Effects of Oral Contraceptives or a Gonadotropin-Releasing Hormone Agonist on Ovarian Carcinogenesis in Genetically Engineered Mice

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Abstract

Although epidemiologic evidence for the ability of combined oral contraception (OC) to reduce the risk of ovarian cancer (OvCa) is convincing, the biological mechanisms underlying this effect are largely unknown. We conducted the present study to determine if OC also influences ovarian carcinogenesis in a genetic mouse model and, if so, to investigate the mechanism underlying the protective effect. LSL-K-rasG12D+/PtensloxP/loxP mice were treated with ethinyl estradiol plus norethindrone, contraceptive hormones commonly used in combined OC, or norethindrone alone, or a gonadotropin-releasing hormone agonist. The combined OC had a 29% reduction in mean total tumor weight compared with placebo (epithelial tumor weight, \(-80\%\)). Norethindrone alone reduced mean total tumor weight by 42% (epithelial tumor weight, \(-46\%\)), and the gonadotropin-releasing hormone agonist increased mean total tumor weight by 71% (epithelial tumor weight, \(+150\%\)). Large variations in tumor size affected the \(P\) values for these changes, which were not statistically significant. Nonetheless, the OC reductions are consistent with the epidemiologic data indicating a protective effect of OC. Matrix metalloproteinase-2 activity was decreased in association with OC, indicating that OC may affect ovarian carcinogenesis by decreasing proteolytic activity, an important early event in the pathogenesis of OvCa. In contrast, OC increased invasion in a K-ras/Pten OvCa cell line established from the mouse tumors, suggesting that OC hormones, particularly estrogen, may have a detrimental effect after the disease process is under way. Our study results support further investigation of OC effects and mechanisms for OvCa prevention.

Despite decades of research, ovarian cancer (OvCa) continues to cause more deaths among women in the United States than any other reproductive cancer (1). Using the best screening methods available (pelvic exam, vaginal ultrasound, and CA-125), the detection of one case of OvCa requires between 11 and 64 unnecessary surgeries (2, 3). It is therefore widely accepted that given the limitations of screening and our current inability to cure OvCa, prevention is key to a reduction in mortality. Until we are able to cure or consistently diagnose early OvCa, strategies for prevention merit at least as much attention as treatment for the disease.

Convincing epidemiologic studies have shown that combined oral contraceptive (OC) use decreases the risk of developing epithelial OvCa more than any other agent studied and the protection conferred is long lasting (4–8). However, the mechanism of this effect is largely unknown. One hypothesis is that OC decreases ovulation resulting in less genetic instability in the ovarian surface epithelial cells from imperfect repair at the ovulation site (9). A second hypothesis is that gonadotropins activate cells, thereby inducing malignant transformation, and that OCs protect against OvCa by decreasing gonadotropin levels (10). There have been few laboratory studies, however, that either confirm the protection conveyed by OC or explore the molecular mechanism responsible. Several studies to date have suggested that the protective effect may be a direct result of progestins, which with...
ethinyl estradiol are one of the two components of combined OC. Ovarian carcinoma cell lines treated with progestins show less proliferation and increased apoptosis (11). Furthermore, treatment with progesterone has been shown to lower tumor burden and prolong survival in a xenograft mouse model of OvCa (12). However, these studies were done either in vitro or in vivo in immunodeficient mice injected with OvCa cell lines that metastasized. Therefore, these studies actually tested the effect of hormones on established OvCa cells.

The lack of adequate animal models has been a major obstacle to OvCa prevention research. Genetic mouse models of OvCa suited to research in prophylaxis have only recently become available (13–15). One such model, the LSL-K-rasG12D/+PtenloxP/loxP model (13), takes advantage of two genetic mutations that play important roles in OvCa. In this model, adenoviral vector expressing Cre recombinase (AdCre) mediates recombination activating oncogenic K-ras and deleting the tumor suppressor Pten selectively in the ovarian surface epithelium resulting in epithelial OvCa. Mutations in K-ras have been reported in up to 12% of invasive OvCa but occur with greater frequency in borderline tumors (16). Loss of heterozygosity of the Pten allele has been shown in up to 45% of epithelial OvCa and is a predominant mutation in the endometrioid OvCa subtype (17, 18). Because there is also evidence that inactivation of Pten is an early event in ovarian tumorigenesis, it may be of particular importance in evaluating chemopreventive agents (17). The K-ras/Pten model produces the endometrioid subtype of epithelial OvCa, rather than the serous subtype, which occurs more commonly. However, the protective effect of OC has been found to be independent of histologic subtype (8, 19).

This model builds on the molecular pathology of OvCa and closely mimics the natural history of the disease, and so we used it to characterize the effect of combined OC on the development of OvCa. Initially, we evaluated tumor burden in mice treated with two common components of combined OC (ethinyl estradiol plus norethindrone) compared with tumor burden in mice treated with a gonadotropin-releasing hormone (GnRH) agonist or placebo. We hypothesized that the GnRH agonist would reduce OvCa risk through effects in decreasing gonadotropin levels and thus ovulation. Then, we evaluated mice treated with norethindrone alone. We also evaluated the secondary outcomes of proliferation, invasion, apoptosis, and changes in cell signaling pathways. To our knowledge, no previous study has investigated antiproliferative actions of OC in a genetic mouse model of OvCa development.

Materials and Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Chicago. We obtained the LSL-K-rasG12D/+PtenloxP/loxP mice from the Massachusetts Institute of Technology (Boston, MA; refs. 13, 20). After weaning, the genotype of each female mouse was determined by PCR of tail clip DNA using primers as previously described (13). Virgin female mice were housed in a modified barrier facility with free access to food and water. A temperature of 37°C and light cycle of 12-h on/off was maintained. At 5 wk of age, mice were treated with s.c. depot pellet (Innovative Research of America) containing either ethinyl estradiol (1.0 mg/kg/d) plus norethindrone (5 mg/kg/d), norethindrone alone, or placebo. A fourth group of mice was treated with a GnRH agonist, Depot-Lupron (3.25 mg/kg/ wk; TAP Pharmaceuticals). The mice were treated for 3 wk before OvCa was initiated. Treatment was continued until the end of the study. To confirm that hormones suppressed the reproductive axis, at the time of necropsy serum estradiol levels were measured using a double antibody RIA kit (Siemens Medical Solutions Diagnostics). OvCa was initiated by intrabursal injection of AdCre virus (University of Iowa Gene Transfer Vector Core) as follows: mice were sedated, the right ovary was exposed, and the ovarian bursa was injected with AdCre (2.5 × 107 plaque-forming units). The left ovary was not injected and served as an internal control. Mice were evaluated weekly for palpable tumor and all were sacrificed 8 wk after the injection of the virus. At the time of sacrifice, the primary tumor was excised, weighed, and measured and the number of metastatic nodules and volume of ascites were recorded. All tissue was fixed in 10% formalin, embedded in paraffin, and stained with H&E. Two pathologists (I.O.G. and T.N.K.) unaware of the treatment regimen identified the percent of the tumor with epithelial architecture. The absolute weight of the tumor was then multiplied by the epithelial percentage to obtain an adjusted epithelial tumor weight for each mouse.

Establishment of a cell line from mouse tumors

A cell line, hereafter referred to as the K-ras/Pten mouse OvCa cell line, was established from the mouse ovarian tumors as previously described (21). Briefly, the primary ovarian mouse tumor was minced immediately after surgical excision and placed into a Petri dish with RPMI 1640 with 1% penicillin/streptomycin and incubated at 37°C for 20 min (95% O2:5% CO2). Tissue was then incubated in RPMI 1640 containing 4 mg/mL collagenases and 0.1 mg/mL bovine pancreatic DNase for 1 h at 37°C (95% O2:5% CO2). The suspension was filtered and brought up in RPMI 1640 and then placed on a preformed Ficoll gradient and centrifuged at 18°C, 400 × g for 30 min. The top layer containing cancer cells was removed, washed with PBS, and cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) supplemented with 1% penicillin/streptomycin, 1% nonessential amino acids, 1% MEM vitamins, and sodium pyruvate.

Effect of treatment on AdCre-induced recombination

Mouse embryonic fibroblasts were derived as previously described (22). Briefly, LSL-K-rasG12D/+PtenloxP/loxP mouse that was 14 d pregnant was euthanized and the embryonic sacs were harvested. The visceral tissue was separated from the embryos, minced, placed in trypsin, and incubated for 30 min at 37°C. The tissue was then incubated overnight at 37°C in mouse embryonic fibroblast derivation culture medium (DMEM, 10% heat-inactivated FBS, 1% nonessential amino acids, and 1% penicillin/streptomycin). When the cells were 90% confluent, they were plated into two plates and treated with norethindrone (1 × 10−6 mol/L). One of the plates was also treated with AdCre virus (1,000 multiplicities of infection). All cells were genotyped for the K-ras locus-containing cassette (550 bp). In addition, DNA was extracted from paraffin-embedded tumor samples from mice treated with norethindrone using DNA extraction kit (DNesy Tissue kit, Qiagen) and genotyped for the K-ras locus-containing cassette. Primer sequences are available on request.

Western blot analysis

Tumor tissue from the mouse experiments was snap frozen, ground, and lysed with ice-cold HB buffer. K-ras/Pten mouse OvCa cells were cultured until 90% confluence and then lysed with radio-immunoprecipitation assay buffer. An equal amount (30 µg) of cell extracts was separated by SDS-PAGE. Proteins were visualized with enhanced chemiluminescence. For matrix metalloproteinase (MMP-2) Western blots, cells were cultured in phenol red–free RPMI 1640 with 10% charcoal-stripped FBS and treated with ethinyl estradiol and/or
norethindrone \((10^{-4} \text{ mol/L, each})\) or placebo (1% ethanol) for 24 h before Western blots were done. All hormones were obtained from Sigma-Aldrich.

**Invasion assay and zymograms**

The activities of MMP-2 and MMP-9 were determined by gelatin zymography as previously described (23). *In vitro* cellular invasion was assayed by determining the ability of cells to invade through a synthetic basement membrane as previously described (24).

**Immunostaining**

For immunohistochemical experiments, mouse ovarian tumor was fixed in 10% formalin for 18 h and paraffin embedded. Five-micrometer sections were mounted on slides. Sections were deparaffinized in xylene and hydrated in ethanol before being placed in 3% \(\text{H}_2\text{O}_2\)/methanol blocking solution, which allowed antigen unmasking. After blocking, the slides were incubated with the designated primary antibody for 1 h at room temperature and then with rabbit anti-mouse biotinylated secondary antibody for 30 min. The slides were stained using the EnVision avidin–biotin–free detection system and counterstained with hematoxylin. Analysis of apoptotic cells was done by visualizing the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using an ApopTag apoptosis detection kit following the recommendations of the manufacturer (Millipore Corp.). Tumors of comparable histology were used for all immunostaining and Western blotting comparisons between placebo and hormone-treated groups.

**Immunostaining quantification**

Cytoplasmic staining with MMP-2 antibody was quantified by using an Automated Cellular Imaging System (ACIS) from Clarient as previously reported (25). ACIS software calculated the average intensity for each region as a measure of the integrated optical density (IOD) in the cytoplasmic compartment. The IOD of each image (region) is a proxy for antigen content and is given as the average optical densities of each molecule (pixel) within the region. Computing of the IOD is directly proportional to the concentration of molecule recognized by the stain according to the Beer-Lambert Law (26). For comparison purposes, the IOD value was normalized to the entire measured area by calculating IOD/10 \(\mu\text{m}^2\). Quantification of Ki-67 and TUNEL immunostaining was done using ACIS by setting color-specific thresholds to determine brown (positive) and blue (negative) nuclei within 12 representative regions per slide and by calculating the ratio of positively stained nuclei to all nuclei, expressed as a percentage for Ki-67 or a region score for TUNEL. The high and low region scores for each slide were excluded and the average region score was taken of the remaining 10 regions.

**Statistical analysis**

An a priori power analysis revealed that with nine mice per group, there would be 82.5% power to detect the 50% reduction in total tumor volume with \(\alpha = 0.05\) and \(\beta = 0.2\). Comparison of medians for all four groups was done using Kruskal-Wallis test with \(P \leq 0.05\) considered significant. Comparison between one treatment group and the placebo group was done using the \(t\) test with unequal variance. For immunostaining analysis, normality assumptions were checked for each outcome and transformations were made accordingly. Square root transformations were necessary to evaluate the percent of cells that stain positive for Ki-67 and MMP-2 expression. A log transformation was necessary for analysis of TUNEL staining. The differences between the mean in the placebo group and individual treatment groups were tested with random effects models, allowing for a random intercept for each mouse. All data analysis was done with STATA 9 (StataCorp).

**Results**

**A genetic mouse model reliably develops endometrioid OvCa**

In the initial study, 73% of mice (35 of 48) developed ovarian tumors ranging from 0.1 to 8 g (Fig. 1A). If a tumor did not develop, it was assumed that the surgery was unsuccessful due to rupture of the ovarian bursa and leakage of the virus. Only mice with tumors were included in the final size and weight analyses. Histologically, the tumor architecture consisted of sheets of poorly defined polyoid and glandular structures, with occasional squamous differentiation consistent with endometrioid OvCa. The epithelial component was often adjacent to an infiltrative mass of pleomorphic spindle cells, with patches of hyaline necrosis (sarcomatous component; Fig. 1B). Cytokeratin 19 and CA-125 immunohistochemical staining confirmed an epithelial origin for the tumor (Fig. 1C and D). Metastatic tumor nodules in the abdominal cavity were noted in 66% of the mice \((n = 33, 2-25)\).

**Tumor burden after hormonal treatment**

Decreased serum estradiol levels were shown with all treatments, indicating that the hormones did suppress the reproductive axis in the mice \((P = 0.04; \text{Fig. 2})\). There was no difference in tumor incidence between the treatment groups (placebo, 13 of 18; ethinyl estradiol plus norethindrone, 12 of 13; Depot-Lupron, 10 of 17; \(P = 0.18\)). Treatment with ethinyl estradiol plus norethindrone resulted in a 29% reduction in tumor weight when compared with placebo \((P = 0.9; \text{Fig. 3})\). For the epithelial component of the tumors, the reduction in tumor weight was even more pronounced \((-80\%)\). The GnRH agonist group had increased tumor weights (absolute tumor weight, +71%; \(P = 0.9; \text{Fig. 3}\); epithelial weight, +150%). There was great variation in tumor sizes (data not shown), which affected the SDs of all tumor weight changes recorded in this and the following paragraph. There was no effect of treatment on metastases, but the number of metastasis was highly variable \((\text{mean, 4.6 \pm 5.7})\). Mice that received ethinyl estradiol plus norethindrone had more ascites \((\text{ethinyl estradiol plus norethindrone, 217 } \mu\text{L}; \text{placebo, 0 } \mu\text{L}; P = 0.02)\).

In a second experiment, mice were treated with norethindrone alone \((n = 15)\) or placebo \((n = 19)\). Here, there was a reduction in tumor weight in mice receiving norethindrone compared with placebo (absolute tumor weight, −42%; epithelial weight, −46%; \(P = 0.5; \text{Fig. 4}\)). The mean number of metastases, the volume of ascites, and the tumor incidence were similar in both treatment groups. To ensure that norethindrone was not inhibiting AdCre-induced recombination, we genotyped mouse embryonic fibroblasts treated with norethindrone and ovarian tumors from mice treated with norethindrone and showed absence of the K-ras lox-stop-lox cassette (Supplementary Fig. S1).

**Hormonal treatment decreases MMP-2 activity**

Given the importance of type IV collagenases (MMP-2/MMP-9) in OvCa biology (23, 27, 28), we sought to determine if hormonal contraception affects protease activity. Zymograms showed a decrease in MMP-2 enzymatic activity in tumors treated with hormones (Fig. 5A). Immunohistochemical analysis did not show a significant change in MMP-2 expression in the tumors (Fig. 5B). MMP-2 expression was decreased
in the K-ras/Pten mouse OvCa cell line after hormonal treatment (Fig. 5C). This decrease in MMP-2 activity indicates that one possible mechanism of the protective effect of hormonal contraception may be through decreasing key proteases in OvCa cells.

**Treatment of ethinylestradiol plus norethindrone increases proliferation**

Ki-67 immunohistochemistry showed increased proliferation in both the epithelial and sarcomatous portions of the tumors in the ethinylestradiol plus norethindrone group compared with the placebo group (placebo, 11% positive cells; ethinylestradiol plus norethindrone, 27% positive cells; $P = 0.03$; data not shown). Treatment with Depot-Lupron or norethindrone alone did not affect Ki-67 staining. These findings suggest that once the tumors are established, combined OC may increase tumor proliferation.

**Hormonal treatment did not affect apoptosis or cell signaling pathways**

TUNEL assays of the OvCa tumor samples showed similar mean region scores in all treatment groups (placebo, 2.7; ethinylestradiol plus norethindrone, 2.4; norethindrone alone, 2.5; Depot-Lupron, 2.4; $P = 0.7$; data not shown). Western blot analysis revealed no effect of hormones on extracellular signal-regulated kinase, signal transducer and activator of transcription 3, or c-Jun NH$_2$-terminal kinase signaling pathways (data not shown).

**Hormones increase invasion of cancer cells**

To further study the effect of combined OC on invasive OvCa cells, we established and characterized an OvCa cell line from several K-ras/Pten tumors (Fig. 6A). Genotyping confirmed the absence of both the K-ras lox-stop-lox cassette and Pten (Fig. 6B). Estrogen receptor $\alpha$ and progesterone receptor type B were present. The cells expressed cytokeratin, but not vimentin, as is consistent with an epithelial origin (Fig. 6C). The cell line did not express the GnRH receptor (data not shown). In vitro invasion experiments showed a significant increase in the number of invaded cells after treatment with combined OC hormones (placebo, $n = 148$; ethinylestradiol plus norethindrone group, $n = 729$; norethindrone alone, $n = 404$; leuprolide, $n = 258$; $P < 0.05$; Fig. 6C). These findings suggest that once the ovarian surface epithelial cell is fully transformed into an OvCa cell, treatment with contraceptive hormones has a detrimental effect, consistent with recent results that women on hormone replacement therapy have a higher rate of OvCa (29).

**Discussion**

There is very convincing epidemiologic evidence that women who use combined OC have a significantly reduced risk of OvCa (4–8), but the biological mechanisms underlying this
The changes in tumor weights were not statistically significant even for the large effects [80% reduction in epithelial tumor weight for combined OC and increases of 71% (absolute) and 150% (epithelial) in tumor weight for the GnRH agonist] that fell within our statistical assumption of a \( \geq 50\% \) treatment effect. A wide variability in tumor sizes resulted in large SDs for all changes, influencing the \( \geq 50\% \) treatment effect.

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We were unable to show in this model a statistically significant reduction in tumor weight with OC treatment. There are several possible explanations for this. First, the timing of treatment may have affected outcomes. Continuing treatment after the need for future studies exploring the role of GnRH in ovarian carcinogenesis (32).

We evaluated the effect of OC on several important events in ovarian carcinogenesis in addition to effects on ovarian tumor burden. Both gelatin zymography and Western blotting detected OC-associated decreases in MMP-2 activity compared with placebo. Increased MMP-2 activity has been associated with increased invasion and poor prognosis in OvCa patients (23, 27, 28) and is believed to play a critical role in OvCa metastasis (28). The effect of OC on OvCa invasion was also evaluated. In vitro invasion assays revealed significantly increased invasiveness of OvCa cells after hormonal treatment, with the greatest increase in the group that received estrogen in addition to the progesterin. Others have also shown increased OvCa cell adhesion and migration after treatment with estrogen (33). Epidemiologic data also suggest a detrimental effect of estrogen on OvCa. A recent meta-analysis found a 22% increased risk of OvCa among women who used hormone replacement therapy of estrogen alone, and this effect was ameliorated by progestins (29). Increased apoptosis has been suggested to play an important role in the protective effect of combined OC. Rodriguez et al. (30) showed increased apoptosis in ovarian surface epithelium in macaques treated with a progesterin. We found no effect of treatment on apoptosis. These collective findings suggest that OC may protect against OvCa development in part through decreasing MMP-2 and thus proteolytic activity, which is an important early event in the pathogenesis of OvCa (28). However, once OvCa is established and invading, estrogen and, to lesser extent, progestins may have a detrimental effect.

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We were unable to show in this model a statistically significant reduction in tumor weight with OC treatment. There are several possible explanations for this. First, the timing of treatment may have affected outcomes. Continuing treatment after
OvCa was initiated may have stimulated cancer growth. In addition, there is epidemiologic evidence that the longer a woman uses combined OC, the greater her reduction in OvCa risk (6). Therefore, if treatment was stopped before the injection of AdCre and mice were treated for a longer period of time before the injection, a greater effect may have been seen. Second, it is possible that activation of the K-ras oncogene and deletion of PTEN had such a powerful effect on the pathophysiology of the disease that they weakened the protective effect of the OC hormones, possibly explaining why the treatment did not affect tumor incidence.

The present study was not designed to evaluate time to tumor development, a very desirable chemopreventive end point that can be analyzed more easily in the highly accessible skin or oral cavity than in the ovaries and some other sites. Hidden in the abdominal cavity, the ovaries have to reach a substantial size before they can be palpated in mice (1). The ovaries have to reach a substantial size before they can be palpated in mice. Hidden in the abdominal cavity, the ovaries have to reach a substantial size before they can be palpated in mice (1). Although we followed a few mice using magnetic resonance imaging (MRI), the ovarian detection limit was still a size of at least 5 mm (data not shown). Because of this limitation and the great expense of MRI in detecting early tumors, we designed the study to assess the end point of tumor weight at 8 weeks after AdCre injection. Our study cannot address the important question of whether OC reduced tumor weight through delaying tumor development or through treating and thus slowing the progression of early tumors or through both means. Recent evidence suggests that chemopreventive agents can function not only by reducing cancer incidence but also by slowing the progression of low-volume, clinically undetectable disease, thus blurring the distinction between cancer prevention and early cancer treatment (34).

Although with limitations, this genetic mouse model still provides advantages over xenograft models in the study of chemoprevention by mimicking the natural history of the disease. One other study in a genetic mouse model found that an inhibitor of the mammalian target of rapamycin delayed ovarian tumor development monitored by biweekly MRI, but this agent acted directly on the cell signaling pathway affected by the genetic mutation of the model (35). Other potential chemopreventive agents in this setting, including cyclooxygenase-2 inhibitors, retinoids, and the retinamide fenretinide, have been evaluated only in xenograft mouse models using established cancer cell lines (36–40).

Based on the strong known preventive effects of OC and the growing cruency of the concept of the early cancer, we believe that the evidence in our study suggests that OC likely both prevented and treated OvCa in our genetically engineered mice and thus supports further mechanistic mouse studies of OC for OvCa prevention. Such studies will need feasible, effective means for detecting true prevention end points, such as time to tumor development, for which small-animal imaging techniques such as ultrasound and MRI show promise. Advances in
molecular imaging, including the ability to detect enzyme activity and follow the effect of molecular alterations in tissue in real time (41, 42), will further improve our understanding of chemopreventive studies in animals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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