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Vascular Endothelial Growth Factor Expression Is Not Regulated by Estradiol or Medroxyprogesterone Acetate in Endometrial Carcinoma

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Objective: To determine whether the expression of vascular endothelial growth factor (VEGF) is altered by treatment of an *in vivo* tumor with 17β-estradiol (E2) or medroxyprogesterone acetate (MPA). Methods: A well-differentiated endometrial carcinoma tumor was isolated from a patient and explanted into the dorsal skin of ovariectomized nude mice, from which it was serially passaged *in vivo.* The explanted tumor retained all the properties of the original tumor, including estrogen and progesterone receptor expression and growth promotion and inhibition by E2 and MPA, respectively. The mice were treated with continuous E2 administration followed by treatment with either a single intramuscular administration of 2 mg MPA or weekly administrations of 2 mg MPA. Untreated tumor-bearing mice served as controls. The tumors were harvested at 0 to 21 days from first MPA administration. RNA from the tumors was isolated and VEGF expression was determined by Northern analysis. Results: VEGF was expressed in the absence of treatment with E2 or MPA, and expression was unaltered by continuous treatment with E2. Additional treatment with a single dose of MPA did not alter expression at Days 1, 2, 3, 7, 14, and 21, and additional treatment with weekly doses of MPA did not alter expression at Weeks 1, 2, and 3. Conclusions: VEGF is constitutively expressed in this *in vivo* model of endometrial carcinoma, and its expression is unaltered by treatment with E2 or E2 + MPA. Regulation of VEGF expression is not a mechanism by which these hormones exert their growth effects on endometrial tumors.

INTRODUCTION

It is well known that many endometrial carcinomas are hormonally responsive. Specifically, the growth of well-differentiated endometrial tumors is stimulated by estrogen in animal models and is often clinically inhibited by progestins [1, 2]. However, the mechanism by which these sex steroids exert their growth effects is unknown. In general, solid tumors are thought to be dependent on angiogenesis, or the formation of new blood vessels, for growth beyond a diameter of a few millimeters [3]. This raises the possibility that sex steroids exert their effects by inducing or repressing the process of angiogenesis in endometrial tumors.

To partially test this hypothesis, we focused our attention on vascular endothelial growth factor (VEGF), a recently described growth factor which appears to be specifically mitogenic for endothelial cells [4]. Recent evidence suggests that VEGF may be an important physiologic mediator of angiogenesis in normal endometrium and in other tissues [5]. One mechanism by which 17β-estradiol (E2) and medroxyprogesterone acetate (MPA) exert their growth-promoting and -inhibiting effects, respectively, on endometrial tumors may be by regulation of expression of VEGF. We used an established *in vivo* model for well-differentiated endometrial carcinoma to determine whether expression of VEGF is altered by either E2 or MPA.

METHODS

In vivo tumor model. Expression of VEGF as a function of sex steroid hormone exposure was studied in an *in vivo* model of human endometrial carcinoma. As previously described [1], a human endometrial carcinoma was obtained from hysterectomy within 15 min of excision. The tumor was immediately minced and divided for histologic examination, determination of estrogen receptor (ER) and progesterone receptor (PR) content, and transplantation. Xenotopic transplantation was accomplished by subcutaneous injection of 50–100 mg of tumor into the postthoracic region of ovariectomized 4- to 6-week-old Balb/c athymic nude mice (Harlan Sprague–Dawley, Indianapolis, IN). Once the tumors reached

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1–2 cm in geometric diameter, they were excised and serially passaged in athymic nude mice. The tumor used in this study was well-differentiated and expressed both ER and PR, and these characteristics remained unchanged with serial passage [1]. Maintenance of PR expression could be achieved by treatment with E2. Previous studies demonstrated that this tumor's growth was enhanced by E2 and inhibited by MPA [1, 6].

Tumor treatment with E2 and MPA. Tumor-bearing mice were treated with E2 using a subcutaneously implanted continuous-release pellet. These pellets consisted of a cholesterol/lactose carrier binder coated with E2 (Innovative Research of America, Rockville, MD) and resulted in steady-state blood concentrations of 200–300 pg/ml of E2 for at least 60 days. MPA (Depo-Provera, The Upjohn Co., Kalamazoo, MI) was administered as a single 2-mg intramuscular injection, a dose previously demonstrated to result in down-regulation of PR in this tumor, implying a significant biologic effect [7].

In the first experiment, a single mouse was injected with tumor cells from the same passage at several separate dorsal postthoracic sites. Tumors were harvested after treatment with E2 alone for 60 days and then after 24 and 48 hr of exposure to a single dose of MPA. Untreated tumor from the same passage was used as a control. In the second experiment, four mice were injected with tumor cells from the same passage at several separate dorsal postthoracic sites. The mice were treated with E2 for 60 days, at which time tumor was harvested from each mouse. The mice were then exposed to a single dose of MPA and tumor from one mouse was harvested after 3, 7, 14, and 21 days of exposure. In the third experiment, five mice were injected with tumor cells from the same passage and treated with E2 for 60 days. Three mice were then treated with weekly injections of saline and two mice were treated with weekly injections of MPA. Tumors were harvested after 1, 2, 3, and 4 weeks of exposure to saline and after 1, 2, and 3 weeks of exposure to MPA. The tumors in the third experiment were measured weekly with vernier calipers and the geometric diameters of the tumors were calculated.

All harvested tumors were immediately snap-frozen in liquid nitrogen and stored at −150°C until RNA extraction.

Northern analysis. Total RNA was isolated from tumors using the guanidium thiocyanate/cesium chloride method. Twenty micrograms per lane was electrophoresed in a 1% agarose–formaldehyde gel and transferred to a nylon membrane (Nytran Plus, Schleicher and Schuell). Prehybridization and hybridization were performed overnight in 50% formamide, 5× SSC, 5× Denhardt's solution, and 0.5% SDS at 42°C, the latter with $>1 \times 10^7$ cpm/ml of random primer-labeled DNA probe prepared from full-length cDNA of VEGF-165 (a kind gift of J. Abraham, Scios Nova, Mountain View, CA). Membranes were washed twice in 2× SSC/1% SDS at RT, then twice in 0.2× SSC/1% SDS at 55°C, and exposed to Kodak XAR film for 1–3 days with an intensifier at −70°C. Membranes were then stripped and rehybridized to random primer-labeled chicken β-actin cDNA.

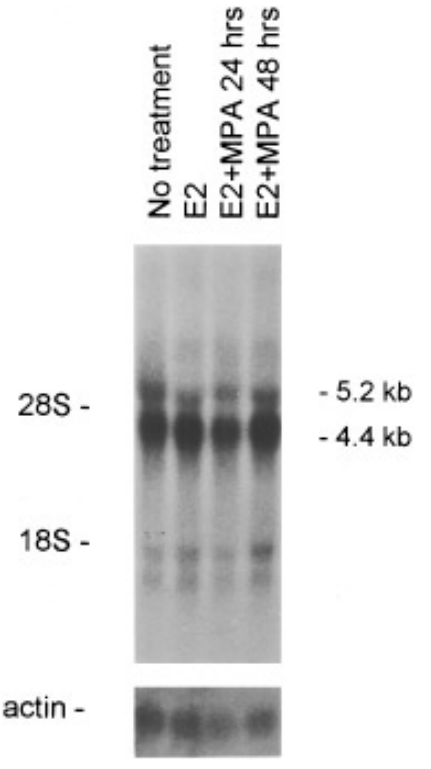

FIG. 1. Effect of sex steroid hormones on expression of VEGF in endometrial carcinoma.

RESULTS

In the first experiment (Fig. 1), VEGF was expressed in the absence of treatment with E2 or MPA, and expression was unaltered by continuous treatment with E2. Additional treatment with a single dose of MPA did not alter expression at 24 or 48 hr. In the second experiment (Fig. 2), VEGF expression was unaltered in E2-treated tumor after a single dose of MPA at Days 3, 7, 14, and 21. In the third experiment (Fig. 2), VEGF expression did not differ between E2-treated tumors after weekly injections with saline and MPA at 1, 2, and 3 weeks. As seen in previous experiments, MPA had a growth inhibitory effect on the tumors compared to saline (Fig. 3).

The major RNA species seen in all tumors were 4.4 and 5.2 kb. Other species were consistently identified at 1.6 and 1.8 kb. To correct for differences between lanes in total RNA loading, densitometry of the RNA signals of VEGF compared to actin was performed. This confirmed no appreciable effect of E2 or MPA on VEGF expression (Table 1).

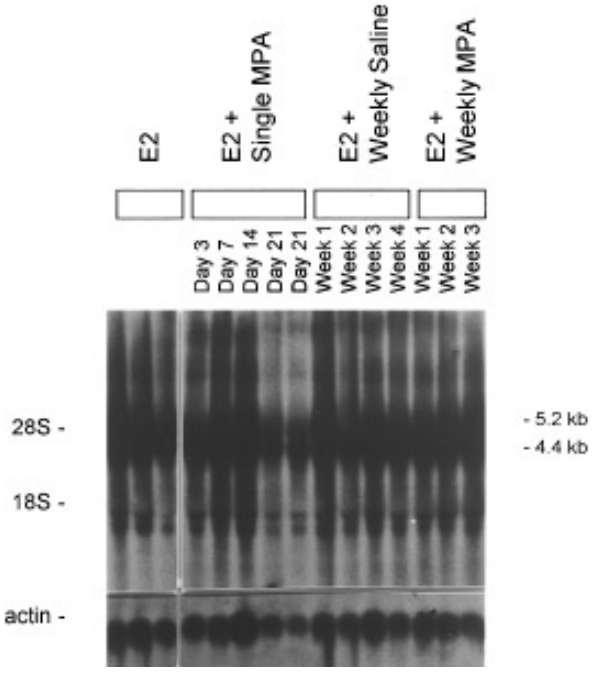

FIG. 2. VEGF expression in endometrial carcinoma after treatment with single or weekly doses of MPA.

TABLE 1
Densitometry Ratios for Standardization of VEGF Signals

Treatment	Standardized signal
Experiment 1	
No treatment	1.41
E2	1.40
E2 + MPA	
24 hr	1.79
48 hr	1.40
Experiment 2	
E2	2.49
E2	2.16
E2	2.11
E2 + MPA	
Day 3	2.15
Day 7	2.24
Day 14	1.52
Day 21	1.78
Day 21	3.22
Experiment 3	
E2 + saline	
Week 1	2.92
Week 2	2.52
Week 3	1.62
Week 4	2.28
E2 + MPA	
Week 1	1.86
Week 2	2.38
Week 3	2.82

DISCUSSION

This study demonstrates that VEGF is constitutively expressed in this *in vivo* model of human endometrial carcinoma and that its expression is unaltered by treatment with biologically significant doses of E2 or E2 + MPA. Therefore, it appears that regulation of VEGF expression is not a mechanism by which these hormones exert their growth effects on endometrial tumors. These results do not exclude the possibility that modulation of angiogenesis is an important effect of sex steroids. A number of other mediators of angiogenesis, including factors that inhibit angiogenesis, may be under the control of E2 or MPA. Given the obligate role of angiogenesis in the growth of other solid tumors, it is possible that sex steroids modulate angiogenesis in endometrial tumors by mechanisms unrelated to VEGF.

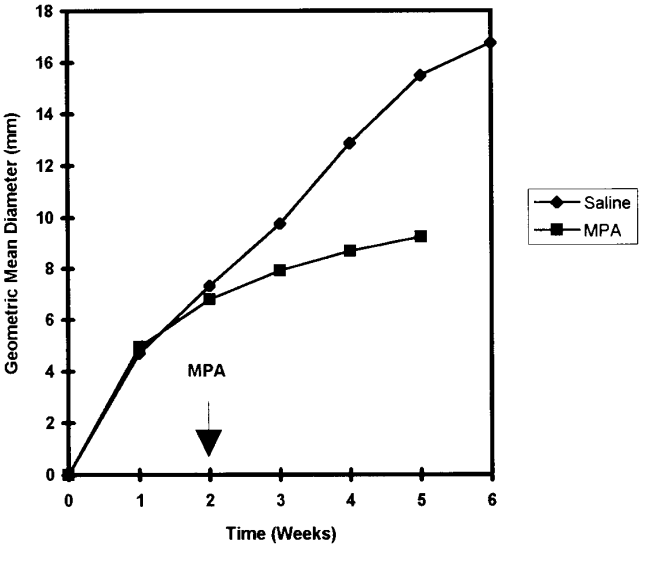

FIG. 3. Effect of saline vs MPA on tumor growth.

Tumor angiogenesis presents an attractive target for antitumor therapy. Angiogenesis is absolutely required for solid tumor growth beyond a diameter of a few millimeters [3]. Tumors appear to secrete growth factors that selectively recruit host endothelial cells to form new blood vessels from existing ones [8]. If the action of these growth factors could be inhibited, tumor growth theoretically could be arrested. Since all solid tumors are angiogenesis-dependent, the same therapy could potentially be effective for many different tumors. Furthermore, specific antiangiogenesis therapies would be expected to have few toxicities, since most normal adult tissues do not undergo angiogenesis as part of their normal physiology.

It was important to use a tumor model which stably ex-

pressed ER and PR. A model which lacks this expression would not be expected to be growth-responsive to E2 or MPA, and therefore no association could be made between VEGF expression and growth. It was also important to use an *in vivo* model, since cell culture models of angiogenesis may be misleading with respect to angiogenic factor expression. Most human endometrial carcinoma cell lines are derived from poorly differentiated tumors and do not express ER or PR. Even those cell lines that initially express ER and PR quickly lose this expression when passaged *in vitro* [9]. Furthermore, the behavior of cell lines in culture may not be reflective of that of cells in solid tumors, especially with regard to complex processes such as angiogenesis. The *in vivo* model used in this study does not have these limitations. This tumor stably expresses ER and PR, and its growth is enhanced by E2 and inhibited by MPA, mimicking the clinical behavior of human endometrial carcinomas. The *in vivo*, three-dimensional nature of this model is ideal for studying a morphogenetic process such as angiogenesis.

We chose to focus on VEGF for several reasons. Preliminary evidence suggested that VEGF is an important mediator of angiogenesis in both endometrium and endometrial tumors. Normal human endometrium and endometrial carcinoma cell lines express VEGF [10]. VEGF is a specific mitogen for endothelial cells, and high-affinity receptors for VEGF are expressed exclusively on endothelial cells [4]. Most significantly, a monoclonal antibody specific for VEGF inhibited the growth of several malignant cell lines in nude mice and resulted in decreased vascularity in these tumors, suggesting a critical role for VEGF in the growth of these tumors [11]. Despite the negative results of this study, the constitutive expression of VEGF in human endometrial carcinoma demonstrated in this study suggests the possibility that VEGF mediates angiogenesis in this tumor.

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