Combination of 4-HPR and oral contraceptive in monkey model of chemoprevention of ovarian cancer

Molly Brewer [1], James Ranger-Moore[2], William Satterfield[3], Zengping Hao[1], Jian Wang [1], Emily Brewer [1], J. Taylor Wharton [4], Robert Bast [4] , Changping Zou [1]

[1] Department of OB/GYN, University of Arizona, College of Medicine, Tucson, AZ 85724, [2] University of Arizona, College of Public Health, Tucson, AZ 85724, [3] Department of Veterinary Sciences Park, M. D. Anderson Cancer Center, Houston, TX, 77030, [4] Gynecologic Oncology, M. D. Anderson Cancer Center, Houston, TX, 77030

TABLE OF CONTENTS

1. ABSTRACT

4-(N-hydroxyphenyl) retinamide (4-HPR) and the oral contraceptives (OCP) are currently being used alone, and in combination, for the prevention of ovarian cancer. However, the mechanism of their effects has not been studied. Non-human primate models are ideal for studying the role of these and other drugs for cancer chemoprevention because of the genetic similarity between primates and humans in respect to hormone regulation and menstrual cycle. 4-HPR and OCP were administered to sixteen female adult *Macacca mulatta* (Rhesus macaques) for three months alone and in combination. Laparotomy was performed before and after treatment, and ovarian biopsies were obtained to evaluate the expression of retinoid and hormone receptors, and apoptosis. ERα was undetectable, but ERβ, PR, RXRα, and RXRγ were constitutively expressed in the ovaries. 4-HPR induced RXRα and RXRγ expression at a low level and, OCP induced expression of ERβ. However, the combination of 4-HPR with OCP had a larger effect on expression of retinoid receptors. Apoptosis was detected in the 4-HPR group (equivalent dose: 200 mg/day). The results provide a rationale for the use of the Rhesus macaque as a model for ovarian cancer chemoprevention.

2. INTRODUCTION

Ovarian cancer is the most common cause of death from gynecologic cancer. There are 23,400 new cases and 13,900 deaths estimated in 2003 in the US (1). Despite aggressive treatment, this cancer has the highest mortality of all gynecologic cancers with a 5 year survival rate for stage III and IV (the most commonly diagnosed stage) of 5-30% (1,2). Over 70% of ovarian cancers are diagnosed after the cancer has spread beyond the ovary (2), partially because there is no acceptable screening test or biomarkers to identify women destined to develop ovarian cancer or identify ovarian cancer at a premalignant or early stage. Late stage disease has an extremely poor prognosis. Given these characteristics, one rationale to reduce the mortality from ovarian cancer is prevention.

Retinoids have been used extensively in laboratory and clinical studies to prevent several human malignancies (3-8). Retinoids, especially the synthetic retinoid 4-HPR, have been shown to reduce ovarian cancer in a large scale clinical trial in Italy (5-8) and is now being used in combination with Tamoxifen in a phase II breast cancer prevention trial (9). It has been proposed that the anticarcinogenic and antitumor effects of retinoids are the result of retinoid-induced changes in cell growth and differentiation caused by changes in the expression of specific genes, such as oncogenes, growth factors, and growth factor receptors. Retinoids exert their effects on gene expression by activating a signal transduction pathway in which nuclear retinoid receptors play a pivotal role (10-16). These receptors are members of the steroid hormone receptor super family (10-16).

From a combined analysis of case control studies, observational data suggest that the oral contraceptive (OCP) reduces ovarian cancer risk by

approximately 10% for each year of use (17) leading to a total reduction around 40% after 4 to 5 years of use. (18,19). It is hypothesized that the biological prevention activity is related to the progestational component of the OCP.

Animal models are developed to reconcile biologic phenomena between species (20) and allow for extrapolation of knowledge from one species to another, usually animal to human. Humans are difficult to study as experimental models because of ethical limitations, cost limitations, and lack of volunteers. Institutional Review Boards (IRB) has limited the scope of chemoprevention trials because the targeted population does not have cancer and thus the tolerance for side-effects is less than if cancer were present. Development of an animal model to mimic a human disease allows experimentation in a strictly controlled laboratory environment, in which experimental subjects can be manipulated in ways that can never be done in humans. Animal models are ideal for developing strategies based on mechanistic understanding of how these agents or interventions work. By understanding mechanisms of action, we can not only develop better strategies for intervention, but also target the most appropriate population. The non-human primate model is ideal for developing strategies because of the genetic similarity between monkey and humans, such as hormone regulation and menstrual cycle, which are lacking in small animal models such as the chicken and rodent.

Multi-targeted therapies or combination of retinoids and hormonal therapies, including OCP, may have an advantage in cancer chemoprevention because they may combine different mechanisms of prevention with a synergistic effect. This is the first study to combine 4-HPR and OCP in chemoprevention of ovarian cancer *in vivo*. Our results will provide important information for human trials.

3. MATERIAL AND METHOD

3.1. Monkeys

Sixteen female adult (age) Macacca mulatta (rhesus macaques) were used in the study as previously described (21). Monkeys used in this study were culled from the specific pathogen free rhesus colony because they developed herpes-B-indeterminate status, they were poor breeders, or they had chronic diarrhea. The monkeys were prevented from eating for 12 hr before surgery. Anesthesia was induced with an intramuscular injection of tiletamine HCl/zolazepam HCl (Telazol; Fort Dodge Laboratories, Inc., Fort Dodge, IA). The monkey was sedated, removed from the cage and intubated, and anesthesia was maintained on 2–2.5% isoflurane gas with oxygen. After each initial procedure, monkeys were maintained in separate housing and were fed the drug in a flavored treat. Menses were recorded daily, and progesterone levels were measured 2 weeks apart to evaluate ovulatory status.

Monkeys were given 35 mg 4-HPR/day, OCP 0.2 mg norethindrone/0.07 mg of ethinyl estradiol/day, combination of 4-HPR+OCP, or no medication for three months as previously reported (21). Laparotomy was performed before and after drug administration, and ovarian biopsy specimens were obtained. Doses of 4-HPR and OCP were calculated by allometric scaling, which calculates a dose based on both weight and basal metabolic rate (21). This is derived from the equation $Y + K(W_{kg}^{0.75})$, where Y is the resting animal's energy output in kcal/24 h (also termed the minimum energy cost), K is a constant based on core temperature, which is specific for each species, and W is mass in kilograms. Doses were calculated for a 7 kg monkey.

3.2. Immunohistochemistry (IHC)

Paraffin-embedded monkey slides were deparaffininzed in xylene, rehydrated through graded alcohols to water, then incubated for 10 min in PBS. The slides were blocked for 30 min with 3% horse serum diluted in PBS, the sections were then blotted and incubated with primary antibody of RARs and RXRs, ERα, β, and PR (Santa Cruse) for 1 hr at room temperature. For immunoperoxidase staining, the avidin-biotin-peroxidase complex (ABC) technique was used (Vector Laboratories). For this technique, the endogenous peroxidase was inactivated by incubation for 30 min in 0.015% peroxide in methanol, then rehydrated for 10 min in PBS. The slides were then incubated with biotinylated horse antibody to the appropriate species, depending on the primary antibody, for 1 hr at room temperature, washed in PBS, then incubated with ABC for 30 min at room temperature. The slides were then washed and the peroxidase reaction developed with diaminobenzidine and peroxide. The slides were then washed in water, counterstained with hemotoxylin, mounted in Aqua-mount, and evaluated on a light microscopy. Appropriate positive and negative antibodies and tissues were included in each assay as controls.

3.3. Real-Time Quantitative RT-PCR

Real time Q RT-PCR was performed utilizing the 7700 Sequence Detector (Applied Biosystems, CA). Specific quantitative assays for RARα, β, γ, and RXRα, β, γ were developed using Primer Express software (Applied Biosystems). Total RNA was extracted from monkey ovary by Tri-reagent. The RT-PCR resulting data were analyzed using SDS software (Applied Biosystems). The final data were normalized to GAPDH (housekeeping gene) and are presented as the molecules of transcript/molecules of GADPH x 100 (% GAPDH). For this type of analysis, it is critical that the housekeeping gene does not change across experimental conditions. Calculate ΔCt for each gene by Excel according to the equations as: ΔCt = Cttreatment condition - Ctcontrol condition. 2^(- ΔΔCt) = Fold Change, which "Ct" means: the cycle at which the reaction crossed the threshold. If fold change > 1.0, the gene is up-regulated
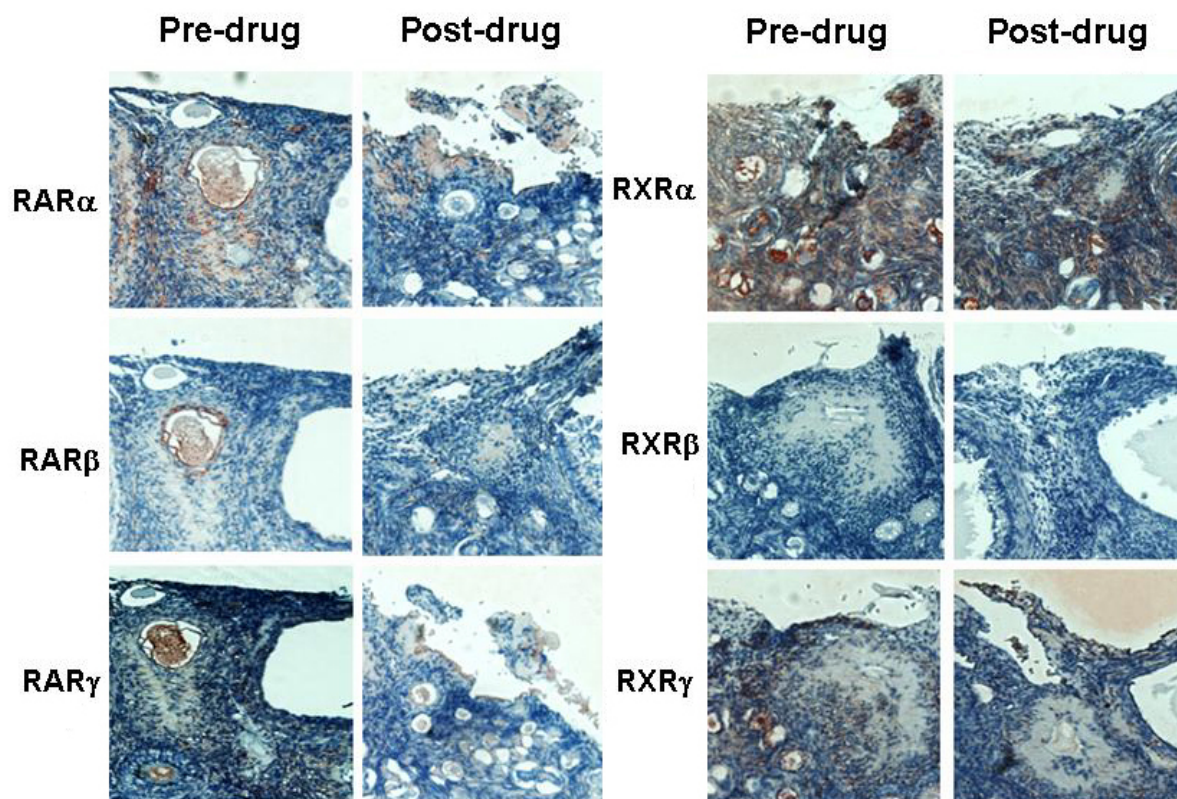
Figure 1. Immunohistochemical staining of retinoid receptors in control group (J465). Monkeys received no medication for three months. Left panel was stained for RAR receptors, and right panel was stained for RXR receptors.

relative to the control (equal to 1). If fold change is <1.0, the gene is down-regulated relative to the control.

3.4. *In situ* Cell Death Detection

Paraffin-embedded monkey slides were deparaffininzed in xylene, rehydrated through graded alcohols to water, then incubated for 10 min in PBS. The slides were then labeled with *In situ* Cell Death Detection kit (Boehringer Mannheim). Briefly, the slides were incubated in 0.5 % triton X100 for 10 min, then washed, and followed by proteinase K digestion in 37°C for 15 min. Following this incubation, 0.3 U/μl TDT and 20 mM biotinylated dUTP in TDT buffer was applied to the slides and incubated for 1 hr at 37°C. Counter staining was done using Avidin-Biotin-peroxidase Complex.

3.4. Statistical Analysis

Results of immunohistochemical analysis were semi-quantitatively scored as 0 (no staining), 1+ (weak staining), 2+ moderate staining), or 3+ (strong staining). The results were ordered using a rank sum test. The equality of the population was tested with Kruschal Wallis and Chi Square where appropriate. Histograms were prepared to visually evaluate differences between groups.

4. RESULTS

4.1. Comparing RARs and RXRs Expression and Induction by 4-HPR, OCP and Combination *in vivo*

The expression of RARs and RXRs were examined in tissue sections. RXRα and RXRγ were found to be consistently present in all monkeys (Figure 1, Table 1).

Treatment with 4-HPR alone consistently increased RXRα expression from a 1+ to 2+ expression. RXRγ expression was increased from 1+ to 2+ in 2/3 monkeys but decreased expression in one monkey from 1+ to 0 (Figure 2 and Table 2). Treatment of OCP alone had little effect on retinoid receptors expression (Figure 3 and Table 3) except for a modest decrease in RARα in two-thirds of monkeys from 1+ to 0. The combination of 4-HPR and OCP increased RXRα significantly, p=0.04 (Figure 4, and Table 4). The combination of 4-HPR and OCP also modulated 4 of the retinoid receptors RARα, RARβ, RARγ, and RXRα expression. RARβ increased consistently (p=0.01). RXRα expression increased in three-fourths of monkeys from 1+ to 3+ (1 monkey) 1+ to 2+ (2 monkeys) and did not change in one monkey (2+). The retinoid receptor RXRγ was found to be increased in 2 monkeys treated with 4-HPR alone and 1 monkey treated with the combination of 4-HPR and OCP (Tables 2 and 4) and increased in 1 monkey treated with the combination.

4.2. Hormone Receptor Expression and Induction by OCP and Combination of 4-HPR and OCP *in vivo*

Hormone receptors ERα ERβ, and PR were examined in monkeys. ERα expression was not detected in the monkey ovaries which were studied (not shown). PR expression was detected in all monkeys (Table 1), and treatment did not change PR expression significantly; however, 4-HPR treatment decreased PR expression in one monkey (Table 2) and OCP increased PR expression in 2 monkeys (Table 3).

Table 1. Semi-quantitative assessment of immunohistochemical staining of receptor expression in control animal group.

Control group	Pre-drug J465-L	Post-drug J465-R1	Pre-drug J937-L	Post-drug J937-R1	Pre-drug J371-L	Post-drug J371-R1
RARα	+	+	++	++	-	-
RARβ	-	-	-	-	-	-
RARγ	-	-	-	-	-	-
RXRα	+	+	++	++	++	++
RXRβ	-	-	-	-	-	-
RXRγ	+	+	++	+	+	+
ER β	+	++	++	++	++	++
PR	++	++	++	++	++	++
EGF	-	-	-	-	-	-
EGF-R	++	++	++	++	++	++

Table 2. Semi-quantitative assessment of immunohistochemical staining of receptor expression in 4-HPR-treated animal group

4HPR Group	Pre-drug J243-L	Post-drug J243-R1/R2	Pre-drug J753-L	Post-drug J753-R2	Pre-drug J153-L	Post-drug J153-L1/L2
RARα	+	++	+	+	+	+
RARβ	-	-	+	++	-	-
RARγ	-	-	-	-	-	-
RXRα	+	++	+	++	+	++
RXRβ	-	-	-	-	-	-
RXRγ	+	++	+	-	+	++
ER β	++	++	+	++	+++	+++
PR	++	-	++	++	++	++
EGF	++	+	+	-	-	+
EGF-R	++	++	++	++	++	++

Table 3. Semi-quantitative assessment of immunohistochemical staining of receptor expression in OCP-treated animal group

OCP group	Pre-drug J261-L	Post-drug J261-R1	Pre-drug J269-L	Post-drug J269-R2	Pre-drug L976-L	Post-drug L976-R1
RARα	+	-	+	-	+	+
RARβ	-	-	+	-	-	-
RARγ	-	-	-	-	-	-
RXRα	++	++	+	+	++	++
RXRβ	-	-	-	-	-	-
RXRγ	++	-	+	+	+	++
ER β	+	++	++	+++	++	+++
PR	++	++	+	++	+	++
EGF	-	-	-	-	-	-
EGF-R	++	++	++	++	++	++

Table 4. Semi-quantitative assessment of immunohistochemical staining of receptor expression in combination treatment animal group

Combin group	Pre-drug J511-L	Post-drug J511-R1	Pre-drug L783-L	Post-drug L783-R2	Pre-drug L581-L	Post-drug L581-R2	Pre-drug L809-L	Post-drug L809-R2
RARα	-	++	-	-	+	+	+	++
RARβ	-	++	-	+	_	++	-	++
RARγ	-	+	-	+	-	-	-	-
RXRα	+	+++	+	++	++	+	+	++
RXRβ	-	-	-	-	-	-	+	+
RXRγ	+++	++	+	+	++	++	+	++
ER β	++	++++	++	++++	+++	+++	++	+++
PR	++	+	+	-	+	++	++	-
EGF	-	-	++	+++	-	+	-	++
EGF-R	++	++	++	++	++	++	+	++

The result from combination group was shown in Table 4. ERβ were found to be expressed in untreated and treated monkeys (Figure 5, Table 1). The control group and the 4-HPR group had no change in ERβ expression (p = 1.0), ERβ showed an increase in expression in the OCP group (Figure 3 and Table 3) and in the combination of 4-HPR and OCP (Figure 4 and Table 4) (p = 0.01). P53 and p21 were not expressed and did not change between groups except for minimal expression in one or two samples (data not shown). EGF and EGF-R expression, the epidermal growth factor receptor, approached significant expression in the combination group with a p value of 0.09.

4.3. Modulation of Retinoid Receptors Expression Detected by Real Time Q RT-PCR

Real-time Q RT-PCR results showed 4-HPR and the OCP/4-HPR combination treatment increased 4 out of 6 retinoid receptors expression, RARα, β, γ, and RXRα (Figure 6). The real-time PCR results are consistent with the IHC result. However, RXR β and γ expression were decreased by exposure to 4-HPR, RXR γ expression was increased in two-thirds of monkeys and decreased in 1 monkey by IHC (Figure 6 and Tables 2 and 4). OCP treatment increased RXRα and decreased RARα expression which were also similar to the IHC result (Figure 6 and Table 3). 4-HPR modulated RARα, β, γ, and RXRα receptor expression; OCP
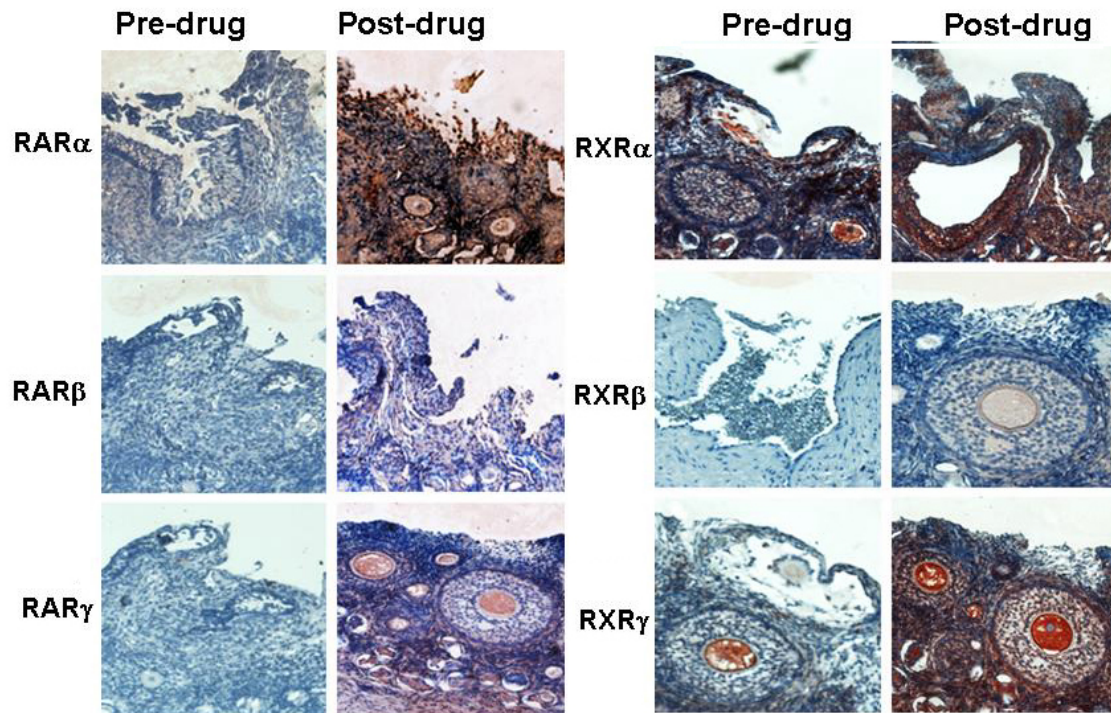
Figure 2. Immunohistochemistry staining of retinoid receptors in 4-HPR treatment group (J243). This group of monkey received 35 mg 4HPR/day for three months. Left panel was stained for RAR receptors, and right panel was stained for RXR receptors. The receptors expression was examined before and after 4-HPR treatment.
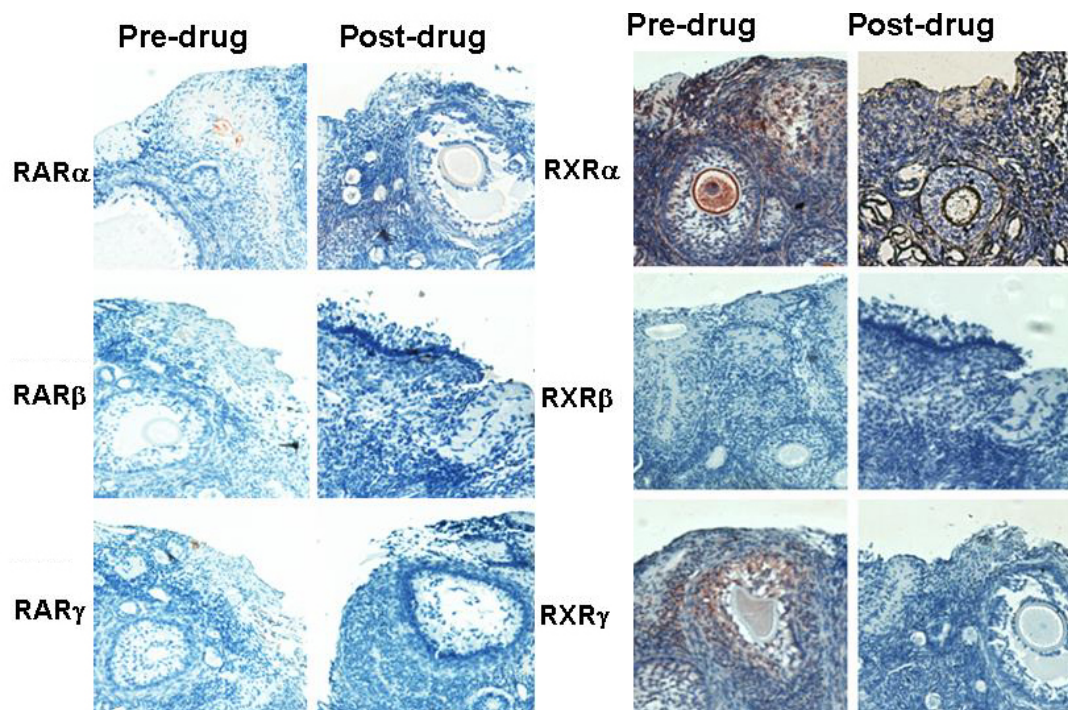

Figure 3. Immunohistochemistry staining of retinoid receptors in OCP treatment group (J261). Monkeys received OCP 0.2 mg norethindrone/0.07 mg of ethinyl estradiol/day. Left panel was stained for RAR receptors, and right panel was stained for RXR receptors. The receptor expression was examined before and after OCP treatment.
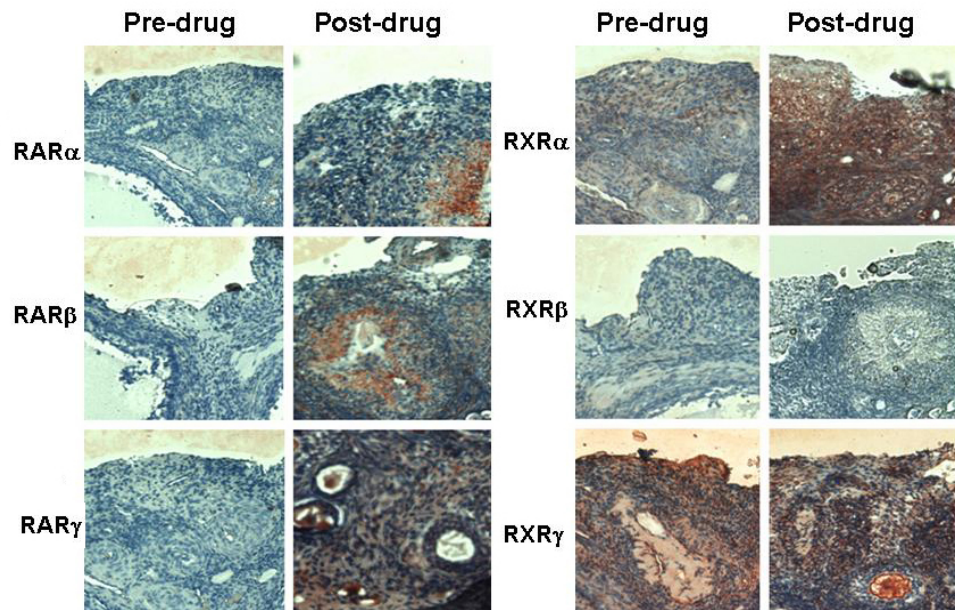
Figure 4. Immunohistochemistry staining of retinoid receptors in combination of 4-HPR and OCP treatment group (J511). Left panel was stained for RAR receptors, and right panel was stained for RXR receptors. The receptor expression was examined before and after combination of 4-HPR and OCP treatment.
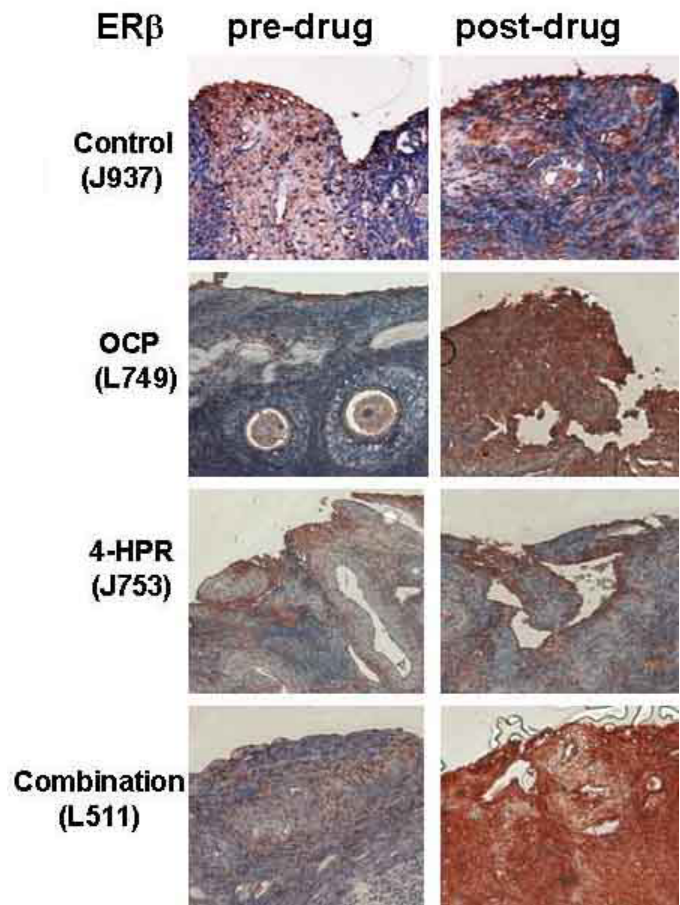

Figure 5. Immunohistochemistry staining of ERβ before and after treatments. Left panel was prior to treatment, and right panel was after treatment.
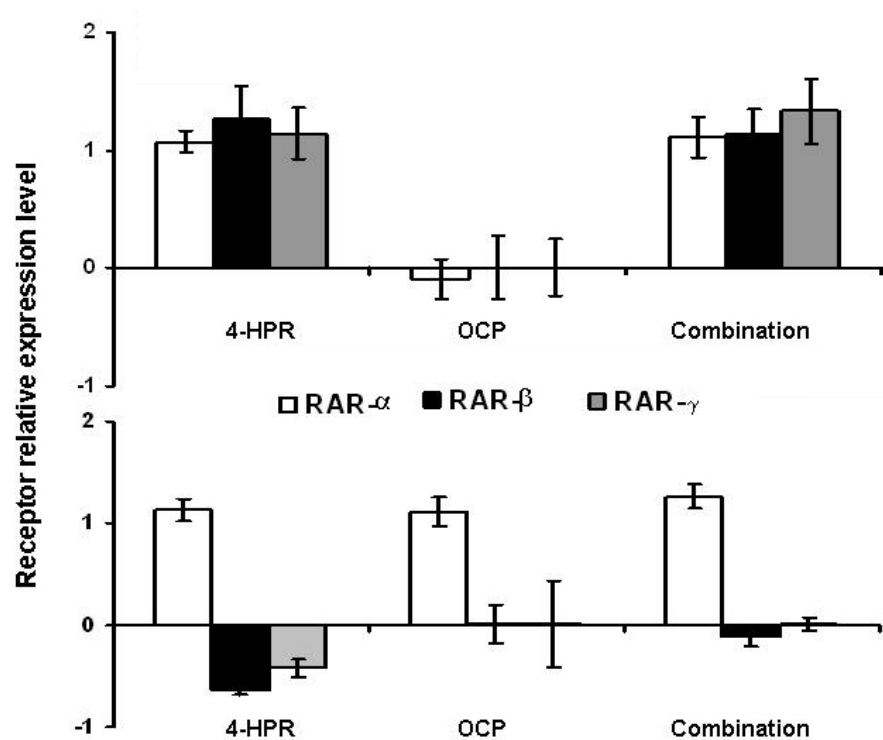
Figure 6. The total RNA was extracted from monkey ovaries and real time Q RT-PCR were analyzed for expression of retinoid receptors. The result represented the fold changes. If fold change > 1.0, the gene is up-regulated relative to the control (equal to 1). If fold change is <1.0, the gene is down-regulated relative to the control.
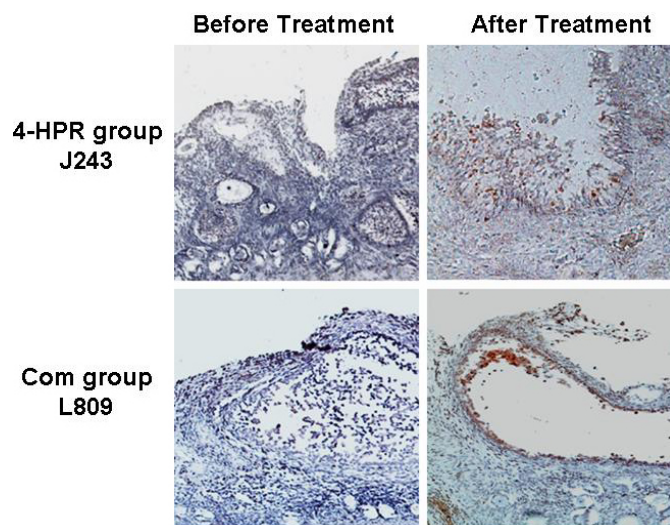

Figure 7. *In-situ* apoptosis staining in 4-HPR and combination treatment animal group. Apoptosis could not be detected in ovarian epithelial. Apoptosis was detected after 4-HPR and combination of 4-HPR and OCP treatment. Apoptotic cell staining was stronger in the combination-treated group than that in the 4-HPR-treated group.

increased RXRα expression; the combination of the two treatments did not show any synergistic or additive effects.

4.4. Apoptosis Induction by 4-HPR and Combination of 4-HPR and OCP *in vivo*

4-HPR at the equivalent human dose of 200mg/day concentration induced apoptosis in monkey ovaries *in vivo* using *in-situ* cell death detection kit staining (Figure 7).

5. DISCUSSION

This is the first report on the combination of retinoid and OCP induced modulation of retinoid

receptors *in vivo* detected by both IHC and real-time Q RT-PCR. A prior human study in oral pre-malignant and cancer of the head and neck showed induction of RARβ by 4-HPR (15, 16) which is consistent with our findings. It is also the first report on the induction of apoptosis *in vivo* using 4-HPR at 200mg/day.

Epidemiologic and laboratory data suggest that retinoids may have a role as preventive or therapeutic agents for ovarian cancer (4-5, 12-14). It has been proposed that the anticarcinogenic and antitumor effects of retinoids are the result of retinoid-induced changes in cell growth and differentiation caused by changes in the expression of specific genes, such as oncogenes, growth factors, and growth factor receptors. Retinoids exert their effects on gene expression by activating a signal transduction pathway in which nuclear retinoid receptors play a pivotal role (9-15). Nuclear retinoid receptors are the proximate mediators of many of the effects of retinoids on gene expression; therefore, it is plausible to assume that changes in their expression and function may cause aberrations in the response of cells to retinoids and thereby alter the regulation of cell growth, differentiation, and expression of the transformed phenotype.

Oral contraceptives (OCP) are well known in their ability to prevent ovarian cancer. Much of the preventive effect is thought to correspond to apoptosis induction, a process that removes cells at risk of undergoing subsequent malignant transformation. This hypothesis is supported by the observation that apoptosis is induced in up to 25% of cells in the ovarian epithelium in cynamolgus monkeys receiving levonorgestrel as a single agent (22). In the macaque setting, the level of apoptosis is associated with progestin-related changes in TGF-β isoforms; *i.e.*, a decrease in TGF-β1 accompanied by an increase in TGF-β2/3. In the human setting, additional support for the activity of progestin is provided by an analysis of data from the Cancer and Steroid Hormone Study (CASH) (23), showing a dose-response relationship. Data from the CASH Study suggests that ovarian cancer risk reduction increases with exposure to progestins of higher potency even for a short duration (24). Dose-response is a question that needs to be further explored in preclinical settings. Historically, there has been wide variation in OCP formulations from the 1960's particularly with regard to androgenicity of the progesterone component, but such changes over time did not appear to affect the ovarian cancer risk reduction associated with oral contraceptives (25, 26). The result from our study showed OCP can induce RXRα as well as ER expression, RXRα could form herterodimer with the ER receptor to regulate gene expression. The preventive capabilities are unclear in high risk women with one study showing a benefit and one study showing an increased risk of breast cancer. For women at no additional risk of breast cancer over the general population, it is an appealing alternative for prevention: however, it is unlikely that a woman is at substantial risk of ovarian cancer without being at risk for breast cancer.

This study suggests that the combination of 4-HPR and OCP may increase the modulation of epithelial cell growth through both the retinoid receptor RXRα and through the ERβ receptor. The retinoid receptors are thought to be associated with the activity of the retinoid. A prior study (27, 28) explored the fluorescence signatures of these drugs on the monkey ovary and found that 4-HPR increased the expression of FAD and OCP decreased the expression NADH associated emission, while the combination had an additive but not synergistic effect. In fact, the combination had a decrease in its effect on both the increased FAD and decreased NADH associated signature suggesting that the combination had less effect than each drug individually. These cofactors of the electron chain are strongly associated with the energy potential of tissue. This study suggests that the combination had more effect on markers associated with growth inhibition and apoptosis. A study with colon cancer cells showed that upregulation of ERβ was associated with inhibition of proliferation in colon cancer cells. A possible mechanism by which ERβ over-expression inhibits proliferation is by modulation of key regulators of the cell cycle; there was a decrease in cyclin E and an increase in the cdk inhibitor p21CIP1. Flow cytometry blocked the G1-S phase progression induced by ERβ over-expression. ERβ modulation is intriguing and bears further study.

6. CONCLUSION

This study evaluates the potential for using the Rhesus monkey as a model for ovarian cancer chemoprevention by using combination of 4-HPR and OCP. It also serves as an initial evaluation of potential intermediate biomarkers for ovarian cancer chemoprevention trials using these drugs. The results of this monkey study suggest that the combination of 4-HPR and OCP up-regulated 4 out of 6 retinoid receptors and ERβ expression, providing a potential mechanism for the effect of these drugs on the ovary. Although numbers of animals were extremely small, this pilot trial supports further study of potential intermediate biomarkers in the Rhesus monkey as a model for human ovarian chemoprevention studies.

7. ACKNOWLEDGMENT

8. REFERENCES

1. Cancer Statistics 2004. American Cancer Society, Inc. 2004.
2. Hoskins, W.J. Prospective on ovarian cancer: Why prevent? *J Cell Biochem Suppl* 23, 189-199 (1995)
3. Kelloff, G.J., Boone, C.W., Crowell, J.A., Nayfield, S.G., Hawk, E., Steele, V.E., Lubet, R.A., Sigman, CC. Strategies for phase II cancer chemoprevention trials:

cervix, endometrium, and ovary. *J Cell Biochem Suppl* 23, 1-9 (1995)

4. Supino, R., Crosti, M., Clerici, M., Warlters, A., Cleris, L., Zunino, F., Formelli, F. Induction of apoptosis by fenretinide (4HPR) in human ovarian carcinoma cells and its association with retinoic acid receptor expression. *Int J Cancer* 65, 491-497 (1996)

5. Brewer, M., Wharton, J.T., Wang, J., Auersperg, N., Gershenson, D., Bast, R., and Zou, CP. *In vitro* model of normal, immortalized ovarian surface epithelium cells and ovarian cancer cells for chemoprevention of ovarian cancer. *Gyn Oncology* 98(2), 182-92 (2005)

6. Veronesi, U., De Palo, G., Marubini, E., Costa, A., Formelli, F., Mariani, L., Decensi, A., Camerini, T., Del Turco, M.R., Di Mauro, M.G., Muraca, M.G., Del Vecchio, M., Pinto, C., D'Aiuto, G., Boni, C., Campa, T., Magni, A., Miceli, R., Perloff, M., Malone, W.F., Sporn, M.B. Randomized trial of fenretinide to prevent second breast malignancy in women with early breast cancer. *J Natl Cancer Inst* 91, 1847-1856 (1996)

7. De Palo, G., Mariani, L., Camerini, T., Marubini, E., Formelli, F., Pasini, B., Decensi, A., Veronesi, U. Effect of fenretinide on ovarian carcinoma occurrence. *Gynecol Oncol* 86, 24-27 (2002)

8. De Palo, G., Veronesi, U., Camerini, T., Formelli, F., Mascotti, G., Boni, C., Fosser, V., Del Vecchio, M., Campa, T., Costa, A. Can fenretinide protect women against ovarian cancer? *J Natl Cancer Inst* 87, 146-147 (1995)

9. Minton, S.E. Chemoprevention of breast cancer in the older patient. *Hematol Oncol Clin North Am.* 14(1), 113-130 (2000)

10. Zou, CP., Kurie, J.M., Lotan, D., Zou, CC., Hong, W.K., Lotan, R. Higher potency of N-(4-hydroxyphenyl) retinamide than all-trans-retinoic acid in induction of apoptosis in non-small cell lung cancer cell lines. *Clin. Cancer Res* 4, 1345-1355 (1998)

11. Sun, S.Y., Kurie, J.M., Yue, P., Dawson, M.I., Shroot, B., Chandraratna, R.A., Hong, W.K., Lotan, R. Differential responses of normal, premalignant, and malignant human bronchial epithelial cells to receptor-selective retinoids. *Clin. Cancer Res* 5, 431-437 (1999)

12. Wu, S., Zhang, D., Donigan, A., Dawson, M.I., Soprano, D.R., Soprano, K.J. Effects of conformationally restricted synthetic retinoids on ovarian tumor cell growth. *J Cell Biochem* 68, 378-388 (1998)

13. Zhang, D., Holmes, W.F., Wu, S., Soprano, D.R., Soprano, K.J. Retinoids and ovarian cancer. *J Cell Physiol* 185, 1-20 (2000)

14. Sabichi, A.L., Hendricks, D.T., Bober, M.A., Birrer, M.J. Retinoic acid receptor beta expression and growth inhibition of gynecologic cancer cells by the synthetic retinoid N-(4-hydroxyphenyl) retinamide. *J Natl Cancer Inst* 90, 597-605 (1998)

15, Sun, S.Y., Li, W., Yue, P., Lippman, S.M., Hong, W.K., Lotan, R. Mediation of N-(4-hydoxyphenyl) retinamide-induced apoptosis in human cancer cells by different mechanisms. *Cancer Res* 59, 2493-2498 (1999)

16. Clifford, J.L., Menter, D.G., Wang, M., Lotan, R., Lippman, S.M. Retinoid receptor-dependent and -independent effects of N-(4-hydroxyphenyl)retinamide in F9 embryonal carcinoma cells. *Cancer Res* 59, 14-18 (1999)

17. Gnagy, S., Ming, E.E., Devesa, S.S., Hartge, P., Whittemore, A.S. Declining ovarian cancer rates in U.S. women in relation to parity and oral contraceptive use. *Epidemiology*11(2), 102-5 (2000)

18. Whittemore AS, Harris R, Itnyre J, Halpern J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. I. Methods. Collaborative Ovarian Cancer Group. *Am J Epidemiol* 15;136(10), 1175-1183 (1992)

19. Whittemore AS, Harris R, Itnyre J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. *Am J Epidemiol* 15;136(10), 1184-1203 (1992)

20. Zupp J. Concern at animal research should not be dismissed. *Nature* 20;437(7062), 1089 (2005)

21. Brewer M, Baze W, Hill L, Utzinger U, Wharton JT, Follen M, Khan-Dawood F, Satterfield W. Rhesus macaque model for ovarian cancer chemoprevention. *Comp Med* 51(5), 424-9 (2001)

22. Rodriguez GC, Nagarsheth NP, Lee KL, Bentley RC, Walmer DK, Cline M, Whitaker RS, Isner P, Berchuck A, Dodge RK, Hughes CL. Progestin-induced apoptosis in the Macaque ovarian epithelium: differential regulation of transforming growth factor-beta. *J Natl Cancer Inst* 2;94(1), 50-60 (2002)

23. Cancer and Steroid Hormone Study (CASH), Centers for Disease Control Cancer and Steroid Hormone Study. The reduction in risk of ovarian cancer associated with oral contraceptive use. *N Eng J Med* 316, 650–655 (1987)

24. Schildkraut, J. M., B. Calingaert, P. A. Marchbanks, P. G. Moorman, and G. C. Rodriguez. Impact of progestin and estrogen potency in oral contraceptives on ovarian cancer risk. *J. Natl. Cancer Inst. (Bethesda)* 94, 32–38 (2002).

25. Risch, HA. Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. *J Natl Cancer Inst* 2;90(23), 1774-86 (1998)

26. Risch, HA. Oral-contraceptive use, anovulatory action, and risk of epithelial ovarian cancer. *Epidemiology* 11(5), 614-5 (2000)

27. Brewer, M., U. Utzinger, E. Silva, D. Gershenson, R. C Bast, M. Follen, Jr., J. T. Wharton, and R. Richards-Kortum. Biomarker modulation in a nonhuman rhesus primate model for ovarian cancer chemoprevention. *Cancer Epidemiol Biomark Prev* 10, 889–893 (2001)

28. M. Brewer, U. Utzinger, Y. Li, E. N. Atkinson, W. Satterfield, N. Auersperg, N. Auersperg, R. Richards-Kortum, M. Follen, and R. Bast, "Fluorescence spectroscopy as biomarker in a cell culture and in a nonhuman primate model for ovarian cancer chemopreventive agents," *J. Biomed. Opt.* 7, 20-26 (2002)

Send correspondence to: Molly Brewer, MD, DVM, The University of Arizona, 1515 N Campbell Ave., Box 245024, Tucson, AZ 85724, Tel: 520-626-9280, Fax: 520-626-9287, E-mail: mbrewer@azcc.arizona.edu