

Pennsylvania State University

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Pennsylvania State University received \$7,628,852 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Vitamin D and Crohn's Disease: From the Bench to the Bedside - Decreased outdoor activity and increased pollution and diets that lack adequate vitamin D have combined to create large fluctuations in vitamin D status, especially in populations that experience winter. Since vitamin D regulates the development and function of the immune system, this may account for the increase in autoimmune diseases like inflammatory bowel disease (IBD). Changes in vitamin D status result in more severe forms of experimental IBD, but vitamin D supplementation completely blocks the development of experimental IBD. Our hypothesis is that Crohn's patients have low circulating levels of vitamin D, which may exacerbate IBD. We propose to give Crohn's patients vitamin D and determine whether this dose is well tolerated, induces an increase in circulating vitamin D levels and/or improves health.

Anticipated Duration of Project

7/1/2009 – 6/30/2012

Project Overview

A) Broad objectives/Aims: The incidence of autoimmune diseases like inflammatory bowel disease (IBD) has increased in developed countries over the last 50 years. We propose that decreased outdoor activity and increased pollution and diets that lack adequate vitamin D have combined to create large fluctuations in vitamin D status in developed countries and especially in populations that experience winter. Experimentally we've shown that changes in vitamin D status result in more severe forms of experimental IBD. In addition, active vitamin D (1,25(OH)₂D₃) completely blocks the development of experimental IBD. The vitamin D hypothesis proposes that vitamin D regulates the development and function of the immune system and that changes in vitamin D status affect the development of the resultant immune response and the development of diseases like IBD. Our hypothesis is that because of low dietary vitamin D intakes and malabsorption of many nutrients, Crohn's patients will have low circulating vitamin D levels that are detrimental for their health. We plan to give

Crohn's patients 1000 IU of vitamin D/d and determine whether this dose is well tolerated, induces an increase in circulating vitamin D levels and has any additional health benefits (improved bone markers, Crohn's disease activity scores, inflammatory markers).

B) Research Design: The plan is to conduct a phase I trial of vitamin D supplementation in 50 adult patients with mild to moderate Crohn's disease. Patients will be recruited from both Hershey and State College. Because the study is a feasibility study no placebo controls will be included. Instead patients will serve as their own controls. Baseline and 6 month data collection will be done on diet, serum vitamin D levels, inflammation, bone mineral density, Crohn's activity scores and quality of life surveys.

Principal Investigator

Margherita Cantorna, PhD
Associate Professor
Penn State University
115 Henning Building
University Park, PA 16802

Other Participating Researchers

Terryl Hartman, PhD, Jill Smith, MD - employed by Penn State University

Expected Research Outcomes and Benefits

The expected outcomes are 1) to identify what the dietary intakes and circulating levels of vitamin D are in the patient pool, 2) to determine whether vitamin D supplementation will improve vitamin D status, and 3) to determine whether increased vitamin D intakes are associated with health benefits in the Crohn's patients including bone mineral densities, Crohn's activity scores, and quality of life. The information we gather in this study will be used to determine whether a larger clinical study is worthwhile, whether additional selection criteria in the recruitment of Crohn's patients are necessary, whether vitamin D is well tolerated and adequate for improving vitamin D status, and whether clinical and immunologic improvement is seen over the 6-month study period. In addition, the clinical data will be used to help develop new mechanistic experiments aimed at determining what aspects of the immune response and/or health of the Crohn's patients are improved with vitamin D supplementation.

Summary of Research Completed

We have successfully recruited, enrolled, and completed the collection of data from 13 Crohn's patients. In addition, we have 5 patients that will have completed the study by the end of September 2011. We are no longer recruiting new patients and once we have all of the samples we will spend the next six months analyzing the data and preparing a manuscript and a new grant proposal based on the data we've collected. Our total number of patients in the study will be 18, which is less than we had hoped for. Because we were using an escalating dose of vitamin D we were required to have multiple clinic/laboratory visits that discouraged participation from

patients in rural Pennsylvania. 1000 IU/d of vitamin D was not adequate for raising 25(OH)D3 levels in any of the 18 individuals in the study. In 13 of the 18 patients or 72% the maximal dose of 5000 IU/d was given for the 24 week study.

Figure 1 shows the effect of vitamin D supplementation on 25(OH)D3 levels. The baseline values were low in all 15 patients and increased significantly with vitamin D supplementation for 24 weeks. Vitamin D supplementation effectively raised 25(OH)D3 levels in the serum. Figure 2 shows the effect of the vitamin D supplementation on the Crohn's disease activity index (CDAI) scores. We recruited patients with mild to moderate Crohn's disease as defined by the Crohn's disease activity index (clinical evaluation scored 0-600 most severe). Scores below 150 indicate quiescent disease, scores from 150-199 indicate mild disease and scores of 200-449 indicate moderate disease. Our preliminary data suggests a potential beneficial role for vitamin D supplementation in Crohn's patients (Figure 2). Patients started out with scores of 210 +/- 14 indicating symptoms that were on the low end of the moderate scale. There was a significant drop in the Crohn's disease scores to 112 +/- 17 that indicates quiescent disease in these preliminary analyses.

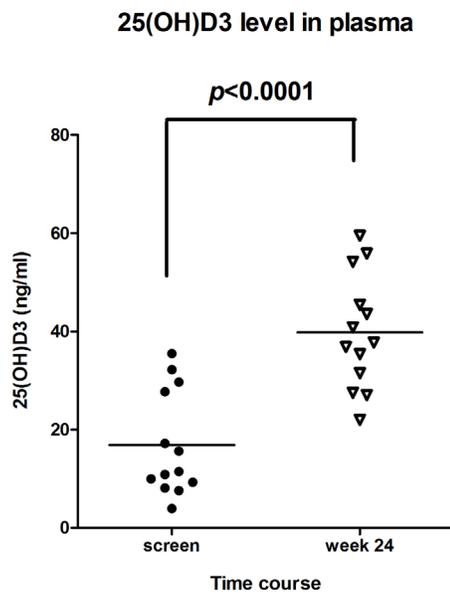


Figure 1.

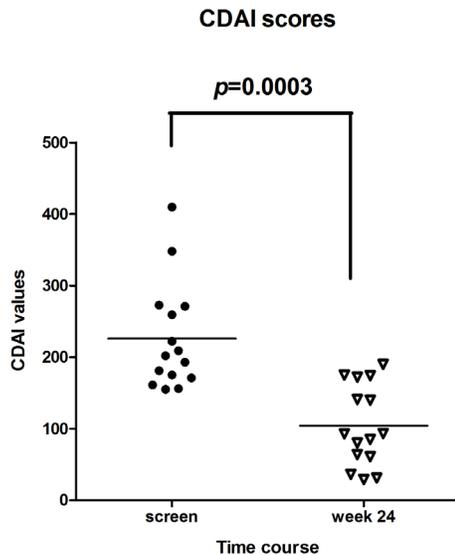


Figure 2.

Research Project 2: Project Title and Purpose

Epigenetic Regulation of Inactive X Chromosome Expression - Personalized medicine, or the tailoring of pharmaceuticals and treatments to specific individuals, is an important goal of genomic medicine in the 21st century. It is increasingly apparent that even subtle gene differences may impact common traits. We are interested in evaluating gene differences that effect the female sex chromosome (X) where most, but not all, genes on the second X must be silenced. The goal of these experiments are to evaluate the variation in gene expression for genes on the X chromosome and to design an assay that can quickly and effectively assess inactive X expression for specific genes to assess their role in common diseases.

Duration of Project

1/1/2009 – 12/31/2009

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>.

Research Project 3: Project Title and Purpose

Role of UGT2B7 Genotype in Patient Response to Tamoxifen - The glucuronidation phase II metabolic pathway is an important factor in breast cancer recurrence and toxicity associated with tamoxifen (TAM) treatment, and genetic mutations in the enzyme responsible for metabolizing TAM may play a key role in determining overall patient response to TAM. We propose to test this hypothesis by measuring levels of TAM metabolites in patients on TAM and correlating

these levels with the presence of genetic mutations. Using detailed clinical questionnaires, we will also examine the potential association between the presence of mutations and patient toxicity and side effects (hot flashes, arthralgias, deep vein thrombosis and uterine cancer). Long-term goals will include studies examining whether these genetic mutations impact breast cancer recurrence and metastasis.

Duration of Project

5/1/2009 - 6/30/2010

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>.

Research Project 4: Project Title and Purpose

Functional Brain Imaging of Memory and Language for Epilepsy Surgery - The purpose of this project is to translate procedures and findings in experimental functional MRI (fMRI) into clinically validated techniques for localizing language and memory systems in the brain of patients undergoing surgery for epilepsy (i.e., temporal lobectomy). The current Wada procedure, which is used to localize systems in the brain, was developed over 50 years ago and is highly invasive, and does not always provide interpretable data. We propose to investigate the feasibility of using fMRI procedures as reliable indicators of language and memory localization to replace the Wada Test.

Anticipated Duration of Project

6/1/2009 - 6/30/2012

Project Overview

The specific aims of this project are:

- (i) Determine the fMRI characteristics most associated with typical left hemisphere language dominance. We hypothesize that areas where there is no fMRI activity detected across three language tasks can be safely resected during anterior temporal lobectomy for intractable epilepsy.
- (ii) Test the hypothesis that an asymmetry index of medial temporal lobe activity during memory retention tasks will be concordant with Wada memory test result asymmetry and of greater predictive value than the Wada for post-surgical memory function.
- (iii) Test the hypothesis that pre-operative fMRI language and memory asymmetries will be predictive of 6-month post-operative re-organization of function.

We plan to study 10 patients who are diagnosed with Temporal Lobe Epilepsy (TLE) and undergo anterior temporal lobe resection (ATLR) for relief of intractable seizures. The fMRI and

cognitive tests will be given to the clinical subjects prior to and 6 month post surgery in addition to the standard clinical tests used for preoperative surgery planning. The memory and language fMRI protocols based on previous studies will be validated with 10 age- and sex- matched healthy volunteers.

Participants will complete three basic language activation tasks including: verbal generation, picture naming, and sentence comprehension. Tasks associated with naming and reading were chosen because these skills are most closely associated with ATR complications. This will be examined in a 6-month follow up fMRI study by identifying the areas of fMRI activation that nonetheless were necessary to resect and comparing both out-of-magnet neurocognitive testing of language function and activation patterns.

Additionally, 3 different memory activation tasks, designed to stimulate material-specific memory and multi-material memory activations in the medial temporal region, will be used. During the encoding phase, stimuli will be presented in a block design format, with the participant responding to a specific question about what the stimulus is. These include: (1) single word with high semantic content; (2) nonverbal-visuospatial patterns; and (3) face-name pairs. The 3 types of stimuli will be intermixed in 3 runs of 45 stimuli (15 items for each category) and participants will respond with whether the stimulus is a work, nonverbal design, or face-name pair. After 10 minutes of rest, stimuli will be presented in event-related formats that are either (a) repeated from the initial encoding set or (b) novel stimuli not previously presented, for participant memory about whether they occurred 10 minutes before.

Principal Investigator

Paul J. Eslinger, PhD
Professor
Milton S. Hershey Medical Center
500 University Drive, Mail code: EC037
Hershey, Pa 17033

Other Participating Researchers

Dan T. Nguyen, MD, Michael Sather MD, Jayant Acharya MD, Vinita Acharya MD, Kevin M. Cockroft, MD, Claire Flaherty-Craig, PhD, Paul Kalapos, MD, James McInerney, MD, and Qing X. Yang, MD - employed by Milton S. Hershey Medical Center

Expected Research Outcomes and Benefits

fMRI has the potential to provide noninvasive brain mapping of critical cognitive functions such as language and memory with less cost, less health risk, higher reliability, and more information for surgical planning. It is the hope of the research team that this research will produce data that will lead to future clinical trials for the replacement of the Wada test for pre-operative planning. Additionally, successful completion of this project will advance the development of similar techniques for other neurosurgical conditions such as brain tumors and vascular malformations.

Summary of Research Completed

Our full study samples have been recruited. All patients have been studied in the pre-operative phase. One TLE patient has completed the 6-month post-operative studies, with a second patient scheduled for 6-month post-operative studies August 16, 2011. The remaining 8 TLE patients are expected to come to surgery and be studied in the post-operative phase in the upcoming academic year. The completion of ATR surgery is not under the control of the principal investigators and is subject to a great number of constraining clinical, personal and even insurance variables such as need for depth electrode and grid insertion recording studies in patients, managing co-morbid conditions, and family support systems. However, the fMRI studies to date have become an increasingly important component of the epilepsy surgical conference discussions. The Epilepsy Surgery Team is committed to completing treatment of the TLE patients and collecting the full sample of post-operative fMRI studies in order to correlate findings with clinical outcomes. For this past year and the upcoming year, remaining funds are strictly for completion of fMRI studies. All investigators and support personnel are completing the study protocol at no additional costs to the program.

Results of analysis for the full samples confirm our earlier reported findings. Measures of language activation showed significant hemispheric asymmetry of activity, such as the predominant left hemisphere activations observed during the Sentence Completion task (see Figure 1). Across the several language tasks, statistical analyses revealed no clear differences between patient and healthy controls in all eloquent language areas, confirming the expected language dominance patterns in TLE patients (see Figure 2).

On the memory tasks, patients showed less activation in the ATL regions (see Figure 3). This was more evident in the right ATL region on the picture recognition task and left ATL regions for the word recognition task. This pattern of results is consistent with expected hemispheric material-specific memory effects.

Figure 4 shows the word memory activation map from a TLE patient who underwent Wada testing with sodium brevitol. The results of the Wada test were ambiguous with regard to memory functions. The activation clusters associated with memory processing are clearly asymmetric in the anterior-medial temporal regions, with greater volume of activation in the left hemisphere (see top 8 slices). The fMRI language lateralization findings were fully consistent with the Wada test results.

Post Operative Test Results

To date, one TLE patient has come to the 6-month post-operative phase where we could re-examine their fMRI data. Despite their right ATR, this patient has shown improvement in word and face-name memory functions, as follows:

Word Recognition:	Pre = 73% vs. Post = 87%
Picture Recognition:	Pre = 90% vs. Post = 83%
Face Name Recognition:	Pre = 38% vs. Post = 44%

The preoperative activation map of a TLE patient taken from the fMRI Face-Name encoding condition is shown in Figure 5. The patient had right anterior medial temporal lobe pathology which is reflected in the asymmetric hypoactivation in that region during this learning task (see top 6 slices where left is on left and right on right).

In Figure 6 you will see the postoperative vs. preoperative activation map of the same ATR patient undertaking the same Face-Name encoding task. The area of surgical resection can be seen in the right anterior medial temporal lobe in the 4 upper left brain slices. As a result of the surgery and relief of seizures, the right anterior and medial temporal lobe is now much more active during the learning process. The patient accuracy score also improved.

A second ATR patient is scheduled for their 6-month postoperative scan on August 16, 2011. We expect to complete the post-operative fMRI studies of all 10 TLE patients within the next year.

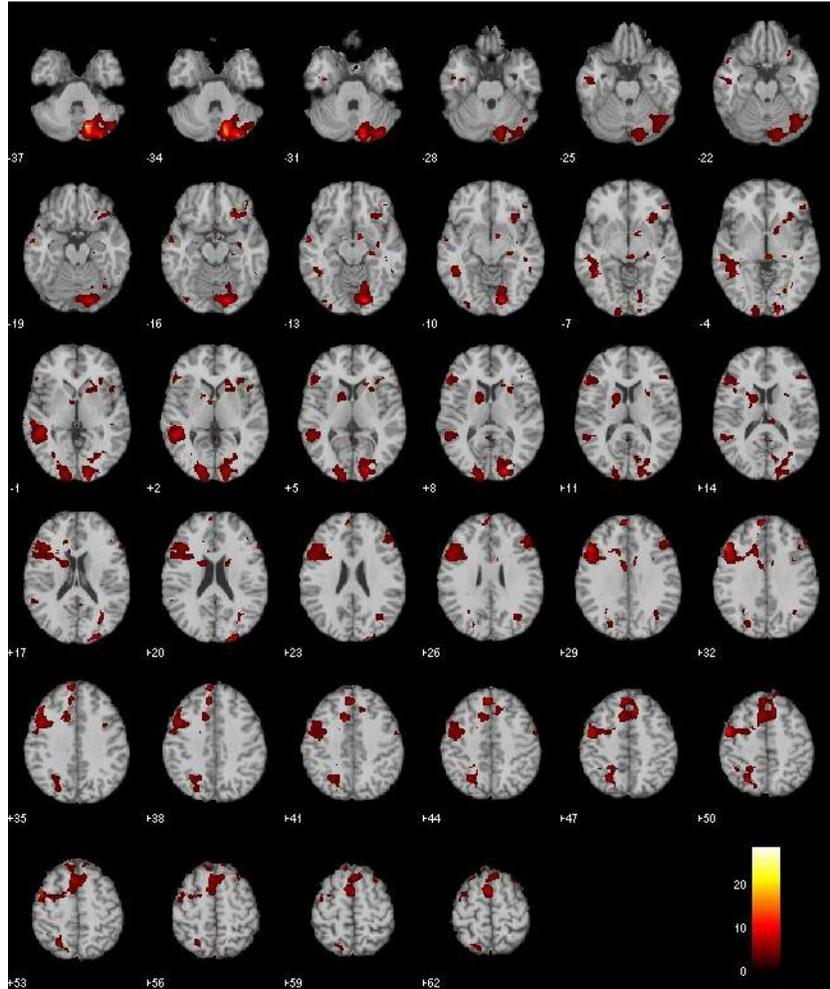


Figure 1. Average activation map of the TLE sample generated during the Sentence Completion Task. These results confirm that specific clusters of activity occurred in the left cerebral hemisphere (left on left in this image), specifically including Broca’s area and Wernicke’s area. Hence, this task produces a reliable and highly significant asymmetry of language activity consistent with expected left hemisphere dominance for language in right-handed patients with TLE.

Category Fluency Task

Sentence Comprehension Task

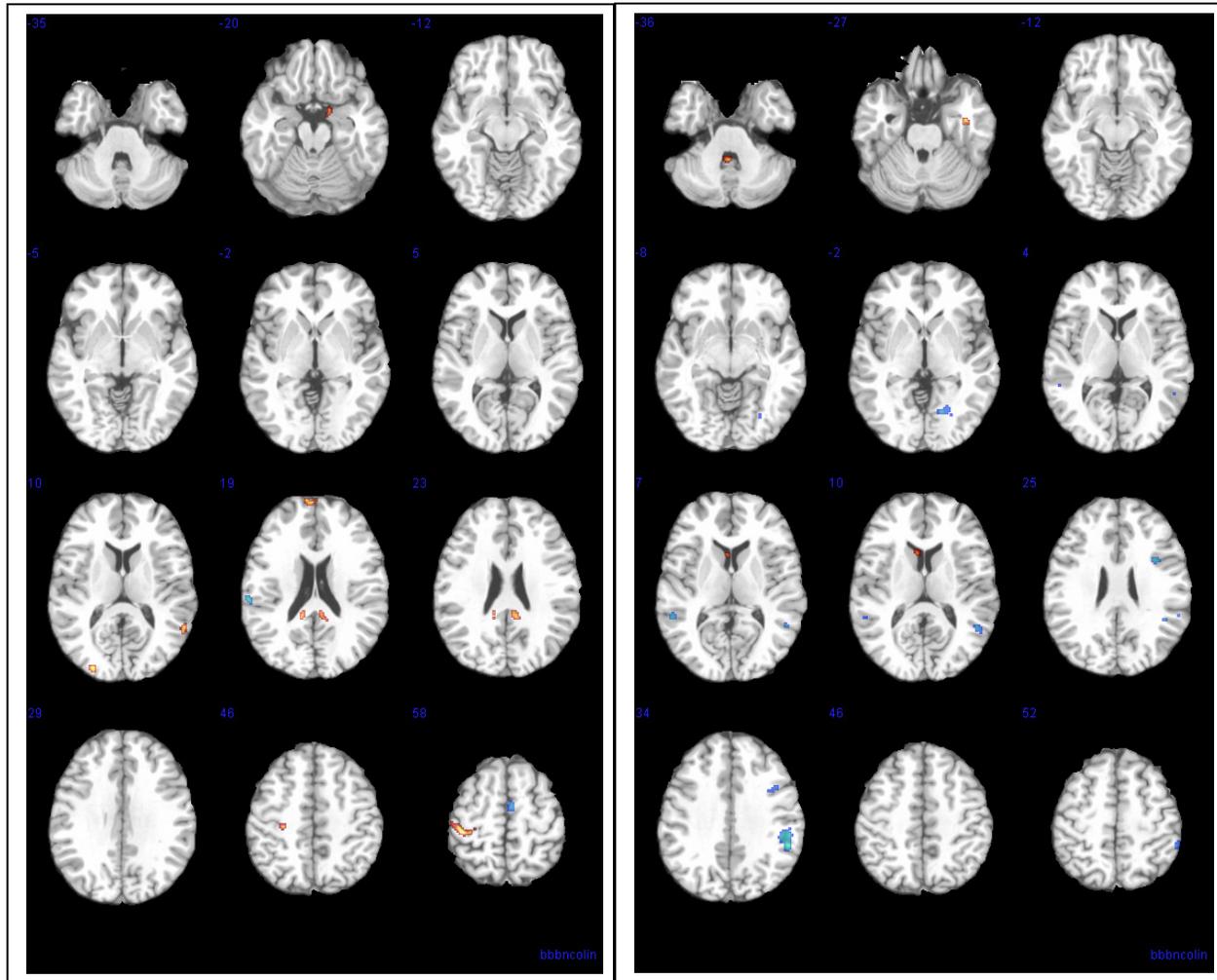


Figure 2. Statistical analysis of activation pattern differences between the TLE sample and healthy control sample on category fluency and sentence comprehension measures of language lateralization ($p < .001$; Red: TLE > Control; Blue: Control > TLE).

Word Recognition Memory

Picture recognition Memory

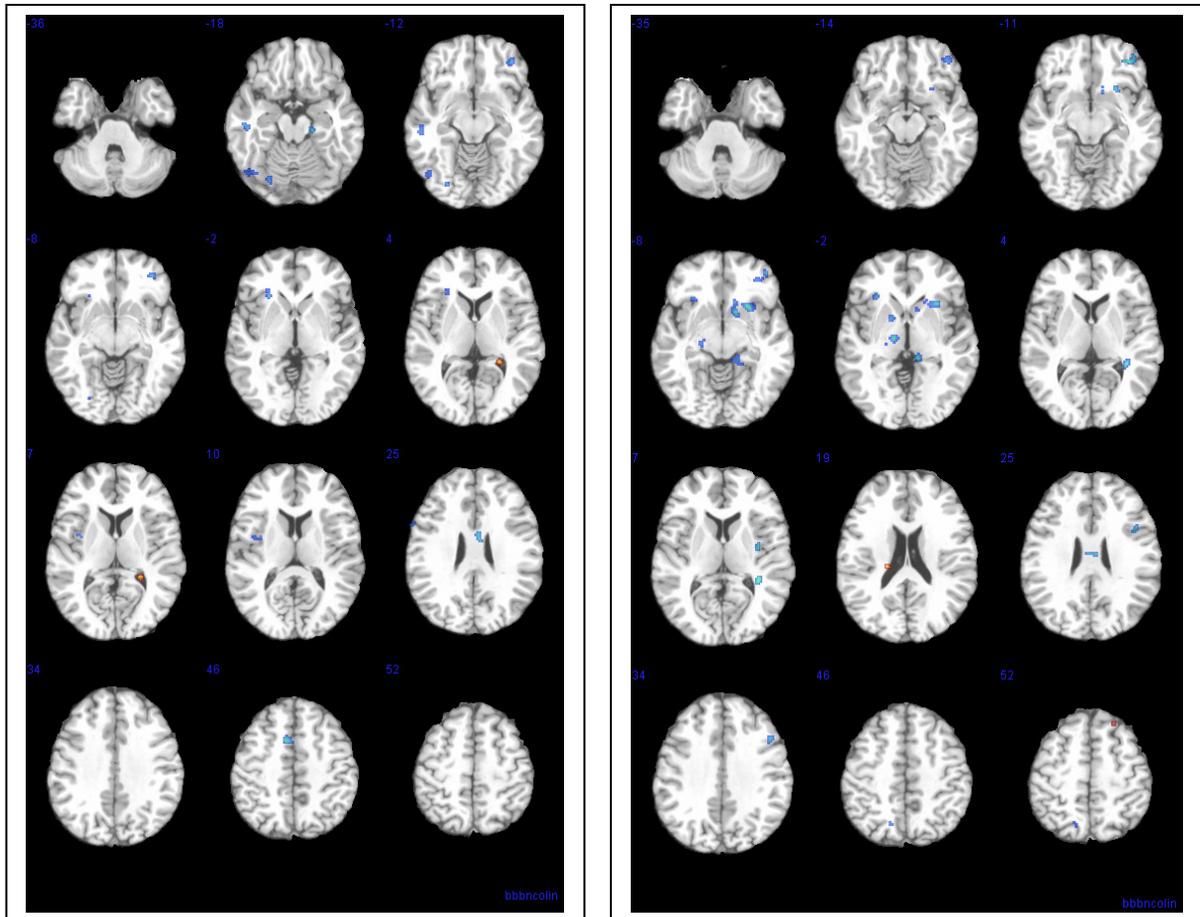


Figure 3. Statistical analysis of activation pattern differences between the TLE sample and healthy control sample on word recognition memory and picture recognition memory tasks. Controls are showing greater activation in frontal-temporal regions than TLE patients. ($p < .001$; Red: TLE > Control; Blue: Control > TLE).

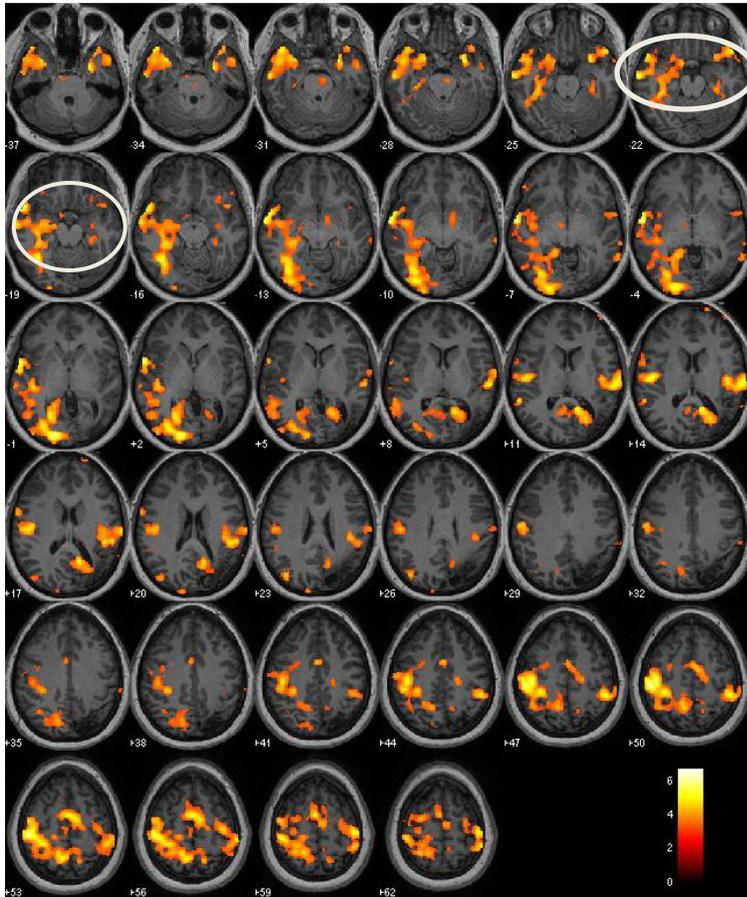


Figure 4. This is a word memory activation map from a TLE patient who underwent Wada testing with sodium brevitol. The results of the Wada test were ambiguous with regard to memory functions. The activation clusters associated with memory processing are clearly asymmetric in the anterior-medial temporal regions, with greater volume of activation in the left hemisphere (see top 8 slices). The fMRI language lateralization findings were fully consistent with the Wada test results.

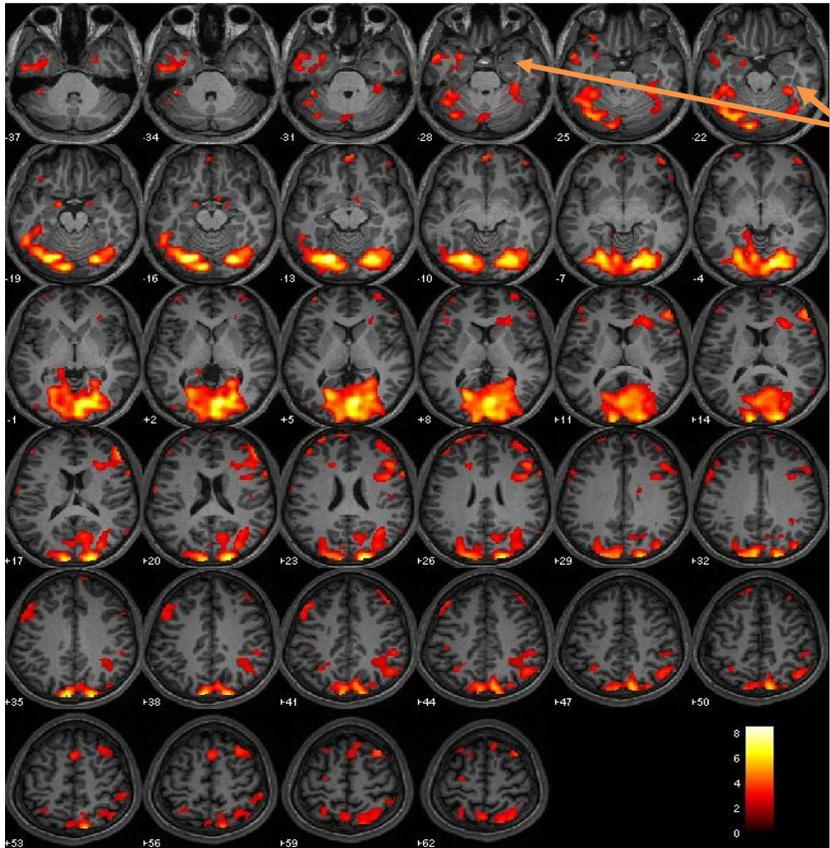


Figure 5. This is the preoperative activation map of a TLE patient taken from the fMRI Face-Name encoding condition. The patient had right anterior medial temporal lobe pathology which is reflected in the asymmetric hypoactivation in that region during this learning task (see arrows and top 6 slices where left is on left and right on right).

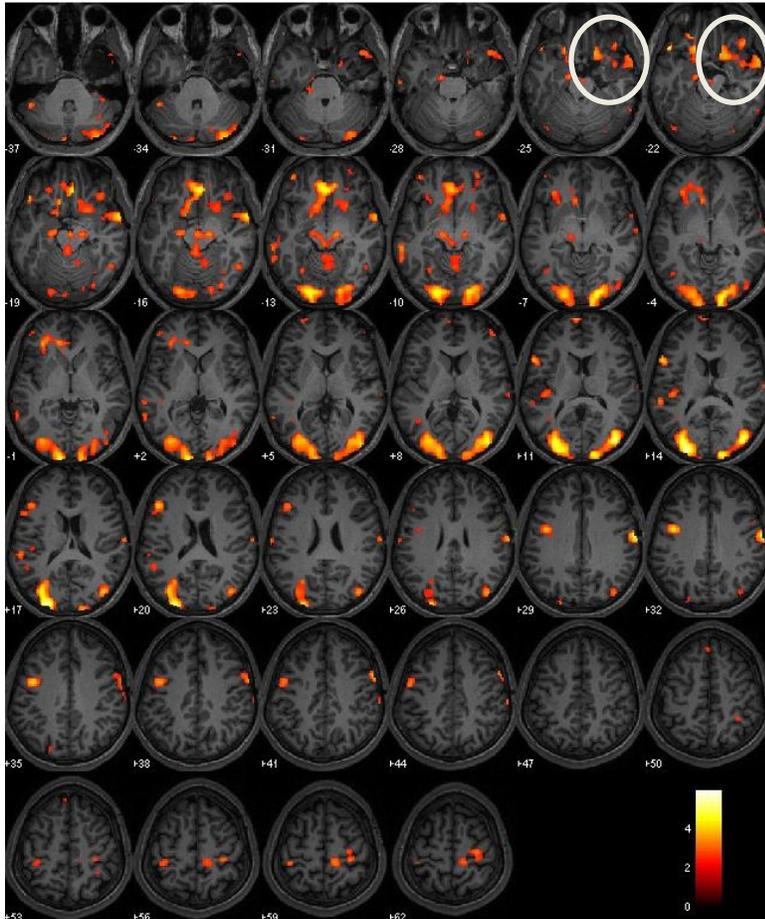


Figure 6. In follow-up to the pre-operative activation map above, this is the fMRI postoperative vs. preoperative activation map of the ATR patient undertaking the same Face-Name encoding task. The region of surgical resection can be seen as a dark area in the right anterior medial temporal lobe in the 4 upper left brain slices. The results show that when post-operative vs. pre-operative brain activation patterns are statistically compared, the right anterior and medial temporal lobe is now much more active during the learning process (see white circle regions). The patient's accuracy of learning also improved.

There is also notable activation in areas connected to ATL including the visual cortex and prefrontal cortex. These regions interact with the ATL during learning and memory tasks. Hence, the improved memory appears to be at the neural network level.

Research Project 5: Project Title and Purpose

Molecular Targets for Preventing Loss of Skeletal Muscle Mass - The loss of skeletal muscle mass that occurs in a number of physiological and pathological conditions has adverse effects on functional strength and mobility, and in many conditions is associated with an increase in morbidity and mortality (e.g., aging, sepsis, cancer). In order to design appropriate therapeutic interventions to ameliorate skeletal muscle atrophy, the molecular mechanisms that regulate skeletal muscle size must be thoroughly understood. Significant strides have been made recently with regard to elucidating the pathways involved in the regulation of both protein synthesis and degradation in muscle, but many gaps still remain in our understanding of the complex processes regulating muscle mass in adults. The purpose of this project is to more fully characterize molecular targets identified as key in the regulation of protein synthesis.

Anticipated Duration of Project

1/1/2009 – 6/30/2012

Project Overview

Eukaryotic Initiation factor 2B (eIF2B), a key regulatory and rate-controlling factor in the initiation of mRNA translation, can affect global rates of protein synthesis and has been recently investigated in an experimental rat model of resistance exercise. Previous results from the Principal Investigator's laboratory indicate that the translational efficiency of the mRNA for the catalytic epsilon subunit of eIF2B (eIF2B ϵ) is uniquely upregulated following resistance exercise in a rapamycin-sensitive manner. Cell culture experiments have further demonstrated that the mammalian target of the rapamycin complex 1 (mTORC1) signaling pathway is both necessary and sufficient for upregulation of eIF2B ϵ . The implication of mTORC1 signaling in the post-exercise signaling in muscle and the time course of increased protein synthesis seem to be consistent between rat and human models of exercise; however, changes in the expression of eIF2B subunits in response to resistance exercise in humans has not been previously reported. Furthermore, the contribution of the eIF2B ϵ expression to increased protein synthesis in muscle and the mechanism of its translational regulation are poorly understood. Considering the potential role of the upregulation of eIF2B ϵ in the increased rate of global protein synthesis observed in the rat model of resistance exercise, it is important to address these questions.

This project seeks to examine the role eIF2B ϵ in skeletal muscle hypertrophy and its regulation in relation to mTORC1 signaling by employing human, animal, and cell culture models. The goal of the project is to establish that resistance exercise in humans results in increased expression of eIF2B ϵ in skeletal muscle, to elucidate the mechanism by which the increased expression occurs using molecular and cell biological techniques, and to demonstrate that increased expression of eIF2B ϵ alone *in vivo* can increase muscle protein synthesis and lead to hypertrophy. The central hypothesis of the project is that activation of the mTORC1 signaling pathway leads to a post-transcriptionally regulated increase in expression of eIF2B ϵ protein and that an increase in eIF2B ϵ expression alone is sufficient to increase muscle protein synthesis and skeletal muscle fiber size. The following Specific Aims are designed to characterize the role and regulation of eIF2B ϵ in skeletal muscle hypertrophy: (1) to validate that an acute resistance exercise bout results in increased eIF2B ϵ protein expression in human skeletal muscle; (2) to demonstrate that *in vivo* modulation of eIF2B ϵ expression directly affects muscle protein synthesis and muscle fiber size; and (3) to validate the role of miR-133 in the translational repression of eIF2B ϵ mRNA.

Principal Investigator

Leonard S. Jefferson, Jr., PhD
Evan Pugh Professor and Chair
Pennsylvania State University College of Medicine
Department of Cellular & Molecular Physiology
500 University Drive, MC H166
P.O. Box 850
Hershey, PA 17033-0850

Other Participating Researchers

Alexander P. Tuckow, MS, Lydia Kutzler, Holly Lacko, Andrew Kelleher - employed by Pennsylvania State University College of Medicine

Expected Research Outcomes and Benefits

The results from this project will advance our understanding of the mechanisms of skeletal muscle hypertrophy and identify a potential target for future physiological or pharmacological interventions aimed at potentiating skeletal muscle hypertrophy and/or ameliorating skeletal muscle wasting in a number of conditions and disease states by favoring net protein synthesis.

Summary of Research Completed

This original project has been expanded to study whether or not dysregulation of the mTORC1 signaling pathway contributes to inactivity-induced loss of skeletal muscle mass, a widespread phenomenon associated with a number of conditions including sedentary life styles, chronic bed rest, casting, limb suspension and immobilization.

Each of these inactivity-related conditions is thought to share a common feature, i.e., a delayed and reduced magnitude in the response of skeletal muscle protein synthesis to an anabolic stimulus, which has been referred to as anabolic resistance. Additional studies have been designed to test the hypothesis that dysregulation of mTORC1 and/or protein synthetic control mechanisms is the underlying cause of the anabolic resistance-induced loss of skeletal muscle mass. To test this new hypothesis, we will pursue an experimental protocol in which control rats and rats subjected to short-term unilateral hindlimb immobilization will be placed on a meal-feeding regimen designed to elicit the anabolic resistance of protein synthesis in skeletal muscle. We will then perform a detailed analysis of components of the mTORC1 signaling pathway as well as selected regulatory mechanisms that mediate control of protein synthesis. Overall, we expect the proposed research to reveal molecular targets that can likely be manipulated pharmacologically resulting in new and innovative approaches to the prevention and treatment of muscle wasting conditions.

Studies performed during the previous reporting period suggested that degradation of the catalytic epsilon subunit of eukaryotic initiation factor 2B (eIF2B ϵ) occurs through a proteasome-dependent process. In studies performed in the past 6 months, we have extended the previous findings to identify amino acid residues in eIF2B ϵ that are subject to ubiquitination. In these studies, C2C12 myoblasts were transfected with a plasmid expressing eIF2B ϵ bearing a FLAG epitope at the N-terminus. The cells were then serum starved for 16 h and treated with or without the proteasome inhibitor MG-132 for 8 h. Interestingly, in the absence of MG-132 the expression of FLAG-eIF2B ϵ was nearly identical to that of the endogenous eIF2B ϵ expression. In contrast, with the addition of the proteasome inhibitor, there was a dramatic increase in FLAG-eIF2B ϵ protein expression. Furthermore, additional bands were identified in Western blots of lysates from cells treated with MG-132 that are presumed to be mono- and multi-ubiquitinated FLAG-eIF2B ϵ respectively.

To provide more direct evidence that eIF2B ϵ is ubiquitinated, lysates from C2C12 myoblasts transfected with two plasmids, one expressing FLAG-eIF2B ϵ and the other expressing HA-Ubiquitin (HA-Ub), were subjected to co-immunoprecipitation using a monoclonal antibody directed against eIF2B ϵ . Immunoprecipitation of eIF2B ϵ enriched the abundance of higher molecular weight bands detectable by Western blot. Probing the membrane with anti-HA (i.e., ubiquitin) antibody revealed a smear at molecular weights greater than the major eIF2B ϵ band. The smear is characteristic of polyubiquitinated proteins and represents HA-ubiquitin proteins that co-immunoprecipitated with eIF2B ϵ . Immunoprecipitation of HA(Ub) was also performed to examine the co-immunoprecipitation of eIF2B ϵ with ubiquitin. HA(Ub) immunoprecipitation followed by Western blot for eIF2B ϵ revealed several distinct bands as well as higher molecular weight smearing. The three most distinct bands were presumed to be eIF2B ϵ with one, two, and three ubiquitins. None of these eIF2B ϵ bands were detected in HA immunoprecipitates from cells that were not transfected with HA-Ubiquitin.

Having established the co-immunoprecipitation of eIF2B ϵ with HA-Ub using either approach (i.e., immunoprecipitation using eIF2B ϵ or FLAG antibody to pull down the modified protein as well as immunoprecipitation using anti-HA(Ub) antibodies and detection of the eIF2B ϵ protein), experiments were next directed towards identification of residues modified by ubiquitination using tandem mass spectrometry. In these studies, C2C12 myoblasts were co-transfected with pFLAG-eIF2B ϵ and pRK5-HA-Ubiquitin and treated with the proteasome inhibitor MG-132 for eight hours, allowing for the accumulation of ubiquitin-modified proteins. The lysates were subjected to FLAG immunoprecipitation followed by HA immunoprecipitation, and the sample was subjected to SDS-PAGE and two major band(s), corresponding to approximate molecular masses of 75-100 kDa and 100-250 kDa, respectively, were excised and analyzed by LC-MS/MS. The analyses resulted in 67.2% overall coverage of the rat eIF2B ϵ protein sequence. Five unique lysine residues containing the -GG modification indicative of ubiquitination were identified in the ~75-100 kDa excised gel slice: Lys56, Lys98, Lys136, Lys212, and Lys500. Two of these residues (Lys136 and Lys212) were also identified as ubiquitinated peptides in the highest molecular weight gel slice, although the overall peptide coverage was lower in that analysis so we cannot rule out the possibility that other lysines are ubiquitinated in the highest molecular weight species. Finally, four of the five ubiquitinated lysine residues and the regions surrounding them are highly conserved in the mouse, rat, and human proteins whereas Lys500 is not conserved in the human protein.

In addition to the ubiquitin modifications, the LC-MS/MS analysis revealed several phosphorylated residues among the rat eIF2B ϵ peptides. Three serine residues (Ser527, Ser535, and Ser539) were identified that have been previously reported and characterized. In addition to the known sites of phosphorylation, three novel residues were identified as phosphorylated residues: Ser22, Ser125, and Thr317. A fourth residue was contained among the contiguous serine and threonine residues (#325-327) but the exact location of the phosphorylated residue was not clear from the spectra. Each of the novel phosphorylation sites we report (Ser22, Ser125, Thr317) are conserved among rat, mouse, and human eIF2B ϵ proteins.

The information provided above forms the basis for a manuscript that is currently being prepared for submission:

Tuckow, A.P., Kimball, S.R., and Jefferson, L.S. Identification of Ubiquitinated Lysine Residues on Eukaryotic Initiation Factor 2B Epsilon

In April 2011 the original project was expanded to include an examination of mechanisms involved in a phenomenon that is referred to as anabolic resistance. The Specific Aims of these studies are:

- 1) Establish optimal time point at which anabolic resistance manifests in an experimental model of inactivity-induced wasting of skeletal muscle.
- 2) Define the composition of the meal that best illustrates anabolic resistance.
- 3) Optimize conditions for ectopic expression of DNA plasmid constructs in skeletal muscle in vivo.

To date we have performed initial studies in which one hindlimb of male Sprague-Dawley rats was immobilized by application of a fiberglass cast on the left leg; control rats were not casted. Six days later, all animals were fasted overnight, and the following day one-half of the animals were fed rodent chow. Fifty min after the start of feeding, [³H]phenylalanine was administered i.v., and ten min later, the plantaris, soleus, and gastrocnemius muscles were removed, weighed, and homogenized. It was found that the relative mass (muscle weight/body weight) of the three muscles was reduced 16%, 22%, and 28%, respectively in the immobilized, compared to the control, leg. Refeeding stimulated global rates of protein synthesis in control rats by approximately 30% in the soleus and 25% in the combined gastrocnemius/plantaris muscles. However, no increase in protein synthesis was observed in either muscle type in the immobilized leg of the casted animals. Signaling through mTORC1 was assessed by changes in phosphorylation of three downstream targets, S6K1, 4E-BP1, and eIF4G. In control animals, refeeding significantly increased the phosphorylation of all three proteins. In contrast, in the immobilized leg of the casted animals, refeeding-induced phosphorylation of S6K1 and 4E-BP1 was attenuated, and phosphorylation of eIF4G was blocked. Overall, the results demonstrate the effectiveness of the casting model in mimicking the anabolic resistance reported in humans. In addition, the results suggest that in the immobilized leg, a defect manifests in the nutrient- and/or hormone-induced activation of the mTORC1 signaling pathway.

Research Infrastructure Project 6: Project Title and Purpose

Research Infrastructure - Biological Research Laboratory Construction - The purpose of this project is to design and build an Animal Biosafety Level three (ABSL-3) research laboratory for the study of immunology and infectious diseases requiring high level Biocontainment. This project is a continuation of the project funded by the 2007-08 formula grant.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

The project was temporarily put on hold while the Penn State design team put together an

expanded design and grant proposal for the National Center for Research Resources (NCRR) Recovery Act Construction Program. The goal of the grant proposal was to expand the ABSL-3 facility to include an insectary, and additional laboratory and animal holding space. In addition the expanded facility will have increased redundancies to meet National Institutes of Health (NIH) construction guidelines. The proposal was successful and an award of \$14.8 million was received to expand the facility. The proposed Biological Research Laboratory will provide space, support, and biocontainment for basic, applied and diagnostic research; national, state and community outreach; and education on important human and / or zoonotic pathogens. It will include state of the art laboratories, an insectary, and animal resources, facilities and services that are recognized within and outside the University as being of the very highest quality consistent with our talents and resources. This facility will be composed of a number of ABSL-3 suites for *in vivo* research using small animal models of human disease (primarily rodents and poultry models). Each suite will have independent air locks to support multiple agent research as well as providing compartmentalization to mitigate cross-contamination concerns. Changing rooms and shower out facilities are included as required. Supporting the ABSL-3 suites will be laboratories for *in vitro* bacteriology, virology, and molecular biology procedures. Outside of the biocontainment area another support laboratory provides preparatory space for the activities within the barrier. A conference/classroom/break room allows for on-site training sessions and staff meetings, and a manager's office and loading dock are also included. This project has been expanded to a \$23,000,000 building project that will encompass 20,000 gross square foot, and will provide laboratories, animal holding space as well as an insectary that will support the critical need for biocontainment research space at the Pennsylvania State University. This facility will be a unique and much needed resource for infectious disease research.

Principal Investigator

Mary J. Kennett, DVM, PhD, Diplomate ACLAM
Director Animal Resource Program and Professor of Veterinary & Biomedical Sciences
101 CBL
The Pennsylvania State University
University Park, PA 16802

Other Participating Researchers

Biao He, PhD, Girish Kirimanjeswara, PhD, Craig Cameron, PhD, Vivek Kapur, PhD -
employed by Pennsylvania State University

Expected Research Outcomes and Benefits

The proposed enhanced Biological Research Laboratory, an Animal Biosafety Level 3 (ABSL-3) facility, presents excellent opportunities for infectious disease research and will greatly enhance the research capabilities on campus. It is not possible to do research with highly infectious agents such as anthrax and avian influenza without proper protection and biocontainment. The new enhanced facility will allow work with vector borne diseases due to the inclusion of an insectary. This facility will provide special air handling capabilities to filter the exhausted air, liquid and solid waste decontamination, high security, and standard operating procedures within

the facility to ensure the safe handling of such agents. The facility is organized into a central spine that connects all of the research spaces to the central decontamination and support areas of the project.

Summary of Research Completed

This project is continuing. The environmental assessment was completed and a signed Finding of No Significant Impact (FONSI) was issued. The design team completed final design documents and submitted them to the NIH technical review team. The NIH architects and engineers have reviewed the project in detail and have approved the construction documents. The technical review process is therefore complete and we should receive a revised Notice of Award shortly. This notice will allow the release of the \$14.8 million in federal funds for the project. Penn state is currently preparing a Notice of Federal Intent that will be filed at the time construction begins.

The following is an outline of progress since the previous report.

- June 2010 – approval granted by NIH to continue using Torcon, Inc. construction management (CM) services as Torcon provided constructability and cost estimating services during the design process.
- October 2010 – Torcon prepared a preliminary Guaranteed Maximum Price (GMP) for use by Penn State officials
- November 2010 – Penn State Board of Trustees approved the preliminary GMP and granted approval for the project
- December 2010 – Penn State and Torcon begin preparing sub contractor packages and sub contractor requirements
- January 2011 – sub-contractors begin submitting pre-qualification information to Torcon. Project bidding requirements were posted on the Penn State construction web site.
- February 2011 – Torcon and Penn State hosted a pre-bid conference for sub-contractors. A second pre-bid opportunity was held two weeks later to accommodate subcontractors who could not attend the first pre-bid meeting.
- Bids were received by Penn State and Torcon on subcontractor packages March 23 and 24, 2011
- The design team, Penn State and Torcon conducted package de-scoping meetings with sub contractors following receipt of bids. The final bid packages are in review and the bid package for a CM at risk, including the final GMP as well as a discussion of the pre-qual, pre-bid, and bidding process, will be submitted soon. Once this is reviewed, NIH will issue a revised Notice of Award.
- The contract will be prepared by Penn State and presented to Torcon for signing. Once signed, Penn State issues the Notice to Proceed, the initial job conference will be held and construction begins.

In addition, we have hired a scientific director to oversee the research mission of the facility. Girish Kirimanjeswara is faculty member with ABSL3 experience who will bring research experience and leadership to the facility. John Henneman has also been hired as the BRL manager. John has 15 years of experience overseeing the construction and operation of BSL3 facilities. He will oversee the construction of the facility, start-up, validation and

commissioning, as well as maintenance and operations once the facility is built.

The building has been designed to meet construction and redundancy standards as described in the NIH Design Policy and Guidelines for ABSL-3 and ABSL-3 enhanced research space for high pathogenicity infections (such as avian influenza). The building will feature numerous security features including a perimeter fence, card or proximity reader access, biometric security to access the containment zone, as well as motion detectors, lights and security cameras. Several levels of security will be in place, only limited access to the facility will be allowed, and all entries will be documented. Training and security clearances will be required prior to entry according to a biosafety manual specific to the BRL. Standard Operating Procedures (SOPs) are in development specifically for the BRL to train and certify all personnel working within containment, to ensure safety and security according to Occupational Health and Safety guidelines. Specific policies are under development for practices such as showering, minimal sharps use, and decontamination. SOPs to minimize the production of aerosols are being written, and all animal specific regulations and biosafety procedures will be followed. Biohazardous materials use authorization (Recombinant DNA or Human Pathogen registration, Select Agent use etc.) will be instituted as appropriate.

Research Project 7: Project Title and Purpose

Regulation of Nutrient Sensing and Muscle Wasting by Alcohol - Metabolic balance is maintained at the cellular and whole-body level by responding to signals from the environment and from inside the body, namely growth factors, hormones, and stress signals. Chronic consumption and acute ingestion of alcohol reduces the muscle mass of those abusing this drug by changing the body's ability to respond to some of these signals. The purpose of the project is to elucidate how alcohol decreases the ability of the amino acid leucine to increase the synthesis of muscle proteins. If we can determine this mechanism, therapeutic interventions can potentially be developed which prevent the loss of muscle under this condition and as a result of other stress conditions, such as immobilization, trauma, infection, which have similar effects on muscle protein balance.

Duration of Project

1/1/2009 – 6/30/2010

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>.

Research Project 8: Project Title and Purpose

Murine Induced Pluripotent Stem Cells: Differentiation and Bone Formation - The goals of the project are to assess the potential of stem like cells called induced pluripotent stem cells (iPS) to differentiate toward mesenchymal stem cells (MSCs) and to make bone and cartilage in vivo,

thus to understand the future application of the cells for the repair and regeneration of musculoskeletal tissues.

Duration of Project

1/1/2009 – 6/30/2010

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>.

Research Project 9: Project Title and Purpose

Evaluation of mTOR as a Chemoprevention Target in Skin Cancer - The object of these studies is to validate the mammalian target of rapamycin (mTOR) as a target for chemoprevention of non-melanoma skin cancer. Since skin cancers are the most common form of malignancies, with increasing numbers world-wide, identification of possible targets for their prevention are particularly relevant to public health.

Anticipated Duration of Project

1/1/2009 – 12/31/2011

Project Overview

The overall goal of these experiments is to validate mTOR as a potential target for chemoprevention of nonmelanoma skin cancer (NMSC). The experiments involve proof-of-principle studies to show the feasibility of conditionally deleting mTOR in the skin of bitransgenic mice expressing Cre recombinase and a floxed mTOR allele (K5CreER/mTOR^{LoxP/LoxP} mice). Additional short-term experiments examining UVB-induced proliferation and apoptosis were also undertaken, since our currently used model of chemical carcinogenesis is less physiologically relevant than a UVB model.

Principal Investigator

Lisa M. Shantz, PhD
Associate Professor
Penn State College of Medicine
500 University Drive
Hershey, PA 17033

Other Participating Researchers

Theresa D. Carr - employed by Penn State College of Medicine

Expected Research Outcomes and Benefits

The central idea of the project is that the mammalian target of rapamycin (mTOR) is an important target for chemoprevention of non-melanoma skin carcinogenesis. mTOR responds to changes in cellular nutrients by controlling the synthesis of proteins essential for multiple processes, including cell proliferation and organization of the cell cytoskeletal. A large number of cancer-promoting mutations are known to result in mTOR activation. A role for mTOR-controlled pathways in skin carcinogenesis has been suggested in recent clinical trials, which have shown that renal transplant patients administered the specific mTOR inhibitor rapamycin as an immune suppressant suffer from significantly fewer non-melanoma skin cancers compared to patients taking calcineurin inhibitors. Our studies, in combination with these promising clinical trials, will provide a strong rationale for continued development of not only rapamycin analogues but also mTOR kinase inhibitors, which would target both mTOR complexes as chemopreventive agents.

Summary of Research Completed

The aim of these studies is the characterization of the epidermis in K5CreER/mTOR^{LoxP/LoxP} mice, both untreated and after UVB exposure. These studies used both adult mice and primary keratinocytes isolated from 1-3 day old pups. In adult epidermis, we verified the ablation of mTOR after treatment with 4-hydroxytamoxifen (4OHT) using the following protocol: The dorsal surface of each mouse was shaved 24-48h prior to treatment. Mice were treated with 4OHT (1mg) every day for a total of 5 days. Whole skin was harvested 14 days after the final treatment for DNA isolation and PCR, and for Western blots examining the phosphorylation of S6-kinase (S6K) to measure mTORC1 activity and AKT to measure mTORC2 activity. A subset of mice were also exposed to 120 mJ/cm² UVB, and analyzed for induction of mTOR-dependent pathways using the same markers. The results show that recombination is observed at the DNA level only in the presence of 4OHT (*Figure 1A*). However, under basal conditions phosphorylation levels of both S6K and AKT were very low, and did not differ between vehicle (DMSO/Acetone; D:A)-treated and 4OHT-treated mice. This is not surprising and reflects the low levels of mTOR activity in adult unstimulated epidermis. Exposure to UVB dramatically induced phosphorylation of both S6K and AKT in vehicle-treated mice, and this was markedly attenuated in 4OHT-treated K5CreERT²/mTOR^{lox/lox} skin, confirming inhibition of pathways downstream of both mTOR complexes in mice treated with 4OHT (*Figure 1B*).

We showed in our previous progress report that treatment with Rapamycin, a specific inhibitor of mTORC1, blocked hyperproliferation of mouse skin in response to UVB exposure (data not shown). Similar experiments were performed with K5CreERT²/mTOR^{lox/lox} mice to determine whether deleting both TOR complexes enhanced this effect. Mice were treated with 4OHT and UVB as described above. Either 24 h or 48 h after UVB exposure, dorsal skin sections were fixed for 4 h in 10% neutral buffered formalin, paraffin embedded, sectioned at 5 μm, and stained with H&E. The epidermal thickness, measured at four non-overlapping skin sections, was averaged for each mouse, using 3 mice for each condition. In the absence of UVB (mock), both vehicle- and 4OHT-treated K5CreERT²/mTOR^{lox/lox} epidermis were 1-2 cells thick, suggesting that in the absence of an external stimulus, mTOR activity is dispensable in the adult epidermis. UVB irradiation caused a significant increase in epidermal thickness in vehicle-

pretreated K5CreERT²/mTOR^{lox/lox} mice within 24 h, but this effect was significantly blunted in mice treated with 4OHT, with an even greater difference observed 48h after UVB exposure (*Figure 2*). These results are comparable to those obtained previously with Rapamycin treatment, and suggest that mTORC1 inhibition is sufficient to block UVB-induced hyperproliferation of the epidermis.

In vitro experiments used mTOR-ablated primary keratinocytes to examine the differential contribution of mTORC1 and mTORC2 to primary keratinocyte survival after UVB exposure. Keratinocytes were isolated from wild-type and K5CreERT²/mTOR^{lox/lox} pups 1-3 days after birth and established in low calcium medium. Briefly, epidermis and dermis were incubated in 0.25% trypsin overnight at 4°C. After removal of the dermis, keratinocytes were isolated and plated in standard growth medium consisting of Ca⁺²- and Mg⁺²-free EMEM (Biowhitaker) and 8% FCS treated with Chelex (BioRad Laboratories). Final calcium concentration was adjusted to 0.05 mM using CaCl₂, and the culture medium was also supplemented with EGF (5ng/ml). After 24 h in culture, cells were treated with vehicle or 4OHT for 3 days. To confirm recombination, DNA was harvested for PCR and protein was harvested for Western blot analysis. To examine the effects of UVB exposure, cells were exposed to 50 mJ/cm² UVB, then harvested at various time points and assayed for proliferation and apoptosis.

To confirm recombination in primary keratinocytes from K5CreERT²/mTOR^{lox/lox} mice, cells were treated with either vehicle or 4OHT as described above. PCR results of DNA isolated from primary keratinocytes show clearly that the recombined allele is present only upon treatment with 4OHT (*Figure 3A*), indicating no leakiness in the system. Experiments examining the activities of mTOR-dependent pathways were also performed. In keratinocytes not exposed to UVB, (0' UVB), basal activities of mTORC1 and mTORC2 were only slightly reduced in response to 4OHT treatment, as measured by phosphorylation status of S6K and AKT, respectively (*Figure 3B*). These results are consistent with those obtained in whole skin, as described above. However, when keratinocytes were exposed to UVB, then harvested 30 min later, a dramatic reduction in phosphorylation of both S6K and AKT was observed in 4OHT-treated keratinocytes compared to those not treated with 4OHT, confirming that both pathways were inhibited (*Figure 3B*). This reduced response to UVB persisted for at least 120 min after UVB exposure. Thus, this experimental system allows us to assess the physiological consequences of inhibiting both TOR complexes in keratinocytes.

To test the hypothesis that mTORC2-dependent pathways exert an anti-apoptotic effect in response to UVB treatment, primary keratinocytes were isolated from K5CreERT²/mTOR^{lox/lox} mice and treated with 4OHT as described above to induce recombination. Cells were then treated with 50 mJ/cm² UVB and assayed for viability 24h later. The results show that deletion of both mTOR complexes appeared to enhance UVB-induced cell death (*Figure 4A*). This result is strengthened by measurements of cleaved Caspase 3, a marker of apoptosis, 9 h after UVB, which showed significantly more cleaved Caspase in mTOR-ablated keratinocytes than in vehicle-treated K5CreERT²/mTOR^{lox/lox} cells (*Figure 4B*). These data are in contrast to those previously obtained with Rapamycin, which suggested that inhibition of mTORC1 had no enhancing effect on UVB-induced apoptosis (data not shown). Thus, these results strongly suggest a role for mTORC2 in control of UVB-induced apoptosis.

Conclusions: The results suggest that mTORC2-dependent pathways maintain the survival of DNA-damaged cells after exposure to UVB, while mTORC1-dependent pathways control their proliferation. These studies underscore the importance of mTORC1 and mTORC2 in mediating UVB-induced pro-survival signaling, validating their potential as prospective chemoprevention targets in non-melanoma skin cancer. The studies described were used as Preliminary Results in an R21 application to the National Institute of Environmental Health Sciences that was recently funded (ES019242).

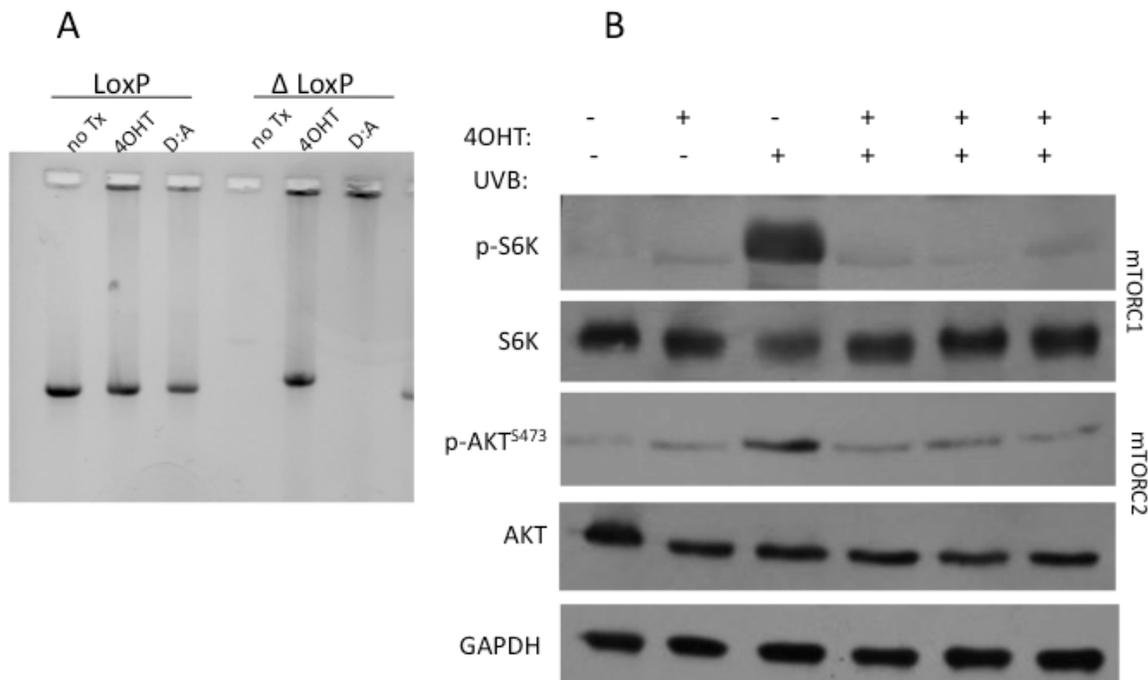


Figure 1. *In vivo* recombination in skin of K5CreER/mTOR^{LoxP/LoxP} mice exposed to 4OHT. **A.** PCR demonstrates the presence of the wild-type allele in all treatment groups (untreated, 4OHT-treated, and vehicle (D:A)-treated), while the presence of the recombined allele is only present after treatment with 4OHT. **B.** Both mTORC1- and mTORC2-dependent pathways are activated by UVB, and this is blocked by ablation of mTOR with 4OHT treatment (treated mice in triplicate).

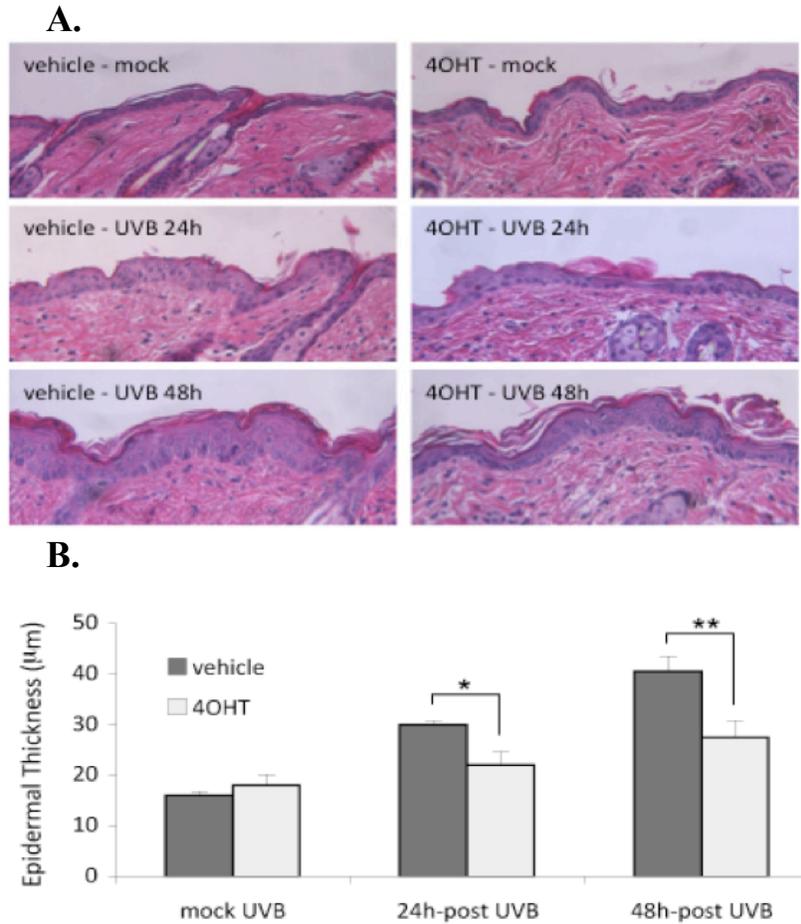


Figure 2. mTOR deletion blocks UVB-induced hyperproliferation of mouse epidermis. Mice were treated with 4OHT and UVB as described in the text and epidermal thickness was measured after 24h and 48h. **A.** Representative H&E sections from each group. **B.** Quantitation of A with N=3 mice, averaging 4 non-overlapping sections from each mouse. *p<0.05; **p<0.005

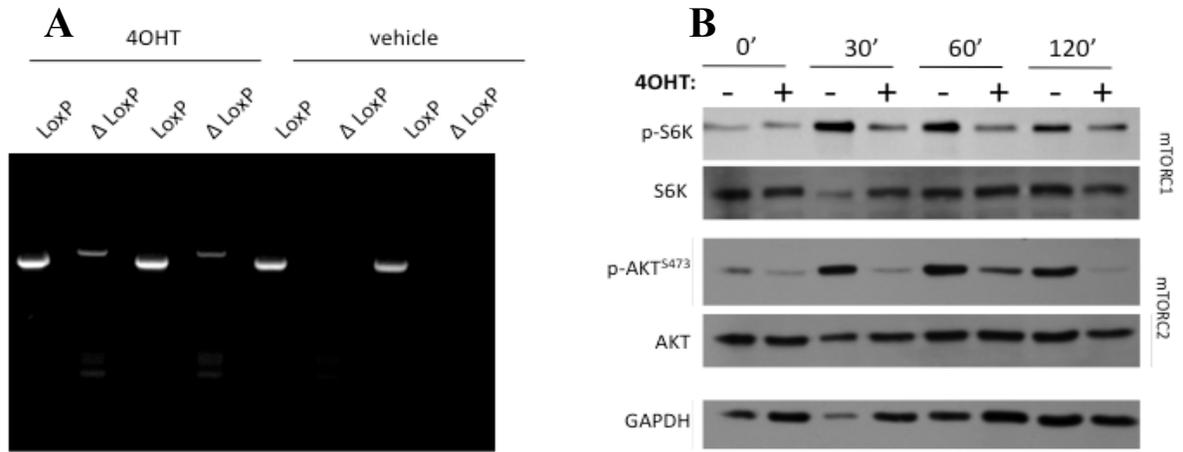


Figure 3. Verification of LoxP recombination in primary keratinocytes from K5CreERT²/mTOR^{lox/lox} mice. *A.* PCR demonstrates the presence of the wild-type allele in all samples (vehicle- and 4OHT-treated), while the presence of the recombined allele is only present after treatment with 4OHT. *B.* Cells were grown and treated as described in the text, and then Western blots were performed for the indicated proteins. Basal levels of mTOR activity are comparable to wild-type, while response to UVB stimulation is attenuated in the absence of mTOR.

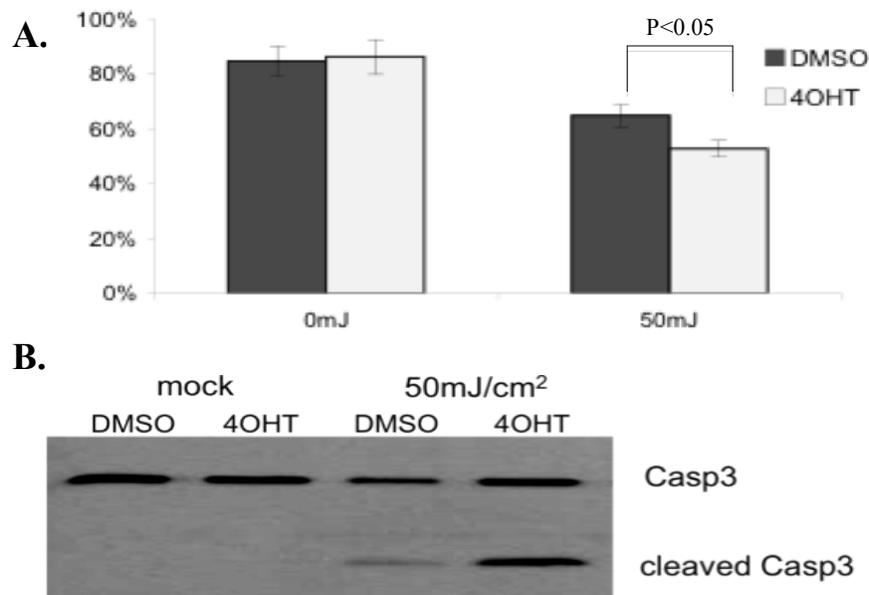


Figure 4. Loss of both TOR complexes in primary keratinocytes increases apoptosis in response to UVB. *A.* Trypan blue exclusion to measure cell viability 24 h post-UVB (50mJ/cm²) in K5CreERT²/mTOR^{lox/lox} keratinocytes treated with 4OHT to induce recombination. *B.* Determination of Caspase 3 cleavage 9 h post-UVB (50 mJ/cm²) in K5CreERT²/mTOR^{lox/lox} keratinocytes treated with 4OHT or vehicle.

Research Project 10: Project Title and Purpose

IRES-Mediated Synthesis of Proteins Integral to Adaptation to Hyperoxia - The purpose of this project is determine if exposure of human lung cells to high concentrations of oxygen alters the processes responsible for synthesis of specific proteins. In particular, our goal is to understand how high ambient oxygen concentrations increase the synthesis of some proteins while decreasing the synthesis of others. We believe, that oxygen “uncovers” alternative synthetic processes inherent in some protein templates, thereby enabling increased synthesis during periods of stress.

Duration of Project

1/1/2009 - 6/30/2010

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>.

Research Project 11: Project Title and Purpose

Stroke Recovery in Type II Diabetes - The overall objective of this project is to determine the role anti-diabetic agents have on stroke outcome. Although it is well established that diabetics are at an increased risk of mortality and morbidity following an ischemic stroke there are no known therapies that are specifically targeted at diabetics to improve stroke recovery. This is a significant public health problem considering that the worldwide diabetic population is growing at an alarming rate and thus the incidence of strokes is undergoing the same dramatic increase. This project will test both the chronic and acute effect of several anti-diabetic agents on stroke outcome with the specific objectives of determining which are the most efficacious in promoting recovery in the diabetic animals and the mechanism(s) by which the respective agents elicit their actions.

Duration of Project

1/1/2009 - 3/31/2010

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>.

Research Project 12: Project Title and Purpose

Modulation of Basal Ganglia Electrophysiology by Dopaminergic Cell Transplant - Cell

transplants have been shown to provide symptomatic relief for Parkinson's disease patients and in parkinsonian animal models. Separately, researchers have highlighted abnormal brain electrical patterns that occur in Parkinson's disease and are partially reversed with levodopa treatment. This project looks at a crucial related question which has not yet been examined: whether cell transplants change the electrical patterns in the brain. The results of the project will shed crucial light on the utility of cell transplants compared to levodopa treatment and the potential to reduce dyskinesias (a major side effect of prolonged levodopa treatment). The results will also shed light on basic neuroscience questions underlying Parkinson's disease pathology.

Anticipated Duration of Project

6/1/2009 – 6/30/2012

Project Overview

The broad research objective is to better understand the normal electrical properties of the brain, how they are altered in Parkinson's disease and whether "normalizing" the aberrant electrical patterns is critical to restore normal behavior. Part of this overall objective is to better understand the mechanisms by which cell transplants ameliorate Parkinson's disease symptoms.

The specific research aim is to answer the question: Do dopaminergic cell grafts modulate basal ganglia (BG) electrophysiology in the anesthetized and unanesthetized hemiparkinsonian (HP) rat? Planned subquestions are: Are the changes different in asleep versus awake conditions, do fetal ventral mesencephalic (FVM) cell grafts cause different changes than retinal pigment epithelium (RPE) cell grafts, and is there a difference between striatal-only grafts versus combined striatal-nigral grafts?

The methods involve comparing the recorded electrical patterns from the brains of rats. Rats are divided into five groups: Normal, Control, and Transplanted (3 different transplant paradigms: FVM-striatal, FVM-striatal/nigral, and (RPE)-striatal). After baseline testing, and induction of hemiparkinsonism via the 6OHDA neurotoxin-injection paradigm, the rats in the chronic group will be transplanted with cells (or vehicle) and implanted with electroencephalogram (EEG) screw electrodes and chronic electrodes for recording local field potentials (LFPs) from the subthalamic nucleus (STN), whereas rats for urethane recordings will receive transplants but no chronic electrodes. After recovery, rats in the chronic group will have EEG and LFP signals recorded weekly for three months under both asleep and awake conditions, whereas rats in the anesthetized-recordings group will undergo electrophysiology solely at the end of the three months. Afterward the brains will be histologically examined. The recorded signals will be examined for differences in specific frequency bands between the different groups.

Principal Investigator

Thyagarajan Subramanian, MD
Professor
Penn State Hershey Medical Center
500 University Drive, MC H109
P.O. Box 850
Hershey, PA 17033-0850

Other Participating Researchers

Timothy Gilmour - employed by Penn State Hershey Medical Center

Expected Research Outcomes and Benefits

The answers to the main research question and subquestions will advance the current understanding and treatment of Parkinson's disease. Knowledge of the extent to which cell transplants normalize the basal ganglia electrophysiology will be crucial to understanding the long-term safety of cell transplantation therapy. Furthermore, if transplants normalize the electrophysiology better than levodopa does, new research avenues would be opened to investigate whether cell transplants are better able to avoid dyskinesias, since dyskinesias are known to be associated with electrophysiological abnormalities.

Each subquestion will also provide essential information. Knowledge of the cell type which produces the best symptomatic amelioration and electrophysiological normalization will directly translate into improving transplant paradigms for human patients. Likewise the answer to the question of whether dopamine replacement into multiple basal ganglia nuclei is necessary for electrophysiological normalization will directly affect transplant paradigms.

In summary, the outcomes of this project will directly affect therapy implementation for human sufferers of Parkinson's disease, and will shed crucial light on electrophysiological questions about the underlying pathology.

Summary of Research Completed

The project had two major branches. The first branch compared anesthetized recordings from grafted and control rats, and the second branch examined chronic awake recordings from identical groups.

Anesthetized Recordings

The anesthetized recording project achieved significant discoveries and has been submitted to a high impact factor journal, *Brain*. Figure 1 shows the histology, including significant graft survival. Figure 2 shows significant normalization of the bursting firing patterns in the rats with large grafts. These results are significant and show that continuous replenishment of dopamine through grafts has the potential not only to ameliorate behavioral symptoms, but also restore electrophysiological firing patterns. This may prove to be crucial in developing Parkinson's

disease therapies that avoid causing motor complications like dyskinesias.

Chronic Recordings

The chronic-recordings project has also had progress in the last year, but is not yet completed and submitted for publication. Table 1 shows the projected number of rats for the entire chronic project, and the number of rats whose experimental procedures were completed during the reporting period 6/1/2010 – 6/30/2011. The analysis of the electrophysiological LFPs and cortico-BG coherence is still in process, as well as the analysis of the histology and the behavior. Planned analyses include custom software written in Matlab (Mathworks) to compute the spectral power of the LFP signals. Analysis of variance (ANOVA) will be performed between groups on the LFP spectral power and coherence with EEG in the alpha, beta, and gamma frequency bands.

Table 1 – Listing of numbers of rats completed for chronic/unanesthetized experiment

Control Groups		Experimental Treatment Groups		
Group 1 Normal	Group 2 HP	Group 3 FVM-single	Group 4 FVM-dual	Group 5 RPE-dual
Projected for entire project				
10 rats	10 rats	20 rats	20 rats	20 rats
Completed prior to 6/1/2010				
5 rats	4 rats	8 rats	5 rats	0 rats
Completed between 6/1/2010 – 6/30/2011				
4 rats	0 rats	9 rats	0 rat	0 rats

Figure 1 (in review for *Brain*) – Histology from anesthetized-recordings project. (A) Sample tyrosine hydroxylase (TH) stained section of HP rat showing lesioned left striatum, (B) sample TH stained section of transplanted rat showing lesioned left striatum with surviving TH-positive cell transplant, (C) Higher magnification of boxed area in (B), (D) TH stained section confirming denervation of left substantia nigra pars compacta, (E) Immunofluorescent staining showing typical graft derived TH-positive cells and their neurites, (F) Immunofluorescent staining of the same area as (E) showing DAT-positive graft cells and their neurites, (G) Composite overlay of (E) and (F), (H) Cresyl violet stained section showing electrode track into subthalamic nucleus (STN), (I) Cresyl violet stained section showing electrode track into substantia nigra pars reticulata (SNR). Scalebars: 1mm for A, B, D, H, I; 250um for C; 100um for E, F, G.

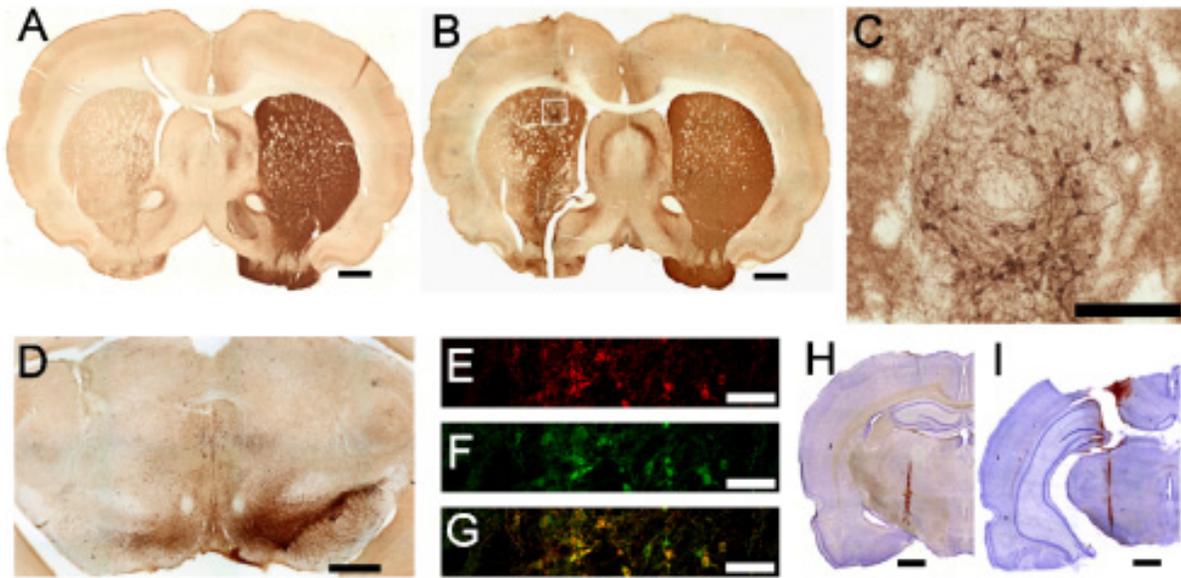
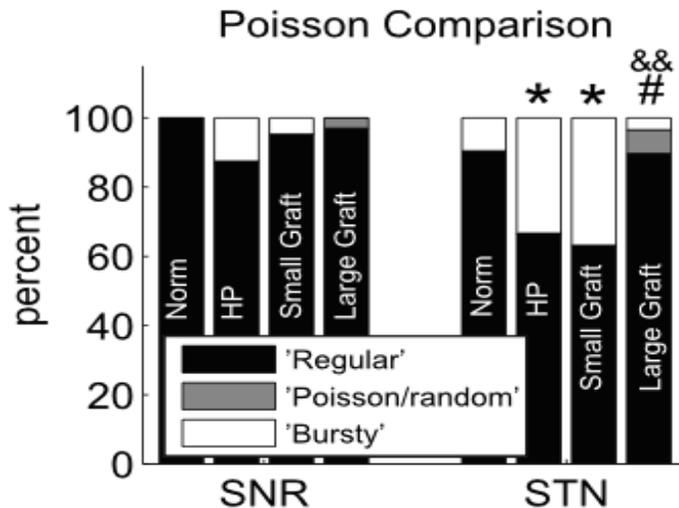


Figure 2 (in review for *Brain*) - Slow wave (SWS/SWA) firing rates and patterns (* $p < 0.05$, *** $p < 0.001$ compared to normal control; # $p < 0.05$ compared to HP control; & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$ compared to small graft group). Legend in E also applies to A, B, C, F, and G.



Research Project 13: Project Title and Purpose

Identification and Analysis of Arterial Blood Pressure Noise in Baroreceptor Denervated Rats -

The arterial baroreceptor reflex rapidly and effectively stabilizes blood pressure. A prominent finding, across many species, is that eliminating the baroreceptor input by sinoaortic denervation (SAD) more than doubles arterial pressure variability (APV). Understanding the sources and basic mechanisms of APV is of clinical importance. Using neuromuscular blocked (NMB) rat preparation, we will specifically examine the obligate roles of the baroreflex key way stations, e.g., the rostral ventral lateral medulla (RVLM), in producing the large post-SAD APV. The funds will be used to collect preliminary data for our NIH R03 2nd re-submission. Our long-term goal is to develop APV analysis into a non-invasive and economical tool for clinical prognosis and diagnosis of cardiovascular diseases.

Duration of Project

1/1/2009 - 6/30/2011

Project Overview

Surprisingly few studies have investigated the neurophysiological sources, and/or regulatory function, if any, of the APV. Understanding APV is of clinical importance. Since the 1980's, despite numerous attempts to catalog APV changes in a range of diseases, the prognostic and diagnostic promise has yet to be realized. A possible reason for this is lack of fundamental understanding of the sources and causes of APV. This current study aims at identifying the potential key central noise sources that are responsible for the large post-SAD APV. Our long-term goal is to further develop APV analysis into a non-invasive and economical tool for the clinical prognosis and diagnosis of cardiovascular diseases.

Previously, using a unique chronically neuromuscular blocked (NMB) rat preparation, we found that SAD more than doubled APV, and that ganglionic block, using chlorisondamine, significantly reduced the large post-SAD APV. These results indicated (1) that the noise sources for APV are endogenous and central, and (2) that normally, APV is attenuated by the baroreflex. Studies have shown that the brain regions rostral to the collicular are not potential noise sources, which suggests that the caudal brain stem is likely to be the source of the noise. Using NMB rats, we will determine the obligate roles of the baroreflex key way stations in the brain stem, e.g., the rostral ventral lateral medulla (RVLM), in the large post-SAD APV. We hypothesize that the RVLM (The Specific Aim) has an important role in the post-SAD APV. The Aim consists of 4 phases: baseline (3 d) -> SAD (3 d) -> bilateral ibotenic lesions of the RVLM (3 d) -> ganglionically block the rat with intravenous chlorisondamine (2 d). A control, of bilateral lesions in a non-cardiovascular area of the brain stem, the cuneate nuclei, will parallel the Aim. The major outcome measurements include the APV, characterized as the standard deviation of the arterial pressure (AP) and the very low frequency (VLF: 0.01-0.2 Hz) power of the AP spectrum. For each variable, separately, a 2 x 3 mixed factorial ANOVA varying experimental conditions (control, lesion) x phases (baseline, SAD, lesion) will be used to determine the RVLM contribution to post-SAD APV.

Principal Investigator

Xiaorui Tang, PhD
Assistant Professor
Penn State College of Medicine
H181 Neural and Behavioral Sciences
500 University Drive
Hershey, PA 17033

Other Participating Researchers

Barry Dworkin, PhD, Ralph Norgren, PhD - employed by Pennsylvania State University

Expected Research Outcomes and Benefits

Expected Research Outcome: The arterial baroreceptor reflex is a potent mechanism to stabilize blood pressure. Eliminating the baroreceptor input to the brain stem by sinoaortic denervation (SAD) more than doubles arterial blood pressure variability (APV). We expect that the key central noise source for the largely increased post-SAD APV resides at the rostral ventral lateral medulla (RVLM) in the brain stem.

Benefits: The APV has been related to a variety of cardiovascular diseases, such as hypertension, as well as brain or spinal cord injury. Understanding the primary origins of APV could let us better catalog APV changes in a range of diseases and better understand regulatory functions of the APV. This current project is the first step towards our long-term goal to develop APV analysis into a non-invasive and economical tool for the clinical prognosis and diagnosis of cardiovascular diseases.

Summary of Research Completed

In this reporting period, our main focus was on disseminating our research findings, which include: (1) we were invited for an oral presentation on research findings supported by this project at the 2010 FASEB Summer Research Conference (at Vermont); (2) two posters were presented at the Experimental Biology meeting in Washington and at the Digestive Disease Week meeting in Chicago last year; and (3) we collected additional data for the manuscript submitted to the European Journal of Applied Physiology. The first revision of this manuscript is currently under review.

Summary of the findings: The baroreflexes stabilize moment-to-moment arterial pressure. Sinoaortic denervation (SAD) of the baroreflexes results in a large increase in arterial pressure variability (APV) across various species. Due to an incomplete understanding of the nonlinear interactions between central and peripheral systems, the major source of APV remains controversial. While some studies suggested that the variability is endogenous to the central nervous system (CNS), others argued that peripheral influences may be the main source. In this study we used a unique, chronic neuromuscularly blocked (NMB) rat preparation that largely constrains peripheral influences to determine the CNS contribution to the post-SAD APV. First,

we confirmed that SAD significantly increased APV in the NMB rat, then demonstrated that post-SAD ganglionic blockade substantially reduced APV and subsequent intravenous infusions of phenylephrine and epinephrine (in presence of ganglionic blockade) only slightly increased APV. These data suggest that the CNS is an important source for the post-SAD APV, and skeletal activity, thermal challenges or other forms of peripherally generated cardiovascular stress are not required. In addition, we showed that bilateral aortic denervation produced a larger increase in APV than bilateral carotid sinus denervation, suggesting that the aortic baroreflex plays a more dominant role in the control of APV than the carotid sinus.

Experimental Protocols and Results:

Protocols were arranged into two main categories in the study: (1) evaluating the CNS contribution to the post-SAD APV; and (2) comparing the role of the aortic and carotid sinus baroreflexes in stabilizing cardiovascular variability.

(1) Protocols for evaluating the CNS contribution to the post-SAD cardiovascular variabilities.

In the baroreflex denervated rats: Baseline → *SAD* → *chlorisondamine administration* → *phenylephrine and epinephrine infusions*: Following recovery from the general surgery, six hours of baseline data were acquired before SAD. Twenty-four hours following the SAD, a bolus of the ganglionic blocker, chlorisondamine (2.5 mg/kg, i.v.), was given. Six hours later, phenylephrine and epinephrine were infused continuously (i.v.) for six hours each in a random order at a dose that restored arterial pressure to, or above, baseline levels, as customized for each NMB rat (phenylephrine, 5 to 73 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; epinephrine, 2.6 to 36 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). To achieve the appropriate dose, the infusion rates of phenylephrine and epinephrine were adjusted from 5 to 33 $\mu\text{l}/\text{min}$ and from 3 to 13 $\mu\text{l}/\text{min}$, respectively. The purpose of using two different pressors, i.e., phenylephrine (an alpha adrenergic agonist) and epinephrine (an alpha and beta adrenergic agonist), was to partially control for idiosyncratic effects of the particular agent on the APV. Saline infusion at a rate of 10 $\mu\text{l}/\text{min}$ was used as a control.

In baroreflex-intact rats: Baseline → *Chlorisondamine administration* → *phenylephrine and epinephrine infusions*: Following recovery from the general surgery, a bolus of chlorisondamine (2.5 mg/kg, i.v.) was given. Six hours later, the pressors phenylephrine and epinephrine were continuously infused (i.v.) for six hours, each in a random order as described above. Saline infusion at a rate of 10 $\mu\text{l}/\text{min}$ was used as a control.

(2) Protocols for comparing the role of the aortic and carotid sinus baroreflexes in stabilizing the APV

Bilateral aortic or carotid sinus denervation: Baseline → *bilateral aortic (or carotid sinus) denervation* → *bilateral carotid (or aortic) denervation, i.e., SAD*: Following recovery from the general surgery, bilateral aortic (or carotid sinus) denervation was performed. This initial denervation was chosen randomly on either pair of the baroreceptors (i.e., half of the rats began with the aortic then the carotid sinus denervation, and another half with the carotid sinus then the aortic denervation). Twenty four hours later, further denervation was conducted on the remaining pair of baroreceptors.

Results:

(1) The CNS has a significant contribution to the post-SAD APV.

Arterial pressure variability (APV) following baseline, SAD, Chlorisondamine, phenylephrine and epinephrine infusion was analyzed using repeated measures of ANOVAs across stage (Fig. 1B2, N=9). The main effect of stage was significant ($F(4, 38) = 84.31, p < 0.05$). Newman-Keuls post hoc test showed that, following SAD, APV was significantly increased from the baseline (from 8.3 ± 1.4 to 25 ± 1.98 mmHg; $p < 0.05$ vs. baseline); the subsequent application of chlorisondamine decreased APV from 25 ± 1.98 to 2.44 ± 0.36 mmHg ($p < 0.05$ vs. every other experimental stage). In presence of chlorisondamine, phenylephrine and epinephrine infusion both only slightly increased APV, from 2.44 ± 0.36 to 7.18 ± 0.47 mmHg for phenylephrine ($p < 0.05$ vs. the chlorisondamine stage) and from 2.44 ± 0.36 to 7.46 ± 0.71 mmHg for epinephrine ($p < 0.05$ vs. the chlorisondamine stage). *These data suggest that the CNS has a significant contribution to the post-SAD APV.*

Arterial pressure variabilities (APV) following baseline, chlorisondamine, phenylephrine and epinephrine infusion in the baroreflex intact and SAD rat were analyzed separately using repeated measures of two-way ANOVAs varying group and stage. There was a significant two-way interaction varying group and stage in APV ($F(3, 33) = 32.93, p < 0.05$, Fig. 2B). Similarly, Newman-Keuls' post hoc test showed that in both SAD and baroreflex intact NMB rats, chlorisondamine significantly decreased APV compared with the pre-chlorisondamine baseline condition ($p < 0.05$ vs. every other experimental stages); the subsequent phenylephrine and epinephrine infusion (in presence of the chlorisondamine) both increased APV compared with the chlorisondamine condition ($p < 0.05$ vs. the chlorisondamine stage). Between the SAD and the baroreflex intact rats, the SAD rats had a higher systolic AP (Fig. 2A) and APV (Fig. 2B) than the baroreflex intact rats during the pre-chlorisondamine baseline stage ($p < 0.05$, for the two variables).

Independent of prior status of the baroreflex system (i.e., SAD or intact), a bolus application of chlorisondamine substantially decreased APV in both SAD and baroreflex intact NMB rats. This suggests that, by interrupting the baroreflex efferent pathway, chlorisondamine blocked the CNS contribution to the APV. Moreover, in presence of the chlorisondamine, subsequent phenylephrine and epinephrine infusion both returned systolic AP to or above the baseline levels in both SAD and intact rats (Fig. 2A, $p < 0.05$). This restoration of systolic AP significantly increased APV (Fig. 2B), VLF (Fig. 2C) and LF powers (Fig. 2D) in both SAD and intact NMB rats, suggesting that the APV is not only modulated by the CNS but by the peripheral system as well.

The aortic baroreflex has a more dominant role in the control of APV than the carotid sinus baroreflex

Arterial Pressor Variabilities following baseline, bilateral aortic or carotid sinus denervation and complete SAD were analyzed using separate two-way analyses of variance (ANOVAs) with repeated measures varying group and stage. There was a significant two-way interaction varying group and stage in the APV ($F(2,20) = 5.09, p < 0.05$, Fig. 3A). Newman-Keuls' post hoc

test showed that, for the APV (Fig. 3A), it was increased from the baseline 7.02 ± 0.66 to 17.27 ± 0.82 mmHg following bilateral aortic denervation ($p < 0.05$ vs. baseline), and further increased to 24.02 ± 2.07 mmHg following the subsequent complete SAD ($p < 0.05$ vs. baseline and bilateral aortic denervation); the APV was increased from the baseline 7.31 ± 0.96 to 12.14 ± 1.83 mmHg following bilateral carotid sinus denervation ($p < 0.05$ vs. baseline), and further increased to 27.29 ± 1.12 mmHg following the subsequent complete SAD ($p < 0.05$ vs. baseline and bilateral carotid sinus denervation). These data suggest that while both aortic and carotid sinus baroreflexes are important in stabilizing APV, the aortic baroreflex has a more dominant role than the carotid sinus baroreflex. These data also suggest that the effect of complete SAD on APV is independent of the sequence of the denervation.

In conclusion, these studies suggest that: (1) the CNS has a significant contribution to the increased APV observed following baroreflex denervation. Although peripheral interference contributes, it is not necessarily essential. (2) Compared with the carotid baroreflexes, the aortic baroreflexes have a more dominant role in stabilizing moment-to-moment APV. With the carotid sinuses intact, only a single aortic limb was required to stabilize the pressure.

Fig. 1 Tang et al.

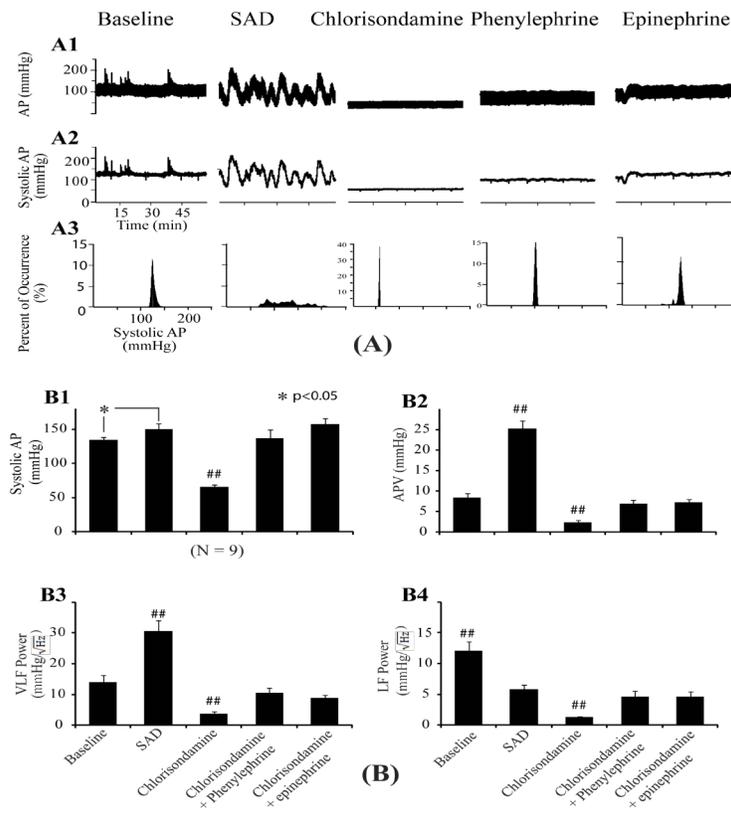


Figure 1: Effects of sinoaortic denervation (SAD), chlorisondamine administration, intravenous infusions of phenylephrine, and epinephrine on the arterial pressure (AP) and arterial pressure variability (APV) in NMB rats. Panel A shows representative traces of AP (A1) and systolic AP (A2) and a frequency histogram of the systolic AP (A3) from each experimental procedure. Panel B summarizes the results of systolic AP (B1), APV (B2), very low frequency (VLF: 0.01-0.15 Hz, B3) and low frequency (LF: 0.15 – 0.6 Hz, B4) powers of the systolic AP spectra at baseline, following sinoaortic denervation, chlorisondamine administration and intravenous infusions of phenylephrine and epinephrine. While SAD significantly increased APV and VLF power, chlorisondamine decreased the values of both variables to below their corresponding baseline levels; subsequent phenylephrine and epinephrine infusions only slightly increased APV. APV was calculated as standard deviation of the hourly systolic AP. Data are mean \pm SEM. Error bars = SEM. * $p < 0.05$. ## Statistically significant difference from every other experimental stage.

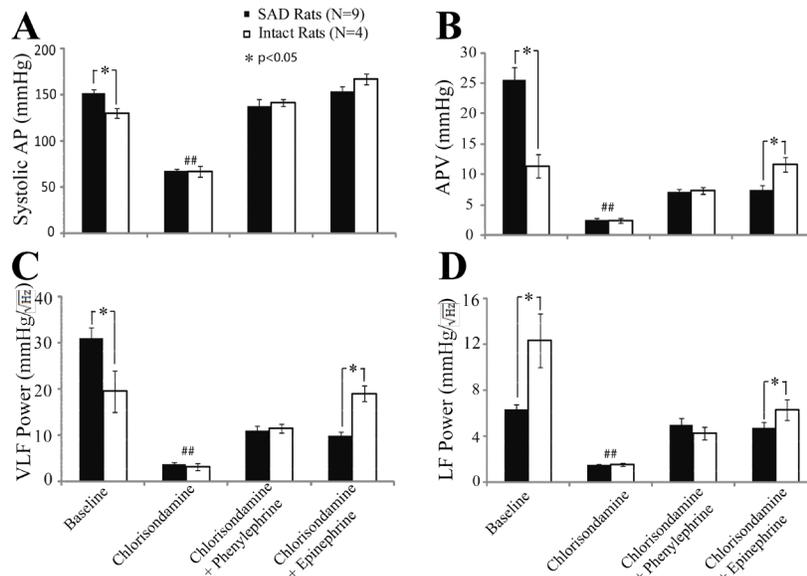


Figure 2: Chlorisondamine administration, intravenous infusions of phenylephrine, and epinephrine produced similar effects on the systolic arterial pressure (AP), arterial pressure variability (APV), very low frequency (VLF: 0.01-0.15 Hz) and low frequency (LF: 0.15 – 0.6 Hz) power of the systolic AP spectra in both SAD and baroreflex-intact NMB rats. (A) The systolic AP. In both SAD and baroreflex intact rats, chlorisondamine significantly decreased systolic AP and subsequent infusions of phenylephrine and epinephrine both returned systolic AP to or above the corresponding baseline levels. (B) The APV. In both SAD and baroreflex intact rats, chlorisondamine significantly decreased the APV and subsequent infusions of phenylephrine and epinephrine both increased APV to the baroreflex intact baseline variability level. (C) VLF power of the systolic AP spectrum. In both SAD and baroreflex intact rats, chlorisondamine significantly decreased the VLF power and subsequent infusions of phenylephrine and epinephrine both increased the VLF power to the baroreflex intact baseline level. (D) LF power of the systolic AP spectrum. In both SAD and baroreflex intact rats, chlorisondamine significantly decreased LF power and subsequent infusions of phenylephrine and epinephrine both increased the power to the SAD level. Moreover, the SAD rat had a higher systolic AP, larger APV and VLF, but a lower LF power than the baroreflex intact rat during the baseline stage. In the baseline stage, the baroreflexes were completely denervated for the ‘SAD Rats’ and un-manipulated for the ‘Intact Rats’. APV was defined as standard deviation of the hourly systolic AP. Data are mean \pm SEM. Error bars = SEM. *p<0.05. ## Statistically significant difference from every other experimental stage.

Fig.3 Tang et al.,

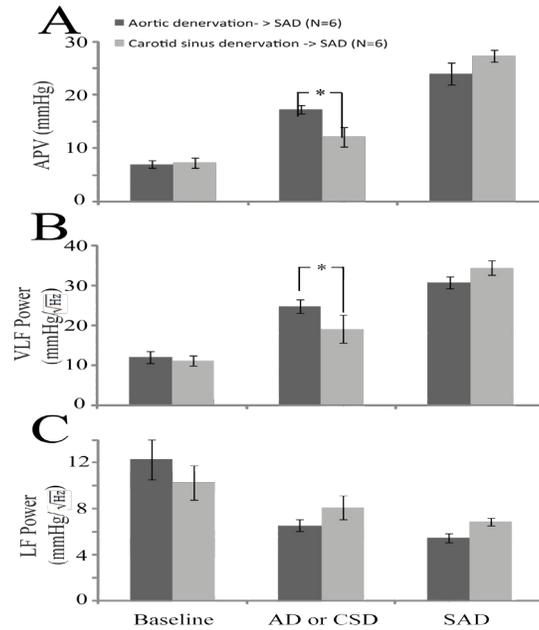


Figure 3: While bilateral aortic and carotid sinus denervation significantly increased APV and VLF power, bilateral aortic denervation produced a larger increase in APV than carotid sinus denervation. Panels A, B and C depicted the effects of different denervation (i.e., AD, CSD or SAD) on APV, VLF and LF, respectively. Bilateral aortic and carotid sinus denervation both significantly increased APV and VLF power compared to the baroreflex intact baseline ($p < 0.05$ vs. baseline, for the APV and the VLF power); the subsequent SAD further increased APV and VLF. ($p < 0.05$ vs. baseline and AD or CSD denervation stages). Bilateral aortic denervation produced a larger increase in APV and VLF than carotid sinus denervation ($p < 0.05$, aortic vs. carotid sinus denervation). Data are mean \pm SEM. Error bars = SEM. * $p < 0.05$. APV was calculated as standard deviation of the hourly systolic arterial pressure. VLF and LF: the very low frequency (VLF: 0.01-0.15 Hz) and the low frequency (LF: 0.15 – 0.6 Hz) power of the systolic arterial pressure spectra, respectively. AD: aortic denervation; CSD: carotid sinus denervation; SAD: sinoaortic denervation.

Research Project 14: Project Title and Purpose

Myocardial Protein Synthesis after Alcohol Intoxication - Although the risk of cardiovascular diseases is reduced with moderate alcohol consumption, chronic alcohol abuse leads to heart dysfunction and potentially heart failure. Excessive use of alcohol has a direct toxic effect on the heart demonstrated by a weakening of the heart muscle and the inability to pump blood efficiently. The degree of dysfunction is proportional to the duration and severity of alcohol consumption, with continued heavy drinking leading to an enlarged heart. The purpose of this project is to understand how excessive, chronic alcohol consumption causes structural and functional damage to the heart at the molecular level. Detailed understanding of the defects caused by alcohol will allow for a better understanding of the molecular events surrounding the onset of alcohol-induced heart disease and may allow for the development of targeted therapies.

Duration of Project

1/1/2009 - 6/30/2010

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>.

Research Project 15: Project Title and Purpose

Development of Nanoliposomal Therapeutics for Leukemia - Leukemia is a cancer of the blood which is expected to cause 21,710 deaths in 2008 in the United States. Research continues to find mutations that cause leukemia. Inhibitory RNA (RNAi) is a silencing mechanism that turns off a gene. RNAi directed at a cancer-causing mutation can specifically kill cancer cells. However, RNAi must be delivered to cells and protected from degradation in order to work. Small vesicles called nanoliposomes create an efficient and non-toxic delivery vehicle for RNAi as well as other drugs. Another drug that selectively kills cancer cells is ceramide. Ceramide is a lipid produced in the body during many chemotherapy treatments that is partly responsible for the action of these drugs. This project seeks to test anti-leukemia treatment with nanoliposomes containing ceramide, RNAi, or both, initially in mouse and ultimately in future projects in human leukemia.

Duration of Project

5/1/2009 – 6/30/2010

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>.

Research Project 16: Project Title and Purpose

P16 Alteration and BRAF Mutation and Patient Outcomes in Papillary Thyroid Cancer - Thyroid cancer represents the most common endocrine malignancy, with papillary thyroid cancer (PTC) accounting for 90% of malignant thyroid tumors. Although outcome is generally favorable, a group of patients develop local recurrence and/or distant metastases and ultimately die of their disease. For these patients with aggressive disease, better diagnostic and prognostic tools are clearly needed. Molecular markers that accurately predict tumor behavior are lacking. In the past, p16 gene alterations and BRAF mutations have individually been implicated in tumor aggressiveness in PTC. We hypothesize that presence of these mutations simultaneously in patients with PTC is a predictor of worse clinical outcomes and tumor aggressiveness.

Anticipated Duration of Project

5/1/2009 – 8/30/2011

Project Overview

Papillary thyroid carcinomas (PTC) represent about 90% of all thyroid cancers. Although, incidence of PTC has increased in recent years, the mortality has remained low. There is a subgroup of high risk patients with PTC that develop local recurrence and/or distant metastases and ultimately die of their disease. Genetic markers are clearly needed to prognosticate disease-specific mortality, risk of recurrence and death risk in PTC. Several studies have shown that BRAF mutations may confer worse clinical prognosis in PTC. Alterations of the tumor suppressor gene p16 are common in human cancers. Its role in thyroid cancer is not clearly defined. Although BRAF mutation clearly indicates poor outcomes in PTC, p16 as it relates to aggressive thyroid cancer is not well studied and certainly the outcome of thyroid cancer patients whose tumor harbors both the genetic aberrations have never been studied. We propose that in the presence of BRAF mutation, the presence of p16 protein expression is associated with tumor aggressiveness and worse clinical outcomes.

Specific Aims: 1) To analyze surgical specimens of PTC for the presence of p16 alterations and BRAF mutations. 2) To correlate the concurrent presence of p16 alterations and BRAF mutations with patient outcomes as manifested by distant metastases, local recurrence and loss of iodine avidity.

Research Design and Methods: Retrospective study. We will collect thyroid tissue from 30 patients with PTC that underwent surgery at HMC along with 30 normal thyroid samples (controls). Tissue samples were cryofrozen after surgery and then held in the Penn State Cancer Institute (PSCI) Tissue Bank. Tissue will be allocated to the basic scientist lab, for p16 and BRAF analysis. AS-PCR will be performed to screen for the (V600E) BRAF mutation. P16 protein expression will be detected by Immunohistochemistry. Subsequently we will make the correlations between the presence of these markers and clinical outcomes.

Principal Investigator

David Goldenberg, MD, FACS
Associate Professor of Surgery
Director, Head and Neck Surgery
Department of Surgery
Penn State University College of Medicine
500 University Drive-H091
Hershey, PA, 17033

Other Participating Researchers

Gavin P Robertson, PhD, Saima Durvesh, MD, Andrea Manni, MD, David Mauger, MD, SubbaRao Madhunapantula, PhD – employed by Penn State College of Medicine, Milton S

Hershey Medical Center

Expected Research Outcomes and Benefits

We expect that 30% of the samples will test positive for both p16 alteration and BRAF mutation and these will be the patients with the poorest clinical outcomes at presentation. These include short term signs of aggressive disease such as large tumor size, extra-capsular spread and local metastasis. With the findings from this pilot study, we anticipate to propose a larger scale, randomized clinical study to further look at the correlation between these mutations, the long term follow up including development of iodine resistance and the effect on long term survival.

Correlation between p16 and BRAF, if confirmed, will serve as a diagnostic and prognostic tool to help us identify the high-risk subgroup of patients with PTC that have the potential to develop aggressive cancers. These are the patients that stop responding to conventional treatment and ultimately die of their disease. These patients may benefit from earlier more aggressive treatment, with potential improvement in overall clinical prognosis. Thus, *our project has the potential to impact human health by providing better tools to diagnose and treat a more deadly form of thyroid cancer.*

Summary of Research Completed

Our stated aims were to analyze surgical specimens of PTC for the presence of p16 alterations and BRAF mutations. This data is to be correlated with patient outcomes as manifested by distant metastases, local recurrence and loss of iodine avidity.

We had previously received twenty-six samples of papillary thyroid cancer and adjacent normal control tissue which were sent to the Robertson Lab for processing. In the past year, we received an additional 14 tumor samples with corresponding 14 normal and control tissue samples. At that time, our study had received a total of 40 tumor and 40 corresponding tissue samples from the Penn State Cancer Institute Tissue Bank. One tumor (along with the corresponding normal) was removed from the study per directive by Dr. Kevin Gleason former chair of the Internal Review Board.

All samples were sent to be analyzed for the BRAF mutation by PCR at the Robertson Lab and the P16 alteration by Immunohistochemistry performed by The Department of Pathology. For BRAF: Tumors were either frozen or formalin fixed paraffin embedded sections. Frozen tumors were ground in a mortar and pestle and split in half. Formalin fixed paraffin embedded sections were dissolved in xylene and then rehydrated with 100% ethanol. Half of each frozen tumor was used as protein for western blot and half was used in Allele Specific PCR for BRaf^{V600E} Mutation screening. DNA was extracted for ASPCR from both the frozen tumor halves and the formalin fixed paraffin embedded sections by way of the DNeasy® Blood and Tissue Kit (Qiagen). Extracted DNA was amplified in a GeneAmp® PCR System 9700 (Applied Biosystems). 100ng DNA samples were mixed with Custom Premium Oligos primers for GAPDH and Mutant BRaf^{V600E} (Invitrogen). Taq Polymerase was used in a premixed formula in Qiagen's Taq PCR Master Mix. Amplified DNA was run on a 2% Agarose gel at 100mV for 1 hour and imaged under UV light in an Alpha Innotech MultiImage™ Light Cabinet.

IHC for p16 was performed using the CINtec p16 Histology kit (mtm Laboratories, Westborough, MA). FFPE sections were first deparaffinized and hydrated through xylenes and a graded ethanol series followed by heat-induced epitope retrieval in the CINtec unmasking solution for 20 minutes. This was followed by a 10 minute 3% peroxidase block at room temperature. Incubation with the p16 primary antibody was then performed at room temperature for 30 minutes followed by incubation with the CINtec Histology kit Visualization Reagent for 30 minutes at room temperature. Staining was completed with development of the kit's DAB chromagen substrate for 10 minutes followed by counterstaining for 6 minutes in Mayer's modified hematoxylin (Dako Cytomation, Carpinteria, CA). Sections were dehydrated, cleared and cover slipped.

All the anonymous clinical data has been collected, compiled and verified by an honest broker. The collected clinical data includes: age, sex, tumor stage, tumor grade, tumor size, extra capsular spread, prior history of thyroid disease, prior history of irradiation, recurrence, regional metastasis, distant metastasis, iodine avidity, thyroglobulin, and treatment received.

On review of the histology on H&E, it was noted that seven samples provided by the tissue bank (as cancer samples) did not contain thyroid cancer and a further four samples were unable to be processed for the BRAF mutation because of insufficient sample size. Due to these various inconsistencies, we went back to the original paraffin block of the subjects. Blocks were re-cut and all tumors re-verified by Dr. Henry Crist. We are now in the final stages of re-analyzing P16 in seven samples and BRAF mutation on four samples.

Research Project 17: Project Title and Purpose

The Interaction of Environmental Agents and LDL-Cholesterol in Parkinson's Disease - This project seeks to characterize the apparently paradoxical observation that low cholesterol (LDL-C) is a risk factor for Parkinson's disease (PD), and to examine some of the possible underlying mechanisms. One aim is to collect pilot data and support the planning of testing the association between low LDL-C and PD further via a prospective study in the "Atherosclerosis Risk in the Communities" (ARIC) cohort. The second aim is to test the idea that lower LDL-C is etiologically-linked to PD because it affects the distribution or toxicity of trace environmental toxicants. The data provided by this pilot study will set the stage for more detailed examination of the health consequences of these findings and their underlying mechanisms with the collaboration of a PD clinician, epidemiologist, and basic neuroscientist.

Duration of Project

5/1/2009 – 6/30/2011

Project Overview

Parkinson's disease (PD) is the second most common, age-related neurodegenerative disorder about which there are many unexplained "paradoxes". PD patients are generally more cardiovascularly-healthy than controls. Smoking, a major cardiovascular risk factor, is protective in PD. Whereas the apolipoprotein E (APOE) ε4 allele is associated with increased

risk of Alzheimer's disease (AD), it is the $\epsilon 2$ allele that we have linked to increased PD prevalence. Interestingly, the $\epsilon 2$ allele is associated with lower plasma low-density lipoprotein-cholesterol (LDL-C), longevity in general, and lower risk of AD. Such paradoxes led to the central hypothesis that lower LDL-C is associated with increased risk of PD. Four studies have provided early support for this hypothesis. If confirmed, this hypothesis may have critical clinical and public health impact, especially if lower LDL-C is etiologically linked to PD. The first aim is to test the central hypothesis with a prospective study using the "Atherosclerosis Risk in the Communities" (ARIC) cohort. The ARIC cohort of ~16,000 participants, with baseline fasting lipid profiles since 1986, could provide a rigorous test of our central hypothesis already supported by four prior studies. We shall perform preliminary data analysis on these PD cases, establish feasible PD case validation procedures, and plan for a successful independent grant application to further the research in this cohort. The second aim is to test the hypothesis that peripheral cholesterol-APOE status affects the metabolism and or central entry of toxicants that can cause Parkinson's like damage. This work will focus on the hypothesis that low cholesterol either increases central nervous system (CNS) availability of environmental toxicants, or affects repair associated with injury. We shall modify cholesterol levels using dietary manipulations and test the effects of rotenone and MPTP, two human-relevant model toxicants. We shall inject these compounds at various doses, and follow the toxicokinetics of the parent compounds and some selected metabolites using high-performance liquid chromatography (HPLC)/mass spectroscopy. In addition, we shall assess the effects of these compounds on brain dopamine neuronal function and integrity initially using neurochemical approaches. Together, these experiments will provide important data that might link genetic and environmental factors in the causation of "idiopathic" PD.

Principal Investigator

Richard B. Mailman, PhD
Professor and College of Medicine Distinguished Senior Scholar
Penn State University College of Medicine
Department of Pharmacology, R130
500 University Dr.
Hershey PA 17033-0850

Other Participating Researchers

Xuemei Huang, MD, PhD – employed by Penn State Hershey Medical Center

Expected Research Outcomes and Benefits

The clinical arm of this study will be an important addition to the existing literature suggesting the surprising idea that lowering cholesterol levels without a specific medical rationale may have unforeseen negative consequences. If this idea is supported by Aim 1 of this study, then low cholesterol may be important in other neurodegenerative diseases as well. If true, it may suggest caution in the practice of giving medication to lower cholesterol when a patient is not at cardiovascular risk.

The second aim is the first attempt to elucidate possible mechanisms that may be involved in the clinical findings. There are three general types of mechanisms by which low cholesterol may predispose to Parkinson's disease, and the proposed studies test one of these hypotheses rigorously.

In total, we expect that the studies will provide further support for the central hypothesis that lower LDL-C is associated with an increased risk of PD, and also provide pilot data that lower LDL-C cholesterol might be etiologically linked to PD via changing the CNS availability of environmental toxicants. These data will have critical clinical and public health impact, and warrants larger scale projects that we shall pursue using National Institutes of Health (NIH) or foundation extramural grants.

Summary of Research Completed

Aim 1: Test the central hypothesis further in clinical cohorts such as the "Atherosclerosis Risk in the Communities" (ARIC) prospective cohort.

The following summarizes progress made for this Aim during the past year that is contained in three publications listed at the end of this section. In last year's Progress Report, we summarized the results of initial analysis of data from the DATATOP cohort. This analysis provided preliminary evidence that higher total serum cholesterol concentrations may be associated with a modest slower clinical progression of PD. The preliminary results summarized last year were consistent with the final analysis done during the past year and now in press.

During the past year, we were also able to access a large environmental cohort and test again our clinical hypothesis. The first result of this research was an examination of the relationship of apolipoprotein E (ApoE) genotype in relation to Parkinson disease (PD) among 786 cases and 1,537 controls, all non-Hispanic Caucasians. After adjusting for year of birth, sex, smoking status, daily caffeine intake, and family history of PD, ApoE ϵ 4-carriers (ϵ 34/ ϵ 44) had significantly lower odds for having PD than did ϵ 33 carriers, but ϵ 2-carriers (ϵ 23/ ϵ 22) did not. In addition, we conducted a meta-analysis which confirmed our primary findings. In PD patients, the prevalence of dementia appeared to be higher among ϵ 4-carriers (compared with ϵ 33) but lower among ϵ 2-carriers, although neither test was statistically significant. Our study suggested that the ApoE ϵ 4 allele may be associated with a lower PD risk among non-Hispanic Caucasians. In summary, in this large population-based study, the presence of ApoE ϵ 4 was associated with a lower occurrence of PD. Although this is somewhat unexpected when viewed in isolation, it is consistent with the primary hypothesis of this project relating higher cholesterol to a lower risk of PD. The three new publications related to the clinical focus of Aim 1 are listed below:

Huang X, Auinger P, Eberly S, Oakes D, Schwarzschild M, Ascherio A, Mailman RB, and Chen H for the Parkinson Study Group DATATOP Investigators. Serum Cholesterol and the Progression of Parkinson's Disease: Results from DATATOP. PLOS One (in press).

Gao J, Huang X, Park Y, Liu R, Hollenbeck A, Schatzkin A, Mailman RB, Chen H. Apolipoprotein E genotypes and the risk of Parkinson disease. Neurobiology of Aging (in press)

Huang X, Chen H, Petrovitch H, Mailman R, Ross W. Reply: Plasma cholesterol and Parkinson's

disease: Is the puzzle only apparent? *Mov Disord.* 2010 Jan 15;25(1):137. PMID: 20077471
PMCID: PMC2815017

In summary, this aspect of the project has tested the hypothesis that low cholesterol is associated with greater occurrence and faster progression of Parkinson's disease. The studies we have completed during the last two years are consistent with the initial hypothesis. This has resulted in the two new publications and the peer-reviewed commentary listed above, and also has set the stage for a long-term study in the ARIC cohort as outlined in the original aims. This latter study will be supported by the NIEHS intramural program, but their interest was made possible by the research of this project.

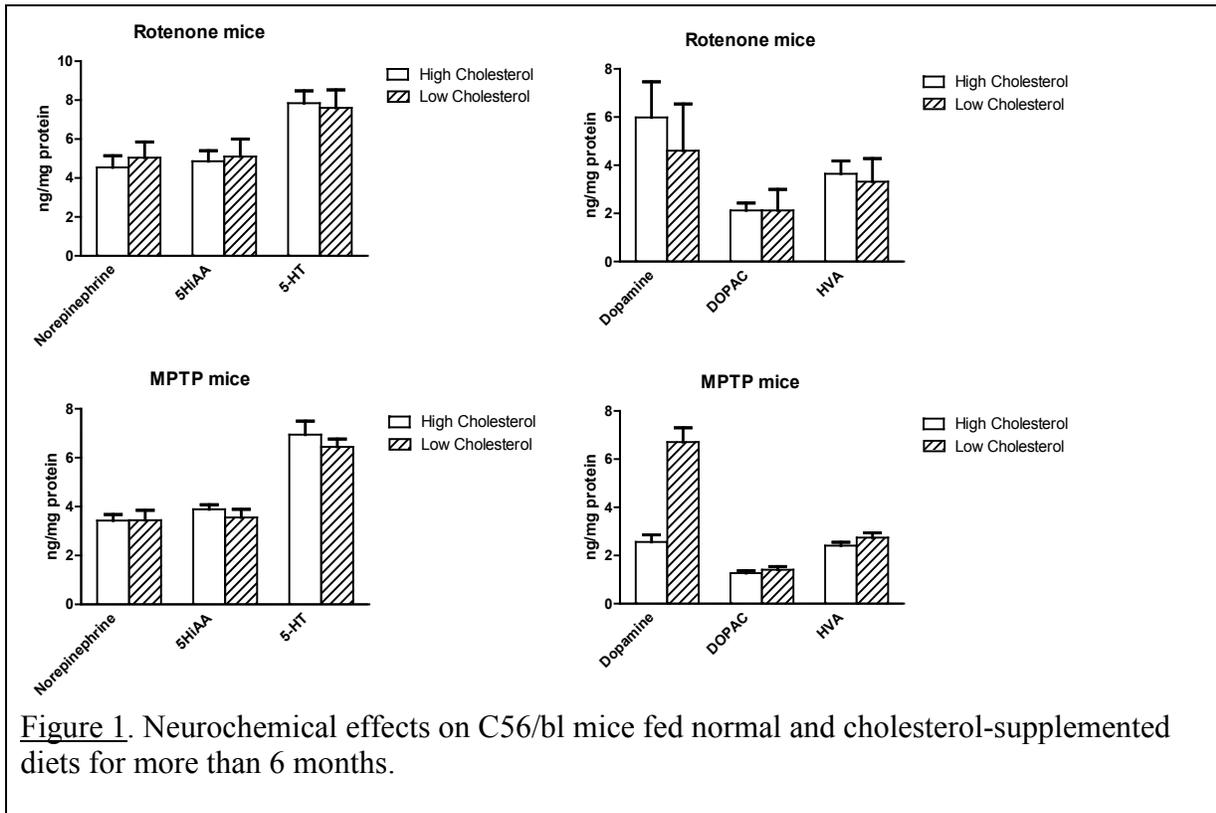
Aim 2 Progress: Testing the hypothesis that peripheral cholesterol-APOE status affects the metabolism and or central entry of toxicants that can cause Parkinson's like damage.

During the past year two independent studies have been completed in mice with the results summarized below. The working hypothesis was that cholesterol status influences the metabolism and/or distribution of trace toxicants to which PD-susceptible people are exposed, or alternately, that cholesterol status influences the distribution of such toxicants, permitting greater entry into the brain. The model toxicants we selected were rotenone (modestly dopamine-neuron selective) and MPTP (highly dopamine-neuron selective). As we noted in last year's progress report, we had to develop assays for cholesterol and also determine how long mice had to be on their diet to achieve meaningful differences in cholesterol. In experiment 1 begun last fiscal year, we established two groups of mice: one fed a high-cholesterol diet and one fed a low-cholesterol diet. We had purchased commercial kits for cholesterol measurements from Biovision because they required only 2 μ L of serum per assay, but as noted, this experiment-limiting test was problematic. Biovision worked with us and improved the kits, and after confirming values with the Penn State Hershey Medical Center clinical laboratory, we intoxicated the animals as planned.

Experiment 1 had started with only 24 animals (six cells of four animals each), and several were lost due to unrelated events (fighting with other mice; etc.) because of the long-time period that was required to work out suitable cholesterol measurements. Because of the loss of animals, and because this was a pilot experiment, we eliminated the control only group. Although unusual, the primary hypothesis was that high cholesterol would be protective against one or both of the toxicants, and although not ideal, this could be tested without a control group. Thus, the effects of MPTP and rotenone were examined in groups of mice with high (296 mg/dL) and low cholesterol (124 mg/dL) treatments. A summary of the results of this experiment is shown in Figure 1. Despite the profound difference in plasma cholesterol, the only significant difference was that depletion of dopamine was actually greater in the high cholesterol group challenged with MPTP. These preliminary data do not support the working hypothesis of Aim 2.

We repeated this study in its entirety, but began with adequate numbers of mice such that we could accommodate for attrition due to causes noted earlier. In addition, because of the issues noted above, the cholesterol dietary treatments ran for more than 7 months in the first experiment, and we had to shorten this to complete the experiments before the end of the grant period. We therefore chose a pre-treatment period of 105 (112 days until the animals were

ethanized and studied). In this case all groups had 5-6 animals, except for the high cholesterol control group in which N =4. An unexpected finding in Experiment 2 was that the high cholesterol group was less elevated than controls (185 ± 16 vs. 140 ± 7 mg/dL) compared to experiment 1; the high cholesterol mice also were markedly heavier (39.4 ± 1.1 versus 33.4 ± 0.5 g). Despite these differences, there is remarkable consistency with the results of experiment 1 as shown in the summary data from the neurochemical analyses in three different brain regions (Figures 2-4). Again, these data refute the working hypothesis. We conclude that the consistent clinical findings that we (and now others) have made (see data from Aim 1 and earlier publications) are not due to either of the two hypotheses we proposed that primarily relate to metabolism and distribution of toxicants as affected by cholesterol. Although “negative”, these are very important data as they now suggest the involved mechanism is likely related to neuronal response or repair, and not simply toxicant concentration.



Striatum

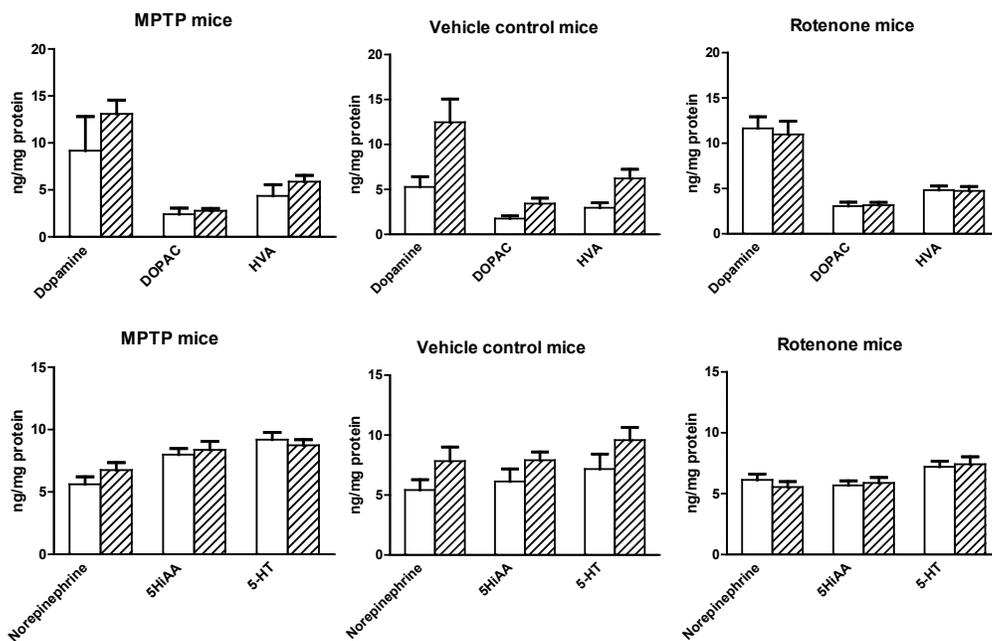


Figure 2. Effects of toxicants on striatal monoamine neurochemistry of mice on high and low cholesterol diets

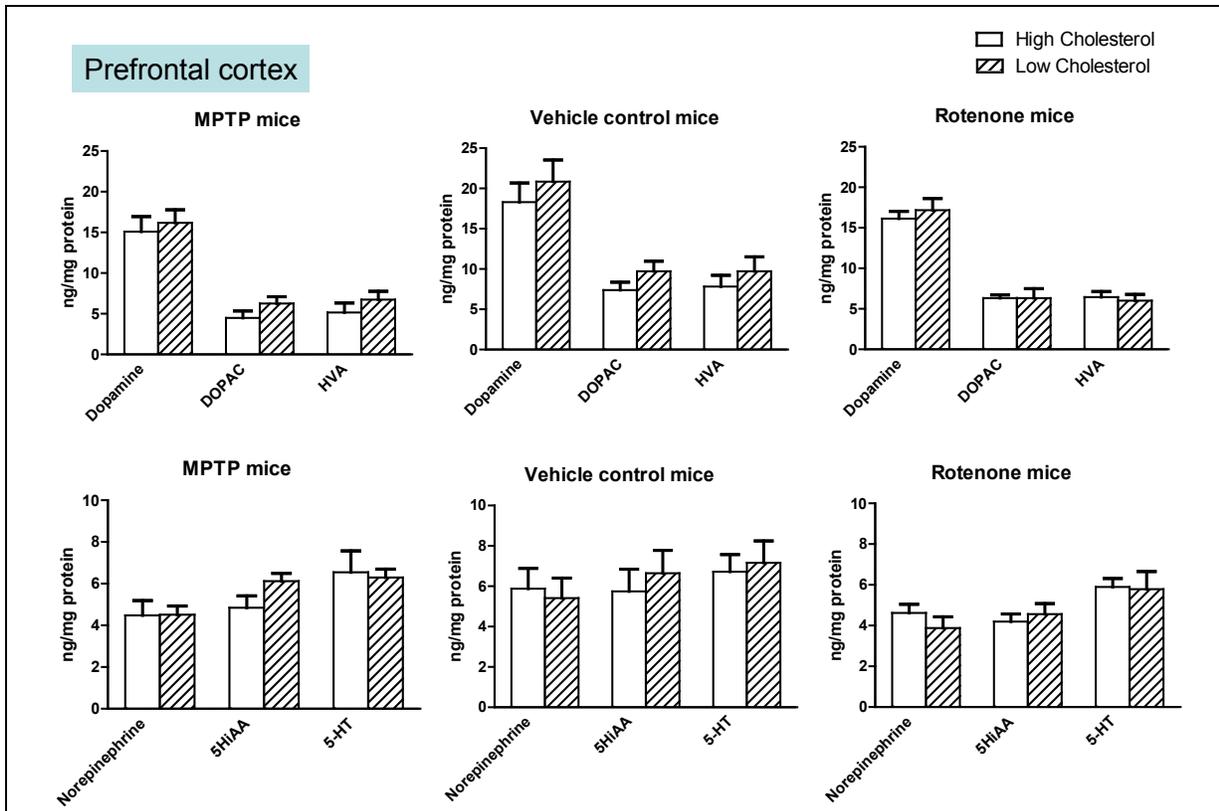


Figure 3. Effects of toxicants on prefrontal cortical monoamine neurochemistry of mice on high and low cholesterol diets

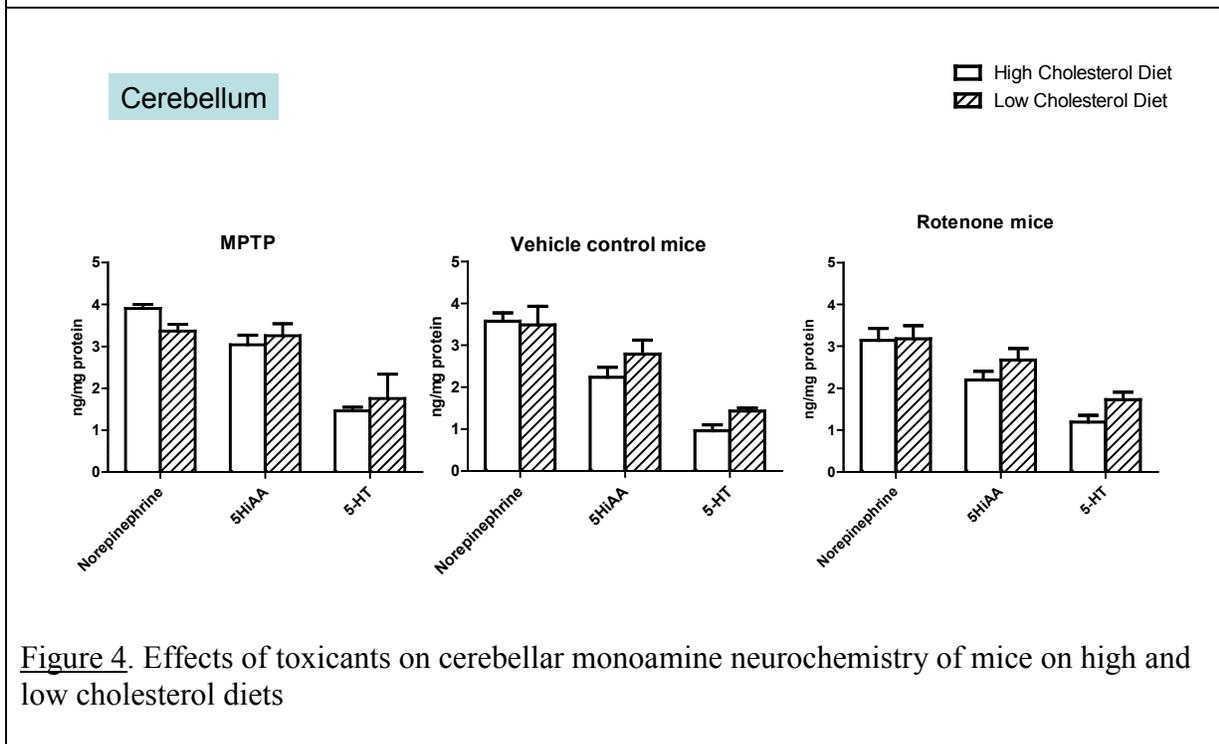


Figure 4. Effects of toxicants on cerebellar monoamine neurochemistry of mice on high and low cholesterol diets

Research Project 18: Project Title and Purpose

Moving Experimental Cancer Therapeutics from the Research Bench to the Clinic - The Penn State Melanoma Therapeutics Program has a Therapeutic Drug Portfolio of ~20 experimental therapeutic compounds at various stages of development. Unfortunately, agents proceed through preclinical development at which point progress halts. The purpose of this project is to establish the necessary expertise and infrastructure to more rapidly move these compounds to the clinic. This involves developing interactions with companies to make clinical grade compound for testing in patients; undertaking necessary toxicological and pharmacological evaluation for an Investigational New Drug (IND) application; and interacting with the Food and Drug Administration (FDA) to enable experimental agents to obtain IND status so that they can be evaluated in the Pennsylvania State University (PSU) Cancer Institute Clinics in phase I trials. Accomplishing these objectives for one of the compounds from the current melanoma drug portfolio is the objective of this project.

Anticipated Duration of Project

5/1/2009 – 6/30/2012

Project Overview

Clinicians treating cancer believe that agents targeting key pathways deregulated in cancer cells are necessary for more effectively treating these diseases. Unfortunately, relatively few agents are available in the clinic to treat cancer in this manner. Malignant melanoma is a prime example of a devastating disease needing more effective agents for treatment of metastatic disease. The hope is that targeted agents inhibiting the activity of aberrantly behaving proteins would be more effective than those untargeted drugs currently available to patients. To develop better cancer therapies to treat melanoma, The Penn State Melanoma Therapeutics Program is identifying genes causing the disease, developing drugs to target these genes and drug delivery systems to get the agents more effectively to the cancer cells, and finally testing the agents in clinical trials in patients. However, the biggest hurdle is the final step, which involves testing the agents in the clinic. Therefore, the objective of this project is to develop the organizational structure necessary to move agents developed within the Melanoma Therapeutics Program from the preclinical to clinical arena. Development of an agent known as ISC-4, which inhibits the Akt3 kinase deregulated in ~70% of melanomas will be emphasized. Therefore, the *objective of this project* is to undertake the necessary characterization to move ISC-4 from the preclinical arena to the clinic. First, a large batch of GMP grade ISC-4 is required, which will be compared to currently used smaller laboratory generated batches. By-products and physical characteristic will be determined. Next efficacy for inhibiting tumor development using the larger synthesis scale will be compared to smaller scale synthesis together with toxicological evaluation. Second, toxicology as well as pharmacokinetic and pharmacodynamic properties of ISC-4 in a second animal model will be evaluated. Collectively, these studies will form the foundation for an IND from the FDA for ISC-4. Once these data are obtained, The Penn State Cancer Institute plans to test the compound's toxicity in humans as well as determine any efficacy in a phase I melanoma clinical trial. Note: A phase I trial testing of the agent in melanoma is not the objective of this project, rather it is to successfully obtain IND status for the compound. Thus, the *central*

hypothesis for these studies is that targeting Akt3 signaling using ISC-4 would be an effective targeted approach for inhibiting melanoma development. This discovery would be highly significant, evaluating the therapeutic implications of targeting a major signaling pathway promoting melanoma development.

Principal Investigator

Gavin P. Robertson, PhD
Associate Professor of Pharmacology, Pathology, and Dermatology
Associate Director for Translational Research
The Pennsylvania State University
College of Medicine
The Milton S. Hershey Medical Center
Department of Pharmacology, R130
500 University Drive, P.O. Box 850
Hershey, PA 17033-0850

Other Participating Researchers

SubbaRao Madhunapantula, PhD, Raghavendra Gowda, PhD – employed by Pennsylvania State University, College of Medicine

Expected Research Outcomes and Benefits

One person in the United States dies from melanoma every hour. Currently, no effective treatment exists for patients suffering from the metastatic stages of this disease. In spite of the widely appreciated magnitude of the problem, there is still a critical gap in knowledge regarding key deregulated signaling pathways causing melanoma and therapies specifically targeted to correct these defects to inhibit tumorigenesis and metastasis. *Availability of ISC-4 as a melanoma therapeutic targeting Akt3 signaling in melanoma has the potential of leading to a more effective rationally targeted therapeutic agent for melanoma patients.* Therefore, as a direct outcome of the project, it is expected that a scaled-up clinical batch of ISC-4 will be generated; the compounds physiochemical and biological characteristics will be evaluated in preclinical models and compared to small-scale lab generated batches. Second, toxicity as well as pharmacokinetics and pharmacodynamics will be determined in a second animal model, which is critical data necessary to apply for an IND for ISC-4 from the FDA so that the compound can be tested in phase I clinical trials. Clinical availability of ISC-4 is predicted to have a significant positive impact on the currently poor prognosis faced by advanced-stage melanoma patients by contributing to availability of more effective therapies, which would increase the length and quality of life for melanoma patients. Therefore, the positive impact for cancer patients suffering from melanoma will be significant.

Summary of Research Completed

The work headed in the direction of developing a topical formulation of the agents. The feasibility of this approach has resulted in a publication detailing this discovery. Furthermore,

clinical topical formulations are being developed and HPLC and LC-MS assays developed for measuring the concentration of ISC-4 in the formulation. Once developed, these assays will be used to assess loading and stability. In this regards, a SBIR phase I grant on which Penn State is a subcontract has been submitted.

A manuscript has been published. The manuscript was featured on the cover of the journal and discovery reported in the lay press on TV, radio and in print. The citation and abstract detailing the discovery are listed below:

[Melanoma chemoprevention in skin reconstructs and mouse xenografts using isoselenocyanate-4.](#) Nguyen N, Sharma A, Nguyen N, Sharma AK, Desai D, Huh SJ, Amin S, Meyers C, Robertson GP. *Cancer Prev Res (Phila)*. 2011 Feb;4(2):248-58.

Abstract: Melanoma incidence and mortality rates continue to increase despite the use of sunscreen as well as screening programs for early surgical excision of premalignant lesions. The steady increase in melanoma incidence suggests that additional preventive approaches are needed to augment these existing strategies. One unexplored area involves targeting genes whose deregulation promotes disease development to prevent melanoma. The Akt3 signaling pathway is one key signaling cascade that plays a central role by deregulating apoptosis to promote development of approximately 70% of melanomas. Isoselenocyanate-4 (ISC-4), derived from isothiocyanates by increasing the alkyl chain length and replacing sulfur with selenium, has been developed to target this important signaling pathway in melanomas; however, its chemopreventive potential is unknown. In this study, the chemopreventive efficacy of topical ISC-4 was evaluated in a laboratory-generated human skin melanoma model containing early melanocytic lesion or advanced stage melanoma cell lines and in animals containing invasive xenografted human melanoma. Repeated topical application of ISC-4 reduced tumor cell expansion in the skin model by 80% to 90% and decreased tumor development in animals by approximately 80%. Histologic examination of ISC-4-treated skin showed no obvious damage to skin cells or skin morphology, and treated animals did not exhibit markers indicative of major organ-related toxicity. Mechanistically, ISC-4 prevented melanoma by decreasing Akt3 signaling that led to a 3-fold increase in apoptosis rates. Thus, topical ISC-4 can delay or slow down melanocytic lesion or melanoma development in preclinical models and could impact melanoma incidence rates if similar results are observed in humans.

A SBIR grant in which Penn State is a significant subcontractor has been submitted in collaboration with PenForGel, LLC. These funds would enable the topical agent to be tested in patients in a clinical trial that would take place at Penn State University.

Abstract: According to the American Cancer Society's 2010 Cancer Facts and Figures report, over 68,000 Americans were diagnosed with melanoma last year, and almost 9,000 died of their disease. Melanoma is the fifth leading cause of cancers in men, and the seventh leading cause of cancer in women. If diagnosed early, treatment can be successful. Because melanoma can often become aggressive, once it metastasizes to other organs, it can become fatal. The incidence of melanoma increases with age with a 28% probability of disease if you are under 40 years of age, and a 70% and higher probability when you are over the age of 60. Current approaches to management include surgery, radiation, immunotherapy, chemotherapy or combinations of

several approaches. Approaches for prevention remain inadequate with sunscreens and avoiding direct sunlight offering the best alternatives. A significant unmet medical need exists for better approaches to manage, and potentially prevent, this cancer. No commercially available agents exist that are effective on melanocytic lesion cells to retard or even prevent skin cancer development by targeting genes deregulated in early melanomas. Akt3 is a key protein kinase activated in ~70% of melanomas. Its function is to reduce cellular apoptosis, thereby promoting melanoma tumor development. If this pathway was targeted in melanocytic lesion cells, it could prevent melanoma development, which would be a very innovative and novel approach for treatment and management, which is supported by studies documented in the recent publication [Cancer Prev Res \(Phila\)](#). 2011 Feb;4(2):248-58. Melanoma chemoprevention in skin reconstructs and mouse xenografts using isoselenocyanate-4. <http://www.ncbi.nlm.nih.gov/pubmed/21097713>.

The *objective of this SBIR proposal* is to develop novel topical clinical formulations of ISC-4 and measure loading efficiency, agent stability, and permeation across organotypic and human skin. Efficacy for inhibiting or decreasing melanocytic lesion development will be tested in laboratory generated skin. Substantial preliminary data developed by PenForGel, LLC demonstrates the potential feasibility of this novel topical agent for preventing melanocytic lesion development using an experimental preclinical topical formulation. It is unlikely that this agent would be used for treating patients with advanced stage melanoma having widely disseminated tumors in distant organs, but rather for early or ongoing management, or possible prevention of melanoma. The *central hypothesis* for these studies is that targeting Akt3 signaling using a novel topical formulation containing ISC-4 would be an effective targeted approach for managing or preventing melanoma development, or for treating unresectable lesions in skin for which surgery is not an option. This hypothesis will be tested and the objectives of the research accomplished by: (1) Development of a topical formulation of ISC-4 for clinical evaluation; and (2) Establishing the inhibitory efficacy of the topical clinical formulation for preventing melanocytic lesion development in laboratory generated human skin containing tumors that resemble early melanomas. Collectively, these discoveries are expected to form a crucial portion of the foundation for an application by PenForGel, LLC to the FDA to obtain IND status for topical ISC-4 to enable evaluation in a phase I clinical trial. ISC-4 for topical applications has been patented and a pre-IND meeting has been arranged with the FDA to discuss the plan for the clinical evaluation and trial in which topical ISC-4 would be tested in humans. Dr. Joseph Drabick, M. D. at Penn State would serve as the PI on the clinical trial

Research Project 19: Project Title and Purpose

Changes in Oxygen-induced Proliferative Retinopathy in 4E-BP1/2 Knockout Mice - The purpose of this project is to determine if dysregulation of protein synthetic pathways contributes to the aberrant development of retinal blood vessels in oxygen-exposed newborn rodents. Using newborn mice deficient in proteins (4E-BP1/2) regulating the initiation phase of protein synthesis, we seek to determine the impact of high concentrations of oxygen to retinal blood vessel growth and vascular endothelial growth factor expression. We believe that the findings from this project will provide a useful framework from which to develop clinical and therapeutic interventions to protect premature newborns from the detrimental effects of oxygen exposure.

Anticipated Duration of Project

5/1/2009 – 12/31/2011

Project Overview

Premature infants exposed to high concentrations of oxygen to treat pulmonary disease often develop retinopathy of prematurity (ROP), a condition associated with long-term visual impairment and blindness. The objective of this project is to determine the contribution of protein synthetic regulatory pathways in the development of oxygen-induced retinopathy in newborn mice. Specifically, we will investigate the role of cap-dependent mRNA translation on the expression of vascular endothelial growth factor (VEGF) protein expression and the subsequent development of neovascularization. We hypothesize that loss of cap-dependent mRNA regulatory proteins, 4E-BP1/2, will reduce retinal neovascularization and retinal VEGF protein expression during the proliferative stage of retinopathy. The project will utilize 7-day-old mice exposed to 75% O₂ for 5 days followed by 5 days of room air recovery; a well-established model of proliferative retinopathy. In the first aim, we will assess the effect of 4E-BP1/2 on the magnitude of oxygen-induced retinal neovascularization using immunohistochemistry. Aim 2 will delineate changes in VEGF protein expression secondary to the altered regulation of cap-dependent mRNA translation induced by the loss of 4E-BP1/2. Specifically, we will determine changes in VEGF and hypoxia-inducible factor-1 (HIF-1) expression by immunoblotting and real-time PCR, respectively. Overall, this project will provide evidence for a role of translational regulation of protein synthesis in the pathogenesis of retinopathy of prematurity.

Principal Investigator

Jeffrey S. Shenberger, MD
Associate Professor
Department of Pediatrics
Hershey Medical Center
500 University Drive, H085
Hershey, PA 17033-0850

Other Participating Researchers

Leonard S. Jefferson, PhD, Tabitha L. Schrufer, MS, Lianqin Zhang, MD – employed by Pennsylvania State University, College of Medicine

Expected Research Outcomes and Benefits

Each year, approximately 15,000 premature infants in the U.S. develop some degree of retinopathy of prematurity (ROP), a form of oxygen-induced eye injury associated with life-long visual impairment. We anticipate that loss of the protein synthetic regulatory proteins, 4E-BP1/2, will diminish abnormal blood vessel growth in the newborn retina induced by exposure to high concentrations of oxygen. Specifically, we anticipate that animals without 4E-BP1/2

proteins will demonstrate a reduction in aberrant blood vessel budding and in the level of blood vessel growth factors within the eye. This information will be used as part of a National Institutes of Health grant proposal investigating how oxygen-mediated changes in protein synthesis modify the expression of factors essential to normal and pathologic retinal growth and development in the newborn. Because effective strategies to prevent or treat abnormal retinal blood vessel development do not currently exist, this project's findings may ultimately lead to the identification of novel therapeutic targets aimed at ameliorating or averting harmful new vessel formation. Given the potential for blindness, a better understanding of ROP may dramatically improve the lives of hundreds of preterm infants and their families.

Summary of Research Completed

We have previously studied the vascular development of wild type (WT) and some 4E-BP1/2 double knockout (DKO) BALB/c mice at varying time points. Our progression in the DKO mice was slowed by the need to reintroduce BALB/c background to the animals in order to enhance breeding. Establishment of the stable background has recently been completed and we are now able to study additional animals. We have completed WT analysis of avascular and neovascular area. This is essential work as it has never been established that BALB/cJ mice undergo neovascularization (NV) in response to the oxygen-induced retinopathy (OIR) protocol.

Methods: Experimental Design. The methods and design were described in last year's Annual report.

Measurement of retinal avascular area (R_a) and NV. Retinas were fixed in 4% paraformaldehyde, flat-mounted on glass slides, and incubated with isolectin B4-Alexa 594 (Invitrogen). Digital images of each quadrant were acquired by laser confocal microscopy (TCS SP2 AOBS, Leica Microsystems) at 5X and merged in Photoshop CS4 (Adobe). Total retinal (R_t) and vascular (R_v) areas will be measured using Photoshop and R_a area determined using $R_a = R_t - R_v$. Times of maximal regression identified by isolectin were confirmed using fluorescein angiography as described by our lab. The extent of NV was determined by counting the number of retinal vascular cell nuclei anterior to the internal limiting membrane in H&E-stained retinal cross-sections using the protocol described by Davies et al. The number of nuclei per 5 μ m section obtained 40- μ m apart was calculated on 15 sections obtained from the mid-portion of each eye.

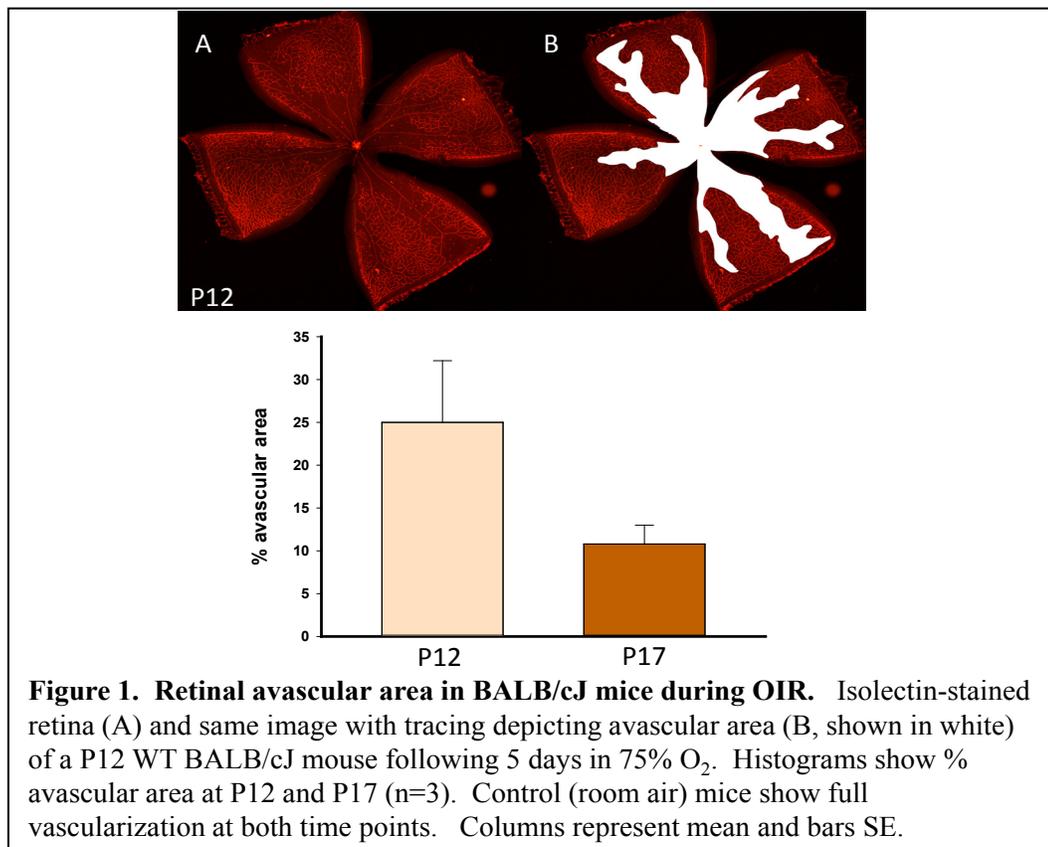
Results. Previous studies found that BALB/cByJ variant mice do not show NV during OIR. In order to develop NV, mice must first undergo retinal vascular regression. This is typically seen beginning at P9 and continued through P12 in the OIR model. As shown in Figure 1, BALB/cJ mice experience retinal vascular regression as depicted by an average of 25% retinal avascular area at P12. Following return to room air, OIR mice begin to revascularize, but still retain approximately 10% avascular area in the retina. As expected, room air (control) mice had fully vascularized retinas at both P12 and P17.

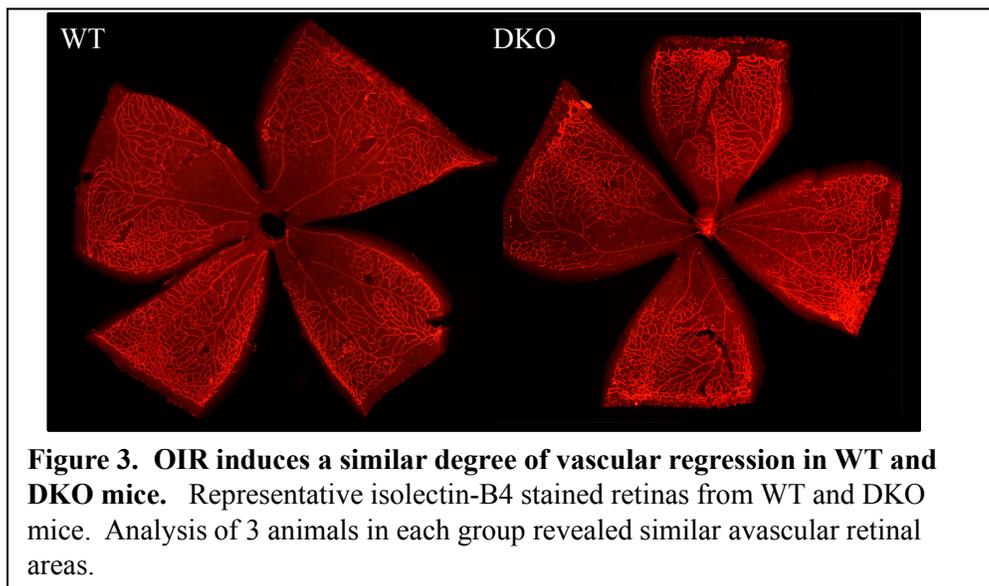
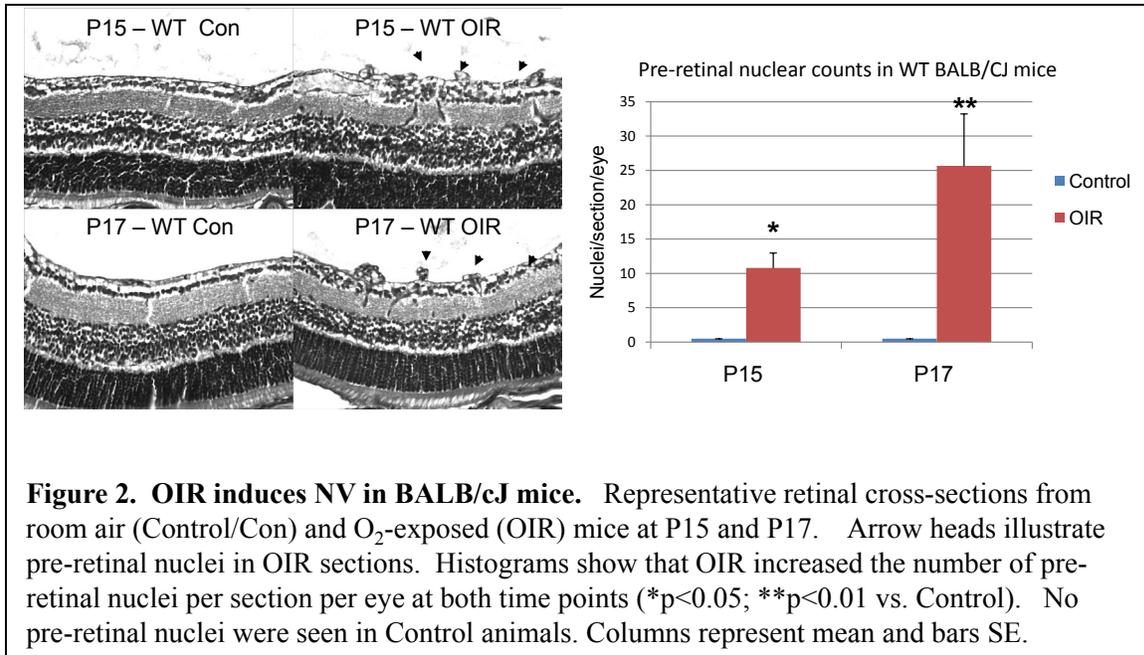
Phase II of the OIR model corresponds to the development of NV. We confirmed that BALB/cJ mice develop NV at both P15 and 17 by counting nuclei anterior to the internal limiting membrane in retinal cross-sections. As expected, pre-retinal nuclei were not found in room air

(control) animals. During OIR, counts averaged 11 and 26 nuclei/section/eye at P15 and P17, respectively (Figure 2). These results indicate that BALB/cJ mice are a suitable OIR model and demonstrate our ability to quantify NV. We are currently in the process of counting nuclei at P19 to determine if the maximal NV response in BALB/cJ mice is similar to that observed in the characteristic OIR strain, C57BL/6.

Preliminary assessment of the avascular area in DKO mice at P12 indicates a similar amount of vascular regression compared to BALB/cJ mice (Figure 3). Analysis of 3 animals in each group found $25.0 \pm 7.2\%$ and $33.9 \pm 1.7\%$ regression in WT and DKO animals (NS), respectively. Additional pups will be analyzed to confirm a lack of difference. In terms of animals at P17, we have yet to have enough DKO pups to investigate, but breeding is ongoing.

In summary, we have now demonstrated that BALB/cJ mice develop NV during OIR. Additionally, we have found similar degrees of retinal vascular regression in WT and DKO during Phase I or OIR. We are working to determine the degree of vascular regression and NV at P17 in the DKO animals and to correlate these changes with alterations in the vascular growth factors FGF2 and VEGF.





Research Project 20: Project Title and Purpose

Molecular Mechanisms of Uninfected Red Cell Phagocytosis in Severe Malarial Anemia (SMA) -
 The purpose of this study is to determine how uninfected red cells are destroyed by macrophages of malaria-infected mice.

Duration of Project

5/1/2009 – 6/30/2010

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>.

Research Project 21: Project Title and Purpose

In Vivo Anti Tumoral Properties of Ceramide Nano-Liposome in Murine Hepatocellular Cancer-
Through the use of nanotechnology, ceramide has been encapsulated in tiny bundles called liposomes. Ceramide is a sphingolipid, and is not soluble in blood. However encapsulation of ceramide into nanoliposome allows ceramide to travel in the bloodstream without any toxicity and thus allows it to target the tumor. Previous studies in murine breast cancer models showed that ceramide preferentially induced apoptosis of breast cancer cells, sparing the surrounding healthy tissue and found that very high doses of ceramide was non-toxic to the animals. In this study we will apply ceramide liposome treatments for cancer therapy using a SV40 T- antigen murine model system aimed at the inhibition of the solid tumor of hepatocellular cancer (HCC). These studies are critical in understanding the importance of the liposomal drug therapy, and may be unique for the experimental model.

Duration of Project

5/1/2009 – 6/30/2011

Project Overview

Hepatocellular cancer (HCC) is the fifth most common cancer in the world with 5-year survival rates of less than 5%. The incidence of HCC is at least one million new patients per year. HCC primarily occurs in individuals with cirrhosis related to either hepatitis C virus (HCV) or hepatitis B virus (HBV) infections. This tumor is refractory to most chemotherapy, and radiation is rarely possible due to liver toxicity. Treatment relies mainly on surgical management: via resection, transplantation, or ablative techniques. Current treatment modalities, including surgery and liver transplantation, offer limited survival benefits, with the number of deaths from the disease in the US (16,780) nearly equal to the number of newly diagnosed cases (19,160). New therapies are desperately needed.

Ceramide liposome (Lip-C6) has been found to be a potential therapeutic agent demonstrating anti-mitogenic and pro-apoptotic effects on various human cancerous cell lines in vitro. The cytotoxic effect of ceramide liposome acts exclusively on cancer cells even at low concentrations (5uM) and has not been noted on the normal epithelial cells even at very high concentrations. This has resulted in the unprecedented interest in ceramide liposome and has prompted the search for new and innovative preclinical studies. Moreover, reports from literature demonstrate

that liposomes play a critical role in reduction of the tumor size in transplantable models. The effects of ceramide liposome on potential chemotherapeutic responses have not been defined in HCC. We have developed a murine model for HCC that spontaneously develops liver tumors under the influence of the large T antigen (T ag) of the SV40 virus. This model represents a unique approximation to human hepatocellular carcinoma, as the mice spontaneously develop malignant tumors through the expression of a viral oncogene. In this model, liver tumors constitutively express the T ag as a tumor-associated antigen and closely resemble the macroscopic and histological characteristic disarrangement seen in liver cancer patients. Therefore, this would be an excellent model to evaluate the liposome treatment effects on liver cancer kinetics. The overall goal of this project is to gain a new understanding of the effects of treatment with ceramide liposomes on tumor growth in a murine model of spontaneous HCC.

Principal Investigator

Hephzibah Rani S. Tagaram, PhD
Research Assistant
Penn State University, College of Medicine
500 University Drive
Hershey, PA-17033

Other Participating Researchers

Kevin Staveley-O'Carroll, MD, PhD, Mark Kester, PhD, Harriet Isom, PhD – employed by Penn State University, College of Medicine

Expected Research Outcomes and Benefits

Neo-angiogenesis is the process of formation of new blood vessels in the tumors. It is well accepted that neo-angiogenesis is a critical step required to maintain tumor growth. Multiple therapeutic strategies targeting tumor vessels have demonstrated benefit in delaying solid tumor progression. We hypothesize that treatment with ceramide liposome (lip-C6) delays the progression of liver tumors in a murine model for HCC.

Ceramide liposome (Lip-C6) has been found to be a potential therapeutic agent demonstrating anti-mitogenic and pro-apoptotic effects on various human cancerous cell lines in vitro. However, those anti-mitogenic and pro-apoptotic effects have not been evaluated specifically for tumor blood vessel cells after exposure to ceramide-liposome. Utilizing immunohistochemical techniques we will simultaneously evaluate the expression of cellular proliferation markers like PCNA and Ki67 and apoptotic markers (TUNEL) on cells expressing CD31 and CD105 markers which are specific for endothelial cells. Treatment with Lip-C6 will result in decreased expression of proliferation markers PCNA and Ki67, and increased expression of TUNEL as an indication of the inhibition in tumor growth. This inhibition may be prominent in mice treated with lip-C6 compared to mice treated with ghost liposome or mice without treatment. We anticipate controlled levels of CD31 and CD105 as an indication of anti-angiogenic ability of lip-C6 compared to ghost liposome. The mechanism and pathways underlying the anti-angiogenic properties of Lip-C6 will be the basis for targeted therapy for the future human studies. These

studies could lead to new avenues intended to evaluate the immunologic effects of liposome treatment on a hepatic tumor antigen-specific fashion. This in turn will lead to a better understanding of the precise mechanisms by which these novel therapeutic approaches mediate apoptosis in HCC.

Summary of Research Completed

The incidence of HCC continues to grow, possibly as a result of chronic liver disease, which often precedes HCC. The long-term survival for patients with HCC is poor and the cancer often becomes metastatic before or during the course of treatment. Liver transplantation is currently a primary treatment option that can offer a cure if the cancer has not become metastatic, but leaves patients with a lifetime of taking immunosuppressive drugs to avoid transplant rejection. However, this surgical option remains limited owing to the scarcity of replacement organs as well as late diagnosis. No promising therapies exist for patients with advanced HCC. Although the multikinase inhibitor sorafenib has been found marginally to prolong survival in patients with advanced HCC, novel treatments are needed. Nanoliposomal C6-ceramide was designed as a therapeutic alternative to common chemotherapeutics. Liposomal formulation of short-chain ceramide analogues allows for the systemic delivery of this very hydrophobic cell-impermeable precipitating pro-apoptotic sphingolipid. Intriguingly, nanoliposomal C6-ceramide was shown to be toxic to cancer cells and not to normal cells, a paradigm arising from distinct ceramide metabolic regulation between these cells. Thus, the therapeutic utility of this cancer cell-selective antineoplastic agent depends on nanoscale delivery modalities.

In vivo administration of nanoliposomal C6-ceramide restricts HCC tumor vascularisation and initiates apoptosis:

To confirm the *in vivo* mechanisms by which nanoliposomal C6-ceramide prevents tumor growth, we assessed cellular proliferation and survival markers in excised tumors. SK-HEP-1 HCC tumors from mice treated systemically with either saline or ghost nanoliposomes had robust PCNA staining (Figure 1A) and nearly no TUNEL-positive staining (Figure 1A). However, tumors from mice treated systemically with nanoliposomal C6-ceramide had virtually no PCNA staining, yet dramatic TUNEL-positive staining was observed. Consistent with increased apoptosis and decreased cellular proliferation, nanoliposomal C6-ceramide-treated animals but not ghost-treated or saline-treated animals had significantly reduced phospho-AKT staining, a marker of pro-survival signaling (Figure 1B). As ceramide has previously been shown to restrict angiogenesis which could also lead to tumor apoptosis, we evaluated tumor sections for CD31 and CD105, both markers of angiogenesis (Figure 1C). Tumors from mice treated systemically with nanoliposomal C6-ceramide had dramatically less CD31 and CD105 staining than tumors from mice treated systemically with ghost nanoliposomes or saline control. Furthermore, significant VEGF staining was observed in tumor sections from mice treated with ghost nanoliposomes or with saline, but not from those treated with nanoliposomal C6-ceramide (Figure 1D). VEGF is a growth factor that promotes vascularisation. These findings suggest that the efficacy of nanoliposomal C6-ceramide *in vivo* is also due to restriction of angiogenesis and therefore to diminished vascularisation of the tumors. Taken together, these results indicate that the efficacy of nanoliposomal C6 ceramide is the result of a decrease in tumor vascularization as well as a decrease in tumor cell proliferation and an increase in tumor cell apoptosis.

Many chemotherapeutics as well as radiation therapy have been shown to induce accumulations

of ceramide. Specific to our study, we showed that nanoliposomal delivery of C6-ceramide in vivo to SK-HEP-1 HCC tumors blocked tumor vascularization, which was visualized in tumor sections as a reduction in CD31 and CD105 staining as well as a reduction in VEGF staining. Tumor vascularization or angiogenesis into and within tumors is necessary to establish nutrient support. It is not coincidental that studies have shown that nutrient deprivation can lead to cellular ceramide generation and cellular death. It is thus plausible that preventing HCC tumor vascularization with systemic nanoliposomal C6-ceramide treatment could induce widespread apoptosis within the tumors. In fact, short-chain ceramide treatment has been shown to generate more physiological long-chain ceramide species within tumors. The efficacy of our nanoliposomal C6-ceramide in the treatment of diverse cancers lends significant therapeutic promise as it translates from the bench to the bedside.

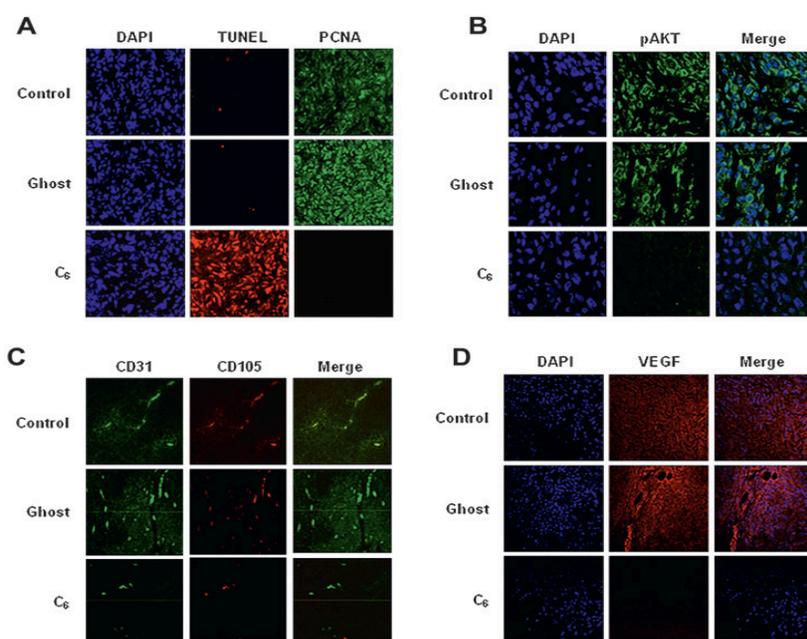


Figure 1. Systemic administration of nanoliposomal C6-ceramide induces widespread apoptosis and decreased proliferation by decreasing pAKT activity in SK-HEP-1 tumors, concomitant with a reduction in markers of angiogenesis. Formalin-fixed paraffin-embedded SKHEP-1 tumor sections were deparaffinised and stained for markers of proliferation, apoptosis and angiogenesis. Tumor sections from mice treated with phosphate buffer solution, ghost nanoliposomes or nanoliposomal C6-ceramide were treated as follows: (A) stained with DAPI to reveal cellular nuclei, stained by the TUNEL method to reveal apoptotic cells, stained with PCNA to reveal proliferating cells; (B) stained for the presence of phospho-AKT; (C) stained for the presence of PECAM-1/CD31 and endoglin/CD105; and (D) stained for the presence of vascular endothelial growth factor. All were then visualized by confocal microscopy. Photomicrographic images are representative of three separate tumor sections per treatment.

Research Project 22: Project Title and Purpose

The Use of Biomarkers to Predict the Onset of Vasospasm in Aneurysmal Subarachnoid Hemorrhage - The purpose is to create a panel of key molecular markers that can be used as a diagnostic test to predict the occurrence of cerebral vasospasm in the setting of aneurysmal subarachnoid hemorrhage (SAH).

Anticipated Duration of Project

7/8/2009 - 12/31/2011

Project Overview

Of those patients that survive an initial aneurysm rupture and/or re-rupture, the primary cause of death and further disability is cerebral vasospasm. There are no clinical warnings that allow the clinician to predict which patients will experience vasospasm. The pathogenesis of vasospasm is not known. There are over twenty cellular and molecular factors that have been implicated. No one has been able to satisfactorily predict which patients will undergo cerebral vasospasm based on the presence of hemorrhage in the subarachnoid space. Our study will allow us to examine a wide selection of interleukins and growth factors during a patient's hospital stay and correlate these with clinical cerebral vasospasm. This approach to vasospasm diagnosis using a panel of pertinent molecular markers has not been previously studied. With these data we will be able to create a diagnostic test that will allow for the early prediction of vasospasm.

Principal Investigator

Kevin M. Cockroft, MD, MSc
Associate Professor
Penn State Hershey Medical Center
30 Hope Drive, EC110
Hershey, PA 10033

Other Participating Researchers

James R. Connor, PhD, Ryan Mitchell, PhD - employed by Penn State College of Medicine
Akshal S. Patel, MD –employed by Penn State Hershey Medical Center

Expected Research Outcomes and Benefits

Stroke is the third leading cause of death and the primary cause of disability in the United States. Aneurysmal subarachnoid hemorrhage (SAH), a form of hemorrhagic stroke, is a particularly devastating disease. The thirty-day mortality rate after aneurysmal SAH is approximately 50% with the majority of survivors left disabled and unable to return to work. Of those patients that survive the initial aneurysm rupture and/or re-rupture, the primary cause of death and further disability is cerebral vasospasm. Despite its unequivocal clinical significance, the complete pathogenesis of post-hemorrhagic cerebral vasospasm remains poorly understood and there is no

accurate, early diagnostic test to predict its occurrence.

We plan to determine a molecular profile that can be used to predict which patients will experience clinically significant cerebral vasospasm. We will use a multiplex immunoassay to evaluate a panel of inflammatory and trophic proteins present in cerebrospinal fluid (CSF) and serum during the course of aneurysmal SAH. Using regression analysis we will develop a panel of biomarkers that can be used to predict cerebral vasospasm.

Summary of Research Completed

This study is being conducted to discover if there is a relationship between physiologic inflammatory mediators and outcome after aneurysmal subarachnoid hemorrhage. Our hypothesis is that inflammatory mediators play a role in the course of aneurysmal subarachnoid hemorrhage and that there is unique biomarker signature or “finger print” that influences neurologic outcomes; including the development of cerebral vasospasm, subsequent delayed ischemic neurologic deficit (DIND) and poor condition at discharge.

Interim Analysis

Of the 34 patients enrolled into this study thus far, 20 have had their serum analyzed for cytokine and growth factor levels. Methods for specimen analysis with multiplex assay have been described previously. This report will focus on specific clinical correlations. A number of clinical variables were collected for each patient as part of the overall data collection. These included demographics, pertinent medical history, hospital course and outcome at discharge.

In the analyzed subgroup of 20 (Table 1), 13 subjects were female and 10 subjects were smokers prior to hospitalization. The majority of patients underwent endovascular embolization of their aneurysms. At the time of admission three major subarachnoid hemorrhage clinical grading scales were recorded; the Hunt-Hess grade (HHG), the Fisher Grade (FG) score and the World Federation of Neurosurgeons Score (WFNS). A good HH grade was defined as 1 to 3 and a good F score was defined as 1 to 2. At time of discharge the modified Rankin Score (mRS) was documented for every subject; a good outcome was defined as mRS of 0 – 2.

Once patients were stabilized in the critical-care unit they were monitored closely for signs of neurologic decline such as stroke like symptoms or deteriorating mental status. Thirteen (of 20 subjects analyzed) required placement of an external ventricular drain (EVD) for relief of cerebral edema and hydrocephalus. Six of the 13 patients with EVDs went on to require placement of a ventriculoperitoneal shunt for chronic/refractory hydrocephalus.

The most pertinent piece of metabolic laboratory data was the serum sodium level. Hyponatremia worsens patient outcome and predisposes to seizures and clinical decline. Ten of the 20 subjects suffered from hyponatremia, which was treated with sodium supplementation agents.

Transcranial Doppler (TCD) provides an indirect measure of vascular diameter by measuring the velocity of blood flow in intracranial vessels. Each patient in this study underwent daily TCD testing for 14 days or until discharge, whichever came first. Eight major vessels were examined, the anterior cerebral arteries, the middle cerebral arteries, the posterior cerebral arteries and the

intracranial internal carotid arteries. Mean flow velocities are measured in centimeters per second and can range from less than 80cm/s to greater than 200cm/s. A TCD velocity of greater than 200cm/s is generally considered to represent severe vessel narrowing or vasospasm. Eight of the 20 patients had velocities greater than 200cm/s in at least one vessel. In addition, 8 of the 20 patients analyzed experienced delayed ischemic neurologic deficits as documented in the medical record. Six of these patients required some form of neurointerventional procedure to treat this symptomatic vasospasm.

Interim Results

The multiplex assay permitted us to examine 24 candidate analytes simultaneously, on a daily basis while the subjects remained in the hospital. A thorough review of the literature revealed a number of cytokines that have been previously studied in the context of aneurysmal subarachnoid hemorrhage. These include: Interleukins (IL) 1a, 1b, 6 and 8; Vascular Endothelial Growth Factor (VEGF), and Tumor Necrosis Factor (TNF). We were able to demonstrate significant variation of these cytokines among subjects during the course of their disease. We therefore examined the relationship between serum levels of these cytokines and nine different clinical measures: gender, tobacco use, Hunt Hess Grade on admission, Fisher Grade on initial head CT, modified Rankin Score on discharge, requirement of EVD (acute hydrocephalus), requirement of ventriculoperitoneal shunt (chronic hydrocephalus), TCD velocities of greater than 200cm/sec (severe TCD vasospasm), incidence of delayed ischemic neurological deficit and modified Rankin Score at discharge. We found that there is a statistically significant relationship between cytokine levels in the serum and a number of these variables. Elevated IL 6 levels, in particular, were significantly associated with poor Hunt-Hess scores, poor modified Rankin Score at discharge, TCD velocities greater than 200, and delayed ischemic neurologic deficit (Table 2). Elevated TNF levels were significantly associated with tobacco use and poor Hunt & Hess Grade as well as acute and chronic hydrocephalus (Table 3). Males and smokers were more likely to have higher IL-8 levels (Table 4). Higher VEGF levels were related to a worse Fisher Grade and higher IL-1b levels were associated with smoking (data not shown). However, we found no association between IL-1a levels and any of the variables tested (data not shown).

Conclusion

Our data thus far continue to support the hypothesis that inflammatory mediators play a role during the course of aneurysmal subarachnoid hemorrhage and may contribute to the pathogenesis of various complications associated with this disease.

Table 1: Patient Demographics and Presenting Characteristics

Gender	Smoking	HHS	FG	WFNS	Treatment	EVD	VPS	mRS on dc	TCDs >200	DIND
F	No	2	2	1	Coiling	Y	N	1	N	N
F	Yes	2	3	1	Coiling	N	N	1	N	N
F	Yes	2	4	2	Coiling	Y	N	1	Y	N
F	No	4	3	5	Clipping	Y	Y	5	Y	N
M	No	2	3	1	Coiling	Y	Y	1	N	N
M	No	1	4	2	Coiling	N	N	2	N	Y
F	Yes	3	3	2	Coiling	Y	Y	4	Y	N
F	Yes	2	4	2	Coiling	Y	N	1	N	N
F	No	1	2	1	Coiling	N	N	1	N	N
M	Yes	1	4	1	Coiling	Y	Y	5	N	N
F	Yes	1	3	1	Coiling	Y	Y	3	Y	Y
F	No	2	3	2	Coiling	Y	Y	4	Y	Y
F	No	5	4	5	Clipping	Y	N	5	Y	N
F	Yes	2	2	1	Coiling	Y	N	2	N	N
M	Yes	3	4	2	Coiling	N	N	2	N	Y
F	No	4	4	4	Coiling	N	N	6	Y	Y
M	Yes	3	4	2	Coiling	N	N	4	N	Y
M	Yes	4	4	4	Coiling	Y	N	4	Y	N
M	No	2	3	2	Clipping	Y	N	6	N	N
F	No	2	2	2	Coiling	Y	Y	3	N	N

(EVD, external ventricular Drain; VPS, ventriculoperitoneal shunt; HHG, Hunt Hess Grade; FG, Fisher Grade; WFNS, World Federation of Neurological Surgeon Score; mRS on DC, modified Rankin Scale at discharge from hospital; DIND, delayed ischemic neurologic deficits; Treatments refer to endovascular coil embolization or open craniotomy with clipping of aneurysm)

Table 2: Serum IL 6 levels (pg/mL) and Correlation to Outcome Variables

Variable	Mean outcome 1	Mean outcome 2	p-value
Sex	M 88.82	F 55.98	0.197
Smoking	Y 64.59	N 70.32	0.439
Hunt Hess Grade (clinical grade)	1-3 43.86	4-5 137.92	0.012
Fisher Grade (CT hemorrhage grade)	1-2 26.81	3-4 80.96	0.098
Placement of EVD (acute hydrocephalus)	Y 83.94	N 29.06	0.082
Placement of VP shunt (chronic hydrocephalus)	Y 79.53	N 62.25	0.335
TCD>200 (Doppler vasospasm)	Y 100.49	N 40.47	0.047
Delayed Ischemic Neurologic Deficit	Y 105.78	N 41.94	0.039
mRS on discharge (clinical outcome)	0-2 32.67	3-6 95.96	0.038

Table 3: Serum TNF alpha levels (pg/ml) and Correlation to Outcome Variables

Variables	Mean outcome 1	Mean outcome 2	p-value
Sex	M 47.36	F 61.25	0.183
Smoking	Y 70.37	N 42.41	0.023
Hunt Hess Grade (clinical grade)	1-3 63.19	4-5 35.98	0.049
Fisher Grade (CT hemorrhage grade)	1-2 53.57	3-4 57.33	0.413
Placement of EVD (acute hydrocephalus)	Y 64.82	N 123.75	0.034
Placement of VP shunt (chronic hydrocephalus)	Y 74.53	N 48.62	0.047
TCD>200 (Doppler vasospasm)	Y 57.80	N 55.33	0.431
Delayed Ischemic Neurologic Deficit	Y 61.66	N 52.88	0.280
mRS on discharge (clinical outcome)	0-2 54.68	3-6 57.79	0.417

Table 4: Serum IL 8 levels (pg/ml) and Correlation to Outcome Variables

Variable	Mean outcome 1	Mean outcome 2	p-value
Sex	M 31.60	F 21.28	0.032
Smoking	Y 29.95	N 19.84	0.028
Hunt Hess Grade (clinical grade)	1-3 23.79	4-5 28.19	0.247
Fisher Grade (CT hemorrhage grade)	1-2 21.82	3-4 25.92	0.261
Placement of EVD (acute hydrocephalus)	Y 26.57	N 20.97	0.173
Placement of VP shunt (chronic hydrocephalus)	Y 22.01	N 26.13	0.248
TCD>200 (Doppler vasospasm)	Y 25.40	N 24.48	0.440
Delayed Ischemic Neurologic Deficit	Y 27.64	N 23.06	0.208
mRS on discharge (clinical outcome)	0-2 22.28	3-6 27.03	0.195

Research Project 23: Project Title and Purpose

Tim-2 Expression on Oligodendrocytes: A New Immune System Target – Recently, we have made a novel and exciting discovery that Tim-2 receptor is selectively expressed on oligodendrocytes. One of the ligands for Tim-2 is Sema4A, which is highly expressed in the immune system, and is critical in regulating immune attacks on the myelin in animal models of multiple sclerosis (MS). We have shown that when oligodendrocytes are exposed to Sema4A in a cell culture model they die. This is an exciting and new finding suggesting that the Sema4A/Tim-2 interaction may be the deleterious relationship between oligodendrocytes and immune cells that scientists have been seeking. Therefore, in this project, we want to investigate mechanism of Sema4A-induced oligodendrocyte death.

Anticipated Duration of Project

7/8/2009 - 12/31/2011

Project Overview

Despite the recent advances in our understanding of disease pathogenesis and treatment of multiple sclerosis, this disease still represents a significant source of neurologic disability. Although immunomodulatory drugs are very effective at treating active disease, they are completely ineffective at treating the chronic neurodegenerative phase of MS. To address this, one approach is finding ways of encouraging remyelination by endogenous oligodendrocyte progenitor cells, allowing them to repair myelin and therefore preventing secondary axonal loss and neurodegeneration. We have recently discovered that Tim-2 protein is selectively expressed on cells of oligodendrocyte lineage. Tim-2 is present on mature oligodendrocytes and oligodendrocyte progenitors and functions as receptor for extracellular H-ferritin and Sema4A proteins. Considering that both Sema4A and Tim-2 have an important function in immune regulation and are critical in development of experimental autoimmune encephalomyelitis (EAE), we propose that existence of Tim-2 receptors on oligodendrocytes may predispose these cells to immune attack. There are two specific aims that we intend to accomplish.

Aim 1: Test the hypothesis that Sema4A can be detected in demyelinating plaques, but not in normal white matter from human tissue collected at autopsy. A second hypothesis will be tested concomitantly, that Sema4A staining in the plaques will be colocalized with markers of infiltrating lymphocytes (T cells and B cells) and local central nervous system (CNS) inflammatory cells (astrocytes and microglia).

Aim 2: Test the hypothesis that there will be higher concentrations of Sema4A protein in the Cerebrospinal fluid (CSF) of MS patients compared to controls which will be predictive of clinical diagnosis of MS.

Principal Investigator

James Connor, PhD
Distinguished Professor and Vice Chair, Research

Penn State College of Medicine
Neurosurgery, H110
500 University Drive, PO Box 850
Hershey, PA 17033-0850

Other Participating Researchers

Padma Ponnuru, PhD – employed by Pennsylvania State University

Expected Research Outcomes and Benefits

Our findings will have the potential of identifying a novel mechanism by which oligodendrocytes fail to remyelinate in MS, as well as identifying a novel diagnostic marker and drug target for treatment of MS. Although it will take some time (several years) to develop drugs to inhibit Sema4A-Tim-2 interaction on oligodendrocytes, discovery of Sema4A as diagnostic marker would benefit MS patients immediately, as screening for Sema4A protein could become part of MS diagnostic procedure soon after we publish our findings.

Summary of Research Completed

To investigate the expression of Sema4A protein in MS plaques, we first demonstrated that the Sema4A antibody recognizes Sema4A recombinant protein on a Western blot (Figure 1A). Because Sema3A and Sema4D have been suggested to play a role in the pathogenesis of MS and have been found to be expressed within MS plaques (Williams et al. 2007, Giraudon et al. 2004), we demonstrated that this antibody did not cross-react with these related semaphorin proteins on a native protein immunoblot (Figure 1B). The Figure 1B shows that anti-Sema4A antibody recognizes only native Sema4A-Fc, but not Sema3A-Fc or Sema4D-Fc. Equal loading of protein is shown with an antibody against the IgG fused domain to rSema4A-Fc, rSema4D-Fc, and rSema3A-Fc. Subsequently, we used the same antibody to evaluate expression and cellular distribution of Sema4A in 5 MS patient plaques and 4 control specimens containing normal human white matter. Table 1 summarizes the histopathological parameters of the specimens used in this study. The MS and control patient population were matched well by age (average age 67 and 68, respectively) and gender (male/female ratio was 20%:80% for MS and 25%:75% for controls). In Figure 2, a representative image of immunostaining for Sema4A demonstrates much higher levels of Sema4A immunoreactivity within the plaque compared to normal white matter. Sema4A immunoreactivity was localized in small cells cuffing the blood vessels, which is pathognomonic for infiltrating lymphocytes within the MS plaque. A second group of Sema4A-positive cells observed in the sections were large bloated cells infiltrated within the myelin. These cells are morphologically typical of microglia/macrophages. In each of 5 plaques examined, the Sema4A immunoreactivity was much higher in the plaque compared to normal white matter. We defined the extent of demyelination and distinguished plaque area from normal appearing white matter by staining for myelin basic protein (MBP; Figure 2, C, D) for each plaque used in the study. Collectively, these data demonstrate that there are higher levels of Sema4A protein within the MS plaque compared to normal white matter, and that the chief cellular sources of this protein within the plaque are infiltrating lymphocytes and activated macrophages/microglia.

We have IRB approval for Aim 2 but no data has been collected to date.

Figure 1. Anti-Sema4A antibody recognizes Sema4A, but not closely related Sema3A or Sema4D. (A) Recombinant Sema4A-Fc chimera protein was loaded onto a denaturing SDS-PAGE gel and either stained with Comassie or probed with anti-Sema4A antibody on a Western blot (WB). The antibody recognizes the Sema4A-Fc chimera recombinant protein (A), which is of appropriate molecular size (115-120 KDa) for the glycosylated protein. (B) Native (non-denatured) rSema4A-Fc, rSema3A-Fc, and rSema4D-Fc were immobilized onto a nitrocellulose membrane and probed with anti-Sema4A antibody via slot blot. A separate blot was probed with anti-IgG antibody against the fused Fc domain of the semaphorin proteins to show total protein. This representative immunoblot demonstrates that the Sema4A antibody recognizes rSema4A-Fc, but not rSema3A-Fc or rSema4D-Fc.

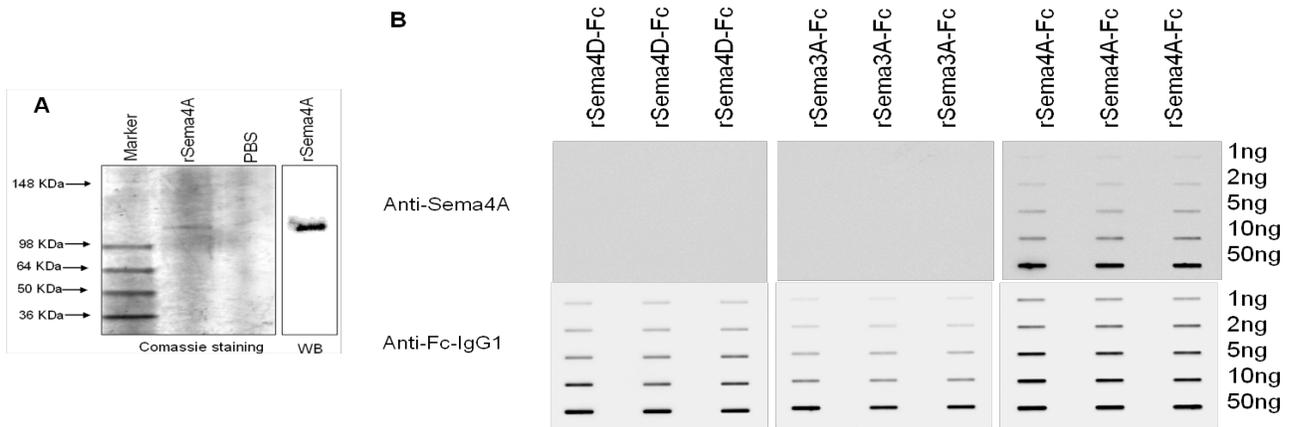
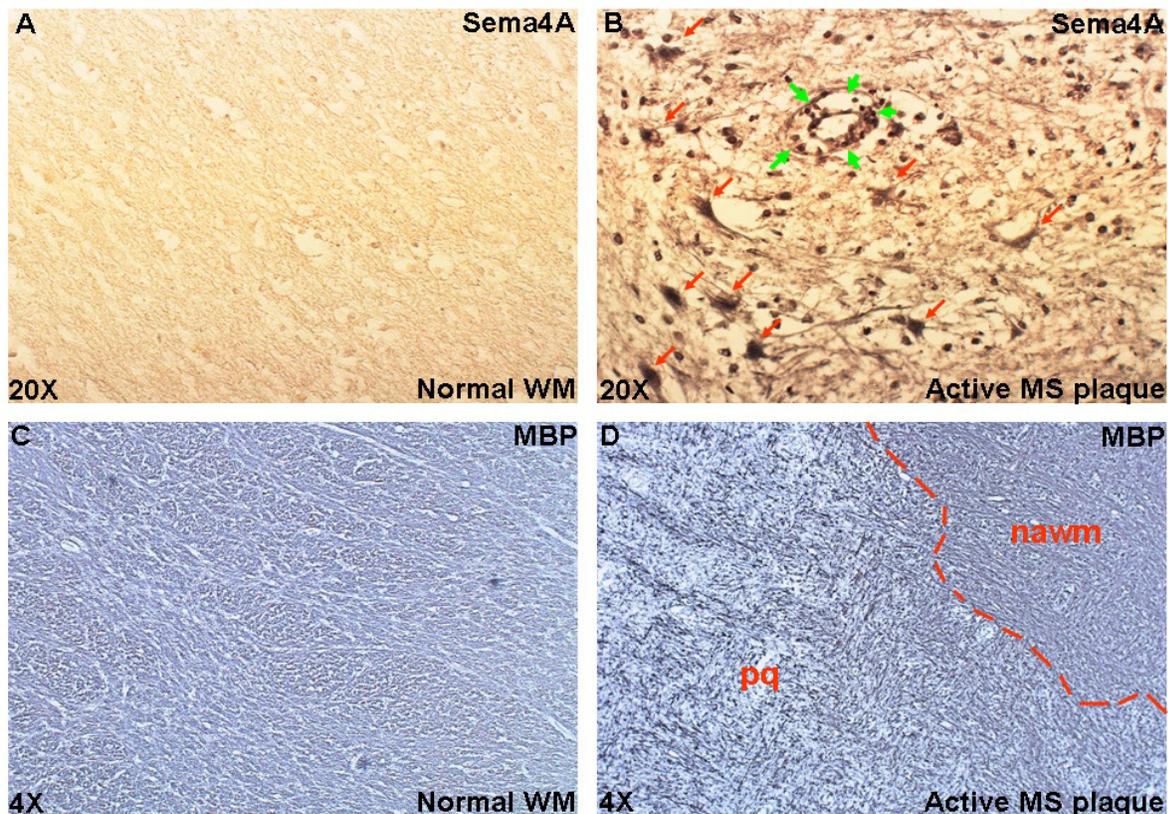


Table 1. Characteristics of MS patients and normal controls used in the study. The tissue specimens from a total of nine patients were used in the study, of which 5 were MS patients and 4 were normal controls. The average age of MS patients and controls were 67 and 68 years, respectively.

MS patient	Primary diagnosis	Age	Gender	Plaque	Path. Classification
1	Multiple Sclerosis	76	F	Active	demyelination, lymphocytic infiltrate (E), microglia present
2	Multiple Sclerosis	84	F	Active	demyelination, lymphocytic infiltrate (D), microglia present
3	Multiple Sclerosis	65	M	Active	demyelination, lymphocytic infiltrate (P, E), microglia present
4	Multiple Sclerosis	62	F	Active	demyelination, lymphocytic infiltrate (E, D), microglia present
5	Multiple Sclerosis	48	F	Active	demyelination, lymphocytic infiltrate (P, E, D), microglia present
Control patient	Primary diagnosis	Age	Gender	Plaque	Path. Classification
1	Normal	66	M	none	Normal white matter
2	Normal	49	F	none	Normal white matter
3	Normal	77	F	none	Normal white matter
4	Normal	80	F	none	Normal white matter

Figure 2. Sema4A is expressed in MS plaques. Sections of normal white matter (A) and those of patients with MS (active plaques, B) were immunostained for Sema4A. Representative images are presented here that demonstrate strong immunoreactivity with Sema4A antibodies in the MS plaque compared to normal white matter. The reaction product was mainly localized in lymphocytes (perivenular cuff, green arrows in B) and in scattered macrophages/microglia (red arrows in B). The size and location of the plaque was determined by immunostaining of the neighboring sections for myelin basic protein (MBP) (C and D). These low powered images demonstrate representative results of MBP immunoreactivity in both normal white matter and MS plaques. Within the given specimen, the demarcation between the region of strong demyelination/plaque (pq) and normal appearing white matter (nawm), was observed and is indicated by the red dotted line.



Research Project 24: Project Title and Purpose

Mechanisms of Microsatellite Mutagenesis in Human Cells – Short, repetitive DNA sequences, called microsatellites, are a characteristic feature of the human genome. Mutations within microsatellite sequences are causally linked to the development of several human diseases, including cancer and cardiovascular illness. We have created a new, interdisciplinary program among computational and experimental investigators at Penn State University to elucidate the mechanisms whereby microsatellites arise, mutate, and disappear within individual human genomes. The purpose of this project is to provide direct experimental evidence in support of our new collaborative model in order to improve the competitiveness of our NIH-R01 application.

Duration of Project

7/8/2009 – 6/30/2010

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>.

Research Project 25: Project Title and Purpose

Epigenetic Therapy of Human B Cell Malignancies – Cancers of B cells including leukemia and lymphoma are common and mostly incurable. We have used a unique combination of chemotherapy and immunotherapy to achieve durable complete response in B cell malignancies like mantle cell lymphoma. Clinical trials have been initiated to test these hypotheses. Clinical samples will be available from these trials for correlative science studies. The purpose of this project is to use these samples to determine the silenced target genes activated by our treatment regimen and to further study these genes in vitro.

Duration of Project

9/1/2009 - 6/30/2011

Project Overview

Epigenetic gene silencing is a common theme in many malignancies. Epigenetic therapeutic agents such as DNA hypomethylating agents and histone deacetylase inhibitors are being tested in a variety of malignancies with some success. B cell malignancies are a group of diseases where improved therapies are needed. We have found that combination therapy with epigenetic agents and the monoclonal antibody rituximab is extremely effective against B cell malignancies such as mantle cell lymphoma, chronic lymphocytic leukemia and other indolent B cell lymphomas. This combination appears more effective and less toxic than other current cytotoxic therapies. We have initiated clinical trials testing these therapies. The objective of this project is to use cell lines and patient samples to demonstrate that the therapy is working via an epigenetic mechanism by identifying genes and microRNAs (miRNA) that are transcriptionally activated with these therapies. We will identify these target genes and further study their roles in the pathogenesis of B cell malignancies.

Thus, the *central hypothesis* for these studies is that epigenetic therapy for B cell malignancies using this combination will be safe and effective and result in the activation of certain silenced target gene(s) that allow rituximab to effect apoptosis much more efficiently. This discovery would be highly significant, highlighting the therapeutic implications of epigenetic therapy for B cell malignancies and other malignancies as well.

Principal Investigator

Elliot M. Epner, MD, PhD
Professor of Medicine
Pennsylvania State University
Penn State Cancer Institute
500 University Drive, P.O. Box 850
Hershey, PA 17033-0850

Other Participating Researchers

Mark Kester, PhD – employed by Penn State University
Samir Parekh, MD – employed by Albert Einstein College of Medicine

Expected Research Outcomes and Benefits

This project will not support the clinical trials, which will be funded through other sources. However, this project will use the clinical samples generated from these clinical trials to do important correlative science to identify the target of the hypothesized epigenetic mechanism of action. Once putative targets are characterized, they will be studied in vitro in tissue culture systems. We predict this combined epigenetic/immuno therapeutic approach to be potentially curable in B cell malignancies. If successful, we also predict that this approach can be tested in a variety of other malignancies. Identification of the target genes involved will add important scientific validity and encourage adaptation of this approach by the oncologic community. Thus, success of these clinical trials and their scientific correlates is predicted to have a significant positive impact on the currently poor prognosis faced by mantle cell and other B cell lymphoma patients by contributing to availability of more effective therapies, which would increase the length and quality of life for these patients.

Summary of Research Completed

During the last year, we have made significant progress in the use of combined epigenetic and immunotherapy for the treatment of B cell malignancies. We have focused on cladribine, a purine analog with epigenetic properties. Cladribine is a hypomethylating agent that inhibits the methyl donation reaction by S-adenosyl methionine. We have initiated a clinical trial and are evaluating combination therapy with SAHA (vorinostat), cladribine and rituximab (SCR) in patients with MCL and other B-cell malignancies. A phase I/II study at Oregon Health and Science (OHSU) and Pennsylvania State University (PSU) is evaluating SCR in 40-50 patients with relapsed B-cell malignancies. Among the 15 enrolled patients with newly diagnosed MCL, SCR was associated with an overall response rate (ORR) of 100%, with 12 patients attaining complete remission (CR) as assessed by metabolic and fluorescent in situ hybridization/flow cytometry after Cycle 2. Toxicities were primarily hematologic and reversible. In vitro studies have confirmed epigenetic changes after cladribine treatment in MCL patients, including DNA methylation changes in vivo, inhibition of histone methylation in vitro, and changes in gene expression in vivo.

Correlative science experiments using leukemic peripheral blood samples from patients treated on trial or off trial with SCR or CR were studied pre and post therapy for epigenetic changes. In collaboration with Dr. Samir Parekh at Albert Einstein, we have assayed patient samples for changes in DNA methylation. We have found widespread genome changes in DNA methylation after cladribine treatment (Figure 1). One potential target gene which we have identified is a dual specificity phosphatase gene DUSP2. We have evidence that this gene is hypomethylated and transcriptionally activated after cladribine treatment in several patients who have achieved a complete response. These genes comprise a family of about 15 genes, and we have evidence that another family member DUSP1, is transcriptionally activated in a few patients. Furthermore, treatment of an MCL cell line with cladribine and/or vorinostat transcriptionally activates DUSP2 and leads to epigenetic changes at the DUSP2 promoter as assayed by chromatin immunoprecipitation assays (Figure 2).

Other genes that emerged from the HELP assay analysis as being hypomethylated in MCL and CLL patients have been examined by quantitative RT-PCR. We could not find clear evidence of any of these hypomethylated genes becoming transcriptionally activated in patients treated on trial.

In many of the patients treated we are seeing rapid response with decline in peripheral WBC counts and rapid resolution of lymphadenopathy and splenomegaly. We have investigated the mechanism for this rapid cell death both in vitro and in vivo. We find that caspase 3 but not caspase 9 is cleaved, suggesting an alternative apoptotic pathway.

Our working conclusion to date is that epigenetic therapy is not activating a specific gene but can potentially activate multiple silenced genes in one or a variety of pathways. Based on the identification of DUSP2, we are initially focusing on the ERK-MEK MAP kinase pathway. We have looked at mRNA levels in some of the responding leukemic MCL patients (Figure 3).

In addition to newly diagnosed MCL, we have also seen responses in previously treated patients with MCL and other B cell malignancies such as follicular lymphoma, marginal zone lymphoma, and chronic lymphocytic leukemia. Most of these have been partial responses; one patient with MZL who achieved a CR after failing RCVP and RCHOP. Our hypothesis is that our combination epigenetic/immunotherapy is most active in the pristine epigenome. Clinical trials that examine this hypothesis in previously untreated patients with other indolent low grade lymphomas and CLL are in preparation.

We have also obtained cells from patients resistant to SCR therapy as well as patients with CLL treated with fludarabine for controls to compare to cells from cladribine treated responders. Gene expression profiling has been performed by Dr Samir Parekh's lab at Albert Einstein and shows distinct differences in the genes induced by these similar purine analogues (Figure 4).

To potentially augment the epigenetic effects of cladribine and reduce the side effect profile, we have collaborated with Mark Kester's group to encapsulate cladribine in nanoparticles. Nano clad appears to have improved cytotoxicity over native cladribine by 10-100 fold and also inhibits histone methylation at 10 fold lower concentrations (Figure 5). Animal studies using MCL xenografts and incorporating rituximab and HDACi into combination nanoparticles are

underway.

We have developed a minimal residual disease assay using quantitative RT-PCR for cyclin D1 and Sox 11 on patient samples from our clinical trial. Previous assays have relied on DNA PCR that has variable breakpoints, thus limiting the assay to 20-30% of patients whose breakpoints are amplifiable (Figure 6). Our assay allows almost 100% of MCL patient samples to be assayed. In conclusion, work during the last year has shown significant progress in the development of a novel, nontoxic combination therapy using epigenetic drugs and monoclonal antibodies for untreated MCL. There are promising results but small numbers for other indolent B cell malignancies as well. Evidence that suggests an epigenetic mechanism of action of cladribine has been accumulated. Potential target genes have been identified; however this therapy has the potential of activating multiple silenced target genes. The potential of this combination with other tumor specific monoclonal antibodies will be examined in future and ongoing studies.

Figure 1 Volcano plot of methylation difference (X axis) vs. Significance (on Y axis) showing selected differentially methylated loci between patients after treatment with Cladribine (CDA) as compared to before it. Probe sets that were differentially methylated are marked in grey ($p < 0.05$). Total 6 patients.

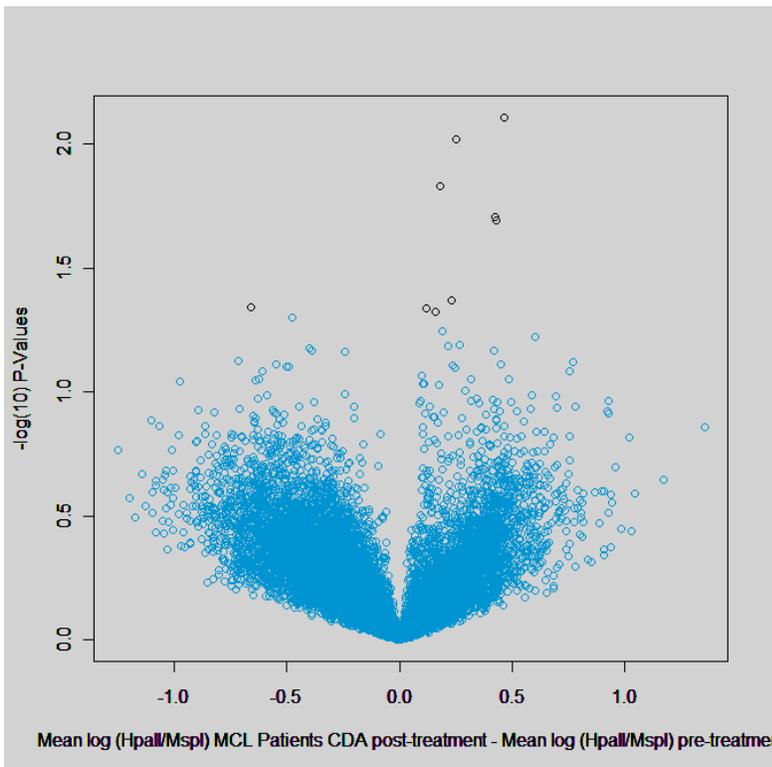


Figure 2. DUSP2 Gene Promoter Histone and DNA Methylation by CHIP assay in Granta cells treated with Cladribine. Granta-519 cells were cultured in 100uM CDA and subjected to CHIP analysis. ChIP assay with Antibodies to 5-methylcytosine show decreased DNA methylation, and with antibodies to methylated histone H3 show decreased histone methylation at the DUSP2 promoter.

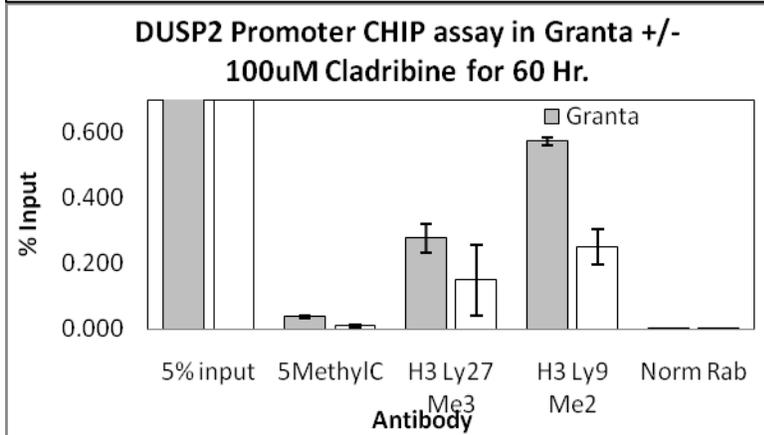


Figure 3. Quantitative RT-PCR of expression levels of several genes in the MEK-ERK pathway from patients treated with cladribine and SAHA. Several genes in treated patients such as DUSP2, MEK1, and ERK2 were upregulated

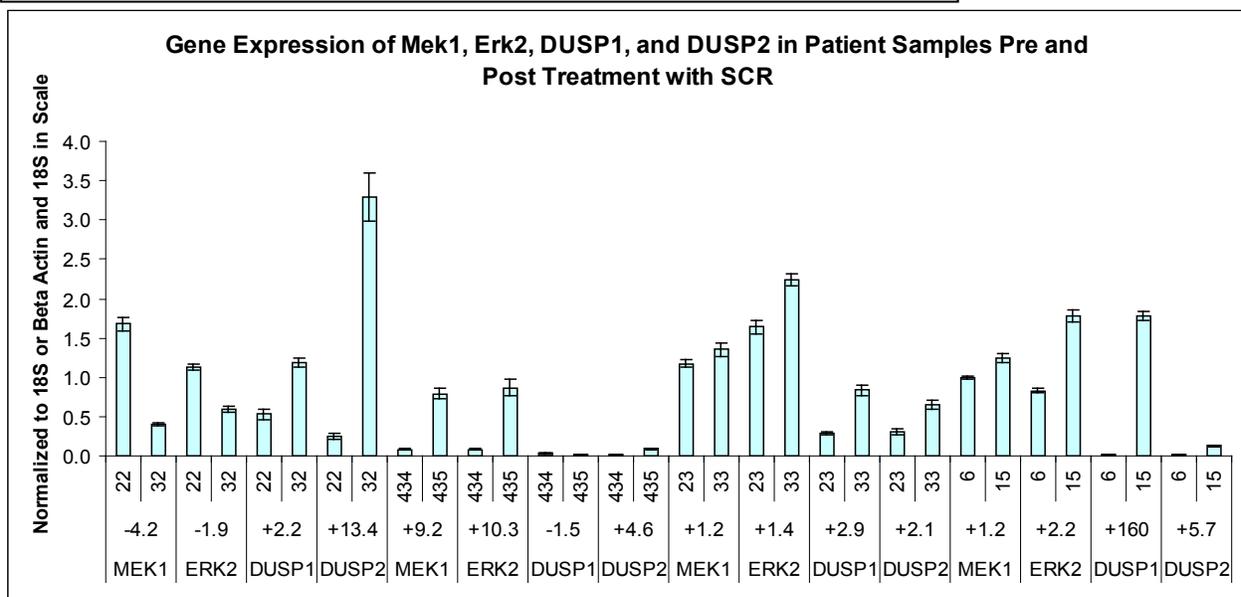


Figure 4 Hierarchical clustering of MCL and CLL patients samples before and after Cladribine and fludarabine/Rituxin treatment using Pearson's correlation (GEP data)

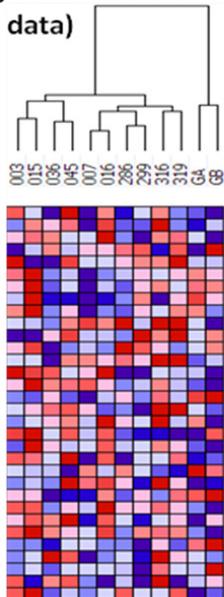


Figure 4. Expression array analysis of leukemic MCL and CLL cells treated with either cladribine or fludaribine. The heat map demonstrates that fludaribine and cladribine are activating different transcriptional programs.

Figure 5 MTS Assay of Granta 519 and IB4 cells treated with cladribine or nanoliposomal cladribine (Nano) for 48 hr. Nano showed a 60 fold increased toxicity in MCL/Granta cells compared to cladribine, while only a 4 fold increased toxicity in EBV immortalized B cells, IB4.

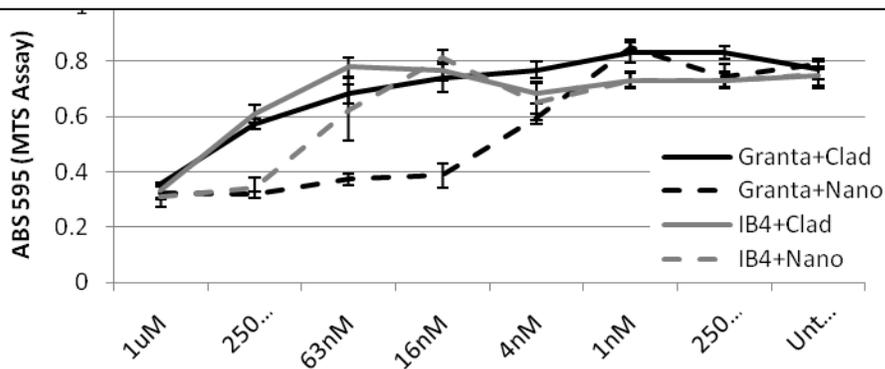
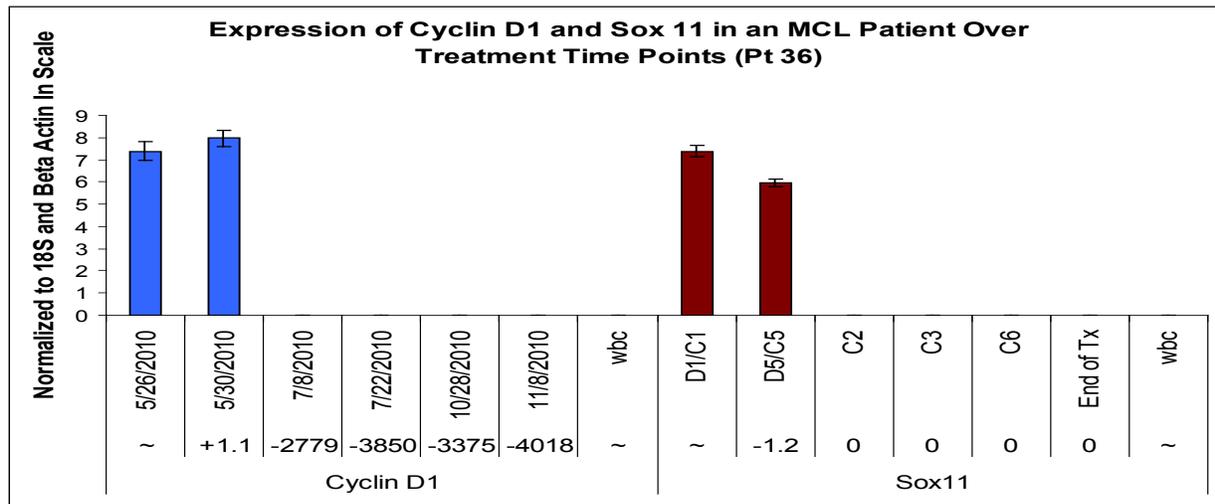
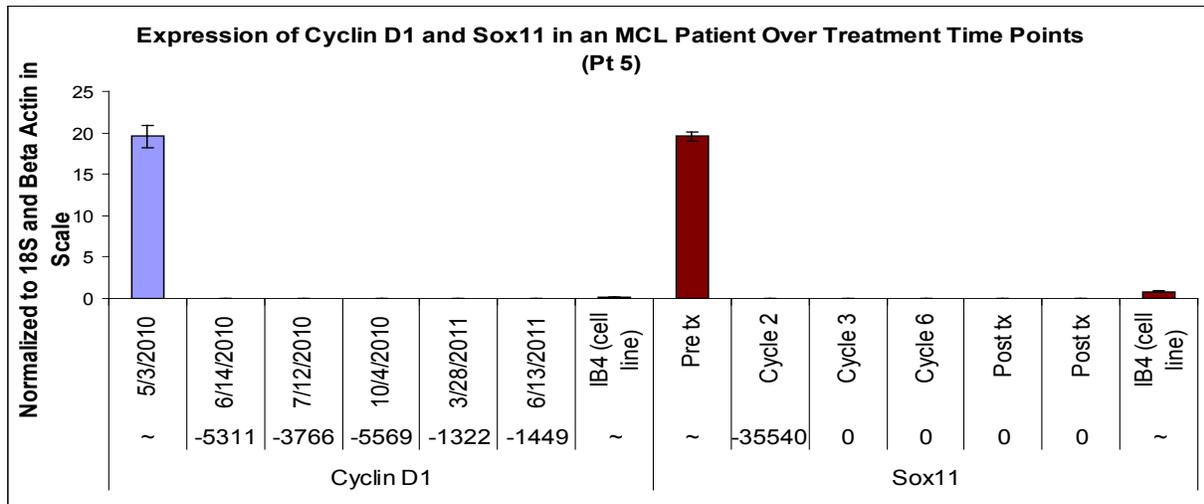


Figure 6



Research Project 26: Project Title and Purpose

Novel Multielectrode Recording Techniques for Assessment of Taste Functions in the Brain – Obesity contributes to a number of life threatening health problems such as non-insulin dependent diabetes, hypertension, and coronary heart disease. The causes of obesity are complex but overconsumption motivated by palatable foods may be an important factor in the etiology of obesity. Since meal size is controlled by sensation originating in the oral cavity, the contribution of oral sensation will potentially provide significant data on ways to control obesity. The present application seeks support to develop novel multielectrode recording techniques necessary to obtain critical pilot data for a future NIH R01 grant application. Specifically, the project will record activity of gustatory-sensory neurons of the hindbrain in high caloric high fat diet-induced obese (DIO) and lean male rats to compare taste responses to palatable sucrose solutions in hungry and sated states.

Duration of Project

7/8/2009 – 6/30/2010

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>.

Research Project 27: Project Title and Purpose

Glycosphingolipids and Diabetic Retinopathy – Diabetic retinopathy is the leading cause of blindness among working age adults. We have found that a class of lipids, glycosphingolipids, is elevated in the diabetic retina. The purpose of this study is to understand the consequences of elevated glycosphingolipids within the retina and determine if systemic inhibition of glycosphingolipid accumulation is therapeutic for diabetic complications.

Duration of Project

7/8/2009 - 6/30/2011

Project Overview

The overall objective of this project is to understand the molecular basis for vision impairment in diabetic retinopathy. The specific objective is to investigate the role of ceramide metabolism and glycosphingolipid-enriched lipid microdomains (rafts) in insulin signaling and cell survival in retinal neurons. The rationale for the project is based upon several novel observations, including: 1) diabetic retinas demonstrate decreased total ceramide levels; 2) with a concomitant increase in glucosylceramides concomitant with; 3) decreased insulin receptor signaling. We hypothesize that diabetes increases glycosphingolipid production in the retina, which decreases activation of pro-survival signaling cascades contributing to cell death. The significance of this work is that identifying glycosphingolipids as a therapeutic target in diabetic retinopathy has the potential to lead to pharmacological and molecular interventions to prevent the progression of vision loss in patients with diabetes.

Specific Aim One: Determine the consequence of glycosphingolipids on insulin signaling and cell death in ex vivo retinas. We will assess the effects of altered glycosphingolipid metabolism upon insulin signaling and resultant cell death in retinal explant models. Here, retinal explants from C56BL/6 mice will be treated with inhibitors to glycosphingolipid metabolism and the effect on the phosphorylation of enzymes within the insulin signaling cascade assessed in addition to caspase 3 activity to assess cell death.

Specific Aim Two: Determine the consequence of glucosylceramide synthase inhibition on the metabolic milieu. Our published studies identify glucosylceramide accumulation within lipid rafts as a therapeutic target for diabetic retinopathy. These *in vitro* data support the hypothesis that inhibitors of glucosylceramide synthase may be effective *in vivo* therapeutic modalities. We

will administer drugs via a systemic approach and directly assess clinical endpoints in a mouse model of diabetes. Taken together, these studies will provide novel insights into dysfunctional lipid metabolism in diabetes and may identify glycosylceramide accumulation as a therapeutic target for diabetic retinopathy.

Specific Aim Three: To determine the metabolic profiles of the vitreous samples from patients with/without diabetes. We will expand on the animal models by next assessing human samples. In this aim, we will undertake a comprehensive assessment of vitreous metabolites by liquid chromatography-mass spectrometry (LC-MS/MS)-based metabolomics approaches. These experiments will identify and monitor soluble non-lipid metabolites that are altered in the vitreous from patients (1) without diabetes, (2) with diabetes, but without proliferative diabetic retinopathy, (3) with proliferative diabetic retinopathy, and (4) with macular edema. Through the analysis of this diverse subset of patients, our objective is to discover factors that change with diabetes and between different severities of diabetic retinopathy.

Specific Aim Four: To determine the lipid profiles of the vitreous samples from patients with/without diabetes. In this aim, we will undertake a comprehensive assessment of vitreous lipids by liquid chromatography-mass spectrometry (LC-MS/MS)-based and infusion-based mass spectroscopy lipidomic methodologies. Using the same vitreous samples as above, the end results of these two aims will be the delineation of the vitreous “metabolome” that will lead to new hypothesis driven projects toward new therapies for diabetic retinopathy.

Principal Investigator

Mark Kester, PhD
Professor of Pharmacology
Pennsylvania State University
500 University Drive
Hershey, PA 17033

Other Participating Researchers

Todd E. Fox, PhD, Megan M. Young, BS – employed by Pennsylvania State University

Expected Research Outcomes and Benefits

Diabetes is a debilitating chronic disease that has no cure and can only be managed by pharmaceutical or nutritional interventions. Worldwide, the incidence of diabetes and diabetic complications is dramatically increasing. This may reflect the incomplete knowledge base underlying the role of inflammatory or nutritional stresses to exacerbate diabetic complications. Despite the knowledge that hyperlipidemia is a cardinal feature of both type 1 and 2 diabetes, the actual lipid species that contribute to complications such as diabetic retinopathy, have not been well defined, or have not elucidated new treatment strategies. Sphingolipids comprise only a fraction of total lipids but a body of evidence has now identified dysfunctional sphingolipid metabolism and/or generation of specific sphingolipid metabolites as contributors to diabetic complications, including retinopathy. Preliminary data suggest that pharmacological therapies

that target dysfunctional sphingolipid metabolism and/or signaling may prove beneficial in decreasing the chronic pathology of retinal disease in diabetes. The project will further investigate these treatment options that may prove beneficial in ameliorating or delaying retinal dysfunction in diabetic patients.

Summary of Research Completed

In the past year, two publications have resulted from efforts made on this project:

1. Fox TE, Young MM, Pedersen MM, Giambuzzi-Tussey S, Kester M, Gardner TW. *Insulin signaling in retinal neurons is regulated within cholesterol-enriched membrane microdomains.* Am J Physiol Endocrinol Metab. 2011 Mar;300(3):E600-9.
2. Fox TE, Bewley MC, Unrath KA, Pedersen MM, Anderson RE, Jung DY, Jefferson LS, Kim JK, Bronson SK, Flanagan JM, Kester M. *Circulating sphingolipid biomarkers in models of type 1 diabetes.* J Lipid Res. 2011 Mar;52(3):509-17.

These two publications serve to delineate the role of lipids in diabetic retinopathy. In paper 1, we show that lipid microdomains, which are enriched in sphingolipids and glycosphingolipids, are critical mediators to insulin signaling. Specifically, we show that these lipid microdomains are necessary for insulin-stimulated insulin receptors and downstream signaling. We also demonstrate that Protein Kinase C-inhibition of insulin signaling occurs downstream of the insulin receptor through the impairment of the translocation of the pro-survival kinase Akt to these lipid microdomains.

In paper 2, we further defined the sphingolipid milieu for diabetes by examining the sphingolipid content of the plasma, as well as the liver and heart. Briefly, we demonstrate two overarching events. First, we demonstrate that circulating sphingosine-1-phosphate is elevated in two distinct models of type 1 diabetes. Secondly, we demonstrate that an omega-9 fatty acid, nervonic acid, is diminished within the sphingolipid class of lipids. Further studies are underway to delineate what reductions of nervonic acid may mean for diabetes and diabetic complications such as retinopathy.

Further defining roles of glucosylceramide synthase:

We have expanded several of our glucosylceramide synthase (GCS) studies to explore potentially new roles within retinal cell types. As inflammation is an underlying event for diabetic retinopathy, we first assessed the contribution of glycosphingolipids within a microglia cell line, BV-2. The specific objective was to assess a role for glycosphingolipids on NFκB signaling. Initially, we utilized a GCS inhibitor, AMP-Deoxynojirimycin (AMP-DNM), to determine the effect on lipopolysaccharide (LPS)-induced NFκB activation. AMP-DNM demonstrated a significant reduction in NFκB activity. To attempt to confirm this, we utilized an siRNA approach to diminish GCS expression. Despite sufficient knockdown of GCS expression, we were not able to observe the same result we observed with AMP-DNM. The AMP-DNM does have a reported off-target effect of inhibiting glucosylceramidase, the enzyme that breaks down inhibitors yielded the same response as AMP-DNM. Thus, we conclude that LPS-induced activation of NFκB is not regulated by GCS. The lack of confirmation of the data obtained with

AMP-DNM (despite reducing glycosphingolipid content) by other inhibitors and glucosylceramide to form ceramide. Targeted knockdown of this protein with siRNA also did not confirm the results of our AMP-DNM studies. We also utilized two other inhibitors of GCS, NB-DGJ and PPMP. NB-DGJ is structurally related to AMP-DNM, whereas PPMP is a structurally distinct inhibitor. Neither of these molecular approaches must be considered when interpreting publications that have shown this inhibitor to reduce several phenotypes such as plasma cholesterol, atherosclerosis, and hepatic steatosis.

We have also begun studies to obtain a better understanding of GCS in diabetes and normal retinal physiology. These studies will take advantage of a *LoxP*-flanked GCS gene, which will allow us to selectively eliminate GCS expression in target tissues. Our initial goals are to selectively knockout GCS expression in endothelial cells (Cre recombinase under the control of a *Cdh5* promoter) to assess the influence on vascular permeability and/or leukocyte adhesion within the retinal vasculature. Similarly, retinal neuron-specific knockout (Cre recombinase under control of *Nes* promoter) will also be utilized. Glial cell knockouts are also a possibility, but is beyond our present scope. These approaches will allow for more targeted hypothesis testing of the roles of GCS than a systemic pharmacological inhibition approach. Presently, we are still establishing our mouse colony and are seeking funding to address these roles of GCS.

Metabolomics/Lipidomics for diabetes and diabetic complications:

In our efforts to delineate the underlying biochemical changes and identify novel therapeutic targets, we have continued to utilize lipidomic and metabolomic approaches to quantify diabetes-induced changes. Unbiased/untargeted retina profiling has revealed several alterations of metabolites. The alterations that contribute the most to the differences between control and diabetic retinas are diabetes-induced elevation of glycerophosphocholine (GPC, a product of phosphatidylcholine breakdown), the branched-chain amino acids leucine and isoleucine, and the polyols, sorbitol and fructose. Additional metabolites that are altered include pantothenate, sialic acid, glutamine and glutathione. These metabolites are involved in lipid metabolism, glycosylation of lipids and prevention of oxidative damage, respectively. The sialic acid pathway is being examined further for our glycosphingolipid studies. The product of GCS can be further metabolized, which can include the addition of sialic acid to form gangliosides.

Though our focus is the retina, we do remove other tissues from our animal studies to maximize the data that we can obtain. An undergraduate student joined our laboratory for a few months and helped us extend our retina studies to measure lipid changes between control and diabetic kidney samples. The “gestalt” of these data is that there is an upregulation of several phosphatidylcholine-, phosphatidylethanolamine-, and triglyceride-species containing saturated palmitic and stearic acids and polyunsaturated docosahexanoic acid (DHA) in diabetics. The increase in the polyunsaturated DHA is intriguing as the retina behaves in the opposite manner, with diabetes reducing DHA-containing lipids. Consistent with our recently published paper on reduced nervonic acid-containing sphingolipids in the plasma, heart and liver of a type 1 diabetic model, we also show that nervonic acid containing sphingomyelin is diminished in the kidney. Interestingly, we also saw an increase in a sphingomyelin that contains a d18:2 backbone. This subtype of sphingolipids is poorly explored in the field and demonstrates the importance of “omic-” based approaches to shed light on metabolites that are typically overlooked. We have also started collecting samples to be able to expand our studies once we obtain sufficient

biological replicates based on patient parameters. These studies will be expanded to encompass a broader spectrum of lipids to obtain a better understanding of diabetes-induced alterations to lipid metabolism.

Research Project 28: Project Title and Purpose

Epithelial/Dendritic Cell Crosstalk in Acute Kidney Injury – The purpose of these studies is to evaluate the interactions between cells of the immune system and kidney cells in causing drug-induced damage in the kidney.

Duration of Project

7/8/2009 - 6/30/2010

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>.

Research Project 29: Project Title and Purpose

Synergistically Acting Targeted Therapeutics for Melanoma – Malignant melanoma remains the most deadly skin cancer with no effective drugs available for the long-term treatment of patients. The melanoma community believes that targeting Akt3, ^{V600E}B-Raf and other key kinases deregulated in melanoma is needed to effectively treat this disease. However, the identity of kinases to target using siRNA for synergistically acting tumor inhibition remains unknown and delivery of siRNA in animals remains a challenge. Therefore, the purpose of this project is to develop therapeutic agents inhibiting proteins deregulated in melanoma to shrink tumors in a synergistically acting manner. To accomplish this nanoliposomes containing siRNA designed to target ^{V600E}B-Raf, Akt3 and other key kinases in melanoma will be developed and efficacy for synergistically inhibiting melanoma development in skin and animals evaluated. Accomplishing these objectives not only identifies key kinases to synergistically inhibit melanoma development but also validates the utility of siRNA-loaded liposomes for treating melanoma patients.

Duration of Project

7/8/2009 - 6/30/2010

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>.

Research Project 30: Project Title and Purpose

Diabetic Changes in Contractile Proteins and Contractility in Arterial versus Venous Grafts – Coronary artery disease is the leading cause of death and disability in patients with diabetes mellitus. Because of this, more than 300,000 patients with diabetes undergo coronary revascularization procedures annually in the U.S. to clear coronary blockages. Unfortunately, the benefit of percutaneous coronary intervention in diabetic patients is limited by their tendency to develop coronary restenosis by nearly 2- to 4-fold compared with nondiabetic patients. To date, the extent of alterations in contractile protein levels and the contractile responses in arterial *vs.* venous grafts remain unclear. Hence, the current project will compare diabetic *vs.* nondiabetic alterations in contractile proteins that regulate smooth muscle contractility in arterial and venous grafts, which will be obtained from patients undergoing coronary artery bypass grafting surgery.

Anticipated Duration of Project

9/1/2009 - 12/31/2011

Project Overview

Broad Research Objectives and Specific Research Aims: The overall goal of this project is to gain new understanding into the contractile properties of arterial *vs.* venous grafts. The specific objective is to investigate the relationship between contractile protein levels and the contractile responses in arterial *vs.* venous grafts in diabetic patients. Saphenous vein grafts have been shown to have worse coronary outcomes compared with left internal mammary artery (LIMA) grafts. We hypothesize that contractile proteins and/or contractile responses in vein grafts are compromised to a greater extent compared with arterial grafts. The Specific Aim of this project will determine diabetic and nondiabetic changes in contractile protein levels and contractile responses using saphenous vein and LIMA tissues from patients undergoing coronary artery bypass grafting surgery (CABG).

Research Design and Methods: Saphenous vein tissues (leftover segments) will be obtained from diabetic (n = 6) and age-matched nondiabetic (n = 6) patients undergoing CABG surgery. In addition, LIMA tissues (leftover segments) will be obtained from diabetic (n = 6) and age-matched nondiabetic (n = 6) patients undergoing CABG surgery. Saphenous vein and LIMA specimens from diabetic and nondiabetic subjects will be cleansed free of blood, adherent fat, and connective tissues. A portion of the tissue will be flash frozen in liquid nitrogen and stored at -80° C until analyses (for protein analysis and contractile protein quantification by immunoblotting). Immunoblot analysis will be performed with the total tissue lysates using primary antibodies specific for SM α -actin, smooth muscle myosin heavy chain, calponin, and caldesmon. For *ex vivo* contractility studies, ring preparations of tissues will be immersed in Krebs-Henseleit solution (pH 7.4) that is gassed with 95% O₂ and 5% CO₂. Contractility studies (isometric tension measurements) with saphenous vein and LIMA will be performed using the myograph chamber. We will determine the contractile responses to cumulative concentrations of serotonin (1 nM to 10 μ M) in the respective ring preparations.

Principal Investigator

Lakshman Sandirasegarane, PhD
Assistant Professor
Penn State College of Medicine
500 University Drive
Hershey, PA 17033

Other Participating Researchers

Mark Kozak, MD, Ann Ouyang, MD – employed by Penn State College of Medicine

Expected Research Outcomes and Benefits

Expected outcomes: From the studies described in the current research project, it is expected that diabetes would decrease the expression of one or more contractile proteins in the blood vessel wall. In this regard, the decreases in contractile proteins would be much greater in saphenous vein grafts compared with left internal mammary artery (LIMA) grafts. Functionally, diabetic decreases in contractile protein expression will be reflected by corresponding alterations in the contractile responses in the blood vessels.

Benefits: The current project will determine the biochemical and functional abnormalities (contractile protein levels and contractile responses) in arterial and venous blood vessel specimens from both diabetic and nondiabetic patients. This will provide a better understanding of blood vessel wall contractile disorder and restenosis in diabetic vs. nondiabetic patients.

Summary of Research Completed

Total number of human subjects enrolled in the study: In the proposed research project, we stated that we would include 6 diabetic subjects and 6 age-matched nondiabetic subjects to compare the contractile protein expression profile and contractile responses between arterial and venous grafts. With the support provided by Dr. Mark Kozak (Collaborator), we recruited 7 subjects for the studies. Of these 7 subjects, six of them were nondiabetic and one was diabetic. The recruited subjects underwent either multivessel or single vessel bypass surgery. From subjects with multi-vessel coronary artery disease, we were able to collect both left internal mammary artery (LIMA) and saphenous vein (SV) segments. This approach would allow us to compare the data between LIMA and SV segments (obtained from the same subject) and would therefore control for inter-patient variations in the results.

Table 1 shows the processing of LIMA and SV tissue segments for the chosen experimental methods, which include immunohistochemistry (IHC), western blot (WB) analysis, and quantitative real-time PCR (qPCR). LIMA and SV tissue segments from nondiabetic and diabetic subjects were cleansed free of blood, adherent fat, and connective tissues using ice-cold PBS. To assess the changes in contractile protein expression in the medial layer, 2-3 mm long vessel segments were fixed in 10% formalin solution (neutral buffered; Sigma) for histochemical and immunohistochemical analysis. To assess the changes in contractile protein expression in

the whole tissue, 40-50 mg of tissue were flash frozen in liquid nitrogen and stored at -80° C until analyses for western blotting. To assess the changes in contractile protein expression at the mRNA level, 25-30 mg tissue was added to RNA stabilization agent and then stored at -80° C until analyses for quantitative real-time PCR.

Immunohistochemistry (IHC): In addition to using whole tissue homogenates for western blot analysis of contractile proteins, we have also prepared formalin-fixed paraffin-embedded tissue sections for immunohistochemical localization of contractile proteins. These studies will further confirm diabetic changes in contractile protein expression in the medial layer of arterial and venous grafts.

Western blot (WB) analysis: For whole tissue protein extraction, 50-60 mg of LIMA or SV tissue segments were homogenized with four volumes of HES buffer (20 mM HEPES, 1 mM EDTA and 250 mM sucrose, pH 7.4) containing protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Homogenization was performed using Tissue Lyser LT (Qiagen) at a setting of 40 Hz for 3 minutes. The homogenized tissues were mixed with urea solubilization buffer (8M urea, 50 mM Tris, 5% SDS and 350 mM DTT) at a fixed ratio of 2.5:1. The above mixture was allowed to stand at room temperature for 1-2 hours with intermittent vortexing and sonication to ensure complete solubilization of proteins. The samples were then centrifuged at 600xg for 10 min to remove tissue debris. The protein concentration in the supernatants was determined using 2-D Quant kit (GE health care).

The supernatants (10-15 µg protein each) of LIMA and SV tissue homogenates were electrophoresed using pre-cast 4-12% NuPage mini-gels (Invitrogen), and the resolved proteins were transferred to nitrocellulose membranes (Hybond C, GE Healthcare). The membranes were blocked in 5% nonfat milk, and probed with the primary antibodies specific for SM α -actin and calponin. After extensive washes, the immunoreactivity was detected using specific HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham Biosciences). Figure 1 shows the western blot analysis of contractile proteins such as SM α -actin and calponin in SV and LIMA segments. The primary antibody used for detection of myosin heavy chain was not specific for human tissues. To address this point, we have also collected tissue samples for qPCR analysis of contractile proteins at the mRNA level.

Quantitative real-time PCR (qPCR): We have optimized the conditions for RNA extraction, cDNA synthesis, and qPCR analysis using Applied Biosystems 7900HT. To date, the samples for qPCR analysis have been collected from 4-6 nondiabetic subjects and 1 diabetic subject. We will compare the mRNA expression profile of contractile proteins between LIMA and SV grafts after obtaining an n value of at least 6 for nondiabetic and diabetic subjects.

Contractility studies: We have optimized the conditions for isometric tension measurements to compare serotonin-induced changes in contractile responses between LIMA and SV ring preparations. The proposed inclusion of additional subjects would provide sufficient arterial and venous segments for statistical analysis of the contractility data.

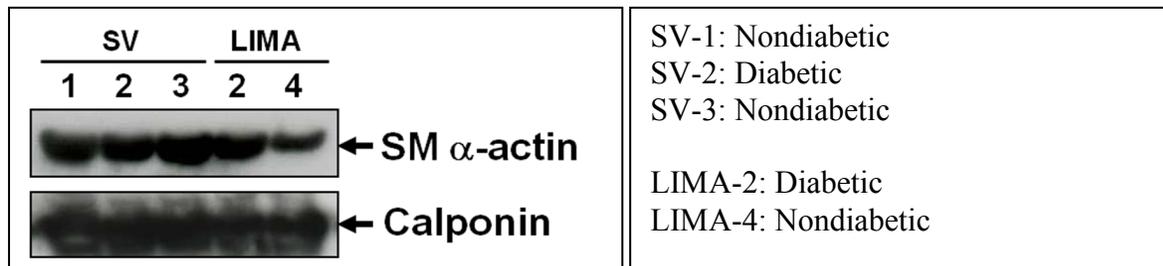
Table 1. Processing of human vascular tissues (LIMA and SV) for immunohistochemistry (IHC), western blot (WB) analysis, and quantitative real-time PCR (qPCR).

	Human subjects	LIMA			SV		
		IHC	WB	qPCR	IHC	WB	qPCR
1	Nondiabetic	√	X	√	√	√	√
2	Diabetic	√	√	√	√	√	√
3	Nondiabetic	X	X	√	√	√	√
4	Nondiabetic	X	√	√	X	X	X
5	Nondiabetic	X	X	√	√	X	√
6	Nondiabetic	√	√	√	√	√	√
7	Nondiabetic	√	X	√	X	X	X

√ denotes the use of tissue segments for IHC, WB, and/or qPCR

X denotes that the tissue segments were insufficient/unavailable for IHC, WB, and/or qPCR

Figure 1. Western blot analysis of contractile proteins in SV and LIMA tissue segments.



Research Project 31: Project Title and Purpose

Autism Indicators: Erythrocyte Membrane Fluidity and/or Lipid Composition – To determine whether fluidity and/or lipid composition of isolated red blood cell membranes differ in children with autism compared to age and gender matched children who are developing normally. If consistent differences are observed, funding to determine whether they are of diagnostic value will be sought.

Duration of Project

7/8/2009 - 5/31/2011

Project Overview

Autism is diagnosed by a set of behaviors most often initially noticed by the parents. While researchers agree that the earlier the diagnosis is made and treatment started, the more likely improvement will be noticed. Children are frequently three years of age or older by the time the diagnosis is able to be made. Recent research has indicated that even though autism affects most

obviously specific areas of the brain, other organ systems may be involved as well. Therefore, abnormalities of membrane function/lipid composition may well be a fundamental difference that could affect neurological functioning and explain the multisystem pathology of autism. For example, a generalized membrane dysfunction could provide a reason for the observation of gastrointestinal malfunction, including diarrhea, in a significant fraction of children with autism. Erythrocytes from children with autism may have membranes that are less “fluid” than those of erythrocytes from normal children. Membrane fluidity reflects its lipid composition. Therefore, the aim of this project is to ascertain whether there is a consistent change in fluidity of membranes isolated from erythrocytes from children with autism relative to that of erythrocytes from age, gender, and geographic residence matched comparison children who are developing typically. If a difference is evident, then the study will determine whether that difference is accompanied by changes in glycerophospholipid (GPL), sphingolipid and/or cholesterol content/composition of the membrane. Fluidity of isolated red blood cell membranes will be measured by monitoring fluorescence depolarization using 1,6-diphenyl-1,3,5-hexatriene as the probe. Lipids will be extracted and aliquots taken for analysis of total lipid phosphorous, amino-glycerophospholipids, and cholesterol and the rest used for sphingolipid isolation and analysis. A significant change in fluidity coupled with a change in lipid content or composition might provide a simple test for predicting at a young age children who are or might become affected with autism.

Principal Investigator

Cara-Lynne Schengrund, PhD
Professor
Penn State College of Medicine
Department of Biochemistry and Molecular Biology H171
500 University Drive
Hershey, PA 17033

Other Participating Researchers

Jeanette C. Ramer, MD – employed by The Milton S. Hershey Medical Center
Fatima Ali-Rahmani - employed by Penn State College of Medicine

Expected Research Outcomes and Benefits

The results of the project should indicate whether the lipid composition of membranes isolated from red blood cells obtained from children diagnosed as having autism differs from those of normally developing, age, gender, and location-matched controls. If a difference is observed it may be possible to 1) develop a simple diagnostic test to identify children with autism and 2) determine what cell function(s) the changes may affect and whether they contribute to the problem.

Summary of Research Completed

Additional analyses of membranes isolated from red blood cells (RBCs) obtained from children

that were diagnosed with autism or from age, gender, and geographic residence matched controls during the first year of this project were kept at -80°C until analyzed for the presence of cholesterol, hematoside [GM3, Sialic acid α 2-3Gal β 1-4Glc β 1-ceramide], monosialoganglioside GM1 [Gal β 1-3GalNAc β 1-4(Sialic acid α 2-3)Gal β 1-4Glc β 1-ceramide] as well as phospholipid composition. These analyses were carried out because anisotropy measurements done on membranes isolated from RBCs from children diagnosed with autism were not significantly different from measurements done on membranes isolated from RBCs from controls (see 2010 annual report), and therefore it was necessary to determine whether differences in specific lipids might offset each other in regards to their effects on membrane fluidity thereby accounting for the similarities observed in anisotropy measurements.

While preliminary data on *cholesterol content* was included in last year's report, the analyses were repeated on those samples plus ones not included in the initial study. This allowed us to analyze all samples under identical conditions. Twenty membrane samples isolated from RBCs of control and 16 membrane samples isolated from RBCs of children diagnosed with autism were analyzed. Each sample analyzed using the Amplex® red cholesterol kit (Invitrogen) contained 0.1mg of protein. The results supported our initial observation that length of time between membrane isolation and analysis did not have a significant effect on results. Significant differences were not observed between samples isolated from RBCs from either males or females, nor were age-related differences seen between samples obtained from 4 to 8 yr olds. Of particular interest was the finding that the null hypothesis (cholesterol content would be similar in the two groups) was rejected ($\sigma=0.0057$) and there was a significant decrease in the average amount of cholesterol found in membranes isolated from RBCs from children diagnosed with autism compared to those isolated from controls (see Figure 1). Gender and age of children from whom samples were used for these assays are given in Table 1. The reduction in cholesterol levels agrees with observations made by Bukelis et al in 2006 indicating that cholesterol levels for 19 of 100 blood samples studied from autistic children (no apparent Smith-Lemli-Opitz Syndrome) were below the 5th percentile for control children over 2 yrs of age.

Determination of GM1 and GM3: In previous work we found that an acute reduction in cholesterol levels of the membranes of N2a murine neuroblastoma cells was accompanied by an increase in expression of the monosialoganglioside GM1 and subsequently that decreases in cholesterol induced by the H63D mutant of the hemochromatosis protein HFE were accompanied by an increase in GM1. Both cholesterol and glycosphingolipids such as GM1 are enriched in lipid rafts, areas of the cell membrane known to function in signal transduction. Therefore, using slot blot analyses, we determined the relative amounts of GM1 and GM3 (a known component of RBC membranes) in membranes of RBCs isolated from both children diagnosed with autism and controls (* next to the age of children indicated in Table 1 indicate samples used in these assays). In each case 2 μg of membrane protein was blotted onto a nitrocellulose membrane according to the manufacturer's (Bio-Rad) directions. Each sample was run in duplicate. Membranes were blocked for 1hr at room temperature using 5% nonfat dried milk in phosphate buffered saline prior to probing overnight at 4°C with horse radish peroxidase conjugate binding subunit of cholera toxin (List Biological Laboratories, Campbell, CA) that binds quite specifically to the carbohydrate moiety of GM1, or a monoclonal anti-GM3 antibody (Associates of Cape Cod, Inc.). Both were applied in PBS containing 2% nonfat dried milk. Blots for identification of GM3 were then exposed to a horse radish peroxidase conjugated

secondary antibody and for both GM3 and GM1 bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate™ and exposure in a Fuji Film LAS 3000. GM3 blots were then stripped using Restore™ western blot stripping buffer prior to probing with an anti-actin monoclonal antibody (Abcam). After exposure to a HRP-conjugated goat anti-mouse secondary antibody bands were visualized as described above. While the sample size was limited, the results from slot blot analyses indicate that there was on the average significantly more ($p < 0.05 > 0.01$, 2-tailed t-test) GM1 (Fig. 2) in the membranes of RBCs isolated from children with autism than controls while that of GM3 (Fig. 3) was reduced ($p < 0.005 > 0.001$, 2-tailed t-test). The observed decrease in cholesterol coupled with the increase in expression of GM1 in RBC membranes from children diagnosed with autism, is in agreement with our previous observations that loss of membrane cholesterol appears to be accompanied by an increase in GM1.

Additional studies were carried out to ascertain whether significant differences might exist in the phospholipid composition of membranes isolated from RBCs from autistic children relative to those from controls. Phospholipids were extracted using the procedure provided by Dr. X. Han, as described in the first year annual report. Samples were then given to Dr. T. Fox for analysis using core facilities in the College of Medicine. Analysis of this data plus that reported in the year one annual report did not show any significant difference in the relative amounts of phosphatidylethanolamine and phosphatidylserine present in samples from autistic children relative to those from controls. Due to shifting the analyses from the lab of Dr. Han to that of Dr. Fox, it was necessary to repeat some samples in order to confirm reproducibility of results. Therefore only six different samples from children with autism and four from controls were analyzed.

In conclusion, our results indicate that while changes in lipid composition seen for membranes isolated from RBCs from children diagnosed with autism did not affect membrane anisotropy (yr. 1 report), there was a significant difference in the average cholesterol and GM1 content of the membranes with the decrease in cholesterol in those from autistic children accompanied by an increase in GM1. This change agrees with previous findings that a significant decrease in membrane cholesterol was accompanied by a significant increase in GM1 in neuroblastoma cells. Cholesterol has been reported to affect the oxytocin receptor, which when absent in mice resulted in aberrant social behaviors, obesity, and lack of body temperature control, and the G-protein coupling of the serotonin_{1A} receptor which functions during development to establish anxiety-like behavior. The fact that GM1 is associated with proteins involved in signal transduction [e.g.: cAMP kinase, and mitogen-activated protein kinase] also supports the hypothesis that these changes may be indicative of lipid-induced changes in signal transduction not only in RBCs but in other cells, including those in the central nervous system.

Autism samples		Controls	
Gender	Age	Gender	Age
M	3*	M	3*
M	3*	F	3
M	3	M	4
M	4*	F	4*
M	4*	F	5*
M	4	F	5*
M	5*	F	6*
M	5	F	6*
F	6*	F	6
M	6*	M	6
M	6*	M	6
M	6*	M	7*
M	6*	M	7*
M	6	M	7
M	7*	F	7*
M	8*	F	7
		F	8*
		F	8*
		F	8
		M	8

Table 1: Ages and sex of children from whom RBC samples were obtained for analyses of membrane-associated cholesterol.

Autism score	Cholesterol
30	14.7
53	15.2
39	16.37
41	15.13
49	10.47
36	10.64
NA	11.48
33	9.52
45	8.16
44	8.84
31	5.08
38	13.3
38	9.33
44	6.75
44	17.78
34	14.21

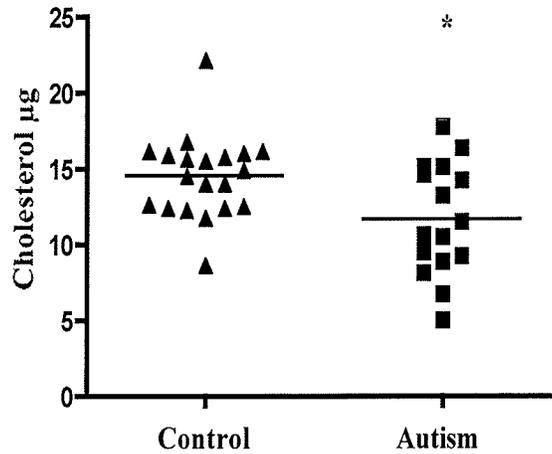


Figure 1: Cholesterol found in RBC membrane samples containing 0.1mg of protein. Cholesterol values are listed next to autism scores of the children from whom samples were obtained. No consistent trend between cholesterol content and autism score was noted. The graphed data indicates that on the average samples from children with autism had significantly less cholesterol than those from controls ($p < 0.05 > 0.01$, two-tailed student t-test).

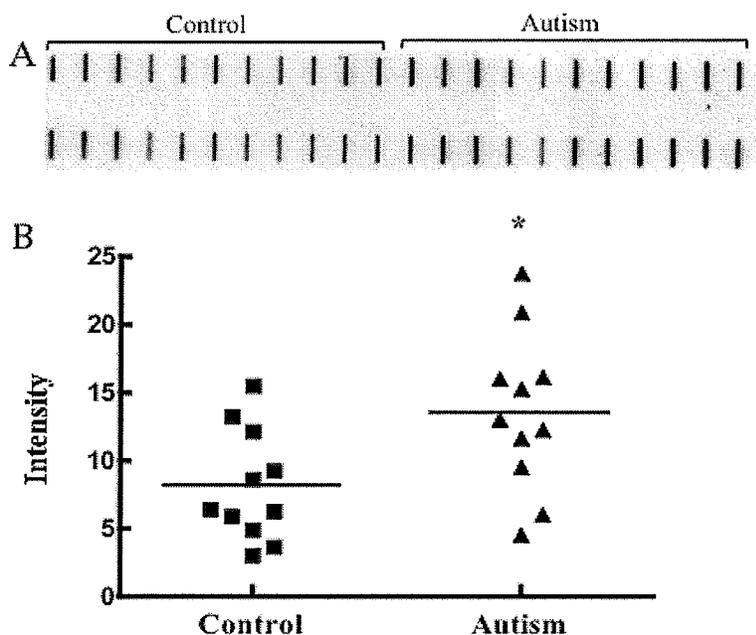


Figure 2: Relative amount of horse radish peroxidase conjugated cholera toxin binding subunit bound to slot blots of RBC membranes isolated from the blood of children with autism relative to those from controls. A) Duplicate blots were prepared using samples of red blood cell membranes containing 2 μ g of protein isolated from blood obtained from eleven different children with autism and a similar number of samples of blood from controls. GM1 was detected using HRP-conjugated binding subunit of cholera toxin and bands visualized using Super Signal West Femto Maximum Sensitivity Substrate™ and exposure in a Fuji Film LAS 3000. B) Intensity is given as the (average arbitrary units obtained for duplicates of each sample minus the arbitrary units for the background) X 10⁻⁵. Analysis of intensity of the bands indicated that on the average samples from children with autism had significantly more GM1 than those from controls (*, $p < 0.05 > 0.01$, two-tailed student t-test).

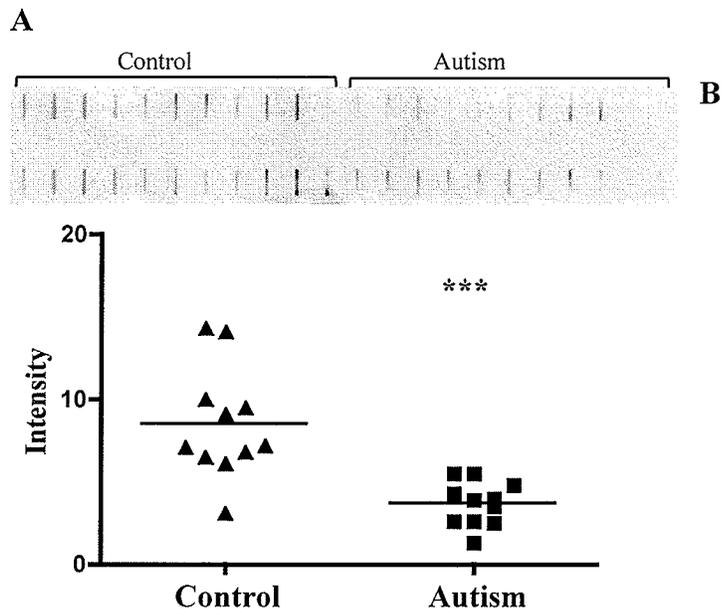


Figure 3: Relative amount of GM3 antibody staining of A) slot blots of RBC membranes isolated from the blood of children with autism relative to that from controls. Duplicate blots were prepared using samples of red blood cell membranes containing 2g of protein each isolated from blood obtained from eleven different children with autism and a similar number of samples of blood from controls. B) Analysis of intensity of the bands indicated that on the average samples from children with autism had significantly less GM3 than those from controls ($p < 0.005 > 0.001$, two-tailed student t-test).

Research Project 32: Project Title and Purpose

Efficacy of Gemcitabine for Pancreatic Cancer: Role of DNA Polymerases – Pancreatic ductal adenocarcinoma is a very aggressive malignancy that is treated by gemcitabine. Its efficacy is thought to be due to inhibition of DNA synthesis. It is our hypothesis that newly discovered DNA polymerases modulate gemcitabine toxicity and thereby decrease its efficacy. The purpose of the present experiments is to test the hypothesis that inter-individual differences in the levels of these polymerases affect incorporation of gemcitabine into the DNA and its efficacy in pancreatic cancer patients receiving gemcitabine.

Anticipated Duration of Project

7/8/2009 - 6/30/2012

Project Overview

The nucleotide analog, gemcitabine, a promising agent for a variety of cancers, is the most active chemotherapy agent for metastatic pancreatic cancer. Its efficacy is thought to be due to inhibition of DNA synthesis by stalling the DNA replication fork. However, it is our central hypothesis that newly discovered DNA polymerases (κ , ι , η , and ζ) can rescue the stalled

hypothesis that newly discovered DNA polymerases (κ , ι , η , and ζ) can rescue the stalled replication forks and incorporate gemcitabine into the DNA and consequently modulate gemcitabine toxicity and efficacy. The long-term goal of this research is to more fully understand the anti-cancer mechanisms of gemcitabine so that we can design a more effective treatment protocol and/or identify which patients will be more likely to respond to gemcitabine treatment. In the present experiments we plan to test the hypothesis that inter-individual differences in the levels of these polymerases affect incorporation of gemcitabine into the DNA and its efficacy. Specifically we will isolate white blood cells from patients who received gemcitabine. From these cells we will measure the levels of DNA polymerases δ , κ , ι , η , and ζ . In addition, we will measure the amount of gemcitabine incorporated into the DNA by an HPLC-MS/MS method, and after two months of treatment, determine the efficacy of the treatment. Upon successful completion of this research, we will have discovered whether levels of DNA polymerases δ , κ , ι , η , and ζ can modulate gemcitabine efficacy and determine whether the measurement of gemcitabine incorporation into the DNA of lymphocytes is a biomarker of efficacy of treatment.

Principal Investigator

Thomas E Spratt, PhD
Associate Professor
Pennsylvania State University
500 University Dr
H171
Hershey, PA 17033

Other Participating Researchers

Yixing Jiang, MD, PhD, Kristin Eckert, PhD, Kwangmi Ahn, PhD, AS Prakasha Gowda, PhD – employed by Pennsylvania State University

Expected Research Outcomes and Benefits

Gemcitabine, while a potent anti-cancer drug, is successful in only a minority of cases. The effectiveness of gemcitabine is believed to be due to the ability of gemcitabine to become incorporated into the DNA and then inhibit DNA synthesis. Our hypothesis is that individuals differ in their ability to incorporate gemcitabine into their DNA and that these differences lead to differences in efficacy. We expect to determine if the ability of an individual to incorporate gemcitabine into the DNA influences the effectiveness of the therapy. If true, then we would have designed a simple diagnostic test to determine whether gemcitabine therapy would be effective in an individual.

Summary of Research Completed

During the past year, we have encountered a serious problem in recruiting eligible patients for this study. We had originally estimated that there would be a pool of approximately 50 eligible pancreatic cancer patients who would receive gemcitabine. Unfortunately, we recruited only

one patient into the study during the past year.

To review the recruitment problem, we had a meeting in early December of 2010 including Thomas Spratt (PI), Dr Yixing Jiang (co-PI), Dr Ernest Johnson (Director for Research Development), Terry Novchich (Director of the Hershey Medical Center Clinical Trials Office), and Cynthia Naret (Director of the Penn State Cancer Center Clinical Trials Office). In response, the group recommended three changes in the protocol, as follows:

1. Include patients with breast and lung cancers, two other cancers that are treated with gemcitabine. We assumed that the mechanism of action of gemcitabine would be the same for each cancer, so that the original hypothesis and methodology would still be appropriate.
2. Include patients who receive drugs in addition to gemcitabine. Since the writing of the original grant proposal, there has been a change in the standard of care in which gemcitabine is given in combination with other agents. Since we would not predict that these agents would change the mechanism of action of gemcitabine, we anticipate that the original hypotheses would remain valid.
3. Include patients who are not on their original dosing of gemcitabine. Patients are typically given gemcitabine once a month for several months. Since the level of gemcitabine in the DNA is expected to be reduced to background levels over the month, we anticipate that this change would not affect our protocol.

With these protocol changes, the number of eligible patients increased. From February to June, we identified approximately 15 patients who met the revised eligibility criteria. However, none of these patients consented to participate in the study.

We recently had another meeting to further review the recruitment experience and discuss ways to increase enrollment. The group identified two problems that need to be addressed, as follows:

First, patients do not want to stay at the clinic for 6 hours and then return the next day to donate blood. They feel ill and do not want to be bothered with waiting around.

To address this problem we decided to alter the protocol, to obtain one blood sample prior to and one immediately after gemcitabine administration. We believe that the patients will be more receptive to this protocol since they do not have to stay at the hospital any longer than necessary and return again the following day.

The pre-gemcitabine blood sample will be used to isolate lymphocytes and treat them in vitro with gemcitabine. We will then follow the incorporation of the gemcitabine into the cells over time. The post-gemcitabine treatment sample will be treated in the normal way and we will determine the levels of gemcitabine in the DNA. Currently a new protocol that incorporates these changes is being prepared for submission to the IRB.

A second conclusion at this meeting was that the PI must participate more actively in communicating with the physicians and nurses in the clinic regarding recruitment of lung cancer

and breast cancer patients into the study, and the PI agreed to do this.

We anticipate that the changes summarized above will increase enrollment of patients into the study during the coming year.

Research Project 33: Project Title and Purpose

Embedded Rural Clinical Research Infrastructure: Utilization of Community Based Nurses and Paramedics – This project establishes the foundation for a research and community health approach that will be applicable to rural Americans nationwide. Rural Pennsylvanians are an underserved and aging population who suffer from an increased rate of elder falls and poor rates of influenza immunization. This project will deploy an innovative strategy to engage this community by using Penn State Cooperative Extension agents working together with newly trained and embedded health assessment staff. Embedded nurses and emergency medical technicians may offer an effective solution to furthering community assessment, improving important health measures, and creating new employment opportunities for these providers. Home safety assessment recommendations will be collected and recommendations for aging in place re-construction will be made with preferred local contractors.

Anticipated Duration of Project

7/8/2009 - 6/30/2012

Project Overview

This project will focus on establishing a Rural Embedded Assessment Community Health (REACH) Network of research coordinators in central Pennsylvania to meet the health care needs of the elderly. Objectives & Specific Aims: This study will test the hypothesis that in-home interventions to make the home safer will reduce the risk of falls and fall-related injuries compared to a control group not receiving the intervention. Embedded central Pennsylvania (PA) regional registered nurses (RN) and emergency medical technicians and paramedics (EMT) can successfully complete training and maintain adequate competency in the good clinical practice of research (GCPR), as established by the World Health Organization. Elder fall prevention and influenza immunization will serve as initial targets of our rural community health improvement efforts. Design & Methods: Distance learning programs delivered via the Penn State University (PSU) will achieve an adequate level of competency on appropriate outcome metrics in these RN&EMT populations in central PA. The density of GCPR graduates within the central PA region will allow representation in targeted central PA communities at a proportion of 1:2,500 population. Community participation to achieve a sample size of 25 elders will be considered as a threshold for participation. Continuing education units will be offered as an incentive to improve student enrollment in the program and coordination with county extension services will be performed. Additionally, selected graduates with a community commitment and excellent skills will be offered employment opportunity in representing their respective communities in this clinical research network. Facilitation will be carried out using computer desk-top based, web-conferencing tools to reduce travel, expense and enhance participation. Initial communities with strong commitment and agreeable participants will be selected for pilot programs, with

regional expansion in future years. Generalizeability for rural and small towns will be assessed. Expected Outcomes: Groups of embedded trained staff will work to identify community needs and engagement by holding standardized meetings focused on eliciting community perception on health needs, coordinating with county extension office staff. Trained community research staff from the embedded pool will work with local community leaders in identifying acceptable methods to improve fall risk and immunization of elder citizens. Outcome measures include percentage of elder homes assessed and approval recommendations and actual modifications accepted by elders. Before and after proportions of community based elders receiving influenza vaccination will be assessed.

Principal Investigator

Thomas E. Terndrup, MD
Associate Dean for Clinical Research
Emergency Medicine
Penn State College of Medicine
500 University Drive
Hershey, PA 17033

Other Participating Researchers

Ted Alter, PhD, Marilyn Corbin, PhD, Gregory Olsen, PhD, MLA – employed by Pennsylvania State University
Claire Flaherty-Craig, PhD - employed by Penn State College of Medicine

Expected Research Outcomes and Benefits

The REACH Network would capitalize on the experience and expertise of nurses and emergency medical technicians (nurse/EMTs) who are part of the social fabric of rural communities, and team these workers with Penn State University cooperative extension staff to perform detailed assessments in the homes of elderly individuals. After completing training, the nurse/EMTs will conduct a safety analysis of living conditions in the home, complete a neurocognitive questionnaire, and make recommendations for preventive health care (eg, immunizations), using an innovative mobile data acquisition device for facilitated and enhanced site data input. This approach should reduce the incidence of fall-related injuries and increase the immunization rates compared with elders who do not receiving this intervention. Our transformational approach to community engaged research will address the challenges of a lack of understanding of local health needs, poor community trust in research, and the absence of trained research personnel domiciling within those communities.

Summary of Research Completed

The REACH Network Community Advisory Board met July, 20, September 14, October 19, and November 16, 2010, as well as January 20, March 25, April 21, and May 19, 2011.

The REACH Network utilized the services of six research-trained Community Health Assistants

(CHAs) during the reporting period, including two local emergency medical technicians (EMTs), three local nurses, and the research coordinator, Leigh Gordon Brown. The CHAs were charged with maintaining cohorts of at least 25 participants and conducting initial and follow-up home visits with participants in accordance with the project timeline.

As of June 30, 2011, 223 participants have been recruited. A total of 159 baseline questionnaires have been completed, as well as 110 timed get up and go tests and mini-cog assessments. CHAs have reported three withdrawals from the program due to death. One participant officially withdrew in writing due to moving. CHAs also reported that eight participants have expressed an intent to withdraw, but have not submitted written notice of withdrawal, as per instructions in the consent form.

Three-month follow-ups conducted between August and December 2010 revealed that 9.75% of participants reported falling in the three months after the initial home visit. Three of the four participants who reported falling also reported at least one fall that caused an injury.

In August 2010, CHAs began six-month follow-ups with participants who received the HomeFast Safety Assessment. The six-month follow-ups involved a personalized questionnaire based on the recommendations made in the brochure given to participants after the assessment. 72 six-month follow-ups were completed as of June 30, 2011. Preliminary data showed that most participants reported making at least some of the recommended changes.

In October 2010, the second phase of the project commenced with the seasonal influenza vaccine survey. CHAs began their visits with participants who were randomized to receive the Seasonal Influenza Vaccine Survey. 41 surveys have been completed. 85% of participants reported receiving an influenza vaccination and 91% of those reported receiving the vaccination from their primary doctor. 39% reported having influenza at some time in their lives.

During this reporting period, the REACH Network also conducted numerous outreach activities. In August 2010, project coordinator Leigh Gordon Brown and CHA Kimbra Shoop attended the Senior Expo at the Lewistown Community Center to present information about REACH and recruit new participants, while Dr. David Schooley, Advisory Board Co-Chair, and Leigh Gordon Brown spoke about REACH to Family Health Associates and the Geisinger Family Health Physicians at their staff meetings. REACH was featured in an article published in the Fall 2010 edition of PSU Outreach magazine. Dr. Schooley and Leigh Gordon Brown also met with Senator Jake Corman regarding the REACH Network and opportunities to secure funding to sustain the project in February 2011.

Presentations and poster sessions conducted during the reporting period included the following: In September 2010, Dr. Schooley gave a presentation on REACH at a meeting of the Lewistown Hospital medical staff. Project coordinator Leigh Gordon Brown provided a brief presentation about REACH for the Mifflin County Cooperative Extension Annual Meeting on November 1, 2010. In June 2011, Dr. Marilyn Corbin also presented a poster about REACH at the Healthy Homes National Conference in Denver, CO.

REACH collaborators Dr. Louis D. Brown and Dr. Theodore Alter submitted a first person

account article to the *American Journal of Community Psychology*. In May 2011, reviewers requested revision and resubmission of the article by July 5, 2011. Revisions were in process at the conclusion of this reporting period.

Research Project 34: Project Title and Purpose

Cytoadherence in Maternal Malaria – Infection by malaria parasite *Plasmodium falciparum* during pregnancy, especially during the first pregnancy, results in several pathological conditions, including low birth weight, spontaneous abortion, still birth, and maternal morbidity and mortality. The pathology is caused by the accumulation of parasite-infected red blood cells in the placenta by the binding of a parasite adhesive protein expressed on the infected red blood cell surface to chondroitin 4-sulfate receptor in the placenta. Knowledge of the structural interactions involved in this adherence process will be valuable for the development of a vaccine and/or therapeutics for pregnancy-associated malaria.

Anticipated Duration of Project

7/20/2010 – 8/31/2011

Project Overview

The overall objective of this research project is to understand the structural interactions involved in the binding of *Plasmodium falciparum* erythrocyte binding protein 1 called VAR2CSA, expressed on the surface of *P. falciparum*-infected red blood cells (IRBCs), to chondroitin 4-sulfate (C4S) in human placenta. The information gained will be valuable in developing a vaccine against pregnancy-associated malaria. The following are the Specific Aims of this project:

Specific Aim 1. To obtain proteins corresponding to individual Duffy binding like (DBL) domains of VAR2CSA. We will express all six DBL domains of VAR2CSA in *E. coli*, purify them by Ni-affinity, ion-exchange and size-exclusion chromatography, and evaluate their secondary structures by CD spectral analysis and by measurement of thermal unfolding.

Specific Aim 2. To study DBL domain-domain interactions. In this Specific Aim, we will test our hypothesis that a high affinity C4S binding by VAR2CSA occurs in an extended pocket/groove formed by domain-domain interactions between DBL domains. The interactions between various VAR2CSA DBL domain pairs will be studied using several biophysical techniques. The results are anticipated to provide a tentative physical map of inter-DBL domain interactions and their organization in VAR2CSA.

Specific Aim 3. Crystallization of DBL domain complexes and NMR analysis of DBL domains. The goal of this Specific Aim is to optimize conditions for the crystallization of interacting DBL domain pairs and perform NMR analysis of DBL domains.

Principal Investigator

Channe D. Gowda, PhD
Professor
Department of Biochemistry and Molecular Biology
Pennsylvania State University College of Medicine
500 University Drive, H171
Hershey, PA 17033

Other Participating Researchers

John M. Flanagan, PhD, Maria C. Bewley, PhD, S. Goel, PhD - employed by The Pennsylvania State University

Expected Research Outcomes and Benefits

Malaria is a huge public health problem in many countries of the world and *Plasmodium falciparum* is the most virulent species among human malaria parasites. To avoid immune defense and splenic clearance, the parasite expresses antigenically variant adhesive proteins on the surface of infected red blood cells (IRBCs), which bind to cell adhesion molecules expressed on the microvascular endothelial surface, and chondroitin 4-sulfate (C4S) in the placenta, sequestering in brain, placenta and other organs. The *var* gene family of proteins, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), is responsible for antigenic variation and different adhesive property of IRBCs. The PfEMP1 proteins are thought to be the key malaria virulence factors and play a central role in the development of cerebral, placental, and other fatal malaria pathologies. This project focuses on domain-domain organization in VAR2CSA, the variant PfEMP1 involved in placental sequestration that contributes to pregnancy-associated malaria (PAM). Women who have acquired anti-VAR2CSA antibodies because of *P. falciparum* infections during prior pregnancies are resistant to PAM, indicating that blocking IRBC adherence is of great clinical relevance. Thus, VAR2CSA is an important vaccine target for PAM. However, how VAR2CSA interacts with C4S chains remains largely unknown. VAR2CSA is a large (~350 kDa) protein, consisting of six Duffy binding like (DBL) domains. In this study, we will begin to address our long-term goal of defining the structural interactions involved in VAR2CSA binding to C4S. Accordingly, we will analyze DBL domain-domain interactions and optimize conditions for crystal structure studies. The information gained will contribute toward our ultimate goal of designing strategies for the development of a vaccine against PAM and other severe malaria illnesses.

Summary of Research Completed

Specific Aim 1. To obtain proteins corresponding to individual Duffy binding like (DBL) domains of VAR2CSA.

We cloned the DBL domains of *P. falciparum* (3D7 strain) VAR2CSA into a pET-28aTEV vector at an *N*-terminal His tag and a tobacco etch virus (TEV) protease-cleavable site and expressed them in *E. coli*. The domain boundaries of cloned DBLs were: DBL1x aa 46-430, DBL2x aa 535-961, DBL3x aa 1214-1562, DBL4ε aa 1574-1957, DBL5ε aa 1983-2291, DBL6ε

aa 2332-2634, and DBL2x-ID2-DBL3x aa 535-1562). All constructs expressed at high levels; DBL3x and DBL6ε were primarily soluble proteins and were purified by Ni-NTA chromatography and size-exclusion chromatography. DBL1x and DBL2x were expressed as inclusion bodies and were isolated by denaturing Ni-NTA chromatography, refolded to obtain their native structures, and further purified by Ni-NTA affinity and cation-exchange chromatography. On size-exclusion chromatography, DBL2x and DBL3x eluted as single monomeric proteins. MS of DBL2x showed a single molecular ion of predicted mass 44.8 kDa; calculated mass 45.3 kDa (Figure 1). The yields were typically 10-15 mg/L culture. CD spectra of the refolded DBLs showed a high degree of α-helical content (Figure 2), and were similar to the spectrum of DBL3x that was previously used for crystal structure studies and that of DBL6ε produced in mammalian cells. Thermal unfolding of DBL-2x and DBL-3x measured by CD (not shown) showed a sharp loss of secondary structure at ~70 °C as expected for compact proteins that contain several disulfide bonds.

We found that DBL2x535-961 protein corresponding to the original construct described above tends to aggregate over time. Homology modeling of DBL2x using I-TASSER (zhanglab.cmb.med.umich.edu/I-TASSER/) and modeller (www.salilab.org/modeller/) programs converged to a common fold. Inspection of these models identified a hydrophobic patch on the exposed surface, which might account for the time-dependent aggregation of DBL2x. We modeled a construct containing 47 additional amino acids at the N-terminus and predicted this to be hydrophilic and mask the exposed hydrophobic region, potentially stabilizing the protein (Figure 3). The protein (residues 499-961) was expressed in *E. coli*, refolded, and purified. Size-exclusion chromatography and multi-angle light scattering detection showed that the purified protein is homogeneous with the expected size 52 kDa. As predicted, it does not aggregate. CD analysis showed that it is properly folded and the spectrum was similar to that shown in Figure 2, but has higher helical content.

Specific Aim 2. To study DBL domain-domain interactions.

(i) *Multiple DBL domains interact to form domain-domain complexes.* We tested the association of DBL domains to form complexes. DBL1x and DBL2x, expressed in recombinant yeast, bound in a dose-dependent manner, to *E. coli*-expressed and purified DBL3x using an ELISA-based reporter assay (Figure 4). The DBL1x expressed in yeast also bound *E. coli*-expressed DBL2x (not shown). By contrast, DBL4ε, DBL5ε and DBL6ε did not interact with each other or with either DBL2x or DBL3x with measurable affinity. DBL2x purified from the recombinant yeast efficiently bound immobilized DBL3x (K_d of 10-20 nM) in an ELISA assay. Furthermore, yeast that expressed DBL2x bound to immobilized DBL3x in a dose-dependent manner (not shown). The interaction between DBL1x, DBL2x and DBL3x was also observed by tryptophan fluorescence (Figure 5) and SPR analyses (not shown). Together, these data suggest that DBL1x, DBL2x, and DBL3x form an interacting unit in VAR2CSA.

(ii) *DBL structure and interaction studies by SAXS analysis.* Scattering measurements were performed on DBL2xL, DBL3x, and the binary complex DBL2xL + DBL3x at the National Synchrotron Light Source (NSLS, beamline X9), Brookhaven National Laboratory, NY. Small and wide-angle data were collected simultaneously using a MarCCD SAXS detector and a Photonic Science, Pilatus 300K pixel array WAXS detector, respectively. Data were processed using the ATSAS program suite (<http://www.embl-hamburg.de/biosaxs/software.html>, version

2.4), a web-supported data analysis package. Radius of gyration (R_g) and zero scattering ($I(0)$) were calculated using both GNOM and Guinier analysis within PRIMUS. Inverse Fourier transform calculations of $I(0)$ to yield $P(r)$ functions, $I(0)$, R_g and the maximum dimension D_{max} were determined from the data between S ($S=4\pi\sin(\theta)/\lambda$) of $0.01 \text{ \AA}^{-1} - 0.3 \text{ \AA}^{-1}$.

For calculating low resolution structural models, *ab initio* shape reconstruction using the program GASBOR with default parameters was used and generated three-dimensional envelopes from the 1D scattering. In each case, 10 independent models were generated, aligned and averaged using the DAMAVER program. In addition, SASREF was used to compare simulated intensity data derived from the crystal structure of DBL3x with the experimental intensity measured at the beamline.

Measurements were made at 3 different concentrations. The scattering curves are flat in the low S range, confirming the absence of aggregates. Profiles obtained at different protein concentrations have the same shape indicating that there was no significant increase in self-association with increasing concentration. Guinier analysis was used to calculate the R_g and determine the effect of protein concentration upon oligomerization state. The plots are linear over the Guinier region at low S where $SR_g < 1$ and indicated that the proteins were monomeric, in agreement with SEC-MALS results. The molecular reconstructions showed characteristic compact structures of DBL2xL and DBL3x, and that DBL2xL and DBL3x interact side by side to form a more extended molecule (Figure 6). This is consistent with the hypothesis that the carbohydrate-binding site is comprised of more than one domain.

Specific Aim 3. Crystallization of DBL domain complexes and NMR analysis of DBL domains. Preliminary crystallization trials using DBL2xL protein produced non-diffraction quality microcrystals. The results are encouraging and further crystallization trials are underway.

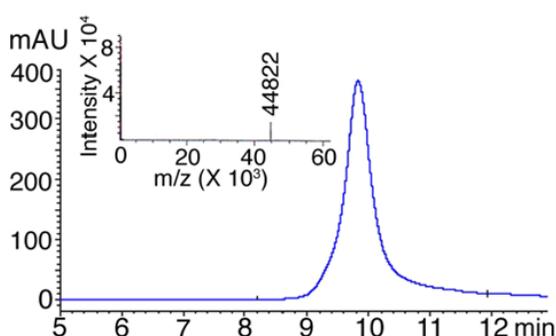


Figure 1. Size-exclusion chromatography of refolded *E. coli* DBL2x; inset, mass analysis by MS. DBL3x also gave symmetrical single peak.

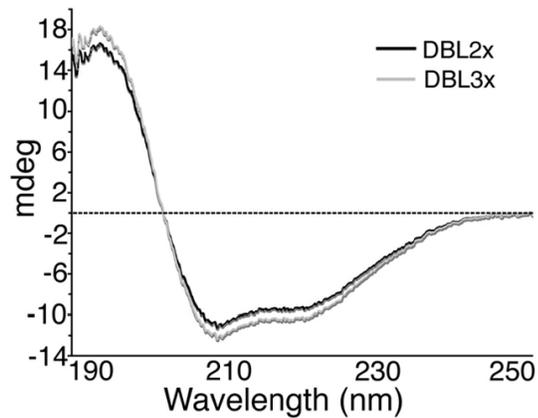


Figure 2. CD spectra of refolded DBL2x and DBL3x

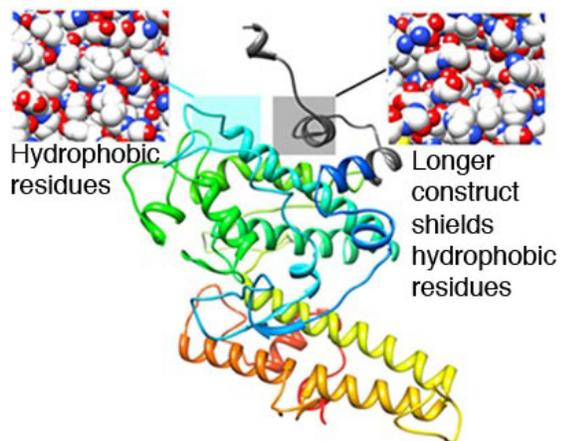


Figure 3. Model of DBL2xL showing that the N-terminus extra sequence (dark gray) masks the exposed hydrophobic surface. The structure is colored rainbow. Gray, carbon; red, oxygen; blue, nitrogen.

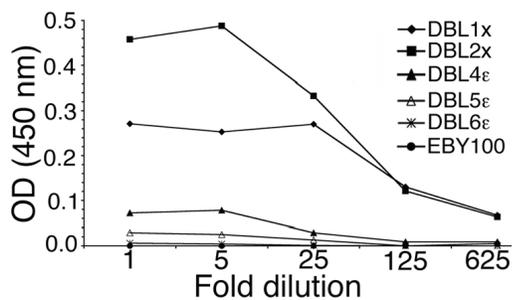


Figure 4. Binding of lysates (at 1:1 to 1:625 dilutions) of yeast expressing various DBLs to recombinant DBL3x.

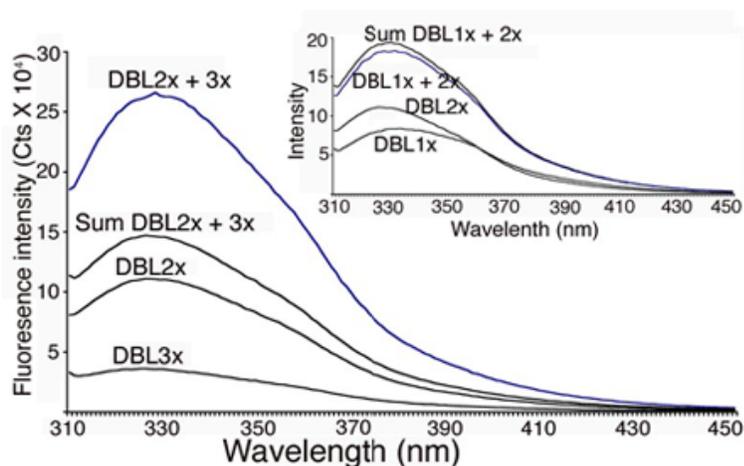


Figure 5. The fluorescence intensity of DBL2x plus DBL3x (1 μ M each) is higher than the sum of individual DBLs; the emission λ was also shifted to a higher region: Inset: DBL1x and DBL3x.

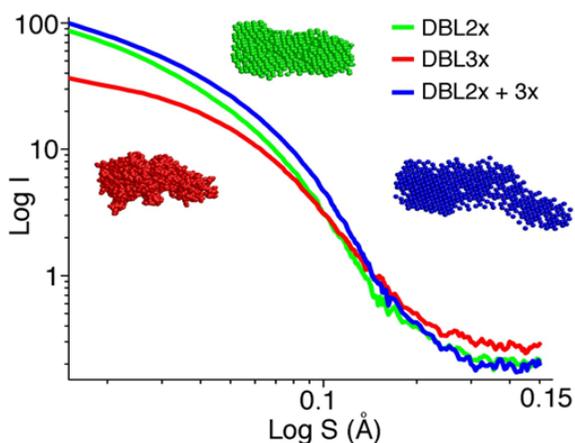


Figure 6. Scattering intensity as a function of the momentum transfer $S = 4\pi\sin(\theta)/\lambda$. SAXS measurements were done in 20 mM Tris, 150 mM NaCl, pH 7.5. The molecular reconstructions are shown.

Research Project 35: Project Title and Purpose

Impact of iPS Cell-derived Highly Reactive Immune Cells on Cancer – Adoptive cell transfer (ACT) of tumor-specific T killer cells that are able to directly or indirectly kill or suppress cancer cells is a promising treatment for cancer. It is known that highly avid T killer cells are the optimal population for ACT-based immunotherapy. However, ACT with such T killer cells is often not feasible due to difficulties in generating large numbers of these cells from patients. Finding a new mechanism to generate these cells is critical for the success of ACT-based cancer immunotherapy. Using a series of complementary approaches, including the combination of genetic modification and *in vitro* development, we will generate and characterize highly avid melanoma-reactive T killer cells from induced pluripotent stem (iPS) cells, and study the impact

of iPS cell-derived T killer cells on cancer.

Duration of Project

7/20/2010 – 6/30/2011

Project Overview

The *objective* of this project is to generate and characterize highly avid melanoma-specific cytotoxic T lymphocytes (CTLs) from human iPS cells. To address this objective, we will test the *working hypothesis* that lentivirally transduced human iPS cells with genes of melanoma-specific T cell receptor (TCR), and cultured via *in vitro* Notch signaling, will differentiate into highly avid melanoma-specific T lymphocytes. We will test our working hypothesis by using the *experimental approach* of lentivirus-mediated TCR transduction and stem cell culture with *in vitro* Notch signaling. Human iPS cells will be genetically modified with MART-1 (Melanoma Antigen Recognized by T-cells) /Melan-A (melanocyte antigen-A)-specific TCR and stimulation with *in vitro* Notch signaling.

1. Generation of highly avid MART-1/Melan-A-specific CTLs derived from human iPS cells.

We will transduce human iPS cells with MART-1/Melan-A-specific-TCR by lentivirus-mediated transduction, and culture the cells with *in vitro* Notch signaling.

2. Characterization of highly avid MART-1/Melan-A-specific CTLs derived from human iPS cells.

We will characterize the generated MART-1/Melan-A-specific CTLs derived from human iPS cells:

(1) *In vitro analysis of the MART-1/Melan-A-specific CTLs.* We will stimulate the human iPS cell-derived CTLs *in vitro* with T2 cells pulsed with the cognate peptide MART-1/Melan-A₂₅₋₃₇ or the control peptide MAGE-3₂₇₁₋₂₇₉ to determine the level of cytokine secretion, proliferation, expansion, and survival. After five days, melanoma-reactive CTLs will be isolated and restimulated to determine recall/memory responses. Cytotoxic potential of CTLs will be measured by *in vitro* chromium release assay.

(2) *In vivo testing of the MART-1/Melan-A-specific CTLs and ACT therapy in a murine melanoma model.* We will use NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (NOD/SCID IL2R γ ^{null}, the Jackson Laboratory), a murine model to evaluate human iPS cell-derived MART-1/Melan-A-specific CTLs.

Principal Investigator

Jianxun Song, PhD
Assistant Professor
Penn State University College of Medicine
Department of Microbiology & Immunology H107
500 University Dr.
Hershey, PA 17033

Other Participating Researchers

Rizwanul Haque, PhD – employed by The Pennsylvania State University

Expected Research Outcomes and Benefits

We expect to generate iPS cell-derived MART-1/Melan-A-specific CTLs, which will be highly avid to MART-1/Melan-A antigen, and suppress human melanoma growth in the murine model.

The proposed studies are significant and will provide new insights regarding the generation of highly avid T killer cells for ACT-based cancer immunotherapy and facilitate the development of the therapeutic strategies for the prevention and treatment of cancer.

Summary of Research Completed

In this study, we have generated and characterized MART-1/Melan-A-specific CTLs from iPS cells by TCR gene transduction and stimulation with *in vitro* Notch ligand.

1. Generation of highly reactive MART-1/Melan-A-specific CTLs derived from human iPS cells.

First, we have generated the construct that contains MART-1/Melan-A-specific TCR genes. We subcloned MART-1/Melan-A-specific TCR α and β chain genes (specific for the HLA-A2-restricted MART-1/Melan-A epitope₂₇₋₃₅: AAGIGILTV) into the lentiviral vector pLVX-IRES-mCherry (Clontech). To ensure that TCR α and β chain genes were expressed equally in iPS cells, we used a 2A sequence to link the two genes. The created lentiviral construct (i.e., pLVX-TCR α -2A-TCR β) was used to generate lentiviruses for TCR transduction that induced the development of MART-1/Melan-A-specific CTLs from human iPS cells.

Second, we have transduced human iPS cells with MART-1/Melan-A-specific-TCR by lentivirus-mediated transduction, and cultured the cells on OP9-DL1/DL4 cells, which express Notch ligands DL1 and DL4. After 50 days, the iPS cell-derived CD8⁺ T lymphocytes were characterized by surface marker expression of TCR β , CD3, CD8, CD1a, CD25, CD44 and CD69 (Figure 1). In addition, we observed to highly express MART-1/Melan-A-specific-TCR on iPS cell-derived CD8⁺ T lymphocytes as determined by flow cytometry (Figure 2).

2. Characterization of highly avid MART-1/Melan-A-specific CTLs derived from human iPS cells.

(1) *In vitro* analysis of the MART-1/Melan-A-specific CTLs.

We found that CD8⁺ T lymphocytes derived from human iPS cells were able to respond to antigen stimulation, proliferated, expanded, and secreted cytokines. In addition, these T cells could generate recall/memory responses when stimulated with antigen again. In addition, we have performed functional characterization of the MART-1/Melan-A-specific CTLs derived from human iPS cells. We found iPS cell-derived mature CTLs were functional in their ability to secrete cytokines and kill target-expressing target cells (Figure 3). Collectively, these data indicated that iPS cell-derived CD8⁺ T lymphocytes were functionally mature.

(2) *In vivo* testing of the MART-1/Melan-A-specific CTLs and ACT therapy in a murine melanoma model.

First, we adoptively transferred the iPS cell-derived CTLs into NOD/SCID mice by an *i.v.* injection through the tail vein. On the following day, we challenged mice by an *i.p.* injection of anti-CD3 plus CD28 Abs. At different time points (i.e., days 3, 7, 14, 35), we analyzed the population of mCherry⁺ cells obtained from the spleens and lymph nodes by flow cytometry. To determine the extent of long-term T-cell persistence, we rechallenged a subset of mice with anti-CD3 plus Abs by *i.p.* injection 5 weeks after the first challenge, and determined the MART-1/Melan-A-specific CTLs still existed as described above.

Second, we used *s.c* injection in the flank to challenge NOD/SCID mice with the human melanoma cell line 526mel, which highly expresses MART-1/Melan-A protein. We analyzed the population of mCherry⁺ cells obtained from the spleens, lymph nodes, and tumor sites by flow cytometry. We observed that adoptive transfer of iPS cell-derived CTLs significantly suppress melanoma growth (Figure 4).

The Figures (Figs. 1-4) contain the main results that we expected.

We also obtained additional results (Figs. 5-8):

A1. Generation of Ag-specific TCR gene-transduced iPS cells

To generate Ag-specific TCR-transduced iPS cells for adoptive transfer, we used the mouse stem cell virus (MSCV)-based retroviral vector pMig in which MHC-I restricted OVA-specific TCR α and β chain genes were linked with a 2A peptide to deliver TCR genes into iPS cells. Since iPS cells express GFP as an endogenous stemness marker, we replaced GFP with DsRed as a new marker for monitoring gene integration, and named the new vector as MiDR (Figure 5a). After transduction, DsRed expression was visualized by fluorescent microscopy on the TCR-transduced iPS cells (Figure 5b). Although the transduction efficiency was low (< 3.5%), we were able to sort for DsRed and GFP double positive cells (Figure 5c). Moreover, we confirmed expression of TCR V β 5 mRNA and DNA integration in the sorted cells by RT-PCR and PCR (Figure 5d). We co-cultured the TCR gene-transduced iPS cells on feeder layers of irradiated SNL76/7 cells in culture plates as previously described, large numbers of TCR gene-harboring iPS cells were generated by using this culture system.

A2. TCR gene-transduced iPS cells differentiated into CD8⁺ T lymphocytes *in vivo*

To determine the outcome of MHC-I restricted OVA-specific TCR gene transduction in CTL differentiation from iPS cells *in vivo*, we adoptively transferred the OVA TCR gene-transduced iPS cells or bone marrow-derived CD117⁺ Lin⁻ HSCs from OT-I TCR transgenic mice into C57BL/6 mice. After six to ten weeks, we analyzed OVA-specific CD8⁺ T cells from the pooled lymph nodes and spleen by flow cytometry. We observed approximately 49% of V β 5⁺ cells in the CD8⁺ population of mice receiving TCR gene-transduced iPS cells or bone marrow-derived CD117⁺ Lin⁻ HSCs from OT-I TCR transgenic mice. In contrast, the CD8⁺ V β 5⁺ cell population was less than 2% in mice receiving vector control gene-transduced iPS cells (Figure 6a). To assess the functionality of CD8⁺ V β 5⁺ cells, we isolated CD8⁺ T cells for phenotype analysis (Figure 6b), and stimulated *in vitro* with irradiated splenocytes from C57BL/6 mice pulsed with the OVA₂₅₇₋₂₆₄ peptide. We were able to observe IL-2 and IFN- γ production from V β 5⁺ cells

gated on the CD8⁺ population as detected by intracellular cytokine staining (Figure 6c). Furthermore, we performed an *in vivo* cytotoxicity assay to determine the proliferation/cytolytic effector potential of CD8⁺ Vβ5⁺ cells, which involves injection of CFSE-labeled, peptide-pulsed syngeneic splenocytes. Interestingly, target cell lysis was approximately 90 times greater in mice receiving TCR gene-transduced iPS cells than in control receiving vector control gene-transduced iPS cells (94% versus 1%) (Figure 6d). Collectively, these data indicate that MHC-I restricted OVA TCR gene-transduced iPS cells have the ability to develop into functional OVA-specific CD8⁺ T lymphocytes *in vivo*.

A3. *In vivo* persistence of Ag-specific T cells derived from TCR gene-transduced iPS cells

To investigate whether *in vivo* programmed CD8⁺ T cells from iPS cells are able to persist, we challenged two groups of mice with E.G7 lymphoma cells that express OVA protein: (i) six weeks after receiving TCR gene-transduced iPS cells; or (ii) one day before with CD8⁺ T cells isolated from OT-I TCR transgenic mice. After fifty days, we found an increased number of OVA-specific CD8⁺ T cells in the pooled lymph node and spleen cells in mice receiving TCR gene-transduced iPS cells than in mice receiving CD8⁺ T cells from OT-I TCR transgenic mice (52.7% versus 12.8%) (Figure 7). This result indicated that *in vivo* programmed CD8⁺ T cells from iPS cells might generate from a time period (e.g., six weeks to twelve weeks after adoptive transfer), which is consistent with previous observations.

A4. Adoptive transfer of TCR gene-transduced iPS cells prevent tumor growth

Because TCR gene-transduced iPS cells are able to differentiate into fully functional Ag-specific CD8⁺ T cells *in vivo*, we hypothesize that adoptive transfer of these iPS cells would generate tumor protection. To test this hypothesis, we adoptively transferred OVA TCR gene-transduced iPS cells into C57BL/6 mice, and challenged mice with E.G 7 tumor cells after six weeks of cell transfer to allow generation of OVA-specific CD8⁺ T cells *in vivo*. In addition, we adoptively transferred CD8⁺ T cells isolated from OT-I TCR transgenic mice into C57BL/6 mice, and challenged mice with the tumor cells on the following day as described in Figure 7. On day 30 after tumor challenge, we found fewer tumor cells in the peritoneal cavity of mice receiving TCR gene-transduced iPS cells than in mice receiving either CD8⁺ T cells from OT-I TCR transgenic mice or control vector-transduced iPS cells (Figure 8b). On day 50, we observed 100% survival of mice receiving TCR gene-transduced iPS cells, compared to 55% survival of mice receiving CD8⁺ T cells from OT-I TCR transgenic mice (Figure 8c). Moreover, we observed a large number of tumor-infiltrating OVA-specific CD8⁺ T cells in mice receiving TCR gene-transduced iPS cells (Figs. 8a - 8c). Collectively, these data strongly support our hypothesis that adoptive transfer of tumor Ag-specific TCR gene-transduced iPS cells are able to induce tumor Ag-specific T-cell persistence, and result in tumor protection.

In conclusion, we developed a novel approach to generate Ag-specific T lymphocytes by programming iPS cells, and the iPS cell-derived CTLs have a great potential in the treatment of disease. One paper based on this study has been published in *Cancer Research* in the issue of July 15, 2011, with a specific introduction by a cover picture.

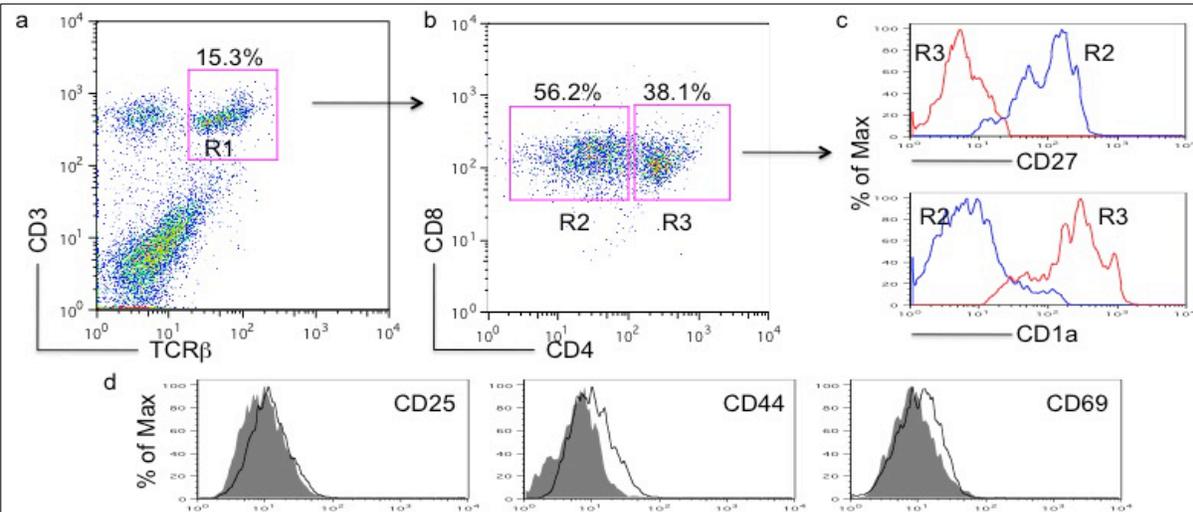


Fig. 1 Characterization of CD8⁺ T cells generated *in vitro*. The data show combined TCR gene transduction and *in vitro* Notch signaling can give rise to mature CD8⁺ T cells from human iPS cells. **Method:** Human iPS cells were transduced with a construct: pLVX-TCR α -2A-TCR β containing MART-1/Melan-A-specific TCR genes. The gene-transduced cells (mCherry⁺) were sorted and co-cultured on OP9-DL1/DL4 cells in the presence of rIL-7, Flt3L and SCF. (a) On day 50 the CD3 and TCR β expression was analyzed by flow cytometric analysis. (b and c) CD3⁺ TCR β ⁺ cells were gated (R1), and analyzed for the expression of CD4 and CD8, with CD27 and CD1a expression shown for cells gated as CD4⁻ CD8⁺ (R2) or CD4⁺ CD8⁺ (R3). (d) The expression of CD25, CD44 and CD69 for cells gated as R2 was shown. Shaded histograms indicate isotype control staining. Data are representative of three experiments.

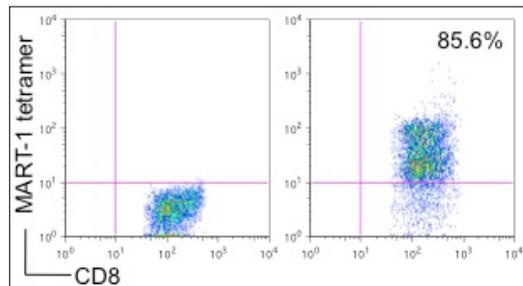


Fig. 2 TCR transduction and stimulation with *in vitro* Notch signaling promote MART-1/Melan-A-specific CTL differentiation from human iPS cells. The data show MART-1/Melan-A⁺ cells after gated on CD8⁺ cells by flow cytometry. **Method:** Human iPSB12-2 cells were transduced with the following construct: pLVX vector control (left) or pLVX--TCR α -2A-TCR β construct that contains MART-1/Melan-A-specific TCR genes (right). mCherry⁺ cells were sorted and co-cultured on OP9-DL1/DL4 cells for seven weeks. Differentiated cells were analyzed for expression of MART-1/Melan-A⁺ by flow cytometry, after gating on the CD8⁺ population.

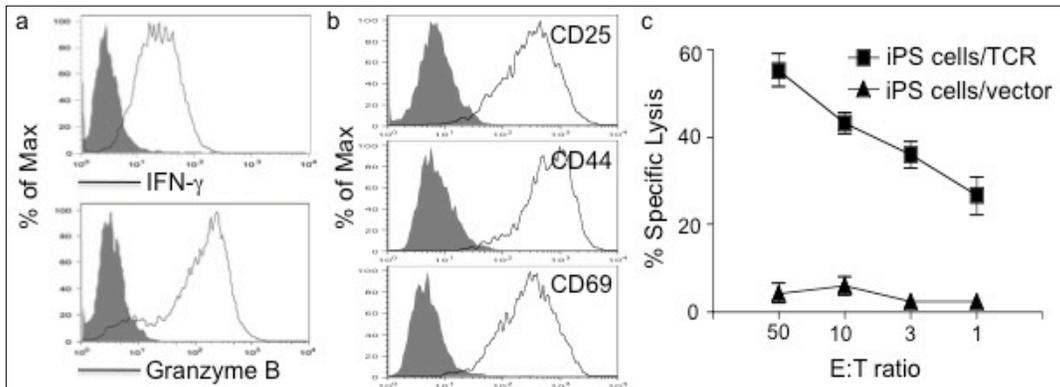


Fig. 3 Functional characterization on MART-1/Melan-A-specific CTLs derived from human iPS cells generated *in vitro*. The data show MART-1/Melan-A-specific CTLs derived from human iPS cells are functional. **Method:** Human IPS cell-derived mature CTLs (CD3⁺ CD8⁺ CD27⁺) as described in Fig. 1 were sorted and stimulated with plate-coated anti-CD3 antibody in the presence of rIL-2 and rIL-7 for three days. (a) CD8⁺ T cells were evaluated for IFN- γ and granzyme B expression by intracellular staining and flow cytometry. (b) CD8⁺ T cells were evaluated for expression of CD25, CD44, and CD69 by surface staining and flow cytometry. Shaded histograms in a and b indicate isotype control staining. The activated CTLs were placed in a standard ⁵¹chromium release assay utilizing MART-1/Melan-A₂₅₋₃₇ peptide coated T2 cells. Graph shows the specific lytic activity of cells at different effector to target cell ratio. Data are representative of three independent experiments.

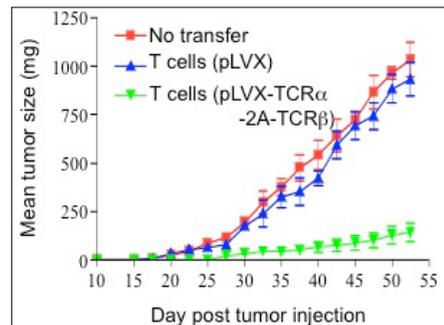


Fig. 4 Effects on human melanoma growth in a murine model. The data show adoptive transfer of iPS cell-derived MART-1/Melan A-specific CTLs derived from iPS cells inhibited human melanoma growth in NOD/SCID mice. **Method:** MART-1/Melan-A-specific CTLs derived from human IPS cells as described in Fig. 1 were adoptively transferred into NOD/SCID mice by *i.v.* injection (2×10^6 cells/per mouse). On the following day, mice were subjected to challenge with 5×10^5 526mel cells by *s.c.* injection. Tumor growth was measured three times a week and tumor weight was calculated. The histograms represent mean \pm SD of tumor weight in 10 animals/point in three different experiments (■ : iPS cell-derived MART-1/Melan-A-specific T cells; ▲ : iPS cell-derived non-specific T cells; ▼ : no T-cell transfer).

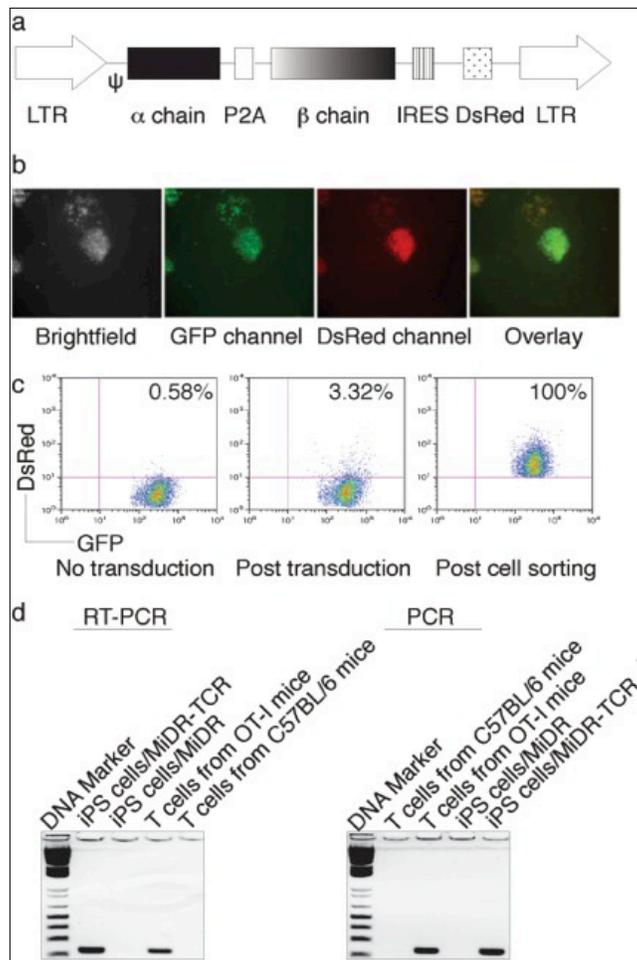


Figure 5. Lentivirus-mediated TCR transduction in iPS cells. iPS cells were transduced with the following constructs: vector control (MiDR), or Ag-specific TCR (MiDR-TCR). (a) Schematic representation of the construct. (b) TCR-transduced iPS cells were visualized by fluorescence microscopy. (c) GFP⁺ iPS cells (left) were transduced with the retroviral construct MiDR-TCR, and GFP⁺ DsRed⁺ iPS cells (middle) were analyzed by flow cytometry and sorted by a high-speed cell sorter (right). (d) GFP⁺ DsRed⁺ iPS cells were sorted and total mRNA and DNA were analyzed for V β 5 gene expression by RT-PCR (left) and for the V β 5 gene by PCR (right). The forward primer is ACGTGTATTCCCATCTCTGGACAT and the reverse primer is TG TTCATAATTGGCCCGAGAGCTG.

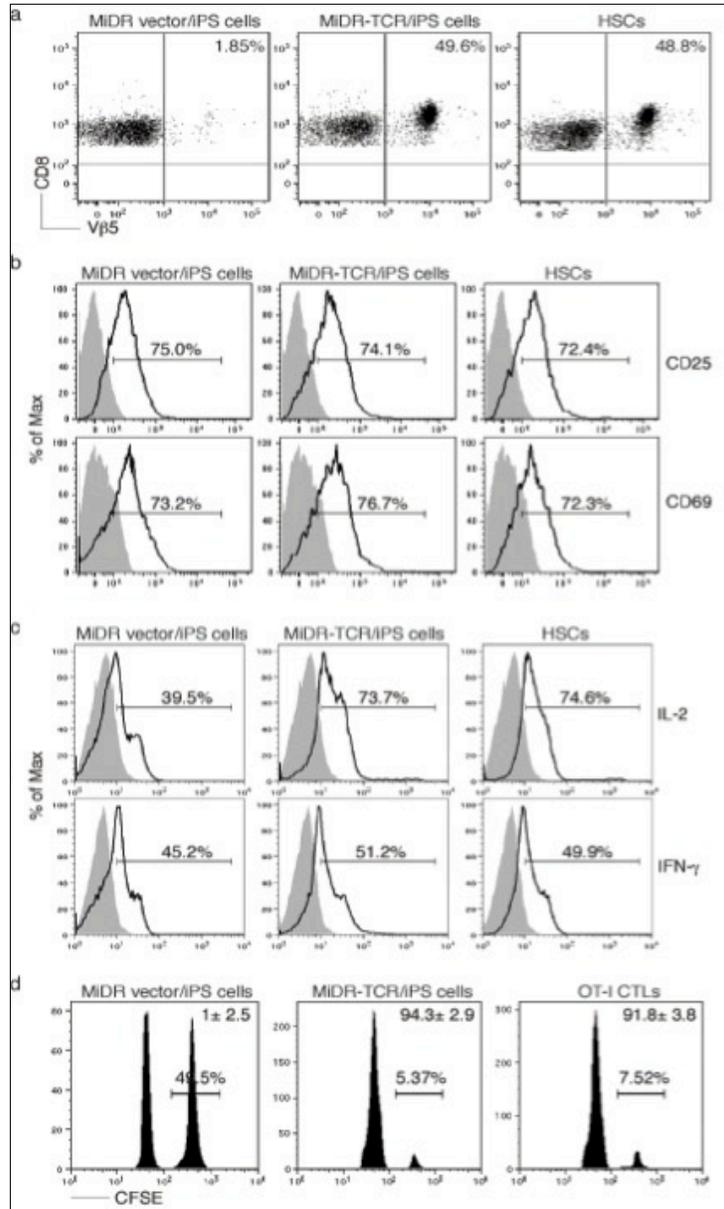


Figure 6. Ag-specific CD8⁺ T-cell development from iPS cells *in vivo*. GFP⁺ DsRed⁺ iPS cells were injected *i.v.* into C57BL/6 mice. After six to ten weeks, Ag-specific Vβ5⁺ CD8⁺ T cell development was determined. (a) CD8⁺ Vβ5⁺ T cells from pooled lymph nodes and spleen were analyzed by flow cytometry. (b) CD25 and CD69 expression was analyzed by flow cytometry, after gating on CD8⁺ Vβ5⁺ T cells (dark lines; shaded areas indicate isotype controls). (c) IL-2 and IFN-γ production from the CD8⁺ Vβ5⁺ population (dark lines; shaded areas indicate isotype controls) were determined by intracellular cytokine staining. (d) *In vivo* proliferation/cytotoxicity assay. CFSE^{hi} (right peaks) and CFSE^{lo} (left peaks) target cells were pulsed with the peptide and the control, respectively, and were injected into mice ten weeks after iPS cell transfer or one day after OT-I CTL transfer. Data are representative of two or three independent experiments.

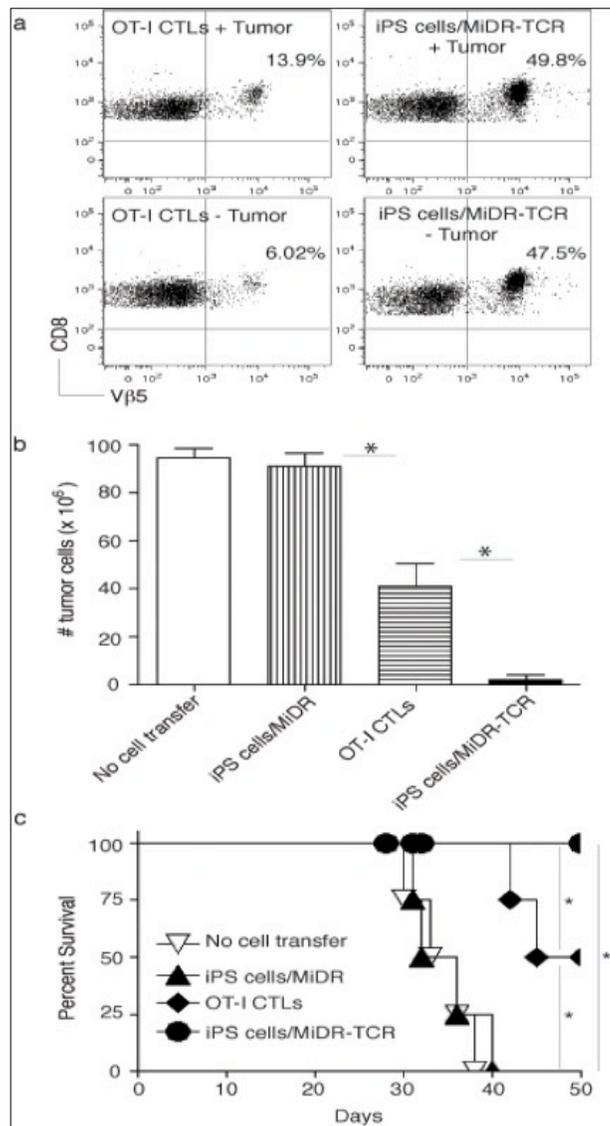


Figure 7. Adoptive transfer of TCR-transduced iPS cells suppresses tumor growth and sustains mouse survival. GFP⁺ DsRed⁺ cells were adoptively transferred into C57BL/6 mice. One group of mice were injected with Ag-reactive CD8⁺ T cells from OT-I TCR transgenic mice, and one group of mice had no cell transfer. After either six weeks or on the following day after the cell transfer, mice were subjected to challenge with tumor cells. (a) Ag-specific T-cell persistence. Seven weeks post tumor challenge or thirteen weeks without tumor challenge, CD8⁺ Vβ5⁺ T cells from the pooled LNs and spleen were analyzed by flow cytometry. (b) On day 20, tumor cells in the peritoneal cavity were enumerated. Data represent mean (±SEM) tumor cell counts from six individual mice. One-way ANOVA test was used for statistical analyses between two groups (*: $p < 0.05$). (c) Mouse survival on day 50. Kaplan-Meier survival curves are shown (n=6). *: $p < 0.05$; **: $p < 0.001$, One-way ANOVA with Newman-Keuls Multiple Comparison Test. Data are representative of three independent experiments.

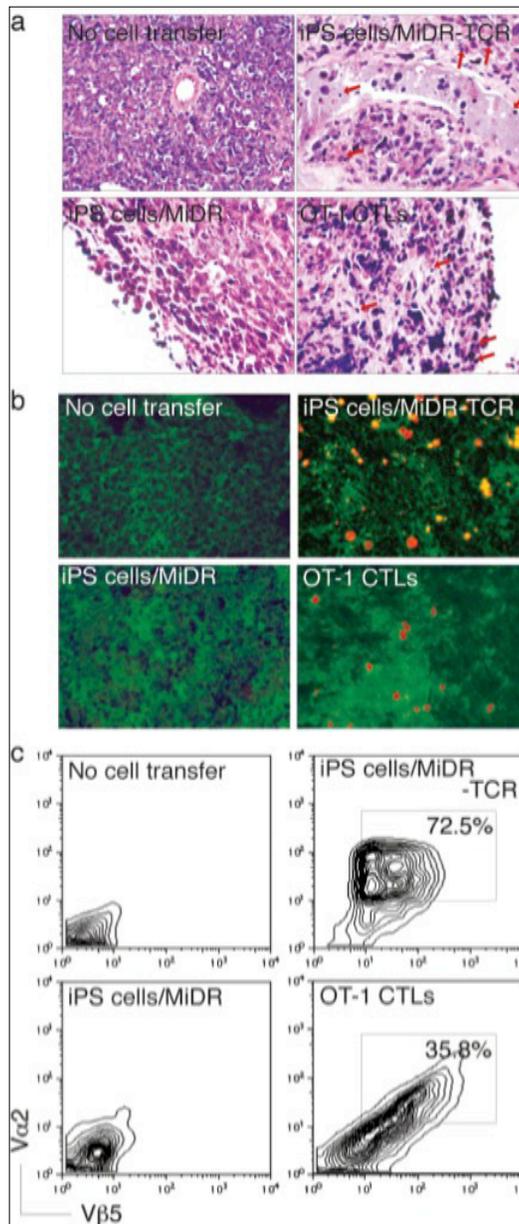


Figure 8. iPS cell-derived Ag-specific CTLs infiltrate into tumor tissues. On day 30 to 35 after tumor challenge, tumor tissues were examined for tumor-reactive T cell infiltration. (a) H & E staining. Inflammatory cells infiltrated in tumor tissues (\downarrow). (b) Immunohistological staining. Ag-specific $V\alpha 2^+$ CTLs (red) infiltrated in Ag-expressing tumor tissues (green). (c) Single-cell suspensions from tumor tissues were analyzed for expression of $V\alpha 2^+$ and $V\beta 5^+$ by flow cytometry, after gating on the $CD8^+$ population. Data are representative of three independent experiments.

Research Project 36: Project Title and Purpose

Cannula Development and In Vivo Testing for Pediatric VAD Development – The purpose of this research is to take the Penn State Pediatric Ventricular Assist Device (VAD) to the next stage of development. The Pediatric VAD is a blood pump, which is intended for infants less than 9 months of age and can take over the work of the heart in cases where the heart function is weakened by disease or congenital defects. The VAD is designed to function for many months, as a bridge to heart transplantation, or as support leading to recovery of native heart function. The specific objectives of this project are to develop cannulae, which are specially designed tubes that connect the VAD to the patient's heart and blood vessels, and to test the VAD/cannulae system in animal studies for 8 weeks in duration. The goal of these studies is to demonstrate minimal risk of clot formation and stroke, leading to pre-clinical studies and eventual clinical trials.

Anticipated Duration of Project

7/20/2010 – 8/31/2011

Project Overview

The Penn State Pediatric VAD is a pneumatically-actuated pulsatile pump based on the Pierce-Donachy adult VAD which was developed at Penn State and commercialized as the Thoratec® PVAD™. The adult pump has a stroke volume of 65 ml and has been used in over 4000 patients worldwide and is approved for bridge-to-transplant and post-cardiotomy recovery. The Infant VAD has a stroke volume of 12-14 ml; a Child VAD with a 25-30 ml stroke volume has been designed but has not yet been built.

The Infant VAD pump assembly has undergone substantial development, under previous NIH support. However, a portable pneumatic driver, suitable for clinical use, has not progressed past an initial design with limited testing of a feasibility prototype. Also, cannulae suitable for human use have not yet been fabricated or tested.

The objective of the research plan is to continue development of the Pediatric VAD in those areas that will strengthen future grant submissions and lead to FDA approval of clinical trials. The specific aims in this project are 1) design and fabrication of new cannulae for use in infants, 2) chronic animal testing in 2 studies of 8 weeks duration to test the new cannulae and to generate additional data regarding biocompatibility of the system. Previous animal studies were limited to 4-5 weeks.

The innovative cannula design uses a modified, extruded PTFE graft to provide flexibility, kink resistance, and resistance to clot formation. Cannulae are being developed to support both left and right ventricles. Studies in animals are required for assessing the biocompatibility of the VAD/cannulae system.

A secondary objective of this research is to better understand the mechanisms of clot formation on artificial surfaces, by performing extensive measurements of coagulation and platelet

activation parameters.

Additional funding is being sought for more extensive, pre-clinical studies leading to clinical trials.

Principal Investigator

William J. Weiss, PhD
Professor of Surgery and Bioengineering
Penn State Hershey Medical Center
College of Medicine
Department of Surgery, Mail code H151
Hershey, PA 17033

Other Participating Researchers

Gerson Rosenberg, PhD, Christopher Siedlecki, PhD, Elizabeth Carney, DVM, Rebecca Peterson, Bryan Fritz, MS, Branka Lukic, MS – employed by The Pennsylvania State University

Expected Research Outcomes and Benefits

The use of the Ventricular Assist Device (VAD) in adults began in the 1970's and is now a viable therapeutic option in advanced heart failure. However, this technology has been slow to develop for pediatrics, in part due to the smaller pediatric market, and in part due to the technical challenges in downsizing adult devices. In infants, there is a need for cardiac support related to congenital heart defects. Approximately 36,000 babies (0.9% of live births) are born with a defect each year in the U.S. Approximately 25% require invasive treatment, and approximately 1800 infants die each year. Acquired heart disease also results in 350 infants (<1 year of age) per year developing cardiomyopathy. As a result, the under 1 year of age group has a much higher rate of heart transplantation than older age groups. Furthermore, the scarcity of donor organs results in waiting times that exceed adult waiting times. The use of a VAD to bridge a patient to transplantation is an accepted approach, although the risks of stroke and infection are significant.

Currently, there is only one VAD available for infants for cardiac support exceeding 1 month duration. The Berlin Heart 10 ml EXCOR® is available in the U.S. via compassionate use procedures or under a 12 center clinical trial. The Berlin Heart has resulted in survival in patients with no alternatives, but this device carries a high risk of blood clot formation, stroke, and pump replacement.

The Penn State Infant VAD being developed in this research project is intended to provide a much safer VAD, by utilizing design approaches developed at Penn State for long term VADs. The device has been successfully tested in animals, but using cannulae (connecting tubes) designed for animals, and for durations of only one month. This project supports the next steps in development: design and testing of new cannulae specifically for use in infants, and testing in animals for 2 months duration. Our long term goal is to provide a VAD which is capable of

safely supporting infants for periods up to one year while they wait for heart transplantation or recover their native heart function.

Summary of Research Completed

The objective of this research is to continue development of the Penn State Pediatric Ventricular Assist Device (PVAD), focusing on the development of a cannulae system that is suitable for clinical use. The Pediatric VAD is currently a device sized for infants less than one year of age, with a stroke volume of approximately 12 cc.

During this past year, we completed the design and fabrication of cannulae and connectors, and performed 2 chronic animal implantations of the PVAD (described below).

During this year, we were awarded, with Minnetronix, Inc. (St. Paul, MN) a Small Business Innovation Research grant award from the National Institutes of Health to develop a portable pneumatic driver for the PVAD. The grant funding will begin in July, 2011. We also submitted an R01 grant proposal and SBIR proposal to NIH to fund further PVAD and cannulae development, but these grants were not funded.

Specific Aim 1. To design and fabricate cannulae for the Penn State Infant Ventricular Assist Device (VAD) for use in human infants, utilizing an innovative ePTFE graft construction to provide flexibility, kink resistance, and biocompatibility.

During the past year, we have taken the proposed cannulae design and developed detailed requirement and specifications. As shown in Table 1, there are 3 different cannulae under development: arterial, apical, and atrial.

A unique aspect of our approach is the use of expanded polytetrafluoroethylene (ePTFE). ePTFE grafts are routinely used for small diameter applications (6-10 mm inner diameter), including arteriovenous conduits for vascular access in dialysis, and as femoral and popliteal artery grafts. In pediatrics, 3-6 mm ePTFE grafts are used as extra-cardiac total cavopulmonary connections, and as systemic-to-pulmonary artery shunts.

The advantages of ePTFE as a vascular graft material are related both to structure and surface chemistry. The porous microstructure consists of fibrils oriented in the axial direction (expansion direction). The pore size is defined by the fibril length, or internodal distance, which ranges from 20-90 μm . The porosity appears to support neointima formation and transmural capillary ingrowth.

The anisotropy allows tensile expansion and compression in the axial direction during bending, so that the graft can be flexed with low force, without buckling. This feature is important in the VAD application. A disadvantage of early grafts was plasma weeping resulting in perivascular seromas; however, newer constructions include hydrostatic layers. Other improvements include integral reinforcing rings (e.g. Gore Intering) and additional layers to increase suture retention, increase radial burst strength, and prevent needle hole bleeding. Chemically, ePTFE is extremely stable and resists biodegradation. It has been shown to be anti-thrombogenic and exhibits low

platelet activation and inflammatory response.

During the past year we completed 3D Solidworks models, engineering drawings, and specifications for the arterial and apical cannulae and connectors. We evaluated a number of ePTFE graft types provided by Atrium Medical Corp. We built 2 complete sets of arterial and apical cannulae, titanium connectors and apical nuts, and Delrin collets for animal testing. The atrial cannulae prototypes will be completed by the end of the project period.

Specific Aim 2. To perform two chronic animal studies of 8 week duration, to test the Infant VAD with the newly developed cannulae, by assessing thrombogenicity of the VAD/cannulae system at the extended (8 week) duration.

Methods. Briefly, the PVAD is implanted in juvenile sheep weighing 20-25 kg. The surgical approach is via a right thoracotomy. The inlet cannula is inserted into the left ventricular apex, while the outlet cannula is sewn to the descending thoracic aorta. Cardiopulmonary bypass is not used. The pump is implanted in the left preperitoneal space. The PVAD supplies approximately 2/3 of the animal's total output. All animal studies were approved by the Institutional Animal Care and Use Committee at Penn State College of Medicine. The animals were housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and veterinary care adhered to *The Guide for the Care and Use of Laboratory Animals*.

A right external jugular catheter was placed at least 2 weeks before surgery to allow platelet function to stabilize, so that the pre-operative measurements reflect normal baseline conditions. Activated partial thromboplastin time (aPTT) and antithrombin III (ATIII) were measured on a CA1500 coagulation analyzer (Dade Behring, Newark, DE) using functional clot-based assays. Heparin levels were measured on the CA1500 using a chromogenic anti-Xa assay. Thromboelastography (TEG) was used to measure the global coagulation kinetics and clot strength. Platelet aggregation was measured using whole-blood impedance platelet lumi-aggregometry (Chrono-log Whole-Blood Lumi-Aggregometer 560-CA with AGGRO/LINK® 5.1, Chrono-log Corp, Haverton, PA, USA). Activation agonists were adenosine diphosphate (ADP) (10µM) and collagen (1 µg/ml). Impedance was recorded 6 minutes after the addition of agonist.

Anticoagulation was by intravenous heparin at approximately 20 units/kg/hr, titrated to achieve a TEG R time of 2 times normal.

The Infant VAD was operated in automatic full-to-empty mode in all cases, using the air flow fill detection system. The automatic mode was set for an end-diastolic delay of 30 msec, which was found in previous *in vitro* studies to minimize hemolysis related to inlet valve closing. The diastolic drive pressure (vacuum) was adjusted to give a mean VAD flow rate of 0.8 – 1.2 liters/min. A fixed systolic drive pressure of 250 mmHg was used and the systolic duration was adjusted as needed for complete ejection with an end-systolic delay of 10-30 msec.

Results. Two animal studies were performed on March 10 and March 13, 2011.

Experiment 1. Animal # 403

This study utilized a 22.7 kg male Dorset-Finn lamb. The implantation and post-operative course were uneventful. The PVAD functioned normally. The study was electively terminated on post-operative day 35.

Figure 1 shows a portion of the hematological data. Note that the heparin levels are quite low, as the aPTT is only slightly elevated above pre-operative levels. Fibrinogen and WBC show a typical acute phase response. The plasma hemoglobin data in Figure 2 shows that hemolysis is not evident. Albumin and total protein are reduced post-operatively but return to normal.

At necropsy, the aortic anastomosis was clean and well-healed, with no grossly evident thromboemboli within the outlet cannula or the aorta to the iliac bifurcation. The inlet cannula was well-positioned in the left ventricular with no obstruction. There were no grossly evident thromboemboli within the cannula lumen. Histologically, there was variable filling of the interstitial spaces of the ePTFE with fibrin and erythrocytes. There was no fibrovascular tissue or organization present. No nuclei were observed.

A grossly observed lesion in the left kidney was consistent with a chronic cortical infarction of unknown duration. The lesion was at least 2-3 weeks old. There were microscopic lesions consistent with (but not specific for) infarction present in both kidneys. Scattered mild individual chronic glomerulosclerosis has been noted in acutely implanted lambs, consistent with a background lesion.

In summary, there was no clinical evidence of thromboembolism or end organ dysfunction during the post-operative course. The new cannulae were judged to be easy to implant, although some improvement is needed for the tools to hold the pump connectors during attachment. The necropsy results indicated excellent biocompatibility. The renal infarcts were minor, of uncertain origin, and consistent with findings in previous studies.

Experiment 2. Animal #424

This study utilized a 20.5 kg male Dorset-Finn lamb. There was difficulty achieving normal tidal volumes after intubation. There was high resistance to inspiratory and expiratory flow, requiring an increase in peak inspiratory pressure. Tidal volumes gradually improved and the implantation was performed. However, respiratory function was not adequate post-operatively, and the animal was euthanized.

Summary. We successfully completed the design of the apical and arterial cannulae for the Infant PVAD. Prototypes were manufactured and tested *in vivo*. Despite the loss of one animal, the results of the successful study demonstrated that the new cannulae are suitable for surgical use, and that thromboembolism is very low, even with low levels of heparin anticoagulation. We do not anticipate any major changes to the cannulae or connector designs at this point.

Table 1. Cannula types to be utilized for uni- and bi-ventricular support modes.

	Inlet Cannulation		Outlet Cannulation	
	Location	Cannula Type	Location	Cannula Type
LVAD	Left ventricle apex	Apical (straight)	Ascending aorta	Arterial
	Left atrium	Atrial (curved)		
RVAD	Right atrium		Pulmonary artery	
Single ventricle	Single ventricle apex	Apical (straight)	Ascending aorta	

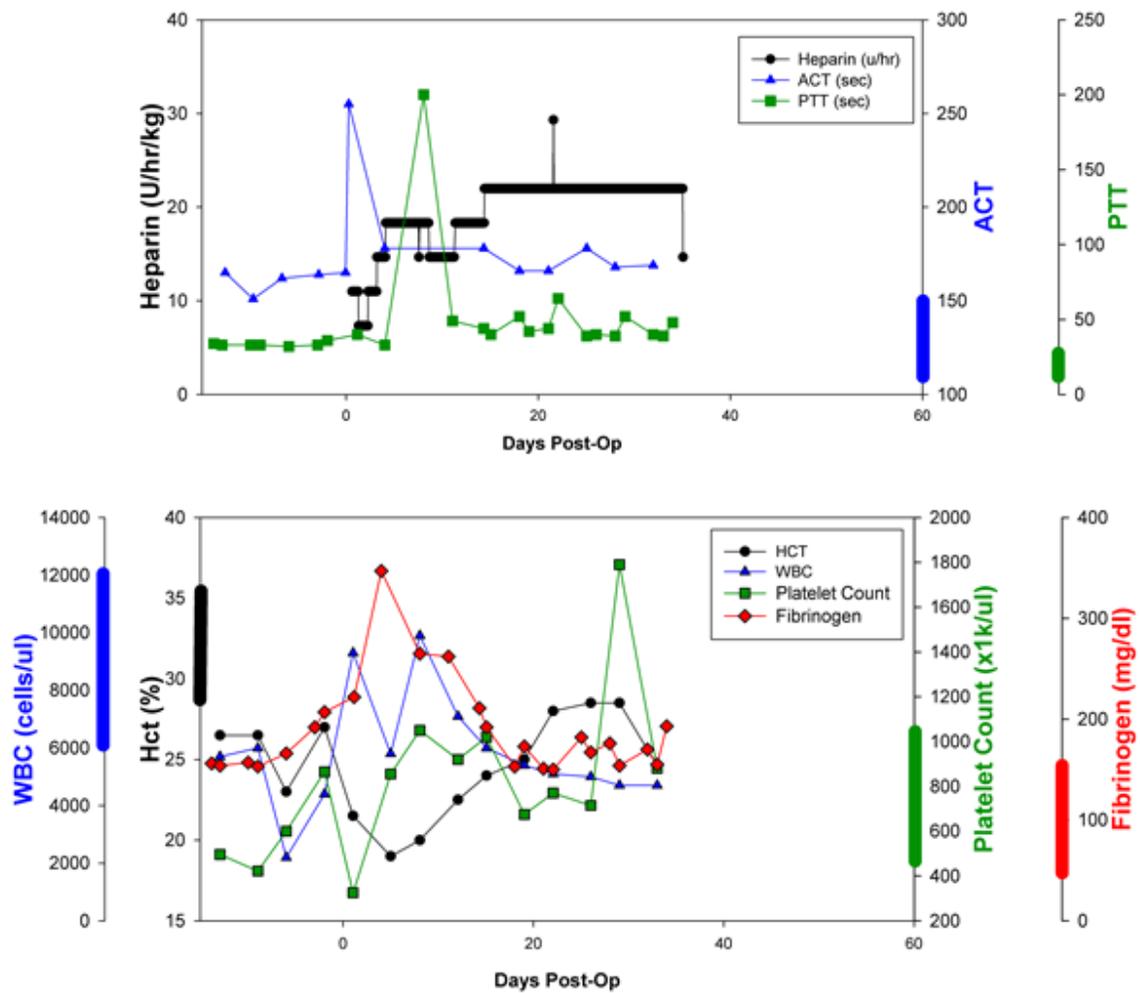


Figure 1. Hematological data for experiment #1.

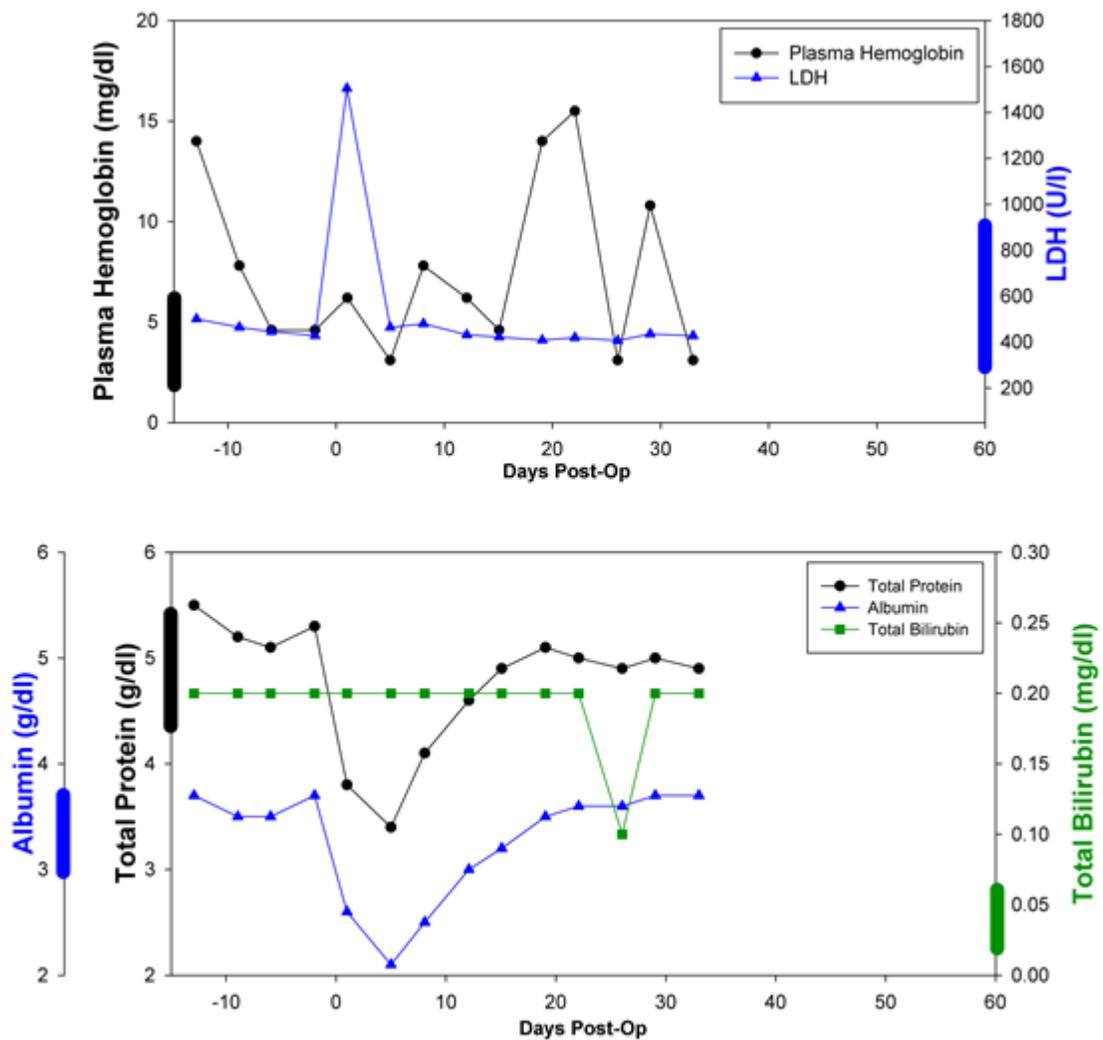


Figure 2. Plasma free hemoglobin and LDH remain within normal ranges (vertical bars are +/-1 standard deviation of pre-operative values pooled from all animals.)