TGFβ-1 Regulation of VEGF Production by Breast Cancer Cells

Declan Donovan, BSc, Judith H. Harmey, PhD, Deirdre Toomey, BSc, D. Henry Osborne, MCh, FRCSI, H. Paul Redmond, MCh, FRCSI, and David J. Bouchier-Hayes, MCh, FRCSI

Background: Angiogenesis is essential for tumor growth and metastasis. Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor identified to date. TGFβ-1 acts as an indirect angiogenic agent.

Methods: VEGF and TGFβ-1 were measured in the serum of breast cancer patients and age-matched controls and in tumor tissue of cancer patients by ELISA. VEGF protein and mRNA expression by breast tumor cell lines were examined, and the effect of TGFβ-1 on VEGF production in these cells was assessed.

Results: VEGF levels were significantly higher (P = .03) in the serum of patients with breast cancer compared to age-matched controls. A positive correlation was found between serum (r = 0.539) and tumor tissue (r = 0.688) levels of VEGF and TGFβ-1. Metastatic MDA-MB-231 breast cancer cells produce more VEGF than do the primary BT474 cells. TGFβ-1 significantly (P < .05) increased production of VEGF.

Conclusions: Breast cancer cells constitutively produce VEGF protein and mRNA. There is a relationship between VEGF and TGFβ-1 levels in breast cancer patients, and TGFβ-1 regulates VEGF expression by breast cancer cells.

Key Words: Vascular endothelial growth factor—Transforming growth factor β-1—Breast cancer—Angiogenesis—Metastasis.

Under normal circumstances, angiogenesis, the sprouting of new capillaries from a preexisting blood vessel, occurs only during reproduction (1), wound healing (2) and embryonic development (3). However, both tumor growth and metastasis depend on angiogenesis. Tumors that lack a blood supply receive limited nutrients by diffusion and cannot effectively eliminate waste products. Tumors in this avascular state reach an equilibrium size of up to 2 mm³ (4). Further growth and metastasis do not occur until the tumor has induced its own blood supply. Tumor vascularization has been associated with tumor aggressiveness and shows promise as a prognostic factor for many forms of cancer, including breast (5), prostate (6), ovarian (7), and gastric cancers (8). Angiogenesis occurs in response to the release of a number of different angiogenic factors. One of the most potent of these angiogenic factors identified to date is vascular endothelial growth factor (VEGF). VEGF is released by tumor cells and macrophages (9) and binds to endothelial cell–specific receptors, Flk-1/KDR and Flt-1, although the KDR receptor signal is believed to be the dominant signal transduction pathway in tumor angiogenesis (10). Binding of VEGF to KDR results in endothelial cell proliferation, chemotaxis, changes of gross morphology, and tubular formation of endothelial cells in vitro (10) and angiogenesis in vivo (11). VEGF also causes hyperpermeability of microvessels, which further promotes angiogenesis by allowing plasma proteins to extravasate from tumor vessels and form an extravascular matrix favoring inward migration of endothelial cells (12). This VEGF-induced permeability may also aid metastasis by facilitating tumor cell migration through the blood vessel wall.

Low oxygen tension, such as exists in solid tumors (13), is known to influence angiogenesis (14). Hypoxia substantially increases VEGF production in a number of
cell types, including tumor cells (15), vascular smooth muscle cells (16), and endothelial cells themselves (17). However, it is not the only regulator of VEGF, and factors such as insulin-like growth factor 1 (IGF-1) (18); interleukin 6 (IL-6) (19); wild-type, but not mutant, p53 (20); estradiol (21); and transforming growth factor β-1 (TGFβ-1) (22) may also play a role in the regulation of VEGF. Unlike many other angiogenic factors, TGFβ-1 does not induce endothelial cell proliferation but is capable of initiating angiogenesis indirectly in vivo (23). TGFβ-1 antibodies (24). This indirect angiogenic activity of TGFβ-1 may be accomplished by the induction of other angiogenic factors such as VEGF. TGFβ-1 has been shown to induce VEGF mRNA in mouse embryo fibroblasts and in a human lung adenocarcinoma cell line (22) and, more recently, VEGF protein in human glioma cell lines (25).

In this study we examined the role of VEGF in breast cancer. We hypothesized that VEGF is elevated in the serum of breast cancer patients and that this elevation may result, at least in part, from high levels of TGFβ-1 in both the serum and tumor tissue. Furthermore, we hypothesized that TGFβ-1 directly increases VEGF production by breast cancer cells. We therefore assayed VEGF and TGFβ-1 in both the serum and tumor tissue of breast cancer patients. VEGF expression in response to TGFβ-1 was assessed in primary and metastatic breast cancer cell lines.

**METHODS**

**Serum Levels**

Preoperative blood was collected from patients undergoing surgery for malignant breast disease (n=26). No patients with a recent history of surgery or chemotherapy were included. Malignancy was histologically confirmed, and the series comprised of 21 ductal and 5 lobular carcinomas. Breast tumors were staged using the TNM staging system (26). Three patients were Tis, and the remaining patients were staged as follows: stage I, n = 8; stage II, n = 11; stage III, n = 3; and stage IV, n = 1. Samples were also collected from a group of healthy age-matched controls with no history of breast or other cancers. Blood was centrifuged at 11,000 x g for 5 minutes and the cleared homogenate collected and assayed for VEGF and TGFβ-1 by ELISA. The levels of VEGF were expressed as pg/ml serum and the levels of TGFβ-1 as ng/ml serum. Statistical analysis of serum VEGF levels was by unpaired Student’s t-test. The level of association between serum VEGF and TGFβ-1 was estimated by correlation, using the Pearson Product Moment Correlation coefficient.

**Tumor Samples**

Samples of tumor tissue were flash frozen in liquid nitrogen within 1 hour of resection and stored at -80°C. Specimens were diced and homogenized in a hand-held homogenizer in 500 μl of PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were maintained at 4°C during the homogenization procedure. Debris was removed by centrifugation at 12,000 x g for 5 minutes and the cleared homogenate collected and assayed for TGFβ-1 and VEGF by ELISA. The level of association between tumor tissue VEGF and TGFβ-1 was estimated by correlation, using the Pearson Product Moment Correlation coefficient.

**Cell Lines**

BT474 cells (ATCC HTB20), a primary breast cancer cell line, and MDA-MB-231 cells (ECACC92020424), a metastatic breast cancer cell line, were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 1% bovine serum albumin (BSA) for 24 hours at a density of 30,000 cells/well in a 96-well plate. In some experiments cells were treated with TGFβ-1 at a concentration of 0, 2.5, 5.0, 7.5, and 10 ng/ml for a 24-hour period. Culture supernatants were removed and assayed for VEGF by ELISA. Cells were washed twice in PBS and lysed by freeze thawing. Total cellular protein was measured using the Coomassie assay (Pierce Biochemicals, Rockford, IL) according to manufacturer’s instructions. VEGF levels were expressed as pg VEGF/μg cell protein. Data were analyzed by Student’s t-test or ANOVA using DataDesk 4.1 (Data Description Inc., Ithaca, NY).

**Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

Cells were grown for 16 hours in DMEM with 1% BSA at a subconfluent density of 150,000 cells/well in a 12-well plate. Total RNA was isolated using TriZol (Gibco-BRL, Paisley, Scotland) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed at 37°C for 3 hours using 0.5 μg of random primers (Promega, Madison, WI) and 200U of SuperScript II RT enzyme (Gibco-BRL). PCR amplification of VEGF cDNA was carried out by 30 cycles of 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 1.5 minute using the following primers as described previously (9):
VEGF IN BREAST CANCER

A.

![Bar graph showing VEGF protein production by BT474 and MDA-MB-231 breast tumor cells following incubation for 24 hours.](image)

**FIG. 1.** (A) VEGF protein production by BT474 and MDA-MB-231 breast tumor cells following incubation for 24 hours. Culture supernatants were assayed for VEGF protein by ELISA. Data is expressed as the mean ± SEM and is representative of 3 independent experiments. Data analysis was by unpaired Student's t-test (*, P < .01, MDA-MB-231 vs. BT474). (B) VEGF mRNA levels in BT474 and MDA-MB-231 cells following incubation for 16 hours. Total RNA was isolated and VEGF and β-actin amplified by RT-PCR. The position of molecular weight markers, HaeIIICX174, is indicated (bp).

VEGF is Produced by Breast Tumor Cells

The breast tumor cell lines BT474 and MDA-MB-231 produced considerable amounts of VEGF protein in the absence of known inducers (Fig. 1A). These experiments were performed in serum-free medium so as to avoid induction of VEGF by agents in the serum, indicating that this represents constitutive expression by these cells. VEGF protein produced was stabilized by the addition of 1% bovine serum albumin to the medium. The metastatic breast cancer cell line, MDA-MB-231, produced significantly (*, P < .05) more VEGF protein (12.1 ± 2.4 pg/µg protein) than did the primary cell line, BT474 (4.09 ± 0.69 pg/µg protein). Tumor cell expression of VEGF was confirmed using RT-PCR analysis of VEGF mRNA. MDA-MB-231 cells contained higher levels of VEGF mRNA than did BT474 cells (Fig. 1B). β-actin, a constitutively expressed message, was also amplified to normalize for differences in the quantity of starting cDNA.

VEGF is Elevated in Breast Cancer Patients

Because breast cancer cell lines were shown to produce VEGF constitutively and metastatic cells produced higher levels of VEGF protein and mRNA, we anticipated that VEGF would be elevated in patients with malignant breast disease. We measured VEGF levels in serum from patients with malignant breast disease (n = 26) and from age-matched controls (n = 15). The mean ages were 57.1 ± 13.7 years in the group with malignant disease and 50.33 ± 7.3 years in the age-matched controls. There was no significant difference in age between the groups as assessed by unpaired Student's t-test (P > .05). VEGF levels in the serum of patients with malignant breast disease were significantly (*, P < .05) higher than in the serum of age-matched controls (Fig. 2). The mean serum VEGF level in the malignant group was 407.67 ± 272.07 pg/ml, whereas in the control group it was 230 ± 127.18 pg/ml. The serum levels of VEGF in patients with stage 2 disease (402.43 ± 245.05 pg/ml) were significantly elevated over control. Patients with stage 3 (771.16 ± 519.34 pg/ml) breast cancer were found to have serum VEGF levels significantly elevated over the levels in patients with carcinoma in situ (370.93 ± 127.95 pg/ml), stage 1 (315.4 ± 167.4 pg/ml) disease, and stage 2 disease (*, P < .05), although the patient num-

---

**Parameters:**

- **A.** VEGF protein production by BT474 and MDA-MB-231 breast tumor cells following incubation for 24 hours.
- **B.** VEGF mRNA levels in BT474 and MDA-MB-231 cells following incubation for 16 hours.

**Primer Sequences:**

- **Forward:** 5'-CGCAAGCTTAGGAGTACCCTGATGAG-3'
- **Reverse:** 5'-CCGTCTAGAACATISGTTGTGCTGT-3'

**PCR Conditions:**

- 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1.5 minutes.

**Molecular Weight Markers:**

- HaeIIICX174

**RT-PCR Products:**

- An amplification reaction with no cDNA template was used as a negative control.
bers need to be increased to fully evaluate the relationship between tumor stage and serum VEGF. Two patients presenting with known metastases were found to have levels of over 1000 pg/ml. We found no significant difference in serum levels of VEGF in patients with estrogen receptor positive or estrogen receptor negative tumors (data not shown).

**TGFβ-1 Levels Correlate With VEGF Levels in Serum and Breast Tumors**

Having identified elevated levels of VEGF in serum of breast cancer patients, we measured levels of TGFβ-1, which has previously been shown to induce VEGF in a number of cell types. Analysis of serum levels of TGFβ-1 and VEGF in a subset (n = 15) of the breast cancer patient cohort showed that a significant (P < .05) positive correlation existed between these two factors (Pearson Product Moment Correlation coefficient = 0.539) (Fig. 3). Furthermore, a significant (P < .05) positive correlation was found between the tumor tissue levels of TGFβ-1 and VEGF (Pearson Product Moment Correlation coefficient = 0.688, n = 9) (Fig. 4).

**TGFβ-1 Increases Expression of VEGF by Breast Cancer Cell Lines**

We identified a positive correlation between VEGF and TGFβ-1 in both serum and tumor tissue of breast cancer patients. To establish whether TGFβ-1 plays a regulatory role in breast cancer cell production of VEGF, we examined the direct effect of TGFβ-1 on VEGF production by breast tumor cells. Cells were treated with 1 to 10 ng/ml TGFβ-1 for 24 hours based on previous studies of TGFβ-1 induction of VEGF in vascular smooth muscle cells (16). The primary cell line, BT474, showed increased production of VEGF protein following treatment with 5 ng/ml TGFβ-1 (6.04 ± 0.68 vs. 4.09 ± 0.69 pg/μg protein, P = .03) (Fig. 5). The metastatic cell line, MDA-MB-231, showed a concentration-dependent increase of VEGF production in response to TGFβ-1 (Fig. 6). This increase was significantly elevated from control cells at 7.5 and 10 ng/ml TGFβ-1 (21.24 ± 2.26 and 25.25 ± 5.5 vs. 12.1 ± 2.4 pg/μg protein, P = .03 and p = .007, respectively). VEGF production was normalized by expressing pg VEGF/mg protein. TGFβ-1 did not affect cell numbers (data not shown).

**DISCUSSION**

We identified elevated VEGF in the serum of patients with breast cancer compared with age-matched controls.
Immunohistologic staining and in situ hybridization studies have previously shown that VEGF is highly expressed in many forms of cancer, including breast (27,28), colon (29), gliomas (15), and malignant ascitic fluid (30). We also identified high levels of VEGF in breast tumor tissue. This is in agreement with previous studies showing VEGF expression to be more abundant in carcinomas than in adjacent nonneoplastic breast tissue (28).

TGFβ-1 is an indirect angiogenic factor in that it does not stimulate endothelial cell proliferation or tubular formation in vitro but causes angiogenesis in vivo (23). This effect could be mediated by the induction of other direct angiogenic factors such as VEGF. TGFβ-1 has previously been shown to increase VEGF expression in fibroblasts and epithelial cells (22), vascular smooth muscle cells (16), and gliomas (25). We found a positive correlation between TGFβ-1 and VEGF levels in the serum of patients with breast cancer. This positive correlation also existed between the tumor tissue levels of these two factors, suggesting a relationship between levels of TGFβ-1 and VEGF. We have shown that TGFβ-1 treatment stimulated VEGF expression in both a primary (BT474) and a metastatic (MDA-MB-231) breast cancer cell line. Although TGFβ-1 inhibits some mammary epithelial cell growth (31), it may promote tumor angiogenesis by increasing tumor cell expression of VEGF, thereby facilitating rapid expansion of the tumor. The mechanism by which TGFβ-1 stimulates VEGF expression is not yet known but is under further investigation. TGFβ-1 has previously been shown to be elevated in the plasma of breast cancer patients (32). Furthermore, plasma TGFβ-1 in these patients was reduced following resection of the primary tumor. TGFβ-1 was found to be preferentially expressed in stromal cells (32). It is also known that TGFβ-1 is constitutively expressed in monocytes and can further stimulate its own production in an autocrine manner (33). TGFβ-1 produced within the tumor is also chemotactic for monocytes (34). Our findings that high TGFβ-1 correlates with high VEGF and that TGFβ-1 induces breast tumor cell production of VEGF implicate TGFβ-1 in the progression of breast cancer.

We show that breast cancer cells constitutively express VEGF. A metastatic cell line, MDA-MB-231, produced significantly higher levels of VEGF protein than did an independently derived primary cell line, BT474. This increase is confirmed at the mRNA level by RT-PCR analysis of MDA-MB-231 and BT474 cells. Taken together with our findings of higher serum levels of VEGF in patients with metastatic disease and previous studies showing that metastatic colon carcinoma cell lines produced more VEGF than did their primary counterparts (29), our results suggest that VEGF may be overexpressed in metastatic cells. Microvessel counts, which represent the extent of angiogenesis within a tumor, have been shown to correlate with the incidence of metastases in breast cancer (35) and in a number of other cancers, including prostate (6), gastric (8), and colon (29) cancer, as well as melanoma (36). The expression of VEGF itself has been correlated with the level of metastasis in human colon carcinomas (29) and experimental melanomas (36). There are a number of mechanisms by which VEGF may aid metastasis. VEGF causes hyperpermeability of blood vessels, which allows plasma proteins to extravasate and form a matrix that assists inward migration of endothelial cells. Hyperpermeability may also allow tu-
growth cells to cross the blood vessel wall and travel in the circulation. VEGF also increases endothelial cell gene expression of proteases, which are required for metastasis, including interstitial collagenase (37), and urokinase-like and tissue plasminogen activators (38). Increased expression of VEGF by metastatic breast cancer cells compared with primary breast cancer cells further implicates VEGF in the metastatic process.

In recent years research has focused on the inhibition of angiogenesis for the treatment of cancer. Clearly, an important step in the design of therapies to inhibit angiogenesis is a complete knowledge of the factors involved and their relationship to each other and their environment. In this study we have shown that TGFβ-1 regulates VEGF expression by breast cancer cells and may therefore play an important role in the initiation and maintenance of the angiogenic phenotype in breast cancer.

Acknowledgements: This research was funded by grants from the Royal College of Surgeons in Ireland, The Beaumont Hospital Cancer Research and Development Trust, and The Charitable Infirmary Charitable Trust.

REFERENCES


学霸图书馆
www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：
图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具