Philadelphia College of Osteopathic Medicine

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

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.

Anticipated 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 pneumonia*e (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 mitochondria cytochrome c pathway since mitochondrial dysfunction has been implicated in AD. It is our hypothesis 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

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Other Participating Researchers and Employers

None

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

Mitochondrial damage is a major characteristic of apoptosis and has been identified in the pathogenesis of Alzheimer's disease (AD). Since apoptosis has been recognized as a form of cell death in AD, experiments performed in this reporting period from July 1, 2009 through June 30, 2010 were aimed at analyzing the mechanisms involved in the mitochondrial damage and in particular the cytochrome c pathway associated with apoptosis in neuronal cells infected with *Chlamydia pneumoniae*. We have previously demonstrated that *Chlamydia pneumoniae* renders neuronal cell resistant to apoptosis, however the cellular targets have not been identified in neuronal cells infected with *Chlamydia pneumoniae*. SKNMC neuronal cells have been substituted for the SH5YSH neuronal cells in order to maximize efforts by correlating ongoing studies that are complimentary to the experiments proposed in this project.

In these studies, apoptosis was experimentally induced with 1μM staurosporine for 4hrs in neuronal cells (SK-N-MC's) that had been infected with *Chlamydia pneumoniae* at an MOI of 1 for 72hrs as well as cells that had not been infected. These cells were analyzed for cytochrome c release which is a direct consequence of mitochondrial damage by using an antibody specific to cytochrome c. The immunolabeled cells were then screened by immunofluorescent microscopy as seen in figure 1. Cytochrome c appeared immunolocalized in a punctate pattern in uninfected neuronal cells as well as the neuronal cells infected with *Chlamydia pneumoniae*. However, the uninfected neuronal cells that had been treated with staurosporine and were undergoing apoptosis had a more diffuse labeling pattern. These preliminary data suggest that *Chlamydia pneumoniae* may be involved in blocking cytochrome c release and/or protect the mitochondria from cellular damage.

Since immunocytochemistry is a qualitative measure of cellular events, western analysis was utilized to determine quantitatively if caspase proteins which are activated by cytochrome c were altered in neurons infected with *Chlamydia pneumoniae* (see figure 2). Neuronal cells were infected with *Chlamydia pneumoniae* for 72hrs and uninfected neuronal cells were treated with 1μM staurosporine for 4hrs and then screened by western analysis for caspase activation using antibodies specific to caspase cleavage and activated caspase. Following incubations with staurosporine, neurons that have been infected with *Chlamydia pneumoniae* resulted in a diminished banding pattern with the caspase antibodies as compared to the robust banding pattern from neurons that had not been infected with *Chlamydia pneumoniae*. As a confirmation of the *Chlamydial* infection within the neuronal cell, cell lysates were screened with an anti-*Chlamydial* antibody (which recognized a single band at ~60KDa). A baseline of caspase activity was not evident from the neurons that had not been exposed to staurosporine in both the infected as well as the uninfected neurons. These data suggest that an infection with *Chlamydia pneumoniae* at 72hrs in neuronal cells either directly or indirectly inhibits a cascade of catalytic activation of caspase at the onset of apoptosis.

To further investigate changes in apoptosis related proteins within our experimental paradigm, studies were performed using human apoptosis antibody arrays (RayBio®; AAH-APO)(see

figure 3). These protein arrays are a nitrocellulose based membrane system spotted with 43 antibodies that recognized apoptosis related proteins. The advantage of this technology allows us to focus on those proteins that appear to be altered in *Chlamydia pneumoniae* infected neuronal cells. These studies focused on the 72hr infection with CPn in neuronal cells and apoptosis was induced with 1µM staurosporine for 4hrs. The results of this apoptosis antibody array revealed that an infection with CPn in neuronal cells does lead to alterations in several pro and anti apoptotic proteins. Proteins that are involved either directly or indirectly with the cytochrome c pathway such as the BCL family, caspases and cytochrome c were decreased in CPn infected cells as compared to uninfected cells. These data confirm our hypothesis that cytochrome c pathway following a *Chlamydial* infection has a physiological relevance in the apoptotic cascade implicated in AD.

Our findings continue to suggest that inhibition of apoptosis may be one of the pathogenetic mechanisms by which a C*hlamydia pneumoniae* infection can mediate the development of chronic diseases such as AD.

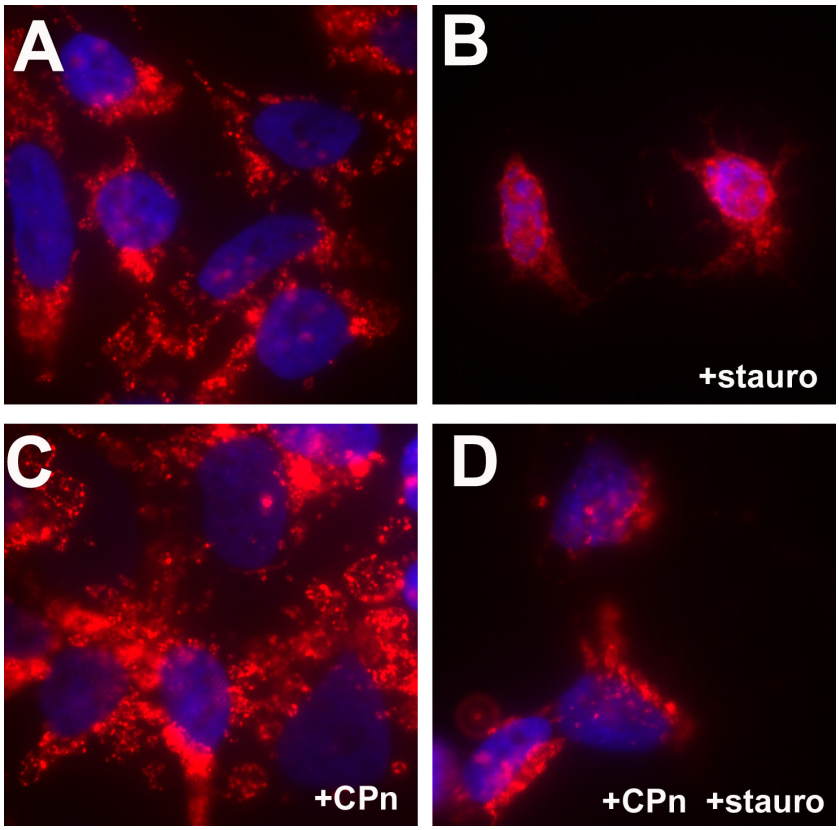

Figure 1: Human neuronal cells (SK-N-MC; HTB-10 ATCC) were infected for 72hrs with *Chlamydia pneumoniae* at an MOI of 1 followed by incubation with 1µM staurosporine for 4 hrs at 37°C in 5% CO_2 and then immunoreacted for cytochrome c (red labeling; Promega #G7421). Note the punctate labeling localized to the mitochondria in neurons that were infected with *Chlamydia pneumoniae (*panel C) as compared to those neurons that were not infected (panel A). Neurons that were infected with *Chlamydia pneumoniae* and exposed to staurosporine (panel D) revealed a more punctate labeling in the mitochondria whereas the neurons that were not infected but exposed to staurosporine (panel B) , displayed a more diffuse cytochrome c immunoreactivity. The nucleus (blue) of neurons infected with *Chlamydia pneumoniae (panel C)* and treated with staurosporine (panel D) maintained a normal nuclear profile as compared to the uninfected neurons incubated with staurosporine (panel B). The nuclear blebbing demonstrates the classic morphology associated with a cell undergoing apoptosis.

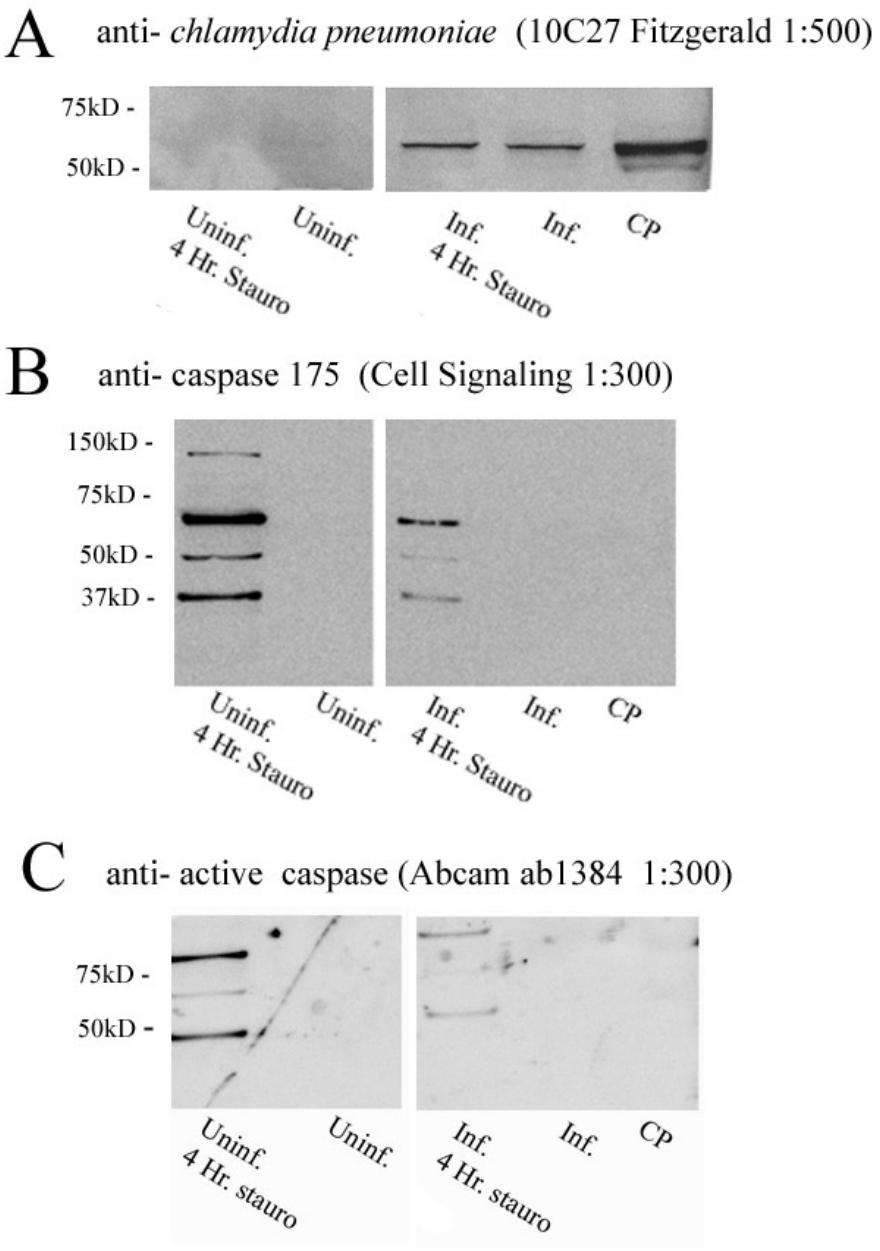

Figure 2**:** Representative Westerns showing caspase activation in human neuronal cells (SK-N-MC;HTB-10 ATCC) that were infected for 72hrs with *Chlamydia pneumoniae* at an MOI of 1 followed by an incubation with 1µM staurosporine to induce apoptosis for 4 hrs and 6hrs. A monoclonal antibody specific for *Chlamydia pneumoniae* was used to verify the chlamydial infection from the cellular lysates of the neuronal cells (panel A). Complexes of caspase are evident in uninfected cells induced with staurosporine into apoptosis at 4 hrs (panels B and C). Cells that have been infected and treated with staurosporine appear to inhibit caspase processing as compared to the uninfected cells treated with staurosporine (panels B and C). Cells were processed in Ripa lysis buffer and 5ug to 10 ug of total protein was prepared for electrophoresis on a 10-20% Tris Glycine gel from Biorad., then transferred onto nitrocellulose membranes.

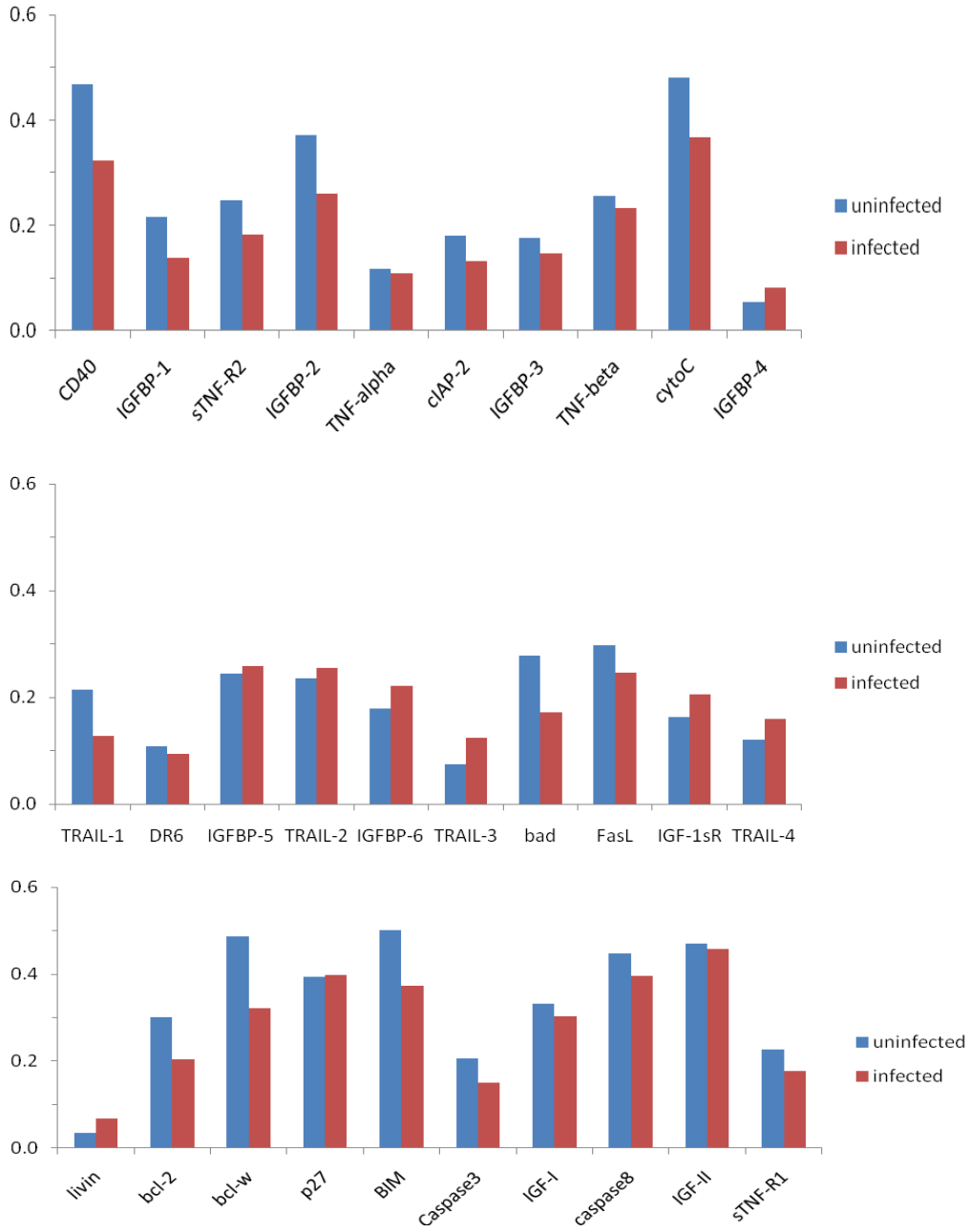

Figure 3: RayBio® human apoptosis antibody array (AAH-APO) was used to screen for apoptosis related proteins. Neuronal cells (SKNMC) were infected with *Chlamydia pneumonia*e at an MOI of 1 for 72hrs and incubated with 1µM staurosporine for 4 hrs. The chlamydial infection appeared to alter several apoptotic related proteins that are both pro and anti-apoptotic. A total of 400ug of cell lysate was used for this assay.