Natural and Synthetic Progestins Accelerate 7,12-Dimethylbenz[*a*] Anthracene-Initiated Mammary Tumors and Increase Angiogenesis in Sprague-Dawley Rats

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Abstract

Purpose: Synthetic progestins are widely used therapeutically; however, there is controversy regarding their proliferative effects. We used a rat 7,12-dimethylbenz[*a*]anthracene (DMBA)–induced mammary tumor model to test the hypothesis that progestins increase angiogenesis and as a result decrease the latency period and increase the multiplicity of mammary tumors.

Experimental Design: Medroxyprogesterone acetate (MPA) pellets were implanted 2, 4, or 6 weeks after DMBA exposure; RU-486 was given 3 days before MPA. Experiments were concluded 70 days after DMBA administration.

Results: MPA exposure 4 or 6 weeks after DMBA reduced the latency period for appearance of tumors in a dose-dependent manner and increased tumor incidence. Administration of MPA 2 weeks after DMBA administration reduced tumor incidence and was protective. Progesterone did not reduce the latency period but significantly increased tumor incidence. RU-486 delayed the latency period and decreased tumor incidence in animals exposed to MPA at 4 weeks after DMBA treatment, indicating that the progesterone receptor may be partially responsible for transmission of proliferative signals. RU-486 also delayed the latency period but failed to reduce overall tumor incidence when animals were exposed to MPA at 6 weeks after DMBA treatment, indicating that other factors may also control MPA-induced acceleration. Whereas MPA-accelerated tumors were both intraductal and tubular, progesterone-accelerated and/or DMBA-induced tumors were tubular. Progestin treatment increased vascular endothelial growth factor expression within tumors in a ligand- and cell type–dependent manner and increased angiogenesis in correlation with vascular endothelial growth factor expression. No mammary tumors or progesterone receptor were detected in DMBA-treated ovariectomized rats regardless of progestin administration.

Conclusions: We propose that progestins can accelerate the development of mammary tumors and that antiangiogenic agents and/or the use of antiprogestins that can reduce tumor incidence might be a viable therapeutic option for treatment of progestin-accelerated tumors. The model described here is a potentially useful preclinical model for rapidly screening such compounds.

Throughout their lives, women are exposed to circulating sex hormones, both natural and synthetic. These hormones are produced by the body during sexual development, ingested in the form of oral contraception, and administered to postmenopausal women (~40% of women in the United States) as hormone replacement therapy (HRT; refs. 1, 2). Ovarian steroids are essential for the normal growth and development of breast tissue; however, they are also associated with abnormal proliferation of cells, resulting in the development of mammary tumors (3). Whereas the role of estrogens in breast cancer has been elucidated through extensive study, the role of progestins remains controversial. Indeed, progestins have been shown to produce both stimulatory and antiproliferative effects in breast cancer cells (4–6).

Recently published data provide a strong link between the use of progestins in HRT and increased breast cancer risk in postmenopausal women (2, 7, 8). The fact that these tumors are detected within 5 years of receiving HRT has led us and others to propose that the progestin component causes progression of preneoplastic tissue or existing, but undetectable, tumors rather than the initiation of new carcinogenic events (4, 8).

Several studies have shown that an increasing angiogenic potential in human breast carcinomas, as assessed by tumor microvessel density measurements, correlates with tumor

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progression and metastasis and poor prognosis (9–11). Expression of vascular endothelial growth factor (VEGF), a multifunctional glycoprotein that is inducible by sex steroids, hypoxia, growth factors, and oncogenes, shows significant correlation with microvessel density (12, 13). Physiologically, VEGF is a critical factor in the developing vascular system and in pathologic angiogenesis, as it stimulates endothelial cell proliferation, migration, and vascular morphogenesis (14, 15). Recent studies have shown its pivotal role in the regulation of normal tissue and in the neovascularization crucial for the survival and growth of tumor cells (16).

Use of chemical carcinogen-based mammary tumor models has facilitated the characterization of compounds as either tumor initiators or promoters. Rat tumors formed in response to either *N*-methyl-*N*-nitrosourea or 7,12-dimethylbenz[*a*]anthracene (DMBA) are hormone responsive, expressing both estrogen receptor (ER) and progesterone receptor (PR; refs. 17, 18). In an earlier mouse study (19), medroxyprogesterone acetate (MPA) was shown to accelerate the development of mammary tumors, although other studies using similar models and the natural hormone progesterone indicated a protective role for progestins (20, 21). These studies involved the administration of MPA before DMBA treatment or following treatment, with discontinuation before tumor formation (19, 20).

Induction of mammary tumors in mice is a lengthy process; however, tumor formation in Sprague-Dawley rats occurs at relatively earlier time points following DMBA treatment. We used Sprague-Dawley rats to test our hypothesis that natural and synthetic progestins accelerate existing tumors, or preneoplastic lesions, to frank mammary tumors. Because we have shown previously that progestin-induced VEGF from human breast cancer cells can stimulate proliferation of endothelial and tumor epithelial cells (4), we also studied alterations in VEGF expression and its association with angiogenesis in this experimental system by analyzing microvessel density in mammary glands following progestin treatment. Here, we report that in the DMBA-induced tumor model in rats progestins do indeed accelerate the development of mammary tumors and that this process is likely brought about by increased VEGF production and angiogenesis.

Materials and Methods

Animals. Unless noted otherwise, 10 animals were used in each experimental group, and each experiment was repeated at least twice. Intact or ovariectomized virgin female Sprague-Dawley rats (Harlan Breeders, Indianapolis, IN), 40 to 45 days old, were housed according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care under conditions of 12-hour light/dark cycles and *ad libitum* access to food and water. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri (Columbia, MO) and were in accordance with procedures outlined in the "Guide for Care and Use of Laboratory Animals" (NIH publication 85-23).

Hormones and antihormones. All hormone and antihormone pellets used were 60-day release formulations purchased from Innovative Research (Sarasota, FL). Animals were anesthetized and MPA, progesterone, RU-486, or placebo pellets were implanted s.c. in the dorsal area.

Tumor induction and hormonal manipulations. The experimental protocol is summarized in Fig. 1. At ages 45 to 50 days, rats were given a single dose of 20 mg/rat of DMBA (Sigma, St. Louis, MO) in peanut oil by gavage (ref. 21; Fig. 1). Control animals received only peanut oil. Two, 4, and 6 weeks later, animals were implanted s.c. with 25 mg MPA pellets to study MPA-induced progression of tumors. For an analysis of dose effects, 0.5, 5, 10, or 25 mg MPA pellets were implanted 4 weeks after DMBA intubations. When used, RU-486 pellets (25 mg) were implanted 3 days before insertion of MPA pellets. Progesterone (10 mg) pellets were implanted 4 weeks after DMBA treatment. Animals were palpated for tumor incidence and tumor size every 2 days following insertion of pellets or starting 24 days after DMBA treatment, whichever came first. The time preceding the appearance of the first tumor (latency period) and the relative size and location of each were recorded. Tumor incidence (number of rats with tumors) and tumor multiplicity (number of tumors/rat) were calculated at the end of the study. Tumor length (L) and width (W) were measured with a micrometer caliper, and tumor size was calculated using the formula: $L / 2 \times W / 2 \times \pi$ (22). Animals were sacrificed after 5-week exposure to DMBA (1 week before placing MPA pellet) or at 8, 10, and 12 weeks after DMBA treatment (2, 4, and 6 weeks after MPA).

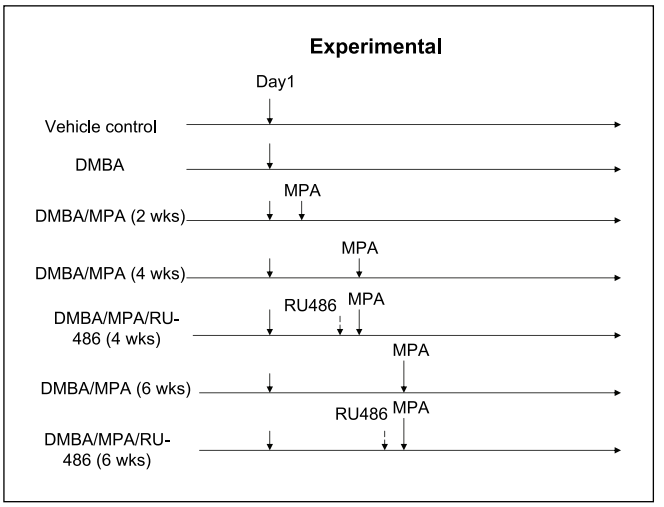

Fig. 1. Experimental protocol. Animals were treated with DMBA by oral gavage (20 mg/kg/rat) at ages 45 to 50 days. MPA pellets (25 mg/60-day release) were introduced at 2, 4, or 6 weeks after DMBA treatment. RU-486 (25 mg/60-day release) was given 3 days before the introduction of MPA pellets. Samples were collected 5, 8, 10, or 12 weeks after DMBA.

Histology and immunohistochemical analysis. The effect of progestins on hormone receptors and angiogenesis was assessed by determining expression levels of the ER, PR, VEGF, and factor VIII using immunohistochemistry. Both axillary and abdominal glands were used for all experiments.

Tissues were fixed overnight in 10% neutral buffered formalin for routine microscopy and 4% paraformaldehyde for immunohistochemistry. Tissues were routinely processed for paraffin infiltration and embedding. Sections (5 μm) were mounted onto ProbeOn Plus microscope slides (Fisher Scientific, Inc., Pittsburgh, PA). Light microscopic examination of serial H&E-stained sections representative of a given tumor was done for classification using previously published criteria (23). Before immunohistochemistry, sections were dewaxed in xylene, rehydrated through graded concentrations of ethanol, then rinsed in distilled water, and, if necessary, stored in PBS at 4°C until use. Sections were subjected to heat-induced epitope retrieval method in 10 mmol/L citrate buffer (pH 6.0) for PR, ER-α, and VEGF immunolabeling and to proteinase K pretreatment [20 μg/mL in TE buffer (pH 8.0)] for the factor VIII immunoassays. Slides were treated with 3% H_2O_2 in absolute methanol (to inactivate endogenous peroxidase activity) before being washed in 3× PBS and then immersed in 10% bovine serum albumin for 20 minutes. Sections were incubated

for 60 minutes at room temperature with each of the following polyclonal antibodies: anti-PR antibody [1:600 dilution of a rabbit anti-human PR polyclonal antibody (A0098) that reacts with the DNA-binding domain (amino acids 533-547); DAKO, Carpinteria, CA], anti-ER-α [1:300 dilution of a rabbit anti-ER-α polyclonal antibody (sc-542) raised against an ER-α peptide of mouse origin; Santa Cruz Biotechnology, Inc., Santa Cruz, CA], anti-VEGF antibody [1:200 dilution of a rabbit anti-VEGF polyclonal antibody (sc-152); Santa Cruz Biotechnology], and anti–factor VIII antibody [1:800 dilution of a rabbit anti–factor VIII polyclonal antibody (A0082); DAKO]. Sections were then washed and sequentially incubated with a secondary antibody [biotinylated swine anti-rabbit IgG (DAKO) and a streptavidin-linked horseradish peroxidase product (BD PharMingen, San Diego, CA)] for 30 minutes at room temperature. Alternatively, some sections were incubated with EnVision+, a horseradish peroxidase–labeled polymer conjugated with anti-rabbit antibodies (DAKO). Bound antibodies were visualized following incubation with 3,3′-diaminobenzidine (0.05% with 0.015% H_2O_2 in PBS) solution (Zymed Corp., San Francisco, CA) for 3 to 5 minutes. Sections were counterstained with Meyer's hematoxylin, dehydrated, cleared, and coverslipped for microscopic examination.

Statistical analysis. Data summaries are presented as mean ± SE. Control and experimental groups were compared with respect to body weight, multiplicity, tumor latency, tumor incidence and cumulative tumor size. At the end of the experiments, latency period differences, tumor multiplicity and microvessel density were compared using unpaired t tests. Final tumor incidence was compared using χ^2 test; $P < 0.05$ were regarded as statistically significant.

Results

General observations. Average body weights of animals in the different treatment groups were similar, and untreated controls remained tumor-free throughout the course of this study. All animals in the study, irrespective of treatment, had a survival of 100% and final body weight ranged between 232 and 250 g.

Acceleration of mammary tumors by MPA is critically dependent on timing of exposure after DMBA treatment. Because there are reports indicating that MPA can protect against DMBA-induced mammary tumors (20, 21), we sought to ascertain the effects of timing of exposure on progression to frank tumors. MPA pellets (25 mg) or placebo pellets were placed 2, 4, and 6 weeks after DMBA treatment (protocol described in Fig. 1). For the 6 weeks after DMBA treatment group, the latency period was 47 ± 1 days, which was significantly shorter than the latency period of 53 ± 1 days in animals treated with DMBA alone in which the placebo pellets were placed ($P < 0.01$, unpaired t test; Fig. 2A). The tumor incidence reached 86 ± 3% in the MPA-treated group at 6 weeks compared with 49 ± 5% in placebo ($P = 0.001$, χ^2 analysis; Fig. 2A). When MPA pellets were implanted 4 weeks after DMBA treatment, tumor latency decreased further to 37 ± 1 days ($P < 0.01$ versus placebos, unpaired t test) and tumor incidence reached 77 ± 3% ($P = 0.02$, χ^2 analysis). These data are summarized in Table 1.

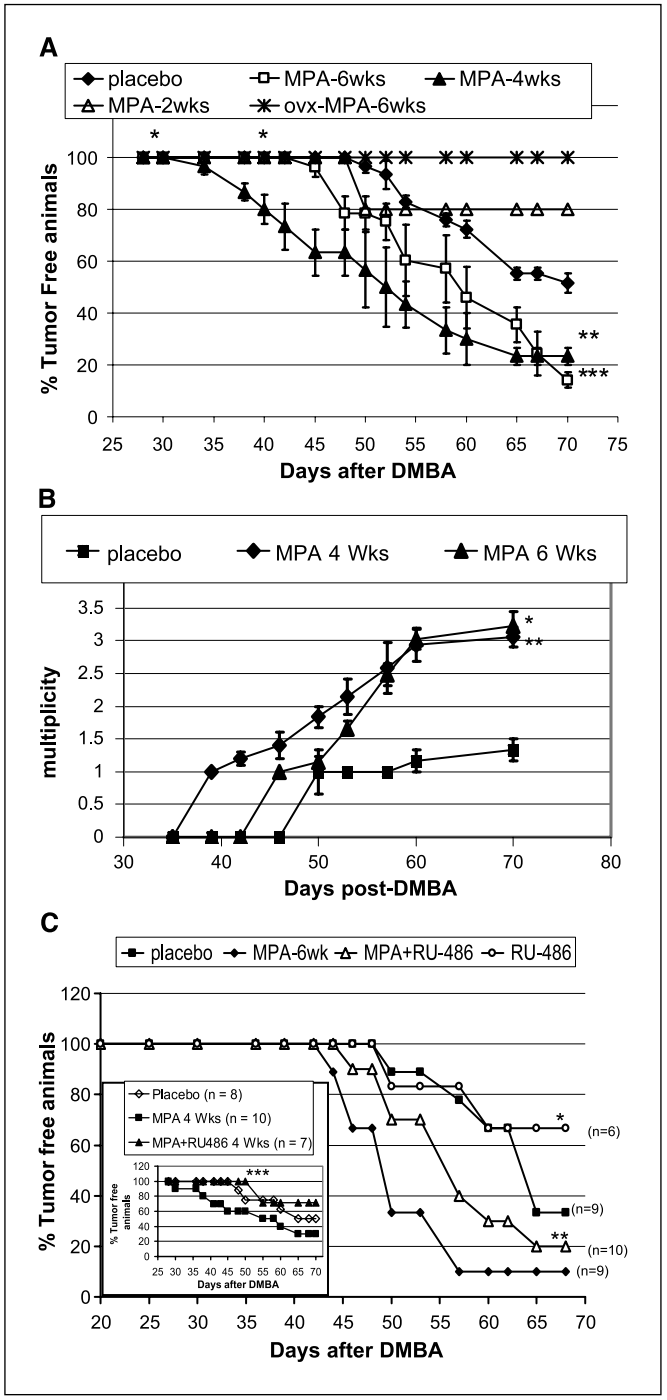

Fig. 2. *A,* MPA accelerates DMBA-induced tumors: MPA pellets were implanted 2, 4, and 6 weeks after DMBA treatment. MPA pellet implantation at 6 weeks after DMBA treatment decreased the latency period (*, $P < 0.01$, unpaired t test) and increased tumor incidence (***, $P = 0.001$, χ^2 analysis) compared with DMBA-treated rats implanted with placebo. The latency period for MPA treatment 4 weeks after DMBA further decreased and was significantly different from placebos and MPA administration 6 weeks after DMBA treatment (*, $P = 0.01$, unpaired t test). Tumor incidence at the end of the study was also significantly different from placebos (**, $P = 0.02$, χ^2 analysis). The tumor latency period for MPA implantation 2 weeks after DMBA was 50 days and tumor incidence reached 20%. *B,* implantation of MPA at 4 and 6 weeks after DMBA increased multiplicity of tumors compared with placebos. Tumor multiplicity values were greater for 6- and 4-week MPA treatment compared with placebos (*, $P = 0.003$; **, $P = 0.04$, respectively, unpaired t test). *C,* RU-486 delays MPA-accelerated tumors and reduces tumor incidence. RU-486 treatment at 6 weeks after DMBA did not reduce the tumor latency period significantly; however, tumor incidence was reduced in the initial phase of the experiment and the final incidence for the MPA and MPA + RU-486 treatment group was similar and significantly different from placebo controls (**, $P < 0.001$, χ^2 analysis). RU-486 alone reduced the incidence of DMBA-induced tumors significantly compared with placebo (*, $P < 0.001$, χ^2 analysis). *Inset,* when MPA was placed 4 weeks after DMBA treatment following RU-486 placement 3 days before, the progesterone antagonist significantly delayed the appearance of tumors ($P < 0.05$, unpaired t test) and also reduced tumor incidence at the end of the experiment. *Points,* mean of three different experiments; *bars,* SE.

Table 1. Growth characteristics of MPA- and progesterone-treated DMBA-induced tumors

Group (wk of treatment)	Mean latency period (d)	Tumor incidence at 8 wk (%)	Final tumor incidence at 10 wk (%)	Multiplicity* at 8 wk	Multiplicity at 10 wk
No treatment	—	—	—	—	—
DMBA control	53 ± 1	11 ± 3	49 ± 5	1.0 ± 0	1.3 ± 0.01
DMBA + MPA (4 wk)	37 ± 1†	57 ± 9‡	77 ± 3.3	2.5 ± 0.3	3.0 ± 0.1†
DMBA + MPA (6 wk)	47 ± 1†	43 ± 13†	86 ± 2.9	2.5 ± 0.1	3.2 ± 0.2†

*Number of tumors/tumor-bearing rat.
†$P < 0.05$, compared with DMBA controls (unpaired t test).
‡$P < 0.05$, compared with DMBA controls (χ^2 analysis).

In contrast to the results discussed above, placement of the MPA pellet at 2 weeks after DMBA treatment did not decrease the latency period (Fig. 2A) and in fact provided partial protection (tumor incidence, 20%) at the end of the study (Fig. 2A). Ovariectomized animals did not develop tumors during the study (Fig. 2A).

As shown in Fig. 2B, MPA also increased tumor multiplicity to 3.0 ± 0.1 ($P = 0.003$, unpaired t test) at 4 weeks of treatment and 3.2 ± 0.2 ($P = 0.04$, unpaired t test) at 6 weeks of treatment compared with 1.3 ± 0.2 in placebo controls.

RU-486 treatment delays MPA-accelerated DMBA-induced tumors. To determine whether the acceleration of progestin-dependent DMBA-induced tumors is dependent on the PR, the antiprogestin RU-486 (25 mg pellets/60-day release) was implanted 3 days before administration of MPA (25 mg) 6 weeks after DMBA treatment. RU-486 did not delay the MPA-accelerated latency period or affect overall tumor incidence significantly; however, it did result in a lower tumor incidence in the early stages of the study (Fig. 2C). At 56 days after DMBA, tumor incidence was lower in the MPA plus RU-486 group (60%) than in the group receiving MPA alone (90%; $P < 0.001$, χ^2 analysis). Tumor multiplicity was also lower (1.3) for the MPA plus RU-486 group than the MPA group alone (2.2; $P < 0.05$, unpaired t test; Table 1). Furthermore, RU-486 alone did not influence the latency period (compared with placebo) and in fact partially blocked DMBA induction (tumor incidence, 33% versus 67% for placebo group; $P < 0.001$, χ^2 analysis). There were no significant differences in tumor multiplicity between RU-486 control and placebo-treated groups. These results indicated that although PR may be partially involved in mediating the MPA effects other factors come into play for increasing tumor progression and overall incidence at this stage.

We next tested the effect of RU-486 to block MPA-induced acceleration of DMBA-induced tumors in the 4-week MPA treatment group. As shown in Fig. 2C (*inset*), RU-486 dramatically delayed the appearance of MPA-accelerated tumors, indicating that PR is likely involved in the progression of DMBA-induced mammary tumors. Treatment of animals with RU-486 alone led to protection of tumor formation in DMBA-treated animals as observed when the DMBA-induced tumors were exposed to RU-486 at 6 weeks. Interestingly, as with the MPA 6-week treatment result, the final incidence of tumor in the MPA- and MPA plus RU-486–treated rats at 4 weeks after DMBA-induced tumors remained low. Thus, the effect of RU-486 in delaying the MPA-driven tumor is stage specific, being pronounced when placed before MPA at 4 weeks after DMBA stage but not negating MPA effects so efficiently when placed before MPA at 6 weeks after DMBA treatment.

MPA accelerates DMBA-induced mammary tumors in a dose-dependent manner. Sixty-day release MPA pellets (5, 10, or 25 mg) were implanted in rats 4 weeks after DMBA treatment. As shown in Fig. 3, these doses reduced the latency period by ~15 days and also increased tumor incidence compared with placebo-treated group. The latency period decreased from ~53 to 37 days ($P < 0.01$, unpaired t test) with a tumor incidence of 60% to 80% on day 70, which is the same as that obtained with 25 mg MPA pellets (Fig. 2A). Although all doses led to significant increase in tumor multiplicity and tumor size compared with controls, there was no statistical difference in these variables at the end point when the various doses were compared with each other (data not shown). Interestingly, as shown in Fig. 3, a lower dose of MPA (0.5 mg/60-day release) implanted at 4 weeks after DMBA also accelerated tumor development ($P < 0.05$, compared with controls, unpaired t test), resulting in a significant increase in tumor incidence of 80% at the termination of this experiment compared with 30% in the placebo group ($P = 0.005$, χ^2 analysis; Fig. 3).

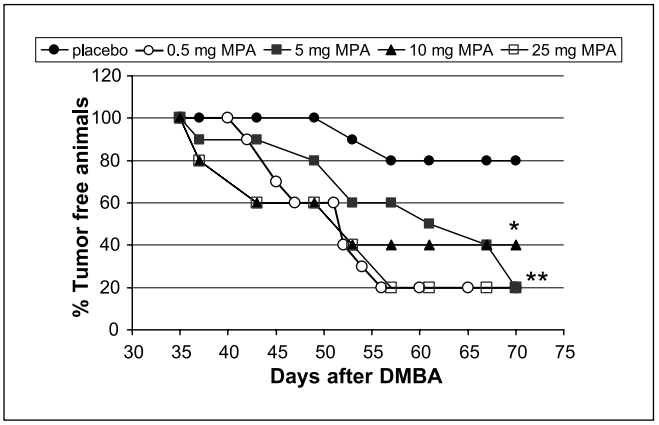

Fig. 3. Dose-dependent response curves. MPA pellets (5, 10, and 25 mg) were implanted 4 weeks after DMBA. Latency periods were reduced to 37 days in 5 and 10 mg MPA treatment (compared with placebo, 53 days). MPA (10 mg) treatment curve was compared with placebos and was significantly different (*, $P = 0.010$, log-rank test). Tumor incidence was 60% to 80% for MPA groups and 20% for placebo group (**, $P < 0.005$, unpaired t test). With 0.5 mg MPA pellets implanted 4 weeks after DMBA, the latency period was 42 days, which was still significantly different from placebo ($P < 0.05$, unpaired t test).

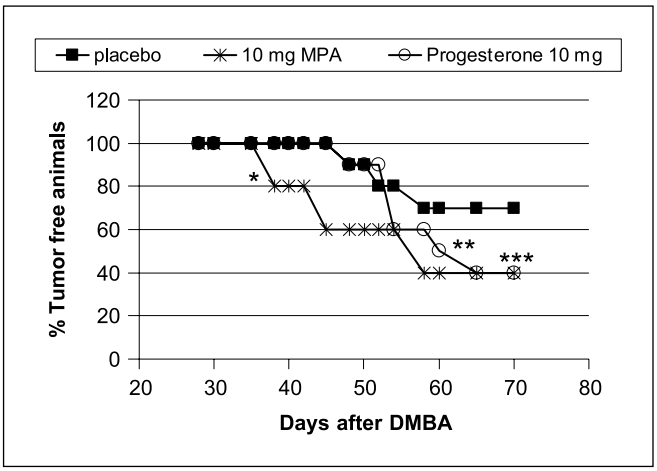

Fig. 4. Progesterone treatment at 4 weeks after DMBA treatment increases incidence of DMBA-induced mammary tumors but does not affect the latency period. Progesterone treatment increased incidence of tumors compared with controls (**, $P < 0.01$, χ^2 analysis). MPA treatment significantly reduced the latency period (*, $P < 0.01$, t test) and increased tumor incidence (***, $P = 0.002$, χ^2 analysis) compared with controls.

Progesterone treatment accelerates development of mammary tumors but does not influence latency period in DMBA-treated animals. We implanted progesterone pellets (10 mg/60-day release) into rats 4 weeks after DMBA treatment to study the effect of the natural hormone on development of mammary tumors. For comparison, we implanted a 10 mg/60-day release pellet of MPA into a second set of animals that we previously showed causes reduction in tumor latency (in Fig. 3). As expected, the latency period was reduced in animals that received 10 mg MPA pellets (compared with placebo, 38 versus 48 days; $P < 0.01$, unpaired t test) and tumor incidence reached 60% versus 30% in the placebo group ($P = 0.002$, χ^2 analysis; Fig. 4). In contrast, 10 mg progesterone pellets implanted 4 weeks after DMBA did not change the latency period compared with placebo, but the tumor incidence remained high (60% compared with 30% in placebo control group; $P < 0.01$, χ^2 analysis).

Tumor morphology following progestin treatment of DMBA-treated rats. Palpable and nonpalpable mammary tumors (found during dissection) were excised either 5 weeks or 8, 10, or 12 weeks after DMBA treatment. In the treatment (non-control) groups, 25 mg MPA or 10 mg progesterone pellets had been placed 6 weeks after DMBA treatment. All tumors were adenocarcinomas. Further histopathologic evaluation revealed that of the 9 tumors excised from the control group, 7 were tubular and 2 intraductal. Progesterone-accelerated tumors were predominantly tubular (Table 2). Of the 15 MPA-accelerated tumors, 8 were classified as intraductal hyperplasia and the remaining were tubular (Table 2). Thus, control (DMBA alone) tumors comprised mainly tubular forms, lesions that were likely the targets of endogenous progesterone. In contrast, the intraductal tumors were the ones that had been more sensitive to MPA. These data indicate that the mechanisms of action of MPA and progesterone are distinct as reported previously (24, 25).

Hormone receptor distribution. We determined the distribution of ER-α and PR in control and MPA- or progesterone-accelerated tumors (Table 3) by immunohistochemistry at 5 and 8 weeks after DMBA. MPA pellets were placed at 6 weeks after DMBA.

As shown in Table 3A as well as in Fig. 5A, intact animals implanted with placebo expressed ER-α in 8.3 ± 0.8% of ductal cells in the mammary gland at 5 weeks after placebo implantation. Five weeks after DMBA treatment, 34 ± 6% cells expressed ER-α. In contrast, DMBA totally suppressed the ER-α expression that was observed in 35 ± 8% of acinar cells in placebo controls. In ovariectomized animals, 52 ± 3% of the ductal cells exhibited ER-α. Following DMBA treatment, there seemed to be a greater proportion of ductal cells (70%) that expressed ER-α. Acinar cells in the untreated mammary glands of ovariectomized animals also expressed ER-α (28 + 5.8%); however, DMBA exposure resulted in complete loss of ER-α from the acinar cells. PR was also expressed in 16% of ductal cells and 23% of acinar cells in the intact placebo-treated animals (Table 3A). However, following DMBA treatment, PR increased in the ductal compartment of intact animals and totally subsided in acinar cells. In ovariectomized animals, PR could only be detected in the acinar cells that were lost following DMBA treatment (Table 3A). Thus, although DMBA treatment generally increased levels of ER- and PR-expressing ductal cells (except for PR in ovariectomized animals), it led to down-regulation of ER and PR in acinar cells.

At 8 weeks after DMBA treatment, cross-sections of mammary gland exhibited ductal and acinar as well as tumor cells. We studied the distribution of ER-α and PR in these compartments. In contrast to the 5-week study, there was no difference in the ductal compartments in terms of ER and PR expression in intact placebo- or DMBA-treated animals (Table 3B; Fig. 5B). When DMBA-treated animals were exposed to MPA or progesterone, both hormones significantly reduced the number of cells expressing ER-α in the ductal cells ($P = 0.02$, Student's t test). ER-β levels did not change following MPA treatment (data not shown). MPA but not progesterone led to a significant decline

Table 2. Total number of intraductal and tubular carcinomas following treatment with MPA and progesterone implanted at 6 weeks after DMBA treatment

Treatment groups	Total tumors examined	Intraductal hyperplasia, *n* (%)	Well and poorly differentiated tubular and papillary adenocarcinoma, *n* (%)
No treatment	—	—	—
DMBA control	9	2 (22.2)	7 (77.8)
DMBA + MPA	15	8 (53.3)	7 (46.7)
DMBA + progesterone	9	1 (11.1)	8 (88.9)

Table 3. Distribution of hormone receptors

(A) 5 wk after DMBA (before hormone pellets)

		Ductal (%)	Acinar/acinar hyperplasia (%)
ER-α	Intact	8.3 ± 0.8	35 ± 7.6
	DMBA	34 ± 6.0*	0
	Ovariectomized	52 ± 2.6†	28 ± 5.8
	Ovariectomized DMBA	70 ± 5.0†	0
PR	Intact	16.6 ± 3.3	23.3 ± 3.3
	DMBA	56.6 ± 6.6*	0
	Ovariectomized	0.6 ± 0.6†	43.3 ± 8.3
	Ovariectomized DMBA	0	0

B. 8 wk after DMBA (2 wk after hormone pellets)

		Ductal (%)	Acinar/acinar hyperplasia (%)	Carcinoma (%)
ER-α	Intact	28.3 ± 1.6	25 ± 1.4	—
	DMBA	26.6 ± 3.3	22 ± 3.0	63.7 ± 4.7
	DMBA + MPA	16.6 ± 3.3‡	23.3 ± 3.3	85 ± 3.8§
	DMBA + progesterone	13.3 ± 3.3†	10 ± 0.0†	45 ± 12.1
PR	Intact	30 ± 2.0	3 ± 1.0	—
	DMBA	42.2 ± 8.1	17.5 ± 2.5‖	70.8 ± 7.7
	DMBA + MPA	12.1 ± 2.1†	12.5 ± 2.5‖	70 ± 20
	DMBA + progesterone	26 ± 1.8	23.3 ± 3.3‖	60 ± 11.4

*$P < 0.05$, compared with intact animals (unpaired t test).
†$P < 0.0009$, compared with intact animals; $P < 0.01$, compared with ovariectomized DMBA animals (unpaired t test).
‡$P < 0.05$, compared with intact animals and DMBA-treated animals (unpaired t test).
§$P = 0.01$, compared with DMBA controls.
‖$P < 0.05$, compared with intact animals (unpaired t test).

in PR content in ductal cells. About 22% to 25% of the acinar cells exhibited ER-α in the mammary glands of placebo- or DMBA-treated animals at this time. Progesterone but not MPA treatment led to a decrease in the ER-α content in acinar cells (Table 3B). In contrast, there were a few cells that exhibited PR in acinar cells at 8-week placebo controls. DMBA treatment with or without MPA or progesterone led to a marked increase in the number of acinar cells expressing PR; however, progesterone treatment led to maximum increase in PR-positive acinar cells (Table 3B). Both ER-α and PR were expressed in majority of the carcinoma cells. MPA but not progesterone increased the number of ER-α-expressing cells, whereas neither hormone influenced the number of tumor cells that stained positive for PR. In ovariectomized animals, there was no change in PR staining in the presence or absence of DMBA at 8 weeks (data not shown).

MPA and progesterone differentially increase VEGF expression in stromal and basal epithelial cells and increase angiogenesis. VEGF protein levels were assessed by immunohistochemistry 8 and 10 weeks after DMBA (following introduction of MPA or progesterone pellets 6 weeks after DMBA). At 8 weeks, many MPA-treated animals bear tumors and the VEGF expression level was elevated in the MPA-driven tumors compared with controls. VEGF was also substantially elevated in the preneoplastic lesions in MPA-treated mammary glands (data not shown) and a slight increase was also apparent at this time in the mammary glands treated with progesterone (Fig. 6A, *top*). Most of the VEGF staining in the MPA-driven tumors was present in both epithelial and stromal cells. These also contained a higher number of blood vessels, correlating with increased VEGF expression (Fig. 6A, *bottom*). At 10 weeks after DMBA, although VEGF labeling was present in both MPA- and progesterone-driven tumors (in epithelial and basal cells), the intensity was higher in the progesterone-driven tumors (Fig. 6B, *top*). Interestingly, basal cells in progesterone-accelerated tumors expressed higher levels of VEGF than did other epithelial cells (Fig. 6A). At 10 weeks, stromal cells in the MPA group expressed VEGF. In contrast to MPA-treated groups, lower levels of VEGF expression were found in the stroma of the progesterone-treated group at all times. In the MPA group, VEGF was uniformly present compared with tumors from the placebo group. Again, associated with changes in VEGF was an increase in the number of blood vessels as detected by factor VIII staining (Fig. 6B, *bottom*). These differences in control of VEGF production in mammary cells indicate that the two progestins studied have distinct modes of action and that both increase angiogenesis in tumor tissues.

To determine whether increased VEGF production in response to progestin treatment-induced angiogenesis in DMBA-induced mammary tumors in rats, we quantified microvessel density by evaluating eight randomly chosen fields that were stained for factor VIII. As shown in Fig. 6C, blood vessel density was higher in the MPA-driven tumors compared with either the control (DMBA alone) group or progesterone-treated group 8 weeks after DMBA. At 10 weeks after DMBA, both MPA and progesterone increased blood vessel density

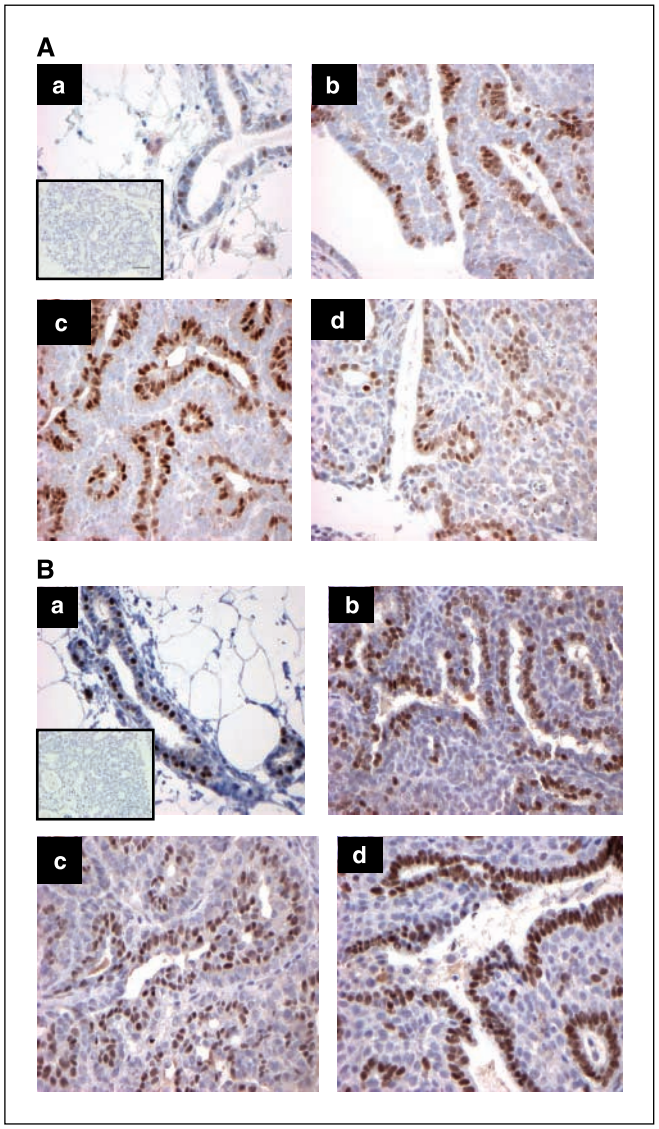

Fig. 5. *A,* ER-α expression in vehicle-treated mammary tissue (*a*) and in DMBA-induced mammary tumors (*b-d*). Mammary tissue and tumors were collected 8 weeks after DMBA (i.e., 2 weeks after implantation of MPA, progesterone, or placebo pellet). *a,* vehicle control; *b,* placebo-treated animals; *c,* MPA-treated animals; *d,* progesterone-treated animals. *B,* PR expression. *a,* vehicle control; *b,* placebo-treated animals; *c,* MPA-treated animals; *d,* progesterone-treated animals. *Inset,* negative controls without primary antibody. Magnification, ×64.

compared with DMBA treatment alone. There was a sharp increase in the number of blood vessels seen at 12 weeks after DMBA in both MPA- and progesterone-driven tumors. This correlated with the time course of VEGF expression in the tissues (Fig. 6A and B).

Discussion

Several studies have shown that estrogen/progestin combination therapy, but not estrogen alone, increases the risk of mammary tumors in women (2, 7, 8). The Women's Health Initiative trial was halted earlier than scheduled due to development of mammary tumors in a significant number of women who were consuming the estrogen/progestin regimen (26). The progestin component in many regimens is MPA, and we hypothesized that progestins increase angiogenesis and as a result decrease the latency period and increase the multiplicity of mammary tumors. The basis for our hypothesis is that the period for detection of tumors following HRT (<5 years) was too restricted for the development or initiation of new tumors. Here, we report that synthetic and natural progestins are capable of accelerating the progression of DMBA-induced mammary tumors in Sprague-Dawley rats. We concentrated on the role of MPA in this process because it is consumed widely for therapeutics as well as for HRT in postmenopausal women.

In our studies using the DMBA-induced mammary carcinoma model, MPA significantly reduced the latency period, tumor progression being dependent on the timing and dose of MPA treatment. When given 2 weeks after DMBA, MPA provided protection against development of tumors, whereas placement of an identical MPA pellet at either 4 or 6 weeks after DMBA led to an earlier appearance of tumors compared with controls. Although we are unable to explain this stage-specific effect of MPA, we speculate that accumulation (or loss) of a critical factor in the mammary gland may be required to interact with MPA-induced effects providing an angiogenic switch for tumor tissues to expand. The reduction in the latency period was observed with 25, 10, and 5 mg MPA pellets. Use of 0.5 mg MPA pellets, considered a therapeutic dose (27), also increased the incidence of DMBA-induced tumors but did not influence the latency period compared with placebo. However, tumor incidence and multiplicity were significantly different from controls in these animals, and tumors were much larger than those in controls. Furthermore, MPA-driven tumors were mostly of the ductal type, whereas the morphology of tumors in controls resembled that of tubular carcinoma, indicating that MPA may be involved in progression of the disease independent of the pathologic status.

Previously, the natural progestin, progesterone, has been shown to reduce the latency period in Sprague-Dawley rats (28, 29). In addition, MPA has been shown to accelerate DMBA-induced mammary tumors in mice (19). However, the latter study involved a protocol in which the DMBA-treated rats were exposed for a short period to MPA rather than to the continuously released form used in the present study. It takes a considerable amount of time to study the effect of progestins on mammary tumor growth in mice; this period was substantially reduced in the model used in this report, which should facilitate the identification of test compounds that may prove useful for clinical studies in the future. Additionally, unlike tumors in mice, mammary tumors in rats retain expression of ER and PR, which are essential for the proliferation of many human breast cancers (30).

Several studies show that progestins can increase the proliferation of breast tumor cells (4, 31). Although estrogens are the primary force driving proliferation in most instances, recognition of the role of progestins in this process is increasing (32, 33). Although DMBA-induced mammary tumors in rats have been studied for over 25 years, to our knowledge, the tumor promoter activity of MPA has not been analyzed previously with respect to dose, timing, developmental stage, and its influence on angiogenesis. Most others have focused on the protective effect of MPA when given earlier than, or soon after, DMBA (19–21). Aldaz et al. (19) used combined MPA and DMBA treatment to obtain mammary tumors, and Russo

et al. (27) concluded that MPA did not increase the risk of carcinoma when given to virgin rats at the clinical dose used for contraception (0.5 mg), but a 10-fold dose increase resulted in a higher tumorigenic response. The latter study involved implantation of low-dose MPA pellets for 21 days, after which pellets were removed before treatment with DMBA. Our experiments involved MPA treatment after DMBA exposure, when carcinogenic events had already taken place. Our data

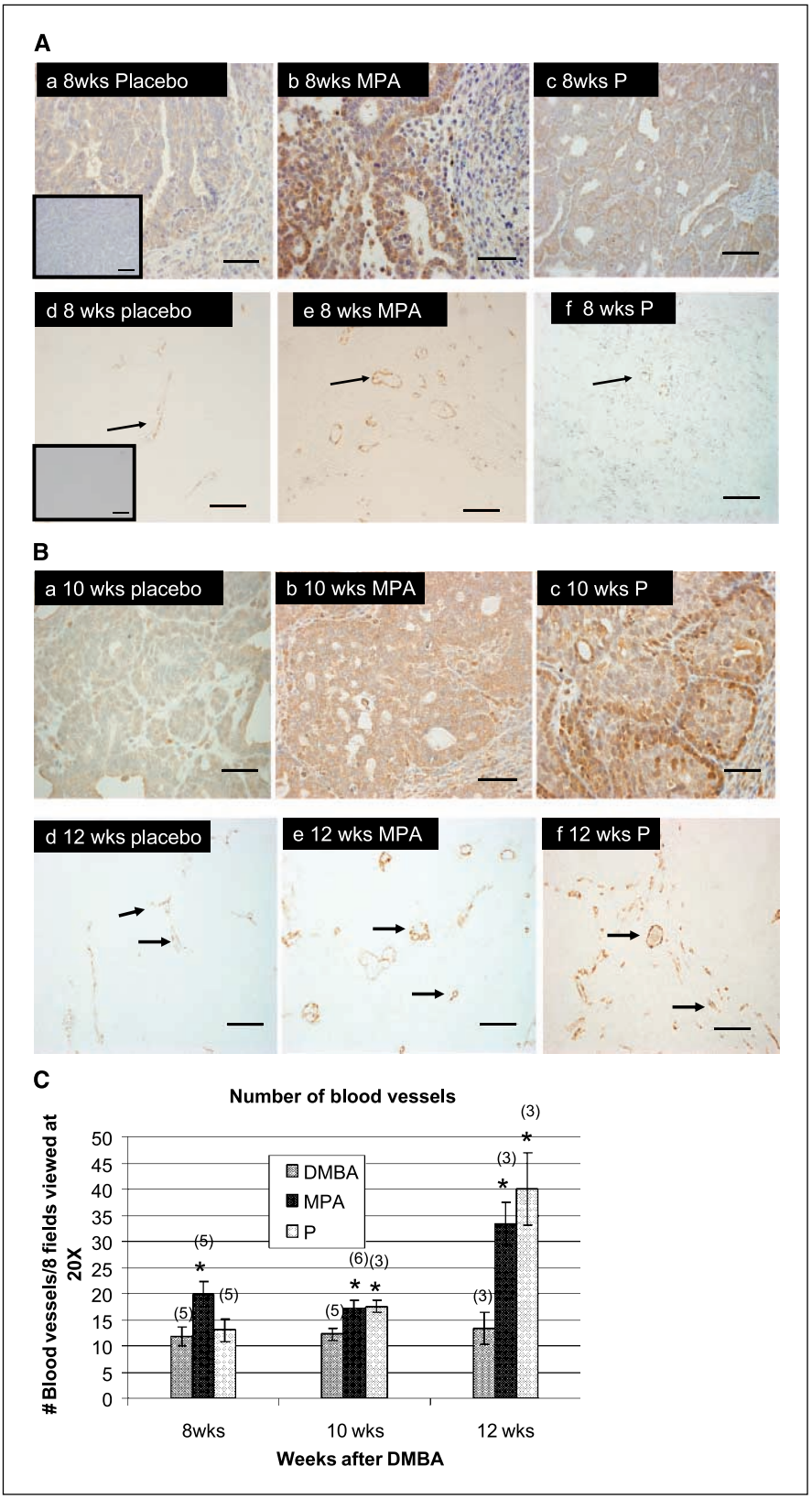

Fig. 6. *A* and *B,* VEGF and factor VIII expression in hormone-treated tumors. DMBA-treated rats were implanted with MPA or progesterone pellets 6 weeks following DMBA treatment. Mammary tumors were excised 8, 10, or 12 weeks after DMBA treatment. *Top,* staining for VEGF; *bottom,* factor VIII. *P,* progesterone. *Black arrows,* blood vessels. *C,* multivessel density analysis of tumor tissue with and without exposure to progestins. Number of blood vessels in DMBA-induced tumors treated with either MPA or progesterone. *, $P < 0.04$, compared with controls (unpaired *t* test). *Inset,* negative controls without primary antibody. Magnification, ×64. Bar, 50 μm.

clearly point to a role for even low doses of MPA influencing the progression of breast disease.

Interestingly, ovariectomized animals did not develop DMBA-induced mammary tumors as also reported previously (34), and MPA had no additional effect. This situation did not change, during the period of the study, even when ovariectomized rats were supplemented with estrogen pellets on day 60 and experiments continued until day 120. No PR could be detected in the mammary glands of ovariectomized animals, indicating that the presence of PR is critical for the promotion of mammary tumors by MPA (data not shown); however, we cannot rule out that an ovarian factor, either independently or estrogen induced, may be required for a complete response for the animals to develop tumors under our experimental conditions. It has been shown previously that progesterone can increase proliferation in tumors that are regressed in response to tamoxifen (21). This effect was blocked by the antiprogestin RU-486, indicating that progesterone effects are mediated by PR. Our results showed that although RU-486 slightly delayed tumor progression in MPA-promoted tumors when MPA was implanted 6 weeks after DMBA, the final tumor incidence did not change, indicating that although PR may have a role in growth promoting effects of MPA it is not the only mechanism by which MPA or progesterone accelerates DMBA-induced tumors. In contrast, RU-486 substantially delayed MPA-driven tumor progression when MPA was implanted 4 weeks after DMBA treatment, indicating that at this stage progesterone may play a significant role in tumor development and the initial phases of tumor progression may be PR dependent. Interestingly, the latter treatment also provided protection against DMBA-induced tumors and tumor incidence was lower than MPA-driven tumor formation during the time of analysis. The question remains as to why RU-486 is relatively ineffective at the 6-week stage. RU-486 has been shown to express estrogenic effects and can also cross-react with other steroid receptors (35, 36). Because rats were continuously exposed to the compound in our study, it is possible that these mechanisms were involved in a stage-specific manner. Interestingly, progesterone has been shown to increase the progression of breast tumors in a *N*-methyl-*N*-nitrosourea-initiated tumor rat model in a synergistic manner with estrone (37). Thus, it is possible that the estrogenic effects of RU-486 may synergize with MPA to accelerate, rather than prevent, tumor formation during the latter stages of tumor progression. It is possible that RU-486 itself may function as an agonist, given that the final tumor incidence in the presence of the progesterone antagonist reached the same level as MPA-driven tumors and was higher than levels observed with placebo. It is also unlikely that the dose of RU-486 was limiting, because both RU-486 and MPA are fairly stable and it was given 3 days in advance of MPA and functioned when tested at the 4-week stage. The effects of RU-486 are complicated, because it can also bind to other receptors, such as the glucocorticoid receptor (35), which could account for its failure to suppress the effects of MPA at later stages of tumor development. Irrespective, our studies clearly show that MPA accelerates mammary tumor development and this effect may depend on PR. The role of PR in DMBA-induced tumorigenesis cannot be ignored, because ovariectomized Sprague-Dawley rats (lacking PR) did not form tumors in our study.

The data reported in this study show that the natural hormone progesterone accelerates the development of DMBA-induced mammary tumors, confirming earlier reports in the literature (28, 29, 38). In this study, progesterone was found to increase the incidence and size of tumors when compared with the placebo control group; however, like the low-dose MPA results, it did not change the latency period compared with controls.

It needs to be pointed out that throughout this study we noticed a wide variation on tumor incidence in the DMBA-induced mammary tumors that were not treated with any hormones. Such a variation has been noticed before and has been associated with season and age in Sprague-Dawley rats (39). The age of the animals in the series of experiments reported remained constant; hence, it is likely that what we have observed is a seasonal variation in tumor incidence. This suggests that other factors besides mutations in tumor cells dictate the final outcome of tumor incidence in DMBA-induced tumor model. Irrespective of final tumor incidence in control animals, the progestin-dependent acceleration of tumor formation remained consistent throughout this study.

Hefflinger et al. have shown previously the importance of angiogenesis in the DMBA-induced tumor model (40). We sought to determine whether progestin-accelerated tumors also undergo increased angiogenesis in response to natural and synthetic progestins. We have shown previously that both MPA and progesterone increase the synthesis and secretion of VEGF in breast cancer cells (41, 42). The current study shows that MPA and progesterone both induce VEGF expression but in different patterns; MPA increased VEGF in the stromal and epithelial compartments, whereas progesterone increased VEGF mainly in the basal layers of tumor epithelial cells, with minimal stromal expression. Thus, progestin-induced VEGF may have both an angiogenic function and a cell survival function, because it is known that secreted VEGF can cause survival of cells in a paracrine and an autocrine fashion (16). Both progesterone and MPA have been shown previously to manifest divergent functions at the molecular level (24, 25, 42) as well as influence the type of tumors induced in mice (43).

The induction of VEGF correlated with increased angiogenesis as determined by increase in factor VIII – positive cells. MPA implantation produced tumors within 2 weeks. These tumors had increased VEGF production as well as an increased number of blood vessels. At this same time point, progesterone exposure did not produce any tumors and did not result in increased numbers of blood vessels over control levels in the mammary glands analyzed. At 8 weeks after DMBA, both MPA- and progesterone-induced tumors bore increased numbers of blood vessels, indicating that angiogenesis is increased in tumors that are accelerated by these compounds. At 12 weeks after DMBA, both MPA- and progesterone-accelerated tumors developed extensive blood vessels corresponding with the excess VEGF observed in the tissues at this stage. These data suggest that the progesterone-dependent increase in mammary tumors in this rat model is associated with increased angiogenesis, indicating that antiangiogenic treatment may be useful for curtailing this kind of breast disease, particularly if a similar mechanism exists in human tumors following progesterone-based HRT regimens.

In summary, our observations suggest that both natural and synthetic progestins can accelerate mammary tumors once a

critical stage of development in DMBA-induced lesions has been reached. This could signify that, in this model, adequate time is required for the accumulation of mutations conferring a growth advantage to certain groups of cells, which can then respond to progesterone stimulation with uncontrolled growth. We believe that an analogous population of cells may be responsible for the accelerated development of tumors in postmenopausal women who are consuming estrogen/progestin-based hormonal therapy (2, 7, 8, 26), because their tumors develop at a rate that cannot be explained by new tumorigenic events. It is thus possible that antiangiogenic treatment may be useful for treatment of progestin-accelerated tumors in women. The model described here may be useful for the identification of "angioprotective" compounds that could potentially prevent acceleration of tumors during therapeutic progestin use.

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Natural and Synthetic Progestins Accelerate 7,12-Dimethylbenz[*a*]Anthracene-Initiated Mammary Tumors and Increase Angiogenesis in Sprague-Dawley Rats

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