Research Article

Cancer Prevention Research

Synthetic Progestins Differentially Promote or Prevent 7,12-Dimethylbenz(*a*)anthracene–Induced Mammary Tumors in Sprague-Dawley Rats

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Abstract

Recent clinical trials show that combined oral dosing with estrogen and progestin increases the incidence of breast cancer in postmenopausal women. Similarly, in a rat model system of mammary carcinogenesis, the synthetic progestin medroxyprogesterone acetate (MPA) decreases latency and increases incidence of 7,12-dimethylbenz(*a*)anthracene (DMBA)–induced mammary tumors. The goal of this study was to compare the effects of four clinically relevant progestins, MPA, norgestrel (N-EL), norethindrone (N-ONE), and megestrol acetate (MGA), on DMBA-induced mammary carcinogenesis in the rat. The experimental protocol involved implantation of 60-day release progestin pellets four weeks after rats were treated with DMBA. In contrast to the effect of MPA, N-ONE, and N-EL, but not MGA, blocked DMBA-dependent carcinogenesis and a dose-dependent effect on tumor growth was shown for N-EL; MGA did not alter tumor growth. Histopathologic studies showed extensive hyperplastic lesions in mammary tissue of progestin-treated animals. Furthermore, following treatment with N-EL or N-ONE, immunohistochemical staining for vascular endothelial growth factor in hyperplastic mammary tissue was lower than in animals treated with DMBA plus MPA or DMBA alone. Expression of vascular endothelial growth factor receptor-1, estrogen receptor α, and progesterone receptor was also lower in hyperplastic mammary tissue in N-EL–, N-ONE–, and MGA-treated animals. Interestingly, N-EL stimulated progression of existing mammary tumors in DMBA/MPA-treated rats, suggesting stage-specific effects of N-EL in this model. Because N-EL and N-ONE prevent tumor growth in the early stages of DMBA-induced mammary carcinogenesis in rats, these progestins may have potential as chemopreventive agents in women with no history of breast disease or family history of breast cancer. *Cancer Prev Res; 3(9); 1157–67. ©2010 AACR.*

Introduction

Breast cancer is the most commonly diagnosed and second leading cause of cancer death in American women (1, 2). Over 200,000 American women, most of whom are postmenopausal, are diagnosed with breast cancer every year (3, 4). Many women are treated with estrogen monotherapy, or a combination of estrogen and progestin [also known as hormone replacement therapy (HRT)] during and after menopause. Previous studies showed that estrogen stimulates endometrial proliferation and increases the risk of endometrial cancer (5), but this effect is not seen in response to combined HRT with estrogen and progestin(6). Nevertheless, recent studies showed that the risk of breast cancer increases in response to HRT with estrogen and progestin (7, 8).

Synthetic progestins as well as the natural hormone progesterone block the proliferative effects of estrogens in the uterus (9). However, synthetic progestins have variable biological effects, due to differences in their structure, stability, pharmacokinetics, and steroid-receptor binding specificity. For example, some progestins bind the androgen and glucocorticoid receptors as well as the progesterone receptor (10, 11). In the United States, most women on HRT are treated with medroxyprogesterone acetate (MPA), the progestin in Prempro (12–14). Other commonly prescribed progestins include norethindrone acetate (N-ONE) for HRT and contraception (FemHRT, Activella, and CombiPatch), norgestrel (N-EL; Ovral; ref. 15), and megestrol acetate (MGA), which has been used clinically for cancer (16).

We recently showed that MPA and progesterone reduce latency and increase the incidence of 7,12-dimethylbenz(*a*)anthracene (DMBA)–induced mammary tumors in Sprague-Dawley rats (17). MPA and progesterone also promote the growth of human breast xenograft tumors in mice (18). However, it has been reported that

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progestins prevent or delay tumor growth in some animal model systems (19, 20). Importantly, these contrasting experimental results were obtained in experimental model systems that used different timing of treatment and dosing regimens. Therefore, we conducted a study to determine and compare the effects of four synthetic progestins on DMBA-induced mammary carcinogenesis in the rat. This cancer model system is very well characterized and is well suited to analysis of the hormonal regulation of carcinogenesis because rat mammary cells express both the estrogen receptor (ER) and the progesterone receptor (PR; ref. 17). The results show that MPA promotes DMBA-induced tumors in rats, but in contrast, when dosed in a similar manner, N-ONE and N-EL block DMBA-induced carcinogenesis in this model system, whereas MGA has little or no inhibitory effect. Furthermore, when dosing with N-EL was delayed until well after DMBA/MPA-induced tumors appeared (i.e., a more advanced stage of tumorigenesis), N-EL stimulated tumor growth instead of inhibiting it as during early tumorigenesis. The implications of these data for clinical treatment of human breast cancer are discussed.

Materials and Methods

Chemicals

DMBA was obtained from Sigma. All placebo and hormone pellets (25 mg/60-d release) were purchased from Innovative Research.

Animals

Forty- to 45-day-old virgin female Sprague-Dawley rats (Harlan Breeders) were housed according to the guidelines of the American Association of Laboratory Animals, with a 12-hour light and dark cycle and *ad libitum* food and water. All animal surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri, Columbia, and were in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (NIH publication 85-23). Unless noted otherwise, 10 animals were used in each experimental group, and each experiment was repeated at least twice.

Experimental design

Forty- to 45-day-old rats were given a single 20 mg dose of DMBA in peanut oil by gavage (reagent-grade DMBA was obtained from Sigma). Four weeks later, animals were anesthetized with isoflurene by inhalation, and pellets containing N-EL, MPA, Norethindrone, MGA, or placebo were implanted s.c. in the dorsal area. Animals were palpated every 2 days, and tumors were measured. The diameter of each tumor was measured with a micrometer caliper, and tumor size was calculated using the formula $L/2*W/2*\pi$ (21). Tumor number, size, and location were recorded for each rat. Breast tissue (inguinal gland) and tumors were collected from animals at the end of the experiment. Tissues and tumors were fixed in 10% buffered formalin for histopathology. Animals were sacrificed on days 65 to 75 depending on the tumor burden. In some experiments, animals treated with N-EL were sacrificed 100 days after dosing with DMBA.

N-EL dose curve

Four weeks after dosing with DMBA, animals were implanted with 60-day release pellets containing 0.5, 10, or 25 mg N-EL or placebo. Animals were palpated every 2 days, and tumors were measured. Tumor diameter was measured with a micrometer caliper, and tumor size was calculated using the formula $L/2*W/2*\pi$ (21). Tumor number, size, and location were recorded for each rat. Animals were sacrificed 65 days after N-EL administration.

N-EL treatment of advanced tumors

DMBA/MPA-treated animals with existing mammary tumors were implanted with N-EL 25 mg/60-day release pellets or placebo pellets 88 days after dosing with DMBA. The slow-release MPA pellet was completely dissolved at this time. Control animals were implanted with pellets containing placebo.

Histology and immunohistochemical analysis

Immunohistochemical analysis was done on paraffin sections using antibodies to ERα, ERβ, PR, vascular endothelial growth factor (VEGF), and VEGF receptors (VEGFR-1/2). Axillary and abdominal glands, and mammary tissues and tumors were fixed overnight in 10% neutral buffered formalin for H&E staining or in 4% paraformaldehyde for immunohistochemistry and then washed thrice in 70% ethanol for paraffin embedding. Five-micrometer sections were mounted onto ProbeOn Plus microscope slides (Fisher Scientific, Inc.). Serial H&E-stained sections were examined by light microscopy and classified according to previously published criteria (22). Processing of tissue sections for probing with antibodies to VEGF and the hormone receptors ERα, ERβ, and PR was as previously described (17). Additionally, tissue sections were also incubated with anti–VEGFR-1 antibody [1:50 dilution of a rabbit anti-VEGFR1 polyclonal antibody (Flt-1, H225, sc-9029); Santa Cruz Biotechnology, Inc.], an anti–VEGFR-2 antibody [1:50 dilution of a rabbit anti–VEGFR-2 polyclonal (Flk-1, ab2349); Abcam, Inc.]. Sections for VEGFR-1/2 were then incubated with EnVision, a horseradish peroxidase–labeled polymer conjugated to anti-rabbit antibodies (DAKO). Bound antibodies were visualized following incubation with 3,3′-diaminobenzidine solution (0.05% with 0.015% H_2O_2 in PBS; DAKO) for 3 o 5 minutes. Sections were counterstained with Meyer's hematoxylin, dehydrated, and coverslipped for microscopic examination.

Histologic staining was quantified using morphometric software (FoveaPro 3.0, 2005 Reindeer Graphics). Images were recorded at ×20 or ×40 magnification, and threshold image intensity was adjusted for measurement in pixels. Nine to 12 images (×20) from three to five sections were analyzed to normalize for cellular density in

the hyperplastic regions within mammary tissues. Staining intensity within hyperplastic regions were recorded, and mean values are shown. Results are expressed in square pixels for VEGF and VEGFRs. PR- and ERα-positive nuclei were counted, and results were expressed as percent cells stained in various sections before and after treatment.

Serum hormones

Serum estradiol was measured in duplicate by enzyme immunoassay per manufacturer's directions (Caymen Chemicals).

Statistical analysis

Statistical significance was tested by one-way ANOVA using SigmaStat Software version 3.5 (Sigstat Software, Inc.). For ANOVA, the assumption of ANOVA was examined and nonparametric measure based on ranks was used, as needed. Values were reported as mean ± SEM. When ANOVA indicated significant effect (F-ratio, $P < 0.05$), the Student-Newman-Keuls' multirange test was used to compare the means of individual groups. When normality using one-way ANOVA failed, significance was determined by Kruskal-Wallis test (one-way ANOVA by ranks) with Student-Newman-Keuls' method for all pairwise multiple comparisons. Serum estrogen levels were analyzed by one-way ANOVA, and all pairwise multiple comparisons were analyzed by fisher LSD method. Animal experiments using progestins and N-EL–accelerated advanced tumors were compared with controls using the Mann-Whitney rank sum test.

Results

Influence of synthetic progestins on progression/prevention of DMBA-induced mammary cancer

We previously showed that MPA accelerates DMBA-induced mammary gland carcinogenesis in the rat (17). Here, the DMBA-induced mammary gland carcinogenesis model was used to compare the effects of MPA, MGA, N-EL, and N-ONE on tumor development. As expected, when MPA was administered 4 weeks after DMBA, tumor latency decreased from 44 to 35 days, and tumor incidence increased from 50% to 70%. In contrast, MGA had no significant effect on tumor latency (47 d) or incidence (40%), whereas N-ONE increased tumor latency to 70 days and decreased tumor incidence (≤10% at day 70). No tumors were detected in the group treated with N-EL in the time frame tested (Fig. 1A). As expected, tumor multiplicity was 2 in the MPA group, whereas it remained 1 in the placebo group as previously observed (17). Multiplicity in the MGA group also remained at 1, whereas only one tumor in one animal was detected in the N-ONE group on day 70 when the experiment was terminated. As previously described, no tumors were detected in the N-EL group; hence, the multiplicity remained 0 in this group throughout the experiment. The effect of N-EL on tumor growth was also evaluated 74 and 100 days after DMBA treatment (Fig. 1B). Tumor incidence in MPA-treated and placebo-treated animals was 86% and 43%, respectively, at day 74. These animals were sacrificed at day 74 due to high tumor burden. In contrast, no tumors were detected in N-EL–treated animals at 74 or 100 days after treatment with DMBA (Fig. 1B).

DMBA-induced mammary tumors in rats require estrogen for growth (17, 23). Therefore, experiments were conducted to determine whether serum estrogen levels were altered in progestin-treated animals. For this purpose, serum was collected from treated and control animals at the end of the experiment shown in Fig. 1A (i.e., on day 61), and estradiol-17β was quantified. The results showed a similar level of estradiol-17β in MPA-treated and control animals, but a higher level of estradiol-17β in animals treated with MGA, N-EL, or N-ONE (Fig. 1C). This indicates that the tumor-suppressive effects of N-EL and N-ONE were not due to a reduction in levels of estradiol-17β.

Dose-dependent effect of N-EL on DMBA-induced mammary tumors

To confirm the observation that N-EL blocks the development of DMBA-induced mammary tumors, the dose dependence of the response to N-EL was determined by exposing DMBA-treated rats to 0.5, 10, or 25 mg/60-d release pellets of N-EL. Our findings showed that 10 or 25 mg/60-d release pellets of N-EL completely blocked DMBA-induced tumors (Fig. 1D), whereas a dose of 0.5 mg/60-d release pellets had no effect, and tumors developed in these animals with a similar latency (41 versus 44 d) and incidence (40 versus 50%) as in placebo-treated animals (Fig. 1D).

Histology and immunohistologic analysis

Histology. At the end of the study shown in Fig. 1A, all animals were sacrificed for analysis of histopathology and expression of relevant markers by immunohistologic methods (see below). Tumors or tumor-free mammary glands were collected from all experimental animals. Tissues were preserved in 4% paraformaldehyde for H&E staining. DMBA-treated animals receiving MPA or placebo pellets developed ductal and lobular carcinomas (e.g., Fig. 2A, top left), and all tumor-free mammary glands from animals treated with progestins exhibited ductal hyperplasia (e.g., N-EL–exposed mammary gland shown in Fig. 2A, right). The single tumor in the N-ONE–treated group was a lobular carcinoma (data not shown). Our previous studies suggested that an elevated level of VEGF might be involved in the mechanism of action of MPA in this model system (17). This hypothesis was confirmed here, as shown by increased VEGF immunostaining in hyperplastic mammary tissue in MPA-treated rats (Fig. 2B, left). However, VEGF staining in the hyperplastic regions of mammary glands of N-EL–treated animals was much lower than in MPA-treated animals or controls (Fig. 2B, bottom right). This prompted a detailed investigation of VEGF expression in hyperplastic regions of mammary glands of DMBA-treated animals exposed to MPA, MGA,

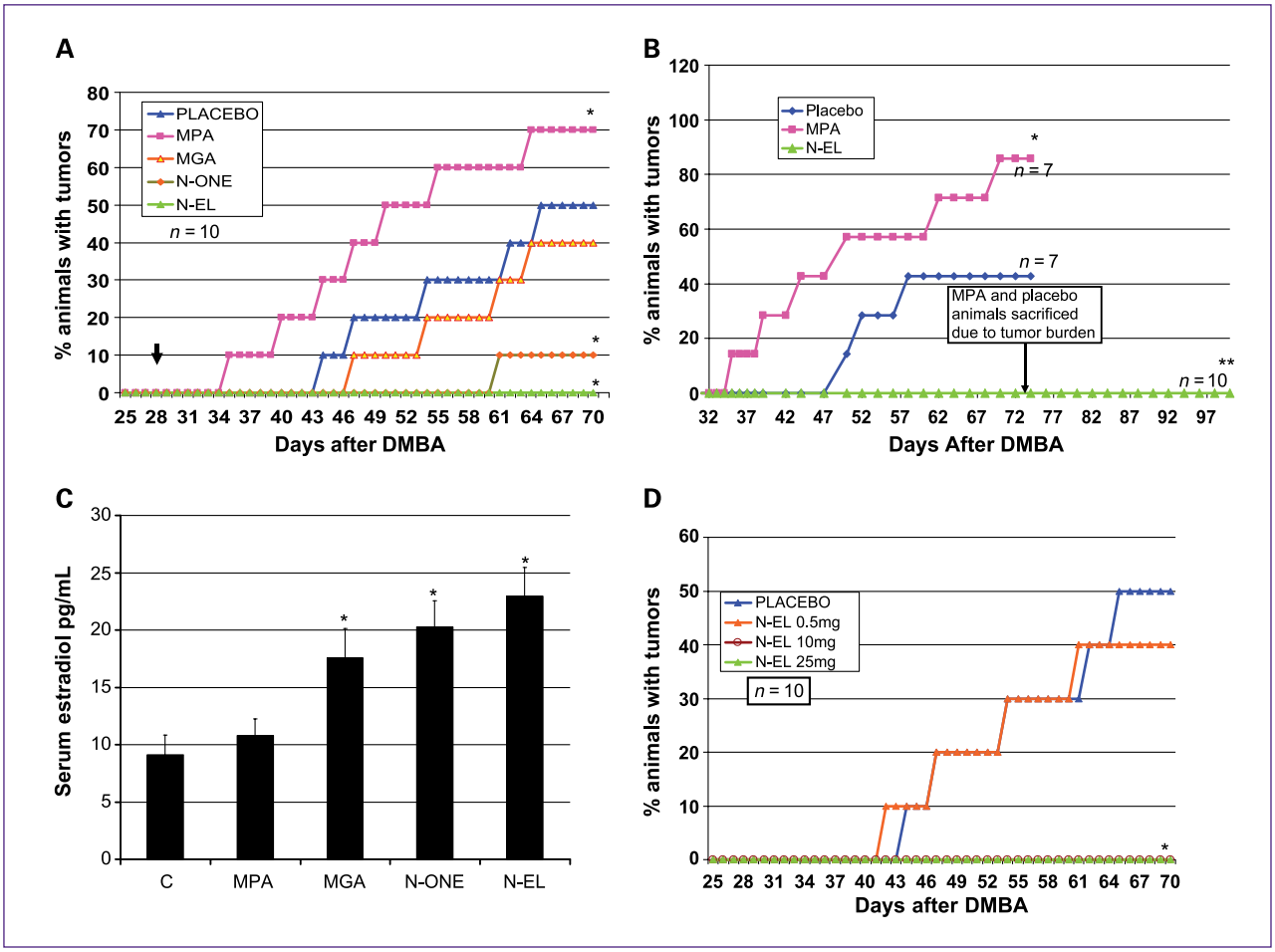

Fig. 1. A, effect of various progestins on acceleration of DMBA-induced mammary tumors in rats. Animals were treated with DMBA and subsequently implanted with a 25-mg/60-d release pellet on day 28 (arrow) as described in Materials and Methods. *, $P < 0.001$, compared with placebo (Mann-Whitney Rank-Sum test). B, preventive effect of N-EL on DMBA-induced mammary tumors in rats. The procedure followed was as described in A, and animals with N-EL were sacrificed on day 100 past DMBA treatment (72 d after N-EL pellet. *, $P < 0.005$; **, $P < 0.001$, compared with placebo; Mann-Whitney Rank-Sum test). C, serum estradiol/whole blood was collected from animals at the end of the study shown in A by heart puncture immediately after euthanizing the animals. The whole blood was centrifuged at 2,000 rpm in glass tubes for 10 min to obtain plasma. Cayman Estradiol EIA kit was used for the assay to determine serum estradiol-17β as described in Materials and Methods. *, $P < 0.05$ compared with Placebo or MPA group by one way ANOVA and further comparison by Fisher LSD. D, dose-dependent suppression of DMBA-induced mammary tumors in rats by N-EL. Animals were treated with DMBA and subsequently with various doses of N-EL as described in Materials and Methods. *, $P < 0.001$, compared with placebo (Mann-Whitney Rank-Sum test).

N-ONE, and N-EL. In addition to VEGF, expression of VEGFRs 1 and 2 (FLT and FLK), and estrogen and PRs was measured. The results of the immunohistologic studies are summarized below. Note that unless otherwise indicated, antigen expression was evaluated in hyperplastic regions of mammary gland tissue.

Vascular endothelial growth factor. As shown in Fig. 3A (staining) and B (quantitation), VEGF was expressed at a higher level in MPA-treated animals than in placebo-treated animals. Interestingly, treatment with MGA did not increase or decrease expression of VEGF relative to placebo-treated animals. In contrast, treatment with N-EL or N-ONE caused a reduction in VEGF staining in the hyperplastic regions of mammary glands (Fig. 3A and B). Because VEGF signaling plays an important role in tumor development and progression (17, 24), it is possible that the ability of N-EL and N-ONE to prevent tumor development is at least in part due to its effect on VEGF expression in preneoplastic rat mammary tissue. The fact that MGA neither promotes nor protects against DMBA-induced mammary tumors in the rat correlates with its inability to increase or decrease VEGF expression in this model.

VEGFR-1/2. VEGF signaling is mediated by the VEGFRs, VEGFR-1 and VEGFR-2. Immunostaining for these receptors showed a similar level of VEGFR-1 in MPA- and placebo-treated rats, but an ~2-fold lower level of expression in MGA-treated and a 4- to 5-fold lower level of expression in N-ONE– and N-EL–treated animals (Fig. 4A and B, left). In contrast, the level of expression of VEGFR-2 was similar in all treatment groups (Fig. 4A and B, right).

These results show a correlation between the antitumor activity of specific progestins and their ability to suppress VEGF signaling by downregulating VEGF and VEGFR-1 (but not VEGFR-2), as observed for N-ONE and N-EL, but not MGA or MPA.

Estrogen receptor α. ERα was expressed at a similar level in MPA- and placebo-treated rats (Fig. 5A and B, left). However, ERα expression was ~2-fold lower in MGA-treated animals and ≥5-fold lower in N-EL– or N-ONE–treated animals (Fig. 5A and B, left). In contrast, the level of expression of ERβ was similar in all treatment groups (data not shown). These results show a correlation between progestin antitumor activity and strong downregulation of ERα, but not ERβ, as observed for N-ONE and N-EL.

Progesterone receptor. PR is highly expressed in the mammary glands of placebo-treated rats (Fig. 5A and B, right). In contrast, PR was significantly downregulated in all progestin-treated animals. However, PR was most strongly downregulated in the epithelial cells of hyperplastic mammary tissues in N-EL– and N-ONE–treated rats. Because MPA also downregulates PR, although it promotes tumor development, a different mechanism (and different biological outcome) may downregulate PR in MPA-treated and N-EL–or N-ONE–treated animals.

N-EL cannot prevent progression of frank tumors induced by MPA

The results presented above indicate that implantation of a 60-day release N-EL pellet 4 weeks after dosing with DMBA completely blocks DMBA-induced mammary tumors. However, this does not rule out the possibility that N-EL might be procarcinogenic in some circumstances, as previously reported for progestins in some experimental systems or clinical situations (25, 26). Therefore, the effect of N-EL on late-stage progression of DMBA-induced mammary tumors was examined. DMBA/MPA-induced tumors were allowed to develop for 89 days (60 d after implantation of MPA pellets), followed by implantation with a 25 mg/60-d release N-EL pellet (or placebo controls; Fig. 6). On day 89, average tumor volume was 28 ± 4 mm^3 versus 30 ± 14 mm^3 in placebo- and N-EL–treated animals, respectively. Animals were then monitored, and tumor volume was measured until day 113. Tumors in animals exposed to N-EL increased in size to 79 ± 15 mm^3, whereas those given placebo decreased to 7 ± 2 mm^3 ($P < 0.01$, ANOVA). This result shows that N-EL strongly promotes growth of advanced DMBA/MPA-induced mammary tumors, thus showing that N-EL can be both an anticarcinogen and a procarcinogen, depending on cancer stage and experimental conditions.

Discussion

In the context of HRT, evidence suggests that progestins counteract the proliferative effects of estrogen in the female uterus, thus reducing the risk of human endometrial cancer (27). However, evidence from recent clinical trials as well as laboratory studies also shows that progestins

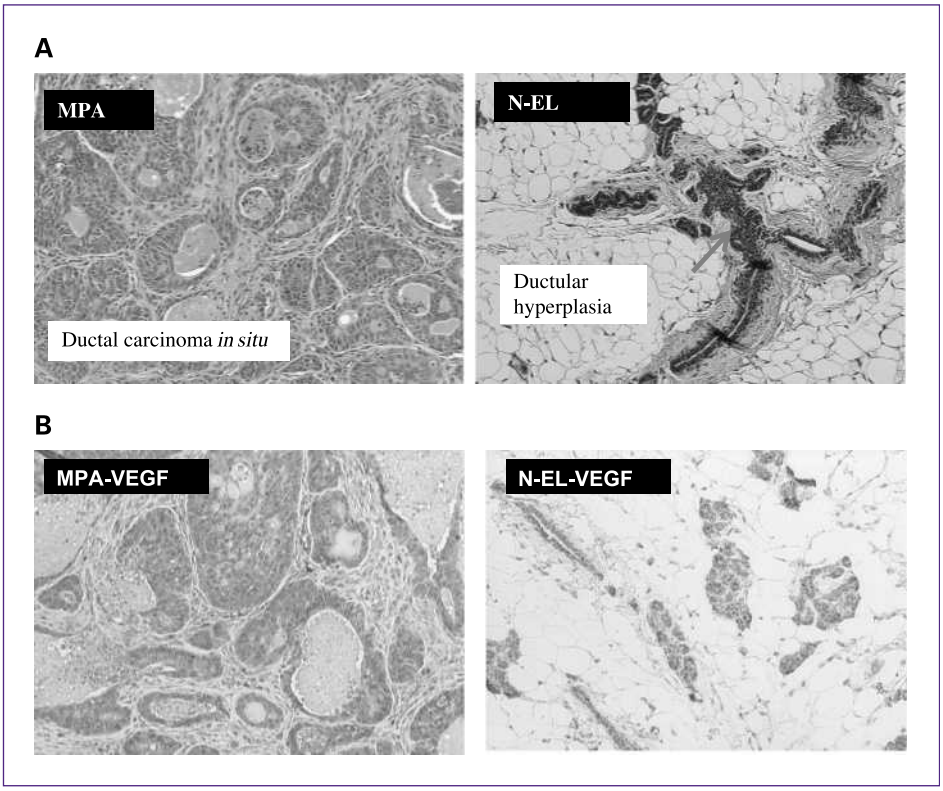

Fig. 2. A, histology of tumors in MPA-treated tumors and N-EL–treated mammary glands following DMBA treatment. Samples were taken from an experiment described in Fig. 1A on day 70. B, VEGF expression in sections taken from tumors in MPA-treated animals, and mammary glands from N-EL–treated animals, following DMBA treatment.

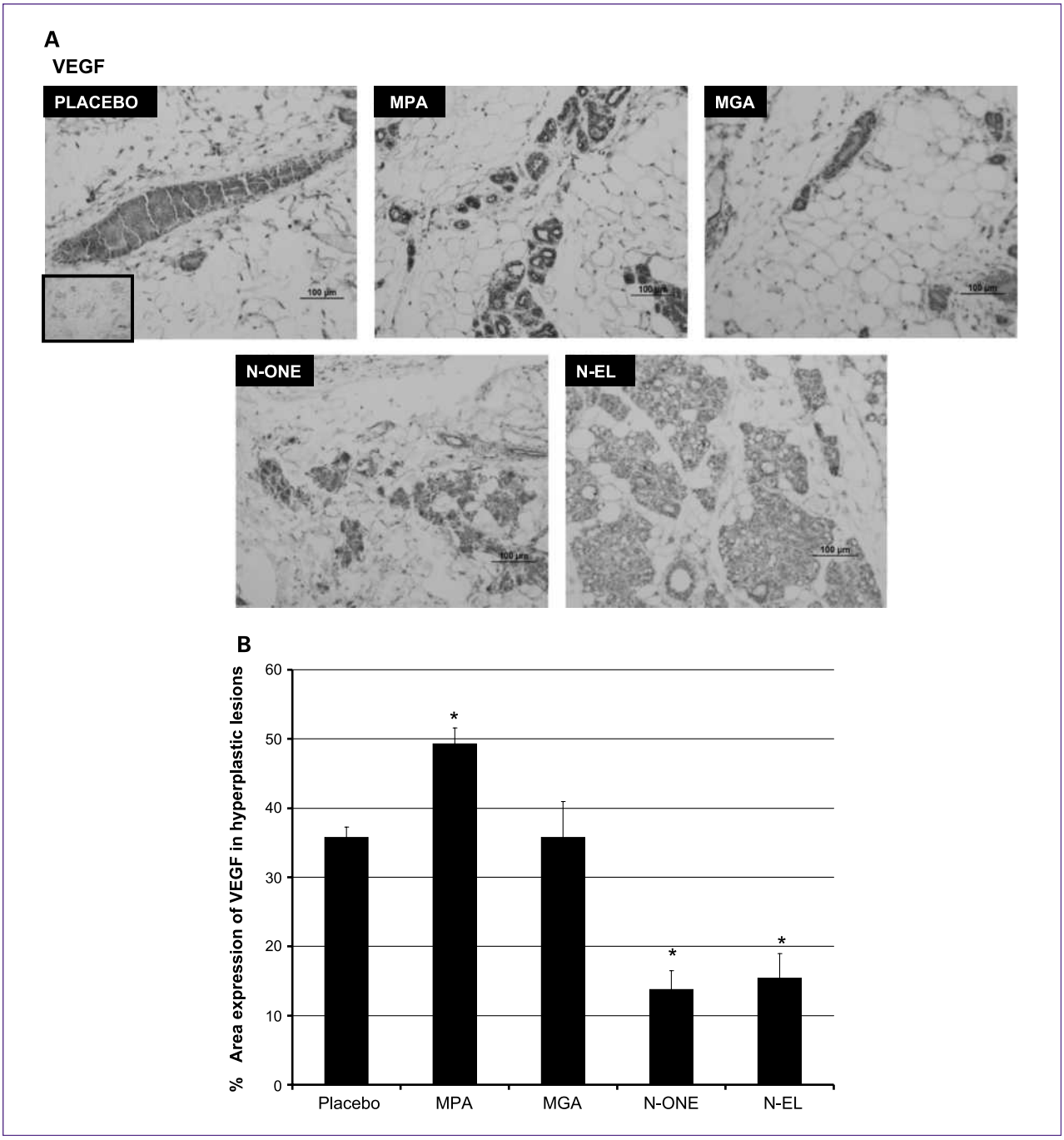

Fig. 3. A, expression of VEGF in DMBA-induced progestin-treated mammary gland. Sprague-Dawley rats were given a single dose of DMBA by gavage on day 1. Twenty-eight days after DMBA treatment pellets (25 mg/60-d release) of placebo, MPA, N-EL, MGA, or N-ONE were placed on the dorsal side of their neck. All animals were sacrificed 70 d after DMBA administration, and mammary glands devoid of tumors were collected. Cross-sections of these tissues show multiple hyperplastic lesions in cross-sections (multiple layers of epithelial cells), which do not progress to frank tumors, as shown in subsequent figures. Paraffin sections from mammary glands were stained for VEGF. Samples were photographed at ×20; scale bar, 100 μm. B, quantitative analysis of VEGF expression in the hyperplastic regions of tissues shown in A. *, significant difference from placebo by one-way ANOVA on ranks, and all pairwise multiple comparison were by Student-Newman-Keuls' method. *, $P < 0.005$.

increase the risk of breast cancer (17, 28, 29). Therefore, the effects of progestins remain controversial (14). The goal of this study was to further explore the effect of different progestins on mammary gland tumor development in the context of the rat model for DMBA-induced mammary tumors. This model allowed us to compare the effects of four progestins in a defined model system and to test the stage specificity of one progestin, N-EL. The

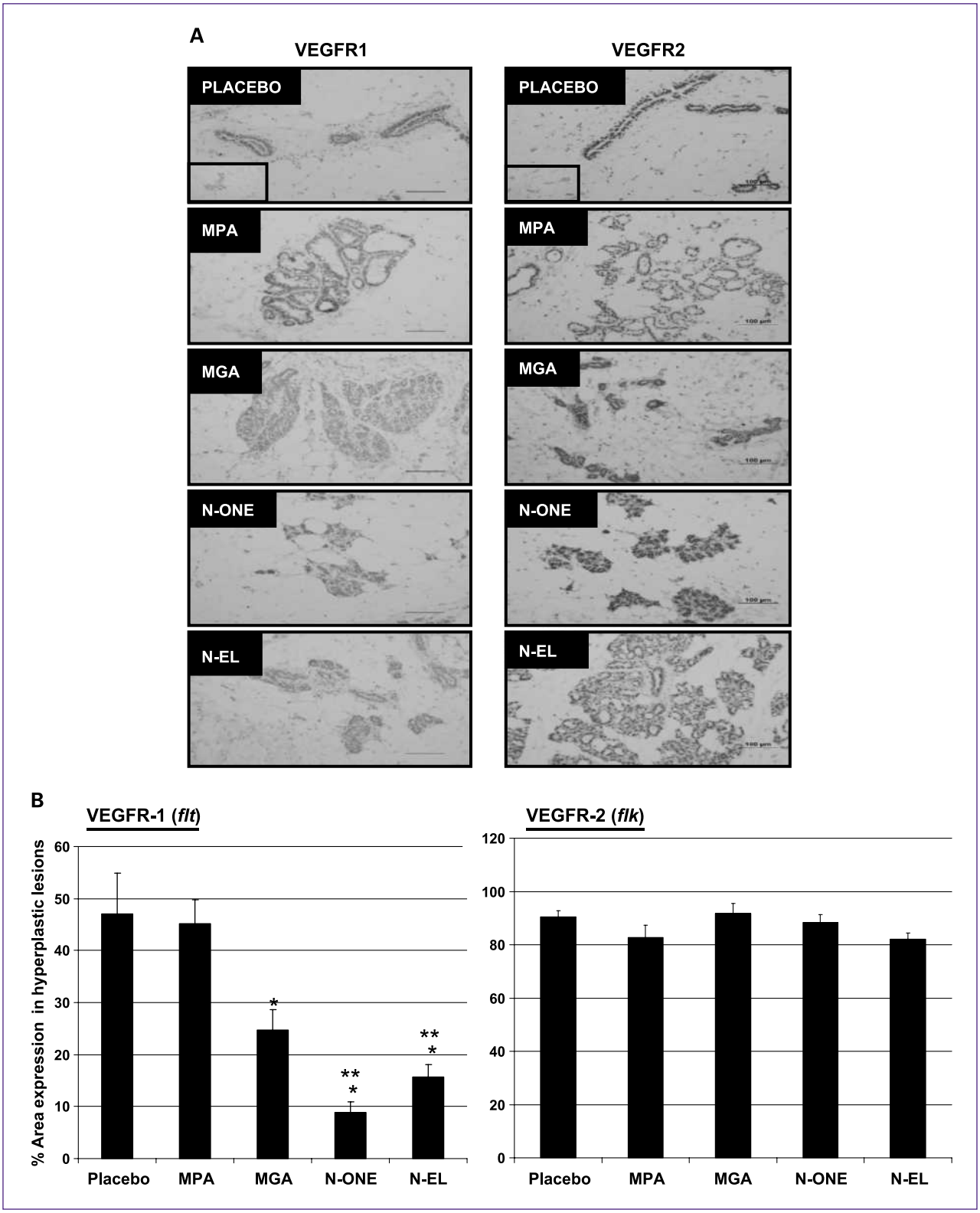

Fig. 4. A, expression of VEGFR-1/2 in DMBA-induced progestin-treated mammary gland. Procedure was as described in Figure 3A above. Insets, no-antibody controls. B, quantitative analysis of VEGFR-1/2 expression in tissues shown in A. *, significantly lower than placebo; **, significantly lower than MGA samples as tested with Krushal-Wallis one-way ANOVA on ranks, and all pairwise multiple comparison were by Student-Newman-Keuls' method (*, $P < 0.05$).

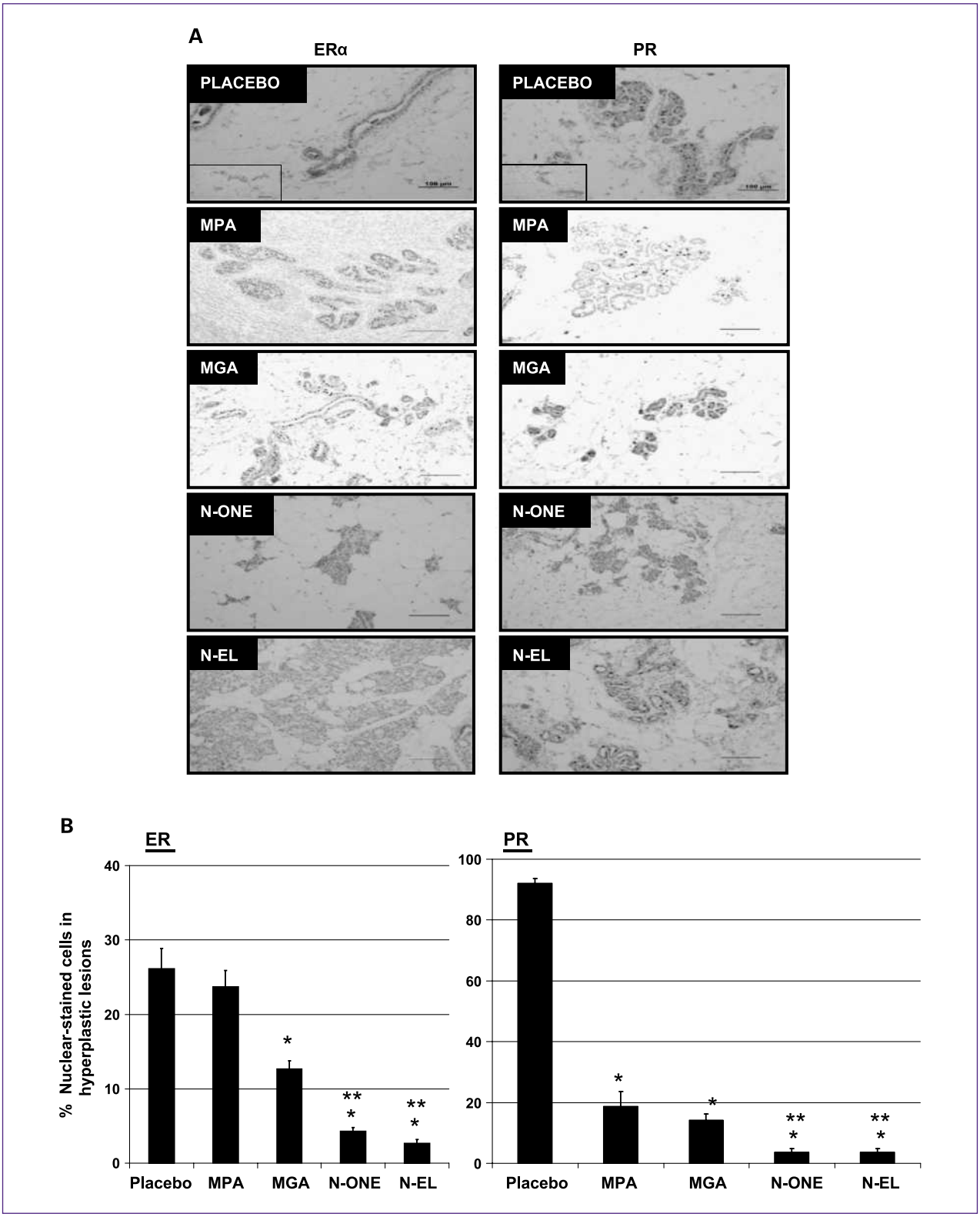

Fig. 5. Expression of PR and ER in DMBA-induced progestin-treated mammary gland. A, samples were stained for PR and ER as described in Fig. 4A above. Images were photographed at 20× magnification and bars represent 100 μm. Insets represent no-antibody controls. B, quantitative analysis of PR and ER expression in tissues shown in (A). *, significantly different ($P < 0.005$) from placebo and **, significantly suppressed compared with MGA using Krushal-Wallis one way ANOVA and all pairwise multiple comparison by Student-Newman-Keuls' method.

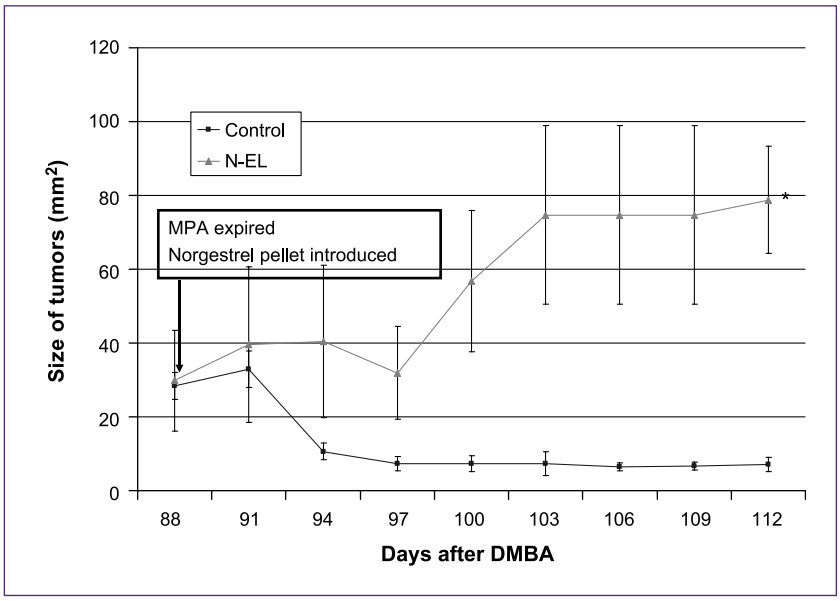

Fig. 6. N-EL stimulates progression of MPA-accelerated DMBA-induced mammary tumors in rats. *, $P < 0.001$, compared with placebo (Mann-Whitney Rank-Sum test).

results confirm the complex nature of hormonal regulation of mammary carcinogenesis but, as described below, suggest potential for successful therapeutic and/or preventive use of specific progestins in a clinical setting.

Although previous studies using the rat model for DMBA-induced mammary cancer showed that MPA functions as a tumor promoter (17, 30), this study shows that N-EL and N-ONE, when administered using the same protocol as used for MPA, strongly inhibit tumor development. To evaluate the possible relevance (and predictive value) of these results for preventive or therapeutic approaches to human breast cancer, it is important to consider whether the biological dose achieved in the rat experimental model is similar to the biological dose achieved in women on HRT. Human pharmacokinetic data for progestins is summarized as follows: MPA is administered at doses of 2.5, 5, or 10 mg/d, whereas N-ONE and N-EL are given as daily doses of 5 and 0.0075 mg respectively. When administered as a single dose of 10 mg, MPA levels peak within 1 to 4 hours and vary between 3 to 5 ng/mL, gradually declining to 0.3 to 0.6 ng/mL after 24 hours (31). Women given either 1,000 μg N-ONE in combination with estradiol or those given 500 to 300 μg N-ONE alone are exposed to a peak of 16, 6, and 4 ng/mL N-ONE, respectively, within 1 to 2 hours posttreatment. These levels then decline to <0.5 ng/mL within 24 hours. Serum N-EL levels following 500 μg of racemic mixture, or 75 to 100 μg N-EL with estradiol, are 6, 3.5, and 2.5 ng, respectively. These levels also decrease to <0.5 ng/mL after 24 hours (31). Rats treated with progestins in this study were implanted with 25-mg 60-day release pellets (0.41 mg/d), resulting in 1 to 2 ng/mL of sustained serum level of progestin. Thus, we argue that comparable biological doses were achieved in the rat experimental model described here as in clinical studies of women on HRT.

Although the demonstration that N-ONE and N-EL strongly inhibit DMBA-induced mammary carcinogeneis when given at early stages is of great interest, the mechanism of this effect is not yet known. Because oophorectomized animals fail to develop DMBA-induced tumors in the absence of exogenous estrogen and progestin (32, 33), it is possible that N-EL and N-ONE reduce availability of estradiol-17β or its receptor, and thus inhibit tumor growth. However, our data show that N-ONE and N-EL increase the level of serum estradiol-17β in DMBA-treated rats. It is interesting to note that a short-term increase in estradiol-17β levels (to ~25 pg/mL) was sufficient to protect rats from methylnitrosourea-induced mammary tumorigenesis (34). An alternative explanation for these data is that the high level of estradiol-17β in N-ONE– and N-EL–treated rats increases the rate of apoptosis in premalignant mammary tissue, as observed in some human cancer cells (35). However, it may also be relevant that N-EL and N-ONE, but not MPA, drastically reduce expression of ERα (but not ERβ) in mammary tissue of DMBA-treated rats. Thus, despite elevated hormone concentration, reduced expression of ERα in DMBA/N-ONE– and DMBA/N-EL–treated rats could ultimately interfere with DMBA-induced carcinogenesis. The change in ratio of ERα/ERβ may also be relevant because elevated levels of ERβ are associated with loss of mammary gland tumor cell proliferation and inhibition of angiogenesis (36). Although all progestins (MPA, MGA, N-ONE, and N-EL) also downregulate PR in the mammary tissue of DMBA-treated rats, this could be a secondary effect of the low abundance of ERα, which regulates expression of PR in mammary tissue (37). Finally, we cannot rule out the effect of progestins on the levels of prolactin in the current model. Early studies indicated that prolactin is essential for the formation of DMBA-induced mammary

tumors (38), and it therefore remains to be established whether progestins lower circulating prolactin levels in the model used in the current study. In contrast, because full term pregnancy in younger women has been associated with reduction of breast malignancies (39), and prolactin levels are elevated during pregnancy and lactation, it is also possible that progestins increase the levels of prolactin in our model and thus provide a protective role for N-EL. These possibilities warrant further investigation.

Ample evidence exists in multiple model systems to show that VEGF-stimulated angiogenesis is essential for tumor development. Thus, it is important to point out that expression of VEGF and VEGFR-1 are drastically reduced in mammary tissue of DMBA/N-EL– and DMBA/N-ONE–treated rats, whereas in DMBA/MGA-treated animals, VEGF expression was unaffected and VEGFR-1 was ~2-fold lower than in placebo controls. Thus, it seems that VEGF-VEGFR1 signaling is strongly suppressed in DMBA/N-EL– and DMBA/N-ONE–treated rats. This is consistent with earlier studies showing that mammary cancer cell lines overexpress VEGFR-1, secrete high levels of VEGF and placental growth factor, and show increased capacity for migration/invasion (40). In contrast, expression of VEGFR-2 did not seem to be altered in DMBA/progestin-treated rats. These results suggest that N-EL and N-ONE are antiangiogenic, and that suppression of angiogenesis may be essential for their anticarcinogenic effect in DMBA-treated rats.

This study exploited the rat model for DMBA-induced mammary cancer to show stage-specific effects of N-EL on mammary tumor development; namely, N-EL is strongly anticarcinogenic when administered to DMBA-treated rats during early stages of tumor development, but strongly promotes development of tumors when administered to tumor-bearing DMBA/MPA-treated rats during the late stages of carcinogenesis. One explanation of this stage specificity is that N-EL could directly or indirectly inhibit the release of growth factors or induce apoptosis of premalignant cells during early tumor development, whereas stimulating the release of growth factors during late stages of tumor development. If this is correct, certain progestins could have beneficial preventive effects when coadministered with estrogen to women lacking precancerous breast tissue. Unfortunately, the same treatment might have undesirable procarcinogenic effects in women with latent breast cancer lesions. However, in the context of the rat mammary cancer model, further studies are needed to determine if there is a safe window for exposure to MPA as has been found for N-EL or N-ONE.

In summary, our study strongly supports the idea that N-EL, and possibly other progestins, exert stage-specific effects during breast tumor formation and development. This and other published studies clearly indicate that progestins exert variable effects in different biological contexts. Therefore, clinical use of progestins requires caution, and these powerful steroids should only be used clinically when the absence of cancer susceptibility (i.e., no latent cancer or family history of cancer) is reliably established. Further studies are needed to determine the exact mechanism of the preventive effects of N-ONE and N-EL in the rat model system, including a molecular explanation for the stage specificity of both the procarcinogenic and anticarcinogenic effects of these compounds.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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