

Progestin Treatment Induces Apoptosis and Modulates Transforming Growth Factor- β in the Uterine Endometrium

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Abstract

Background: Epidemiologic, animal, and human data suggest that progestins are potent endometrial cancer preventive agents. In the ovarian surface epithelium, progestins have been hypothesized to confer a cancer preventive effect via apoptosis and modulation of transforming growth factor- β (TGF- β). Given that the ovarian epithelium and endometrium share a common embryologic origin and similar reproductive and hormonal risk factors for malignancy, we tested the hypothesis that progestins confer biological effects in the endometrium similar to those in the ovary.

Methods: Postmenopausal female macaques ($n = 78$) were randomized into four groups to receive a diet for 36 months containing no hormone versus conjugated equine estrogen (CEE), medroxyprogesterone acetate (MPA), or CEE + MPA. The endometrium was then examined immunohistochemically for treatment-specific changes using antibodies to activated caspase-3

(for apoptosis), Ki-67 (proliferation), and the TGF- β 1, TGF- β 2, and TGF- β 3 isoforms.

Results: Percentages of caspase-positive endometrial glandular cells were 3- to 5-fold higher in CEE + MPA-treated animals compared with all others ($P < 0.05$). Caspase-expressing cells were six times more numerous in the endometrial stroma of animals treated with MPA alone relative to other groups ($P < 0.0001$). Induction of endometrial glandular cell apoptosis in the CEE + MPA-treated group was associated with a dramatic increase in expression of TGF- β 2 and TGF- β 3 in the stromal compartment of the endometrium ($P < 0.0001$). **Conclusion:** Progestin treatment activates chemopreventive biological effects in the endometrium that are similar to those in the ovarian surface epithelium. These data may facilitate identification of a chemopreventive approach that dramatically lessens the risk of both uterine and ovarian cancer. (Cancer Epidemiol Biomarkers Prev 2008;17(3):578–84)

Introduction

Approximately 150,000 new cases of endometrial cancer are diagnosed every year worldwide, making endometrial cancer the fifth leading cancer in women globally. In the United States, it is estimated that 41,200 new cases of endometrial cancer were diagnosed in 2006 (1, 2). Several risk factors for endometrial cancer have been identified including nulliparity, early menarche, late menopause, high body mass index, and the administration of unopposed estrogen for the treatment of climacteric symptoms (3–9).

In the past, endometrial cancer has not garnered as much attention as ovarian cancer. This may be secondary to the better overall prognosis of endometrial cancer, as the majority of women diagnosed with endometrial

cancer are cured. Yet, stage for stage, survival rates for women with endometrial cancer are similar to those for ovarian cancer (1). In addition, similar to ovarian cancer, highly effective treatments for advanced-stage endometrial cancer are lacking. With an aging and increasingly obese population in the United States, the incidence and mortality from endometrial cancer is likely to become a worsening public health problem, thereby providing a strong rationale for the development of effective methods to prevent the disease (10).

A strong body of evidence suggests that progestins are highly effective endometrial cancer preventive agents. In premenopausal women, use of oral contraceptives that contain both an estrogen and a progestin significantly lowers subsequent endometrial cancer risk. Use of oral contraceptives for a period of at least 12 months confers as much as a 30% to 50% reduced risk of endometrial cancer, a protective effect that lasts for 10 to 20 years (11–15). In addition, progestin-potent oral contraceptives appear to have enhanced endometrial cancer protective effects compared with oral contraceptives containing weak progestins (16, 17), and use of progestin-releasing intrauterine devices, which release potent dosages of progestins locally in the endometrial cavity, leads to a marked reduction in endometrial cancer risk (18, 19). In menopausal women, the addition of a progestin to estrogen replacement therapy decreases the risk of

Received 6/15/07; revised 10/31/07; accepted 12/27/07.

Grant support: Department of Defense grant DAMD17-02-1-0183; National Heart, Lung and Blood Institute, NIH grants HL-490852 and HL-45666; and Hertel-Satter Foundation, Bear's Care, and Denyse King.

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doi:10.1158/1055-9965.EPI-07-0551

precancerous endometrial hyperplasias and abrogates the cancer-causing effect of exogenous estrogen (4, 5, 9), suggesting that progestins have a chemopreventive biological effect on the endometrium. Finally, high-dose progestin therapy has been shown to reverse preexisting PTEN-inactivated endometrial latent precursors as well as endometrial hyperplasias and even low-grade endometrial cancers in some women (20-22).

The biological mechanism(s) that underlie the chemopreventive effect of progestins on the endometrium have not been well characterized. In the ovary, progestins have been shown to induce programmed cell death and differentially regulate transforming growth factor- β (TGF- β) in the ovarian surface epithelium, leading to the hypothesis that progestin-mediated biological effects lead to the arrest or reversal of carcinogenesis via clearance of genetically damaged ovarian epithelial cells. This biological mechanism may explain the marked reduction in ovarian cancer risk associated with oral contraceptive use (23). It is interesting that cancers arising from the ovary and endometrium share common epidemiologic risk factors, including marked risk reduction associated with use of oral contraceptives (11-15, 24, 25) and that both the endometrium and the ovarian surface epithelium share a common embryological precursor (26). It is thus plausible that progestins activate similar molecular pathways relevant to cancer prevention in both of these organ sites. To test this hypothesis, we examined the endometria of primates treated with hormonal interventions, including progestin for evidence of induction of apoptosis and for regulation of expression of the TGF- β isoforms.

Materials and Methods

Animals/Randomization. For this study, we used 78 female adult cynomolgus macaques (*Macaca fascicularis*), with an average age of 7.5 years at the study's end. The cynomolgus macaque is an excellent animal model for yielding experimental results that are pertinent to human reproductive biology. This nonhuman primate has a 28-day menstrual cycle that is similar to that of humans (27, 28). The study was a prospective, randomized, controlled trial designed for the primary endpoint of evaluating the effects of postmenopausal estrogens and progestins on the cardiovascular system, breast, and reproductive tracts. The macaques had previously undergone bilateral oophorectomy 3 months before commencement of the study.

The macaques were randomly assigned to receive one of four hormonal interventions for 3 years: control ($n = 19$), conjugated equine estrogen (CEE; $n = 24$; Premarin; Wyeth Ayerst), medroxyprogesterone acetate (MPA; $n = 18$; Provera; Wyeth Ayerst), and CEE and MPA combined ($n = 17$). Test compounds were administered daily in the diet throughout the study period at human equivalent doses on a caloric basis to 0.625 mg/d/woman for CEE and 2.5 mg/d/woman for MPA. This dosing strategy is metabolically based and thus results in relative doses similar to those produced by allometric calculations (29); most importantly, it produces serum concentrations in the range of those seen in women taking hormone therapy (30, 31). The

duration of treatment was 36 months. The base diet was modeled on a typical moderately atherogenic North American diet (40% of calories from fat and 0.2 mg/kcal cholesterol) and commenced at the beginning of the hormonal interventions, which were given on a continuous basis until the end of the study. At the conclusion of the trial, animals were humanely euthanized by sedation with ketamine (10 mg/kg) and administration of 100 mg/kg pentobarbital i.v. Complete necropsy examinations were done, including examination of multiple organ systems, as reported previously (31). The study was approved by the Animal Care and Use Committee at the Wake Forest University School of Medicine.

Tissue Preparation and Immunohistochemistry. From each animal in the study, the uterus was fixed in 4% formaldehyde for 24 h and then stored at 4°C in 70% ethanol. Thereafter, uterine sections were trimmed to 3 mm thickness and embedded in paraffin.

Immunostaining. Briefly, 5 μ m full sections through the uterine wall (endometrium to the serosa) were mounted on charged slides, and tissues were immunostained using our previously published methods (23, 32). Antigen retrieval with heat and citrate buffer (pH 6.0) was used for all antibodies. Appropriate quenching procedures (heat treatment and peroxidase treatment) were used to remove endogenous enzymatic activity. Staining for apoptosis was done using a monoclonal antibody to cleaved caspase-3 (2 μ g/mL; Cell Signaling Technology); sections of benign macaque early menstrual phase endometrium were used as a positive control for apoptosis. Staining for TGF- β isoforms was done using antibodies specific for TGF- β 1 (0.67 μ g/mL anti-TGF- β 1 antibody; Santa Cruz Biotechnology), TGF- β 2 (4 μ g/mL anti-TGF- β 2 antibody; Santa Cruz Biotechnology), and TGF- β 3 (0.25 μ g/mL anti-TGF- β 3 antibody; Santa Cruz Biotechnology). For TGF- β staining, normal human umbilical cord was used as a positive control. Staining for proliferating cells was done using a monoclonal antibody to Ki-67 (1.25 μ g/mL clone MM1; Novocastra Laboratories). For caspase and Ki-67 staining, the enzyme detection system used alkaline phosphatase and the chromogen used was Vector Red (Vector Laboratories); for TGF- β staining, the enzyme system used was peroxidase and the chromogen was diaminobenzidine (DAKO). Sections were counterstained with Mayer's hematoxylin.

Caspase-stained and Ki-67-stained cells were quantified by counting the percentages of stained and unstained cells in sections by an observer blinded to treatments. The percentages of immunopositive uterine glandular cells as well as stromal cells in both the superficial (functionalis) and deep (basalis) layers of the endometrium were quantitated. TGF- β staining was also assessed in glands and stroma of the functionalis and basalis. Staining was graded for each cell type and endometrial location as 0 (unstained) to 3+ (heavily stained) by three reviewers, including a veterinary pathologist (J.M.C.). All observers were again blinded to treatment.

Statistical Analysis. Both the percentage of caspase-3-expressing cells and the semiquantitative assessments of immunostaining for TGF- β isoforms were analyzed for treatment group as continuous variables using ANOVA

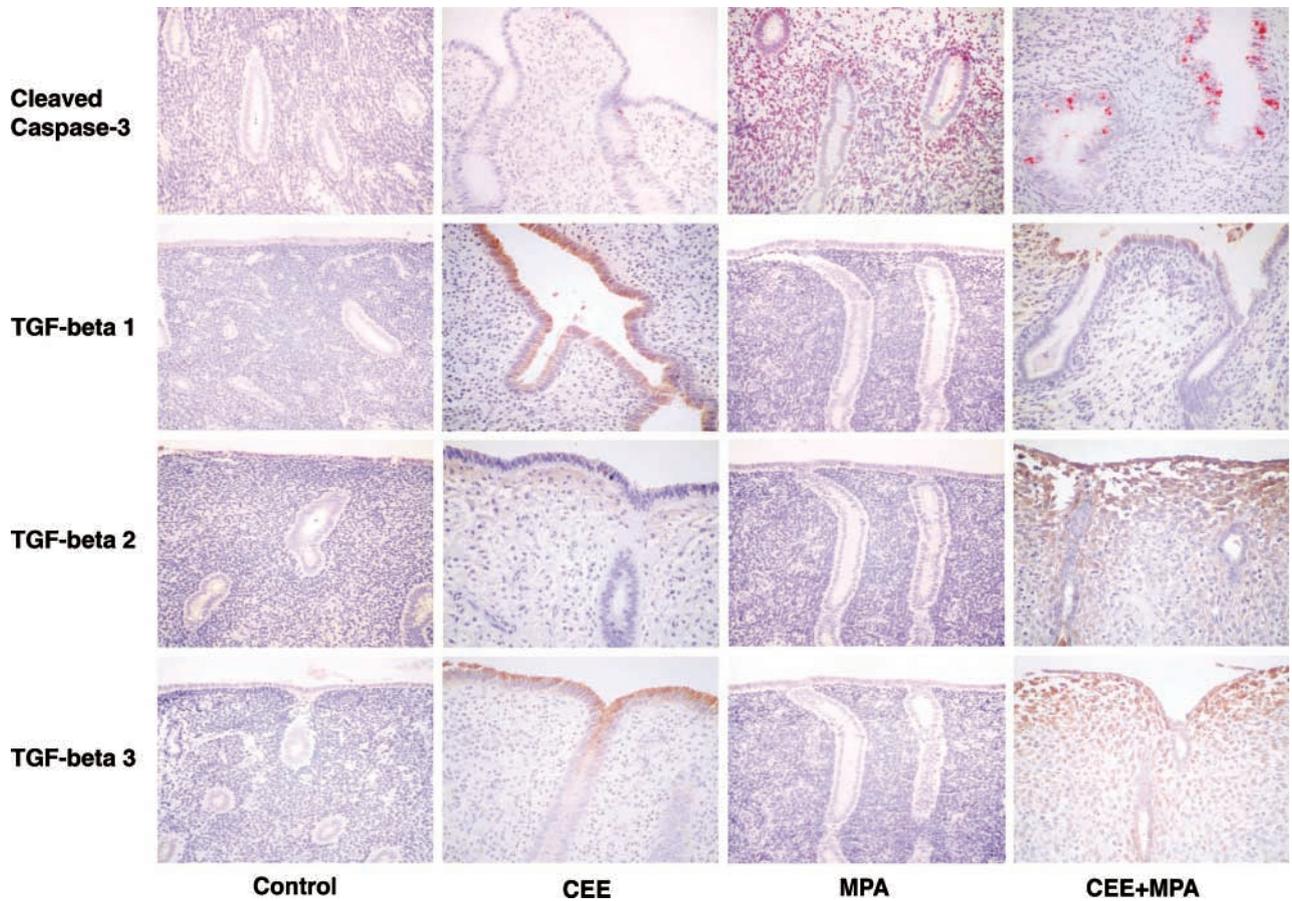


Figure 1. Representative sections of superficial endometrium (functionalis) from each of the treatment groups, immunostained for activated caspase-3 and the TGF- β isoforms.

and a two-tailed significance level of 0.05. Pairwise *t* tests were used to identify specific group differences if the overall ANOVA showed significance. Within-group correlation analysis was done using Pearson correlation coefficients to identify associations between dependent variables. In addition, the data for TGF- β were also analyzed via a binary method. For dichotomized TGF- β [low intensity of staining or low expression (0-1+) versus high intensity of staining or high expression (2-3+)], Fisher's exact test was used to investigate the effects of treatment group to TGF- β expression. Logistic regression modeling was used to determine the importance of the effects (33). Tests were two tailed with significance level of 0.05. Bonferroni-adjusted significance level was applied while the effect to TGF- β was compared between treatment groups.

Results

Hormone treatment-related changes in apoptosis, proliferation, and TGF- β expression were confined primarily to the functionalis (superficial) compartment of the endometrium. The data presented therefore represent findings confined to the superficial endometrial glands and stroma.

Effect of Hormone Treatment on Apoptosis in Endometrium. In general, few apoptotic cells were noted in the endometrium from either the control or CEE-only-treated monkeys. In contrast, progestin treatment with MPA, either in combination with CEE or alone, was associated with significant increases in apoptosis in both glandular and stromal cells (Fig. 1). Percentages of caspase-positive glandular epithelial cells

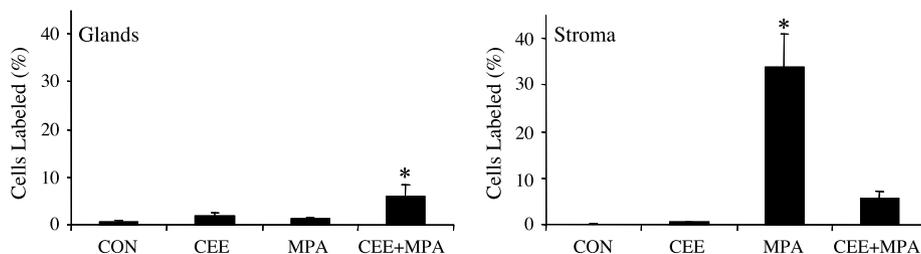


Figure 2. Cleaved caspase-3 immunostaining expressed as a percentage of cells counted in glandular and stromal compartments of the superficial (functionalis) endometrium. Bars: SEM; *: groups whose means differ from controls at *P* < 0.05.

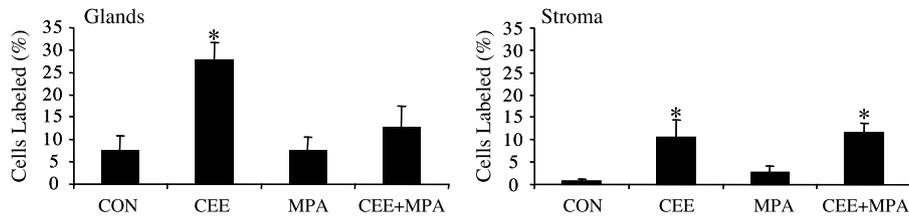


Figure 3. Ki-67 immunostaining expressed as percentage of cells counted in glandular and stromal compartments of the superficial (functionalis) endometrium. Bars: SEM; *: groups whose means differ from controls at $P < 0.05$.

were 3- to 5-fold higher in CEE + MPA-treated animals compared with all others ($P < 0.05$). In addition, caspase-3-expressing cells were six times more numerous in the stroma of animals treated with MPA alone relative to other groups ($P < 0.05$; Fig. 2).

Effect of Hormone Treatment on Proliferation in Endometrium. Expression of the proliferation marker Ki-67 was strongly induced in the glandular epithelium by treatment with CEE ($P < 0.05$). This effect was antagonized by the addition of MPA to the treatment regimen. For both CEE and CEE + MPA, proliferation was increased in the stromal compartment ($P < 0.05$; Fig. 3).

Effect of Hormone Treatment on Expression of TGF- β . Expression of the TGF- β isoforms varied throughout the endometrium relative to both treatment group and location within the endometrium. In endometrial glands, expression of TGF- β 1 was scant in both the untreated monkeys and those receiving MPA alone.

There was a nonsignificant increase in TGF- β 1 expression in CEE-treated monkeys, which was abrogated by the addition of MPA ($P < 0.01$). Stromal expression of TGF- β 1 was absent across all treatments (Figs. 1 and 4). Expression of TGF- β 2 was scant in the glandular compartment and not affected by treatment. However, expression of TGF- β 2 was markedly increased in the endometrial stroma of animals treated with CEE + MPA relative to all other groups ($P < 0.0001$; Figs. 1 and 4). For the TGF- β 3 isoform, expression in the glandular compartment was generally low and variable. MPA-treated and CEE + MPA-treated animals had lower glandular TGF- β 3 expression than CEE-treated animals ($P < 0.01$). In contrast, TGF- β 3 expression in the stroma was markedly elevated in CEE + MPA-treated animals, differing from all other groups ($P < 0.0001$; Figs. 1 and 4). Of note, analysis of the TGF- β scoring data as ordinal (stepwise) data rather than continuous variables yielded similar results.

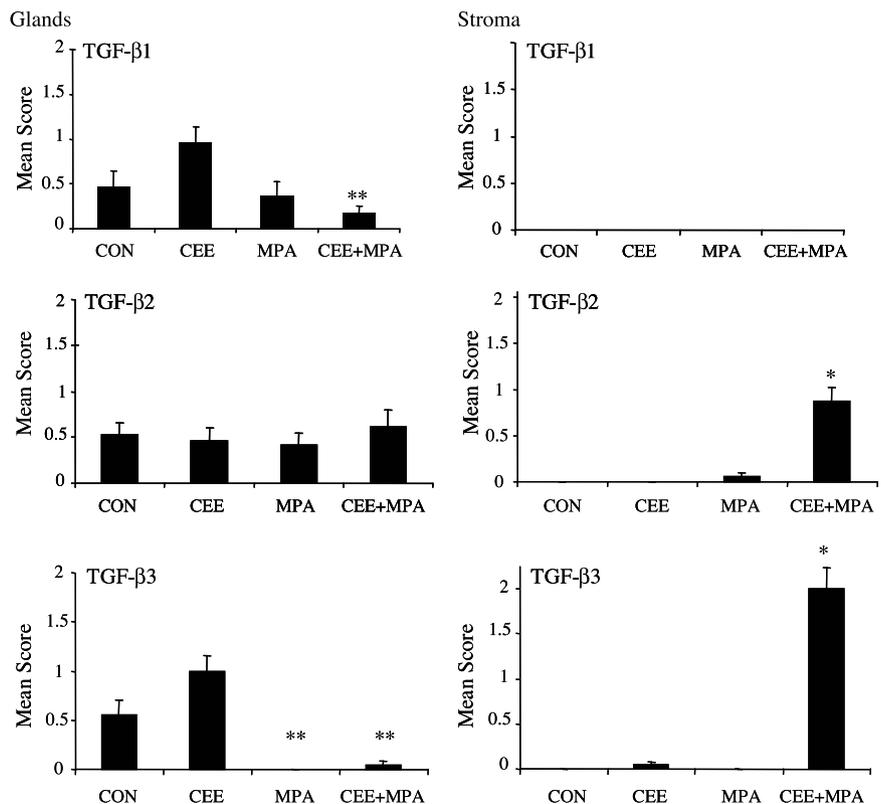


Figure 4. TGF- β 1 immunostaining expressed as mean staining score in superficial (functionalis) endometrial glands and stroma. Bars: SEM; *: groups whose means differ from controls at $P < 0.0001$ or **: groups whose means differ from CEE at $P < 0.01$.

Discussion

In this study, we have characterized the complex opposing effects of estrogen and progesterin on proliferation, apoptosis, and TGF- β signaling in the uterine endometrium. In an ovariectomized menopausal primate model, we have shown that estrogen induces proliferation in endometrial glands while having no effect on apoptosis. In contrast, progesterin inhibits estrogen-induced proliferation and differentially regulates expression of TGF- β , causing a decrease in expression of TGF- β 3 in endometrial glands while dramatically increasing expression of the TGF- β 2 and TGF- β 3 isoforms in the stroma. In addition, progesterin markedly induces apoptosis in both endometrial glands and superficial stroma. Areas of marked apoptosis in both endometrial glands and stroma associated with progesterin treatment occurred concurrently with marked increased expression of TGF- β 2 and TGF- β 3 in the superficial stroma, suggesting that these two biological events may be related. Finally, progesterin-mediated apoptotic effects in endometrial glands occurred only in the presence of estrogen, suggesting the requirement for estrogen priming, whereas estrogen was not required for progesterin to induce marked apoptotic effects in the endometrial stroma.

Overall, our findings are consistent with published data regarding the effect of progestins on apoptosis and TGF- β signaling in the endometrium. Progestins have been shown to induce apoptosis in endometrial glands and stroma, an effect that is modulated by several well-known apoptosis-related proteins (22, 34-39). In the mouse, an increase in the Bax/Bcl-2 ratio and associated increase in apoptosis have been shown in endometrial stromal cells in response to progestins, suggesting that progesterin-mediated apoptotic effects contribute to the remodeling changes in the uterus associated with decidualization (35). In women, up-regulation of Fas/Fas ligand expression has been shown in endometrial cells in response to progestins; conversely, dysregulation of Fas/Fas ligand expression in hyperplastic endometrium may underlie failure of progestins to reverse endometrial hyperplasia (37). In addition, progestins have been shown to increase the Bax/Bcl-2 ratio and induce apoptosis *in vivo* in women undergoing treatment for endometrial hyperplasia. Moreover, the degree of change in Bax, Bcl-2, and apoptosis has been shown to be correlated with the potency of progesterin therapy and in turn with the likelihood of response to therapy (22). Thus, activation of apoptotic pathways may be an important mechanism underlying the chemopreventive effects of progestins in the endometrium. With regard to TGF- β , both TGF- β 2 and TGF- β 3 have been shown to be increased in the endometrium during the secretory phase or in response to progestins *in vivo* (40-43), whereas expression of TGF- β 1 was unchanged (40). Similarly, in the primate endometrium in response to MPA, we found overall levels of TGF- β 2 and TGF- β 3 to be dramatically increased without a significant effect on TGF- β 1.

Our findings in the endometrium bear remarkable similarities to what we described previously in the ovary. Previously, we have shown that progesterin induces apoptosis and differentially regulates expression of TGF- β in the ovarian surface epithelium (23). We

observed a 4-fold increase in apoptosis in the ovarian epithelium in primates treated with the progesterin levonorgestrel. This was associated with a significant increase in expression in the ovarian surface epithelium of the TGF- β 2 and TGF- β 3 isoforms and a decrease in expression of TGF- β 1. In the endometrium, our findings were similar, except for the absence of an effect of progesterin on TGF- β 1 expression, although the addition of MPA to CEE lessened expression of TGF- β 1 in endometrial glands, a trend similar to our prior findings in the ovarian surface epithelium. In addition, in the ovary, changes in expression of TGF- β were localized specifically in the ovarian surface epithelium, whereas the predominant change in expression of TGF- β in the endometrium was primarily in the superficial stroma. Thus, in the ovary, changes in expression of the TGF- β isoforms in the ovarian surface epithelium may have an autocrine and paracrine effect on the ovarian surface epithelium, whereas, in the endometrium, stromal changes in expression of TGF- β may have an autocrine effect in stromal cells and a paracrine effect in adjacent glandular cells. In addition, in the ovarian surface epithelium, increases in expression of TGF- β 2 and TGF- β 3 were noted with progesterin administered alone, whereas, in the endometrium, induction of expression of these two isoforms in the superficial stroma by progesterin required the presence of estrogen. It is possible however that estrogen priming is necessary for optimization of progesterin effects on TGF- β in both the endometrium and ovary. For example, our earlier study evaluating progesterin in the ovarian surface epithelium used premenopausal monkeys who had not undergone ovariectomy and thus received progesterin in the setting of an adequate endogenous estrogenic milieu. Conversely, the subtle differences in progesterin effect in our prior ovarian study and the current study may be due to differences in the progestins administered. Our prior study involved levonorgestrel, a potent gonane progesterin that is a nortestosterone derivative typical in oral contraceptives. However, the current study used MPA, a pregnane progesterin typically used in hormone replacement therapy regimens that has significantly less potency than oral contraceptive progestins. To illustrate the potential differences between these classes of compounds, use of oral contraceptives markedly reduces endometrial cancer risk (11-15), whereas the addition of MPA to hormone replacement therapy estrogens mainly abrogates the cancer-causing effect of estrogen replacement therapy rather than markedly reducing endometrial cancer risk (4, 5, 9). It is possible that a hormone replacement therapy regimen that contains a potent progesterin such as those in oral contraceptives might actually lower endometrial cancer risk. Nonetheless, the finding that progestins have similar biological effects in the ovarian surface epithelium and endometrium suggests a common biological mechanism that may underlie the cancer preventive effect of progestins in both tissues.

The design of our study does not allow us to prove a causal relationship between changes in TGF- β expression and apoptosis in endometrial glands and stroma. However, it is possible that the two are related. Among the growth factors, TGF- β has been implicated as an important regulator of apoptosis (44-46). In addition, in cells derived from the ovarian and uterine epithelium, TGF- β has been shown to induce apoptosis (47-50).

Furthermore, in hormone-sensitive tissues, such as the breast and prostate, TGF- β has been shown to mediate the apoptotic effects of steroid hormones, including the antiestrogens, retinoids, and vitamin D (51-55). Finally, TGF- β is related to Müllerian inhibitory factor, a peptide that causes complete apoptotic regression of the Müllerian system (the precursor to the uterus, fallopian tubes, and upper vagina) in the developing male embryo *in vivo* (56-59), and which has been shown to inhibit growth and induce apoptosis in cells derived from the ovarian and uterine epithelium *in vitro* (60-62). Given the marked inhibitory effect that the members of the TGF- β family have on the Müllerian system, it is interesting to speculate that the uterine endometrium may be uniquely susceptible to a TGF- β -induced apoptotic response *in vivo*. Moreover, agents which selectively regulate TGF- β in the endometrium, may be excellent candidates for further research as endometrial chemopreventive agents.

A growing body of laboratory and *in vivo* evidence has implicated TGF- β as a potent tumor suppressor and cancer preventive agent (63-66). Transgenic mice have shown both resistance to induced mammary tumors when TGF- β 1 is constitutively expressed as well as increased susceptibility to carcinogens when TGF- β genes are deleted (67, 68). In humans, mutations have been described in the TGF- β signaling pathway in a variety of tumors, including cancers of the colon, gastric, pancreatic, uterus, and lymphoid system (65, 69-73). Furthermore, several cellular oncogenes are known to inhibit TGF- β activity. Finally, TGF- β has been implicated as a mediator of the biological effects of several chemopreventive agents, including tamoxifen and retinoids (64, 73). Taken together, these data provide compelling evidence that TGF- β plays an important role as an inhibitor of carcinogenesis and that agents which exploit this pathway may have utility for the chemoprevention of cancer.

Similarly, the apoptosis pathway also holds great promise as a target for chemoprevention. Activation of apoptosis leads to the efficient disposal of cells that have undergone irreparable genetic damage and that are prone to neoplastic transformation (74). Pharmacologic agents that selectively enhance apoptosis have been shown to lower the risk of a variety of cancers in animals and in humans (74, 75). In addition, both in animal models of cancer and in humans, the efficacy of cancer preventive agents has been shown to correlate with the degree of apoptosis induced (22, 74, 76, 77). Conversely, mutations in the genes involved in apoptotic pathways have been shown to be associated with enhanced cancer risk (77).

In light of the known association between activation of TGF- β and apoptotic molecular pathways and cancer prevention, the observation that progestins markedly activate these pathways in the endometrium opens the door toward development of a progestin-based strategy for the effective chemoprevention of endometrial cancer. Moreover, given that progestins have similar chemopreventive biological effects in both the ovary and the endometrium, it is interesting to speculate that a potent progestin-based strategy may effectively prevent both ovarian and uterine cancer, thereby decreasing the incidence and mortality from the two most common gynecologic malignancies.

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Cancer Epidemiol Biomarkers Prev 2008;17:578-584.

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