

# Progesterin Regulation of Vascular Endothelial Growth Factor in Human Breast Cancer Cells<sup>1</sup>

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## Abstract

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor associated with the degree of vascularity, progression, and metastasis of breast cancer, and cases of this disease with increased vascular density have a poor prognosis. We show that in T47-D human breast cancer cells, progesterone induces a dose-dependent increase of 3–4-fold in media VEGF levels, with a maximum response occurring at a concentration of 10 nM. This effect is blocked by the antiprogesterin RU 486. In addition to progesterone, a number of synthetic progestins used in oral contraceptives (e.g., norethindrone, norgestrel, and norethynodrel), hormone replacement therapy (medroxyprogesterone acetate), and high-dose progestin treatment of breast cancer (megestrol acetate) also increase VEGF in the media of cultured T47-D cells. This effect is hormone specific and is not produced by estrogens, androgens, or glucocorticoids. Collectively, these observations suggest that the increase in VEGF caused by progestins is mediated by progesterone receptors present in T47-D cells. The induction of VEGF by progestins is also cell type specific and does not occur in human breast cancer cell lines MCF-7, ZR-75, or MDA-MB-231, nor in Ishikawa cells derived from a human endometrial carcinoma. This is the first report that progestins regulate VEGF expression in human breast cancer cells and raises the possibility that increased angiogenesis in response to endogenous progesterone or its therapeutically used analogues may play a role in cell growth or metastasis in a subset of human breast tumors.

## Introduction

Angiogenesis is an important process for the growth and metastasis of many types of cancer, including breast cancer (1). For example, tumors may not grow past a certain size unless the density of microvessels in their vicinity increases (2), and the density of blood vessels near a tumor can influence its ability to metastasize (3). With regard to breast cancer, it is known that: (a) the production of angiogenic factors such as VEGF<sup>3</sup> is high in many human breast cancers (4); (b) there is a correlation between microvessel density and VEGF expression in primary breast tumors (5); (c) the relapse-free survival rate of breast cancer patients is inversely related to VEGF expression in their tumors (6); and (d) patients with the highest VEGF levels have the poorest prognosis (7). Understanding the regulation of angiogenesis in breast cancer may thus provide important insights into the mechanisms responsible for tumor growth and metastasis, pharmacological treatments, and measures to decrease tumor incidence.

VEGF is the most potent angiogenic factor known (8). It stimulates the proliferation of endothelial cells for the formation of new blood

vessels, and it also has a potent effect on vascular permeability (8). It thus stimulates the growth of new vessels as well as increasing their permeability, which may also be important for the provision of nutrients to tumors. As noted above, VEGF is expressed in many human breast tumors, and the level of its expression seems to correlate with a number of important clinical features of the disease. Elucidating the mechanisms that control VEGF expression in breast cancer cells is thus essential to understand several key features of this disease.

We and others have recently reported that steroid hormones control expression of VEGF. For example, estrogens regulate VEGF expression in the uterus (9, 10) and in rat 7,12-dimethylbenz(a)anthracene-induced mammary tumors (11), and progestins also seem to regulate VEGF expression in the uterus (10). The studies to date suggest that this effect is likely to be a direct action of these steroids mediated by their respective nuclear receptors (9, 10). It is well established that: (a) many human breast cancers contain ERs and PRs; (b) steroid hormones are involved in the etiology and growth of breast cancer; and (c) endocrine therapy is important in the treatment of this disease (12). These observations prompted us to initiate a program to investigate the effects of ovarian steroids on the regulation of angiogenic peptides and related factors in breast cancer. In this work, we now report the induction of VEGF by progestins in human T47-D breast cancer cells.

## Materials and Methods

**Materials.** Phenol red-free DMEM/F12 was purchased from Life Technologies, Inc. (Gaithersburg, MD), and FCS was obtained from JRH BioSciences (Lenexa, KS). Progesterone (4-pregnene-3,20-dione), MPA (17 $\alpha$ -hydroxy-6 $\alpha$ -methyl-4-pregnene-3,20-dione-17-acetate), and MGA (17 $\alpha$ -hydroxy-6-methyl-4,6-pregnadiene-3,20-dione-17-acetate) were purchased from Sigma (St. Louis, MO), and RU 486 was a gift of Roussel-Uclaf. All other synthetic progestins used in this work were purchased from Steraloids (Wilton, NH). The synthetic progestins tested were norgestrel (4-estren-17 $\alpha$ -ethynyl-18-homo-17 $\beta$ -ol-3-one), norethindrone (4-estren-17 $\alpha$ -ethynyl-17 $\beta$ -ol-3-one), norethynodrel (5(10)-estren-17 $\alpha$ -ethynyl-17 $\beta$ -ol-3-one), and caproxyprogesterone (4-pregnen-17-ol-3,20-dione caproate).

**Cell Culture.** All the cell lines used in this work were kindly provided by Dr. David Sirbasku (Department of Biochemistry and Molecular Biology, University of Texas, Houston Health Sciences, Houston, TX). These were initially obtained from American Type Culture Collection. These included the PR-rich T47-D cell line, the ER-rich MCF-7 cell line, the ER- and PR-positive ZR-75 cell line, the ER- and PR-negative MDA-MB-231 cell line, and the Ishikawa human endometrial carcinoma cell line. All cell lines were grown in phenol red-free DMEM/F12 supplemented with 10% FCS and treated as follows. Cells were plated in 100-mm dishes in DMEM/F12 supplemented with 10% serum and grown to approximately 50% confluence. Media were then replaced with phenol red-free DMEM/F12 supplemented with 5% charcoal-stripped serum as described previously (13). After 24 h, the charcoal-stripped serum-supplemented media were changed once again. The indicated concentrations of hormones were added, and the cells were allowed to grow for an additional 18 h. Aliquots of media were collected for measurement of VEGF content by ELISA (see below), and the cells were collected by scraping with a rubber policeman. The cells were taken through three freeze-thaw cycles

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<sup>3</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; PR, progesterone receptor; ER, estrogen receptor; MPA, medroxyprogesterone acetate; MGA, megestrol acetate.

and centrifuged, and supernatant was collected for the determination of protein concentration, measured as described previously (14).

**Measurement of VEGF by ELISA.** VEGF levels in the cell culture media were measured using a Quantakine kit from R&D Diagnostics (Minneapolis, MN), using the procedure recommended by the supplier. Human recombinant VEGF included with the kit was used to construct a standard curve and to obtain absolute values of VEGF protein content. The values were then normalized to the total protein concentration in each dish and compared statistically using the two-tailed Student's *t* test. Values with *P* < 0.05 were considered significant.

**Results**

Because our aim was to investigate the effect of progesterone on VEGF production in human breast cancer cells, we selected T47-D cells, which contain high levels of PR and are thus widely used by many investigators to examine the effects of progestins (15). As shown in Fig. 1, progesterone causes a 3–4-fold increase in the level of VEGF in the cell media. This increase is completely abolished by the antiprogestin RU 486, suggesting that the response is PR-mediated, and the antiprogestin alone decreases the basal level of VEGF in the media. In contrast, none of the other classes of steroid hormones tested altered VEGF levels, indicating that this effect of progesterone in T47-D cells is hormone specific under the experimental conditions we have used.

We next determined the progesterone dose-response curve for increasing VEGF levels to determine whether effective concentrations

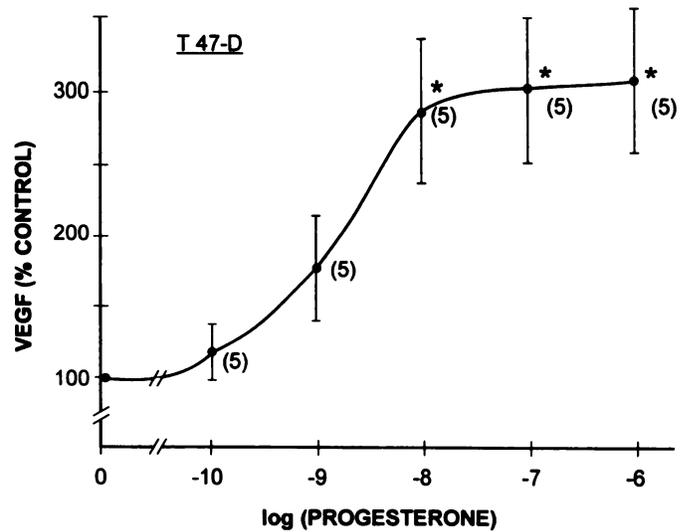


Fig. 2. Progesterone dose-response curve for induction of VEGF. Cells were cultured as described in "Materials and Methods" and then treated with various concentrations of progesterone for 18 h. Media were then analyzed for VEGF by ELISA as described in "Materials and Methods." Values were calculated as pg VEGF/mg total cellular protein and expressed as a percentage of the control for the number of determinations indicated. \*, significant differences compared to control (*P* < 0.01).

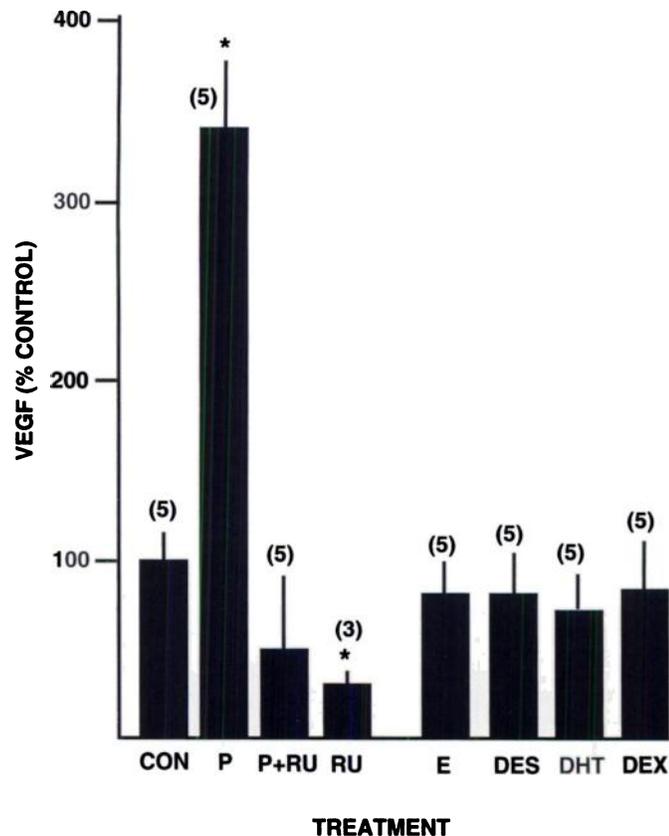


Fig. 1. Progesterone increases VEGF in culture media from T47-D cells. Cells were cultured as described in "Materials and Methods" and then treated with various steroids ( $10^{-8}$  M) for 18 h. RU 486 was used at  $100\times$  excess ( $10^{-6}$  M). Media were then harvested for analysis of VEGF by ELISA. Values were calculated as pg VEGF/mg total cellular protein and expressed as a percentage of the control for the number of determinations indicated. \*, values significantly different from control (*P* < 0.01). CON, control; P, progesterone; E, estradiol; DES, diethylstilbesterol; DEX, dexamethasone; DHT, dihydroxytestosterone.

**VEGF (% CONTROL)**

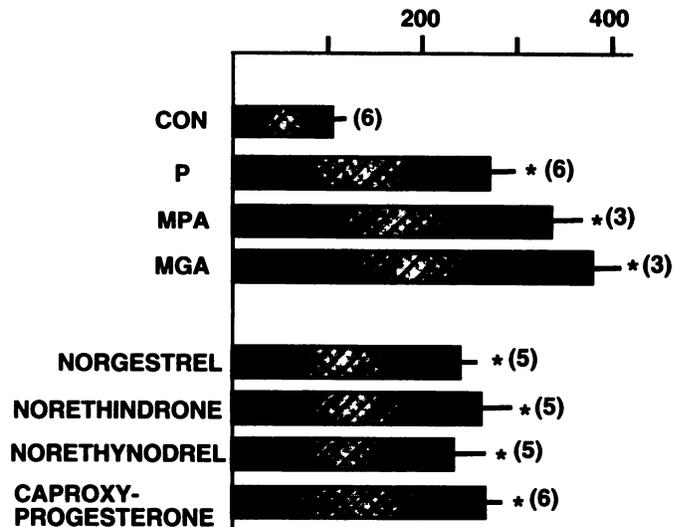


Fig. 3. Induction of VEGF by synthetic progestins. The procedure for measurement of VEGF was the same as described in Figs. 1 and 2. All steroids were used at  $10^{-8}$  M. \*, values for VEGF levels significantly different than control (*P* < 0.01).

of the hormone were in the physiological or pharmacological range. As seen in Fig. 2, progesterone induces a dose-dependent increase of VEGF in the media. A maximal response is produced by  $10^{-8}$  M progesterone, and half-maximal induction was seen at a dose of approximately  $10^{-9}$  M. These doses are in the range that elicits expression of several other genes in this cell line (16, 17) and are generally considered to be in the physiological range of hormone concentration.

The experiments above indicated that progesterone itself increases VEGF levels, and we next sought to determine whether synthetic progestins commonly used in oral contraceptives and postmenopausal hormone replacement regimens had a similar effect on the content of this growth factor. As seen in Fig. 3, all of the synthetic compounds

Table 1 Progesterone induction of VEGF in different cell lines

Cells were grown as described in "Materials and Methods" and then incubated with  $10^{-8}$  progesterone for 18 h. VEGF and cellular proteins were then determined as described in "Materials and Methods." Values are mean  $\pm$  SE for three determinations with all the breast cancer cell lines and two determinations for the Ishikawa cells.

Cell line	Control (pg VEGF/mg total cellular protein)	Progesterone (pg VEGF/mg total cellular protein)
T47-D	3,154 $\pm$ 290	6,885 $\pm$ 786 <sup>a</sup>
MCF-7	4,354 $\pm$ 1,258	4,127 $\pm$ 848 <sup>b</sup>
ZR-75	9,518 $\pm$ 2,606	9,556 $\pm$ 1,195 <sup>b</sup>
MDA-MB-231	26,495 $\pm$ 5,893	29,529 $\pm$ 2,568 <sup>b</sup>
Ishikawa	18,317 $\pm$ 3,283	24,047 $\pm$ 3,270 <sup>b</sup>

<sup>a</sup>  $P < 0.01$  versus control.

<sup>b</sup> Not significantly different from corresponding controls.

tested increase VEGF levels when present at a concentration of  $10^{-8}$  M.

In the final set of experiments, we sought to determine whether the effect of progesterone on VEGF levels is specific for T47-D cells, or whether a similar effect occurs in other breast cancer cell lines or in cell lines derived from other estrogen-responsive tumors. As seen in Table 1, progesterone did not increase media levels of VEGF in cultures of Ishikawa cells that are derived from a human endometrial carcinoma or in other human breast cancer cell lines, including ER- and PR-positive MCF-7 cells, ER- and PR-positive ZR-75 cells, or ER- and PR-negative MDA-MB-231 cells. This result indicates that the effect of progesterone on VEGF is cell specific as well as hormone specific.

### Discussion

Progestins have previously been reported to increase VEGF expression in the uterus of both humans (18) and rodents (9, 10), but to our knowledge, this is the first report that this class of hormones increases VEGF in human breast cancer cells. In T47-D cells, this induction is completely blocked by the antiprogestin RU 486, suggesting that it is mediated by the classical PR system. This is also consistent with the doses of progesterone required to produce this effect, which are in the range generally associated with PR-mediated events in this and other cell lines, and with the fact that a variety of progestins but not other hormones elevate VEGF. In addition, increases in extracellular levels of VEGF have generally been found to be associated with transcriptional activation of the VEGF gene (8) in systems in which molecular mechanisms have been investigated. Collectively, these observations thus suggest that the increase in VEGF we have observed in T47-D cells is likely to result from transcriptional activation of the gene, although this remains to be established by additional studies.

Although the specific biochemical mechanism remains to be elucidated, the ability of progestins to elevate VEGF in T47-D cells raises important questions about the possible effects of these agents on VEGF production by normal breast tissue as well as breast cancer cells, and about whether this effect plays any role in the human disease. The doses of progesterone that produce a maximal increase (*i.e.*, 10 nM) are within the range of circulating hormone levels in the luteal phase of the menstrual cycle. Peak plasma levels in the nanomolar range of norethindrone, norgestrel, and norethynodrel also occur after ingestion of currently used combination oral contraceptives, after administration of MPA for hormone replacement, and certainly after high-dose treatment with MPA or megestrol acetate for breast cancer (19). In addition, a recent report has shown that a number of environmental agents also possess progestin-like activity in experimental test systems (20). Breast cancer is obviously a multifactorial disease, but our findings are the first to suggest the possibility that progestin exposure from any source, *e.g.*, physiological, pharma-

cological, or environmental, could play a role in this disease by increasing the levels of a key angiogenic factor.

It is important to note, however, that the effect of progestins on VEGF is specific for T47-D cells, at least of the cell lines we have tested. If VEGF plays any role in the etiology of breast cancer, it thus seems probable that it would occur only in a subset of tumors. T47-D cells have the highest PR content of the cell lines tested, and it may be that differences in PR levels account for the cell specificity observed. Alternatively, it is also interesting to note that T47-D cells have the lowest basal level of VEGF in their media (see Table 1). This may be purely circumstantial, or it could imply that progestins act by removing an inhibitory stimulus present only in T47-D cells, or that VEGF production is already maximally stimulated by different regulatory factors in the other cell lines tested. Furthermore, whereas only progestins increase VEGF in T47-D cells, estrogens increase this factor in 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors (11). Taken together, these observations suggest that regulation of VEGF expression in mammary tumors is likely to be quite complex and to exhibit species, hormone, and cellular specificity.

One would normally anticipate that an increase of an angiogenic factor such as VEGF is likely to be associated with increased tumor growth, and this seems contradictory to the effects of progestins on T47-D cell proliferation. Whereas the effects of progestins on breast tumor cells are complex and highly dependent on the system studied, the overriding effect of progestins on T47-D cells in culture seems to be an inhibition of proliferation (21). One possible explanation of this apparent discordance is that an increase in VEGF production could actually represent an attempt by tumor cells to escape from an inhibition of proliferation by progestins. In other words, an inhibition of cell proliferation might trigger an internal signal in the cells to secrete a factor that would increase their nutrient supply secondary to angiogenesis. Murphy *et al.* (22) previously suggested this possibility to explain the observation that progestins stimulate growth factor production by T47-D cells while decreasing their proliferation. This would also be in line with the observation that hypoxia, which could be viewed as inhibiting proliferation by depriving cells of a factor essential for growth, is one of the most potent stimuli of VEGF production known (8).

Another point to consider is that increases in angiogenic factors such as VEGF may have important effects in a disease such as breast cancer other than the growth rate of the primary tumor. For example, angiogenesis is expected to increase metastasis of a primary tumor (3). It is thus possible that progestins could increase the metastasis of a tumor by increasing VEGF-stimulated angiogenesis while actually decreasing tumor cell proliferation by a separate mechanism. This possibility may be particularly important to explore in future studies, because progestins are already known to increase expression of cell-matrix adhesion molecules such as the 67-kDa laminin receptor and  $\beta_1$  integrin that are thought to play important roles in metastasis by facilitating the transit of tumor cells across basement membranes to facilitate their eventual entry into the circulation (23).

Our observations may also have therapeutic implications for the endocrine therapy of breast cancer. Progestins and antiprogestins have both been used in the treatment of breast cancer. These agents seem to inhibit the disease by different mechanisms, although both seem to produce their effects via PR-mediated interactions (24). It is thus noteworthy that the antiprogestin RU 486 completely suppresses the ability of progesterone to increase VEGF and decreases the basal levels as well. This may indicate that decreasing levels of VEGF may be one potential mechanism by which antiprogestins inhibit breast tumor growth *in vivo*. In the case of progestin agonists, treatments such as high-dose megestrol acetate are effective in the treatment of some tumors (25). If this compound induces VEGF levels in a subset

of human tumors, as we have observed with T47-D cells in culture, than combining it with inhibitors of angiogenesis such as TNP-470 (26) might improve the response rate in those cases.

In summary, we have demonstrated that progestins increase VEGF in a human breast cancer cell line. Whereas the physiological, pharmacological, and pathological implications of this observation remain to be determined, this is the first report of this action and thus raises previously unrecognized questions about the role of endogenous and therapeutically used progestins in breast cancer. Several studies are underway in our laboratories to pursue these questions and elucidate the molecular basis of this effect.

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