Fox Chase Cancer Center

Annual Progress Report: 2006 Formula Grant

Reporting Period

July 1, 2009 – December 31, 2009

Formula Grant Overview

The Fox Chase Cancer Center received $2,768,335 in formula funds for the grant award period January 1, 2007 through December 31, 2009. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Clinical-Grade Anti-HER2 Diabody for Radioimmunodiagnosis of Breast Cancer - We are preparing to initiate a clinical trial to determine the ability of a diabody, a novel antibody-based molecule, to target breast cancer in patients. The C6.5 diabody specifically targets human HER2 expressing tumors and is capable of delivering radioactive particles for cancer detection and treatment in our animal model. Clinical grade (GMP) diabody will be produced by our collaborator, Dr. Richard Begent of the University College London. We will assess the quality of the GMP diabody and evaluate its ability to bind to recombinant HER2 and to cell-associated HER2 both in vitro and in vivo. Preclinical toxicology studies will be performed by a contract organization as per FDA guidelines. These studies are required prior to the submission of an application to conduct clinical trials with the C6.5 diabody.

Duration of Project

7/1/2007 – 12/31/2008

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 2: Project Title and Purpose

Effects of Estrogen on Tobacco Smoke-Mediated Female Lung Cancer - The incidence of lung cancer has increased dramatically in women over the past decades. Lung cancer now exceeds breast cancer as the leading cause of cancer-related death among women. The reason why women develop more lung cancer than men remains unknown. Studies continue to suggest that the hormone estrogen contributes to the formation of lung tumors. This group is the first to detect estrogen within mouse lung tissue and has shown that smoke hastens estrogen conversion...
to cancer-causing substances. The purpose of this project is to investigate the response of mouse lung tissue to estrogen treatment. Findings from this project are anticipated to provide insight into the contribution of estrogen to female lung cancer. Results could aid in the development of a cancer preventive therapy for current and former smokers.

**Duration of Project**

1/1/2007 - 12/31/2008

**Summary of Research Completed**

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at [http://www.health.state.pa.us/cure](http://www.health.state.pa.us/cure).

**Research Project 3: Project Title and Purpose**

*A Physical Activity Intervention to Prevent Weight Gain in Breast Cancer Patients* - Weight gain following breast cancer diagnosis is common and typically averages 2.5 kg to 6.2 kg, but greater gains are not unusual. Women who gain more weight following a diagnosis of early-stage breast cancer are more likely to relapse and/or have decreased survival. The underlying basis for weight gain by breast cancer patients is not clearly understood. Although dieting is more effective than exercise for weight control in the general populations, a major detrimental side effect of weight loss achieved by diet restriction is loss of lean body mass. Physical activity, including strength training and aerobic activity, may be most effective for weight control among breast cancer patients. This project proposes to study the feasibility of a structured physical activity program for breast cancer patients to prevent weight gain.

**Duration of Project**

1/1/2007 - 6/30/2008

**Summary of Research Completed**

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement Annual C.U.R.E. Reports on the Department's Tobacco Settlement/Act 77 web page at [http://www.health.state.pa.us/cure](http://www.health.state.pa.us/cure).

**Research Project 4: Project Title and Purpose**

*Identification of Biomarkers of Breast Cancer Risk* - To develop and validate a panel of blood-based markers to assess *BRCA1* and/or *BRCA2* mutation status and detect breast cancer early in its development.
Duration of Project


Project Overview

Mammography has been shown in several trials to decrease breast cancer mortality, primarily in post-menopausal women. However, in post-menopausal women, 10% of cancers will be missed by routine screening mammography and 5.9% of women will have a false positive mammogram requiring further investigation and attendant economic cost, morbidity, and decreased quality of life. The efficacy of mammography screening is worse in pre-menopausal women where 25% of cancers will be missed by routine screening mammography and 6.2% of women will have a false positive mammogram. The sensitivity and specificity of mammography is relatively poor in young women with dense breasts who are typical of the high-risk population. Thus, mammography misses a significant number of cancers and results in a large number of false positives in both pre- and post-menopausal women, which leads to unnecessary interventions and undue anxiety.

The applicability of new imaging technologies such as breast MRI or PET may eventually prove useful as a complementary approach for symptomatic women or women with an abnormal mammogram, but it is unlikely that they can be used as a screening approach. Further, their utility in particular subpopulations remains to be determined. Optimally, biomarkers, because of their ease of use and applicability, could be used to identify the women most likely to have or not to have breast cancer -- guiding patient management. However, a biomarker or set of biomarkers that has the required sensitivity and specificity for screening for breast cancer has not yet been identified. Many candidate markers have been, and continue to be, identified due to the revolution in molecular approaches to gene and protein discovery. Cancer screening of the future will likely involve heuristic algorithms that can accommodate many sources of information to make screening decisions that are individualized to the patient. Epidemiological risk factors, family history, biomarker concentrations, changes over time in panels of tumor markers, mammography results, and potentially other imaging approaches will be most effective if integrated to predict the likelihood of breast cancer in a woman. This could direct women to biopsy, additional investigation or potentially chemoprevention. A robust biomarker panel for breast cancer, particularly one that complements mammography in high-risk women, is needed, but has not yet been identified.

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Expected Research Outcomes and Benefits

Cancer screening of the future will likely involve modern algorithms that incorporate risk factors, family history, biomarker concentrations, changes over time in panels of tumor markers, and imaging results. For example, the risk of prostate cancer has been estimated using information about the results of digital rectal examination (DRE) and prostate specific antigen (PSA) in combination with race and age. All four of the variables contribute significantly to the prediction of cancer at biopsy in an individual patient. In ovarian cancer, recent approaches to disease detection in asymptomatic women focus on estimation of the probability that a woman has cancer given all of the available information about her including her risk factors and screening history, as well as the results of her current screens. A similar approach in breast cancer is feasible, as risk factors for breast cancer are well known, and mammographic assessment codes are routinely used to predict breast cancer. Breast density, another mammographic finding, is a risk factor for breast cancer. This approach could identify “at risk” women who should be directed to breast ultrasound, MRI, PET, chemoprevention management, or to novel chemoprevention trials. Early detection of risk is the key to implementing these innovative programs of cancer prevention. A simple blood test could provide a fast and effective means to assess cancer risk. Little, however, is known about the potential of serum markers to complement mammography and risk factors in predicting breast cancer. The goal of these studies is to evaluate the utility of markers in blood to detect women at increased risk of developing breast cancer, as well as to detect cancer early in its clinical course.

Summary of Research Completed

We continue to develop an important and focused cohort of samples to aid in evaluating biomarkers for early detection of breast cancer. In an effort to construct a breast cancer reference panel of samples to identify and validate biomarkers, we focused on the accrual of blood samples collected at the time of screening mammogram. Each blood collection can be directly correlated to breast health as determined by mammography. This has created an essential cohort that can be used to detect and discriminate biomarkers specific to breast cancer. For each sample collected, 8 vials of plasma, 3-4 vials of serum, 5-6 vials of leukocytes, 2 vials of erythrocytes, 2 vials of whole blood, and 1 blood spot card, 5 spots per card are banked. During the past 6 months, an additional 160 women donated blood samples. Based on clinical diagnosis, these samples will most likely be incorporated into our early detection studies.

Finally, we measured circulating levels of six proteins (IL-1beta, IL-6, CRP, fibrinogen, sTNFR1, and sTNFR2) using multiplexed, bead-based Luminex assays to evaluate the associations among stress, inflammation, breast density, and the role of candidate pro-inflammatory genes. Several of these markers are of significant interest given their crucial role in the inflammatory process and their associations with breast disease. The cohort of women, collected in collaboration with Marilyn Tseng (California Polytechnic State University) and
Carolyn Fang (Fox Chase Cancer Center) for this study consisted of 430 foreign-born U.S. Chinese immigrants, representing a unique population in transition, well suited for studies addressing these issues of psychosocial and acculturative stress as determinants of inflammation, breast density and cancer risk. The study participants are part of a separate longitudinal study on diet and breast density. They are pre-/peri-menopausal, of mammography screening age, with US residence <=20 years. We measured the six serum markers from approximately 470 blinded serum samples (430 samples + 10% replicate samples to assess reproducibility). The data are currently being unblinded and analyzed to evaluate associations among the different variables listed above and cancer risk.

**Research Project 5: Project Title and Purpose**

*Novel Molecular Mechanisms of TSC Pathogenesis* - This project addresses the functions of two proteins, NEDD9 and Aurora-A, that our preliminary data and other published studies have implicated as extremely relevant to renal cell carcinoma (RCC) and polycystic kidney disease (PKD). The project also addressed additional proteins, (TSC1/TSC2/Rheb) as potentially associated with these serious diseases. Promising preliminary results have caused us to focus specifically on NEDD9/Aurora-A interactions in kidney pathology.

**Duration of Project**


**Project Overview**

The overarching theme of this project is to better understand the etiology of renal cell cancer (RCC) and other serious pathologic states of the kidney, such as polycystic kidney disease (PKD). The preliminary data motivating the original study were exciting observations in the Golemis, Henske, and Chernoff laboratories that suggested previously undetected interactions between the TSC, NEDD9, and Pak proteins, which may be important for two different aspects of renal disease. First, aberrant formation and function of the renal cell cilia leads to the formation of renal cysts and may be linked to the ultimate generation of RCC. The basal bodies forming renal cell cilia are closely linked to centrosomes and share common proteins. We had recently identified novel important functions of TSC, NEDD9, and Pak proteins at the centrosome and direct interactions between some of these proteins and ciliary proteins lead us to hypothesize TSC, NEDD9, and Pak affect cilia integrity and signaling. Second, loss of TSC proteins causes upregulation of Rheb signaling, causing histologically benign cells to metastasize *in vivo.* Preliminary data suggested direct interactions between Pak and Rheb, and NEDD9 regulation of Pak; while upregulation of either Pak or NEDD9 proteins induces increased cellular migration and invasiveness. We hypothesized that a signaling axis involving TSC, Pak, and NEDD9 promotes metastasis in RCC.

Substantial progress was made on the original aims of this project. These aims were: *Aim 1,* analyze the roles of TSC, NEDD9, and Pak proteins in the formation and signaling properties of renal cell cilia. *Aim 2,* analyze the physical and functional interactions between TSC, NEDD9, and Pak proteins in control of Rheb activity. *Aim 3,* analyze the expression and activity of TSC,
NEDD9, Pak, and Rheb proteins in metastatic and primary RCC. In 2008, two specific events led us to modify these aims for the current reporting cycle. One event was the departure of two key project investigators to other institutions (Dr. Naomi Haas to the University of Pennsylvania Hospital, and Dr. Elizabeth Henske to the Brigham and Women's Hospital/Harvard Medical School). These departures necessarily reduced the emphasis of the research program on TSC, as this was the specific contribution of Drs. Henske and Haas. The second event was the exceptional progress in the study of NEDD9 and Aurora-A, which have now emerged as important components in the response to transient environmental signals in the kidney, clearly relevant to both RCC and PKD, and potentially providing the basis for new clinical management of these diseases. In 2008, the specific aims were revised for the remainder of the proposal as follows:

Aim 1 - to understand the mechanism by which NEDD9-Aurora-A interactions govern signaling in kidney cells, and how this signaling is altered in pathological conditions (merged from previous Aims 1 and 2).

Aim 2 - to analyze the expression and activity of NEDD9 and Aurora-A in metastatic and primary RCC, and in PKD (adapted from previous Aim 3).

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**Expected Research Outcomes and Benefits**

This research is likely to yield a number of important benefits. The study will elucidate the cellular defects that lead to diseases that are clinically very significant. One likely outcome is the ability to improve prediction of disease prognosis in individuals at risk for RCC or PKD, by assessing changes in expression of NEDD9 and Aurora-A kinase as a predictor of more aggressive disease. As a second outcome, drugs have recently been developed that target the activity of Aurora-A, and these are advancing through the clinic. Our progress, summarized below, suggests these drugs may be extremely useful for PKD and potentially RCC. Third, pathological signaling at cilia does not only affect kidneys, but plays a major role in a number of serious developmental disorders including Kartagener’s syndrome, Bardet-Biedl syndrome, and others, and is increasingly being linked to cancer susceptibility. Our results are likely to inform future studies of all of these diseases.
Summary of Research Completed

During this six-month period, we focused on completion of the project, analysis of data, and the preparation of a manuscript. During the first two months, we consulted with the Fox Chase Cancer Center biostatistics facility to ensure all reportable data was supported by robust P values. In some cases, this caused us to revisit earlier experiments and perform additional repetitions of assays. The remainder of the period focused on the creation of figures and text for manuscript submission. A paper describing key findings of the study is currently in review at Nature Cell Biology. This study focuses on defining interactions between the oncogenes NEDD9 and Aurora-A in kidney biology. This study has identified and mechanistically characterized a novel means of Aurora-A activation in response to increases in cytoplasmic calcium. These provide a rationale for reports of Aurora-A action in interphase cells, relevant to Aurora-A dependent activation of proteins such as RalA, which are key effectors in core cancer signaling cascades: to date, almost all other reported studies of Aurora-A focus on its long-term activation during mitosis. Our work also defines Aurora-A as a kinase for the PC2 transmembrane calcium channel, which is an important regulator of calcium homeostasis and signaling in polycystic kidney disease, and in normal cell function. Together, these observations greatly inform the application of Aurora-A-targeting small molecule inhibitors in the clinic, where they are now being commonly applied in Phase II trials.

Research Project 6: Project Title and Purpose

Loss of Oxidative Defense Mechanisms in Ovarian Oncogenesis - Ovarian cancer is the leading cause of death from gynecological cancer in Pennsylvania and in the United States. During 2006, approximately 20,180 new cases were diagnosed with 15,310 women in the US and 820 in Pennsylvania dying from the disease. Cellular defense mechanisms against environmental stresses are important in preventing cancer and other diseases. The purpose of this study is to determine the capacity of the ovary to defend against cellular stresses and how these defense mechanisms are weakened during the process of malignant transformation.

Duration of Project

1/1/2007 - 12/31/2009

Project Overview

The broad objectives of the proposal are to see what capacity normal ovarian epithelia has to detoxify oxygen radicals and how these defenses are compromised during the process of transformation. Cells use various systems such as antioxidants, detoxification enzymes, and glutathione to reduce the burden of mutagenic oxygen radicals. There is some evidence that the maintenance of vitamin A (retinol) homeostasis is an important antioxidant defense mechanism of the ovarian surface epithelial cell and that this ability may be lost during the process of malignant transformation. Using the vitamin A deficient condition as a model for oxidative stress, the proposed specific aims of the research project are:
Specific Aim 1. Determine the ability of ovarian surface epithelium to protect against oxidative stress and the impact that loss of vitamin A homeostasis has on these defenses:

a) Isolate ovarian surface epithelial (OSE) cells from mice with and without defects in vitamin A homeostasis, and determine the sensitivity toward hydrogen peroxide and the extent of oxidative damage.

b) Determine catalase and superoxide dismutase activities and glutathione levels in these cells.

Specific Aim 2. Analyze the alterations in oxidative defenses throughout the process of transformation:

a) Generate a panel of mouse cell lines exhibiting various degrees of transformation by repeated subculturating of mouse ovarian surface epithelial cells from normal and genetically modified mice with defects in vitamin A homeostasis.

b) Determine the sensitivity toward hydrogen peroxide, the extent of oxidative damage, catalase and superoxide dismutase activities, and glutathione levels in this model of spontaneous transformation.

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Expected Research Outcomes and Benefits

A cell’s defense mechanisms against oxygen radicals are suggested to be important for the prevention of cancer. Vitamin A is an essential antioxidant, which protects cells from damaging free radicals. Since the early stages of ovarian cancer development have indicated decreases in vitamin A utilization, the goal of this project is to find what ability the ovary has to detoxify free radicals, and whether this ability is lost during ovarian carcinogenesis. The expected outcome of the project is that vitamin A and other cell defenses against oxygen radicals are reduced in the development of ovarian cancer. The benefit of the proposed work is that it could reveal new basic mechanisms of ovarian tumor progression that eventually could be used as targets to develop a form of preventive therapy for this fatal disease.

Summary of Research Completed

Specific Aim 1. Due to the limited number of cells obtained from mouse ovaries, and therefore limited material to conduct enzyme assays, we decided to concentrate our efforts on further experimentation and expanded subculturing schema as outlined below (Specific Aim 2a and 2b).
Specific Aim 2a. In the previous subculturing attempt, we had reported that $\text{MOSE}^{\text{CRBP1}+/+ \text{VAD}(60S)}$ produced the higher number of colonies (130) than $\text{MOSE}^{\text{CRBP1}+/+ \text{VAS}}$, which was contrary to our original hypothesis that the CRBP1 null MOSE cultured under vitamin A deficiency (VAD) would result in the most soft agarose colonies. In order to verify this result we re-genotyped MOSE$^{\text{CRBP1}+/+ \text{VAD}}$ cells that gave rise to these 130 colonies (60S) and discovered that they were indeed null and not wild type for CRBP1. Therefore, the corrected values for colony agar formation are presented in Table 1. These results are in line with our original hypothesis. Additionally, we confirmed as anticipated that the MOSE cells capable of growth in soft agar were tumorigenic when injected subcutaneously (s.c.) in immuno-deficient mice. Histological analysis of these MOSE$^{\text{CRBP1}+/+ \text{VAS}}$ s.c. tumors revealed poorly differentiated carcinoma of the predominantly clear cell type (Figure 1).

During this reporting period we have performed repeated subculturing of mouse ovarian surface epithelial (MOSE) cells from normal and genetically modified mice with defects in vitamin A homeostasis, attempting to verify the first experiment and test the frequency of malignant transformation. As shown in Figure 2, six independent cultures were created from each of MOSE$^{\text{CRBP1}+/+ \text{VAS}}$ and MOSE$^{\text{CRBP1}+/+ \text{VAD}}$ cells grown in either vitamin A-sufficient or deficient media (MOSE$^{\text{CRBP1}+/+ \text{VAS}}$, MOSE$^{\text{CRBP1}+/+ \text{VAD}}$, MOSE$^{\text{CRBP1}+/+ \text{VAS}}$, MOSE$^{\text{CRBP1}+/+ \text{VAD}}$) and cultured for 60 passages as described in previous reports. These 4x6 cell lines were assessed for substrate independent growth in soft agar at passage 30 (30S). Namely, $5 \times 10^5 \text{MOSE}^{\text{CRBP1}+/+ \text{VAS}}$, MOSE$^{\text{CRBP1}+/+ \text{VAS}}$, MOSE$^{\text{CRBP1}+/+ \text{VAD}}$, and MOSE$^{\text{CRBP1}+/+ \text{VAD}}$ cells were suspended in 3 ml of 0.35% sterile agarose in complete media, and layered over 4 ml of 0.5% agarose as a support layer in 60 mm dishes in triplicate. The dishes were incubated at $37^\circ \text{C}$ in 5% CO$_2$ in a humidified incubator for three weeks. Every five days, three to four drops of fresh media was added to the dishes. To visualize and quantitate colonies, dishes were fixed in 70% ethanol for 1 hour at room temperature, then stained for 20 minutes with 0.1% methylene blue in 50% ethanol. Dishes were finally destained with ddH$_2$O. Colonies consisting of >50 cells were scored on a Nikon Stereo Dissecting Microscope. None of the tested 24 MOSE cell lines at passage 30 produced any colonies in soft agar. We therefore continued subculturing these cells and tested them again for capacity for substrate independent growth in soft agar at passage 60. Once more, none of the tested 24 MOSE cell lines (60S) produced any colonies in soft agar. These findings are not in agreement with the results from the first MOSE subculturing experiment, in which only MOSE$^{\text{CRBP1}+/+ \text{VAD}}$ cells underwent malignant transformation based on substrate independent growth and tumorigenicity in immuno-deficient mice (Table 1). These results are also not consistent with the literature on MOSE and rat OSE cell transformation. We are repeating these studies with additional controls to validate the results.

Specific Aim 2b. Catalase activity. Our results with an inducible CRBP1 system in the human ovarian carcinoma line (OVCAR3) suggested that CRBP1 could protect cells from an oxidative insult generated from either acute hydrogen exposure or prolonged vitamin A deficiency. This cytoprotective effect was not due to increased activity of redox enzymes. Our overall hypothesis was that loss of ability to utilize vitamin A as an antioxidant would alter the redox status of the OSE in such a way as to facilitate a mutagenic environment. To test this hypothesis, we attempted to transform MOSE cells (null or wild type for CRBP1) under vitamin A sufficient or deficient conditions. The CRBP1 null MOSE cells grown under vitamin A deficient conditions...
(MOSE$^{\text{CRBP1-/-VAD}}$) were able to clone in soft agarose and developed the transformed phenotype (Figure 1 and Table 1). Therefore we wished to examine if an altered redox state existed in these cell lines. As a first step we measured catalase activity, the main enzymatic system responsible for eliminating peroxide radicals. As shown in Table 2, the MOSE$^{\text{CRBP1-/-}}$ cells, grown under VAD conditions, had significantly higher catalase activity than MOSE$^{\text{CRBP1+/+}}$ cells, regardless of media conditions under which they were grown. In addition, MOSE$^{\text{CRBP1+/+}}$ cells grown under VAD conditions had higher catalase activity relative to wild type cells grown under VAS conditions ($p<0.002$), yet the catalase activity of wild type cells in VAD were still significantly lower than the null cells grown under similar condition ($p<0.0013$). Therefore it is possible that increased stress as a result of VAD may evoke some compensatory mechanism, especially in the context of loss of CRBP1.

Conclusions

We had hypothesized that loss of the ability to metabolize vitamin A could set the stage for initiation of carcinogenesis. Our results from Specific Aim 1 suggest this to be the case since the only MOSE cells that gained the features of experimental malignancy were MOSE$^{\text{CRBP1-/-}}$ cells grown under VAD conditions. However, the frequency of this event was lower than anticipated. This makes us cautious with regard to stating that our hypothesis is proved. The results from Specific Aim 2 are quite interesting as they show that cells lacking one way to control oxidative stress can compensate. It will be worthwhile to determine the mechanism of catalase activity in MOSE$^{\text{CRBP1-/-}}$ cells.

Table 1. Determination of substrate-independent growth capacity of MOSE cells isolated from CRBP1-null and wild type mice and cultured in vitamin A-sufficient and vitamin A-deficient media

<table>
<thead>
<tr>
<th>Passage number</th>
<th>MOSE$^{\text{CRBP1+/+VAD}}$</th>
<th>MOSE$^{\text{CRBP1-/-VAD}}$</th>
<th>MOSE$^{\text{CRBP1+/+VAS}}$</th>
<th>MOSE$^{\text{CRBP1-/-VAS}}$</th>
</tr>
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<tr>
<td>10S</td>
<td>-</td>
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<td>20S</td>
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<td>30S</td>
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<td>45S</td>
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<tr>
<td>55S</td>
<td>-</td>
<td>40</td>
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<tr>
<td>60S</td>
<td>-</td>
<td>130</td>
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Figure 1. Hematoxylin and Eosin (H&E) staining of the s.c. MOSE$^{CRBPI-/- \text{VAD}}$ (45S) cell tumor in an immunodeficient mouse: poorly differentiated carcinoma, predominantly clear cell type.

Figure 2. Schema for subculturing of MOSE cells isolated from CRBP1-null and wild type mice

- Primary MOSE cell isolation
  - 10 cm$^2$/well
  - Passage 1
  - 10 cm$^2$/well
  - Passage 10
  - T25
  - 6 individual cell lines
  - Test for colony formation in soft agar
  - Passage 30
  - Test for colony formation in soft agar
  - Passage 60
Table 2. Catalase activity of MOSE CRBP1 wild type and null cells grown under vitamin A-sufficient or vitamin A-deficient conditions. Catalase activity is calculated as the rate of decrease in OD/minutes and normalized to cell protein, represented as mean ± SEM. All catalase measurements are done in quadruplicate (n=4).

<table>
<thead>
<tr>
<th>Passage number</th>
<th>Catalase activity (rate/mg protein)</th>
<th>MOSE CRBP1+/+VAS</th>
<th>MOSE CRBP1+/+VAD</th>
<th>MOSE CRBP1-/VAS</th>
<th>MOSE CRBP1-/VAD</th>
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<td></td>
<td></td>
<td>0.00998 ±0.0028</td>
<td>0.013586 ±0.006</td>
<td>0.0016 ±0.0002</td>
<td>0.0401 ±0.0032 * *</td>
</tr>
</tbody>
</table>

(** p<0.0002)

Research Project 7: Project Title and Purpose

Immune Response Interactions Following Concurrent Pathogenic Challenges - The vast number and diversity of microbes in the environment pose continual challenges to the human immune system. Much of what we know about immunity following infection—including the activation of immune cells, recruitment of immune cells to the target organ, mechanisms of pathogen clearance, and maintenance of immunological memory—has been gleaned from mouse models. However, such models have typically evaluated immune response parameters after a challenge with a single pathogen, whereas expanding literature in human immunology indicates that humans are often bombarded with multiple pathogens simultaneously. The purpose of this project is to utilize a novel mouse model to determine how immune responses to two concurrent viral challenges interact, and how pathogen clearance is affected under these conditions.

Duration of Project

1/1/2007 - 12/31/2009

Project Overview

Measles virus (MV) is a well-known human pathogen that continues to result in greater than a million deaths worldwide each year. Despite the availability of an effective vaccine, poor vaccination rates—especially in our nations’ cities—have left many children unprotected against this infection, and periodic outbreaks in the U.S. are a reminder of how rapidly this infection can spread in an unvaccinated population. While MV causes a disease that is usually self-limiting and relatively harmless, in some circumstances, the virus can migrate to neurons of the central nervous system (CNS) where it can initiate massive neuronal destruction leading to the death of the patient. Why some individuals develop CNS complications and most others do not is poorly understood. Therefore, to study the pathogenesis of MV-triggered CNS diseases, a novel mouse model was established, whereby only CNS neurons could become infected, allowing for a precise evaluation of the virus-neuron interaction and the anti-viral immune response in the CNS.
As in humans, immunocompetent mice were fully protected from CNS disease by a rapid and robust immune response. In contrast, immuno-compromised mice (that are a model for immuno-compromised humans including the very young, the elderly, HIV-1 positive people, and cancer patients undergoing chemotherapy) developed severe neurological disease owing to unrestricted infection. Interestingly, when otherwise immunocompetent mice were challenged simultaneously with a second viral pathogen (lymphocytic choriomeningitis virus; LCMV), greater than 50% of the mice showed signs of severe neurological disease, characterized by weight loss, seizures, and paralysis.

The overall goal of this project is to pursue this last observation by determining how concurrent viral infections impact on the generation of anti-viral immune responses and subsequent neuropathogenesis. Using this novel mouse model, we will test the hypothesis that immune cells, specific for a peripheral infection, are mis-recruited to the brain under conditions of concurrent viral challenge and that such aberrant recruitment then triggers immunopathology. We will establish the profile of immune cells that enter brains under conditions of single or concurrent infection, identify the factors that govern their entry into the brain, and characterize how these cells induce cell damage and disease in the CNS.

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Expected Research Outcomes and Benefits

The studies proposed in this project are aimed at exploiting a well-established mouse model of virus infection to more closely model how humans respond to environmental agents that trigger an immune response, including viruses, bacteria, molds, and allergens. An important underpinning of this work is that immune responses to concurrent immunological challenges can interact with each other and potentially result in immunopathology that do not occur when those challenges are introduced individually. While these studies are specifically focused on immunopathology within the central nervous system (given the large number of CNS diseases of unknown etiology), it is expected that the results from these studies may reveal common principles of immune cell interactions in vivo. Identification of novel means by which viruses can trigger disease may reveal new candidates for intervention to prevent or resolve pathogen-induced diseases in humans.
Summary of Research Completed

In the final six months of the project, we focused much of our attention on the completion of our initially stated objectives, repeating experiments for validation, data analysis, and preparation of manuscripts for publication. One manuscript ("Lymphocytic choriomeningitis virus-induced mortality in mice is triggered by edema and brain herniation" by Matullo, C.M., K. J. O'Regan, M. Curtis, H. Hensley, and G. F. Rall) was published in Journal of Virology in January 2010. A second manuscript ("Lymphocyte misrecruitment into the central nervous system following concomitant viral infections triggers antigen-independent neuropathogenesis" by C. K. Matullo, K. J. O'Regan and G. F. Rall) was submitted to PLoS Pathogens in February 2010 for review.

Some specific scientific achievements:

3.1 Specific Aim 1: Characterize mis-recruitment of T cells into the CNS.
3.1.3. Sub-aim 3: Is there increased blood-brain barrier permeability in doubly infected mice?

With the observation that sick, doubly-infected mice showed increased blood-brain barrier permeability, we then turned to a histological analysis of these tissues, in collaboration with our neuropathology colleague, Dr. Mark Curtis at Thomas Jefferson University in Philadelphia. As expected, the neuropathology in moribund mice was identical to that seen in mice infected with LCMV by the lethal IC route. Healthy doubly-infected mice showed no significant neuropathology as compared to uninfected controls.

3.2. Specific Aim 2: Define the contribution of the mis-recruited T cells to the increased pathogenesis observed in the doubly infected mice.
3.2.2. Sub-aim 2: Which immune cell populations are the main contributors to the illness seen in the doubly infected mice?

Our previous results indicated that T cell-mediated neuropathology was dependent on expression of class I MHC antigens. We have since validated and refined this observation using adoptive transfer experiments in which T cells from LCMV-infected, class I MHC competent mice were transferred into MV-infected, class I MHC null recipients. As before, no neuropathology was seen in the doubly infected, MHC-I null recipients, despite a significant infiltration of LCMV-specific T cells into the brain parenchyma. This more rigorous test for the role of class I MHC again substantiates that MHC-I expression is essential for the observed neuropathogenic phenotype.

Research Project 8: Project Title and Purpose

Genome-wide siRNA Screens to Identify Targets for Chemotherapy - One of the major challenges in cancer treatment is the ability of the cancer to develop resistance to the drug that is used to treat the patient. The reasons why cancer cells develop drug resistance are complex, but this is nevertheless an issue that must be tackled to improve treatment outcomes. We believe that drug therapy can be improved by identifying genes in the cancer cell that are responsible for this resistance. In the last several years, a new experimental tool, called siRNA, was discovered that allows scientists to inhibit virtually every single gene in any human cell type, including
cancer cells. We propose to use robotics and high-throughput instruments to block the expression of all of the known human genes (~22,000), one at a time, to identify the genes that enhance cell killing by existing drugs that are used to treat pancreatic cancer.

Duration of Project


Project Overview

Pancreatic adenocarcinomas are highly resistant to conventional chemotherapies. Gemcitabine is the only cytotoxic drug shown to improve survival and quality of life in patients with metastatic pancreas cancer. The sobering statistic, however, is that approximately 75% of patients with advanced disease will not survive more than one year, and few will survive two years even with treatment. Phase III trials of various cytotoxic agents in combination with gemcitabine did not show improvement over gemcitabine alone. The modest improvement attained with erlotinib, which is an inhibitor of EGFR tyrosine kinase, provides hope that drug response can be improved by targeting multiple pathways. The Clinical Trials Planning meeting in pancreatic cancer was convened at the National Cancer Institute. The conclusions from this meeting were that multiple approaches must be implemented to identify new drug targets, vaccines, improve clinical trial strategies, establish biorepositories and identify biomarkers.

My lab is currently using siRNA technology to screen the genome (~23,000 genes) in pancreatic cancer cells to identify new drug targets and potential gene signatures of drug response in pancreatic cancer patients. Recent genome sequencing studies showed that pancreatic cancers exhibit a high degree of variation with respect to the number, as well as the class of genes that are mutated. Combinations of these mutant genes may directly or indirectly affect tumor formation as well as resistance to chemotherapy. High-throughput genome-wide siRNA screening is an effective pseudogenetic approach to identify genes and pathways in tissue culture cells that sensitize cells to killing by drugs or oncogenic states. By the end of September 2009, we will have completed Aim One, our first genome-wide siRNA screen to identify genes that enhance killing of a pancreatic cancer cell line by gemcitabine. The second phase will be to validate and categorize the candidates from the primary screen using various secondary assays. The expectation is that our functional genomics screen will yield candidate drug targets that will enhance sensitization of pancreatic cancers to genotoxic agents such as gemcitabine, and genetic markers that can be used for diagnosis and improve therapy selection for patients.

Specific Aim 1 is to employ a genome-wide siRNA screen to identify genes and pathways that either sensitize or inhibit cell death caused by these agents. Potential targets will be validated by RT-PCR analysis.

Specific Aim 2 will test whether candidate target genes identified from the screen affect drug sensitivity of cell lines in vitro.
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**Expected Research Outcomes and Benefits**

We hypothesize that pancreatic cancers are refractory to cytotoxic drugs, such as gemcitabine and taxanes, because of altered patterns of gene expression that promote drug resistance and survival. Inactivation of these genes should disrupt the survival pathways, and thus, raise drug sensitivities and also expand the repertoire of drugs used for treatment. A genome-wide siRNA screen will largely achieve this goal, as we will query the contribution of each gene to cellular response to drug treatment. The biological pathways regulated by these genes would then be targets for pharmacological intervention that would lead to sensitization of the cancer cells.

We expect to identify a fairly large number of genes whose inhibition promotes pancreatic cancer cell cytotoxicity in the absence or presence of clinically employed chemotherapy agents (Specific Aim 1). Accordingly, we will follow up with several complementary studies to assess the potential significance of the observations. First, the genes of interest must demonstrate either overexpression (if their inhibition promotes cytotoxicity) or under expression. Thus, the clinical disease becomes the benchmark for the laboratory studies. Second, the expression profiles of any candidate genes must be confirmed in the cell lines in order to provide a rationale for preclinical therapeutic studies.

This project will identify candidate genes and pathways that can be disrupted to improve chemotherapy treatment of pancreatic cancer. This work will directly lead to improved pancreatic cancer therapy in three ways: 1) by identifying gene expression “signatures” that predict for chemotherapy treatment benefits; 2) by identifying new targets, the inhibition of which by existing or new drugs may improve existing therapies; and 3) by identifying new targets, the inhibition of which by new drugs will work alone to kill pancreatic cancer cells.

**Summary of Research Completed**

Specific Aim 1 is to employ a genome-wide siRNA screen to identify genes and pathways that either sensitize or inhibit cell death caused by these agents. Potential targets will be validated by RT-PCR analysis.

At the beginning of this report period (7/1/2009), we initiated the genome-wide siRNA screen that consisted of testing a library of ~22,600 genes. The entire genome-wide screen was
completed as of February 2010. The delay was due in part to the fact that we had to introduce and develop some of the methodologies to optimize the use of the high throughput instruments in our facility. There were also brief delays during the screen as we changed lots of cells, reagents, plasticware and growth medium. From a technical standpoint, the screen was completed in a timeframe that is consistent with other groups worldwide, with whom we stay in monthly contact through the ThermoFischer Dharmacon RNAi Global consortium.

The results of our genome-wide synthetic lethal screen are presented here. We used Panc1 cells as they were amenable to siRNA transfection and were relatively resistant to killing by gemcitabine. Our screen was conducted in 384 well plates where 300 siRNAs from the library (Dharmacon SMARTpool) are tested on each plate, with the remaining 84 wells serving as positive and negative controls (Figure 1). The controls include not only killer and non-killer siRNAs but they are plated in a pattern that allows us to normalize against edge effects (cells plated in wells at the edges of a plate may behave anonymously due to evaporation, temperature variances). A typical readout from a screen is shown in Figure 1. siRNAs were tested in duplicate in the presence and absence of a sublethal dose gemcitabine (4x384 plates). This dose is sufficient to induce a biological response as determined by DNA damage induced $\gamma$H2AX foci and S-phase arrest (Figure 2). An inhibitor of the Chk1 kinase, which has been reported, and confirmed by us, to sensitize killing of gemcitabine treated cells is included as one of the positive controls. Following siRNA and drug treatments, cell viability is quantitated with CellTiterGlo, a fluorescence-based assay that has a wide dynamic range and a signal that remains stable while the samples are in the queue for the microplate reader. Each screen generated 2400 datapoints per week that were analyzed by our in-house Biostatistician (Yan Zhou, Ph.D.). The datapoints are the CellTiterGlo readouts from duplicates of the 600 siRNAs tested in the presence and absence of gemcitabine. Our efforts make a difference as seen by our high z-scores (>0.6), a statistical value that is used to evaluate the quality of HTS data. Z-scores of <0.5 means there is low confidence in the datapoints and a screen has to be repeated. Z-scores of >0.5 validate any differences in datapoints between drug and no drug treatment.

In addition to the z-score, we also calculated the false discovery rate (FDR) for each siRNA that was tested. FDR is a statistical method that is used to reduce false positives in screens that rely on comparison between different treatment conditions (+/- gemcitabine). The FDR threshold is ascertained from the observed $P$ value distribution and is adaptive to the differences in the signal level in our data from experiment to experiment. FDR values between 0.0 to 0.2 indicate that the difference in the readouts between drug and control treatments is significant. Using a cutoff FDR value of <0.2, we identified 122 genes whose knockdown increase killing of Panc1 cells to a non-lethal dose of gemcitabine.

Although these hits remain to be validated, a subset of them were found by microarray analysis to be over or under expressed in pancreatic tumors (http://www.pancreasexpression.org/), and mutated in the pancreatic cancer genome sequencing project. We will prioritize our efforts based on these criteria.

Specific Aim 2 will test whether candidate target genes identified from the screen affect drug sensitivity of cell lines in vitro.
Of the 122 primary hits we have identified from our screen, there are about a dozen with obvious ties to DNA damage and mitosis. We have therefore initiated studies to target these genes to separately test for chemosensitization by gemcitabine. We have conducted experiments with siRNAs that target two proteins that are essential for base excision repair. Given that gemcitabine blocks replication fork progress, the resultant DNA damage response is likely to involve the base excision repair pathway. Furthermore, increased expression of one of the BER proteins is a prognostic indicator of lung cancer resistance to cisplatin, an agent that also blocks DNA replication, albeit through a different mechanism than gemcitabine.

In collaboration with Dr. H.Y. Fan at Fox Chase Cancer Center we are studying how loss of BER proteins sensitizes pancreatic cancer cells to killing by gemcitabine. The important observation at this point is that the siRNA’s seem to sensitize pancreatic cancer cells but not another cell line from different origin. Perhaps pancreatic cancer cells are “addicted” to BER because of their inherent genetic instability. Timelapse microscopy of Panc1 cells stably expressing gfpH2B (chromatin marker) showed that cells treated with a non-lethal dose of gemcitabine delay in metaphase but eventually separate their chromosomes and exit mitosis (Figure 3). In contrast, cells depleted of the BER protein enter mitosis after gemcitabine treatment, and eventually died before exit.

Separately, we have conducted siRNA knockdowns of a protein that encodes an essential subunit of an E3 ubiquitin ligase complex that is required for mitotic exit. We found that loss of this protein subunit caused cells treated with gemcitabine to enter mitosis and arrest for an extended period of time. As with depletion of BER, microscopy studies clearly showed that these cells also died in mitosis (data not shown). This is an important observation as the ability to kill cells while they arrest in mitosis eliminates any possibility of generating progeny cells that can develop resistance or are the source of a recurrent tumor.

Ongoing efforts are to systematically validate the remaining list of targets using the existing assays. Regardless of the outcomes from the validation screen, we have already validated three targets in the BER pathway and mitotic exit pathway.
Figure 2. Panc1 cells treated and not treated with gemcitabine were fixed and stained with the indicated antibodies. CENP-F is a marker of S phase, and p-H2AX is a marker for DNA damage.

Figure 3. Timelapse videomicroscopy of Panc1 gfpH2B cells treated with the indicated conditions. The gfp images show chromosome alignment as cells progress through mitosis. Images were collected at 5 minute intervals.