Role of Vascular Endothelial Growth Factor in the Stimulation of Cellular Invasion and Signaling of Breast Cancer Cells[1]

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Abstract

The expression of vascular endothelial growth factor (VEGF) by breast tumors has been previously correlated with a poor prognosis in the pathogenesis of breast cancer. Furthermore, VEGF secretion is a prerequisite for tumor development. Although most of the effects of VEGF have been shown to be attributable to the stimulation of endothelial cells, we present evidence here that breast tumor cells are capable of responding to VEGF. We show that VEGF stimulation of T-47D breast cancer cells leads to changes in cellular signaling and invasion. VEGF increases the cellular invasion of T-47D breast cancer cells on Matrigel/fibronectin-coated transwell membranes by a factor of two. Northern analysis for the expression of the known VEGF receptors shows the presence of moderate levels of Flt-1 and low levels of Flk-1/KDR mRNAs in a variety of breast cancer cell lines. T-47D breast cancer cells bind ^{125}I-labeled VEGF with a Kd of 13×10^{-9} M. VEGF induces the activation of the extracellular regulated kinases 1,2 as well as activation of phosphatidylinositol 3′-kinase, Akt, and Forkhead receptor L1. These findings in T-47D breast cancer cells strongly suggest an autocrine role for VEGF contributing to the tumorigenic phenotype.

Introduction

VEGF[3] is widely recognized to be significant as a stimulator of tumor angiogenesis (1–5). A variety of studies have shown the importance of VEGF as a prognostic indicator of the severity of breast cancer (6–10). VEGF occurs in a number of isoforms, including polypeptides of 121, 145, 165, 189, and 206 amino acids, which are produced by the alternate splicing of a single gene containing eight exons (11–14). Although VEGF-121 and VEGF-165 are the isoforms most commonly secreted by tumor cells, it is the VEGF-165 isoform that acts most strongly on endothelial cells, leading to the formation of new capillaries (15, 16). This effect of VEGF-165 on endothelial cells has been shown to be through defined cytoplasmic receptors (Flt-1, Flk-1/KDR, and Neuropilin-1; Refs. 17–22). Among its other effects, the stimulation of endothelial cells by VEGF-165 is known to lead to cell proliferation and migration (20, 23). These functions are likely to be important in the formation of neovasculature during tumor formation. In support of this, murine embryonic fibroblasts with targeted deletion of VEGF were significantly less tumorigenic in an *in vivo* model, and this was shown to be related to decreased vascular density and decreased vascular permeability (1).

VEGF has been shown to be present in breast tumors at levels that are, on average, 7-fold higher than in normal adjacent tissue (24). Expression of the VEGF receptor, Flt-1, was not increased in these tumors. Other investigators have found selective expression of VEGF and Flk-1/KDR in breast carcinomas (25). Immunocytochemistry showed that Flk-1/KDR was primarily present in the endothelium and epithelium of the mammary ducts. A number of studies have shown that VEGF secretion by the tumor cells is a prerequisite of tumor development. It was shown recently by Yoshiji *et al.* (26) that VEGF was required for the initial stages of breast cancer tumorigenesis, and that this initial effect was related to the development of neovascular stroma. Other studies have shown that the inhibition of vascular angiogenesis by such agents as angiostatin and endostatin resulted in reduced tumorigenesis and even regression of established tumors (27–30).

Although the significance of VEGF in the development of tumor vasculature is well documented, there is also a great amount of information to suggest an autocrine effect of VEGF on the tumor cells. There have been reports of VEGF signaling in melanoma cells (31, 32) and in prostate carcinoma cells (33). Both VEGF and Flt-1 have been shown to be expressed in angiosarcoma cells by immunohistochemistry and *in situ* hybridization (34). In another study, De Jong *et al.* (35) have used immunohistochemistry to measure VEGF and VEGF receptors in breast cancer. They also investigated EGF, PDGFα and -β, TGFβ, and their respective receptors. By carrying out double staining for the receptor/ligand combinations, they were able to distinguish possible autocrine and paracrine mechanisms for VEGF acting on the cells of the tumor. These investigators concluded that in 22–24% of cases, VEGF could act in an autocrine manner, whereas in 38–40% of the cases, it would be able to act in a paracrine

Received 11/30/00; revised 2/9/01; accepted 2/9/01.
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[1] This paper is supported by NIH Grants CA 76226 and R21CA87290, DAMD 17-98-1-8032, and DAMD 17-99-1-9078, by Experienced Breast Cancer Research Grant 34080057089, by the Milheim Foundation, by the Massachusetts Department of Public Health (to H. A.), and by DAMD 17-001-0152 (to T. M.). This work was done during the tenure of an established investigatorship from the American Heart Association (H. A.).
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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; HRG, heregulin; MTR, Matrigel; PI 3-kinase, phosphatidylinositol 3′-kinase; ERK, extracellular regulated kinase; FKH, Forkhead; FKHRL1, Forkhead receptor L1; HUVEC, human vascular endothelial cell; MAP, mitogen-activated protein; GSK-3, glycogen synthase kinase-3.

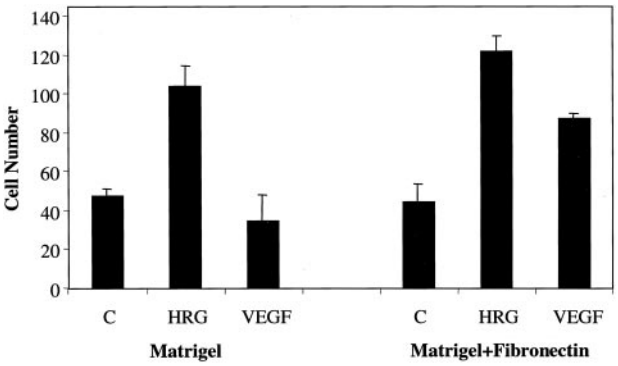

Fig. 1. Invasion of T-47D cells in response to VEGF or heregulin. Comparisons are to medium alone (*C*). Migrations were conducted on membranes coated with Matrigel alone (*left*) or Matrigel plus fibronectin (*right*).

manner. In the studies presented here, we show that VEGF-165 is able to stimulate the invasion of T-47D breast cancer cells into Matrigel. However, there was no effect of VEGF-165 on T-47D cell proliferation. We also show the presence of Flk-1/KDR and Flt-1 mRNAs in a number of breast cancer cell lines. Stimulation of T-47D cells with VEGF-165 led to tyrosine phosphorylation of multiple proteins in crude extracts, activation of ERK1,2 and also activation of the PI 3-kinase signaling pathway. Because T-47D cells are known to secrete VEGF (26), this effect on T-47D cells suggests a possible autocrine component for VEGF, leading to increased tumorigenesis.

Results

VEGF-165 Stimulation Leads to Increased Invasion of T-47D Cells. It was recently reported that VEGF[4] modulates the chemotaxis and migration of endothelial cells (20). In addition, cellular invasion of MCF7 breast cancer cells in response to heregulin has been shown to be mediated through a PI 3-kinase-dependent pathway (36). Therefore, we asked whether VEGF signaling in T-47D breast cancer cells might also affect the invasion of these cells. As is shown in Fig. 1 (*left panel*), initial experiments on Matrigel alone showed no invasion in response to VEGF-165, whereas a >2-fold increase in invasion in response to heregulin was observed. However, when fibronectin was added to the Matrigel coating on the transwell membrane, VEGF-165 caused an ~2-fold increase in invasion that was ~72% of the invasion observed in response to heregulin under these conditions (Fig. 1, *right panel*). These results indicate that VEGF-165 induced the invasion of breast cancer cells in the presence of fibronectin.

Breast Cancer Cell Lines Express Primarily Flt-1 mRNA. We tested for the possible expression of VEGF receptor mRNAs in human breast tumor and normal breast cell lines. Using Northern blotting, we analyzed the expression of Flt-1, Flk-1/KDR, and Neuropilin-1 mRNAs in a number of cell lines. These included SK-BR-3, T-47D, MCF7, MDA-MB-231, and MDA-MB-453 breast cancer lines as well as an HBL-100 nonmalignant breast line and HUVECs. As shown in Fig. 2*A*, all of the breast cancer lines except SK-BR-3 and MCF7 expressed a moderate level of Flt-1. HUVECs expressed a comparatively low level of this mRNA. HUVECs expressed a comparatively high level of Flk-1/KDR, whereas all of the breast cancer cells expressed low levels of this mRNA (Fig. 2*B*). MDA-MB-231 cells expressed a high level of Neuropilin-1, and MCF7 cells expressed a lower level of this mRNA (Fig. 2*C*). Other breast cancer lines failed to express Neuropilin-1 mRNA. These findings indicate that there is variable expression of VEGF receptors (Flt-1, Flk-1/KDR, and Neuropilin-1) in breast cancer. Although T-47D cells and three other breast lines expressed primarily Flt-1, only one of the cell lines (MDA-MB-231) expressed a high level of Neuropilin-1. Flk-1/KDR expression was uniformly low in all of the breast cell lines studied.

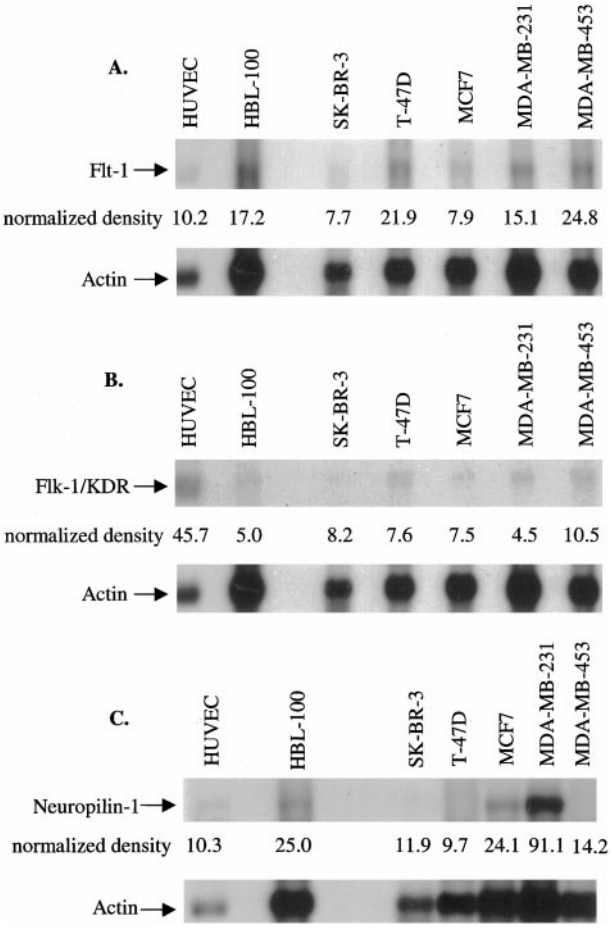

Fig. 2. Northern blotting for (*A*) Flt-1, (*B*) Flt-1/KDR, and (*C*) Neuropilin-1 mRNAs. Cell lines tested are indicated above. Control actin mRNA probe is shown in each *panel*. Densitometry of the Flt-1, Flk-1/KDR, and Neuropilin-1 bands was normalized to the corresponding actin signals.

VEGF-165 Binds to T-47D Cells with a Lower Affinity Compared with the Known VEGF Receptors. To characterize the possible cellular receptors for VEGF on breast

[4] Notation: Unless otherwise stated, all notation of VEGF refers to the VEGF-165 isoform.

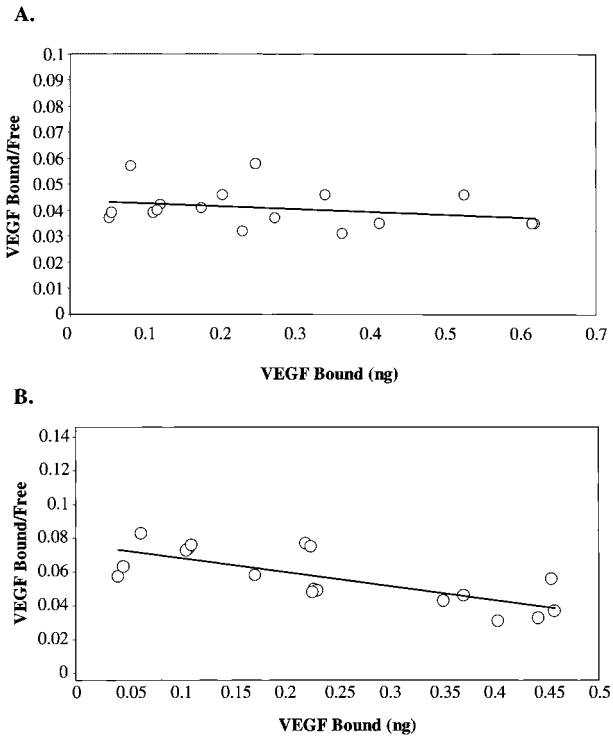

Fig. 3. Scatchard analysis of binding of ^{125}I-VEGF to MDA-MB-231 cells (*A*) and T-47D cells (*B*). Lines indicate a least square fit of the data points.

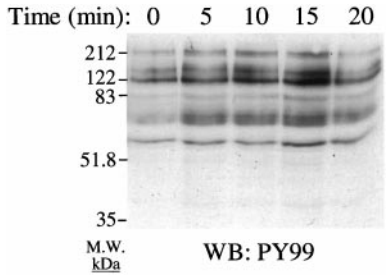

Fig. 4. Stimulation of T-47D cells with VEGF and western immunoblotting of total cell extracts with an anti-phosphotyrosine antibody.

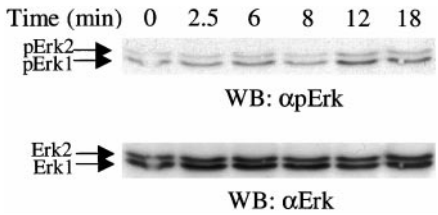

Fig. 5. Stimulation of T-47D cells with VEGF and Western immunoblotting of total cell extracts with anti-phospho-ERK antibody (*upper panel*). *Lower panel* indicates immunoblotting with an antibody to total ERK 1 protein.

cancer cells, we determined the binding of ^{125}I-labeled VEGF to either T-47D cells or to MDA-MB-231 cells as a control. From Scatchard analysis of this data (Fig. 3), we calculated the binding of VEGF to T-47D cells as having a Kd of $\sim 13 \times 10^{-9}$ M and $\sim 0.63 \times 10^5$ binding sites/cell. Binding of VEGF to MDA-MB-231 cells showed a Kd of $\sim 17.4 \times 10^{-10}$ M and $\sim 1.53 \times 10^5$ binding sites/cell. Thus, MDA-MB-231 cells had a VEGF binding that was similar to that determined previously by Soker *et al.* (Ref. 21; Kd $\sim 2.8 \times 10^{-10}$ M; 0.95–1.1 $\times 10^5$ binding sites/cell) reflecting the binding primarily to Neuropilin-1. To confirm our binding data obtained for detached cells, we repeated the experiments following more closely the method of Soker *et al.* (21), who determined binding to cells on tissue culture wells. Using this method, we obtained Kd values for VEGF binding to MDA-MB-231 and T-47D cells that were similar (within a factor of 2–3) to the values obtained by our method with detached cells (data not shown). Waltenberger *et al.* (20) have characterized VEGF binding to Flt-1 to have a Kd of 1.6×10^{-11} M and VEGF binding to Flk-1/KDR to have a Kd of 7.6×10^{-10} M. Our experiments with T-47D cells, on the other hand, showed a binding that was lower in affinity as compared with all of the known VEGF receptors.

VEGF-165 Stimulates the Tyrosine Phosphorylation of a Number of Proteins in T-47D Breast Cancer Cells. We next asked whether VEGF might have an effect on the signaling of receptor tyrosine kinases to intracellular components in T-47D cells. Thus, we stimulated these cells with VEGF-165 and measured the changes in total tyrosine phosphorylation. When serum-starved T-47D cells were treated with 100 ng/ml VEGF-165, we found increased tyrosine phosphorylation of a number of proteins as shown by anti-phosphotyrosine Western blotting of total cell extracts (Fig. 4). These included proteins of molecular weight M_r 60,000, 75,000, 122,000, and 200,000.

VEGF-165 Stimulates the MAP Kinases ERK 1,2 in T-47D Breast Cancer Cells. To analyze whether VEGF might modulate MAP kinase activity in the breast cancer cells, extracts from T-47D cells activated with VEGF were resolved on SDS-PAGE, and transfers were probed to detect activation of ERK 1,2 (Fig. 5). We observed a slight increase in the phosphorylation of ERK 1,2 that was attributable to VEGF at 15–20 min. Blotting for total ERK 1,2 showed that the differences seen were not attributable to differences in protein loading.

VEGF-165 Treatment Leads to Stimulation of PI 3-kinase and Related Pathways in T-47D Breast Cancer Cells. Inasmuch as PI 3-kinase has been shown to be induced by VEGF in endothelial cells (37), we next determined whether VEGF also might activate this pathway in breast cancer cells. PI 3-kinase activity was measured by an *in vitro* kinase assay of extracts from the VEGF-treated cells (Fig. 6*A*, *left panel*). There was a clear stimulation of phosphatidylinositol phosphorylation by VEGF at 5–20 min (see *arrow*). There was no phosphorylation seen in the normal serum control precipitate. As a control, we stimulated T-47D cells with heregulin and measured the PI 3-kinase activity (Fig. 6*A*, *right panel*). This showed the position of phosphatidyl inositol 3-phosphate in the chromatogram.

Because Akt is known to be a down-stream target of PI 3-kinase (38), we then measured Akt activation in VEGF-

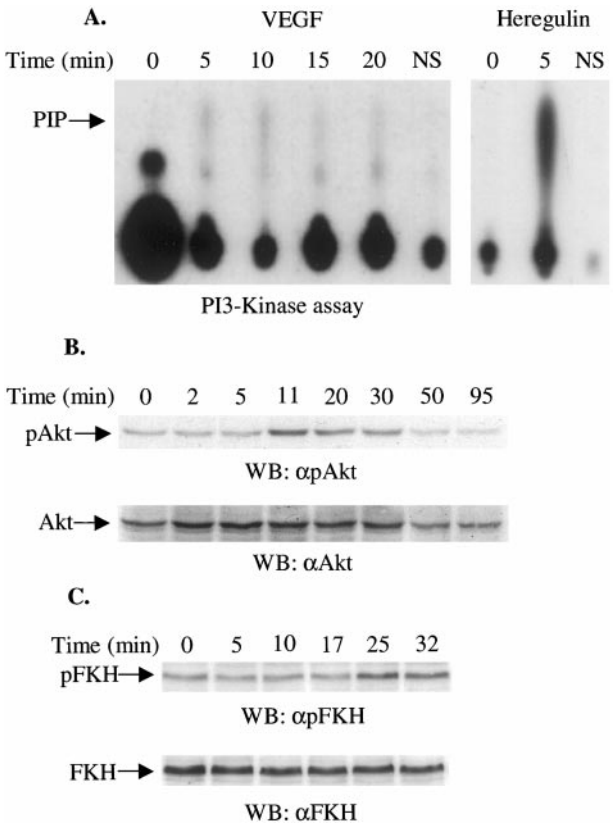

Fig. 6. (*A*) Stimulation of T-47D cells with VEGF and assay for PI 3-kinase (*left panel*). *Right panel* indicates the PI 3-kinase assay of T-47D cells stimulated with heregulin. NS indicates normal serum control precipitate. (*B*) Stimulation of T-47D cells with VEGF and immunoblotting of extracts with anti-phospho-Ser253 Akt. (*C*) Stimulation of T-47D cells with VEGF and immunoblotting of extracts with anti-phospho-FKH antibodies. *Below* each is shown the corresponding blotting of the non-phosphorylated protein.

treated T-47D cells by Western blotting using a phospho-Akt antibody (Fig. 6*B*). VEGF produced a small but detectable Akt phosphorylation signal that was first seen at 25 min, decreased at 50 min, and reached basal levels by 95 min.

We next tested for possible substrates of Akt, including GSK-3, p70 S6 kinase, and FKHRL1. Whereas stimulation of GSK-3 was seen after heregulin treatment, no stimulation of GSK-3 was detectable after VEGF treatment (data not shown). No change was seen in the phosphorylation of p70 S6K at Thr-421 or Ser-424 after either heregulin or VEGF treatment (data not shown). We then tested to see if there was a change in the phosphorylation of FKHRL1, a Forkhead family member known to be involved in the transcription of apoptosis-related proteins (39–41). We saw increases in the phosphorylation of FKHRL1 at Ser-253 in extracts from cells stimulated by VEGF (Fig. 6*C*). A similar increase in phosphorylation was also seen at Thr-32 of FKHRL1 (data not shown). These changes in FKHRL1 phosphorylation appeared to follow the changes in Akt phosphorylation, indicating that FKHRL1 was the substrate for Akt upon VEGF stimulation of breast cancer cells.

Taken together, the results indicate that VEGF induces the activation of ERK1,2 and PI 3-kinase signaling pathways in breast cancer cells, leading to an increased invasiveness of the cells.

Discussion

In this study, we have presented results showing the VEGF-induced invasion and signaling in T-47D breast cancer cells. These studies demonstrate the importance of VEGF in stimulating effects on breast tumor cells in contrast to its effects on endothelial cells. Our studies with mRNA expression show that all breast tumor lines examined, except SK-BR-3, expressed moderate levels of Flt-1 and lower levels of Flk-1/KDR. Thus, we postulate that breast cancer cell lines are representative of human breast tumors in terms of their expressing VEGF receptors. Our studies with T-47D breast cancer cells support the conclusion that these cells are capable of responding to VEGF in terms of changes in intracellular signaling and cellular invasion. Data from our laboratory[5] and from other investigators (26), have shown that T-47D cells secrete VEGF at the levels that are required for this stimulation. Thus, we postulate that, in many breast cancers, the elements are present for stimulation of an autocrine mechanism leading to increased cell invasion.

We tested to see if VEGF receptors other than Flt-1 and Flk-1/KDR might account for the signaling in the breast cancer cells. By Northern blotting, the VEGF receptor Neuropilin-1 was seen to be expressed in two of the breast cancer cell lines, MDA-MB-231 and MCF7 cells. MDA-MB-231 cells contain the highest level of Neuropilin-1, but VEGF fails to stimulate tyrosine phosphorylation or ERK 1,2 activation.[6] Thus, it is unlikely that Neuropilin-1 is involved in the effects that we have seen in T-47D cells. Our Northern blotting of breast cancer cell lines showing the presence of Flt-1 and Flk-1/KDR are consistent with the finding of Speirs and Atkin (42), who found that these receptors were present in human breast cancer tumor epithelial cells. It was similarly shown by De Jong *et al.* (35) that in nearly 50% of the breast tumors, there was significant expression of Flt-1 and Flk-1/KDR in the tumor epithelial cells, correlating with the expression of VEGF by these cells. These investigators postulated that VEGF secreted by these epithelial cells could have both autocrine and paracrine roles. The paracrine mechanism for this action is likely to be through the stimulation of endothelial cells, leading to a development of the neovasculature (17–19, 22). However, the mechanism for the autocrine action of VEGF on the epithelial cells of the tumors has not been characterized. On the basis of the results presented here, we propose that VEGF acts in an autocrine manner by stimulating signaling, leading to cellular invasion in breast cancer epithelial cells. The cellular signaling in T-47D cells stimulated by VEGF leads to the stimulation of ERK1,2 and PI 3-kinase pathways. Stimulation of the PI 3-kinase pathway in particular is often related to cellular invasion. As mentioned above, MCF7 breast cancer cells are known to migrate in response to heregulin through a PI 3-kinase-mediated process (36). We have observed the invasion of T-47D cells in

[5] D. J. Price, H. Kawai, and H. Avraham, unpublished results.
[6] D. Price, unpublished data.

response to VEGF only when fibronectin is present on the transwell membrane. This is an indication that both the growth factor, VEGF, and the extracellular matrix component, fibronectin, are important in potentiating the invasion of the tumor cells. Fibronectin is known to contain binding domains that interact with cell surface heparan sulfate proteoglycans to promote focal adhesions and stress fiber formation (43). Heparan sulfate is known to potentiate the binding of VEGF to its receptors (31). It may be that fibronectin, in conjunction with heparan sulfate proteoglycans, also leads to an increased interaction of VEGF with its receptor. Thus, fibronectin, in cooperation with VEGF, appears to provide the signaling that is required for cellular invasion, whereas VEGF alone is unable to stimulate this process.

To date, we have no indication that other cellular functions might be stimulated in these cells, leading to increased tumorigenesis. There appeared to be little effect on cell survival or proliferation upon VEGF treatment of the T-47D cells (data not shown). Although phosphorylation of the Forkhead transcription factor is often connected with effects on the Fas ligand leading to cell survival (41), there may be other functions of this pathway. Another important question raised by these results is whether or not the endogenously secreted VEGF is sufficient to stimulate the effects that we have observed. As noted above, we and other investigators have shown that VEGF is secreted by the T-47D cells. An argument that could be made about the significance of the effect of VEGF on tumor cells is that because the tumor is secreting VEGF, there may be a higher local concentration of VEGF relative to other growth factors. Thus, *in vivo*, the effect of VEGF on the invasion of these cells may be much greater as compared with the effects of other growth factors that are present at subthreshold concentrations.

In summary, VEGF stimulated the increased invasion of T-47D cells through Matrigel/fibronectin-coated membranes. Northern analysis showed the expression of primarily the VEGF receptor, Flt-1, in a variety of breast cancer cell lines. However, binding of ^{125}I-labeled VEGF to T-47D cells indicated an affinity that was lower than that expected for the known VEGF receptors, suggesting the possibility of an as-yet unidentified VEGF receptor in these cells. VEGF stimulated signaling in T-47D breast cancer cells through the PI 3-kinase/Akt pathway and also through the ERK 1,2 pathway. This observation may indicate an effect of VEGF on tumorigenicity independent of its effects on the vasculature. Future studies will be aimed at characterizing the *in vivo* significance of VEGF signaling in the tumor cells as compared with its signaling in endothelial cells.

Materials and Methods

Materials. Antibodies used in immunological analysis were as follows: anti-phosphotyrosine antibody (PY99), phospho-ERK (E-4) antibody, anti-ERK 1 (K-23), anti-Flk-1 (N-931), and horseradish peroxidase-labeled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt, and anti-phospho-Ser-473 Akt antibodies were from New England Biolabs (Beverly, MA). Anti-Flt-1, anti-phospho-Ser-253 FKH, and anti-phospho-Thr-32 FKH antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). LY294002 was from Sigma Chemical Co. (St. Louis, MO). VEGF-165 and heregulin were a generous gift of Genentech (San Francisco, CA). γ^{32}P-ATP and α^{32}P-CTP were from New England Nuclear (Boston, MA). All other chemicals were from Fisher Scientific (Norcross, GA), unless otherwise noted.

Cell Culture. T-47D cells were an estrogen receptor-positive clone provided by Iafa Keydar, Tel Aviv University (Ramat Aviv, Israel). These cells were cultured in RPMI 1640 (Life Technologies, Inc., Bethesda, MD) supplemented with 7 μg/ml insulin, 10% fetal bovine serum (Life Technologies, Inc.), and penicillin/streptomycin. MCF7 cells (American Type Culture Collection, Rockville, MD) were grown in MEM (Life Technologies, Inc.) supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 μg/ml insulin, 10% fetal bovine serum, and penicillin/streptomycin. MDA-MB-231 and MDA-MB-453 (American Type Culture Collection) were grown in DMEM supplemented with 10% fetal bovine serum, 0.2 mM glutamine, and penicillin/streptomycin. HUVECs were from Clonetics (San Diego, CA) and were cultured in EGM complete medium (Clonetics). HBL-100 (American Type Culture Collection) were cultured in McCoy's 5a medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.

Iodination of VEGF-165. ^{125}I-labeled VEGF-165 was prepared using IODO-GEN, as described previously (31). The protein was separated from free iodine by heparin Sepharose affinity adsorption (Amersham-Pharmacia Biotech, Piscataway, NJ) and elution with 0.8 M NaCl. Specific activity of the ^{125}I-labeled VEGF-165 was ~100,000 cpm/ng protein.

Binding of VEGF-165 to Cells. For quantification of the binding of ^{125}I-labled VEGF-165 to cells, the cells were detached briefly with trypsin/EDTA, washed in full medium, then suspended in binding buffer [20 mM MOPS (pH 7.4)/2 mM $MgCl_2$/140 mM NaCl, and 0.2% gelatin/2 mg/ml glucose). Cells were then incubated with a range of concentrations of VEGF-165 containing a fixed amount of ^{125}I-labled VEGF-165 in binding buffer at a final concentration of 1×10^5 cells/ml on ice. Aliquots of 0.15 ml were pipetted onto a 0.9 cushion of fetal bovine serum. After centrifugation in a microcentrifuge (5 min; 7.5 $\times$ 1000 rpm), tubes were frozen on dry ice. The cell pellet was isolated by clipping the tip of the tube with a canine toenail clipper. Bound (pellet) and free (supernatant) counts were quantified in a Beckman gamma counter. Kd values and the number of binding sites/cell were calculated from Scatchard plots (44) by doing a least square fit of the data using the Microsoft Excel program.

Immunoprecipitations and Western Analysis. After growth factor stimulation, cells were lysed in 20 mM Tris-HCl (pH 7.4)/150 mM NaCl/1% NP-40/0.25% deoxycholate/1 mM Na_3VO_4/1 mM EGTA and a cocktail of protease inhibitors (Complete, EDTA-free; Roche, Indianapolis, IN). Protein was normalized by Bio-Rad protein assay (Bio-Rad, Hercules, CA), and lysates were precipitated overnight with the addition of 1 μg of the specified antibody. The next day, protein G-Sepharose (Pierce, Rockford, IL) was added and the precipitates were washed 3 times with lysis buffer. Precipitates were treated with SDS-sample buffer and run on polyacrylamide gels, followed by transfer to nitrocellulose membranes

(Bio-Rad). Membranes were immunoblotted with primary antibodies as indicated in the figure legends, and with the appropriate horseradish peroxidase-linked secondary antibodies, before chemiluminescent development and exposure to X-ray film.

PI 3-kinase Assay. Assay of PI 3-kinase was carried out after growth factor stimulation of cells and precipitation of lysates by PY99 antibody/Protein G-Sepharose. Precipitates were subjected to an *in vitro* kinase reaction using γ^{32}P-ATP and phosphatidylinositol (Sigma Chemical Co.) as substrates, according to Derman *et al.* (45). ^{32}P-labeled samples were applied to oxalate-coated cellulose/acetate plates and subjected to chromatographic separation (solvent, $CHCl_3$: methanol:H_2O:NH_4OH [60:47:11.3:2]).

Northern Analysis. mRNAs were isolated from cellular extracts by oligo(dT) chromatography using a kit (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. mRNAs were separated on an agarose gel and transferred to a Hybond N membrane (Amersham Pharmacia Biotech). The membrane was hybridized with probes to Flt-1, Flk-1/KDR, and Neuropilin-1 (a generous gift of Dr. Michael Klagsbrun, Children's Hospital, Boston, MA). Blots were prehybridized for 4 h at 42° in 50% formamide/5× SSC-10× Denhardts/0.3% SDS/100 μg/ml ssDNA/10 μg/ml yeast tRNA. Specific ^{32}P-labeled probe DNA was added, and the incubation was continued for 4 h at 42°. The blots were washed twice in 2× SSC-1% SDS at room temperature, and then in 0.2× SSC-0.1% SDS at 42° followed by 0.2× SSC-0.1% SDS at 60°. After washing, the blots were then exposed to X-ray film. Blots were also stripped and reprobed for actin mRNA as a control.

Invasion Assay. Transwell membranes (8-μm pore size, 6.5-mm diameter; Corning Costar Corporation, Cambridge, MA) were coated with Matrigel (2.5 mg/ml) or Matrigel plus fibronectin (2.5 mg/ml), and dry coatings were reconstituted in DMEM for 1–2 h before cell passage. Cells were trypsinized, centrifuged, and resuspended at ~10^7/ml in DMEM containing 0.2% BSA. Cells were seeded onto the upper wells of precoated transwells in the same medium alone [control or in medium supplemented with HRG (20 nM)] or VEGF-165 (100 ng/ml). Lower wells of the transwells contained 600 μl of DMEM and 0.2% BSA. After 24 h, membranes were swabbed with a Q-tip, fixed with methanol, and stained with crystal violet before counting under phase-contrast microscopy.

Acknowledgments

We thank Dr. Shalom Avraham for advice on this project. We also thank Dan Kelley for preparation of figures and Janet Delahanty for assistance with editing.

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