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Estrogen Regulates Vascular Endothelial Growth/Permeability Factor Expression in 7,12-Dimethylbenz(a)anthracene-Induced Rat Mammary Tumors*

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ABSTRACT

Vascular endothelial growth/permeability factor (VEG/PF) is expressed in some normal tissues and at high levels in a wide range of tumors. This growth factor is believed to be a key mediator of angiogenesis. Recent reports have shown that VEG/PF mRNA in the normal rat uterus is stimulated by estradiol (E_2). In this study, we investigated the expression of VEG/PF in the mammary gland and 7,12-dimethylbenz(a)anthracene (DMBA)-induced, hormone-dependent mammary tumor of the rat model, and also whether VEG/PF is regulated by E_2. VEG/PF mRNA from tumor extracts was amplified by RT-PCR with VEG/PF primers and generated two main products which corresponded in size to those expected for VEG/PF 164 and 120. In some cases, a third product corresponding in size to that expected for VEG/PF 188 was also generated. No such PCR products were generated from equal amount of RNA from normal mammary tissue, rat brain, or liver. Using immunocytochemistry, VEG/PF expression was detected in the epithelial cells of the tumors. We developed an ELISA assay to measure VEG/PF protein concentrations and found a 4-fold difference between normal mammary glands (1.3 ± 0.11 ng/mg protein) and tumors (4.44 ± 0.66) ($P < 0.01$). E_2 treatment (5 μg/rat, sc) of rats 24 h after ovariectomy, greatly enhanced the expression of RT-PCR products in tumors within 2 h, which reached a maximum at 6–8 h but declined by 48 h. VEG/PF concentrations were also increased 8–12 h after E_2 injection. When rats were given two injections of aromatase inhibitor 4-hydroxyandrostenedione (4-OHA 10 mg/rat sc) 24 h apart, to reduce estrogen concentrations, a low level of RT-PCR products was maintained for at least 96 h. After a single injection of 4-OHA, RT-PCR products remained low until 36 h when an increase occurred corresponding with a rise in plasma E_2 levels. Injection of E_2 2 h after 4-OHA treatment, caused a rise in RT-PCR products in 6–8 h. However, there was no significant change in VEG/PF concentrations. An increase in VEG/PF protein concentrations followed the increase in mRNA levels by 4–6 h. Thus, it appears that E_2 causes a rapid induction of VEG/PF expression in mammary tumors that is similar to that observed in the normal uterus. These findings suggest that one mechanism by which estrogen acts as a mammary tumor promotor is by stimulating VEG/PF, leading to increased tumor angiogenesis and/or permeability of the microvessels to allow tumor cell migration. (*Endocrinology* **137:** 5589–5596, 1996)

ANGIOGENESIS, the growth of new blood vessels, is a vital process in normal development, wound healing, and reproduction (1). Angiogenesis is also essential for solid tumor growth (2) and plays an important role in the metastatic process (3). Migration of tumor cells from the primary site usually takes place *via* intravasation of cells into tumor blood vessels because the tumor interstitium generally lacks lymphatics (3). In breast cancer patients, high vascular counts have been found to correlate with node metastasis (4, 5). The intensity of tumor angiogenesis in breast carcinomas has been found to predict metastatic spread, either to axillary lymph nodes or to distant sites (4). Furthermore, a high vessel count correlates with reductions in relapse-free survival and overall survival (6). Thus, there is clinical evidence for a coordinated pathway involving angiogenesis in the emergence of metastatic potential (7).

A number of growth factors, cytokines, enzymes, lipids, and miscellaneous other factors have been found to have angiogenic activity *in vivo* and/or to stimulate endothelial cell growth, migration, or protease synthesis *in vitro* (8). However, it is unclear which of these factors play specific and important roles in either physiological or pathological angiogenesis. Vascular endothelial growth factor (9) or vascular permeability factor (10) (VEG/PF) is both a mitogen for endothelial cells and a potent stimulator of microvascular permeability. VEG/PF is known to be expressed at high levels in a wide range of tumors and tumor cell lines (11) and is believed to be a key mediator of tumor angiogenesis (12, 13, 14) and the high blood vessel permeability characteristic of tumors (15, 16). VEG/PF, due to its angiogenic and permeability-enhancing effects, could be an important factor enabling tumors to metastasize.

VEG/PF is expressed in a wide range of normal tissues in which angiogenesis does not occur, including pituitary, brain, kidney, lung, adrenal gland, heart, stomach mucosa, liver, and spleen (17). VEG/PF is also expressed in the normal ovary and uterus in which angiogenesis has an important role. VEG/PF expression in the uterus has recently been

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shown to be rapidly and strongly stimulated by estrogen (18), suggesting that VEG/PF mediates the normal, estrogen-induced increase in vascular permeability and blood vessel growth in the uterus. Because of the important role of estrogen in promoting growth of breast cancers, we have investigated the effect of estrogen on VEG/PF expression in carcinogen-induced, hormone-dependent mammary tumors of the rat model.

Materials and Methods

Animal model

Sprague-Dawley female rats (Charles River, Wilmington, MA), 50–55 days of age, were gavaged with 20 mg 7,12-dimethylbenz(a)anthracene (DMBA) (Sigma Chemical Co., St. Louis, MO) in 2 ml peanut oil per rat as described previously (19). The animals were maintained for the first month after treatment in the Biohazard area of the University of Maryland at Baltimore Animal Facility. Throughout the experiments, the rats received food and water *ad libitum* and were kept under controlled conditions of light (12 h) and temperature. Animals were inspected weekly for mammary tumors. When the first tumor appeared, it was removed as well as the ajoining pectoralis major muscle. The rat was then killed. The tissues were weighed and stored at −70 C until assayed. A total of 32 tumors were collected at various times after DMBA administration for measurement of VEG/PF mRNA and protein concentrations. In addition, two rats with multiple tumors were ovariectomized under fluorothane anesthesia. After 24 h, these animals were injected with E_2 (5 μg/rat sc), and then one tumor removed under anesthesia at 0, 2, 4, 6, 8, 12, and 24 h. Another six intact rats with tumors were injected with a suspension (10 mg/rat sc) of aromatase inhibitor, 4-hydroxyandrostendione (4-OHA) in 3% hydroxypropylcellulose (Sigma). The aromatase inhibitor was synthesized in our laboratory, as previously described (19). One tumor per rat was removed from two of these animals at 0, 24, 30, 36, and 48 h after 4-OHA injection. Two rats received E_2 injections 24 h after administration of 4-OHA, and tumors were removed 0, 2, 4, 6, 8, 12, and 24 h later. Two rats with eight tumors had one tumor each removed just before 4-OHA injection. After 24 h, a second injection of 4-OHA was administered, and the remaining tumors were removed 0, 2, 4, 6, 8, 12, and 24 h later. Blood samples of approximately 1 ml were collected when tumors were removed at 0, 24, 30, 36, and 48 h after the first injection of 4-OHA from all six inhibitor treated rats. One milliliter saline was infused after each collection. All surgical procedures were carried out under flourothane anesthesia and in accordance with NIH guidelines. Animals were killed when the last tumor was removed. Tumors and serum were stored frozen until assayed. Serum concentrations of E_2 were measured by RIA using a commercial kit (Diagnostic Products Corporation, CA) with modification, as previously described (20).

Extraction of RNA

Total RNA was isolated from the tumors using the method of Cathala et.al. (21) with minor modifications. In brief, tumors were homogenized with GT/Tris buffer [4 M GT, 500 mM Tris-HCl (pH7.5), 10 mM EDTA, 0.5% sodium *N*-lauroylsarcosine, and 8% 2-mercaptoethanol; 1 ml/100 mg tissue]. All above reagents were purchased from GIBCO (Grand Island, NY). After one tenth volume of 3 M sodium acetate was added, the homogenate was extracted sequentially with equal volumes of phenol and chloroform/isoamyl alcohol (24:1). The resulting aqueous phase (10,000 × *g* for 15 min) was transferred to a new tube, and an equal volume of isopropylalcohol was added. After 1 h at −20 C the RNA was pelleted by centrifugation (10,000 × *g*, for 15 min), washed with 70% ethanol, and dissolved in water. The absorbance was measured at 260 nm and the concentration determined.

Oligonucleotide primers

Oligonucleotide primers for VEG/PF were obtained from the Biopolymer Laboratory of the Department of Microbiology and Immunology, University of Maryland School of Medicine. The 5′-VEG/PF primer (5′-^{68}GCTCTCTTGGGTGCACTGGA^{85}-3′) and the 3′-VEG/PF primer (5′-^{576}CACCGCCTTGGCTTGTCACA^{627}-3′) were specific for the rat VEG/PF sequence (21). The sequence of the primers for rat β-actin was the same as reported previously (18).

RT-PCR

RT-PCR was carried out according to the manufacturer's instructions with minor modifications (22, 23) using a GeneAmp DNA amplification reagent kit (Perkin-Elmer/Cetus, Norwalk, CT). PCR incubations were carried out in a programmable thermal controller (MJ Research, Cambridge, MA). During each cycle, the samples were heated to 94 C for 30 sec, cooled to 65 C for 30 sec, and heated again to 72 C for 90 sec. Thirty PCR cycles were performed on each sample. A 2-μl sample of each 100 μl PCR solution was added to 0.5 μl 5× loading buffer and fractionated by electrophoresis in a 7 × 8 cm, 0.75 mm thick, 8% polyacrylamide gel at 100 V. Gels were stained in ethidium bromide (0.5 μg/ml) (Sigma) and examined on a 312-nm UV transilluminator. Gels were photographed using Polaroid 665 film. To estimate the relative concentrations of target mRNA in tumors removed at different times after treatment, equal aliquots of the RT solutions from the samples to be compared were serially diluted and then amplified for a fixed number of cycles (18). This semiquantitation of RT-PCR was repeated twice on the RNA of tumors after treatment of the ovariectomized rats with estrogen.

Tumor extraction for VEG/PF protein measurement

Tumors and normal tissue were pulverized in liquid nitrogen and diluted to 100 mg tissue per ml in 0.1 M PBS buffer, pH 7.4. The homogenate was then centrifuged for 30 min at 30,000 × *g* and supernatant collected and stored at −70 C until used for measurement of VEG/PF concentrations.

Indirect ELISA for VEG/PF measurement

VEG/PF concentrations in tissue extracts were determined by an indirect ELISA method developed in our laboratory. Aliquots of 100 μl tissue extracts or samples of known concentrations of human recombinant VEG/PF (Sigma) were placed in duplicate wells of multiwell ELISA plates with enhanced protein binding (Corning, NY) for 16 h at 4 C to absorb the cytosolic proteins completely. The wells were then washed three times with 0.1 M PBS and incubated with 2% BSA in 0.1 M PBS at 37 C for 1 h. After another three washes, 100 μl of primary specific rabbit polyclonal antibody to human recombinant VEG/PF (SantaCruz Biotechnology, Santa Cruz, CA) in 0.1 M PBS with 0.05% Tween-20 (Sigma) was added to the wells for 1 h at room temperature. The plates were washed again three times and incubated with 100 μl of peroxidase conjugated goat-antirabbit antibody (GIBCO) in 0.1 M PBS with 0.05% Tween-20. After three final washes, 100 μl of peroxidase substrate 3,3′5,5′-tetramethylbenzidine (GIBCO) was added to the wells. The reaction was stopped after approximately 15 min at room temperature by the addition of 30 μl 50% sulfuric acid. The intensity of the color reaction was measured by reading the optical density at 450 nm. The concentration of VEG/PF in the samples was calculated using a calibration curve plotted from the optical density of samples containing known concentrations (in the range of 0.1–10,000 ng/ml) of human recombinant VEG/PF analyzed on the same plate. The linear part of the calibration curve was from 1 to 100 ng VEG/PF per ml of sample. The intraassay coefficient of variation did not exceed 4.1% and interassay variation was 8.3%. This antibody, as reported by the manufacturer, has no significant cross-reaction with PDGF and FGF, and some other growth factors. The protein content of each sample was measured as described by Lowry *et al.* (24).

Immunohistochemistry

Five-micrometer sections were cut from paraffin-embedded tissues and mounted on slides coated with chrome alum gel. After deparaffination and rehydration in xylene and ethanol, they were placed in 10 mM citrate buffer (pH 6.0) and processed in a microwave oven for three periods of 5 min each. After cooling, the sections were washed twice in distilled water. After incubation for 20 min in 1% hydrogen peroxide/methanol, the slides were washed twice in distilled water and twice in 0.05 M Tris-HCl buffer (pH 7.4). No specific sites were blocked by

incubating with 1% normal goat serum in 0.05 M Tris-HCl buffer. After decanting the reagents, the sections were covered with anti-VEG/PF rabbit polyclonal antibody (Santa Cruz) used in 1:500 dilution. The samples were incubated overnight in a humid chamber at 4 C. After three washes in 0.05 M Tris-HCl buffer, the slides were incubated for 30 min with biotinylated secondary IgG (Dako Corp., Carpenteria, CA) at room temperature. Sections were washed again and incubated with streptavidin peroxidase (Dako) for 30 min at room temperature. After three more washes, the sections were incubated with 3-amino-9-ethylcarbozole (Sigma) for 9 min, washed, counterstained with Mayer's hematoxylin (Sigma) and coverslipped. Control sections were incubated with 0.01 M PBS and normal mouse IgG instead of primary antibody.

Results

Expression of VEGF mRNA and protein in the normal rat mammary gland and in mammary tumors

RT-PCR amplification of total RNA from DMBA-induced mammary tumors and adjoining pectoralis major muscle with VEG/PF primers generated two to three major products. The sizes of these products corresponded closely to those expected from the transcripts for VEG/PF188, VEG/PF164, and VEG/PF120 [635, 563, and 431 bp, respectively]. Bands corresponding to VEG/PF 120 and 164 were observed in all tumors. In addition, the band corresponding to VEG/PF 188 was seen in some tumors. There was no indication of a larger product corresponding to the transcript for the 206-amino acid form of human VEG/PF. In all cases, the number of PCR products in the tumor was the same as in the adjacent pectoralis major muscle. No transcripts were detected in either normal mammary gland or the adjacent pectoralis major muscle (Fig. 1) when identical amounts of total RNA and an equivalent number of PCR cycles were used.

Measurement of VEG/PF protein content showed up to a 4- to 5-fold increase in VEG/PF levels in mammary tumors (4.42 ± 0.41 ng/mg cytosol protein, n = 32) compared with normal mammary glands (1.01 ± 0.08 ng/ml, n = 12), or to some other normal tissues, such as brain and liver from rats not treated with DMBA ($P < 0.01$) (Fig. 2). There was no apparent correlation between the concentration of VEG/PF and either tumor weight or time after DMBA administration.

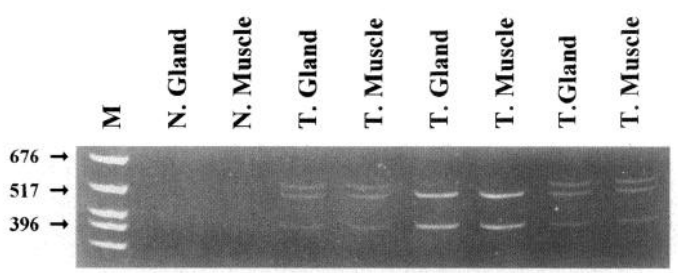

FIG. 1. RT-PCR products of VEG/PF mRNA in mammary tumors and tissue. Three representative mammary tumors adjacent pectoral muscle and mammary gland of rats treated with carcinogen DMB A RT-PCR amplification of total RNA with VEG/PF primers generated two to three products which correspond to VEG/PF 188, VEG/PF 164 and VEG/PF 120. Lanes M, Marker; 1, normal mammary gland; 2, adjacent pectoral muscle; 3, mammary tumor; 4, adjacent pectoral muscle; 5, mammary tumor; 6, adjacent muscle; 7, mammary tumor; 8, adjacent muscle.

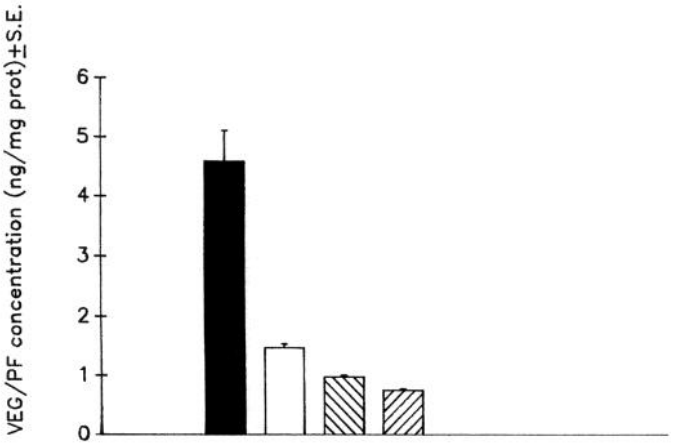

FIG. 2. VEG/PF concentrations in mammary tumors mammary glands and other normal tissues of rats. The concentration of VEG/PF was measured by an indirect ELISA method as described in *Materials and Methods* using a polyclonal antibody to human recombinant VEG/PF. ■, Mammary tumors (n = 15) were removed from rats treated with carcinogen DMBA; □, mammary glands from untreated rats; □, liver from untreated rat; □ brain from untreated rat.

Time course of VEG/PF expression in DMBA-induced mammary tumors of rats treated with E_2 after ovariectomy or administration of 4-OHA

To determine whether estrogen regulates VEG/PF expression in the tumor, we administered E_2 (5 μg/rat sc) to rats in which endogenous estrogen production was first eliminated by either ovariectomy or by administration of the aromatase inhibitor 4-OHA (10 mg/rat sc). One tumor was then removed from each of two ovariectomized rats at 0, 2, 4, 6, 8, 12, and 24 h after E_2 injection (5 μg/rat sc) (Fig. 3A). Two hours after E_2 administration, increased amounts of VEG/PF transcripts were observed (Fig. 3B (*right*) and Table 1). The level of β-actin in the same sample was compared to determine the specificity of any changes in levels of mRNA VEG/PF. The maximal increase in these products was about 8-fold and occurred between 6 and 8 h after E_2. The yield of PCR products then declined gradually up to 12 h, but was still higher than that before treatment. By 24 h after E_2 treatment, the yield was indistinguishable from that at 0 h. No change was observed in the expression of β-actin mRNA in any of the tumor samples shown in Fig. 3B (*left*) and Table 1, indicating that changes in VEG/PF were not caused by loading different amounts of sample onto the gel.

VEG/PF protein levels measured in the same samples paralleled the estrogen-induced increase in VEG/PF mRNA expression, after a short delay. Thus, the first signs of increased VEG/PF protein was observed 6 h after E_2 treatment. The level of VEG/PF protein increased from 4.42 ± 0.41 ng/mg protein to a maximum of 8.84 ± 1.13 ng/mg·protein) 10–12 h after E_2 injection. VEG/PF concentrations were significantly higher, up to 2-fold, in tumors from E_2- treated animals *vs.* tumors from untreated rats ($P < 0.05$). After 12 h, VEG/PF concentrations in tumors tended to decrease. However, they were still higher (6.62 ± 0.41 ng/mg protein) 24 h after administration (Fig. 4) than those from untreated animals (4.42 ± 0.41 ng/mg protein).

We have previously reported that treatment with aromatase (estrogen synthetase) inhibitor, 4-OHA, significantly

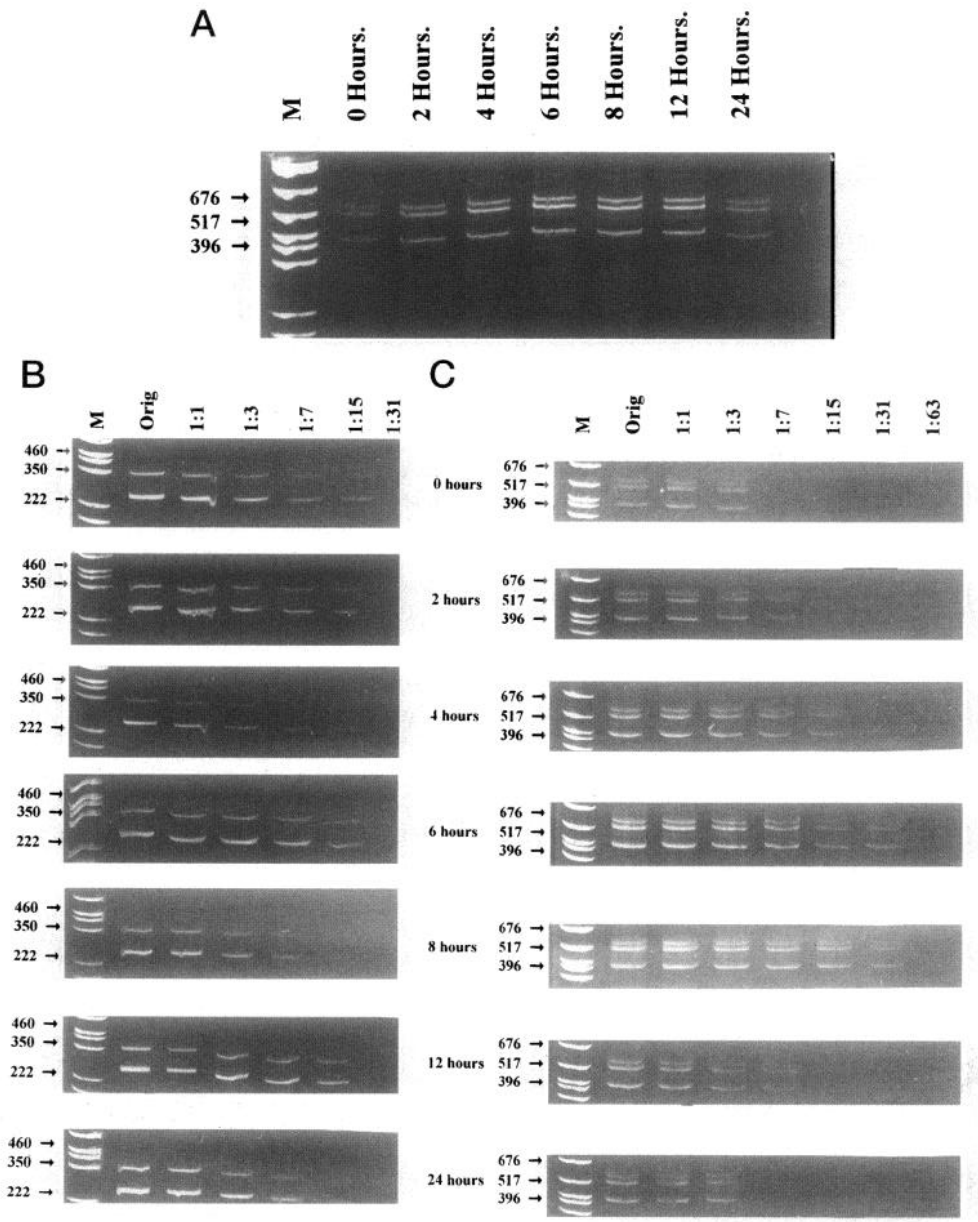

FIG. 3. A, RT-PCR products of VEG/PF mRNA in mammary tumors from ovariectomized rats in response to E_2. Lanes M, Marker; 1, 24 h after ovariectomy; 2, 2 h after E_2 injection (5 mg/rat sc); 3, 4 h after E_2 injection; 4, 6 h after E_2 injection; 5, 8 h after E_2 injection; 6, 12 h after E_2 injection; 7, 24 h after E_2 injection. B, Semiquantitative measurement of RT-PCR products of β-actin (*left*) and VEG/PF (*right*) mRNA in mammary tumors from ovariectomized rats. Equal aliquots of tumor mRNA (sample #1, 24 h after ovariectomy shown in Fig. 3A) were diluted and then amplified for a fixed number of cycles. Lanes: 1, no dilution; 2, 1:1; 3, 1:3; 4, 1:7; 5, 1:15; 6, 1:31; 7, 1:63; 8, 1:127.

decreases serum E_2 levels in the female rat (19, 25). Therefore, the effect of reducing estrogen secretion by treatment with 4-OHA on VEG/PF mRNA and protein concentrations was determined in rat mammary tumors. Rats that developed DMBA-induced mammary tumors were injected with 4-OHA (10 mg/rat, sc). Twenty-four hours later, two of these animals were administered E_2 (5 μg/rat sc) and one tumor was removed from each after 0, 2, 4, 6, 8, 12, and 24 h. E_2 treatment enhanced the yield of VEG/PF transcripts, which began within 2 h, and was maximal between 6 and 8 h, then declined after 12 and 24 h (Fig. 5). No change was observed in β-actin levels. These results corresponded with those of the ovariectomized animals shown in Fig. 3 and Table 1. The concentration of VEG/PF protein in the same samples also increased at 6 h after E_2 injections and reached a maximum at 10–12 h, as shown in Fig. 4. In animals treated with 4-OHA alone, the amount of the products after 30 PCR cycles in tumors removed up to 30 h was unchanged. However, after 36 and 48 h after 4-OHA injection, increased yields were observed (Fig. 6), whereas β-actin levels were unchanged. In these animals, the levels of serum E_2 were reduced about 30% (from 4.15 ± 0.21 pg/ml to 3.25 ± 0.18 pg/ml) up to 30 h after injection of 4-OHA but then increased to about twice the control level (8.9 ± 0.48 pg/ml) by 48 h (Fig. 7). VEG/PF protein content in tumors of these rats increased slightly but not significantly by 48 h after treatment (Fig. 7). In contrast, when tumors were removed at 0, 24, 28, 30, 32, 36, and 48 h from two rat that had been treated with a second injection of 4-OHA 24 h after the first administration, no change in VEG/PF mRNA was found from 0–48 h after first 4-OHA injection (Fig. 8). In this case, serum E_2 levels in samples collected at 0, 24, 30, 36, and 48 h remained below that at the initial time (2.9 ± 0.17 pg/ml)

TABLE 1. Semiquantitative RT-PCR determination of relative VEG/PF mRNA levels in mammary tumors from ovariectomized rats in response to E_2 treatment

Time after E_2	Dilution / Primer	Undiluted	1:1	1:3	1:7	1:15	1:31	1:63	1:127
0 h	VEG/PF	●	●	●	X	X	X	X	X
	β-actin	O	O	O	O	O	X	X	X
2 h	VEG/PF	●	●	●	●	X	X	X	X
	β-actin	O	O	O	O	O	X	X	X
4 h	VEG/PF	●	●	●	●	●	X	X	X
	β-actin	O	O	O	O	O	X	X	X
6 h	VEG/PF	●	●	●	●	●	●	X	X
	β-actin	O	O	O	O	O	X	X	X
8 h	VEG/PF	●	●	●	●	●	●	X	X
	β-actin	O	O	O	O	O	X	X	X
12 h	VEG/PF	●	●	●	●	X	X	X	X
	β-actin	O	O	O	O	O	X	X	X
24 h	VEG/PF	●	●	●	X	X	X	X	X
	β-actin	O	O	O	O	O	X	X	X

Semiquantitative measurement of mRNA VEG/PF and β-actin in serially diluted samples shown in Fig. 3. mRNA VEG/PF concentrations increased within 2 h and were maximal between 6 and 8 h after injection of E_2. ●, mRNA VEG/PF detected.

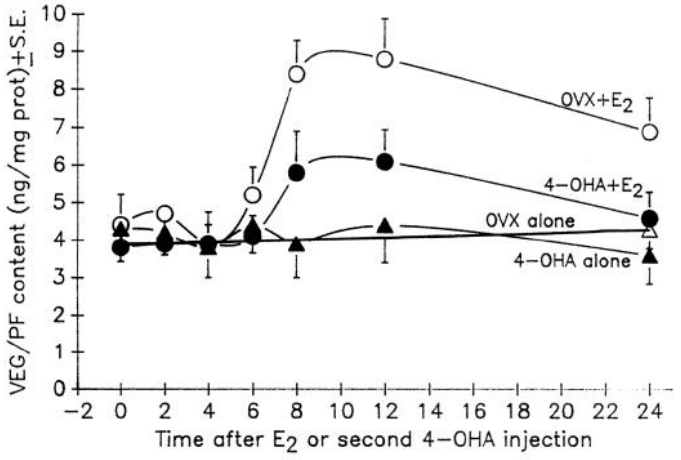

FIG. 4. VEG/PF concentrations in mammary tumors of rats either ovariectomized or treated with 4-OHA, and 24 h later with E_2. Rats were either ovariectomized or injected with 4-OHA (10 mg/rats sc). After 24 h, tumors were removed and rats injected with E_2 (5 μg/rat sc). Samples were from the same rats as in Figs. 3 and 5.

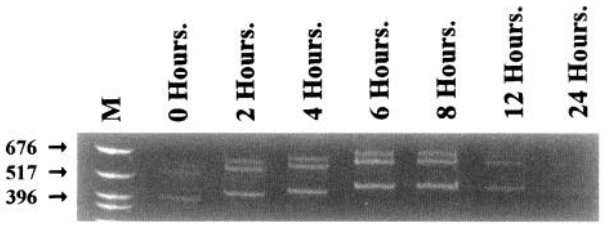

FIG. 5. RT-PCR products of VEG/PF mRNA mammary tumors from rats treated with 4-OHA. Rats were injected with 4-OHA (10 mg/rats sc). After 24 h, tumors were removed and rats injected with E_2 (5 μg/rat sc). Tumors were collected at the times indicated. Lanes: 1, 24 h after 4-OHA; 2, 2 h after E_2; 3, 4 h after E_2; 4, 6 h after E_2; 5, 8 h after E_2; 6, 12 h after E_2; 24 h after E_2. β-actin levels (not shown) were unchanged.

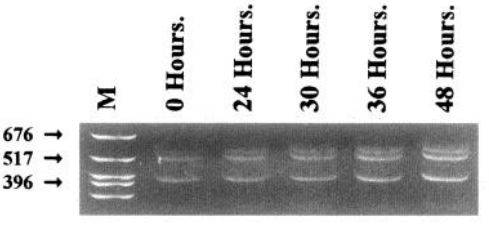

FIG. 6. RT-PCR products of VEG/PF mRNA in mammary tumors from rats treated with 4-OHA (10 mg/rats sc). Lanes: 1, no 4-OHA; 2, 24 h after 4-OHA; 3, 30 h after 4-OHA; 4, 36 h after 4-OHA; 5, 48 h after 4-OHA. Levels of β-actin (not shown) were unchanged.

(Fig. 7). Concentrations of VEG/PF were unchanged throughout the periods of observation (Fig. 7).

Immunohistochemical study in the normal mammary gland and in DMBA-induced mammary tumors

Intense, positive immunoreactivity to the specific antibody against VEG/PF was observed in rat DMBA-induced mammary tumors (Fig. 9-A). The VEG/PF immunoreactivity was located in the cytoplasm of lobular and ductal epithelial cells of the tumors. Little or no immunoreaction was observed in normal mammary gland (Fig. 9-B). When anti-VEG/PF antibody was replaced by irrelevant mouse IgG, no immunoreaction was seen in the same tumors (Fig. 9).

Discussion

VEG/PF mRNA measured by RT-PCR amplification was expressed in all DMBA-induced mammary tumors examined, but was not detected in normal mammary tissue. In addition, VEG/PF protein content in the same tumors was significantly higher than in the normal mammary glands. Angiogenesis is considered to be essential to tumor development (26–29) and appears to play an important role in the ability of tumors to metastasize (30). VEG/PF is thought to be involved in the initiation of angiogenesis (17, 31). Although we have not measured new blood vessel formation in these studies, we found increased expression of VEG/PF mRNA and protein in these well vascularized tumors compared with normal mammary tissue. Although the number

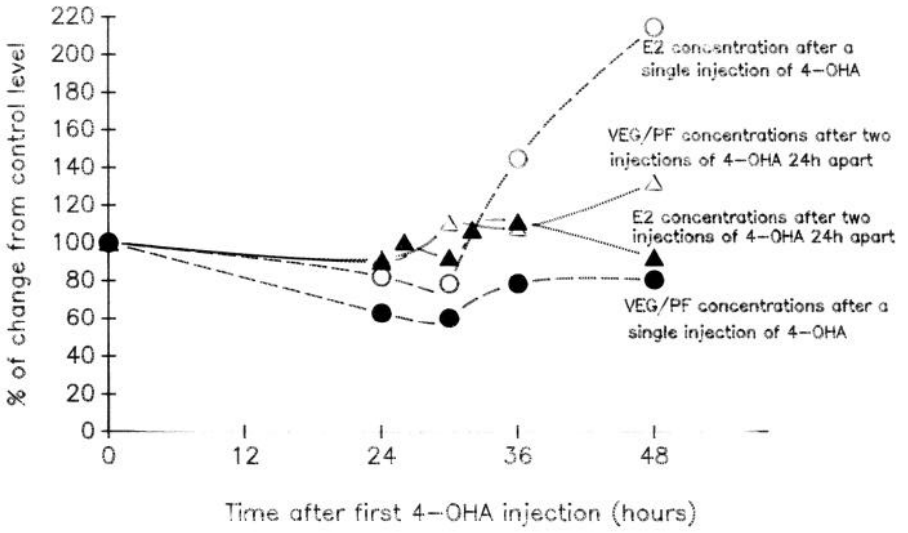

FIG. 7. The effect of 4-OHA on percent change in concentrations of VEG/PF in mammary tumors and of serum E_2. Samples were collected at times indicated from rats after treatment with 4-OHA (10 mg/rat sc). O E_2 concentrations after a single injection of 4-OHA. ● VEG/PF concentrations after a single injection of 4-OHA. △, VEG/PF concentrations after two injections of 4-OHA 24 h apart. ▲, E_2 concentrations after two injections of 4-OHA 24 h apart.

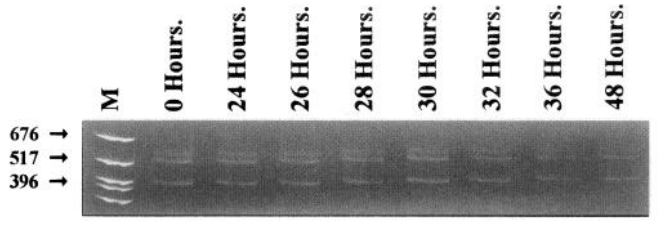

FIG. 8. RT-PCR products of VEG/PF mRNA in mammary tumors from rats treated with 4-OHA (10 mg/rats sc). A second injection of 4-OHA was administered 24 h later and tumors collected at the indicated times.

of PCR products generated by RT-PCR amplification from the pectoral muscle corresponded with those from the tumor that was adjacent to it, the VEG/PF protein concentration was low and similar to that of the normal mammary gland. A possible explanation for this finding could be that transcription is increased in muscle and tumors, but that much greater protein expression occurs in the tumor to stimulate angiogenesis.

VEG/PF has been reported to be expressed in a wide range of normal tissues in which angiogenesis does not occur, including pituitary, kidney, lungs, and heart (17). This indicates that VEG/PF may have multiple normal functions. Although no products were detected in the normal mammary gland in samples amplified for 30 cycles, after 45 cycles of RT-PCR amplification of normal mammary gland with VEG/PF primers, three major transcripts were visible on the gels (data not shown). Only faint expression of VEG/PF around blood vessels could be detected by immunohistochemistry. This finding is consistent with our ELISA results and indicates that very little VEG/PF is expressed in the normal mammary gland of the rat.

VEG/PF was recently reported to be expressed in the normal uterus of the rat and was found to be rapidly and strongly stimulated by estrogen. This suggested that VEG/PF could mediate the normal, estrogen-induced increases in vascular permeability and blood vessel growth in this tissue (18). Endothelial cells have been found to possess estrogen receptors (32), and estrogen has been reported to increase endothelial cell proliferation (33, 34). Because of the important role of estrogen in stimulating the growth of breast cancers in both pre- and postmenopausal women (35), we were prompted to investigate whether estrogens influence VEG/PF expression in the well vascularized (36), DMBA-induced, hormone-dependent rat mammary tumors. Fukeda *et al.* (37) had observed that growth of the capillary endothelial cells in the DMBA-induced tumors is estrogen dependent. Thus, when rats were treated with E_2 after ovariectomy, tumor necrosis was prevented and a high rate of endothelial cell proliferation was maintained (37). Our results demonstrate that the level of VEG/PF, preceded by an increase in mRNA, is rapidly stimulated by estrogen in DMBA-induced mammary tumors of ovariectomized rats. The difference in time between the increase in VEG/PF mRNA and protein level does not exceed more than 4 h and could represent the time required for immunodetectable proteins to be synthesized.

It has been reported that the estrogen-induced increase in VEG/PF mRNA in the rat uterus is inhibited by actinomycin D, but not by cycloheximide, suggesting that the effect is due, at least in part, to an increase in the rate of transcription (18, 38). Our results suggest that similar regulation of VEG/PF may occur in estrogen dependent mammary tumors as in the rat uterus. Inhibition of estrogen synthesis by aromatase inhibitor treatment was also used to confirm that estrogens have a role in regulating the expression of VEG/PF mRNA in DMBA-induced mammary tumors. A recent study reported that tamoxifen inhibited angiogenesis in the chick chorioallantoic membrane, although it was not entirely clear that this compound was acting by directly inhibiting estrogen action (39). In our studies, we used 4-OHA, a selective aromatase inhibitor that reduces circulating estrogen concentrations and causes significant regression of breast cancers in both the rat model (19, 25) and postmenopausal patients (40). The level of VEG/PF mRNA and protein in DMBA-induced mammary tumors from rats treated with 4-OHA was stimulated rapidly when E_2 was administered. Although we did not identify the VEG/PF isoforms, E_2 enhanced VEG/PF in animals with low E_2 levels due either to

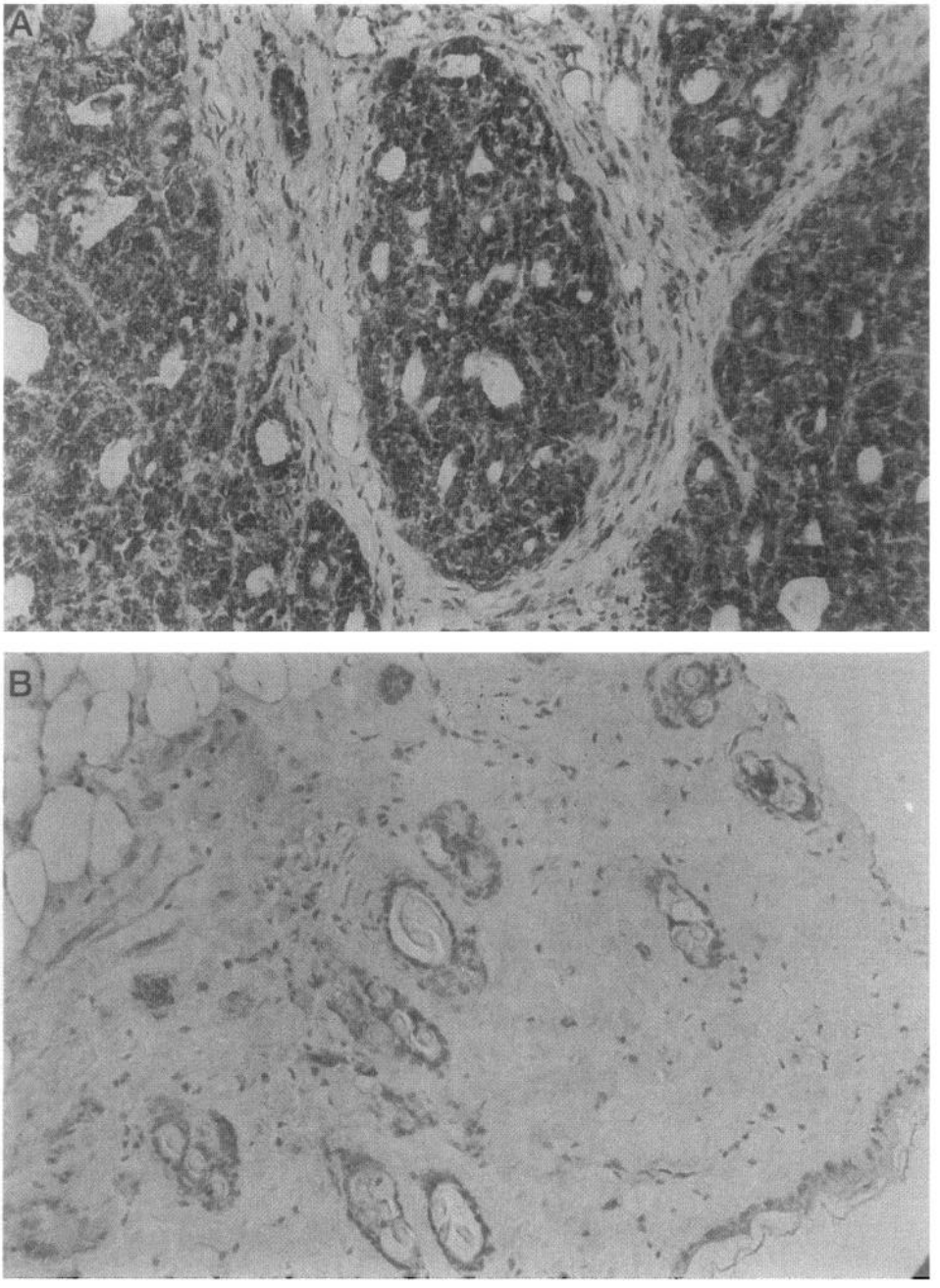

FIG. 9. A, Immunocytochemical staining of mammary tumors of rats with VEG/PF antibody. VEG/PF expression is seen in tumor nests and around blood vessels. B, Immunocytochemical staining of normal mammary tissue of rats with VEG/PF antibody. VEG/PF expression is seen around blood vessels.

ovariectomy or suppression by 4-OHA. However, the effect of 4-OHA is reversible, unlike ovariectomy. Thus, no change was observed in amount of PCR products of tumors removed from rats from 0 to 30 h after 4-OHA treatment, whereas E_2 levels remained suppressed. Subsequently, increased yields occurred 36 and 48 h after 4-OHA treatment when E_2 levels began to rise. Although VEG/PF protein did not follow the increase in mRNA in this case, a slight increase in protein levels was observed at 48 h. This suggests that, after a certain time delay, increases in VEG/PF protein expression (via VEG/PF mRNA) may subsequently occur. Repeated treatment with 4-OHA 24 h after the first injection prevented these increases in both mRNA and protein concentrations, further demonstrating that expression of VEG/PF could be regulated by the level of E_2. Measurements of the level of serum E_2 are consistent with these results. However, although the level of VEG/PF is stimulated by E_2, no decrease was apparent when the level of E_2 was reduced below normal in 4-OHA treated rats.

VEG/PF expression identified by immunohistochemistry was located in the lobular and ductal epithelium of DMBA-induced mammary tumors. Epithelial cells in other tissues have been shown to express VEG/PF (17). In our recent study, aromatase mRNA and protein were also localized in the lobular and ductal epithelium in human breast cancer (41). The results demonstrated that the cancer epithelial cells can catalyze significant amount of E_2. Furthermore, histocultures of tumor tissue provided evidence that tumor aro-

matase can convert testosterone to E_2 in amounts sufficient to produce a growth response in human breast cancer. This effect could be inhibited by 4-OHA. Taken together, our results suggest an autocrine mechanism, whereby E_2 produced by the tumor epithelial cell could directly stimulate the synthesis of VEG/PF in the same cells. Thus, one mechanism contributing to the effectiveness of antiestrogens and aromatase inhibitors in treating estrogen-dependent mammary cancer, could be their ability to limit expression of VEG/PF mRNA by inhibiting estrogen synthesis or action.

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