Progestin-Induced Apoptosis in the Macaque Ovarian Epithelium: Differential Regulation of Transforming Growth Factor-β

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Background: **Oral contraceptive (OC) use is associated with a reduced risk of ovarian cancer. An OC component, progestin, induces apoptosis in the primate ovarian epithelium. One regulator of apoptosis is transforming growth factor-β (TGF-β). We determined the effect of progestin on TGF-β expression in the primate ovarian epithelium and examined the relationship between TGF-β expression and apoptosis.** ***Methods:*** **Female cynomolgus macaques were randomly assigned to receive a diet for 35 months containing no hormones (n = 20); the OC Triphasil (n = 17); or each of its constituents, ethinyl estradiol (estrogen, n = 20) or levonorgestrel (progestin, n = 18), alone. Ovarian sections were immunostained with monoclonal antibodies against TGF-β1 or TGF-β2 plus TGF-β3 (TGF-β2/3) isoforms. The expression of TGF-β isoforms in four ovarian compartments (epithelium, oocytes, granulosa cells, and hilar vascular endothelium) was compared among treatment groups. The association between TGF-β expression and apoptosis, as determined by morphology and histochemistry, was examined in ovarian epithelium. All statistical tests were two-sided.** ***Results:*** **Compared with ovaries from the control and estrogen-only-treated monkeys, the ovaries of progestin-treated monkeys showed 1) a marked decrease in the expression of TGF-β1 and a concomitant increase in the expression of the TGF-β2/3 isoforms in the ovarian epithelium ($P<.001$), 2) an increase in the expression of TGF-β2/3 in the hilar vascular endothelium ($P<.001$), and 3) a marked decrease in TGF-β2/3 expression in granulosa cells ($P<.001$). The apoptotic index of the ovarian epithelium was highly associated with the change in expression from TGF-β1 ($P<.001$) to TGF-β2/3 ($P\leqslant.002$) induced by progestin treatment.** ***Conclusions:*** **Progestin induces differential regulation in the ovarian epithelium of TGF-β, a change in the expression of which is highly associated with apoptosis. These data suggest a possible biologic mechanism for the protective association between OC use and reduced ovarian cancer risk. [J Natl Cancer Inst 2002;94:50–60]**

Epithelial ovarian cancer remains an important public health problem. It is the fourth leading cause of cancer-related deaths among women in the United States and causes over 100 000 deaths annually worldwide *(1,2).* Despite intensive research efforts over the past decade directed toward improved detection and treatment of ovarian cancer, the long-term survival of women with ovarian cancer has improved only modestly. Progress in the fight against ovarian cancer has been hampered by a number of factors, including late diagnosis, the molecular heterogeneity of ovarian tumors, the absence of highly curative chemotherapy, and the lack of a valid animal model for the disease.

The development of effective chemopreventive agents for ovarian cancer may represent our best hope for decreasing the ovarian cancer mortality rate in the future. A potent preventive agent already exists in the estrogen–progestin combination oral contraceptive (OC). Routine use of OCs for as little as 3 years confers as much as a 50% reduction in risk of ovarian cancer. The protective association increases with the duration of use and lasts for as long as 20 years after the discontinuation of use *(3–7).* It has been our belief that, if the mechanism(s) underlying the remarkable protective effect of the OC can be elucidated, it may be possible to develop a pharmacologic chemopreventive strategy that is even more protective against ovarian cancer than OCs. Moreover, it may be possible to develop a chemopreventive strategy that is more broadly applicable than the use of OCs, potentially extending the benefits of chemoprevention beyond the reproductive age group to include those women who are menopausal, a group that currently lacks a nonsurgical approach for ovarian cancer prevention.

Although the biologic mechanism underlying the protective association between OC use and reduction in the risk of ovarian cancer remains unproven, two previously cited theories have focused on the known inhibitory effect of OCs on ovulation and on the inhibitory effect of OCs on the secretion of the pituitary gonadotropins follicle-stimulating hormone and luteinizing hormone. In the first theory, the inhibition of ovulation is presumed to reduce ovarian surface trauma and thereby to reduce the potential for genetic damage in the ovarian epithelium, while the second theory suggests that lowering gonadotropin levels potentially decreases a stimulus to proliferation in the ovary *(8–11).* The ovulation-suppression theory has been challenged because the amount of risk reduction conferred by OCs far exceeds what would be predicted on the basis of the number of ovulations inhibited *(12).* Similarly, the gonadotropin theory has been criticized because of the lack of evidence of an ovarian cancer-

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See "Notes" following "References."

protective effect associated with noncontraceptive estrogen use (which lowers gonadotropin levels) and because of the absence of an association between serum levels of follicle-stimulating hormone and luteinizing hormone and ovarian cancer risk *(12,13).* Both of these theories fail to consider that the ovarian epithelium contains receptors for estrogen, progesterone, and androgen and that reproductive factors may affect ovarian cancer risk via a potent biologic interaction of sex steroid hormones with the ovarian epithelium *(14).*

Recently, we performed a study in primates demonstrating that a combination estrogen–progestin OC has a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component *(15).* Primates randomly assigned in a 3-year trial to receive either combination estrogen and progestin or progestin alone had a fourfold to sixfold increase in the proportion of apoptotic ovarian epithelial cells as compared with control or estrogen-only-treated monkeys. The apoptosis pathway is one of the most important *in vivo* mechanisms that function to eliminate cells that have sustained DNA damage and, thus, are prone to malignant transformation *(16).* In addition, a number of well-known chemopreventive agents have been demonstrated to activate the apoptosis pathway in the target tissues that they protect from neoplastic transformation *(17–32).* The finding that progestins activate this critical pathway in the ovarian epithelium suggests that the protective effects afforded by OCs against ovarian cancer may at least in part be caused by progestin-mediated apoptosis. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer.

The regulation of apoptosis is complex and is influenced by numerous families of transcriptional factors, tumor suppressor genes, oncogenes, and growth factors *(33).* Among the growth factors, transforming growth factor-β (TGF-β) has been implicated as an important regulator of apoptosis and as a mediator of the apoptotic effects of steroid hormones *(34–37).* An association between the degree of TGF-β expression and apoptosis has been shown in cells derived from the breast *(38)* and prostate *(39),* and the apoptotic activity of hormones such as the retinoids has been shown to be mediated at least in part by the activity of TGF-β *(18,19,40).* Notably, multiple members of the steroid hormone superfamily, including the retinoids, vitamin D, and sex steroids, have been shown to modulate the expression of TGF-β, and the promoter region for specific TGF-β isotypes such as TGF-β2 and TGF-β3 contains response elements suggesting hormonal and developmental regulation *(41–51).*

Given the link between TGF-β molecular pathways and apoptosis and evidence suggesting unique regulation of TGF-β by steroid hormones, we sought to determine in the current study whether there is an association between progestin-induced apoptotic effects in the primate ovarian epithelium and expression of TGF-β.

Materials and Methods

Animals/Randomization

As described previously *(15),* 130 young adult female cynomolgus macaques (*Macaca fascicularis*), with an average age of 4.75 years, were randomly assigned into a study designed to evaluate the long-term biologic effects of the contraceptive Triphasil (Wyeth Ayerst, St. Davids, PA). 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 *(52–54).* The study was a prospective, randomized, controlled trial designed for the primary endpoint of evaluating the effects of Triphasil and its individual components (ethinyl estradiol and levonorgestrel) on the cardiovascular system. Secondary outcomes to be analyzed included the biologic effects of Triphasil on the reproductive organs and breast. The randomization process was based on the serum lipid responses (total plasma cholesterol, triglycerides, and high-density lipoprotein-C) of animals to challenge with an atherogenic diet (44% of calories from fat, 0.28 mg of cholesterol per kilocalorie). After randomization, there were no differences between study groups with regard to body weight or age.

Forty of the animals were killed early in the study for baseline cardiovascular and lipoprotein studies, and an additional 14 animals died during the course of the study, primarily from trauma and diarrheal diseases. One animal was excluded because its ovarian tissue was not available for study. The remaining 75 animals were necropsied at the completion of the thirty-fifth month of the study and form the basis for this investigation. The study was approved by the Animal Care and Use Committee at the Wake Forest University School of Medicine, Winston-Salem, NC.

The macaques were prospectively randomly assigned via the lipid response parameters noted above into four groups to receive a diet for 35 months that contained 1) no hormones (control); 2) the oral combination contraceptive Triphasil, which is composed of estrogen (ethinyl estradiol) and progestin (levonorgestrel); 3) the estrogenic component of Triphasil (ethinyl estradiol) alone; or 4) the progestin component of Triphasil (levonorgestrel) alone. Hormones in the latter two groups were administered in the same dosage and schedule that occurs in a typical Triphasil regimen. Doses were scaled on the basis of caloric intake, which takes into account species differences in metabolic rate; this is the generally accepted way to achieve dosages comparable to those in women. The human-equivalent doses were given as follows: 6 days of 0.030 mg ethinyl estradiol plus 0.050 mg levonorgestrel per day, followed by 5 days of 0.040 mg ethinyl estradiol plus 0.075 mg levonorgestrel per day, followed by 10 days of 0.030 mg ethinyl estradiol plus 0.125 mg levonorgestrel per day, followed by 7 days of no hormone treatment. This cyclic regimen was repeated every 28 days continuously for 35 months. During the third week of the last month of the study, the animals were killed and their ovaries were carefully removed and preserved.

Tissue Preparation and Immunohistochemistry

From each animal in the study, one ovary was flash frozen by immersion in liquid nitrogen and saved for future molecular studies, and the other was formalin fixed and paraffin embedded.

Apoptosis. The median proportion of apoptotic ovarian epithelial cells associated with each treatment group had been quantified previously *(15).* Briefly, 5-μm sections taken from the middle of each paraffin-embedded ovary were mounted on charged slides, and the ovarian epithelium was examined for morphologic and immunohistochemical evidence of apoptosis after staining with the APOPTAG-plus kit (Oncor, Gaithersburg, MD). Dark-brown, nuclear staining easily identified cells undergoing apoptosis. Tonsillar and deoxyribonuclease-digested tissue sections were used as positive controls. To calculate the

percentage of ovarian epithelial cells undergoing apoptosis, we counted both the total number of ovarian epithelial cells and the number undergoing apoptosis on each 5-μm section. The median proportion of cells undergoing apoptosis was calculated for each treatment group. At each step in this study, including the histologic examinations of the ovaries, the investigators were blinded with regard to the treatment group associated with each ovary.

TGF-β expression. Immunohistochemical expression of TGF-β was performed as previously described, with slight modification *(55).* Briefly, 5-μm sections taken from the middle of each paraffin-embedded ovary were cut and mounted on charged slides. Two slides from each specimen were placed in a 60 °C oven for 1 hour. One slide was used as the negative control, while the other was used as the study specimen. The sections were deparaffinized, immersed in 0.3% hydrogen peroxide to quench endogenous peroxidase, hydrated, placed in Antigen Retrieval Citra solution at pH 6.0 (BioGenex Laboratories, Inc., San Ramon, CA), and then heated with an electric pressure cooker (Biocare Medical,Walnut Creek, CA) for 5 minutes. The sections were then cooled and rinsed with three washes of phosphate-buffered saline, preincubated in Power Block (BioGenex Laboratories, Inc.) for 10 minutes, and then incubated for 18 hours (overnight) at 4 °C in a humid chamber with primary antibody. For TGF-β1 expression, sections were immunostained with a monoclonal antibody that reacts with TGF-β1 but not TGF-β2 or TGF-β3 (2.5 μg/mL anti-TGF-β1 monoclonal antibody, catalog No. MAB 240; Research and Development Systems (Minneapolis, MN). To evaluate TGF-β2 and TGF-β3 (TGF-β2/3) expression, we stained sections with a mouse monoclonal antibody that reacts with the N-terminal region of both TGF-β2 and TGF-β3 but has no cross-reactivity with TGF-β1 (0.25 μg/mL TGF-β3 mouse monoclonal antibody; Oncogene Research Products, Cambridge, MA). For negative control specimens for TGF-β1 and TGF-β2/3 staining, mouse immunoglobulin G antibody (Coulter Corporation, Miami, FL) was applied at concentrations of 2.5 and 0.25 μg/mL, respectively. Slides were then washed three times with phosphate-buffered saline for 5 minutes each. Application of a biotinylated secondary antibody (Multi-Link Super Sensitive Detection System; BioGenex Laboratories) was performed at room temperature in a humid chamber for 20 minutes, then followed by three washes in phosphate-buffered saline for 5 minutes each. Peroxidase-conjugated streptavidin (Multi-Link Super Sensitive Detection System) was applied to sections and allowed to incubate for 20 minutes in a humid chamber, then followed by three washes in phosphate-buffered saline for 5 minutes each. Slides were incubated with freshly prepared 3,3-diaminobenzidine (D5637; Sigma Chemical Co., St. Louis, MO) chromogen solution (0.5% 3,3-diaminobenzidine, 0.6% hydrogen peroxide, and 0.05% Tris buffer) for 4 minutes and then washed in deionized water for 5 minutes to stop the reaction. This was followed by a 5-minute incubation in a 0.1 *M* sodium acetate solution and then staining with 1.5% methyl green for 5 minutes. Sections were dipped 10 times each in a serial fashion in the following solutions: 95% acetone, 95% acetone, 100% acetone, 100% acetone, 100% xylene, 100% xylene, and 100% xylene; then coverslips were placed on the slides. Umbilical cord sections, stained in a similar fashion, were used as positive control *(56).*

The ovarian sections were examined by two independent sets of reviewers, all of whom were blinded to the hormone administration data (R. C. Bentley and K. L. Lee for TGF-β1 staining; R. C. Bentley and N. P. Nagarsheth for TGF-β2/3 staining). Staining for TGF-β was evaluated in four separate ovarian compartments of each study slide (ovarian surface epithelium, primordial oocyte cytoplasm, granulosa cells of tertiary follicles, and endothelium in ovarian hilar vessels) and graded according to the degree of staining intensity from 0 to 3+ (TGF-β1) and from 0 to 4+ (TGF-β2/3). High expression of TGF-β1 was defined by the slide reviewers as 2+ to 3+ staining intensity, whereas high expression of TGF-β2/3 was defined as 3+ to 4+ staining intensity. Three ovarian sections in the TGF-β1 staining group and two ovarian sections in the TGF-β2/3 staining group were excluded from grading because the samples were technically insufficient for evaluation.

Statistical Analysis

Quantitation and comparison of the median proportion of apoptotic cells in the ovarian epithelium had been performed previously *(15).* Briefly, the Kruskal–Wallis test was used to perform multiple comparisons of all paired treatments *(57),* and the statistical analysis was carried out with the use of the BMDP statistical software package (Biomathematical Data Package Statistical Software, Inc., Los Angeles, CA) *(58).* For this study, the association between expression of the TGF-β isoforms and treatment was analyzed with the use of an overall approximate exact test for contingency tables *(59).* In addition, each 2 × 2 table involving treatment and control was analyzed by use of Fisher's two-sided exact test. The relationship between treatment, amount of expression of TGF-β in the ovarian epithelium, and the mean proportion of apoptotic ovarian epithelial cells was analyzed by use of the general linear model (PROC GLM in the SAS statistical package; SAS Institute, Cary, NC) *(60).* Multiple comparisons were performed with the use of Dunnett's two-sided test for each treatment compared with the control. The relationship between the proportion of high TGF-β expression and the mean proportion of apoptotic cells across treatments was analyzed by use of standard correlation analysis. The association between the TGF-β isoforms with respect to overexpression was analyzed with the use of the κ statistic *(61).* All statistical tests were two-sided.

Results

Effect of Hormone Treatment on Expression of TGF-β

In general, in ovarian sections from the control group of monkeys, the pattern of expression of TGF-β was qualitatively similar to the pattern described previously in the human ovary *(62–65).* In untreated monkeys (Fig. 1, A) and in estrogen-treated monkeys (Fig. 1, B), TGF-β1 expression was abundant in the ovarian epithelium and low to moderate in the stroma (structural tissue under epithelium) and the oocyte cytoplasm. Exposure to progestin either with estrogen (Fig. 1, C) or alone (Fig. 1, D) was associated with a marked decrease in the expression of TGF-β1 in the ovarian epithelium and in the oocyte compartment (*see* arrows in Fig. 1, B and D). The endothelial cells of the vascular hilum had little detectable expression of TGF-β1 (data not shown). Panels E–H in Fig. 1 represent the staining controls for monkey ovaries from four treatments, respectively.

The pattern of expression of TGF-β2/3 in untreated monkey ovaries was distinctly different from that of TGF-β1. Expression

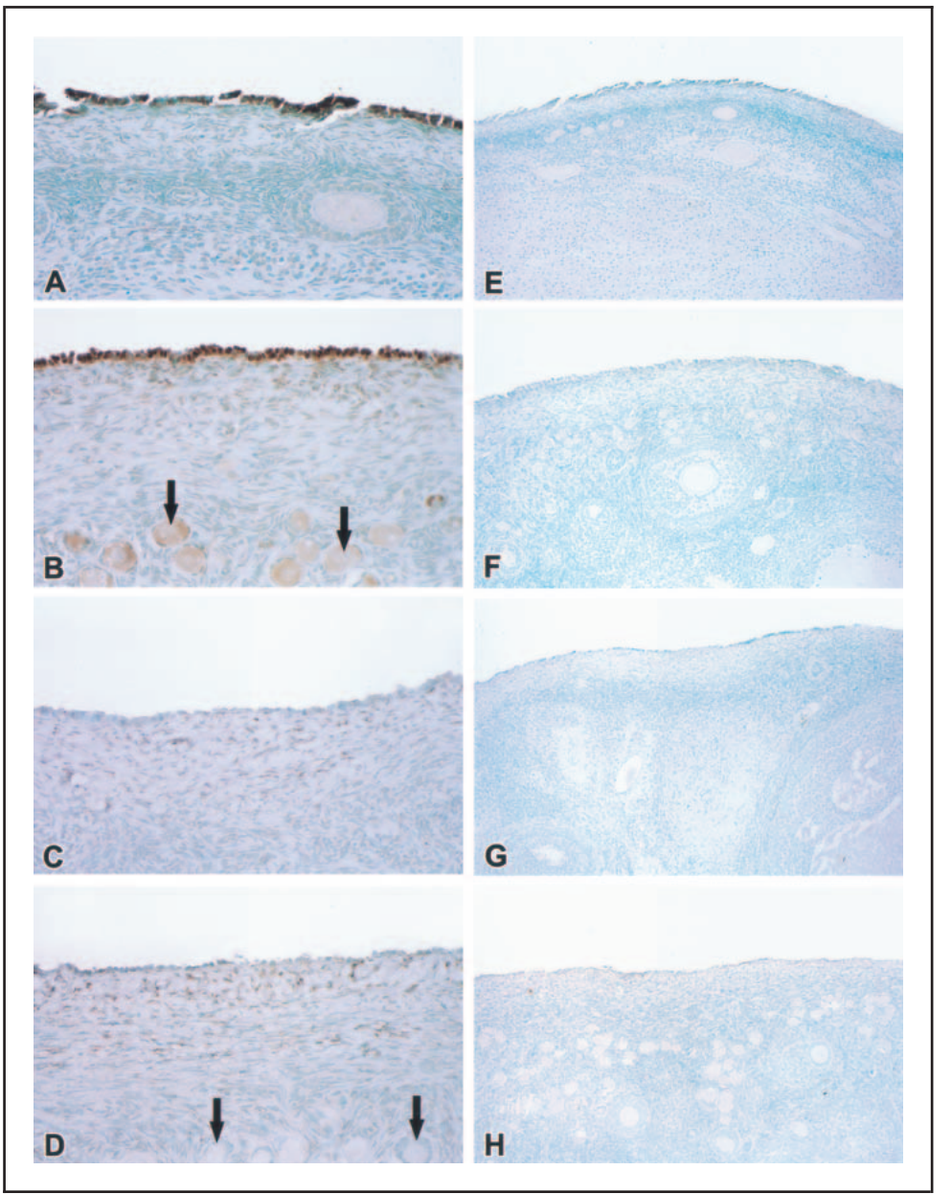

Fig. 1. Representative sections (original magnification ×80) of ovaries from macaques receiving four different hormone treatments were immunostained with anti-transforming growth factor (TGF)-β1 antibody (**A** = control [no treatment]; **B** = ethinyl estradiol alone; **C** = ethinyl estradiol plus levonorgestrel; **D** = levonorgestrel alone). TGF-β1 expression is abundant in the surface layer of ovarian epithelial cells in control (**A**) and estrogen-only-treated monkeys (**B**), and expression was markedly decreased in the progestin-treated monkeys (**C, D**). Progestin treatment (**D**) compared to estrogen treatment (**B**) was also associated with decreased expression of TGF-β1 in the oocyte compartment (*see arrows*). Negative controls for **A**–**D** stained with isotype-matched nonspecific mouse immunoglobulin G are shown in **E**–**H,** respectively.

of TGF-β2/3 was absent to scant in the ovarian epithelium, was high in the primordial oocyte cytoplasm, and was high in granulosa cells in large developing follicles (Fig. 2, A and B). Ovaries from estrogen-treated monkeys showed similar expression of TGF-β2/3 in epithelium and oocyte compartment (Fig. 2, D and E). Panels C and F in Fig. 2 are the respective staining controls for ovaries from control and estrogen-treated monkeys.

Weak signals for TGF-β2/3 expression were detected in endothelial cells in the ovarian hilum (Fig. 3, A) of untreated monkeys. The estrogen treatment resulted in practically no change (Fig. 3, B) in this expression. However, progestin treatment when either given in combination with estrogen (Fig. 3, C) or alone (Fig. 3, D) was associated with a marked increase in the expression of TGF-β2/3 in endothelial cells. The progestin treatment with or without estrogen also was associated with a marked increase in the expression of TGF-β2/3 in the ovarian surface epithelium but a decreased expression in granulosa cells in large, developing follicles (Fig. 4, A and B, and D and E, respectively, and asterisks Figs. 2, D, 4, A, and 4, D). Panels C and F in Fig. 4 were the staining controls for Fig. 4, A and B, and for Fig. 4, D and E, respectively.

The pattern of expression of TGF-β in the ovaries of primates receiving estrogen alone was similar to that in the control group (*see* Fig. 1, A, versus 1, B, for TGF-β1 in surface epithelium; Fig. 2, A and B, versus Fig. 2, D and E, for TGF-β2/3 in primordial oocytes and granulosa cells, and Fig. 3, A, versus 3, B, for TGF-β2/3 in hilar endothelial cells), suggesting that estrogen does not regulate expression of TGF-β in the ovary.

Effect of Hormone Treatment on Apoptosis in Ovarian Epithelium

In general, few apoptotic cells were noted in the ovarian epithelium from either the control or estrogen-only-treated monkeys (Fig. 5, A and B). In contrast, in progestin-treated monkeys, either those treated with combination ethinyl estradiol and levonorgestrel or with levonorgestrel alone, the ovarian epithelium contained numerous brown-staining apoptotic cells (Fig. 5, C and D). Additional morphologic findings in the ovarian epithe-

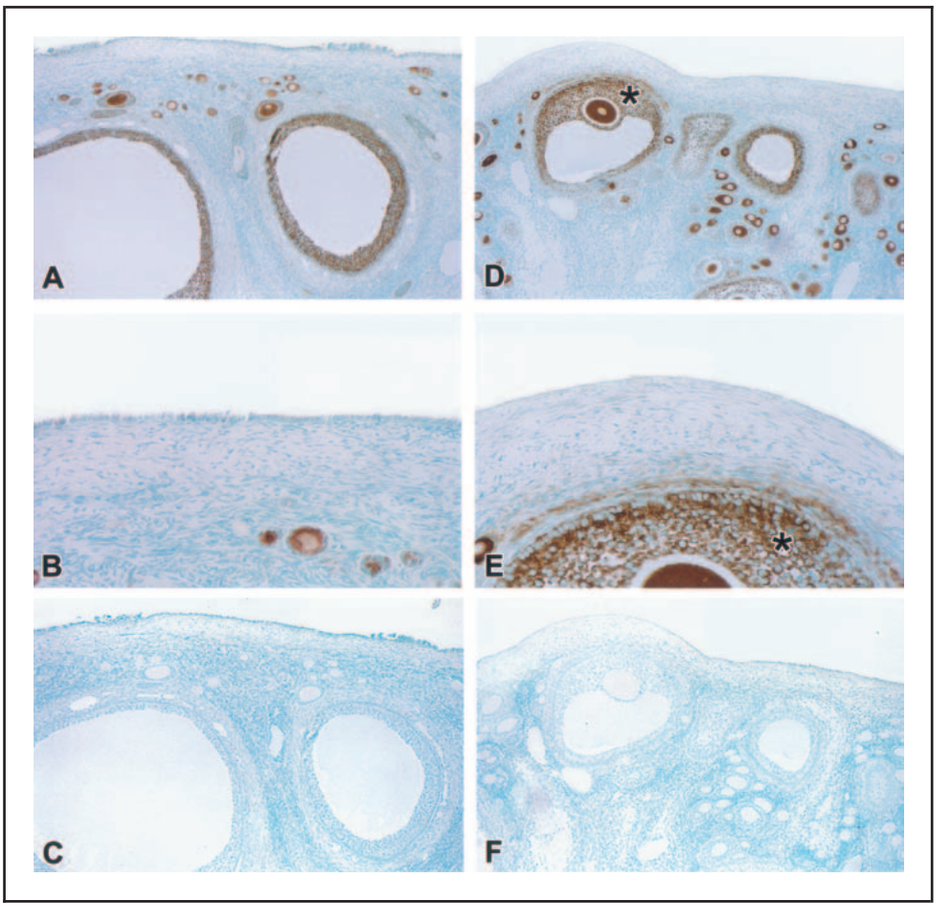

Fig. 2. Representative ovarian sections from control (no treatment) (**A,** original magnification ×25; **B,** original magnification ×80) and ethinyl estradiol-only treated (**D,** original magnification ×25; **E,** original magnification ×80) macaques immunostained with anti-transforming growth factor (TGF)-β2/3 antibody showing marked expression of TGF-β2/3 in primordial oocytes and granulosa cells (*) in large, developing follicles and little detectable expression of TGF-β2/3 in the ovarian surface epithelial layer. **C** and **F:** negative controls for **A** and **B** and **C** and **D,** respectively, stained with isotype-matched nonspecific mouse immunoglobulin G.

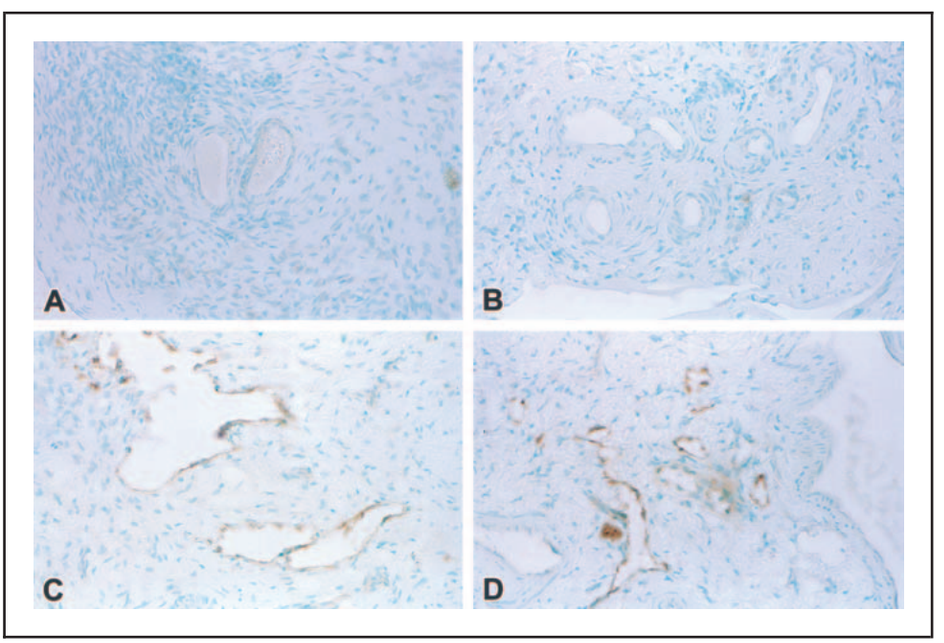

Fig. 3. Representative sections of the macaque ovarian hilum from the four hormone treatment groups immunostained with anti-transforming growth factor (TGF)-β2/3 antibody (**A** = control; **B** = ethinyl estradiol alone; **C** = ethinyl estradiol plus levonorgestrel; **D** = levonorgestrel alone). **C** and **D:** progestin treatment associated with a marked increase in expression of TGF-β2/3 in endothelial cells.

lium of progestin-treated monkeys included patches of ovarian surface devoid of epithelium, epithelial cells with sparse cytoplasm that appeared to be detaching from the surface, areas of epithelial denudation, and brown-staining apoptotic cells containing apoptotic bodies. The apoptotic changes noted in the ovarian epithelium of progestin-treated monkeys were not associated with any change in the proliferative index of the ovarian epithelium via staining for Ki-67 (data not shown).

Semiquantitative Determination of TGF-β Expression

Tables 1–3 summarize semiquantitative measurements of the hormonal regulation of TGF-β1 and TGF-β2/3 expression *in*

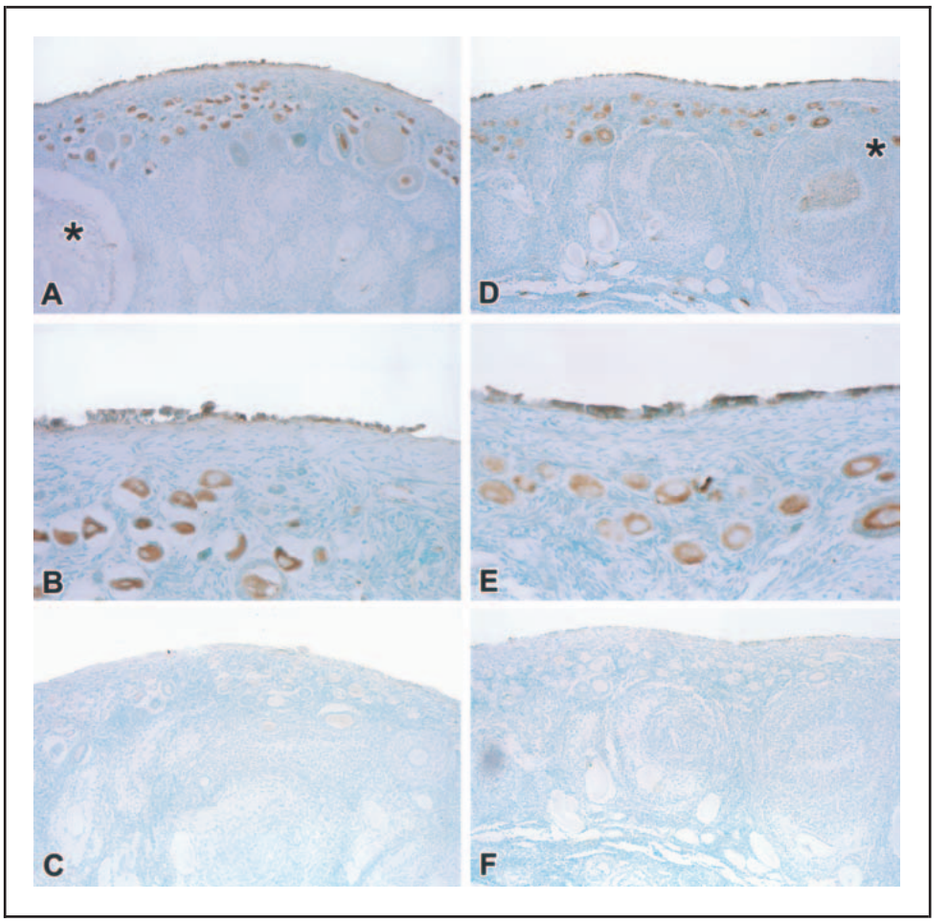

Fig. 4. Representative ovarian sections from combination estrogen–progestin-treated (**A,** original magnification ×25; **B,** original magnification ×80) and levonorgestrel-only treated (**D,** original magnification ×25; **E,** original magnification ×80) macaques immunostained with anti-transforming growth factor (TGF)-β2/3 antibody showing marked expression of TGF-β2/3 in the ovarian surface epithelium and decreased expression of TGF-β2/3 in granulosa cells (*) in large developing follicles. **C** and **F:** negative controls for **A** and **B** and **D** and **E,** respectively, stained with isotype-matched nonspecific mouse immunoglobulin G.

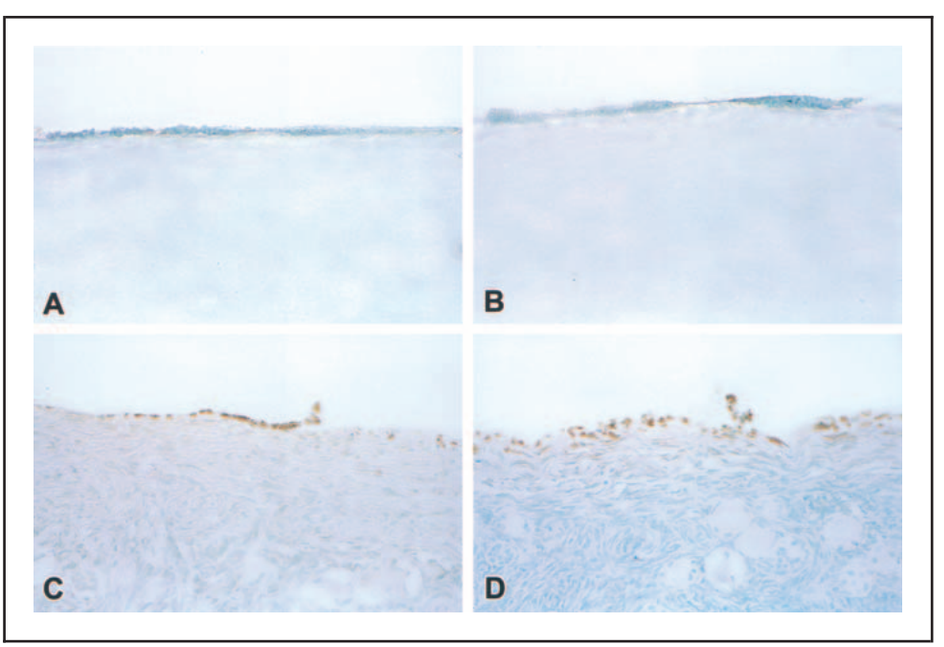

Fig. 5. Apoptag staining of representative macaque ovarian sections from the four hormone treatment groups (**A** = control; **B** = ethinyl estradiol alone; **C** = ethinyl estradiol plus levonorgestrel; **D** = levonorgestrel alone) showing marked apoptosis in the ovarian epithelium associated with progestin treatment (**C, D**) (original magnification ×80).

vivo. Progestin treatment, either combined with estrogen (Triphasil group) or administered alone (levonorgestrel group), was associated with a striking and highly statistically significant decrease in the expression of TGF-β1 in the ovarian epithelium (P<.001) and a moderate decrease in the expression of TGF-β1 in the oocyte cytoplasm (P = .002) (Table 1). In contrast, progestin treatment was associated with a marked increase in the expression of TGF-β2/3 in the ovarian epithelium (P<.001) (Table 2). Without exception, TGF-β2/3 expression in the ovarian epithelium was high (3+ to 4+ staining) in every monkey on

Table 1. Hormonal regulation of expression of transforming growth factor (TGF)-β1 in the macaque ovary

Treatment group	No. (%) ovaries/treatment group with high TGF-β1 expression (2+ to 3+) in each ovarian compartment				
	No.	Epithelium	Granulosa cells	Oocytes	Endothelium
Control	20	18 (90%)	7 (35%)	7 (35%)	0 (0%)
Ethinyl estradiol	19	16 (84%)	4 (21%)	2 (13%)	0 (0%)
Triphasil	16	3 (19%)	2 (13%)	0 (0%)	0 (0%)
Levonorgestrel	17	1 (6%)	2 (12%)	0 (0%)	0 (0%)
Overall approximate exact test		$P<.001$	$P = .31$	$P = .002$	$P = 1.00$
Control vs. ethinyl estradiol		$P = .66$	$P = .48$	$P = .13$	$P = 1.00$
Control vs. Triphasil		$P<.001$	$P = .24$	$P = .01$	$P = 1.00$
Control vs. levonorgestrel		$P<.001$	$P = .14$	$P = .009$	$P = 1.00$

Table 2. Hormonal regulation of expression of transforming growth factor (TGF)-β2/3 in the macaque ovary

Treatment group	No. (%) ovaries/treatment group with high TGF-β2/3 expression (3+ to 4+) in each ovarian compartment				
	No.	Epithelium	Granulosa cells	Oocytes	Endothelium
Control	19	6 (32%)	12 (63%)	14 (74%)	5 (26%)
Ethinyl estradiol	20	2 (10%)	8 (38%)	17 (81%)	3 (23%)
Triphasil	17	17 (100%)	1 (6%)	16 (94%)	16 (94%)
Levonorgestrel	17	17 (100%)	1 (6%)	14 (82%)	16 (94%)
Overall approximate exact test		$P<.001$	$P<.001$	$P = 0.45$	$P<.001$
Control vs. ethinyl estradiol		$P = .13$	$P = .11$	$P = .72$	$P = .72$
Control vs. Triphasil		$P<.001$	$P<.001$	$P = .18$	$P<.001$
Control vs. levonorgestrel		$P<.001$	$P<.001$	$P = .70$	$P<.001$

Table 3. Relationship between treatment, transforming growth factor (TGF)-β expression, and apoptosis in the macaque ovarian epithelium

Treatment	TGF-β1			TGF-β2/3		
	No.	% 2+ to 3+	Mean proportion of apoptotic cells in ovarian epithelium (95% CI*)	No.	% 3+ to 4+	Mean proportion of apoptotic cells in ovarian epithelium (95% CI*)
Control	20	90	6.3 (3.0 to 9.6)	19	32	6.4 (2.8 to 10)
Ethinyl estradiol	19	84	6.2 (1.8 to 10.6)	20	10	4.5 (1.2 to 7.8)
Triphasil	16	19	22.3 (13.6 to 31.0)†	17	100	21.2 (12.7 to 29.7)‡
Levonorgestrel	17	6	25.1 (16.0 to 34.2)†	17	100	26.4 (17.7 to 35.1)†

*CI = confidence interval for the mean.

†$P<.001$ by Dunnett's test comparing mean apoptotic index seen after treatment with that seen in controls (no treatment).

‡$P = .002$ by Dunnett's test comparing mean apoptotic index seen after treatment with that seen in controls (no treatment).

progestin (n = 34). Similarly, there was a significant increase in TGF-β2/3 expression in the ovarian hilar endothelial cells in monkeys on progestin ($P<.001$). In contrast, progestin treatment was associated with a marked decrease in TGF-β2/3 expression in granulosa cells ($P<.001$) (Table 2).

Within the ovarian epithelial compartment, comparison of the apoptotic index with the degree of change in the expression of the TGF-β isoforms revealed a highly significant correlation between changes in TGF-β expression and apoptosis ($P<.001$) (Table 3). With the use of the general linear model, for TGF-β1 there was a statistically significant treatment effect ($P<.001$) with respect to the mean proportion of apoptotic cells. Table 3 gives the mean proportion of apoptotic cells for each treatment group and shows that the Triphasil and levonorgestrel groups differ significantly from the control group ($P<.001$ for both comparisons with the use of Dunnett's test). Similarly, for TGF-β2/3 there was also a statistically significant treatment effect ($P<.001$), and Table 3 shows that the Triphasil and levonorgestrel groups differ statistically significantly from the control group ($P = .002$, and $P<.001$, respectively, by Dunnett's test). The Pearson correlation coefficients between the proportion of high TGF-β expression and the mean proportion of apoptotic cells across treatments were –0.998 ($P = .002$) for TGF-β1 and 0.973 ($P = .03$) for TGF-β2/3. Finally, overall, there was a negative association between TGF-β2/3 overexpression and TGF-β1 overexpression ($\kappa = -0.62$; $P<.001$). Taken together, these data demonstrate the novel finding that progestin-induced apoptosis in the ovarian epithelium is associated with an isoform switch in expression of TGF-β.

Discussion

To the best of our knowledge, this is the first study to demonstrate regulation of TGF-β expression in the primate ovarian epithelium *in vivo* by a contraceptive steroid. We found TGF-β expression to be differentially regulated in the ovarian epithelium of primates that received progestin, administered either in the form of an estrogen–progestin combination pill or alone. Progestin treatment was associated with a marked decrease in expression of TGF-β1 concomitant with a marked increase in expression of TGF-β2/3. In addition, the progestin-induced change in TGF-β isoform expression was highly correlated with

an increase in apoptosis in the ovarian epithelium. Estrogen treatment appeared to have no impact on TGF-β expression in the ovary.

The mechanism underlying progestin regulation of TGF-β expression in the ovary remains to be determined. It is possible that progestins induce factors in the ovarian stroma that regulate TGF-β pathways at sites throughout the ovary via a paracrine effect. Conversely, it is possible that progestins act directly through classic progesterone receptor-mediated pathways to effect TGF-β expression. The results of this study suggest that progestin regulation of ovarian TGF-β expression occurs via a direct effect in that changes in TGF-β expression associated with progestin treatment were primarily localized to sites in the ovary known to express progestin receptors. These include the ovarian epithelium *(66)* and granulosa cells of large follicles *(67).* In addition, the finding that progestins increase the expression of TGF-β2/3 in the ovarian epithelium while at the same time decreasing expression of TGF-β2/3 in granulosa cells supports the notion not only that progestin induction of TGF-β2/3 in the ovarian epithelium is a direct effect but also that the end effect of progestins in the ovary is site specific. It is interesting that we also noted increased expression of TGF-β2/3 in the endothelial cells of the vascular hilum in progestin-treated monkeys. It has been shown recently that endothelial cells contain functional progesterone receptors and that progesterone inhibits endothelial proliferation *(68).*

There is mounting evidence that differential regulation of peptide growth factors by steroid hormones contributes to the diverse end effects of these hormones in target tissues. Among the growth factors, TGF-β has been shown to be differentially regulated by estrogens, retinoids, androgens, and vitamin D compounds. In bone, raloxifene increases the expression of TGF-β3 while having no effect on the expression of TGF-β1 and TGF-β2 *(69).* In cells derived from the breast, estradiol decreases the expression of TGF-β2 and TGF-β3 while having no effect on the expression of TGF-β1 *(70),* whereas tamoxifen has been shown to increase the expression of TGF-β1 *(71).* In chondrocytes, vitamin D increases the expression of TGF-β2 and decreases the expression of TGF-β1 and TGF-β3 *(72).* Glucocorticoids differentially regulate TGF-β in healing wounds, leading to the suppression of TGF-β1 and TGF-β2 and the increased expression of TGF-β3 *(73).* In the palates of mice, retinoids have been shown to decrease the expression of TGF-β1 while having no effect on the expression of other TGF-β isoforms *(74),* whereas in keratinocytes, induction of TGF-β2 is a major mechanism underlying the biologic effects of retinoids *(51,75).* Finally, in the male accessory organs, androgen withdrawal is associated with both apoptosis and differential regulation of TGF-β *(76).* Thus, the TGF-β isotypes appear to be differentially regulated in a tissue-specific manner. Although the mechanism underlying the complex regulation of TGF-β by hormones is not completely understood, differences in the promoter region among the TGF-β isoforms or in post-transcriptional events may be means by which TGF-β is differentially regulated *(77–80).*

Although the design of our study does not allow us to prove the causal relationship between TGF-β expression and apoptosis, the finding that changes in TGF-β expression were highly associated with apoptosis in the ovarian epithelium in primates on progestin is strongly supportive of the hypothesis that progestin-induced apoptosis may be occurring via a TGF-β-mediated molecular pathway. In addition to the findings of this study, other lines of evidence support this hypothesis. First, in hormone-responsive 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 *(40,81,82).* Second, in tissues that are progesterone receptor positive, such as the breast and endometrium, progestins have been shown to be associated with both induction of TGF-β and apoptosis *(37,41,83–88).* Third, both our group and others *(89–91)* have shown previously that some ovarian cancer cell lines undergo apoptosis when treated with TGF-β. It is interesting that, in our laboratory, we were not able to demonstrate induction of apoptosis in normal ovarian epithelial cells treated with TGF-β. It is possible, however, that the assay techniques used in our study were not sufficiently sensitive to detect apoptosis in a limited sample of normal human ovarian epithelial cells. Alternatively, it is possible that our *in vitro* experiments lacked an important cofactor present *in vivo* that is required for TGF-β-mediated apoptosis to occur or that our *in vitro* conditions failed to simulate the complex interrelationship of TGF-β isoform expression required for apoptosis to occur in nonmalignant human ovarian epithelial cells. A fourth line of evidence is that TGF-β is related to müllerian inhibitory factor, a peptide that causes complete regression of the müllerian system (the precursor to the uterus, fallopian tubes, and upper vagina) in the developing male embryo *(92–95).* In the embryo, the müllerian tract develops from an invagination of the celomic epithelium and, therefore, is derived from the same embryonic precursor tissue as the ovarian epithelium *(96).* Given the marked inhibitory effect that the müllerian inhibitory factor has on the müllerian system, it is interesting to speculate that the ovarian epithelium may be uniquely susceptible *in vivo* to undergoing apoptosis in response to TGF-β and that agents that selectively regulate TGF-β in the ovarian epithelium may be potent apoptosis-inducing agents and cancer preventive agents.

A growing body of laboratory and animal evidence has implicated TGF-β as a potent tumor suppressor and cancer preventive agent *(97–99).* Transgenic mice that have a constitutively active form of TGF-β1 are resistant to 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors *(100).* Conversely, mice with heterozygous deletions of one copy of the TGF-β gene have an increased susceptibility to chemical carcinogenesis *(101).* In humans, mutations have been described in the TGF-β signaling pathway in a variety of tumors, including cancers of colon, cancers of the gastric, pancreatic, and uterine systems, and cancers of lymphoid organs *(99).* Furthermore, a number of cellular oncogenes are known to inhibit TGF-β activity. Finally, TGF-β has been implicated as a mediator of the biologic effects of a number of chemopreventive agents, including tamoxifen, which induces expression of TGF-β1 in stromal cells in the breast *(71),* as well as retinoids, which induce TGF-β in the prostate and respiratory tract *(98).* Taken together, these data provide compelling evidence that TGF-β plays an important role as an inhibitor of carcinogenesis.

In light of the known association between TGF-β and cancer prevention, the observation that OCs markedly alter expression of TGF-β in the ovarian epithelium implicates TGF-β as possibly mediating the ovarian cancer-protective effects of the pill. The finding that OCs induce both apoptosis and TGF-β in the ovary suggests that OCs may be acting as true chemopreventive agents by activating molecular pathways known to arrest or

reverse the process of carcinogenesis, rather than simply by limiting ovulation-induced damage in the ovarian epithelium. Moreover, the finding that activation of apoptosis and differential regulation of TGF-β are related specifically to the progestin component of the OC provides strong evidence in support of the notion that biologic effects produced by the progestin component may be major mechanisms underlying the marked protection conferred by OCs against ovarian cancer.

The discovery that contraceptive progestins activate cancer-preventive molecular pathways in the ovarian epithelium opens the door to the development of a highly effective pharmacologic preventive strategy for ovarian cancer that may be more effective and more broadly applicable than OCs. For example, if the protective effects of OCs were solely a result of ovulation inhibition as previously believed, then there is little potential for designing improved OC formulations that have enhanced ovarian cancer-protective effects, and the protective effects can only be beneficial for premenopausal women who are ovulating. However, if OCs confer marked ovarian cancer protection through a biologic effect unrelated to ovulation inhibition, then it may be possible to design OC formulations that maximize these biologic effects to achieve enhanced ovarian cancer protection. In addition, it may be possible to develop a pharmacologic preventive strategy that can be applied to all women, including administration of a pharmacologic regimen that has ovarian cancer-preventive effects in postmenopausal women who are anovulatory.

The ideal preventive agent for ovarian cancer may be composed of a combination of agents that act in an additive or synergistic fashion to maximally activate molecular pathways that inhibit carcinogenesis in the ovarian epithelium, thereby maximizing ovarian cancer prevention while minimizing side effects. In this regard, agents selected from the steroid hormone superfamily on the basis of their ability to activate TGF-β are uniquely attractive. Steroid hormones would be expected to specifically target only cells expressing appropriate steroid ligand receptor. In addition, given the short half-life of active TGF-β *in vivo,* rapid clearance of TGF-β at target sites would limit the systemic toxicity associated with chemoprevention *(75).* It is interesting to speculate that the combination of a progestin, which regulates TGF-β in the ovarian epithelium, and a retinoid and/or vitamin D might achieve synergistic or additive effects on TGF-β pathways in the ovarian epithelium, leading to a powerful cancer preventive agent. Synergistic effects on both growth inhibition and apoptosis have been described *in vitro* in cells derived from ovarian epithelium with the use of the combination of vitamin A derivatives and TGF-β *(102).* Similarly, cross-talk has been described between vitamin D and TGF-β signaling pathways, and the combination of vitamin D and TGF-β has been shown to have synergistic effects *in vitro (103,104).* These approaches will be the subject of further investigation as we work toward the development of optimal chemopreventive strategies.

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NOTES

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