Effects on the apoptotic marker Cytochrome c following a chlamydial infection in neurons and astrocytes: Implications for Alzheimer's Disease

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Abstract

Neurodegeneration has been well documented in the CNS of Alzheimer individuals and evidence suggests that apoptosis may be a contributing factor in the pathogenesis of Alzheimer's disease. Initiating events that occur in apoptosis have been identified in Alzheimer brains; however, completion of apoptotic processes is not well understood. In earlier studies *Chlamydia pneumoniae* which is an intracellular respiratory pathogen was identified and isolated from brains of patients that had been diagnosed with sporadic AD [1]. Our initial hypothesis suggested that *Chlamydia pneumoniae* could involve the apoptotic process. *Chlamydia pneumoniae* has been found to inhibit apoptosis in neuronal cells [2] and monocytes [3], although the precise apoptotic pathway inhibited is-ill defined. Inhibition of apoptosis may be one mechanism by which *Chlamydia pneumoniae* can sustain an infection in the host to maintain an optimal intracellular environment. This infection may influence the contradictory findings of the completion of the apoptotic process in the Alzheimer's brain.

Given the previous data, our current hypothesis is that astrocytes and neuronal cells infected with *Chlamydia pneumoniae* can sustain an infection that renders the cells resistant to apoptosis. Since mitochondrial damage has been identified in the pathogenesis of Alzheimer's disease, the focus of this study was to determine whether cytochrome c, an electron carrier protein that is essential to the mitochondrial repiratory process, was affected following *Chlamydia pneumoniae* infection of astrocytes and neuronal cells. Following damage to the mitochondria, Cytochrome c is typically translocated from the mitochondria to the cytoplasm whereby cytochrome c activates the apoptotic process . In our studies, apoptosis was experimentally induced by staurosporine in astrocytes and SK-N-MC neuronal cells that were both uninfected and infected with *Chlamydia pneumoniae* for 72hrs. Cytochrome c production was analyzed by immunofluorescent microscopy utilizing an antibody specific to cytochrome c. Our results suggest that *Chlamydia pneumoniae* infected neuronal cells differentially activate cytochrome c as compared to infected astrocytes. In both infected neurons and astrocytes, induction of apoptosis with staurosporine did not appear to induce the apoptotic event. Thus our data appear to indicate that an infection of both cell types blocks apoptotic induction with staurosporine which may be independent of the cytochrome c pathway.

Introduction

Neurodegenerative disorders such as Alzheimer's disease (AD) are characterized by the chronic deterioration of synaptic function and a progressive loss of cortical neurons. Cytoskeletal changes occur within cortical neurons such as the formation of paired helical filaments (PHFs) into neurofibrillary tangles (NFTs), signature pathologies of AD. Accompanying these structures are extracellular deposits of β-amyloid, in particular the Aβ -1-42 peptide that provides a nidus for the formation of senile plaques.

Recently, apoptosis has been implicated as a mechanism in the degeneration of selective neuronal populations in AD. Several studies have shown that a large percentage of cells contain DNA fragmentation and an incomplete cell cycle activation in post-mitotic neurons in AD brains [4]. Other reports confirm that the AD brain provides a pro-apoptotic environment, though has been seen no evidence of the apoptotic process leading to terminal completion[5]. While a plethora of evidence validates neurodegeneration as a major etiology in AD, the initiating event(s) or stimulus has not yet been identified in the sporadic form of this disease. One plausible candidate is Aβ -1-42 peptide that has been found to induce apoptosis-related changes in neurons and is cytotoxic to neurons [5]. Resident CNS cells such as microglia, astroglia, and neuronal cells can generate Aβ -1-42 peptides when stimulated by proinflammatory molecules. However, stimuli other than Aβ -1-42 may trigger a proinflammatory response in the brain that could result in the production or processing of Aβ -1-42. These "triggers" have been ill defined with regard to the pathogenesis of sporadic AD.

In a previous study, *Chlamydia pneumoniae* was identified in areas of neuropathology from brains of individuals who had previously been diagnosed with sporadic AD [1]. More recently, in vitro studies suggest that neuronal cells are less vulnerable to apoptosis when infected with *C. pneumoniae* [2]. Previous studies have shown that monocytes also are resistant to apoptosis when infected with *C. pneumoniae* [3]. How *C. pneumoniae* infection affects the apoptotic process has yet to be fully elucidated though mitochondria appear to undergo stress and damage in both C. pneumoniae infections and Alzheimer's disease. Cytochrome c release from mitochondria has been postulated to be one mechanism by which *C. pneumoniae* may alter the apoptotic mechanism. Therefore, our *in vitro* studies have addressed infection of neurons and astrocytes with C. pneumoniae. We are specifically addressing the extent to which the viability of these cells are enhanced or compromised during an infection with *C. pneumoniae* as well as how infection affects the cytochrome c apoptotic cascade.

Methods

Tissue Culture
The SK-N-MC (HTB-10 ATCC) human neuroblastoma cell line was cultured in Eagle's Minimum Essential Medium (MEM) containing non-essential amino acids and 10% heat-inactivated FBS. The C8-DIA (ATCC CRL-2541) murine astrocyte cell line was cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS). All cell lines were maintained at 37°C in 5% CO_2.

Infection of Cells
Chamber slides (4 wells/slide CultureSlides (BD Falcon) were seeded with approximately 1 x 10^5 cells. Within 24 hours of seeding the chamber slides, cells were infected with *Chlamydia pneumoniae* AR-39 (ATCC) at an MOI of 1. Cells were then and incubated in 500µl of growth medium for 72 hours.

Induction of Apoptosis :
Cells were incubated in chamber slides in 1µM staurosporine solution (1mM stock staurosporine solution from Sigma Aldrich, St. Louis, MO. Cat # S6942) in growth media for 4 hrs at 37°C in 5%CO_2. The cells were washed with PBS at pH 7.4 and processed for immunofluorescence.

Immunofluorescence
Cells were rinsed with HBSS and then fixed with cytofix/cytoperm (BD Bioscience) for 30 min. The cells were washed in phosphate buffer saline (PBS) and then blocked in cytoperm (BD Bioscience)for 15 minutes at room temperature (RT). The slides were incubated with primary antibodies diluted in PBS for 1 hour at 37°C, then washed in PBS. Secondary antibodies diluted in PBS (if required) were added to the slides and incubated for 1 hour at 37°C. Following incubation, the slides were washed in PBS, and followed by distilled water and then incubated at RT for 5 minutes with Bis-benzimide. The slides were coverslipped with anti-fade aqueous mounting media (Biomeda).

Primary Antibody	Company	Dilution
Cytochrome C	Promega G7421	1:2000
Chlamydia 61C75	Fitzgerald	1:50

Secondary Antibody	Company	Dilution
Alexa Fluor 594 Goat Anti Mouse	Molecular Probes	1:2000

Image Capture:
Slides were viewed on a Nikon E800 epifluorescence microscope . Images were captured with a Spot RT camera (Diagnostic Instruments) and analyzed using Image Pro Plus 4.5 software (Media Cybernetics).

Results

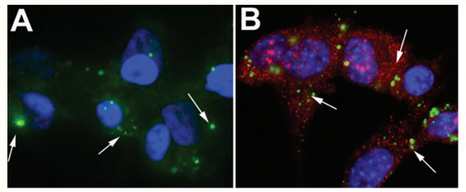

Figure 1: Neuronal cells (panel A) and astrocytes (panel B) infected with *Chlamydia pneumoniae* (green). Several inclusions (arrows) can be identified within the cells cytoplasm (red).

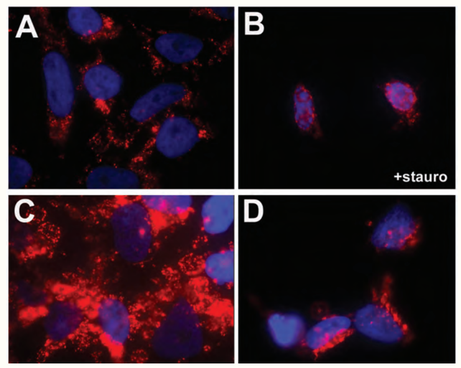

Figure 2: Neuronal cells were infected with *Chlamydia pneumoniae* (CPn) and analyzed for Cytochrome c labeling (red). Apoptosis was induced by staurosporine in neuronal cells that were both uninfected (panel B) and infected (panel D) with CPN for 72hrs. Note the increased labeling for cytochrome c in neurons infected with CPn in panel C as compared to the uninfected neurons in panel A. The neuronal cell infected with CPn (panel D) do not appear to be undergoing apoptosis as compared to the uninfected nuclear fragmentation in panel B following staurosporine included apoptosis. Nuclear profiles (blue).

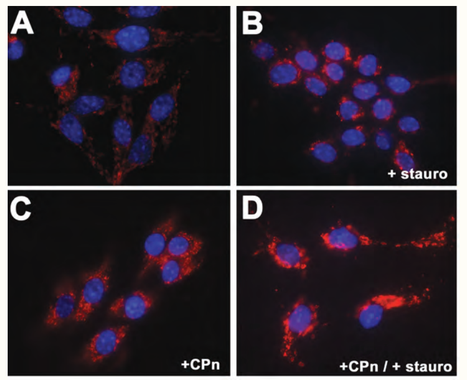

Figure 3: Astrocyte cells were infected with *Chlamydia pneumoniae* (CPn) and anaylized for Cytochrome c labeling (red). Apoptosis was induced by staurosporine in astyrocytes that were both uninfected (panel B) and infected (panel D) for 72hrs with CPn. The cytochrome labeling demonstrates an increase in cytochrome c protein in the cytoplasm in CPn infected cells (panel C and D) as compared to the uninfected cells (panel A and B). Nuclear profiles (blue).

Conclusion

- Induction of apoptosis with staurosporine did not appear to induce the apoptotic event in both infected neurons and astrocytes.
- Chlamydia pneumoniae infected neuronal cells differentially activate cytochrome c as compared to infected astrocytes.
- Infection of both neurons and astrocytes appear to block apoptotic induction with staurosporine which may be independent of the cytochrome c pathway.

References

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