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13. ABSTRACT (Maximum 200 Words)

The overarching hypothesis of this program project is that 4-HPR (a synthetic vitamin A) and oral contraceptives (OCP) induce apoptosis, possibly through induction of TGFb production by stromal cells, as well as by direct interaction with the surface epithelial cells, and these two cell types may act synergistically. In Project 1, 18 adult Rehesus monkeys were give 4-HPR, OCP, the combination, or no medication for 3 months. There were consistent differences in the absolute fluorescence intensities and relative contributions noted between pre- and post-drug measurements in each drug group. A second study involving 30 Cynomolgus macaques and using a crossover design has been completed; immunohistochemical analysis of several biomarkers and analysis of the fluorescence spectroscopy data are ongoing. Project 2 has been transferred to the University of Arizona with the relocation of Dr. Molly Brewer. Now that a supply of 4-HPR has been obtained from the NCI, this study should be ready for patient accrual within the next few weeks pending final approval of revisions by HSRRB. In Project 3, we have focused on understanding the mechanism of action of 4-HPR in tissue culture using both normal and immortalized epithelial cells. Studies are ongoing, and results to date are inconclusive.

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INTRODUCTION

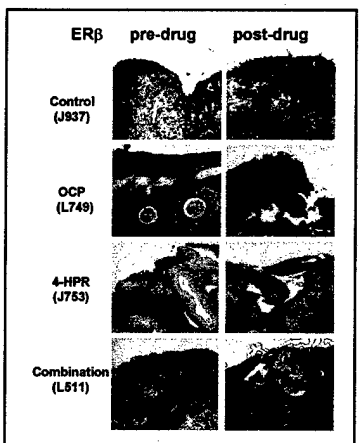
Ovarian cancer is the second most common malignancy of the female genital tract in the United States. No effective screening tool exists. Consequently, over 70% of cases are diagnosed after the cancer has already spread beyond the ovary. For women with stage III epithelial ovarian cancer—the most common stage—the 5-year survival is no higher than 20%. Clearly, early detection and prevention of this disease are critical issues. The overall goals of this program project are: 1) to develop innovative strategies for prevention of ovarian cancer through the assessment of the potential effect of oral contraceptives (OCP) and retinoids (Vitamin A derivatives) on the ovary and identification of molecular markers and mechanisms associated with the chemopreventive activity of these compounds, and 2) to assemble a multidisciplinary team that will become a world leader in the field of ovarian cancer prevention. A large body of epidemiologic evidence supports the fact that OCP can reduce a woman's risk of ovarian cancer as much as 50%. Similarly, preliminary data from a large Italian randomized chemoprevention trial for secondary breast cancers suggests that retinoids may have preventive activity against ovarian cancer. In addition, retinoids have been shown to induce apoptosis in normal ovarian surface epithelial cells in the laboratory. The major overarching hypothesis of this program project is that 4-HPR and OCP induce both growth inhibition and apoptosis. Data on 4-HPR suggests this activity is mitochondrial mediated which can be assessed using fluorescence spectroscopy which is sensitive to changes in NADH and FAD, both electron carriers active in the mitochondria. Changes in mitochondrial permeability are thought to be one of the changes in 4-HPR. Evaluating gene expression is another way of understanding the action of these molecules and the combination of these results should help us discern better methods of prevention of ovarian cancer.

BODY

PROJECT 1

Chemoprevention of Ovarian Cancer Using a Rhesus Primate Model

The second study, using 30 cynomolgus monkeys in a cross over design has been completed and is being analyzed; the increased numbers of monkeys should overcome the limitations of the numbers in the first study. This protocol was approved by the Animal Care Use Committee at The University of Texas M.D. Anderson Cancer Center and was conducted at the Department of Veterinary Sciences in Bastrop, Texas, where all animals were caged separately. The animals were given 4-HPR (4 monkeys), OCP (5 monkeys), the combination of 4-HPR+OCP (5 monkeys), or no medication (4 monkeys) daily for 3 months. Doses of 4-HPR and OCP were calculated by allometric scaling (1). The OCP used was Ortho-Novum 1/35, a medium-dose oral contraceptive with 1 mg norethindrone and 35 ug ethinyl estradiol in each pill. The 4-HPR dose was calculated in the same manner from the accepted human dose of 200 mg daily. Prior to starting medication and following 90 days of medication, monkeys underwent laparotomy, spectroscopy, and ovarian biopsies. Following the first 3 months of treatment, the group receiving 4-HPR crossed over to the OCP arm, the monkeys receiving OCP crossed over to the 4-HPR arm and the control monkeys crossed over to the combination group. There was a 1 month washout period between crossovers. Following completion of the initial portion of this study, the 9 monkeys that were negative on Herpes (Monkey B) serology were continued on OCP. A study by Gus Rodriquez (2) has shown that monkeys receiving OCP and progestin for 2 years showed a 5 fold increase in the rate of apoptosis of the ovarian surface epithelial cells over the controls suggesting that the chemopreventive activity of the OCP may be induction of apoptosis of the surface epithelial cells. However, this rate of apoptosis was not observed in our study. We have continued the OCP, changing to the Tri-phasil which was used in the Rodriquez study hypothesizing that the



ERβ expression

increase in progestin in the third week may have caused the increased apoptosis. Monkeys have undergone ovarian biopsy every 6 months to assess the cumulative response to the OCP. This study was completed in December 2002 and the data is being analyzed.

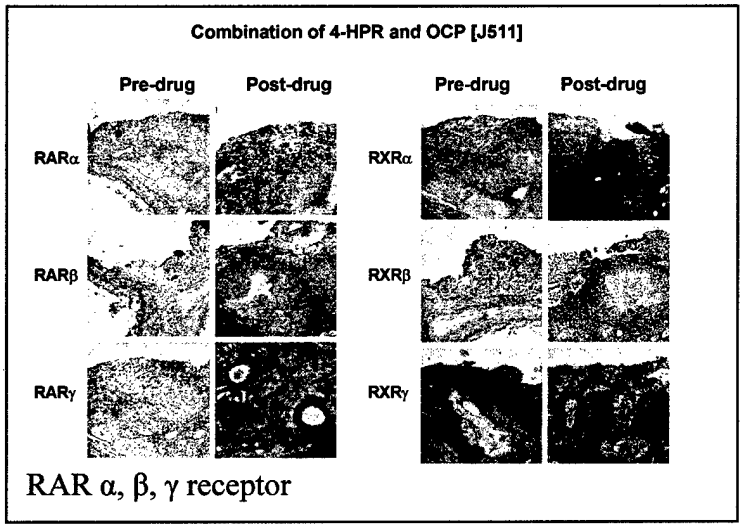
Immunohistochemical markers have been analyzed that suggest that the combination of the OCP and 4HPR have more activity than either alone. ERα was negative as has been reported in other papers. However, ERβ was positive in the OCP and 4-HPR groups but strongly positive in the combination group suggesting that these 2 drugs have synergistic activity. We saw the same synergism in the results of our fluorescence spectroscopy (3) in the combination group. These findings suggest that these drugs may have activity via activation of the ERβ receptor as well as an effect on mitochondrial metabolism. RAR α, β, γ evaluation suggests that RAR α and γ are upregulated with the combination but ERβ is not. There has been much work, particularly in cell lines, evaluating the effect of retinoids on the RAR's. 4-HPR has been suggested to be receptor independent but induce apoptosis and growth inhibition through changes in the mitochondria. However, this data from monkeys suggests that some of the activity of this drug may be mediated through the nuclear receptors. Interestingly, OCP seems to increase this activity, again suggesting a synergistic response between the 2 drugs.

PROJECT 2

Chemoprevention of Ovarian Cancer: Modulation of Biomarkers in Women at Low- and High-Risk for Ovarian Cancer Using Fenretinide (4-HPR) and Oral Contraceptives

The clinical trial in Project 2 has been activated at the University of Arizona following approval by both the University of Arizona IRB and the Human Subjects Protection at the Department of Defense. We have received the OCP from Ortho-McNeil and the 4-HPR from the NCI. The FDA has approved release of the drug with an investigator approved IND. The protocol was modified by the FDA and is currently being re-reviewed by the DOD. We anticipate this trial will have recruited patients within the next 6 weeks. We

have applied for a no-cost extension due to the extreme difficulty in gaining the FDA and IRB approvals needed to start this project.



RAR α, β, γ receptor

PROJECT 3

Chemoprevention of Ovarian Cancer: Molecular Mechanisms and Markers Laboratory Investigations of 4-HPR and OCP

Task1

Experimental Design: OVCA420 and normal ovarian epithelial cells are transferred to 96-well microplates at a concentration of 2000-3000 cells/well and treated with 2, 5 and 10μM 4-HPR; 2, 5 and 10ng/ml TGFβ; 10^{-8}M Progesterone; and 10^{-8}M 17β-Estradiol. Cells are incubated for 72 hours at 37°C and 5%CO_2, fixed and stained by SRB assay, and read at 490nm by a Versa Max (Molecular Devices) microplate reader. To determine the effects of 4-HPR and TGFβ in OVCA420, NOE 115 and NOE 106 in inducing apoptosis we have utilized the Tunel Assay (Apoptag®) and DNA Fragmentation Assay (Clontech®).

Initially we found that NOE 106 and 115 cell lines, under normal growth conditions (1:1 MCDB 105 and Medium 199; 10%FBS) and treated with TGFβ, 4-HPR and combination, do not undergo significant changes in cell growth as compared to

OVCA420 control. We have found that using the same treatment scheme but under different growth conditions (DMEM; Gibco BRL Cat.#11965-092; 5% FBS) we can produce a marked decrease in cell number over a 3 day period with 2-10μM 4-HPR but not with TGFβ alone. This finding has been highly repeatable and may be linked to the reduced growth potential these cell lines exibit in the modified medium. We therefore have focused our efforts in determining the mechanism by which 4-HPR can reduce cell proliferation.

Under the above stated conditions we are currently looking at the effects of 4-HPR in combination with OCP components progesterone and 17β-estradiol, specifically in the NOE 115 cell line. Initial immunohistochemical analysis proved the existence of both progesterone and estrogen cell surface receptors. Both progesterone and 17β-estradiol independent of 4-HPR have no effect on NOE 115 cell growth but some data suggests when treated in combination they reduce cell growth only modestly. Initial experiments using treatments of progesterone or 17β-estradiol in combination with 2 or 5μM 4-HPR, demonstrate no evidence of interaction or synergy between the compounds in reducing cell growth.

We investigated the effects of 5μM TGFß, 4-HPR, and the combination on growth of the IOSE29 series of transformed cell lines (IOSE29 with SV40; IOSE29 with SV40 and telomerase; IOSE29 with SV40, telomerase, and H-ras; IOSE29 with SV40, telomerase, and K-ras) under normal growth conditions (as described above) 3 and 5 days after treatment. There was no growth inhibition in response to any of the above treatments after 3 days, but we found that 4-HPR alone could inhibit growth 5 days post-treatment in all four cell lines, whereas TGFß alone did not suppress growth. The combination of TGFß and 4-HPR could not significantly inhibit growth better than 4-HPR alone. We also found that the transformed cells became resistant to the TGFß treatment when compared to NOE 72 cells. We will be conducting the same experiment with the IOSE80 series of transformed cells in the near future.

Work in progress and in the planning stages include studies that focus on determining the effects of 4-HPR and TGFβ in inducing apoptosis and quantifying signaling in NOE cells treated with TGFβ, 4-HPR and OCP components. Our initial examination of induction of apoptosis in OVCA420, NOE 115 and NOE 106 treated cells have been inconclusive and currently are in the process of being repeated using the Tunel Assay (Apoptag®).

Task2

Experimental Design: Isolated RNA's from NOE 115 cells treated with 5μM 4-HPR in both MCDB 105/Medium 109 and DMEM media have been analyze by Affymetrix© GeneChip© in order to study changes in gene expression that occur after 24 hours of treatment. RT PCR will be utilized to confirm genes of interest found to be either up or down regulated. Similarly, protein samples have been sent to Kinexus Corp (Vancouver, Canada) for an analytical screen of twenty-five apoptotic cell-signaling proteins (Kinetworks™ Apoptosis Screen). This analysis is both qualitative and semi-quantitative.

If it is feasible, we may be able to elucidate the mechanisms underlying the differences in 4-HPR sensitivity reported in NOE 115 cells when treated in either medium in addition to changes in gene expression that occur when comparing 4-HPR treated and untreated subsets. We are currently examining gene array data and determining which genes are of most interest in respect to 4-HPR response. For example, we have found that when NOE 115 cells are grown in DMEM media the retinoic acid receptor responder (RARRES1) gene, whose function is to mediate growth inhibition and cell differentiation activities in response to retinoids, is upregulated approximatedly 4.5 fold when compared to cells grown in normal NOE media. This may account for the major decrease in cell proliferation we have observed when NOE 115 cells are grown in DMEM media and treated with 5μM 4-HPR. In addition, when NOE 115 cells are grown in DMEM media and treated with 5μM 4-HPR, 31 probe sets representing 25 unique genes are upregulated when using a cutoff of 3-fold change in the lower bound when compared to cells grown in normal DMEM media (control). The three most upregulated genes in this category include IL-6, pentaxin-regulated gene, and prostaglandin-endoperoxide synthase 2. When NOE 115 cells are grown in NOE media and treated with 5μM 4-HPR, 239 genes

(244 probe sets) are upregulated when using a cutoff of 3-fold change in the lower bound when compared to cells grown in normal NOE media (control). The three most upregulated genes in this category include IL-6 receptor, HEF-like protein, and CD6 antigen. Interestingly only two genes are down regulated in this category: collagen (type1, alpha1) and ras homology gene family (member 1). Furthermore, the Kinexus protein screen should provide us information about the detailed changes occurring in response to 4-HPR, specifically as it pertains to the apoptotic pathway. According to the Kinexus results, FasL, proCASP7, CASP12 prodomain, PERP, and DFF45-S proteins were significantly elevated in response to 4HPR treatment.

Task3

The experiments outlined in task3 have not been undertaken at this time but have been reported previously.

Administrative Core

During this reporting period, there have been no changes in key personnel. Dr. David Gershenson, PI, continues to meet with the administrative assistant on a weekly basis. The administrative assistant coordinates and schedules all grant-related meetings and conference calls, facilitating interactions and communications between investigators. Conference calls are conducted once a month to 1) review research activities and discuss scientific issues related to grant activities and 2) identify any problems or barriers associated with research and to assure that all goals are being met within realistic time and budget constraints.

Financial accounts have been established for the University of Arizona's subcontract with Dr. Molly Brewer and all projects and cores at The University of Texas M. D. Anderson Cancer Center. The administrative assistant monitors each account on a monthly basis to ensure that there are no problems or discrepancies.

The administrative assistant is also responsible for timely submission of all reports to the Department of Defense.

Histopathology Core

The purpose of the Histopathology Core is to provide central and uniform histopathologic, immunohistochemical, in-situ hybridization, and apoptosis assay support to the projects in this grant. Histopathologic evaluation, immunohistochemistry, in-situ hybridization, and evaluation of apoptosis have a central role in the design of these projects. The Histopathology Core, in using one central lab for this purpose, will promote uniformity of results by controlling variables associated with specimen handling, and with the technical performance and interpretations of these tests.

For Project 1, biopsies from 18 Rhesus monkeys before treatment and after treatment (for a total of 53 specimens) were fixed, processed, and embedded in paraffin blocks. These blocks were sectioned and stained with hematoxylin and eosin for histologic evaluation. A pathologist associated with the core (M.D.) reviewed these H&E slides. Immunohistochemical staining was then performed on all of the specimens. The markers included BAX, BCL-X, EGFR, ER, Her-2, Ki-67, p21, p53, PR, TGF beta, TGF beta-RI, TGF beta-RII. The 636 immunohistochemical stains were then evaluated and quantified. These results were then given to the investigator. Next the 53 specimens were evaluated for apoptosis using APO-TAG. These assays were reviewed and evaluated with the results forwarded to the investigator. In-situ hybridization for RAR-beta on these specimens is pending. An additional 30 primates have undergone three surgeries each

with two biopsies being obtained at each surgery (180 specimens). These specimens have been processed and the immunohistochemical stains have been partially completed.

As Project 2 is not yet underway, no specimens have been processed by the Histopathology Core.

In support of the Idea Grant, immunohistochemical staining was reviewed and evaluated on seven cell lines. These cell lines included NOE 71, 72, 78, 79, 80, 83, and 86. Cytospins and smears from each of these lines were stained with AE1/AE3 and vimentin immunohistochemistry. The stains were reviewed and evaluated with the results forwarded to the investigator.

Please refer to the individual project summaries for the incorporation of the results of these assays and their implications.

CONCLUSIONS

Project 1:

The Statement of Work for this project has been completed. However, we have completed a second study involving 30 cynomolgus monkeys in a crossover study design. The results of this study are currently being analyzed.

Project 2:

Last year, this project was transferred to the University of Arizona, where Dr. Molly Brewer relocated. The protocol was subsequently approved by both the University of Arizona IRB and the Human Subjects Protection at the Department of Defense. The 4-HPR supply has recently been received from the National Cancer Institute. Because of regulatory issues that required clarification, there was a rather lengthy delay in moving forward with final approval. However, we are now in the final stages of revisions that should be approved by both the University of Arizona IRB and the HSSR. We anticipate that we will be ready to accrue patients within a few weeks.

Project 3:

In Task 1, using normal ovarian epithelial cells, after changing growth conditions, 4-HPR produced a marked decrease in cell number compared with controls, whereas TGFß alone produced no such effect. This finding has been highly repeatable. We are currently investigating the effects of 4-HPR in combination with OCP components, progesterone and 17ß-estradiol, in normal ovarian epithelial cell lines. Initial experiments have demonstrated no evidence of an interaction or synergy between these hormones and 4-HPR in reducing cell growth. In addition, 4-HPR alone inhibited cell growth 5 days post-treatment in four transformed cell lines, whereas TGFß alone did not suppress growth. Furthermore, the combination of TGFß and 4-HPR had no greater effect than 4-HPR alone.

In Task 2, isolated RNA from normal ovarian epithelial cells treated with 4-HPR has been analyzed by Affymetrix technology to study changes in gene expression that occur after treatment. We are currently examining gene array data and determining which genes are of most interest with respect to 4-HPR response.

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REPORTABLE OUTCOMES

4-HPR-Induced Inverse Relationship in Mitochondrial Potential Correlated with Apoptosis Induction and Growth Inhibition in Ovarian Cancer Cell Line OV CA 433 Cells. Molly Brewer, J. Taylor Wharton, Jian Wang, Amanda McWatters, Reuban Lotan, Kenneth Hatch, Robert Bast, Changping Zou.
In Progress, 2003

Effect of 4-HPR in Normal, Immortalized Human Ovarian Epithelial Cells and Ovarian Carcinoma Cells. Changping Zou, Molly Brewer, Jian Wang, Amanda McWatters, J. Taylor Wharton, Robert Bast

Submitted 2003, *Cancer Epidemiology, Biomarkers, and Prevention*