences were analyzed for specificity of gene detection using the NCBI Blast module by first derivative primer melting curve software supplied by Perkin Elmer/Applied BioSystems. Quantitative analysis of gene expression was generated using an ABI Prism 7700 Sequence Detection System (TaqMan) and the SYBR Green master mix kit. The sequences of the PCR primer pairs (5' to 3') that were used for each gene are as follows: VEGF forward, 5'-GGAGATCCTTCGAGGAGCACTT-3'; VEGF reverse, 5'-GGCGATTTAGCAGCAGATATAAGAA-3'; cyclophilin forward, 5'-CAGACGCCACTGTGCGCTT-3'; and cyclophilin reverse, 5'-TGTCTTTGGAACCTTGTCTGCAA-3'.

Apoptosis Assays. Apoptosis was assessed as described previously (12) using annexin V-FITC (Biosource) and PI (Biosource), annexin V-phycoerythrin (Promega), or the Apoptag kit (Oncor).

VEGF Receptor Expression. To assess VEGF receptor expression, proteins were extracted from cells in lysis buffer [20 mM Tris (pH 7.4), 0.14 M NaCl, 1% NP40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 50 μg/ml leupeptin, and 1 mM sodium orthovanadate]. Cellular debris was removed from these extracts by centrifugation at 12,000 rpm for 10 min at 4°C, and the concentration of total cellular protein was determined in these samples using the Biorad reagent (Biorad). Equivalent amounts of total cellular protein were subjected to reducing SDS-PAGE (6%), transferred to nitrocellulose, and probed with mouse monoclonal antibodies specific for either neuropilin or KDR, followed by HRP-conjugated goat

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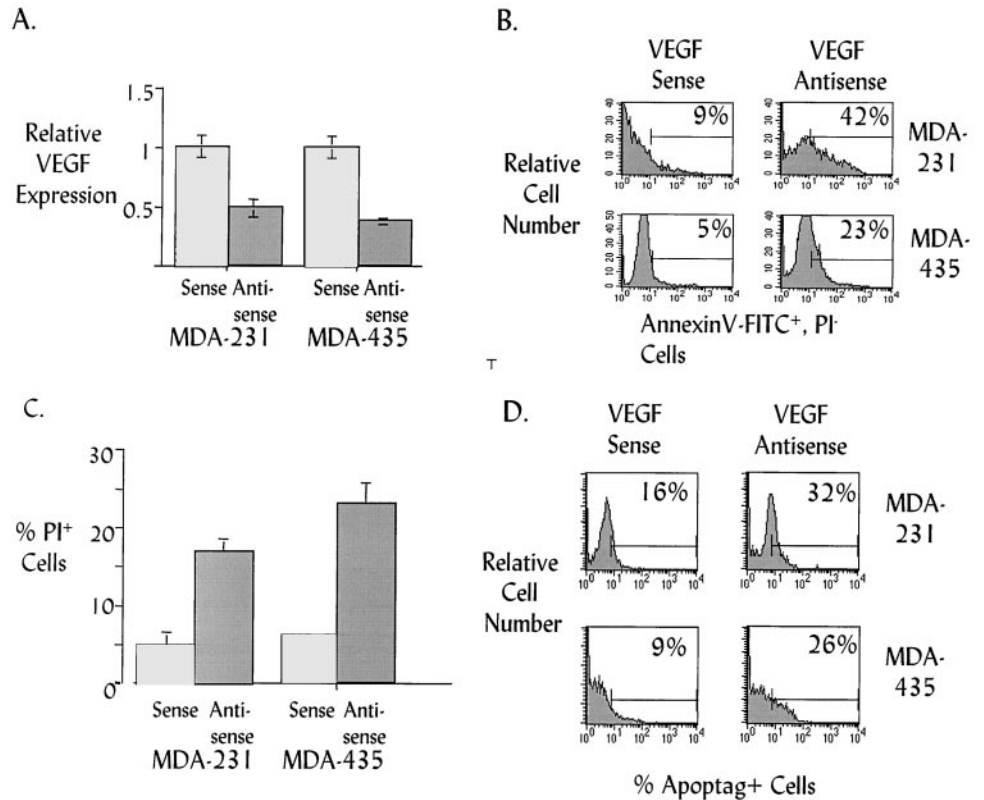
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⁵ The abbreviations used are: VEGF, vascular endothelial growth factor; PI3-kinase, phosphatidylinositol 3'-kinase; RT-PCR, real-time PCR; PI, propidium iodide; PKB, protein kinase B; HRP, horseradish peroxidase.

Fig. 1. Decreasing VEGF expression in metastatic breast carcinoma cells promotes their apoptosis. MDA-MB-231 and MDA-MB-435 cells were transfected with either a VEGF antisense or a control sense oligonucleotide and harvested 15 h (MDA-231) or 40 h (MDA-435) after transfection. **A**, the amount of VEGF mRNA in extracts from these cells was quantified by RT-PCR. The data are presented as the mean VEGF:cyclophilin mRNA ratio (\pm SD) obtained from triplicate samples. **B**, the level of apoptosis is indicated as the percentage of annexin⁺, PI⁻ cells. Similar results were obtained in five separate trials. **C**, the percentage of PI⁺ cells (\pm SD) is indicated. Similar results were obtained in three independent experiments. **D**, the level of apoptosis in these transfectants was measured by performing terminal deoxynucleotidyl transferase-mediated nick end labeling-based reactions using the Apoptag kit (Oncor). The data are presented as the percentage of Apoptag-positive cells.



antimouse IgG. These receptors were then detected by enhanced chemiluminescence (Pierce).

Results and Discussion

The Survival of Metastatic Breast Carcinoma Cells *in Vitro* Is Dependent on VEGF. To assess the importance of VEGF in breast carcinoma survival, we examined the effects of reducing VEGF expression on the survival of two well-characterized metastatic cell lines (MDA-MB-231 and MDA-MB-435). Both of these cell lines express VEGF (13) and exhibit spontaneous metastasis to lungs upon orthotopic injection in the mammary fat pad (14). These cells were transfected with VEGF antisense or sense oligonucleotides and allowed to recover in culture medium containing 10% FBS. As shown in Fig. 1A, expression of the antisense oligonucleotide in these cells reduced the steady-state levels of VEGF mRNA by approximately 50% compared with the sense oligonucleotide, as measured by quantitative RT-PCR. The effect of decreased VEGF expression on cell survival was then assessed by incubating these cells with annexin V-FITC and PI. A 4-fold increase in annexin V binding was observed in antisense-transfected cells relative to both sense-transfected (Fig. 1B) and parental cells (data not shown). Also, a 3-fold increase in cell death in antisense-transfected cells relative to sense-transfected cells was observed, as determined by PI staining (Fig. 1C). Finally, we confirmed that these VEGF antisense-transfected cells were apoptotic using a terminal deoxynucleotidyl transferase-mediated nick end labeling-based assay (Apoptag) (Fig. 1D). Our ability to induce apoptosis in these metastatic cells by inhibiting VEGF expression, even in the presence of serum, indicates an essential function for this cytokine in their survival. The contribution of VEGF to tumor progression has been attributed exclusively to its angiogenic function. Our findings suggest that VEGF can also sustain breast carcinoma survival independently of angiogenesis by stimulating autocrine survival signaling in these cells.

VEGF Promotes Breast Carcinoma Survival by Stimulating the PI3-kinase Pathway. MDA-MB-231 cells exhibit constitutively elevated PI3-kinase activity (15).⁶ Based on data in Fig. 1 and reports that VEGF stimulates PI3-kinase in endothelial cells (16–18), we hypothesized that VEGF maintains constitutively elevated PI3-kinase activity in MDA-MB-231 breast carcinoma cells and that this signaling is critical for their survival. To address this hypothesis, we assessed the effect of reducing VEGF expression in MDA-MB-231 cells on PI3-kinase activity. Cells were transfected with antisense or sense oligonucleotides, and PI3-kinase activity was measured 15 h after transfection. As shown in Fig. 2A, a significant reduction in PI3-kinase activity was detected in antisense-transfected cells relative to sense-transfected cells. Densitometry of chromatograms from three experiments indicated that PI3-kinase activity was reduced by an average of 49% (\pm 7.6% SD). Importantly, PI3-kinase activity was restored in antisense-transfected cells by the addition of exogenous VEGF (Fig. 2A). As a control, we observed that mitogen-activated protein kinase activity was not decreased in antisense-transfected cells (data not shown). These results demonstrate that the elevated level of PI3-kinase activity in MDA-MB-231 cells is the result of VEGF autocrine signaling. We also observed that treatment of these cells with the PI3-kinase inhibitor LY294002 (100 μ M) induced their apoptosis, as indicated by a 2-fold increase in annexin-V-FITC-positive cells, as well as a 3.6-fold increase in PI-positive cells (Fig. 2B). These data indicate the importance of the VEGF-dependent activation of PI3-kinase in MDA-MB-231 cell survival.

Hypoxia Promotes Carcinoma Cell Survival by Increasing VEGF Expression. Given that VEGF is important for the survival of MDA-MB-231 cells in normoxia (Fig. 1), we predicted that hypoxia would provide a survival advantage for these cells by increasing VEGF expression. In addition to increasing their level of VEGF

⁶ L. Shaw, unpublished data.

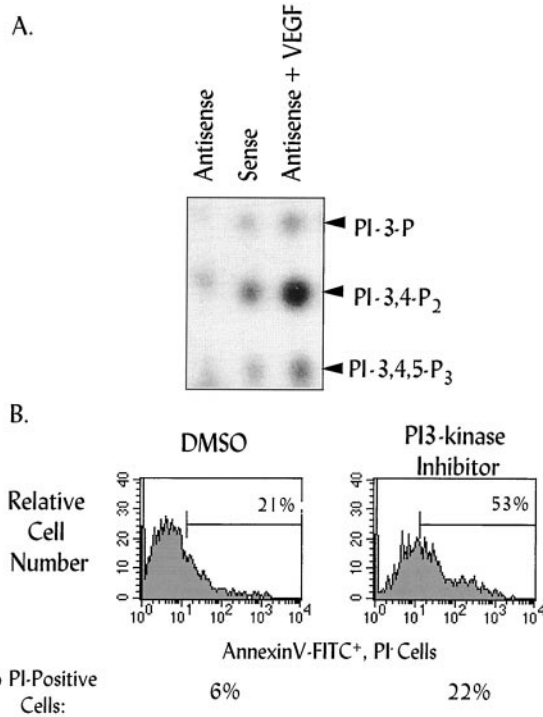


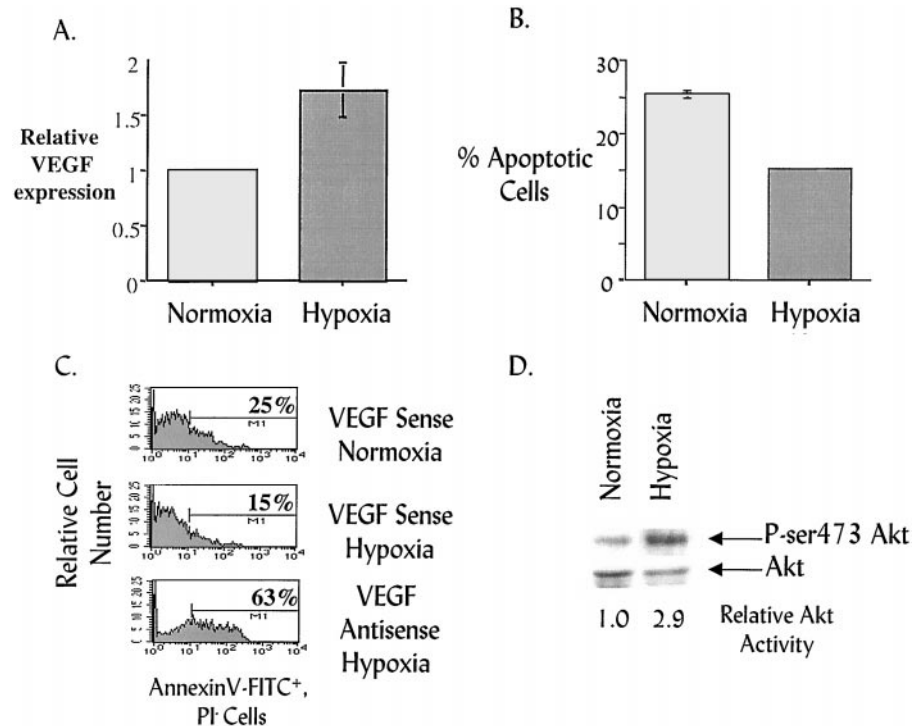
Fig. 2. VEGF is responsible for the constitutive PI3-kinase activity in MDA-MB-231 cells that is important for their survival. *A*, MDA-MB-231 cells were transfected with either the VEGF sense or antisense oligonucleotides and recovered in complete medium containing either no exogenous VEGF or recombinant VEGF₁₆₅ (100 ng/ml). PI3-kinase activity was assessed in extracts from these cells 15 h after transfection using methods described previously (22). The phosphorylated lipid products were resolved by thin layer chromatography as shown in this figure. The D3-phosphoinositides are indicated at the right. The relative amounts of radiolabeled PtdIns-3,4,5-P₃ were determined by densitometry (see "Results and Discussion"). *B*, MDA-MB-231 cells were incubated with DMSO (1:2500 dilution) or LY294002 (100 μM) in low-serum (0.5% FBS) culture medium for 48 h, and new drug-containing medium was added after 24 h. The data are presented as the mean percentage of cells from triplicate samples that were both annexin V-FITC positive and PI negative. The percentage of PI-positive cells is also indicated. Similar results were obtained in three trials.

expression (Fig. 3A), the exposure of serum-deprived MDA-MB-231 cells to hypoxia reduced their level of apoptosis significantly (Fig. 3B). Importantly, using the VEGF antisense oligonucleotide, we also demonstrated that the enhanced survival of these cells in hypoxia is VEGF dependent (Fig. 3C). Based on our observation that VEGF stimulates the PI3-kinase pathway in these cells in an autocrine manner (Fig. 2), we predicted that hypoxia would increase the activity of the serine/threonine kinase Akt/PKB, a downstream target of PI3-kinase, in these cells. The exposure of MDA-MB-231 cells to hypoxia significantly enhanced their level of Akt/PKB activity, as assessed by immunoblotting extracts from these cells with an antiserum specific for Akt/PKB molecules phosphorylated on serine residue 473 (Fig. 3D). These results provide evidence that hypoxia, a toxic stimulus, actually enhances the survival of breast carcinoma cells *in vitro* by augmenting VEGF expression and autocrine signaling.

Evidence for the Involvement of Neuropilin in VEGF Survival Function. In addition to the classical VEGF receptors KDR and Flt-1, neuropilin is a receptor that promotes VEGF function in endothelial cells (19). Interestingly, neuropilin expression in tumor cells may facilitate an angiogenic response by a mechanism that remains to be elucidated (20). Previous studies have indicated by Northern blotting that MDA-MB-231 (19, 21) and MDA-MB-435 (19) cells express neuropilin but not KDR. Based on these findings, we hypothesized that this novel VEGF receptor supports the autocrine survival function of VEGF in these breast carcinoma cells. First, we confirmed by immunoblotting that MDA-MB-231 and MDA-MB-435 cells express neuropilin but not KDR at the protein level (Fig. 4A). As a control, we demonstrated the presence of neuropilin and KDR proteins in an endothelial cell lysate by immunoblotting with these same antibodies (Fig. 4A).

To address the hypothesis that neuropilin promotes the VEGF-dependent survival of breast carcinoma cells, we compared the relative ability of VEGF splice variants that differ in receptor specificity to promote the survival of MDA-MB-231 cells. Specifically, we used VEGF₁₆₅, which binds to all of the three known VEGF receptors, and VEGF₁₂₁, which binds to KDR and Flt-1 but not to neuropilin (19).

Fig. 3. Hypoxia enhances the survival of MDA-MB-231 cells by increasing VEGF expression. MDA-MB-231 cells were maintained in low-serum (0.5% FBS) culture medium in either normoxic or hypoxic conditions. *A*, after 48 h, VEGF mRNA levels were quantified by RT-PCR. The data are presented as the mean VEGF:cyclophilin mRNA ratio (±SD) obtained from triplicate samples. *B*, the level of apoptosis after 48 h is indicated as the mean percentage of cells from triplicate wells that were annexin⁺, PI⁻ (% Apoptotic Cells). Similar results were obtained in two additional trials. *C*, MDA-MB-231 cells were transfected with either the VEGF antisense or sense oligonucleotides. Fifteen h after transfection, these cells were cultured in either normoxia or hypoxia in low-serum (0.5% FBS) culture medium for an additional 48 h. The percentage of annexin V⁺, PI⁻ cells for each sample is indicated. Similar results were obtained in three independent experiments. *D*, after 6 h, Akt/PKB activity was assessed in extracts from these cells using methods described previously (12). Relative Akt activity (ratio of serine 473 phosphorylated to total cellular Akt) was determined by densitometry. Similar results were obtained in two additional trials.



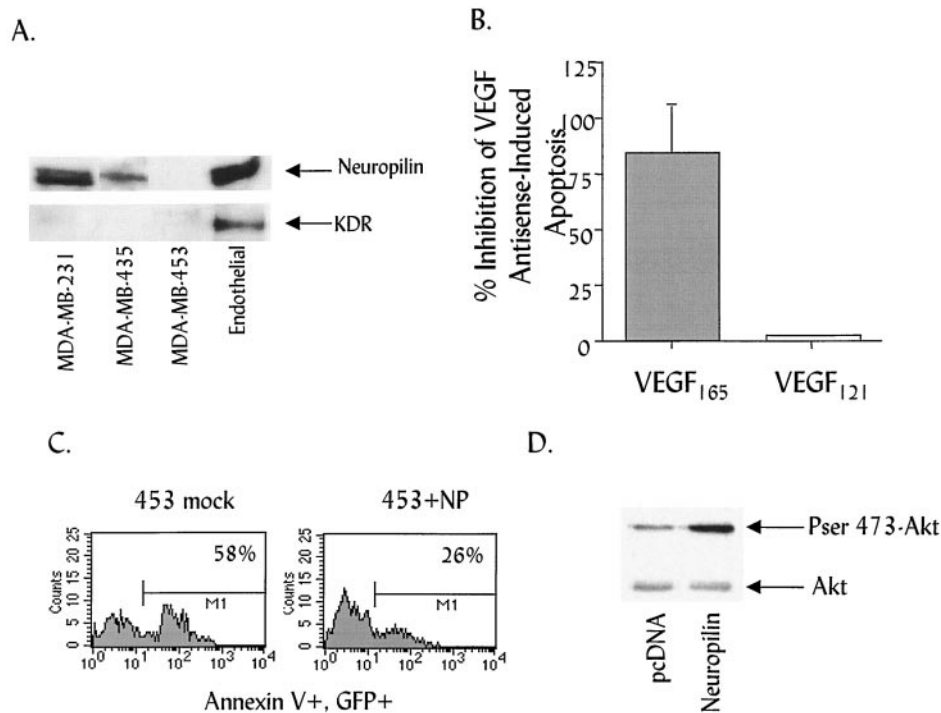


Fig. 4. Breast carcinoma cell expression of VEGF receptors. *A*, the expression of neuropilin and KDR in MDA-MB-231, MDA-MB-435, MDA-MB-453, and primary human endothelial cells was assessed by immunoblotting extracts from these cells with rabbit antisera specific for KDR (Sigma) or a monoclonal antibody specific for neuropilin (Santa Cruz), followed by HRP-conjugated goat antirabbit or antimouse IgG, respectively. KDR and neuropilin were visualized by enhanced chemiluminescence. *B*, MDA-MB-231 cells that had been transfected with the antisense and sense oligonucleotides were incubated in low-glucose DMEM containing heparin (1 $\mu\text{g}/\text{ml}$) and either no VEGF, recombinant VEGF₁₆₅ (R&D Systems; 100 ng/ml final concentration), or recombinant VEGF₁₂₁ (R&D Systems; 100 ng/ml final concentration). After 15 h, these cells were harvested, and the level of apoptosis was determined by staining with annexin-V-FITC and PI. The data are reported as the mean percent inhibition from three samples of VEGF antisense-induced apoptosis. Similar results were obtained in three trials. *C*, MDA-MB-453 cells (3×10^5 cells/well of a 6-well tissue culture plate) were transfected using the LipofectAMINE reagent (Life Technologies, Inc.) with either a myc-tagged plasmid encoding for full-length chicken neuropilin (provided by Dr. Jonathan Raper; 1 μg) or a control plasmid (1 μg), and a vector expressing green fluorescent protein (1 μg of pGFP). These cells were allowed to recover in complete culture medium for 12 h and then exposed to hypoxia in low-serum (0.5% FBS) medium for 48 h. The level of apoptosis in these cells was then assessed by annexin V-FITC and PI staining. The data represent the mean percentage of cells from three samples that were annexin positive and PI negative. Similar results were obtained in three separate trials. Importantly, neuropilin expression in these transfectants was confirmed by immunoblotting extracts from these cells with a HRP-conjugated antibody specific for the myc tag (data not shown). *D*, MDA-MB-453 cells were transfected with either a control plasmid or chicken neuropilin as described in *C*. These cells were allowed to recover for twelve hours and then exposed to hypoxia in low-serum (0.5% FBS) medium for 48 h. Akt/PKB activity in these cells was assessed using previously described methods (12).

MDA-MB-231 cells were transfected with VEGF sense or antisense oligonucleotides and maintained in medium containing either no exogenous VEGF, VEGF₁₆₅, or VEGF₁₂₁. The level of apoptosis in these cells was then assessed by annexin V-FITC and PI staining. Importantly, the incubation of the antisense-transfected cells with VEGF₁₆₅, but not with VEGF₁₂₁, inhibited their apoptosis significantly (Fig. 4*B*). The ability of a neuropilin-binding splice variant of VEGF, but not a variant lacking neuropilin specificity, to augment the survival of MDA-MB-231 cells suggests the importance of neuropilin in VEGF autocrine signaling in these breast carcinoma cells.

Neuropilin has been detected in metastatic but not in nonmetastatic tumors (19). In agreement with this observation, we found that neuropilin is expressed in the metastatic cell lines MDA-MB-231 and MDA-MB-435, but not in the nonmetastatic cell line MDA-MB-453 (Fig. 4*A*). Based on these data and our results showing that a neuropilin-binding VEGF splice variant enhances breast carcinoma survival (Fig. 4*B*), we hypothesized that cells lacking neuropilin expression, such as the nonmetastatic breast carcinoma cell line MDA-MB-453, cannot support VEGF survival signaling. To test this prediction, we compared the relative abilities of mock-transfected and neuropilin-transfected MDA-MB-453 cells to survive in hypoxia. We observed that the exposure of mock-transfected MDA-MB-453 cells to hypoxia induced a significant level of apoptosis in these cells (Fig. 4*C*). In contrast, neuropilin-expressing MDA-MB-453 cells were protected from hypoxia-induced apoptosis (Fig. 4*C*). Finally, as evidence that neuropilin activates the PI3-kinase pathway in these cells, we ob-

served that neuropilin-expressing MDA-MB-453 cells exhibit higher levels of Akt/PKB activity than do mock transfectants (Fig. 4*D*).

Studies on neuropilin function have highlighted its role in endothelial cells as a critical KDR coreceptor that facilitates VEGF-mediated signaling through this tyrosine kinase-linked receptor (19). Our studies are the first to identify a specific function for neuropilin in tumor cells, namely, its importance in maintaining breast carcinoma survival. In addition, these studies demonstrate that neuropilin supports this VEGF autocrine function in cells lacking KDR expression by stimulating the PI3-kinase pathway. These findings raise the exciting possibility that neuropilin functions either alone or in concert with other tyrosine kinase-linked receptors to transduce VEGF signaling in metastatic tumors. This involvement of neuropilin in breast carcinoma survival and the finding that neuropilin is expressed in metastatic but not in nonmetastatic tumor cells (19) suggest that neuropilin may be an important determinant of metastasis because it promotes tumor cell survival. The implications of this hypothesis with respect to both the mechanism of metastasis and therapeutic intervention are significant.

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