Estrogen-Mediated Angiogenesis in Thyroid Tumor Microenvironment Is Mediated Through VEGF Signaling Pathways

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Objectives: To explore the induction of a proangiogenic phenotype in endothelial cells in the thyroid tumor microenvironment by estrogen-treated thyroid cancer cells and to define the role of vascular endothelial growth factor (VEGF) in this interaction.

Design: Cell-based in vitro systems analysis.

Subjects: Thyroid tumor cell lines (BCPAP [papillary thyroid cancer] and ML-1 [follicular thyroid cancer]) were cultured with estradiol with and without an estrogen receptor (ER) inhibitor (fulvestrant or ICI) and used to treat human umbilical vein endothelial cells (HUVECs).

Interventions: Immunofluorescence was used to confirm the presence of ERα/H9251 and ERβ/H9252 in BCPAP cells. Conditioned medium was then used to evaluate the induction of HUVEC tubulogenesis and migration. Secretion of VEGF in this medium was evaluated by Western blot analysis. The expression of phosphoinositide 3-kinase (PI3K), the initiator of a proangiogenic pathway, was evaluated with Western blot analysis of HUVEC lysates.

Results: Estrogen receptor α and ERβ are expressed in thyroid cancer cells. Estrogen-stimulated ML-1 cells secreted an increased amount of VEGF likely as a result of ER signaling. In contact with this environment, HUVECs demonstrate enhanced tubulogenesis and migration. Western blot analysis documented estrogen-mediated up-regulation of PI3K in HUVECs. These effects were mitigated by an ER inhibitor (fulvestrant/ICI) and a neutralizing VEGF antibody.

Conclusions: Our data provide evidence that estrogen can induce a proangiogenic endothelial cell phenotype in the thyroid tumor microenvironment through ER and VEGF signaling. Our findings suggest that the effect of antiestrogenic therapy targeting tumor angiogenesis can be enhanced through VEGF inhibition.


According to the American Thyroid Association, the incidence of thyroid cancer is 3 to 4 times higher in women. In fact, a woman’s risk of developing a thyroid disorder is 1 in 8, which is comparable to a woman’s risk of developing sporadic breast cancer. Pregnancy and early menopause increase this risk, with a decrease in thyroid cancers after menopause.1-3 These epidemiological trends have led to the hypothesis that estrogen (estrone, estradiol [E2], and estriol), through its primary receptors ERα and ERβ, plays a critical role in thyroid proliferative disease.1-3 Previous research in our laboratory has supported this hypothesis with data suggesting that estrogen enhances the mitogenic, migratory, and invasive properties of thyroid cancer cell lines.1 Furthermore, we have shown that in addition to these processes angiogenesis is initiated through paracrine signaling between cancer cells and endothelial cells. Through this interaction, there is also specific mobilization of progenitor stem cells from the bone marrow called bone marrow–derived endothelial progenitor cells, or BM-EPCs. These BM-EPCs are characterized by cell surface expression of CD133, CD34, and acyl-LDL, suggesting their endothelial differentiation and stem cell origin. These cells interact with tumor cells to initiate the sprouting of new blood vessels. Interestingly, the BM-EPCs are known to express estrogen receptor (ER) and are increasingly mobilized by estrogen supplementation.4 To further examine this fundamental interaction between thyroid cancer cells and endothelial cells and to uncover potential therapeutic targets, we used conditioned media from treated thyroid cancer cell lines to create an in vitro representation of the thyroid tumor microenvironment.
Tumor growth beyond 2 mm in diameter is dependent on the process of angiogenesis. It provides essential nutrients such as oxygen, proteolytic enzymes, hormones, and other growth factors that are necessary for tumor expansion and potential metastasis. The intricacies of this multistep process have been the subject of much research in the past 2 decades. It has been shown that angiogenesis is a complex process that is regulated by a balance between proangiogenic and antiangiogenic agents in the tumor microenvironment. The key proangiogenic factor in both physiologic and pathologic angiogenesis is vascular endothelial growth factor (VEGF). Not unlike other cancers, VEGF has been shown to play an integral role in thyroid cancer growth. In culture, thyroid cancer cell lines are known to secrete more VEGF than normal thyrocytes. Activation of VEGF’s principal proangiogenic receptor (VEGFR-2) causes homodimerization and oligomerization, triggering its intrinsic tyrosine kinase activity. This process initiates a series of pathways that result in endothelial cell survival, mitogenesis, migration, and tubule formation. The intracellular pathways downstream of VEGFR-2 include the Akt/PKB pathway, which is initiated through increased phosphoinositide 3-kinase (PI3K) activity.

The objective of this study was first to define the role of estrogen in the interaction between thyroid cancer cells and endothelial cells. We hypothesized that the ability of estrogen to promote a metastatic phenotype in thyroid cancer might be initiated through initiation of a proangiogenic phenotype in endothelial cells. Given the key role of VEGF in angiogenesis in other cancers, we also hypothesized that it has a similar function in endothelial cells in the thyroid tumor microenvironment. We believe that by elucidating the cellular interactions of the tumor microenvironment, new and potentially effective targets of clinical therapy and prevention can be identified.

METHODS

CELL CULTURE

Thyroid cancer cell lines used in this study—BCPAP (human papillary thyroid cancer cell line) (DSMZ) and ML-1 (human follicular thyroid cancer cell line) (DSMZ)—were cultured in RPMI-1640 (Mediatech) and Dulbecco modified Eagle medium (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), penicillin (10,000 IU/mL), streptomycin (10,000 µg/mL) (Mediatech), and 2 mM L-glutamine (Mediatech). An endothelial cell line (human umbilical vein endothelial cells [HUVECs]) (DSMZ) was cultured in F-12K 1X (Mediatech) supplemented with 10% FBS (Atlanta Biologicals), penicillin (10,000 IU/mL), streptomycin (10,000 µg/mL) (Mediatech) for endothelial growth factor supplement, and heparin.

ERα AND ERβ IMMUNOFLUORESCENCE IN BCPAP CELL CULTURE

Cells were seeded in 8-chamber slides (BD Biosciences) at a density of 15,000 cells per well. The cells were allowed to adhere at 37°C overnight and subsequently processed for immunofluorescence. They were fixed with a 4% paraformaldehyde–phosphate-buffered saline solution, followed by permeabilization with 0.2% Triton X-100, and then the cells were blocked using a solution of 0.1% Triton X-100, 1% bovine serum albumin, and 10% goat serum. The cells were then incubated with the ERα and ERβ antibody (1:300 dilution) (Santa Cruz Biotechnology) overnight at 4°C, followed by incubation for 45 minutes at room temperature with Alexa Fluor 488–labeled goat anti-rabbit antibody (1:250) (Invitrogen). The slides were then mounted with Vectashield containing 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). The cells were visualized using an inverted-stage confocal microscope (Axiovert 200M; Zeiss).

ENDOTHELIAL CELL TUBULOGENESIS

The HUVECs were harvested by trypsinization and plated (50,000 cells per slide) on growth factor–reduced basement membrane matrix–coated (300 µL) (Matrigel; Beckon Dickinson) dishes. Tubulogenesis was monitored for 24 hours after the addition of conditioned media from BCPAP and ML-1 cells that were treated with 10^{-8} M E_2 with or without E_2 inhibitor. This treatment was then duplicated with the addition of a neutralizing antibody. The individual wells were evaluated qualitatively using an inverted-stage confocal microscope (Axiovert 200M).

ENDOTHELIAL CELL MIGRATION

BD Biocoat Control Inserts (BD Biosciences) with 8-µm pore membrane filters were used for the migration assay. The HUVECs were harvested by trypsinization and 1 × 10^6 cells were seeded in upper chamber in 100 µL of medium containing 1% FBS. The lower chamber contained 600 µL of conditioned media (untreated, 10^{-8} M E_2±ER inhibitor [fulvestrant/ICI]) from BCPAP or ML-1 thyroid cells±neutralizing VEGF antibody (1 mg/mL) (R&D Systems). After 18 hours of incubation, gentle scrubbing with a cotton-tipped swab was used to remove the nonmigrating cells from the upper surface of the membrane. The cells on the lower surface of the membrane were then fixed with methanol and stained with 1% toluidine blue–1% borax stain followed by 2 washes with distilled water. Inserts were then allowed to air-dry and counted in 3 different ×10 fields by 2 different observers. The data were expressed as numbers of migrated cells per ×10-field micrograph for each sample well and normalized to cell counts obtained from an untreated control.

WESTERN BLOT ANALYSIS

The HUVECs were treated overnight with neutralizing VEGF antibody (R&D Systems) in conditioned media from 10^{-8} M E_2±ER inhibitor (fulvestrant/ICI)–supplemented BCPAP cells. Whole-cell lysates of these cells were prepared using 1 × 10^6 cells/100 µL of radioimmunoprecipitation assay buffer (50 mM Tris-hydrochloride [HCl] [pH, 7.4], 150 mM sodium chloride [NaCl], 0.2% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.5%, NP-40, and 1 µM Pefabloc) and incubated on ice for 30 minutes with intermittent vortexing. The lysates were then centrifuged at 14,000 rpm for 30 minutes at 4°C, and the supernatants were collected. Cell lysates (5-µg protein) were subjected to 12% SDS–polyacrylamide gel electrophoresis under reducing conditions (presence of β-mercaptoethanol). Briefly, the proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore Corp) at 220 mA for 2 hours, and the membranes were blocked with 5% dried milk in TBS-T (200 mM Tris-HCl [pH, 7.4], 150 mM NaCl, and 0.01% Tween 20 added fresh per liter of 1X TBS-T) for at least 2 to 3 hours on a shaker at room temperature. Subsequently, the membrane was incubated overnight at 4°C with P3K (Cell Signal-
ing Technology #4292) and tubulin (Cell Signaling Technology #2148) primary antibody (in TBS-T) on a shaker. The membranes were washed 3 times with TBS-T and incubated with respective secondary antibody for 2 hours at room temperature on a shaker. After 4 washes with TBS-T and 1 wash with TBS, the membranes were developed with a Western blotting substrate (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific) and detected on x-ray film, which was then analyzed with densitometry and normalized to loading controls.

Conditioned media from ML-1–treated cells (10−8 ME 2±ER inhibitor [fulvestrant/ICI]) were also subjected to Western blot analysis in similar fashion as described above. Briefly, the conditioned media were subjected to 12% SDS–polyacrylamide gel electrophoresis under reducing conditions (presence of β-mercaptoethanol). The proteins were transferred to polyvinylidene fluoride membranes at 220 mA for 2 hours, and the membranes were blocked with 5% dried milk in TBS-T (200mM Tris-HCl [pH, 7.4], 150mM NaCl, and 0.01% Tween 20 added fresh per liter of 1X TBS-T) for at least 2 to 3 hours on a shaker at room temperature. Subsequently, they were incubated overnight at 4°C with VEGF primary antibody (Santa Cruz Biotechnology #SC-152) (in TBS-T) on a shaker. They were washed 3 times with TBS-T and incubated with respective secondary antibody for 2 hours at room temperature on a shaker. After 4 washes with TBS-T and 1 wash with TBS, they were developed with a Western blotting substrate (ECL) and detected on x-ray film.

STATISTICAL CALCULATION

A paired t test was used to assess statistical significance of the migration data. P ≤ .05 was used to reject the null hypothesis and was considered statistically significant.

RESULTS

THYROID CANCER CELL LINES (BCPAP) EXPRESS BOTH ERα AND ERβ

Before treating thyroid cancer cell lines with estrogen, we first confirmed that these cell lines expressed both ERα and ERβ by immunofluorescence (Figure 1). Compared with the control without primary antibody, the BCPAP cells were shown to express both ERα and ERβ.

E2 INDUCES BCPAP AND ML-1 CELLS TO SECRETE PARACRINE FACTORS THAT LEAD TO ENDOTHELIAL CELL TUBULOGENESIS

The HUVECs that were plated on basement membrane matrix–coated chambers were observed for tubulogenesis for 24 hours after the addition of conditioned media from E2±ER inhibitor (fulvestrant/ICI)-treated BCPAP and ML-1 cells (Figure 2). We observed qualitatively that there was an increase in organized netlike tubule formation with the addition of conditioned media from E2-treated thyroid cancer cells. This increase was not observed after the addition of an ER inhibitor (fulvestrant/ICI). Endothelials cells in this setting were found to form short stublike structures without organized tubule formation. This finding suggests that the paracrine secretion of factors that induce tubulogenesis was mediated through the ER.

E2 INDUCES ML-1 CELLS TO SECRETE VEGF INTO CONDITIONED MEDIA

To relatively quantify the degree to which estrogen stimulates thyroid cancer cell lines to secrete VEGF, we evaluated conditioned media from E2±ER inhibitor (fulvestrant/ICI)-treated ML-1 cells by Western blot analysis (Figure 3). We observed that 24-hour treatment of ML-1 cells with E2 caused an increase in secretion of VEGF when compared with the untreated control. This effect was mitigated by the presence of an ER inhibitor (fulvestrant/ICI), suggesting that VEGF secretion was at least partially mediated through the ER.

NEUTRALIZING VEGF ANTIBODY CAN REDUCE E2-INITIATED ENDOTHELIAL CELL TUBULOGENESIS IN THE THYROID TUMOR MICROENVIRONMENT

With the addition of VEGF-neutralizing antibody to the basement membrane matrix–coated chambers, we observed a decrease in the amount of tubule formation (Figure 4). Similar to the effect with an ER inhibitor,
these endothelial cells were found to form fewer tubules with more short stublike projections. This finding suggests that tubule formation may be modulated through VEGF secretion by the thyroid cancer cell lines. Endothelial cells treated with conditioned media from thyroid cancer cells exposed to ER inhibitor and neutralizing VEGF antibody were found to have no organized tubule formation.

**E2 INDUCES BCPAP AND ML-1 CELLS TO SECRETE PARACRINE FACTORS THAT LEAD TO ENDOTHELIAL CELL MIGRATION**

The HUVECs were loaded in a transwell migration chamber with conditioned media from E2+ER inhibitor (fulvestrant/ICI)-treated BCPAP and ML-1 cells as the chemotactants (Figure 5). We observed that the migratory ability of thyroid cells was enhanced with E2. This significant increase in migration ($P < .001$ for ML-1 and $P = .01$ for BCPAP) was 11.4% for BCPAP-conditioned media and 11.7% for ML-1-conditioned media compared with untreated samples. This increase in cell migration was abrogated when an ER inhibitor (fulvestrant/ICI) was used ($P < .001$ for ML-1 and $P = .002$ for BCPAP), further suggesting that the paracrine secretion of promigratory cytokines was mediated through the ER.

**NEUTRALIZING VEGF ANTIBODY CAN REDUCE E2-INITIATED ENDOTHELIAL CELL MIGRATION IN THE THYROID TUMOR MICROENVIRONMENT**

With the addition of neutralizing VEGF antibody to the conditioned media, we observed a significant decrease in endothelial cell migration ($P = .002$ for ML-1 and $P = .01$ for BCPAP) when compared with conditioned media from just E2-treated cells (Figure 5). In combination with the ER inhibitor (fulvestrant/ICI), neutralizing VEGF antibody was found to decrease cell migration by 10% in ML-1 and 5% in BCPAP, a near significant decrease in ML-1-conditioned media ($P = .05$) and a significant decrease in BCPAP-conditioned media ($P = .02$) when compared with conditioned media without neutralizing VEGF antibody.

**ENDOTHELIAL CELLS TREATED WITH E2-SUPPLEMENTED BCPAP-CONDITIONED MEDIA INITIATE A VEGFR-2–MEDIATED PATHWAY**

VEGFR-2 has been found to activate PI3K, leading to the initiation of the Akt/PKB pathway in endothelial cells (Figure 6). This pathway leads to increased cell survival, vascular permeability, and cellular migration.9 To
Estrogen has been shown to play a key role in the growth and metastatic potential of differentiated thyroid cancer. Previous findings in our laboratory have documented that the interaction between estrogen and its receptor modulates thyroid cell growth, adhesion, migration, and invasion. However, for solid tumor growth beyond 2 mm, the process of angiogenesis must be initiated in the thyroid tumor microenvironment. With the importance of angiogenesis in mind, we postulated that estrogen initiates a cascade of events that lead to a proangiogenic microenvironment, possibly through VEGF, a growth factor that is upregulated when it is exposed to a variety of hormones, growth factors, and extracellular matrix components. After recruitment to a cell membrane–bound tyrosine kinase receptor such as VEGFR-2 and activation, PI3K phosphorylates phosphatidylinositol-4,5-biphosphate, generating phosphatidylinositol 3,4,5 triphosphate. While not directly activating Akt/PKB, a serine/threonine kinase, phosphatidylinositol 3,4,5 triphosphate alters its conformation for subsequent activation by phosphoinositide-dependent kinase 1. After activation, Akt/PKB has been found to promote proliferation and increased cell survival through antiapoptotic gene transcriptional regulation, direct deactivation of proapoptotic protein pathways, and direct regulation of cell cycle progression. In relation to angiogenesis specifically, evidence suggests that Akt/PKB activation in response to proangiogenic factors, ie, angiopoietin-1 and VEGF, decreases endothelial cell apoptosis. Furthermore, the Akt/PKB pathway has been found to activate endothelial nitric oxide synthase, which is a key regulator of increased vascular permeability, cytoskeletal reorganization, and migration. Our data suggest that the expression of PI3K may be increased in endothelial cells when thyroid cancer cells are supplemented with estrogen and subsequently decreased with the addition of an ER inhibitor (fulvestrant/ICI). Interestingly, this decrease in PI3K expression was lower than that found in the untreated control, which may be an indication of high PI3K basal expression rates in these endothelial cells with the addition of conditioned media from ER inhibitor–treated thyroid cancer cells may subsequently have caused a decrease in this basal rate. Also, with the addition of a neutralizing VEGF antibody, suggesting a role for VEGF in estrogen-mediated induction of a proangiogenic phenotype in endothelial cells.

To corroborate our data, we also evaluated a VEGFR-2–initiated proangiogenic pathway. The Akt/PKB pathway is mediated through PI3K activation. It is upregulated when it is exposed to a variety of hormones, growth factors, and extracellular matrix components. After recruitment to a cell membrane–bound tyrosine kinase receptor such as VEGFR-2 and activation, PI3K phosphorylates phosphatidylinositol-4,5-biphosphate, generating phosphatidylinositol 3,4,5 triphosphate. While not directly activating Akt/PKB, a serine/threonine kinase, phosphatidylinositol 3,4,5 triphosphate alters its conformation for subsequent activation by phosphoinositide-dependent kinase 1. After activation, Akt/PKB has been found to promote proliferation and increased cell survival through antiapoptotic gene transcriptional regulation, direct deactivation of proapoptotic protein pathways, and direct regulation of cell cycle progression. In relation to angiogenesis specifically, evidence suggests that Akt/PKB activation in response to proangiogenic factors, ie, angiopoietin-1 and VEGF, decreases endothelial cell apoptosis. Furthermore, the Akt/PKB pathway has been found to activate endothelial nitric oxide synthase, which is a key regulator of increased vascular permeability, cytoskeletal reorganization, and migration. Our data suggest that the expression of PI3K may be increased in endothelial cells when thyroid cancer cells are supplemented with estrogen and subsequently decreased with the addition of an ER inhibitor (fulvestrant/ICI). Interestingly, this decrease in PI3K expression was lower than that found in the untreated control, which may be an indication of high PI3K basal expression rates in these endothelial cells.
The role of VEGF expression in differentiated thyroid cancer has been reported earlier. In 1996, Soh et al documented through enzyme-linked immunosorbent assay that there is similar basal VEGF secretion in normal thyroid tissue and differentiated thyroid cancer. They found that basal VEGF secretion in all cell lines is in-

Figure 4. Human umbilical vein endothelial cell (HUVEC) tubulogenesis after treatment with conditioned media from thyroid cancer cell lines. The arrow in the left-hand panels indicates tube formation. A, HUVEC tubulogenesis after treatment with conditioned media from estradiol (E$_2$) and estrogen receptor (ER) inhibitor (fulvestrant/ICI)-treated BCPAP cells with and without (±) neutralizing vascular endothelial growth factor (VEGF) antibody. Note the network of tubules indicated by the arrows in the center panel. B, HUVEC tubulogenesis after treatment with conditioned media from E$_2$ and ER inhibitor (fulvestrant/ICI)-treated ML-1 cells ± neutralizing VEGF antibody. Note the network of tubules indicated by the arrows in the center panel.
Increased in response to thyroid-stimulating hormone. Interestingly, thyroid cancer cells secreted significantly more VEGF in response to thyroid-stimulating hormone than normal thyroid cells. In 1997, the same group investigated the expression of VEGF messenger RNA and the production of VEGF in cultured cell lines from human primary and metastatic follicular and papillary thyroid cancer. When they compared the differentiated thyroid cancer cell lines with normal thyroid cell lines, they found that the VEGF messenger RNA and VEGF protein levels were higher in the differentiated thyroid cancer cell lines. In 2000, they furthered their investigation using a human dermal matrix inoculated with follicular thyroid cancer cells xenografted into nude mice and then treated with recombinant human VEGF and neutralizing VEGF antibody. They showed that the number of blood vessels and tumor size directly corresponded with the VEGF concentration. They also showed that the number of blood vessels and tumor size were negatively affected by a neutralizing VEGF antibody.

Although tubulogenesis is an in vitro representation of angiogenesis, the qualitative nature of the assay poses some limitations. Efforts to quantify the degree of endothelial cell tubulogenesis with branch point measurement for HUVECs and primary endothelial progenitor cells are under way. However, the qualitative effect of the various treatments is nevertheless a good measure of cellular interactions and the resulting phenotypic changes. It is also plausible that the estrogen's modulation of the proangiogenic phenotypic changes in endothelial cells may also be seen in both normal and thyroid goiter cell lines. In fact, many studies have documented that angiogenesis does occur in normal thyroid tissue in similar fashion to some degree. However, our objective was not to show that angiogenesis increases in thyroid cancer when compared with noncancerous cell lines. Instead, we sought to identify potential targets within the thyroid tumor microenvironment that initiate and enhance tumor growth and metastasis through angiogenesis. It is likely that there are molecular differences between tumor- and normal thyroid cell–induced angiogenesis. To characterize and eventually target angiogenesis safely and effectively, these differences will surely require in-depth analysis.

Our investigation centered on the proposition that angiogenesis in the tumor microenvironment is dependent on the secretion of paracrine factors that alter the disease pathogenesis. Clinical therapy can only be initiated once these factors are identified and their significance is evaluated. Despite the limitations, our findings demonstrate that potential targets to impede the growth of well-differentiated thyroid cancer may be found in the interaction of estrogen and VEGF with their receptors. Our data show that the phenotypic changes that were observed in endothelial cells are at least partially initiated through estrogen-stimulated VEGF secretion in the thyroid tumor microenvironment. The utility of the combination of antiestrogen and anti-VEGF therapy is also suggested by our findings with both an ER inhibitor (fulvestrant/ICI) and a neutralizing VEGF antibody. However, further experimentation using varying concentrations of these inhibitors will need to be conducted to elucidate a practical role of combination therapy.

![Image](http://archotol.jamanetwork.com/pdfaccess.ashx?url=/data/journals/otol/22563/ on 06/19/2017)
Furthermore, these data can now be used to investigate whether cells of endothelial lineage, ie, BM-EPCs, which are found to migrate from bone marrow to initiate neovascularization,4 can also be altered phenotypically by estrogen-stimulated thyroid cancer cell lines. We hypothesize that if VEGF secretion can initiate a proangiogenic cascade in endothelial cells, it may also cause mobilization and the integration of BM-EPCs in the tumor microenvironment. As our in vitro model continues to be modified, we hope that our findings can be extrapolated to the development of an animal model of the thyroid tumor microenvironment. An animal model can help in the elucidation of the signaling mechanisms between human thyroid cancer cell lines, endothelial cells, and BM-EPCs, with the goal of evaluating a targeted approach of combination therapy with antiestrogens and neutralizing VEGF antibodies in thyroid proliferative diseases.

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