Philadelphia College of Osteopathic Medicine

Annual Progress Report: 2008 Formula Grant

Reporting Period

January 1, 2012 – December 31, 2012

Formula Grant Overview

The Philadelphia College of Osteopathic Medicine received $17,036 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

Dissecting the Apoptosis Pathway Affected by C. pneumoniae in Alzheimer's Disease - 

Alzheimer’s disease (AD) is thought to be the leading cause of dementia in the elderly population, and is recognized clinically in early-onset (familial) and more prevalent late-onset forms, both of which manifest similar neuropathologies. This project will examine the detailed events that lead to neuronal cell death in late-onset AD, specifically the role in initiating these events played by the bacterium Chlamydia pneumoniae (Cpn), a common pathogen that infects the respiratory system and has been found in the autopsied brains of late-onset AD patients. We focus on the apoptotic process that has been implicated in the death of neurons in AD. Understanding these mechanisms may lead to treatment strategies for the growing numbers of AD patients.

Duration of the Project

1/1/2009 – 12/31/2012

Project Overview

There is increasing evidence to suggest that apoptosis plays a role in neurological disorders such as Alzheimer’s disease (AD). Recent developments in understanding the mechanisms leading to neuronal cell loss observed in AD have implicated apoptosis as being the link interconnecting amyloid and tau pathologies. The apoptosis hypothesis proposes that β-amyloid activates caspases that cleave tau as has been shown in recent studies. In addition, β-amyloid-induced neuroinflammation is recognized as a major factor in AD etiology. Consistent with these data, we hypothesize that a pathogen such as Chlamydia pneumoniae (Cpn) stimulates β-amyloid processing or production in neuronal cells, leading to synaptic dysfunction, neuroinflammation and apoptosis in neighboring cells, while the infected host is paradoxically protected from apoptosis.
We have preliminary data suggesting that neuronal cells infected with Cpn have increased levels of β-amyloid, specifically oligomeric forms which may be amyloid β-derived diffusible ligands, or ADDLs, shown to disrupt synaptic function in neurons. In our most recent publication we have demonstrated that Cpn can sustain a chronic infection by inhibiting aspects of the apoptotic process in neuronal cells.

Within the scope of this project, we will further analyze the cellular mechanisms by which Cpn inhibits apoptosis. It is our aim to examine the mitochondrial cytochrome c pathway since mitochondrial dysfunction has been implicated in AD. We hypothesize that Cpn mediates a cellular process that blocks the mitochondrial cytochrome c release and thus inhibits the biochemical process of apoptosis. Our approach will include immunocytochemistry and flow cytometry to analyze for mitochondria cytochrome c release following an infection with Cpn in human SHSY-5Y human neuronal cells from ATCC (American Type Culture Collection). Specific antibodies will be used to screen for the Cpn infection and the cytochrome c in this paradigm. Additionally, a cytochrome c inhibitor, methazolamide will be used to determine if an alternate pathway is being utilized by Cpn in the neuronal cell. This research will add to our knowledge of factors affecting the apoptotic cellular mechanism observed in AD.

Principal Investigator

Denah M. Appelt, PhD
Professor
Philadelphia College of Osteopathic Medicine
4170 City Avenue
Philadelphia, PA 19131

Other Participating Researchers and Employers

Juliana Zoga, MS

Expected Research Outcomes and Benefits

Apoptosis, a form of cell death, is being investigated as one of the primary mechanisms leading to the death of neurons in Alzheimer’s disease (AD). Several risk factors have been identified in the development of sporadic AD such as neuroinflammation and mitochondrial dysfunction. Furthermore, the environmental pathogen *C pneumoniae* (Cpn) has been associated with AD, having been found in cerebrospinal fluid (CSF) and brains of patients diagnosed with sporadic AD. Cpn infection has been shown to cause mitochondrial dysfunction and to inhibit apoptotic pathways in other diseases such as atherosclerosis. In this regard, Cpn infection could underlie aspects of mitochondrial dysfunction through the modulation of apoptotic process seen in sporadic AD.

We have presented evidence that Cpn can sustain a chronic infection by inhibiting apoptosis in neuronal host cells. Our data suggest that Cpn may be a key factor in modulating neuronal cell death. Since the mitochondrial dysfunction is suspect in AD etiology and pathogenesis, the cytochrome c apoptotic pathway is thus a sensible target for investigation. The knowledge gained
from this project may be useful in targeting the key cellular alterations identified for the development of clinical therapeutic modalities aimed at treating or preventing AD.

**Summary of Research Completed**

The data from our previous experiments funded from this grant support our hypothesis that an acute infection from *Chlamydia pneumoniae* in neuronal cells can suppress or inhibit the initial stages of apoptosis. At the molecular level, apoptosis and autophagy initiate many genes common to both pathways as well as common proteins. In Alzheimer’s disease, the relationship between apoptosis and autophagy is complex, in that they are both involved in alternative cell-death pathways.

Autophagy has been linked to Alzheimer’s pathogenesis through its merger with the endosomal-lysosomal system, which also has been shown to play a role in aberrant amyloid processing. The exact role and level of autophagy regulation in the pathogenic mechanism of Alzheimer’s disease has yet to be determined. Studies have suggested that aberrant autophagy induction may result in an accumulation of autophagic vacuoles containing amyloid-β leading to the pathogenesis seen in Alzheimer’s disease. Infections with *Chlamydia pneumoniae* escape autophagolysosomal fusion and therefore subvert lysosomal degradation creating a host friendly environment for the intracellular pathogen. The focus of these studies is to determine if there is a relationship interconnecting protein processing and genes regulated in autophagy following *Chlamydia pneumoniae* infection in neuronal cells.

For the infection process, neuronal cells, SKNMC (ATCC, HTB-10), were plated at 3x10⁴ to 1x10⁵ on round 18mm coverslips (Fisher, 12-545-100) for immunofluorescence or 1x10⁵ for Westerns in 12 well plates (Bioexpress, T-3026-6) in total volume of 500µl growth media (ATCC, Eagle's Minimum Essential Medium 30-2003+10%FBS). The cells were infected at an MOI=1 with *Chlamydia pneumoniae* AR39 (ATCC, 53592) and incubated at 37°C for 24-72hrs.

For immunofluorescence, coverslips with attached cells were rinsed with HBSS and fixed with Cytofix/Cytoperm (BD Biosciences, 554722) for 30 minutes at RT. Cells were rinsed with PBS pH 7.4 / blocked with Perm/Wash (BD Biosciences, 554723) for 30 minutes at RT and rinsed with PBS. Cells were incubated with unconjugated or conjugated antibodies for 1hr at 37°C, rinsed in PBS. The unconjugated 1o antibodies were incubated with 2o antibodies for 1hr at 37°C, rinsed in PBS. Following PBS, coverslips were rinsed in distilled water, mounted with Fluoro-Gel II Mounting Media containing DAPI (EMS,17985-50) onto slides. 1o antibodies used were Lamp1(Abcam: Ab25630), Beclin-1 (Cell Signaling : 3738S), and *Chlamydia pneumoniae* (60-C19:Fitzgerald) . 2o antibodies used were Alexa Fluor 594 Goat Anti-mouse and rabbit Rhodamine (Invitrogen).

For Westerns analysis, cells lysates were prepared with Ripa buffer. Equal amounts of protein, as determined by Coomassie Plus Protein Assay (Pierce, Rockford, IL), were subjected to electrophoresis through 10-20% gradient polyacrylamide gels under reducing conditions, and transferred to nitrocellulose membranes (Invitrogen, IB3010-02). The blots were blocked with
PBS/Casein (Biorad, 161-0783) for 30 minutes at RT and incubated with 1° antibodies for 24hrs at 4°C, rinsed with PBS, and blocked with PBS/Casein for 30 minutes. The blots were incubated with 2° antibodies for 2hrs RT before processing with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34080) and captured with the Kodak Imaging System. 1° antibodies used were Beclin-1 (Cell Signaling : 3738S), ATG7 (Cell Signaling: 2631), ATG12 (Cell Signaling: 2010), and Chlamydia pneumoniae (10C27A:Fitzgerald). 2° antibodies used were HRP Goat Anti-mouse IgG.

Cells were analyzed by immunocytochemistry and Western immunoblotting using antibodies targeting *Chlamydia pneumoniae* and autophagy proteins, such as Lamp1, Beclin-1, Atg7, and Atg12. Immunocytochemistry and Western blot analyses indicate that autophagy was induced from 24 to 72 hrs post-infection in neuronal cells. By immunocytochemistry, punctuate Lamp1 labeling was localized principally in the axonal processes of uninfected cells as compared to infected cells at 24-72hrs post-infection; punctate labeling in infected cells was observed most predominantly in the cell’s perikaryon at 72hrs post-infection (figure 1). *Chlamydia pneumoniae* infected neuronal cells exhibited an increased immunostaining with Beclin-1 relative to the minimal labeling observed in uninfected cells at 72hrs post-infection (figure 2). By Western blot analysis, at 48hrs post-infection, there was an increase in immunolabeling with Beclin-1, Atg7, and Atg12 as compared to the uninfected neuronal cells (figure 3).

We have demonstrated that *C. pneumoniae* is capable of inhibiting apoptosis in neuronal cells, thereby prolonging the viability of the infected neuronal cell and maintaining a persistent infection. Data presented in this report give a rationale for alternative cell-death pathways such as autophagy that have been recognized in the pathology associated with AD. These data suggest an interconnection between infection and alterations in proteins associated with autophagy, as has been observed in AD. Therefore, from our data, we have generated a working model that integrates *Chlamydia pneumoniae* as the initiator of the alterations in autophagy and apoptotic pathways that could lead to neurodegeneration as observed in AD (Model 1).

![Model 1 Diagram](image)

**Model 1:** Infection with *Chlamydia pneumoniae* alters key mechanisms in the host cell, leading to effects in neighboring cells as well as gross neuropathology.
Figure 1: Neuronal cells were screened for the presence of autophagosomes following an infection with *Chlamydia pneumoniae* for 24 to 72hrs. An increase in autophagic vacuoles is observed within the cytoplasm of the infected neuronal cells that had been infected with *Chlamydia pneumoniae* (green) for 24 to 72hrs as compared to uninfected cells. Immunofluorescent labeling with Lamp1 antibody (red) was used to assess autophagic vacuoles.

Figure 2: *Chlamydia pneumoniae* (green) appeared to induce autophagy in neuronal cells that had been infected for 24 to 72hrs as revealed by the Beclin 1(red) immunofluorescence labeling.
Figure 3: Western analysis of neuronal cells infected with *Chlamydia pneumoniae* for 48hrs at an MOI of 1. Infected cells demonstrated an increase in immunostaining for autophagy proteins ATG7, Beclin, and ATG12. The cells lysates were screened for the presence of a *Chlamydial* infection as observed by the 60Kd band.