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PRINCIPAL INVESTIGATOR: Hava Avraham, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

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Hava Avraham, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

E-MAIL:
havraham@caregroup.harvard.edu

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Our proposal aims to investigate the function of VEGF in breast cancer cell growth and signaling. Our data suggest that breast cancer cells secrete various levels of VEGF and express in addition to the Flt-1 and Flk-1/KDR receptors, a novel VEGF receptor type which needs to be identified and characterized. These data lead us to hypothesize that VEGF, secreted by breast cancer cells, is a multi-functional protein which acts in an autocrine fashion and can simultaneously activate specific VEGF receptor signaling pathways in breast cancer cells, thereby regulating breast cancer cell growth, tumor angiogenesis and subsequently tumor growth. In order to test this hypothesis, we propose to focus on two basic aims: (a) To identify and characterize the VEGF receptors expressed in breast cancer cell, and to analyze their expression in primary breast tissues. We will elucidate the signaling events upon VEGF stimulation in breast cancer cells and identify which activated signaling molecules are essential for the VEGF-mediated effects on breast cancer cell growth; and (b) To study the effects of VEGF and VEGF receptor expression on the regulation of tumor angiogenesis and signal transduction pathways in breast cancer cells.

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Hava [signature] 7/14/2000

PI - Signature Date

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INTRODUCTION

Angiogenesis is a hallmark of many types of cancer including breast cancer. Angiogenesis is an important process for growth and metastases of cancer. All solid tumors depend on angiogenesis-the development of new blood vessels-both for growth and malignant progression. Angiogenesis is required for expansion of solid tumors beyond microscopic sizes (e. g. 1-2 mm in diameter) and for sustaining tumor viability. Examination of diverse human tumors has confirmed the relationship between increasing tumor microvessel density and malignant progression. Crucial to tumor angiogenesis is the balance between tumor-associated angiogenesis stimulators, such as vascular endothelial growth factor (VEGF), and endogenous angiogenic inhibitors.

Angiogenesis is a significant prognostic factor in breast cancer, but the factors that control angiogenesis *in vivo* are not well defined. It is known that the production of angiogenic factors such as VEGF is high in many human breast cancers and that there is a correlation between microvessel density and VEGF expression in primary breast tumors. Furthermore, the relapse-free survival rate of breast cancer patients is inversely related to VEGF expression in their tumors, and patients with the highest VEGF levels have the poorest prognosis. While there are accumulating data about VEGF signaling in endothelium, there is much less information on pathways triggered by VEGF in cancer cells, including breast cancer cells.

Thus, the overall goal of our proposal is to investigate the function of VEGF in breast cancer cell growth and signaling. The first step in VEGF action is binding to its high affinity tyrosine kinase receptors Flt-1 and Flk-1/KDR, found primarily in endothelial cells. Our data suggest that breast cancer cells secrete various levels of VEGF and express in addition to the Flt-1 and Flk-1/KDR receptors, a novel VEGF receptor type which needs to be identified and characterized. These data lead us to hypothesize that VEGF, secreted by breast cancer cells, is a multi-functional protein which acts in an autocrine fashion and can simultaneously activate specific VEGF receptor signaling pathways in breast cancer cells, thereby regulating breast cancer cell growth, tumor angiogenesis and subsequently tumor growth. In order to test this hypothesis, we propose to focus on two basic aims: (a) To identify and characterize the VEGF receptors expressed in breast cancer cell, and to analyze their expression in primary breast tissues. We will elucidate the signaling events upon VEGF stimulation in breast cancer cells and identify which activated signaling molecules are essential for the VEGF-mediated effects on breast cancer cell growth; and (b) To study the effects of VEGF and VEGF receptor expression on the regulation of tumor angiogenesis and signal transduction pathways in breast cancer cells. Specifically, we will investigate effects of conditional ectopic expression of VEGF and VEGF receptors in MCF-7 breast cancer cells and in MCF-10A normal mammary epithelial cells and will analyze the effects of their overexpression on the regulation of tumor angiogenesis and signal transduction pathways in breast cancer cells.

BODY

RESULTS

Detection of VEGF, Flt-1 and Flk-1/KDR in breast tumor sections.

To investigate whether breast tumor cells as well as endothelial cells may express VEGF and its receptors, we performed some immunohistochemical staining using antibodies against VEGF, Flk-1/KDR, and Flt-1. The staining was performed on paraffin block sections from 5 patients with diagnosed breast carcinoma at different stages of the disease (Stages I-III). In addition we

tested non-involved breast tissue ("normal") from two of the diseased patients as well as breast tissue from two patients that underwent reduction mammoplasty ("normal"). Figure 1 is representative of the staining expressed by 4 out of 5 breast tumors tested. VEGF is expressed both in the tumor cells as will as in the endothelial cells of blood vessels (Fig. 1B). Flk-1/KDR is expressed in all tumors tested at a higher level (Fig. 1C) than Flt-1. Figure 2 shows non-involved tissue ("normal") tested by the same antibodies. No detectable expression of the proteins was observed. No staining was observed in the absence of the primary antibody indicating the specific expression of these proteins in the breast tumor cells (not shown).

Breast cancer cell lines express Flt-1 and other VEGF receptor mRNAs.

We then 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 non-malignant breast line and HUVEC endothelial cell line. As shown in Figure 3, all of the breast cancer lines except SK-BR-3 expressed a moderate level of Flt-1, while HUVEC cells did not express this mRNA. HUVEC cells expressed Flk-1/KDR, while all the breast cancer cells except SK-BR-3 expressed low levels of this mRNA. MDA-MB-231 cells expressed a high level of Neuropilin-1 and MCF7 cells expressed a lower level of this mRNA. Other breast cancer lines failed to express Neuropilin-1 mRNA.

VEGF-165 binds to T-47D cells with a lower affinity compared to the known VEGF receptors.

In order to characterize the possible cellular receptors for VEGF on breast cancer cells we determined the binding of ^{125}I-VEGF to either T-47D cells or MDA-MB-231 cells (Fig. 4). From this data, we calculated the binding constant of VEGF for T-47D cells to be Kd~ 5.2×10^{-9} M and the binding constant of VEGF for MDA-MB-231 to be Kd ~10.5×10^{-10} M. VEGF receptor numbers were determined to be 5.49×10^4 binding sites/cell for T-47D cells and 1.55×10^5 binding sites/cell for MDA-MB-231 cells. Thus, MDA-MB-231 cells had a VEGF binding that was similar to that previously determined by Soker et al., 1996 (Kd ~2.8×10^{-10} M, 0.95-1.1 x 10^5 binding sites/cell) reflecting the binding to primarily Neuropilin-1. Waltenberger et al., 1994, have characterized VEGF binding to Flt-1 to be with a Kd of 1.6×10^{-11} M and VEGF binding to Flk-1/KDR to be with a Kd of 7.6×10^{-10} M. Our experiments with T-47D cells, on the other hand, showed a binding which was of a lower affinity than all of the known VEGF receptors.

VEGF-165 stimulates tyrosine phosphorylation of a number of proteins stimulates PI 3-kinase activity in T-47D breast cancer cells.

In order to determine if VEGF might stimulate cellular signaling in T-47D breast cancer cells, we stimulated these cells with VEGF-165 and measured 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. 5A). These included proteins of molecular weight 60, 75, 122, and 200 kDa. We compared this activation with that produced by heregulin in the same cells. Heregulin stimulated tyrosine phosphorylation of proteins of the same molecular weights, although the intensities of the stimulation were clearly greater, and one protein of ~50 kDa was stimulated by heregulin, but was not seen at all in the stimulation by VEGF-165. Also, in comparing the time-course of activation by the two growth factors, VEGF was slower in reaching a maximal stimulation (15 min.) compared to heregulin (5 min.). In the

same experiment, PI 3-kinase activity was measured by an *in vitro* kinase assay of extracts from the heregulin and VEGF treated cells (Fig. 5B). There was a clear stimulation of PI phosphorylation by VEGF at 5 to 20 minutes, even though the level of PI 3-kinase stimulated by heregulin was greater. There was no phosphorylation seen in normal serum control precipitates.

VEGF-165 stimulates the MAP kinases ERK 1, 2 in T-47D breast cancer cells.

In a similar experiment, SDS-PAGE transfers of extracts from T-47D cells activated with VEGF or heregulin were probed to detect activation of ERK 1,2. We observed a slight increase in phosphorylation of ERK 1,2 due to VEGF at 15-20 minutes and a much greater activation of ERK 1,2 due to heregulin at between 5 and 15 minutes (Fig. 6). Again, blotting for total ERK 1,2 showed that the differences seen were not due to differences in protein loading.

VEGF-165 treatment leads to stimulation of PI 3-kinase-related pathways in T-47D breast cancer cells.

We then measured Akt activation in VEGF and heregulin-treated T-47D cells by western blotting using a phospho-Akt antibody (Fig. 7A). A strong stimulation of Akt was seen with heregulin beginning at 5 minutes and this level of stimulation was observed up to 32 minutes. VEGF produced a smaller but detectable signal of Akt phosphorylation that was first seen at 25 minutes, decreasing at 50 minutes, and reaching basal levels by 95 minutes. We tested for possible substrates of Akt, including glycogen synthase kinase-3 (GSK-3), p70 S6K, and FKHRL1. While stimulation of GSK-3 was seen after heregulin stimulation, no stimulation of GSK-3 was detectable after VEGF stimulation (not shown). No change was seen in the phosphorylation of p70 S6K at Thr-421 or Ser-424 for either heregulin or VEGF (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 (Hillion et al., 1997, Anderson et al., 1998, and Brunet et al., 1999). We saw increases in the phosphorylation of FKHRL1 at Ser-253 in both the VEGF and the heregulin stimulation (Fig. 7B). A similar increase was also seen at Thr-32 of FKHRL1 (not shown). These changes in FKHRL1 phosphorylation appeared to follow the changes in Akt phosphorylation, indicating that FKHRL1 was the substrate for Akt in both cases.

VEGF-165 treatment leads to increased T-47D cell survival.

Our initial efforts to identify the possible cellular function of VEGF on T-47D cells indicated no detectable effect on cell proliferation (not shown). Growth factor stimulated PI 3-kinase has been shown to stimulate cellular survival in a wide variety of neoplastic and normal cells (for a review see Datta et al., 1999). Since our previous experiments had shown the importance of the PI 3-kinase pathway in VEGF signaling in T-47D cells, we asked whether VEGF stimulation might be able to bring about increased survival of cells that were driven toward apoptosis by the PI 3-kinase inhibitor LY294002. As is described in the Materials and Methods, T-47D cells were treated with VEGF-165 in the presence or absence of LY294002 and survival was measured after 2-4 days by the counting of the viable cells. As can be seen in Fig. 8, after two days of treatment, LY294002 reduced the cell number by 45% compared to the cells that were untreated. When cells were treated simultaneously with VEGF-165 and LY294002, the survival of the cells was increased by 33% compared to the cells treated with LY294002 alone. This was an indication that the VEGF signaling through the PI 3-kinase pathway produced a significant effect in terms of the survival of the T-47D cells. Similar results were seen after 4 days of LY294002 treatment (not shown). The result shown was typical of three separate experiments.

Another indication of the importance of VEGF in stimulating cell survival came from treating the T-47D cells with the apoptotic agent camptothecin (Litvak et al., 1999) and the measurement of apoptosis by the detection of DNA/histone fragments characteristic of apoptosis. As is shown in Fig. 9, when cells were treated with VEGF-165 prior to the treatment with camptothecin, VEGF-165 significantly reduced the degree of apoptosis compared to that produced by the camptothecin alone. The effect of VEGF-165 appeared to be maximal at 100 ng/ml as increasing the concentration to 500 ng/ml did not increase the survival (not shown). When cells were treated with heregulin prior to camptothecin, an even greater effect was seen in terms of antagonizing the effect of the apoptotic agent. This partial protective effect of VEGF-165 is consistent with that seen when the cells were treated with LY294002 in the presence and absence of VEGF-165. Thus, modulation of the PI 3-kinase pathway can bring about increased survival of the T-47D cells. Also, even when apoptosis is induced by other non-PI 3-kinase-related pathways, the stimulation of the PI 3-kinase survival pathway by VEGF-165 can antagonize this apoptosis.

VEGF-165 stimulation leads to increased migration of T-47D cells.

Since it was recently reported that cellular migration of MCF7 breast cancer cells in response to heregulin was mediated through a PI 3-kinase dependent pathway (Adam et al., 1998), we asked whether VEGF signaling in T-47D breast cancer cells might also affect the migration of these cells. As is shown in Fig. 10 (left panel), initial experiments on matrigel alone showed no migration in response to VEGF-165, while a greater than two-fold increase in migration in response to heregulin was observed. However, when fibronectin was added to the matrigel coating on the transwell membrane, VEGF-165 caused an approximately two-fold increase in migration that was ~72% of the migration observed in response to heregulin under these conditions (Fig. 10, right panel).

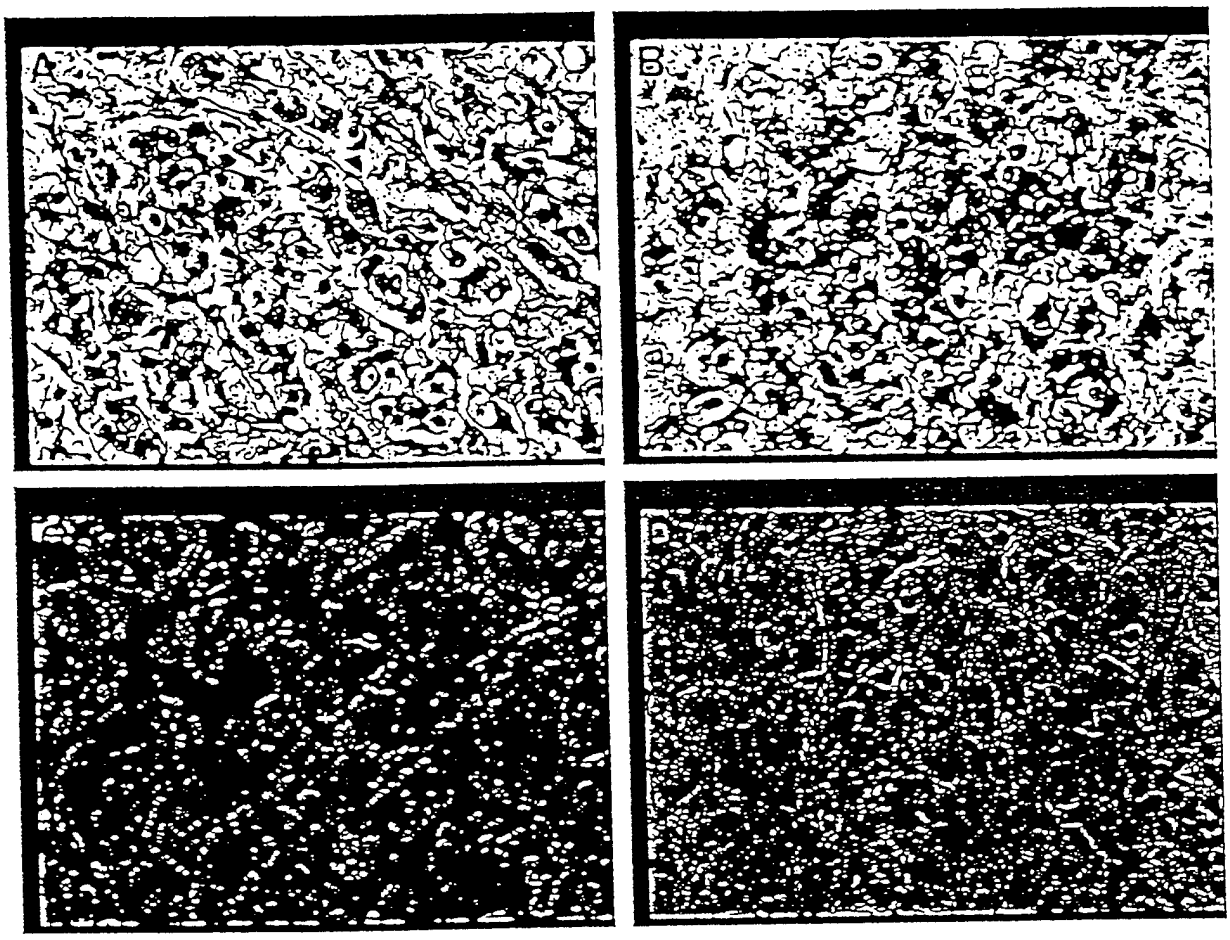


Figure 1. Immunohistochemistry of paraffin sections of a breast tumor. A. Stained with hematoxylin/eosin; B. Stained with rabbit anti-VEGF; C. Stained with mouse monoclonal anti-Flk-1/KDR; D. Stained with mouse monoclonal anti-Flt-1.

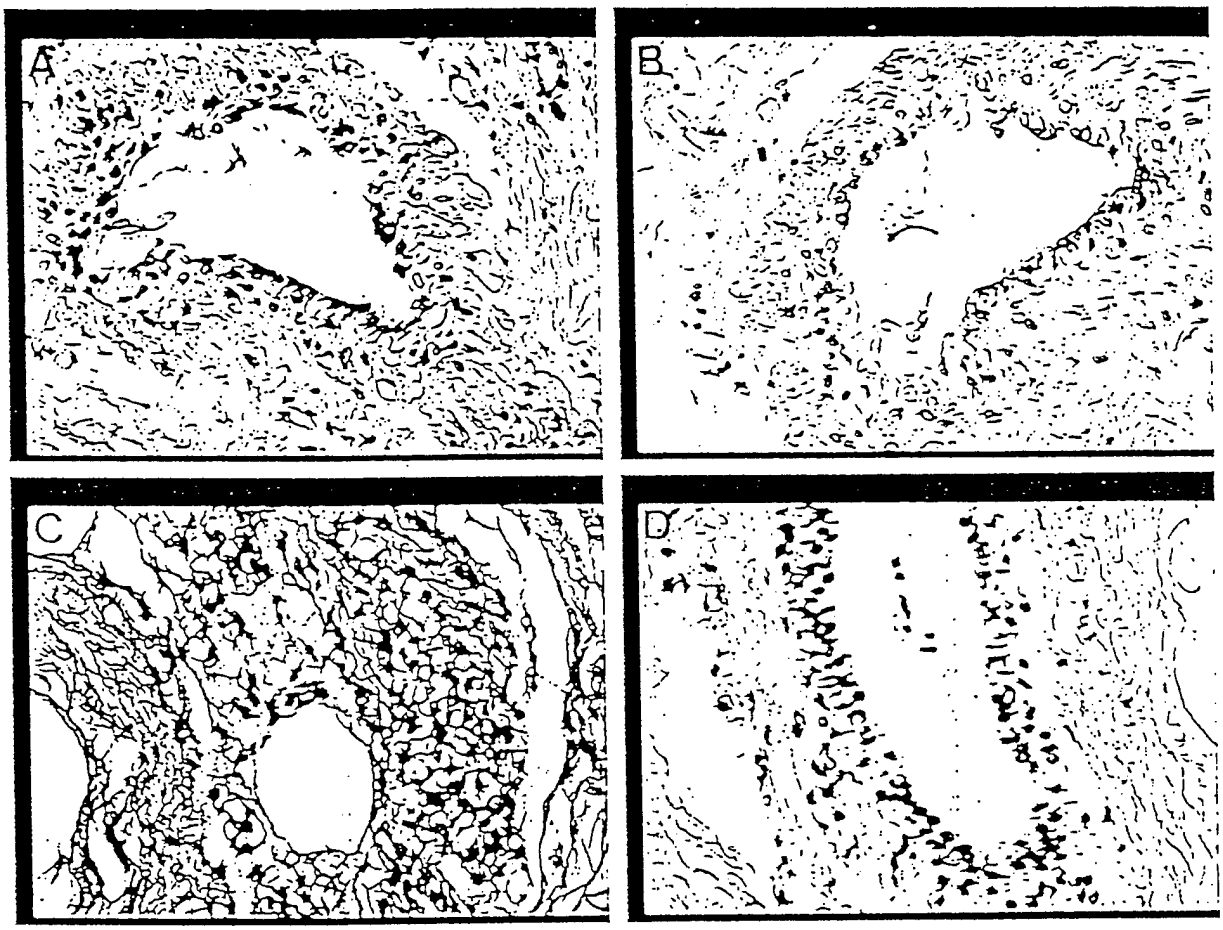


Figure 2. Immunochemistry of paraffin sections of normal adjacent breast tissue (same patient as Fig. 1) A. Stained with hematoxylin/eosin; B. Stained with rabbit anti-VEGF; C. Stained with mouse monoclonal anti-Flk-1/KDR; D. Stained with mouse monoclonal anti-Flt-1.

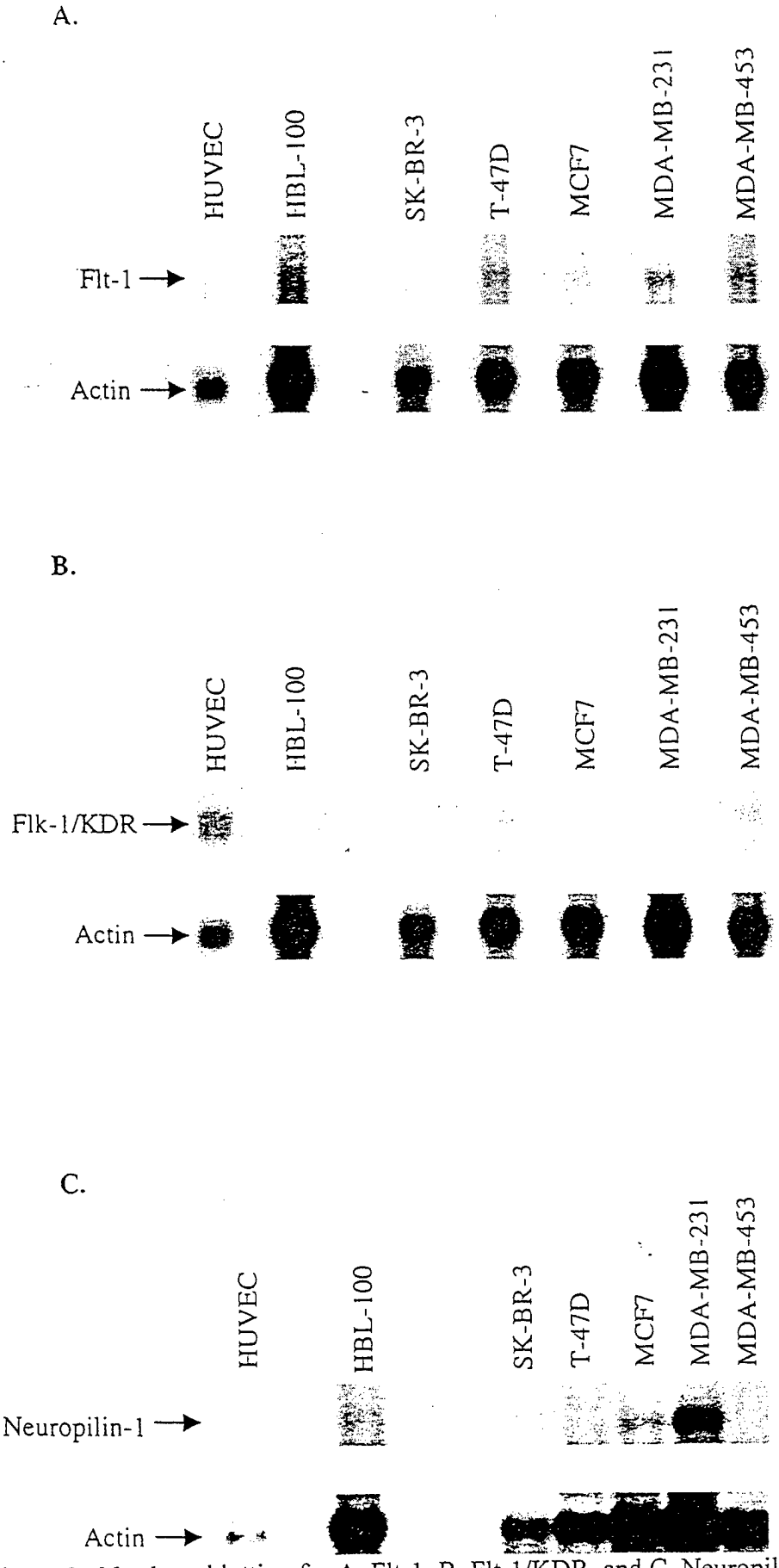
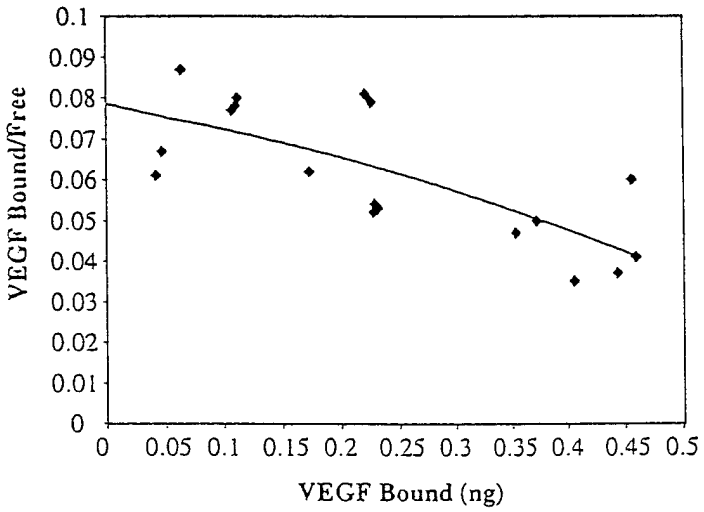


Figure 3. Northern blotting for A. Flt-1, B. Flt-1/KDR, and C. Neuropilin-1 mRNA. Cell lines tested are indicated above. Control actin mRNA probe is shown in each panel.

Fig 4

A. MDA-MB-231



B. T-47D

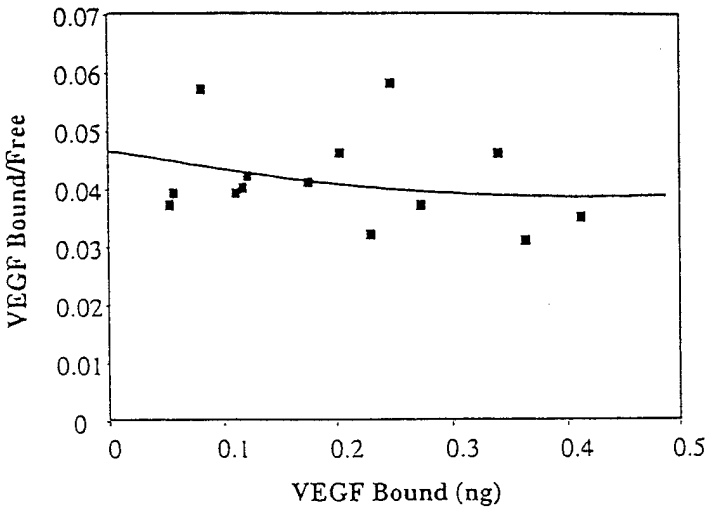
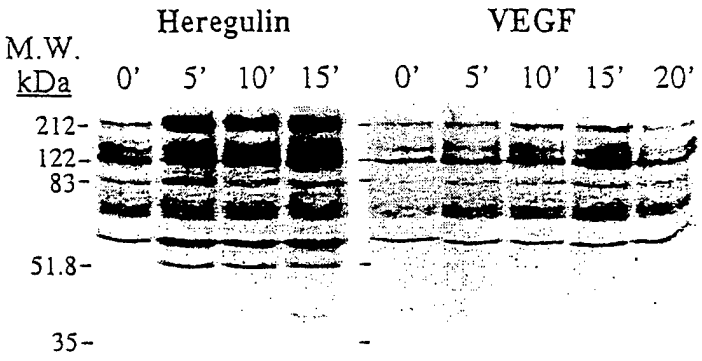


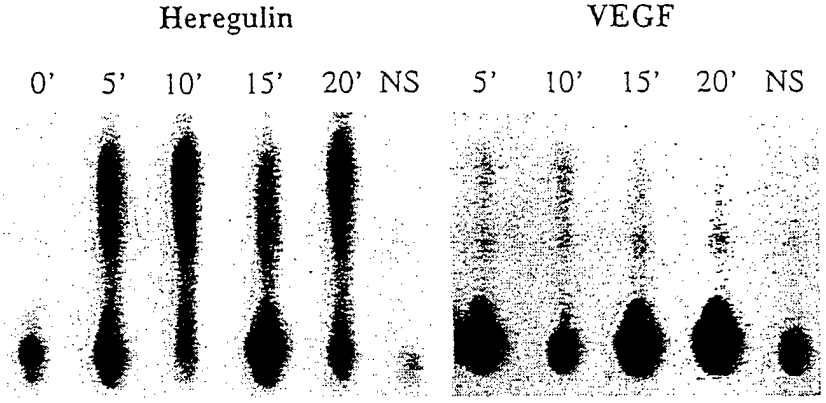
Figure 4. Scatchard analysis of ^{125}I-VEGF-165 binding to breast cancer cells. Binding to cells was determined as described in Materials and Methods. A. ^{125}I-VEGF-165 binding to MDA-MB-231 cells. B. ^{125}I-VEGF binding to T-47D cells.

A.



WB: PY99

B.



PI-3 Kinase assay

Figure 5. Stimulation of T-47D cells with heregulin (left) and VEGF (right). A. Western immunoblotting of total cell extracts with PY99 antibody. Arrows indicate major phosphorylated proteins. B. Assay of PI 3-kinase. Arrow indicates ^{32}P-PI.

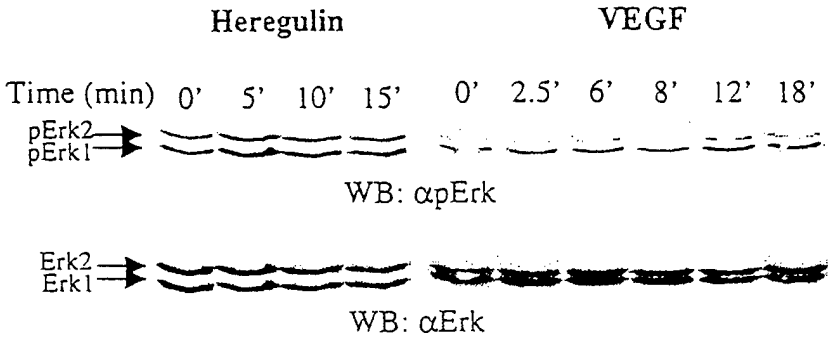


Figure 6. Stimulation of T-47D cells with heregulin (left) or VEGF (right) and western immunoblotting of total cell extracts with anti-phospho-ERK antibody (upper panel). Lower panel indicates immunoblotting with an antibody to total ERK 1 protein.

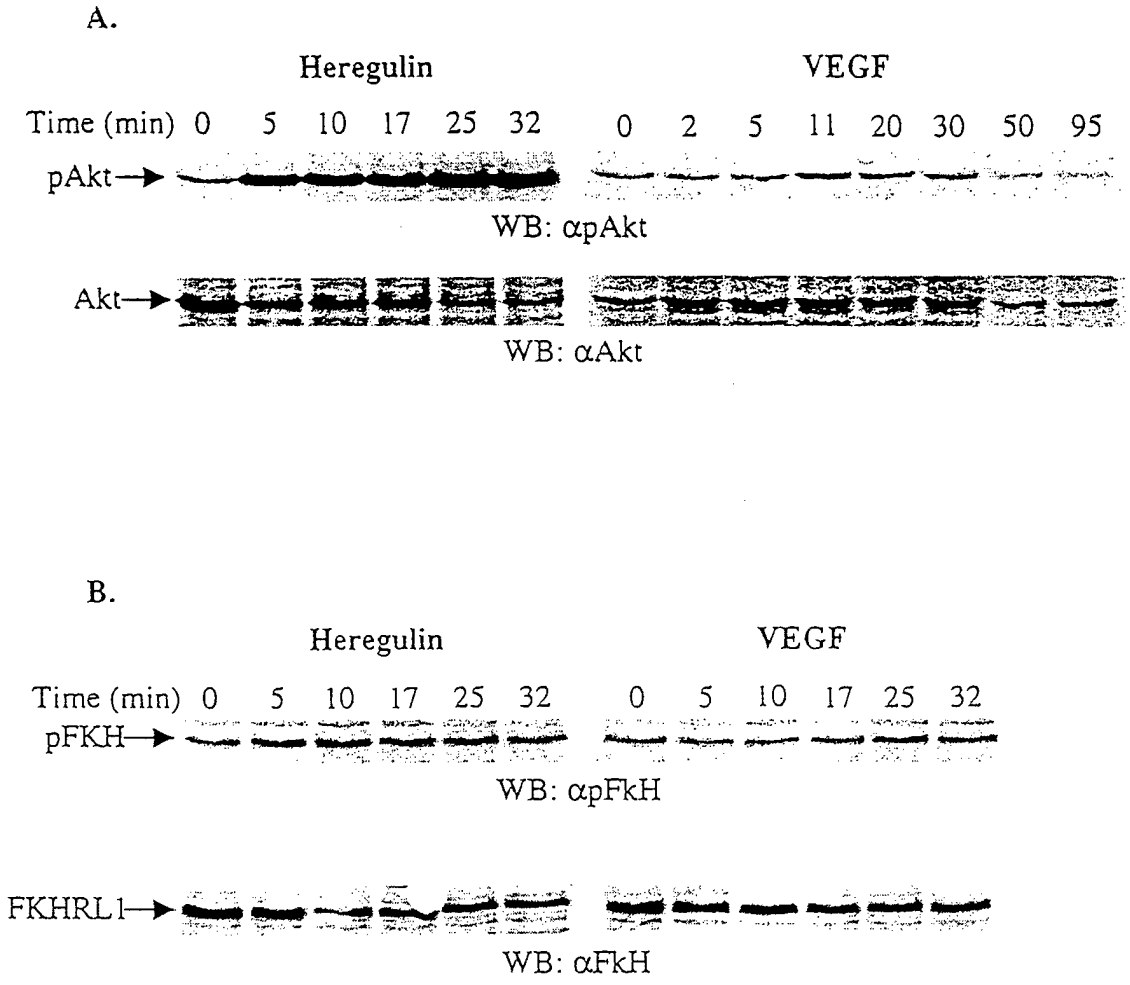


Figure 7. Stimulation of T-47D cells with heregulin (left) or VEGF (right) and immunoblotting with (A) anti-phospho-Ser473 Akt, or (B) anti-phospho-Ser253 FKH antibodies. Below each is shown the corresponding blotting of the non-phosphorylated protein.

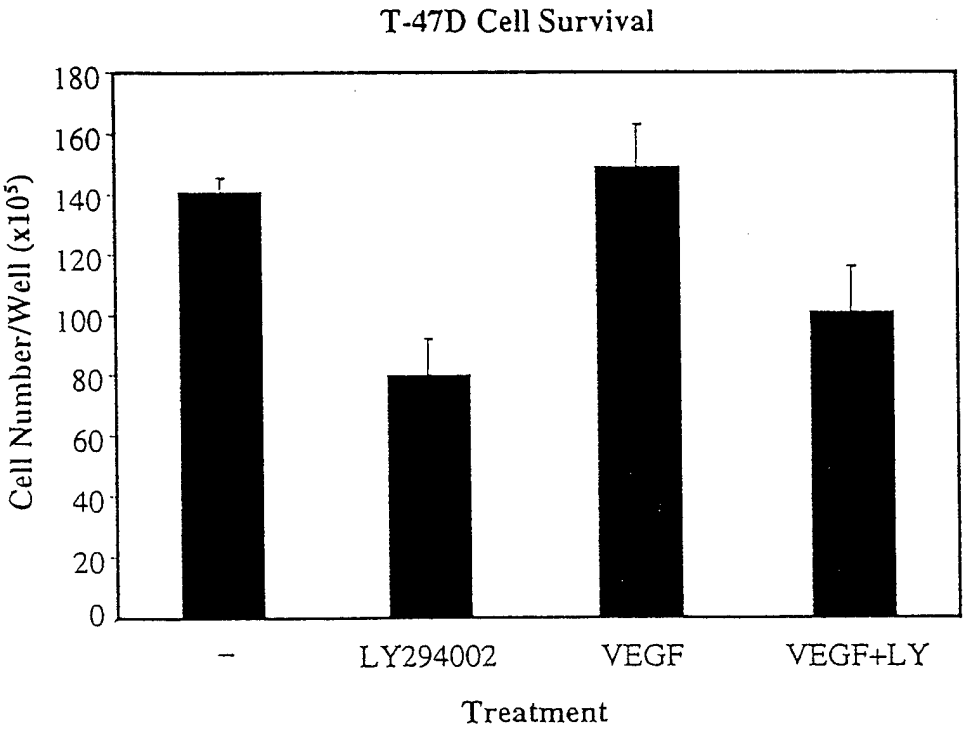


Figure 8. Survival of T-47D cells after treatment with LY294002 in the presence or absence of VEGF. Ordinate represents counting of live cells by trypan blue exclusion.

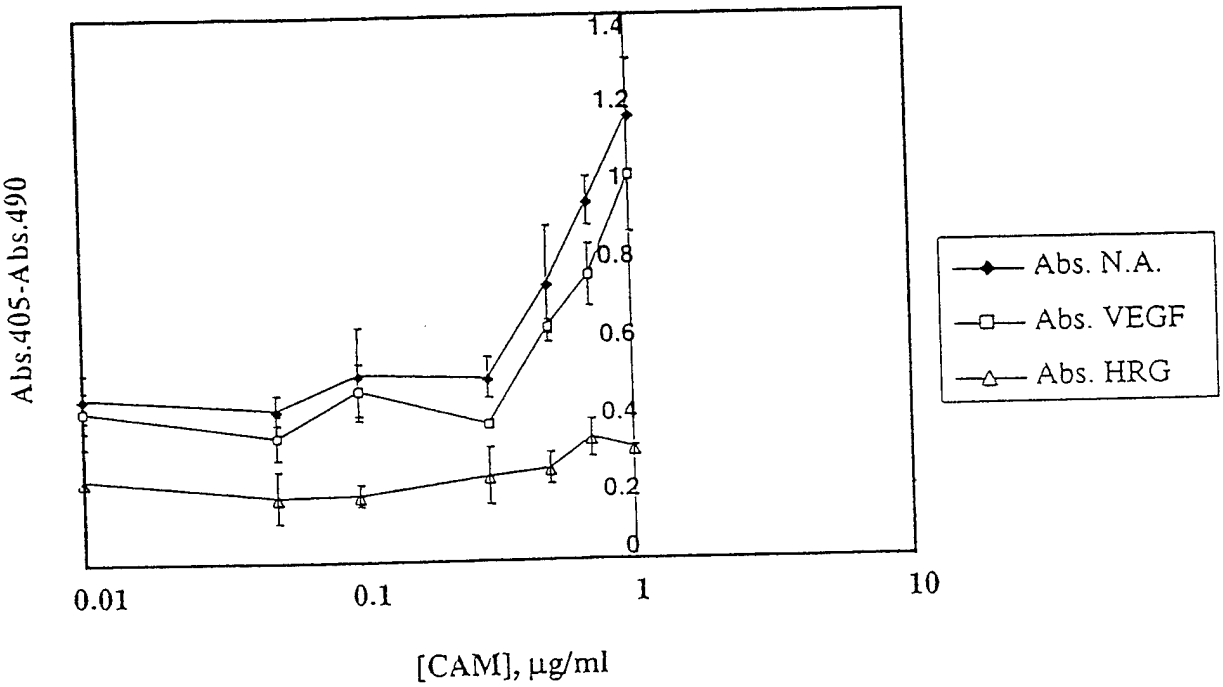


Figure 9. T-47D cell apoptosis after treatment with increasing concentrations of camptothecin alone (♦), camptothecin + VEGF (□), or camptothecin + heregulin (Δ).

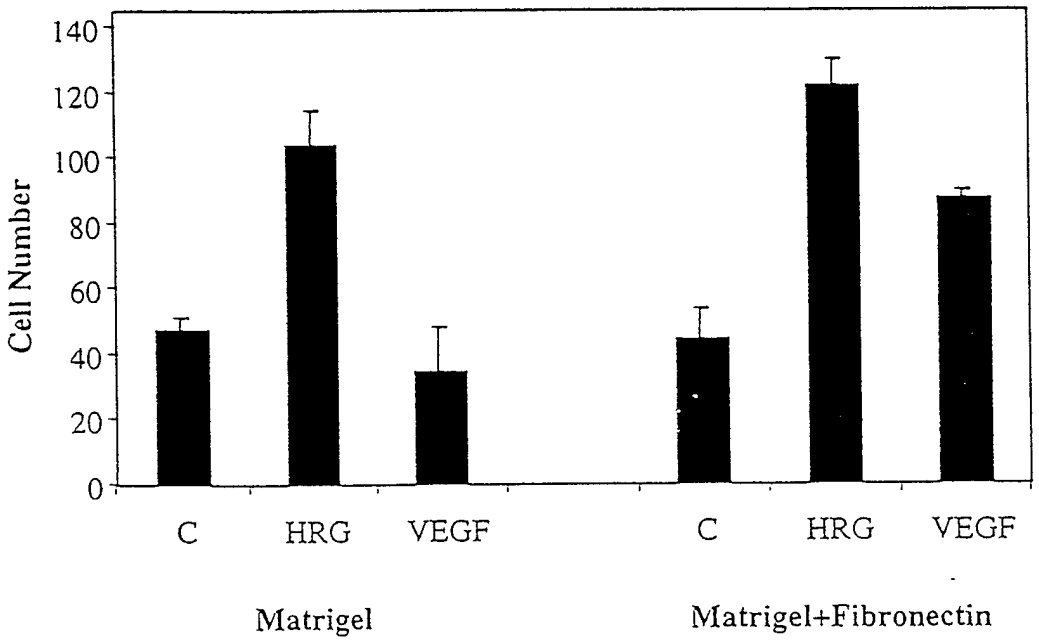


Figure 10. Migration of T-47D cells in response to VEGF or heregulin. Comparisons are to medium alone (C). Migrations were conducted on membranes coated with matrigel alone (left) or matrigel plus fibronectin (right).

KEY RESEARCH ACCOMPLISHMENTS:

Effect of VEGF on:
1) Signaling
2) Migration
3) Survival
has analyzed in breast cancer cells.

REPORTABLE OUTCOMES

We currently have a manuscript in preparation

CONCLUSIONS:

To elucidate the role of VEGF in breast cancer, we have carried out immunohistochemical staining of VEGF and VEGF receptors in primary human breast tumors. Positive expression of VEGF, Flt-1, and Flk-1/KDR was found in four out of five primary breast tumors. Northern analysis for the expression of the known VEGF receptors showed 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 bound ^{125}I-VEGF with Kd of 5.2 x 10^{-9}M. The effect of VEGF compared to heregulin on the stimulation of signaling in T-47D breast cancer cells was shown by changes in tyrosine phosphorylation of total cellular proteins. The MAP kinases ERK 1,2 were increased in their phosphorylation in response to VEGF and heregulin in T-47D cells. Both VEGF and heregulin stimulated the PI 3-kinase pathway, leading to phosphorylation of Akt and FKHRL1. Treatment of T-47D cells with VEGF resulted a 33% increase in survival by two different methods. Furthermore, VEGF increased the cellular migration of T-47D breast cancer cells on transwell membranes. These findings strongly suggest an autocrine role for VEGF contributing to the tumorigenic properties of breast cancer cells.