Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones

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Abstract

Increased levels of vascular endothelial growth factor (VEGF) are associated with a poor response of breast cancer to anti-hormone treatment. Although VEGF is regarded as an endothelial-specific growth factor, recent reports have shown that VEGF can promote proliferation of other cell types, including breast tumor cells. We have characterized the proliferative effects of VEGF in breast cancer cell lines that are commonly used for understanding the role of estrogens, progestins, and anti-hormones on tumor growth. Since steroid hormones can increase the level of VEGF in certain breast cancer cells, we evaluated the effects of exogenous VEGF on the growth-suppressive effects of anti-estrogen (ICI 182,780) and RU-486 (anti-progestin mifepristone) in human breast cancer cells. VEGF₁₆₅ and VEGF₁₂₁ increased the proliferation of tumor cell lines that expressed VEGFR-2 (VEGF receptor 2) (flk/kdr) via the extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) pathway. Furthermore, VEGF induced the expression of the anti-apoptotic protein Bcl-2 and blocked down-regulation of Bcl-2 by ICI 182,780 and induced Bcl-2 in BT-474 and T47-D cells even in the presence of RU-486. Increased Bcl-2 levels in response to VEGF were associated with increased proliferation and survival of tumor cells even in the presence of anti-hormones. These results suggest that VEGF stimulates proliferation of VEGFR2-positive tumor cells, promotes survival via the expression and activity of Bcl-2 and overrides the growth-suppressive effects of anti-hormones. This represents a potential explanation for anti-hormone resistance and tumor progression in clinical samples. Thus, it may be useful to use combined modality treatment involving anti-hormones and anti-angiogenic agents to treat breast cancers that express elevated levels of VEGF.

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Introduction

Angiogenesis, the formation of new blood vessels, is essential for the development, progression, and metastasis of malignant tumors (Folkman 1995, Ferrara 2002). In the absence of angiogenesis, tumors cannot grow beyond 1–2 mm³ in size (Bergers & Benjamin 2003). Vascular endothelial growth factor (VEGF) is a primary stimulus of angiogenesis in tumors and functions through binding VEGF receptor-2 (VEGFR2; also known as flk/kdr) and VEGFR1 (also known as flt) expressed on endothelial cells (Ferrara *et al.* 2003). Two

secretory forms of VEGF are typically produced by endothelial and breast tumor cells: VEGF₁₆₅ and VEGF₁₂₁ (Folkman 1995, Ferrara 2002). VEGF also acts as a survival or an anti-apoptotic factor and has been shown to induce Bcl-2 in endothelial cells as well as in breast cancer cells (Pidgeon *et al.* 2001). Although VEGF was initially thought to be an endothelial cell-specific growth factor, recent reports have identified VEGF receptors on non-endothelial cells (Brown *et al.* 1995, Kuroda *et al.* 1995, Liang & Hyder 2005). Although VEGF receptors have been implicated in the proliferation

of breast tumor cells in response to VEGF, these results are inconsistent and the lack of such an effect has also been reported (Price *et al.* 2001).

Many human breast cancers contain estrogen receptors and progesterone receptors, which cause proliferation of tumor cells (Spicer & Pike 1994). Steroid hormones have been shown to regulate VEGF in breast cancer cells (Hyder *et al.* 1998, Hyder & Stancel 1999). This has raised the hypothesis that tumor cells may produce VEGF not only for proliferation of endothelial cells but also for proliferation and/or survival of breast tumor cells in an autocrine and/or paracrine manner. We have recently shown that progestin-induced VEGF secretion from three different breast cancer cell lines can cause tumor cell proliferation in an autocrine and paracrine manner (Liang & Hyder 2005). Other researchers have demonstrated a similar role for VEGF in estrogen-induced tumors (Nakamura *et al.* 1996, Masood *et al.* 2001). Hence, VEGF probably affects the progression of breast tumors by impacting on tumor cell proliferation and survival as well as through induction of angiogenesis.

Both anti-estrogens and anti-progestins have been shown to cause regression of hormone-dependent tumors; however, some tumor cells invariably become resistant to the anti-hormones and continue to grow (Gutierrez *et al.* 2005). In certain cases, anti-hormones can even stimulate tumor growth (Clarke *et al.* 2001). It is not known what specifically causes the resistant cells to continue to proliferate, although it has been suggested that growth factors may be involved (Nicholson *et al.* 2005). Interestingly, clinical studies have shown that tumors with high levels of VEGF fail to respond to hormone therapy (Foekens *et al.* 2001, Manders *et al.* 2003) suggesting that VEGF production might be involved in anti-hormone resistance.

The objectives of this study were to characterize the growth-inducing properties of VEGF on proliferation and survival in a variety of breast cancer cell lines that are routinely used to study the effects of hormones and anti-hormones on tumor growth. We also sought to identify the signal transduction pathway responsible for VEGF-induced proliferative response, and to determine if the presence of increased levels of VEGF interferes with the growth-suppressive effects of anti-hormones in breast cancer cells.

Materials and methods

Materials

The breast cancer cell lines T47-D, BT-474, HCC-1428, MCF-7, HCC-1500, MDA-MB-231, and ZR-75, and RPMI-1640 medium were obtained from ATCC

(Manassas, VA, USA). All cell lines except HCC-1428 and HCC-1500 express Her-2/neu (Konecny *et al.* 2003 and data sheet from ATCC) with BT-474, expressing the highest level of HER-2/neu protein. Phenol red-free DME/F12 medium, PBS, and 0.05% trypsin-EDTA were purchased from Invitrogen Corporation & Life Technologies and fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). Recombinant human VEGF (rhVEGF₁₆₅ and rhVEGF₁₂₁), anti-VEGF antibody (catalog number AF-293-NA, neutralizes both VEGF₁₆₅ and VEGF₁₂₁), IgG control antibody, and the VEGF ELISA Kit were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). The antibody 2C3 that blocks interaction of VEGF from binding to VEGFR2 (flk) was raised against recombinant human VEGF as described previously (Brekken *et al.* 2000). C44, an IgG2a mouse anti-colchicine monoclonal antibody (Rouan *et al.* 1990) served as a negative control and was from ATCC. The antibody against VEGFR2 (flk/kdr) was from Santa Cruz, CA (catalog number SC-6251). The inhibitor for VEGFR2 Tyr kinase activity (SU1498), the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, and the MAPK/ERK inhibitor U0126 were obtained from CalBiochem (La Jolla, CA, USA). The ELISA kit to detect BrdU (bromodeoxyuridine) incorporation in cells was from Roche Diagnostics Corporation and progesterone (P), medroxyprogesterone acetate (MPA), and sulforhodamine B (SRB) were obtained from Sigma. All the primers used in the study were synthesized at Integrated Device Technology (IDT) (Coralville, IA, USA). The anti-Bcl2, anti-flk/kdr, and secondary horseradish peroxidase-conjugated antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Protein was quantified using the Bicinchoninic acid (BCA) assay (PIERCE, Rockford, IL, USA) and absorbance was measured at 562 nm with a SpectRA MAX 190 Microplate Reader (Molecular Device, Sunnyvale, CA, USA).

Cell culture

T47-D, BT-474, MCF-7, ZR-75, and MDA-MB-231 cells were grown in phenol red-free DME/F12 medium supplemented with 10% FBS (T47-D, BT-474, MCF-7, and ZR-75) or 5% FBS (MDA-MB-231). HCC-1428 and HCC-1500 were grown in RPMI-1640 medium supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-Gln, 4500 mg glucose/l, and 1500 mg sodium bicarbonate/l. All cells were grown in 100×20 mm² tissue culture dishes and harvested with 0.05% trypsin-EDTA.

RT-PCR for VEGF receptor flk/kdr and flt-1

Cells were grown in DME/F12 medium supplemented with 5% dextran-coated, charcoal-treated serum for 24 h. RNA was prepared using UltraSpec (Biotecx, Houston, TX, USA) according to the manufacturer's protocol. Reverse transcription (RT)-PCR was carried out using Platinum PCR Supermix (Invitrogen) in an Applied Biosystems 2700 thermocycler. PCR parameters were as follows: 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 7 min. MDA-MB-231 cDNA was used as a VEGFR2-positive control. The PCR product was analyzed on a 2% agarose gel containing 1 µg/ml ethidium bromide. Electrophoresis was carried out in 0.5X TBE (pH 8.0) at 80 V for 2 h. The gel was photographed under UV light. Primer sequences were as follows: VEGFR2 (flk/kdr) (400 bp product), sense: CATCACATCCACTGGTATTGG, antisense: GCCAAGCTTGTACCATGTGAG; VEGFR1 (flt) (300 bp product), sense: TACACAGGGGAAGAAATCCT, and antisense: ACAGAGCCCTTCTGGTTGGT. Primers for the internal 18S control were obtained from an Ambion kit (Austin, TX, USA).

Cell proliferation assay by BrdU incorporation, ELISA

Cells were seeded into each well of a 96-well plate and incubated overnight at 37 °C with 5% CO₂. Twenty four hours prior to the experiment, the medium was replaced with serum-free DME/F12. The cells were then incubated under various conditions (VEGF, anti-VEGF antibody, progesterone, MPA, or SU1498) for 24 h. BrdU (100 µM) was added to each well, 3 h before termination of the treatment as described before (Liang & Hyder 2005) and the measurement was completed using the manufacturer's protocol. Absorbance was read at 370 nm with a SpecTRA MAX 190 Microplate Reader. Each experimental point was assayed in six different wells, and each study was carried out in duplicate or triplicate.

Cell proliferation assay by SRB

The SRB assay was used to measure cell viability as previously described (Rubenstein *et al.* 1990, Skehan *et al.* 1990). This cell protein dye-binding assay measures the protein content in surviving cells as an index to determine cell growth and viability (Rubenstein *et al.* 1990, Skehan *et al.* 1990). Briefly, 5×10^3 T47D cells or 8×10^3 BT-474 cells were seeded in each well of 96-well plates in 100 µl culture medium (DME/F12 + 10% FBS) and incubated

overnight at 37 °C with 5% CO₂. The next day, the medium was switched to serum-free DME/F12 medium and incubated for 18–24 h. The medium was then replaced and various concentrations of VEGF, anti-VEGF antibody, 10 nM progesterone, or MPA were added for 72 h. VEGF was replaced after 48 h of incubation. Surviving or adherent cells were fixed *in situ* by withdrawing the growth medium and adding 100 µl of PBS and 100 µl of 50% cold trichloroacetic acid and then incubated at 4 °C for 1 h. Cells were washed in ice water and then dried. Cells were then stained in 50 µl of 4% SRB for 8 min at room temperature. Unbound dye was removed by washing five times with cold 1% acetic acid and cells were dried at room temperature. Bound stain was solubilized with 150 µl 10 mM Tris. The absorbance of samples was measured at 520 nm with a SpecTRA MAX 190 microplate reader. Six wells were used for each concentration and each experiment was performed at least twice.

Cell apoptosis and death assay

Annexin V-FITC apoptosis detection kit (Biovision Research Products, Mountain View, CA, USA) was used for detecting early stages of apoptosis. Propidium iodide was used for detection of DNA fragmentation or cell death. Cells were grown in six well plates and treated with VEGF, VEGF with or without anti-VEGF antibody, 2C3, and SU1498 for 48 h. The percentage of Annexin V-FITC positive and PI-positive cells were determined from the fluorescence of 10 000 cells by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The experiments were performed twice.

Whole cell extracts and Western blots

Breast cancer cells were grown and treated with various reagents in 100 mm dishes. At the end of incubation, cells were washed with 3 ml of ice-cold PBS and harvested by gentle scraping. Cells were centrifuged for 5 min at $200 \times g$ at 4 °C and the pellet was re-suspended in either 200 µl radioimmuno precipitation assay (RIPA) buffer for flk/kdr detection (1 mM dithiothreitol (DTT; Sigma) and 1% protease inhibitor cocktail was added prior to use) or in 100 µl complete lysis buffer with protease inhibitor (Active Motif) for Bcl-2 detection by Western blot. The mixture was then incubated on ice for 30 min with shaking. Samples were centrifuged at $14\,000 \times g$ for 15 min, and the supernatant was transferred to microcentrifuge tube, aliquoted, and stored at –80 °C. The supernatant was adjusted to the desired protein concentration as needed.

For western blotting, samples containing 30–50 µg of protein were separated by SDS-PAGE in NuPAGE 3–8% Tris–Acetate Gel (Invitrogen) for flk/kdr detection and in NuPAGE 10% Bis-Tris Gel for Bcl2. Electrophoresis was performed at 100 V for 2 h using NuPAGE Tris–Acetate SDS or 2-(N-Morpholino)ethane-sulfonic (MES) SDS running buffer. Separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) at 30 V for 1.5 h. The blots were blocked for 1 h at room temperature (RT) in 5% non-fat dry milk in Tris buffered saline (TBS) containing 0.1% Tween 20 (TBS-T), and incubated with primary anti-flk/kdr antibody (1:400 dilution) or anti-Bcl-2 antibody (1:200) for 2 h at RT. The blots were washed three times with TBS-T and incubated with secondary antibody for 1 h at RT. The blots were then washed six times (8 min each) with TBS-T and immunoreactive bands were visualized using an ECL plus detection kit (Amersham, Pharmacia Biotech). Membranes were stripped and re-blotted for β -actin (Sigma), which was used as a control for protein loading.

Statistical analysis

Differences among groups were tested using one-way ANOVA with repeated measure over time. The assumption of ANOVA was examined and non-parametric measure based on ranks was used if needed. Values were reported as mean $\pm$ S.E.M. When ANOVA indicated significant effect (F-ratio, $P < 0.05$), the Student–Keuls multirange test was employed to compare the mean of the individual groups using SigmaStat software (Systat Software Inc., Richmond, CA, USA).

Results

VEGF increases proliferation of breast cancer cells

We initially tested the effect of VEGF₁₆₅ on the proliferation of BT-474 and T47-D cells in serum-free conditions. As shown in Fig. 1A, VEGF increased BrdU incorporation in both cell lines. This process was inhibited significantly by an anti-VEGF antibody, but not by non-specific immunoglobulin (IgG). Neither antibody nor IgG alone had any effect on cellular proliferation. VEGF also increased cell viability as determined by the SRB assay, which detects cellular protein (Fig. 1B; Rubenstein *et al.* 1990, Skehan *et al.* 1990). This increase in cell viability was also inhibited by an anti-VEGF antibody. Interestingly, the addition

of an antibody that specifically sequestered the exogenous VEGF consistently showed that T47-D cells were more strongly dependent on VEGF than the BT-474 cells, since sequestration of VEGF led to a loss of viability in T47-D cells. However, the VEGF antibody alone did not reduce cell viability, suggesting that factors other than VEGF produced by tumor cells must be involved in cell viability. The loss of cell viability when VEGF and VEGF antibody were present at the same time suggests that this complex must also sequester some other factors that are required for the viability of T47-D cells, as we also reported in our earlier communication (Liang & Hyder 2005). No such effect was observed in BT-474 cells. The serum-free conditions used in these experiments were optimal for observing VEGF-induced proliferation of breast cancer cells, since our preliminary studies showed that addition of either steroid deprived media containing 5% dextran-coated charcoal treated serum or addition of growth factor supplements into serum-free medium inhibited VEGF-induced proliferation of tumor cells (data not shown).

VEGF-dependent induction of cell proliferation varies across different breast cancer cell lines and is dependent on VEGFR2 (flk/kdr)

To test whether the VEGF-dependent proliferation of breast cancer cells was dependent on the presence of VEGF receptors, we used RT-PCR to amplify VEGFR2 (flk/kdr) or VEGFR1 (flt) in seven different human tumor cell lines commonly used in breast cancer research. As shown in Fig. 2A, four of the seven breast cancer cell lines expressed both VEGFR2 (flk/kdr) and VEGFR1 (flt), and one expressed only VEGFR2 (MDA-MB-231). The VEGF receptor-positive tumor cell lines included BT-474, T47D, HCC-1428, and MCF-7, which express the ER and PR, as well as MDA-MB-231 cells, which lack the ER and PR. Cell lines that lacked expression of either VEGF receptor included HCC-1500 and ZR-75 cells (Fig. 2A). Western blot analysis indicated that tumor cells that lacked VEGF RNA expression also lacked the VEGFR2 protein (Fig. 2B). We next tested these cell lines for VEGF-induced proliferation. As shown in Fig. 2C, only the VEGF receptor-positive cells dose dependently proliferated in response to VEGF. The VEGF receptor-negative HCC-1500 and ZR-75 cell lines did not exhibit a proliferative response to VEGF (Fig. 2B). The effects of VEGF were variable across the VEGFR2-positive cells lines, whereas T47-D and BT-474 cells continued to respond to VEGF with increasing concentrations of the growth factor (1–200 ng/ml), HCC-1428 and MCF-7 cells exhibited a

Figure 1 VEGF-induced cell proliferation of BT-474 and T47-D cells. (A) Cells were treated with 100 ng/ml VEGF in the presence or absence of 2 μ g/ml anti-VEGF antibody (Ab), or IgG for 24 h. Cell proliferation was determined by BrdU incorporation. (B) Cells were treated with 100 ng/ml VEGF in the presence or absence of 1- (Ab1) or 10- (Ab10) μ g/ml anti-VEGF antibody (Ab) for 72 h. Cell viability was determined by the SRB assay. C, control *Significantly different compared with control group, **significant inhibition compared with VEGF 100 ng/ml group ($P < 0.05$ using ANOVA). All experiments were done at least twice.

maximal response at about 50 ng/ml, and MDA-MB-231 cells reached the maximum response at about 100 ng/ml (Fig. 2B). In this limited series of tumor cells, there did not appear to be any correlation between the expression levels of VEGF receptors as determined by RT-PCR analysis, the amount of protein detected and the degree of VEGF-induced cellular proliferation.

Several studies have reported that VEGF-induced proliferation is mediated by the interaction of VEGF

with VEGFR2 (flk/kdr) in both breast cancer cells and in endothelial cells (Masood *et al.* 2001, Mercurio *et al.* 2004, Liang & Hyder 2005, Schoeffner *et al.* 2005). Thus, we used an antibody (2C3) raised against human VEGF that blocks interaction of VEGF with VEGFR2 (flk) but not with VEGFR1 (flt) (Brekken *et al.* 2000), to determine whether blocking the VEGF-VEGFR2 interaction will prevent VEGF-mediated cellular proliferation. As shown in Fig. 2D, 2C3 prevented

Figure 2 VEGF-induced cell proliferation is dependent on VEGFR2 (flk/kdr) in breast cancer cell lines. (A) RT-PCR analysis of VEGF receptor expression (flk/kdr and flt) in various cell lines used in the study. 18S RNA levels were analyzed as a control. (B) Western blot analysis of VEGF receptor expression (flk/kdr) in various cell lines used in the study. β -actin was analyzed as a loading control. (C) The breast cancer cells tested in (A) were treated with 1–200 ng/ml VEGF for 24 h under serum-free conditions and cell proliferation was evaluated by analysis of BrdU incorporation, as described in 'Materials and methods'. (D) Cells were treated with 100 ng/ml VEGF in the presence or absence of 20 μ g/ml 2C3 or C44 (BT-474) or 10 μ g/ml 2C3 or C44 (T47-D) for 72 h. Cell viability was determined by the SRB assay. (E) SU1498, an inhibitor of flk/kdr blocked VEGF-induced cell proliferation in a dose-dependent manner (0.1–20 μ M) in BT-474 and T47-D cells. *Significantly different compared with control group, **significant inhibition when compared with VEGF 100 ng/ml group ($P < 0.05$ using ANOVA).

VEGF-induced proliferation of both BT-474 and T47-D cells; control antibody C44 raised against colchicine and had no effect. This result strongly suggests that VEGF protects breast cancer cell viability via VEGFR2 (flk/kdr).

In addition to the antibody study described above, we used SU1498, an inhibitor of the tyrosine kinase activity of VEGFR2, to determine whether VEGFR2 is also responsible for VEGF-mediated proliferation in T47-D and BT-474 cells. As shown in Fig. 2E, SU1498 blocked VEGF-stimulated BrdU incorporation in both tumor cell lines in a dose-dependent manner, indicating that VEGF-mediated proliferation of these breast tumor cells was dependent on VEGFR2. Interestingly, following SU1498 treatment, BrdU incorporation reached values below those of control cells. This might be explained by additional interactions of SU1498 at higher concentrations with platelet derived growth factor (PDGF)

receptor, epidermal growth factor (EGF)-receptor and HER-2 kinases (Calbiochem data sheet).

Influence of VEGF isoforms on proliferation of tumor cells

Other studies have clearly established that the two secreted forms of VEGF, VEGF₁₆₅ and VEGF₁₂₁, induce proliferation of endothelial cells (Soker *et al.* 1997). We tested the two isoforms separately to determine their effects on the proliferation of BT-474 and T47-D cells. As shown in Fig. 3, both isoforms stimulated the proliferation of breast tumor cells. However, VEGF₁₆₅ appeared to be more potent as previously reported for endothelial cells (Graells *et al.* 2004). Quantitatively, both cell lines responded similarly to increasing concentrations of VEGF₁₆₅ and VEGF₁₂₁, though with 200 ng/ml, VEGF₁₆₅

Figure 3 The VEGF₁₆₅ and VEGF₁₂₁ isoforms induce dose-dependent induction of cell proliferation. BT-474 and T47-D cells were seeded into 96-well plates with 10% FBS DME/F12 medium overnight. The culture medium was then removed and serum-free media was added for 24 h. The medium was then replaced with serum-free medium containing different concentrations of VEGF₁₆₅ and VEGF₁₂₁ (1–200 ng/ml) for 24 h. Cell proliferation was analyzed by BrdU incorporation, as described in 'Materials and Methods'. *Significant difference compared with corresponding control group ($P < 0.05$; one-way ANOVA); Δ significant difference in proliferation by VEGF isoforms between the two breast cancer cell lines ($P < 0.05$ by Student *t*-test).

BT-474 cells responded significantly better than T47-D cells (as also observed in Fig. 2C). These results demonstrate that both VEGF isoforms induce breast cancer cell proliferation, though high concentrations of VEGF₁₆₅ elicit an increased response in BT-474 cells.

VEGF-induced cellular proliferation involves the MAPK pathway

As shown in Fig. 2D, VEGF-dependent proliferation of breast cancer cells was dependent on VEGFR2. Other investigators have shown that the interaction of VEGF with VEGFR2 can activate the PI3-kinase/Akt-dependent signaling pathway and the mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signal transduction pathway (Graells *et al.* 2004, Svensson *et al.* 2005). We used specific inhibitors of four different signaling modules to identify the pathway involved in mediating VEGF-induced tumor cell proliferation in BT-474 and T47-D breast cancer cells. The inhibitors tested included LY294002 (a PI3K inhibitor), U0126 (a MAPK/ERK inhibitor), SB202190 (a MAPK/p38 inhibitor) and SP600125 (a MAPK/c-jun inhibitor). Cells were incubated with increasing concentrations of inhibitor in the presence or absence of 100 ng/ml

VEGF. The results demonstrated that the MAPK/ERK inhibitor, U0126 blocked proliferation of both T47-D and BT-474 cells (Fig. 4A and B). A similar level of inhibition was also observed with the MAPK/p38 pathway inhibitor SB202190 (data not shown). However, inhibitors for the PI3-kinase dependent signal transduction pathway (Fig. 4A and B) and MAPK/c-jun pathway (data not shown) were without effect.

VEGF induces Bcl-2 protein in breast cancer cells

Since VEGF can induce proliferation of breast cancer cells, it was expected that VEGF would induce expression of the anti-apoptotic protein Bcl-2. We tested this hypothesis in BT-474, T47-D, and MCF-7 cells by incubating the cells overnight with VEGF (50 or 100 ng/ml) in the presence or absence of an anti-VEGF antibody. As shown in Fig. 5A, VEGF at both 50 and 100 ng/ml increased Bcl-2 levels in the three cell lines tested and in all cases the induction was blocked by the anti-VEGF antibody. For reasons that remain unclear, incubating cells with VEGF plus the anti-VEGF antibody reduced Bcl-2 expression levels below basal levels in both T47-D and MCF-7 cells. This was similar to the results shown in Fig. 1B, in which VEGF led to loss of cell viability in the presence

Figure 4 VEGF-induced proliferation of breast cancer cells involves the MAPK/ERK signaling pathway. (A) BT-474 cells and (B) T47-D cells were seeded into 96-well plates in DME/F12 with 10% FBS overnight. Cell culture medium was then replaced with serum-free DME/F12 medium for 24 h. Media was replaced and cells were pre-treated with 1, 10, or 20 μ M PI3K inhibitor (LY294002) or MAPK/ERK inhibitor (U0126) as indicated for 2 h and then VEGF (100 ng/ml) was added where indicated for 24 h. VEGF antibody (Ab; 2 μ g/ml) was added to the control set for determining specificity of VEGF response. Cell proliferation was evaluated by analysis of BrdU incorporation as described in 'Materials and methods'. *Significant induction compared with control group, **significant inhibition compared with control VEGF group, ^ significant induction compared with the values obtained with inhibitor alone. ($P < 0.05$ using one-way ANOVA).

of the anti-VEGF antibody, as compared with the anti-VEGF antibody alone.

Next, we tested whether VEGF protects T47-D cells from undergoing apoptosis and cell death and whether this effect occurs via VEGFR2 (flk/kdr). As seen in Fig. 5B and C, placing the cells in SFM dramatically induced apoptosis and cell death, an effect, which was prevented by adding VEGF to the medium. However,

simultaneous presence of antibody 2C3 prevented the VEGF-induced protection of cellular apoptosis and cell death. We next confirmed the effect of VEGF in preserving cell viability by adding an inhibitor of VEGFR2 tyrosine kinase activity, SU1498, to the culture media. As seen in Fig. 5B and C, SU1498 blocked VEGF induced protection of cell death. Intriguingly, the simultaneous presence of SU1498

Figure 5 VEGF increases Bcl-2 expression and cell survival in breast cancer cells. (A) BT-474, T47-D, and MCF-7 cells were grown in 100 mm culture dishes overnight in DME/F12 + 10% FBS. The medium was then replaced with serum-free medium for 24 h. Cells were then treated in serum-free medium with 50 (VEGF50) or 100 ng/ml (VEGF100) VEGF for 48 h in the absence or presence of an anti-VEGF antibody (2 μ g/ml, Ab). Cells were then harvested and whole cell extracts were prepared. Bcl-2 protein levels were measured by Western blot analysis and β -actin levels were used as a loading control. (B) Suppression of VEGF induced cell survival by anti-VEGF antibody (Ab), 2C3, and SU1498. Cells were grown in six-well plates and treated with 100 ng/ml VEGF, or VEGF with or without 2 μ g/ml anti-VEGF antibody (Ab), or 10 μ g/ml 2C3, and 2 μ M SU1498 for 48 h. Percentage of Annexin V-FITC-positive and PI positive cells were determined from the fluorescence of 10 000 cells by a FACScan flow cytometer and a representative experiment is shown. Numerical values shown represent combined proportion of dead cells (PI positive; upper right) + cells undergoing apoptosis (Annexin V positive; lower right). (C) Bar graph representing dead cells (PI positive) + cells undergoing apoptosis (Annexin V positive) from 3 to 6 determinations as described in (B). *Significant differences compared with control group (C2-SFM), **significant differences compared with VEGF 100 ng/ml group (SFM + VEGF, $P < 0.05$ using ANOVA). FBS; fetal bovine serum; SFM, serum-free medium.

and VEGF consistently led to higher levels of apoptosis and cell death compared with cells incubated with SU1498 alone.

VEGF inhibits the anti-proliferative effects of anti-hormones in ER-and PR-containing breast cancer cell lines

Bcl-2 is an anti-apoptotic protein that functions in cell survival, tumor progression, and drug resistance, and is induced in response to VEGF in breast cancer cells. Consequently, we wondered whether the pro-proliferative hormone estrogen might also induce Bcl-2 in estrogen-responsive MCF-7 cells. Fig. 6A shows that estradiol increased Bcl-2 expression in the MCF-7 cell line, an induction which was blocked by the anti-estrogen ICI 182,780, which is known to be anti-proliferative and pro-apoptotic for these cells (Ellis et al. 1997). In contrast, the ICI 182,780-mediated down-regulation of Bcl-2 was blocked in the presence of VEGF (Fig. 6A).

We next asked if the presence of extracellular VEGF, in addition to the anti-hormone ICI 182,780, could override the anti-proliferative properties of ICI 182,780. MCF-7 cells were seeded into 96-well plates in DME/F12 medium +10% FBS. The next day, culture medium was replaced with serum-free DME/F12 medium and cells were incubated for another 24 h. Cells were then treated with 10 nM estradiol in the presence or absence of 100-fold excess of ICI 182,780 and increasing concentrations of VEGF. As shown in Fig. 6B, VEGF significantly abrogated the anti-proliferative effects of ICI 182,780 at all concentrations tested (10, 50, and 100 ng/ml).

We also tested the effects of RU-486 on Bcl-2 expression in T47-D and BT-474 cells, both of which proliferate in response to progesterone. RU-486 is an anti-progestin that suppresses cellular proliferation. As shown in Fig. 7A, progesterone and RU-486 did not induce Bcl-2 expression in either BT-474 or T47-D cells. However, VEGF induced the expression of Bcl-2 even in the presence of RU-486, which prevents cellular proliferation. When we tested the effects of VEGF on the anti-proliferative actions of RU-486 in the two cell lines, we found that it promoted the proliferation of both BT-474 and T47-D cells in a dose-dependent manner, even in the presence of progesterone and 100-fold excess RU-486 (Fig. 7B).

Discussion

VEGF is a potent angiogenic growth factor and a prognostic factor in breast cancer (Gasparini et al.

1997, Obermair et al. 1997, Borgstrom et al. 1999). Recent reports have shown that VEGF is elevated in patients who fail to respond to hormone therapy or in those who have an early recurrence, suggesting that VEGF may be directly involved not only in the formation of new blood vessels which are required for tumor expansion, but also that it may have direct effects that induce the proliferation and survival of tumor cells. In this respect, it is interesting that many reports have now indicated that hormone-dependent tumors secrete VEGF in response to either estrogens or progestins (Hyder & Stancel 2000), which could provide a partial explanation for the growth-promoting effects of steroid hormones in breast cancer.

The present investigation was undertaken to determine whether VEGF is a proliferative and/or a survival factor in various breast cancer cell lines that are frequently used to investigate the effects of steroid hormones on cellular proliferation. Furthermore, we wished to determine if the presence of excess VEGF overrides the anti-proliferative effects of anti-hormones that are known to mediate apoptosis of breast cancer cells and thus control proliferation of breast tumors (Ellis et al. 1997). Our results demonstrate that VEGF dose dependently drives proliferation of various breast cancer cells and that this effect is dependent on the presence of VEGFR2 (flk). Interestingly, the large panel of breast cancer cells utilized indicated that although all cells that expressed VEGFR2 responded to VEGF with proliferation, there were differences in sensitivity in the various cell lines. This could be due to the presence of different levels of VEGF receptors on the cell surface or due to interactions of receptors with other proteins and receptors, e.g., neuropilin receptors (Barr et al. 2005) that could affect the sensitivity of tumor cells to VEGF-induced proliferation. Our findings also show that VEGF likely protects the cells from undergoing apoptosis in serum-deprived conditions by up-regulating the expression of Bcl-2.

There is considerable interest in inhibiting tumor cell growth by reducing VEGF levels and thereby inhibiting endothelial cell growth and/or promoting apoptosis of tumor cells. Therefore, we examined a large panel of breast cancer cells utilized in hormone-related cancer research to observe the extent to which VEGF serves as a proliferative growth factor. We concentrated mainly on ER- and PR-positive cells, since the majority of breast cancer patients whose tumors express PR and ER are treated with anti-hormones, and the tumors subsequently become resistant to endocrine therapy, while retaining their receptor status (Ali & Coombes 2000). Recent reports have hinted that excess VEGF production could

Figure 6 VEGF blocks ICI 182,780-mediated down-regulation of Bcl-2 and overrides the anti-proliferative effects of ICI 182,780 in MCF-7 cells. (A) MCF-7 cells were grown in 100 mm culture dishes overnight in DME/F12 + 10% FBS. The medium was then replaced with serum-free medium for 24 h. Cells were then treated with 10 nM estradiol (E2) in the absence or presence of 1 μ M ICI 182,780 or 1 μ M ICI 182,780 plus 100 ng/ml VEGF for 24 h. Cells were washed and whole cell extracts were subjected to Western blotting for Bcl-2 and β -actin. (B) MCF-7 cells were seeded into 96-well plates with 10% FBS DME/F12 medium overnight. The medium was then replaced with serum-free medium for 24 h. Cells were then treated with VEGF, with or without VEGF antibody (Ab), 10 nM estradiol (E2), with or without ICI 182,780 (ICI) and with or without 10, 50, and 100 ng/ml VEGF (V10, V50, or V100 respectively) for an additional 24 h. Cell proliferation was analyzed by BrdU incorporation. Results showed that VEGF significantly abrogated the effects of ICI 182,780 on inhibition of MCF-7 cell proliferation at all concentrations tested (10, 50, and 100 ng/ml). *Significantly different when compared with control group, ^ significantly different than E2 + ICI group ($P < 0.05$; ANOVA).

contribute to the acquisition of anti-hormone resistance (Ryden *et al.* 2005). Leptin has also been shown to exert similar effects and to override the effects of ICI 182,780 in breast cancer cells (Garofalo *et al.* 2004). Thus, one mechanism that drives the resistance of tumors to anti-hormones may include excess production of growth-promoting factors from tumor cells.

Previous studies have shown that VEGF can induce proliferation of breast cancer cells (Liang & Hyder 2005). However, other studies failed to observe this effect (Price *et al.* 2001). Our own studies indicate that cell culture conditions can have a profound effect on VEGF-induced proliferation; we found that serum inhibited the proliferative response (data not shown). Under serum-free conditions, however, VEGF induced proliferation in five out of the seven breast cancer

cell lines tested. The common feature amongst the VEGF-responsive cells was the presence of VEGFR2, which has previously been shown to mediate the proliferative response in other cell types (Ferrara *et al.* 2003). A recent study has implicated VEGFR1 in breast tumor cell growth and survival also (Wu *et al.* 2006a). Thus, further studies are needed to clarify the role of VEGFR1 and VEGFR2 in the proliferation and survival of breast cancer cells. Nevertheless, future therapeutic strategies will most likely involve strategies to block both receptors for significant inhibition of VEGF-dependent breast cancer growth and proliferation *in vivo*.

Our data suggest that one mechanism for VEGF-induced survival and progression of breast cancer cells could be the expression of the anti-apoptotic protein

Figure 7 VEGF blocks RU-486 mediated anti-proliferative effects in BT-474 and T47-D breast cancer cells. (A) BT-474 and T47-D cells were grown in 100 mm culture dishes overnight in DME/F12 + 10% FBS. The medium was then replaced with serum-free medium for 24 h. Cells were then treated with 10 nM progesterone (P) in the absence or presence of 1 μ M RU-486. One set of plates also contained progesterone, RU-486 and 100 ng/ml VEGF with or without 2 μ g/ml VEGF antibody (Ab). Cells were washed and whole cell extracts were subjected to Western blotting for Bcl-2 and β -actin. (B) BT-474 or (C) T47-D cells were seeded into 96-well plates with 10% FBS DME/F12 medium overnight. The medium was then replaced with serum-free medium for 24 h. Cells were then treated with 10 nM progesterone or the synthetic progestin MPA in the absence or presence of 1 μ M RU-486 (RU) with or without 10, 50, or 100 ng/ml VEGF (V10, V50, or V100) for an additional 24 h. Cell proliferation was determined by BrdU incorporation. Results showed that VEGF at all concentrations significantly abrogated the anti-proliferative effects of RU-486 on both BT-474 and T47-D cells at all concentrations tested (10, 50, and 100 ng/ml). *Significantly different than control group (C), and ^, significantly different as compared with P+RU or MPA+RU groups ($P < 0.05$; ANOVA).

Bcl-2 (Fig. 5–7A). Bcl-2 has previously been shown to be increased in response to VEGF in MDA-MB-231 cells (Pidgeon *et al.* 2001) and has recently been implicated in angiogenesis and expansion of tumors (Karl *et al.* 2005), although its role in breast cancer cells is not clear. Our data show that VEGF-induced Bcl-2 expression is a general phenomenon in breast cancer cells that may have a broader role than simply serving as an anti-apoptotic protein. Interestingly, Bcl-2 was also induced by estradiol in MCF-7 cells; an estrogen response element that has previously been mapped in this gene (Perillo *et al.* 2000). ICI 182,780 blocked E2-induced Bcl-2 expression, indicating that part of the anti-proliferative response of ICI 182,780 could be due to decreasing the expression of this survival protein. When MCF-7 cells were exposed to VEGF and ICI 182,780 simultaneously, the loss of Bcl-2 was blocked and proliferation was increased (Fig. 6A and B). Since ICI 182,780 has been shown to down-regulate ER (Wu *et al.* 2006b), it is possible that Bcl-2 induction seen with E2, ICI 182,780, and exogenous VEGF is ER independent and is mediated by the exogenous VEGF alone. Since ER has been reported to induce VEGF in MCF-7 breast cancer cells by some investigators (Hyder 2006), it is possible that such an induction of VEGF is directly responsible for Bcl2 induction and cell survival. Similar results were observed for counteracting the anti-proliferative effects of RU-486 in breast cancer cells (Fig. 7). Taken together, these findings suggest that an increased production of VEGF by tumor cells could contribute to anti-hormone resistance in tumor cells and may also protect them from apoptosis by increasing Bcl-2 levels.

In summary, the findings from this study suggest that VEGF can induce proliferation and survival of breast cancer cells that express VEGFR2 (flk). This contrasts with the view that VEGF is primarily an endothelial cell-specific mitogen. The proliferative response is dependent on the MAPK/ERK signaling pathway. The VEGF-dependent proliferative response overrides the anti-proliferative effects of anti-hormones in breast cancer cells. This might result from the direct effects of VEGF via its receptors and also from VEGF-dependent induction of anti-apoptotic Bcl-2 protein, which has recently been shown to induce angiogenesis (Karl *et al.* 2005). These observations support the view that combined treatment modalities, including anti-hormones and anti-angiogenic compounds, should be considered for treating ER- and PR-positive breast cancers, especially those that express high levels of VEGF. The concept of trimodal cancer therapy has recently been proposed (Huber *et al.* 2005). It would seem beneficial to consider trimodal therapy in breast

cancer, targeting not only VEGF production, but also VEGF receptors and the MAPK/ERK signaling pathway. Such modalities may not only overcome anti-hormone resistance but may also block proliferation of tumors lacking steroid receptors but expressing VEGF receptors and an active MAPK/ERK pathway that may mediate VEGF-dependent proliferation of tumor cells.

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