ABSTRACT

The progesterone receptor (PR) is a ligand-dependent transcription factor that promotes progestin-stimulated expression of target genes. Two functional PR isoforms, PRA and PRB, are expressed in progestin-responsive cells. PRA and PRB have distinct roles in gene expression and in mammmary gland development. One role of PRs in T47-D cells is regulating expression of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor. This study explores the isoform specificity of this PR function using parental T47-Dco cells that express both PRA and PRB and clonal derivatives that express either PRA (YA cells) or PRB (YB cells) or lack PR (Y cells). Treatment with progesterone induces VEGF mRNA and protein 2-fold in T47-Dco and YA cells and 3–7-fold in YB cells, suggesting that PRB inhibits PRB-dependent induction of VEGF. This is consistent with the observation that clinically relevant progestins induce a much higher level of VEGF in YB cells than in YA cells. Another novel finding in this report is that estradiol (10−8 M) induces VEGF production from YB cells. However, this induction is not blocked by 100-fold excess tamoxifen or ICI-182,780. Moreover, both tamoxifen (10−8 M) and ICI-182,780 (10−8 M) function as agonists for VEGF in YB cells. Small interfering RNA against PR or estrogen receptor abrogated estradiol and tamoxifen induction, indicating that the agonist-like response of these compounds in YB cells is estrogen receptor and PR dependent. Estradiol, tamoxifen, and ICI-182,780 also induce VEGF in BT-474 cells when their PRB levels were elevated by transfecting an expression plasmid for PRB, but not when the cells were transfected with vector alone. These results indicate that PRB preferentially regulates VEGF expression in breast cancer cells and PRB-enriched tumor cells may produce more VEGF, have a better developed vasculature, and potentially are more resistant to tamoxifen and ICI-182,780 than cells that express an equivalent or higher level of PRA than PRB. These results imply that PRB-enriched breast tumors may respond well to anticancer therapies that include inhibitors of angiogenesis.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor that promotes growth, expansion, and metastasis of breast cancer (1, 2). The role of VEGF in breast cancer expansion and inverse correlation with patient prognosis is well established (3–5). VEGF transcripts include several splice variants that produce distinct isoforms of VEGF (1, 6) including secretory (VEGF165 and VEGF 121; numbers denote amino acids) and nonsecretory isoforms (VEGF188 and VEGF180). These isoforms convey the mitogenic and permeability functions of VEGF to the endothelial cells via the two receptors f/k/f and f/k/f (2, 6). The mitogenic effect of VEGF isoforms on endothelial cells allows formation of new blood vessels, and permeability effects allow nourishment of growing tumors, which ultimately lead to their expansion (2). The role of angiogenesis in breast cancer is extensively studied because of interest in restricting this process by inhibiting VEGF, thereby preventing or controlling cancer growth (7).

Proliferation of many breast cancer cells is under control of the sex steroids estrogen and progesterone. Such proliferation allows expansion of tumor tissue that requires new blood vessels for nourishment. Whereas the effects of hormones on proliferation of breast cancer cells have been well studied, little attention has been paid to the role of sex steroid hormones and their receptors in controlling the process of angiogenesis for nourishment of tumor tissue. We previously published (8, 9) that both natural and synthetic progestins used in oral contraception or hormone replacement therapy induce VEGF in T47-D breast cancer cells through the progesterone receptor (PR). Several other steroids, including estradiol, were without any effect on VEGF expression in T47-D breast cancer cells. Based on this observation, we speculated (8, 9) that progesterone-dependent increase in VEGF could provide a partial explanation for increased incidence of breast cancer among women consuming progestin formulations in clinical trials for hormone replacement therapy (10).

The cellular effects of progestins are generally conveyed through two isoforms of the PR, PRA and PRB, that are expressed in most human breast cancer cells (11). PRA and PRB are expressed in nearly equimolar amounts in normal breast, but synthesis of PRA and/or PRB tends to be dysregulated in breast cancer cells, such that the two isoforms are differentially expressed (11–13). Previous studies provide evidence that the majority of advanced-stage tumors are rich in PRA; however, PRB is the predominant isoform in ductal carcinoma in situ (13). Importantly, a +331 G/A polymorphism in the PR gene was recently linked to increased expression of PRB in breast cells and increased risk of breast cancer (14). Collectively, these results indicate that PRB may play an essential role in breast tumor progression, most likely by dysregulating expression of selected genes. PRA lacks NH2-terminal amino acids 1–164 of the PRB isoform (11). PRA and PRB are functionally distinct, influencing distinct aspects of mammary gland differentiation (15). In addition, PRA has a dominant negative effect on PRB and controls transcription of nuclear receptors including the estrogen receptor [ER (16)]. PRA can also function as a strong transcriptional activator in certain cases (17).

Because recent studies indicate that individual PR isoforms have a distinct progestin-dependent gene expression pattern (18), we undertook the present study to examine the PR isoform specificity for VEGF induction using T47-D cells that express PRA (YA cells), PRB (YB cells), or PRA and PRB (T47-D parental cells) or do not express PR (Y cells (19)). We also examined the effects of estrogens and antiestrogens on VEGF induction in the cells expressing individual PR isoforms. We provide evidence that PRB is the dominant isoform regulating VEGF in breast cancer cells and that the presence of PRB alone permits the ER to induce VEGF in breast cancer cells in response to estradiol or tamoxifen treatment. The role of angiogenesis in breast cancer autonomy is also discussed.

MATERIALS AND METHODS

Chemicals. Estradiol [1,3,5(10),6-estratetraen-3,17β-diol], progesterone (4-pregnene-3,20-dione), and medroxy progesterone acetate (17a-hydroxy-oxymethyl-4-pregnene-3,20-dione17-acetate) as well as RU-486 were purchased.

Received 9/26/03; revised 1/3/04; accepted 1/27/04.
Grant support: Grants from the NIH (Grant CA-86916) and Susan G. Komen Breast Cancer Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Notes: S. M. Hyder is the Zalk Missouri Professor of Tumor Angiogenesis.

Requests for reprints: Salman M. Hyder, Dalton Cardiovascular Research Center, 134 Research Park Drive, University of Missouri-Columbia, Columbia, Missouri 65211. Phone: (573) 882-1261; Fax: (573) 884-4232.
from Sigma (St. Louis, MO). Other synthetic progestins used in this work were purchased from Steraloids (Wilton, NH). Additional synthetic progestins tested were norgestrel (4-estren-17α-ethyl-18-homo-17β-ol-3-one) and noretindrone (4-estren-17α-ethyl-17β-ol-3-one). The antiepileptics 4-hydroxytria- moxifen was purchased from Calbiochem (La Jolla, CA), andICI-182,780 was from Tocris Cookson (Eisvilleville, MO). All other chemicals used were of the highest reagent grade available.

**Cells and Cell Culture.** The wild-type PR-positive parental T47-Dco breast cancer cell line and isolation of its PR-negative clonal derivative T47-D-Y and construction of PR-positive T47-D-YA and T47-D-YB cells (referred to henceforth as to Y, YA, and YB cells) have been described previously (19). All cell lines were grown in phenol red-free DMEM:Ham’s F-12 (Invitrogen, Carlsbad, CA), supplemented with 5% FCS (JRH Bioscience, Lanexa, KS). Cells were routinely cultured in 100-mm dishes and incubated in 5% CO₂ at 37°C in a humidified environment. The T47-D-YA and T47-D-YB cells were grown in medium containing 200 μg/ml G418 (Sigma) to maintain selection for stable expression of PRA and PRB.

**VEGF ELISA Assay.** VEGF was measured with a Quantikine kit from R&D Diagnostics (Minneapolis, MN) using the supplier’s protocol as described previously and normalized to total cellular protein in each dish (8). Human recombinant VEGF was used as a standard. Data were analyzed for statistical significance using a two-tailed Student’s t-test. Values of P < 0.05 were considered significant. Inter- and intra-assay coefficients of variance, as described previously and normalized to total cellular protein in each dish (8).

**Western Blot.** Cells were lysed after 20 h, and luciferase activity was measured using the luciferase reporter construct as described previously and normalized to total cellular protein in each dish (8).

**Cells and Cell Culture.** The wild-type PR-positive parental T47-Dco breast cancer cell line and isolation of its PR-negative clonal derivative T47-D-Y and construction of PR-positive T47-D-YA and T47-D-YB cells (referred to henceforth as to Y, YA, and YB cells) have been described previously (19). All cell lines were grown in phenol red-free DMEM:Ham’s F-12 (Invitrogen, Carlsbad, CA), supplemented with 5% FCS (JRH Bioscience, Lanexa, KS). Cells were routinely cultured in 100-mm dishes and incubated in 5% CO₂ at 37°C in a humidified environment. The T47-D-YA and T47-D-YB cells were grown in medium containing 200 μg/ml G418 (Sigma) to maintain selection for stable expression of PRA and PRB.

**VEGF ELISA Assay.** VEGF was measured with a Quantikine kit from R&D Diagnostics (Minneapolis, MN) using the supplier’s protocol as described previously and normalized to total cellular protein in each dish (8). Human recombinant VEGF was used as a standard. Data were analyzed for statistical significance using a two-tailed Student’s t-test. Values of P < 0.05 were considered significant. Inter- and intra-assay coefficients of variance, as given by the manufacturer for cell culture supernatant assay, are 5.0–8.5% and 3.5–6.5%, respectively.

**Plasmid Transfection and Luciferase Assays.** The luciferase reporter was constructed as follows. The PvuII-Smal fragment of pPRE/GRE.E1b.CAT (20) was excised and inserted into the Smal site of pGL3Basic from Promega (Madison, WI). pPRE/GRE.E1b has two copies of the consensus PRE linked as described previously and normalized to total cellular protein in each dish (8).

**RESULTS AND DISCUSSION**

We demonstrated recently (8, 9) that VEGF is under progesterin control in T47-D human breast cancer cells and directly controlled by the PR because antiprogestins blocked this effect. However, other steroids, including estrogens, did not influence VEGF induction, indicating that natural and synthetic progestins are probably the main sex steroids that allow tumor expansion by promoting angiogenesis in a subset of breast cancer cells. Our recent observations have shown that VEGF induction by progesterone is not restricted to T47-D cells but occurs in three of seven PR/ER-positive cell lines.3 This suggests that VEGF is regulated by progesterone in a cell type-specific manner and that progesterone-dependent VEGF expression may be widespread in breast cancer cell lines. Because expression of PR isoforms is dysregulated in human breast tumors, leading to excess in one PR isoform (11–13, 22), we explored the PR isoform specificity of PR-dependent VEGF expression in progesterin-stimulated breast tumor cells expressing individual PR isoforms. Our results presented in this communication identify PRB as the dominant PR isoform controlling VEGF induction in response to natural and synthetic progestins and also provide evidence that predominant presence of PRB in breast cancer cells permit ER to induce VEGF in breast cancer cells.

**Small Interfering RNA (siRNA) Treatment.** The siRNA kit for suppressing ER-α message was from Upstate Biotechnology (Waltham, MA). The sequence for ER-α specific and nonspecific siRNA is proprietary and was not provided by the manufacturer. YB cells were transfected with 100 nM siRNA or control siRNA in 6-well plates using LipofectAMINE 2000 reagent (Invitrogen) as suggested by the manufacturer. The PR-siRNA and control siRNA oligonucleotide were purchased from Qiagen. The siRNA sequences were as follows: PR sense strand, 5'-UCAACAUAGGCCCAGCAGCAGAdTdT; and antisense strand, 5'-UUCGCCCUUCGCCAUUGAdTdT (GenBank accession number NM_000926). Cells were transfected with 0.5 μg of siRNA duplexes using RNAi Starter Kit (Qiagen). Transfected cells were incubated in DMEM:Ham’s F-12 media with 5% dextran-coated charcoal medium for 48 h and harvested for detection of ER-α and PR by Western blot. A second set of cells was used for VEGF ELISA. After the first 48 h incubation with ER-siRNA and PR-siRNA, media were replaced with DMEM:Ham’s F-12 with 5% dextran-coated charcoal serum, and cells were incubated for 16 h with various steroids. Media from these cells were collected, and VEGF was quantified by ELISA.

**Small Interfering RNA (siRNA) Treatment.** The siRNA kit for suppressing ER-α message was from Upstate Biotechnology (Waltham, MA). The sequence for ER-α specific and nonspecific siRNA is proprietary and was not provided by the manufacturer. YB cells were transfected with 100 nM siRNA or control siRNA in 6-well plates using LipofectAMINE 2000 reagent (Invitrogen) as suggested by the manufacturer. The PR-siRNA and control siRNA oligonucleotide were purchased from Qiagen. The siRNA sequences were as follows: PR sense strand, 5'-UCAACAUAGGCCCAGCAGCAGAdTdT; and antisense strand, 5'-UUCGCCCUUCGCCAUUGAdTdT (GenBank accession number NM_000926). Cells were transfected with 0.5 μg of siRNA duplexes using RNAi Starter Kit (Qiagen). Transfected cells were incubated in DMEM:Ham’s F-12 media with 5% dextran-coated charcoal medium for 48 h and harvested for detection of ER-α and PR by Western blot. A second set of cells was used for VEGF ELISA. After the first 48 h incubation with ER-siRNA and PR-siRNA, media were replaced with DMEM:Ham’s F-12 with 5% dextran-coated charcoal serum, and cells were incubated for 16 h with various steroids. Media from these cells were collected, and VEGF was quantified by ELISA.

**Small Interfering RNA (siRNA) Treatment.** The siRNA kit for suppressing ER-α message was from Upstate Biotechnology (Waltham, MA). The sequence for ER-α specific and nonspecific siRNA is proprietary and was not provided by the manufacturer. YB cells were transfected with 100 nM siRNA or control siRNA in 6-well plates using LipofectAMINE 2000 reagent (Invitrogen) as suggested by the manufacturer. The PR-siRNA and control siRNA oligonucleotide were purchased from Qiagen. The siRNA sequences were as follows: PR sense strand, 5'-UCAACAUAGGCCCAGCAGCAGAdTdT; and antisense strand, 5'-UUCGCCCUUCGCCAUUGAdTdT (GenBank accession number NM_000926). Cells were transfected with 0.5 μg of siRNA duplexes using RNAi Starter Kit (Qiagen). Transfected cells were incubated in DMEM:Ham’s F-12 media with 5% dextran-coated charcoal medium for 48 h and harvested for detection of ER-α and PR by Western blot. A second set of cells was used for VEGF ELISA. After the first 48 h incubation with ER-siRNA and PR-siRNA, media were replaced with DMEM:Ham’s F-12 with 5% dextran-coated charcoal serum, and cells were incubated for 16 h with various steroids. Media from these cells were collected, and VEGF was quantified by ELISA.

3 Y. Liang, J. Wu, and S. M. Hyder, manuscript submitted.
PRE-luciferase-transfected T47-Dco, YA, or YB cells, luciferase was induced 190-, 20-, or 950-fold by progesterone, respectively. This result suggests that PRA may repress the PRB in the PRA:PRB heterodimer, possibly by recruiting inhibitory corepressors as suggested by others (16). A 100-fold excess of RU-486 completely suppressed progesterone-dependent luciferase activity in T47-Dco and YA cells and suppressed it >95% in YB cells (Fig. 1B). This observation is consistent with the report that RU-486 is a weak PRB agonist (23, 24). In control Y cells, neither progestin nor RU-486 induces luciferase activity.

We tested the ability of YA and YB cells to regulate VEGF expression individually. Cells were treated with 10^{-8} M progesterone for 6 h, and VEGF mRNA was measured by Northern blot analysis (Fig. 2A). Progesterone induced VEGF transcript 1.7 ± 0.1-fold (mean ± SE; range, 1.6–1.9; n = 3) in both YA cells and parental T47-Dco cells (Fig. 2A). In contrast, progesterone induced VEGF transcript 3.5 ± 0.3-fold (range, 3.3–3.9; n = 3) in YB cells. RU-486 (100-fold excess) suppressed induction of VEGF mRNA in YA and YB cells and did not alter VEGF transcription in the absence of progesterone (Fig. 2A). These results demonstrate that PRB is the dominant inducer of VEGF mRNA in breast cancer cells and that PRA may suppress PRB-dependent induction of VEGF. It is likely that progesterone stimulates transcription of the VEGF gene in both cell lines in a PR-dependent manner because actinomycin D completely blocks progesterone-dependent transcription of VEGF in T47-D cells, as we have documented previously (9).

We previously published that progesterone stimulates synthesis of VEGF message that encodes for truncated diffusible forms of VEGF (VEGF_{165} and VEGF_{121}), as well as a low amount of the nonsecretory form [VEGF_{165} (9)] in T47-D cells, and ELISA confirmed an increase in secreted VEGF protein in cell culture media (9). These diffusible molecules stimulate directional growth of adjacent vascular networks toward the tumor (2). We have determined that progesterone also stimulates a similar increase in VEGF transcripts in YA and YB cells (Fig. 2B; Ref. 9), producing a higher level of secretory VEGF than nonsecretory VEGF.

Having established that cells expressing either the A or the B isoform of PR synthesize VEGF message in response to progesterone, we next used ELISA to assess the levels of VEGF protein released from these cells in response to progesterone, in the presence or absence of 100-fold excess antagonist RU-486 (Fig. 3A). After growth in the presence of 10^{-8} M progesterone for 18 h, VEGF protein increased 6–7-fold in YB cells, 2–3-fold in YA cells, and 2–3-fold in T47-Dco cells. This result is consistent with the differences in the levels of VEGF mRNA levels described and discussed above (Fig. 2A). However, the basal level of VEGF protein was approximately 3-fold higher in Y cells than in T47-Dco, YA, or YB cells. One interpretation of this result is that unliganded PRA may suppress VEGF synthesized and released from cells; however, additional experiments are needed to test this possibility. In the presence of progesterone, the anti-progestin RU-486 was inhibitory. However, with RU-486 treatment alone, VEGF expression increased 2–3-fold in YB cells, reaching levels that are achieved by hormonal treatment of YA or T47-Dco cells (Fig. 3A). Because RU-486 alone does not increase VEGF transcript levels in YB cells (Fig. 2A), this suggests that RU-486 may have other as yet unknown effects on VEGF.

REGULATION OF VEGF BY PROGESTERONE RECEPTOR B

**Fig. 1.** Expression of progesterone receptor (PR) A and PRB isoforms in T47-Dco cells and clonal derivatives. A. Western blot of PR from T47-Dco parental cells and clonal derivatives expressing PRA (YA) and PRB (YB) and lacking PR (Y). Western blot analysis was performed as described in “Materials and Methods.” Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for equal loading. B. cells were transiently transfected with 2 μg of PRE-luciferase vector and treated with 10^{-8} M progesterone, 10^{-8} M RU-486, or both for 18 h. Protein extracts were prepared for measuring luciferase activity as described in “Materials and Methods.” C: control; P: progesterone; RU: RU-486. Data shown represent the mean of three determinations ± SE (note that luciferase activity is plotted on a log scale).

**Fig. 2.** Induction of vascular endothelial growth factor (VEGF) mRNA by progesterone in progesterone receptor A and progesterone receptor B cells. A. cells were treated with progesterone (10^{-8} M) in the absence or presence of 100-fold excess of RU-486 for 6 h. RNA was prepared and analyzed by Northern blot as described in “Materials and Methods.” 28S rRNA was used as an internal control for loading efficiency. Data are the mean ± SE from three determinations. Asterisk indicates values significantly different from controls (P < 0.05, two-tailed Student’s t test). B. spliced variants of VEGF mRNA in progesterin-treated T47-D cells. RNA was isolated from progesterone-treated cells (10^{-8} M) and analyzed by reverse transcription-PCR using primers as described in “Materials and Methods.” VEGF isoforms (VEGF_{121}, VEGF_{165}, and VEGF_{189}) are indicated.
secretion. Nevertheless, this result implies that RU-486 may not be an effective inhibitor of tumor growth in PRB-enriched tumors.

Previous studies show that different synthetic progestins induce VEGF to different extents in T47-D cells (9). This effect was also examined and compared in YA and YB cells. All progestins induced VEGF in YA and YB cells (Fig. 3B) and uniformly stimulated severalfold higher expression in YB cells than in YA cells. For example, the androgenic compound nandrolone (10–8 M) induced VEGF approximately 3-fold, whereas the progestin used in hormone replacement therapy, medroxy progesterone acetate, induced VEGF approximately 7-fold in YB cells (Fig. 3B), and induction with both compounds was blocked by RU-486, indicating involvement of PR (data not shown). These results could reflect metabolic differences for specific progestins in YA or YB cells. Alternatively, ligand-specific cofactor interaction with PR may influence the extent of induction and/or release of VEGF from breast cancer cells.

Previous studies show that VEGF is inducible by a protein kinase C-dependent mechanism that involves AP-1 sites in the VEGF promoter (25). For comparison with progestin-induction, protein kinase C-induced expression of VEGF was analyzed by ELISA as described in “Materials and Methods.” Results are expressed as pg VEGF/mg cellular protein. B, induction of VEGF by synthetic progestins. The procedure for measurement of VEGF was the same as that described in A. Synthetic progestins were used at 10–8 M. Data are the mean ± SE of six determinations. Asterisk indicates induced values that show a statistically significant difference from controls (P < 0.05). C, induction of VEGF by 12-O-tetradecanoylphorbol-13-acetate. Cells were treated with 12-O-tetradecanoylphorbol-13-acetate (10–7 M) for 18 h. Data are the mean ± SE of six determinations. Asterisk represents induction values that show a statistically significant difference from controls (P < 0.05, two-tailed Student’s t test). Double asterisk represents a value that is not significantly different from controls within the same group (Y cells).

The results of the present study could have a broader implication and may relate to the recent clinical trial data indicating that combined estrogen-progestin hormone replacement therapy is associated with higher risk of breast cancer than use of estrogen alone or placebo controls and leads to detection of much larger tumors in patients (26, 27). Because of the short time frame involved with the clinical trials, we previously speculated that progestins may be influencing molecular mechanisms that allow nondetectable pre-existing lesions in women with tumorigenic potential (or occult tumors) to expand in a subset of women (8, 9). One possible explanation for this observation is that progestins regulate potent angiogenic growth factors in a subset of tumors to permit tissue expansion to a greater extent than estrogens. A recent study has shown that normal mammary gland biopsies from primates undergoing estrogen/progestin hormone replacement therapy contain higher levels of PRB than those from primates given estrogens alone (28). If such a situation was to exist in women receiving estrogen/progestin combination therapy, who may already have "pre-cancerous" tissue, then it could be speculated that these cells will acquire a growth advantage due to excess VEGF production not only from tumor cells but also from the normal surrounding tissue in response to progestins. However, it remains to be shown whether VEGF is under progestin control in normal cells, although a recent study indicates that VEGF levels are higher in the normal breast in
luteal phase when the progesterone levels are high (29). Thus, progestins may create a cancer-permissive environment in which tumor growth is supported by formation of new vasculature. Collectively, these results suggest that hormone replacement therapy with progestins may increase breast cancer risk in a subset of cases by stimulating angiogenesis and increasing tumor growth. It will be of interest in the future to determine whether the larger tumors detected after hormone replacement therapy (27) express excessive PRB that allows tumor expansion due to increased VEGF production. Although admittedly speculative, future experiments to address this hypothesis would be of considerable interest. Also, whereas most invasive breast tumors preferentially express PRA (22), animal experiments have shown that PRB-enriched tumor xenografts tend to be larger than those containing PRA (30). The results presented here noting that PRB-enriched tumor cells express more VEGF than PRA-enriched tumor cells suggest that PRB-enriched tumors may metastasize more readily than PRA-enriched tumors, even though the latter may be more invasive locally. This effect might depend on the existence of distinct sets of genes that are up- or down-regulated by PRB or PRA in progestin-stimulated cells (18).

The role of estrogen in regulating VEGF in breast cancer cells is controversial: some studies indicate that VEGF is not induced by estrogen in breast cancer cells (8, 31, 32), whereas other studies indicate that estrogen has agonist-like effects on breast cancer cells both in vivo and in cell culture (33, 34). In contrast, estrogen does regulate expression of VEGF in the uterus (35, 36), indicating that VEGF is regulated in a tissue- and cell-specific manner. An estrogen response element in the VEGF gene has been identified (37). In addition, some evidence suggests that PRA represses transcription of ER (16). In light of these results, the effect of estrogen was examined in T47-Dco, YA, and YB cells in the presence and absence of progestin to study the influence, if any, of the PR isoforms on estradiol-mediated VEGF induction in breast cancer cells.

PR-positive YA, YB, and T47-Dco cells express ER at a similar level, whereas PR-negative Y cells express ER at a lower level (Fig. 4A). In addition, VEGF expression was comparable in cells treated with progestin or progestin plus estradiol (Fig. 4B). However, unexpectedly, estradiol induced VEGF significantly in YB cells but not in YA, T47-Dco, or Y cells (Fig. 4B). The effect of estradiol on VEGF expression in YB cells was not inhibited by 100-fold excess of tamoxifen or ICI-182,780, a pure antagonist for ER (Fig. 4C). This result could indicate that estradiol induces VEGF in an ER-independent manner or that tamoxifen and ICI-182,780 act as ER agonist under these experimental conditions, similar to that described previously in uterine and breast cells (31, 36). To further assess the role of ER in ligand-induced VEGF expression in YB cells, we used ER-specific siRNA to down-regulate ER in YB cells.

As shown in Fig. 5A, inset, siRNA treatment for 48 h abolished ER expression by >90% as determined by Western blot analysis. A nonspecific siRNA was without any effect in reducing ER levels.
In the absence of progestin, but not in YA cells (even though the YA estrogen, tamoxifen, and ICI-182,780 are VEGF agonist in YB cells), PRB may potentiate the estrogen effect in an unknown manner. The latter possibility is consistent with the fact that PRB alone may potentiate the estrogen effect in an PRB-containing cells in the absence of PRA, suggesting that the presence of PRB was essential for both progesterone and estrogen responsiveness in YB cells. This observation suggests that PRB may be involved in cross-talk with ER to allow estrogen-dependent VEGF induction in YB cells. This may indicate a direct interaction between PR and ER or activation of PRB-dependent signal transduction pathways that activate ligand-bound ER for regulation of the VEGF promoter. Such cross-talk between ER and PR has been reported previously (37, 39).

We investigated the PR dependence of estrogen-stimulated expression of VEGF in YB cells. The experiment used PR-specific siRNA to reduce the levels of PRB in YB cells. After siRNA treatment, we treated cells with progesterone, estradiol, or tamoxifen to determine whether the cells were still responsive to progesterone and estrogen. As shown in Fig. 5B, siRNA treatment reduced PR levels by >80% in YB cells compared with the controls. Interestingly, loss of PR eliminates responsiveness to progesterone, estradiol, or tamoxifen (i.e., no enhancement of VEGF secretion occurred). These results indicate that the presence of PRB was essential for both progesterone and estrogen responsiveness in YB cells. This observation suggests that PRB may be involved in cross-talk with ER to allow estrogen-dependent VEGF induction in YB cells. This may indicate a direct interaction between PR and ER or activation of PRB-dependent signal transduction pathways that activate ligand-bound ER for regulation of the VEGF promoter. Such cross-talk between ER and PR has been reported previously (40).

To explore the possibility that an elevated ratio of PRB to PRA in breast cancer cells creates a permissive environment for estrogenic responsiveness, we transfected the ER/PR-positive BT-474 cells with human PRB expression plasmid. The inset in Fig. 6 shows that transient transfection of BT-474 cells led to a 2–3-fold increase in VEGF secretion. We then treated the transfected cells with progesterone and ligands for the ER and monitored VEGF secretion. Interestingly, ER-specific ligands induced VEGF in these cells (Fig. 6). Progesterone, ER agonists, and ER antagonists induced VEGF in PRB-transfected cells, but only progesterone induced VEGF from vector-transfected or from nontransfected BT-474 cells (Fig. 6). These results suggest that increased PRB expression may lead to estrogen sensitivity in human breast cancer cells. Although we have shown such an effect to occur in two breast cancer cell lines, additional breast cancer cell lines should be examined to determine whether this is a general phenomenon.

In summary, our results suggest that PRB predominantly regulates expression of VEGF in breast tumor cells. In contrast, PRA plays a critical role in the estrogenic regulation of VEGF in breast cancer cells.
minor role, if any, in this process and may antagonize PRB-dependent induction of VEGF. PRB-enriched tumors are therefore likely to express a higher level of VEGF, and this may give such tumors a significant growth advantage if the tumors are stimulated by hormone. Also, whereas progestins stimulate PR-A-dependent expression of VEGF to a small extent, both estrogen and progestin stimulate PRB-dependent VEGF expression (Fig. 4). This study examines a limited number of breast cancer cell lines; thus, there is a need for more extensive studies to determine whether PRB dominance in human breast tumor biopsies correlates with elevated levels of VEGF and whether excess PRB is associated with increased breast cancer risk. We predict that one of the consequences of elevated PRB could be elevated secretion of VEGF and greater tumor expansion. Finally, our data show that whereas antiprogestins reduce the levels of VEGF in both PR-A and PRB-containing tumor cells, antiprogestins such as RU-486 as well as antiestrogens, including tamoxifen and ICI-182,780, function as partial agonists of VEGF induction in cells that are rich in PRB. Therefore, it is anticipated that tumors containing elevated levels of PRB may benefit significantly from antiangiogenic therapeutic protocols.

ACKNOWLEDGMENTS

We are grateful to Dr. Zafar Nawaz for provision of PRE-luciferase plasmid and human PRB expression and control vectors.

REFERENCES


REGULATION OF VEGF BY PROGESTERONE RECEPTOR B


Progestin-Dependent Induction of Vascular Endothelial Growth Factor in Human Breast Cancer Cells: Preferential Regulation by Progesterone Receptor B

Jianbo Wu, Jennifer Richer, Kathryn B. Horwitz, et al.

Cancer Res 2004;64:2238-2244.

Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/64/6/2238

Cited articles This article cites 39 articles, 13 of which you can access for free at: http://cancerres.aacrjournals.org/content/64/6/2238.full.html#ref-list-1

Citing articles This article has been cited by 8 HighWire-hosted articles. Access the articles at: /content/64/6/2238.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.