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Chlamydophila (Chlamydia) pneumoniae promotes Aβ 1-42 amyloid processing in Neuronal Cells: A Pathogenic Trigger for Alzheimer's Disease

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Abstract

Background: Previously, our laboratory identified *Chlamydophila (Chlamydia) pneumoniae* (Cpn) in autopsied sporadic AD brains. Furthermore, we have developed a BALB/c mouse model that demonstrated infection-induced amyloid plaques similar to those found in AD, and demonstrated that Cpn infection of neuronal cells inhibited apoptotic pathways of cell death.

Hypothesis: Our current studies address whether infection with Cpn in neuronal cells triggers abnormal cleavage of the beta amyloid precursor protein (βAPP) into Aβ1-42, thereby contributing to amyloid plaque formation characteristic of the pathology identified in AD.

Materials and Methods: Human neuroblastoma cells were infected with the respiratory strain AR39 Cpn in vitro, then amyloid processing was analyzed and quantitated using immunocytochemistry, Western blotting and ELISA assays.

Results: Cpn was shown to infect neuronal cells and induce intracellular amyloid processing. Cpn infection yielded cytoplasmic labeling of Aβ 1-42 that was increased relative to uninfected cells. The ELISA assay revealed that in neuronal cell lysates, Aβ 1-42 in the infected cells was increased 3 to 16-fold over the uninfected cells, from 24 to 72hr post infection. Western blot analysis confirmed an increase in Aβ 1-42 in the infected neuronal cell lysates.

Conclusions: These data suggest that infection of neuronal cells with *Chlamydophila (Chlamydia) pneumoniae* alters the processing of βAPP, thereby producing Aβ1-42. Therefore, these studies and previous research reported by our laboratory support the implication of Cpn as a pathogenic agent in perpetuating the hallmark amyloid plaque formations observed in AD. This concept holds major therapeutic considerations for future studies.

Introduction

Amyloid plaques are a pathological feature of Alzheimer's disease (AD) that are formed by the abnormal deposition of fragments of amyloid precursor protein (APP). Proteolysis by secretases of APP near its carboxyl terminus leads to the generation of 40-43 amino acid peptides that comprise beta-amyloid (Aβ) (Selkoe et al., 1988). These peptides undergo conformational change into β-sheets that deposit into plaques in regions of the brain particularly susceptible to this damage. This deposition appears to be critical in the neuronal degeneration observed in AD (Schellenberg, 1995). There is a hereditary form of the disease, called Familial AD (FAD), that accounts for a small percentage (~5%) of total AD cases world-wide, while sporadic AD accounts for the vast majority of AD cases (estimates of 95% or more). Specific mutations have been recognized in FAD that are associated with the increased proteolysis of APP to form Aβ. However, with sporadic AD, few genetic factors have been implicated, and thus, environmental triggers for amyloid deposition are under investigation.

Infection within the central nervous system (CNS) by *Chlamydophila (Chlamydia) pneumoniae* has been suggested to play a role in sporadic AD. In two separate studies, polymerase chain reaction detected *C. pneumoniae* DNA in 80 - 90% of postmortem brain samples examined from sporadic AD (Balin et al., 1998, Gerard et al., 2006), but in only 5 - 11% of postmortem brain samples from age-matched, non-AD, control individuals. Furthermore, a murine model has been developed in which non-transgenic mice infected with *C. pneumoniae* demonstrate deposits of amyloid in areas of the brain typically affected in AD (Little et al., 2004).

The role that *C. pneumoniae* plays in "triggering" events resulting in AD pathology has been further analyzed following in vitro infection of a human neuroblastoma cell line, SK-N-MC- HTB-10. Experiments were designed to determine whether infection with *C. pneumoniae* influences the production and/or processing of Aβ amyloid oligomers from the beta amyloid precursor protein (βAPP), thereby associating *C. pneumoniae* infection with the formation of toxic Aβamyloid species that may contribute to the pathology of Alzheimer's disease.

Immunocytochemistry, using antibodies specific for the 1-42 fragment of Aβ, suggested that infection in neuronal cells resulted in an increase in Aβ 1-42 immunoreactive peptides. The results from these studies indicated that intracellular amyloid processing was induced in neuronal cells infected by *C. pneumoniae* producing cytoplasmic labeling of 1-42 β-amyloid oligomers. ELISA assays revealed that in neuronal cell lysates, 1-42 β-amyloid in the infected cells was increased 3 to 16-fold over the uninfected cells from 24 to 72 hrs post-infection, Western blot analysis confirmed an increase in higher molecular weight (>50kDa) 1-42 β-amyloid in the infected neuronal cell lysates at all time points post-infection.

These data suggest that infection of neuronal cells with *C. pneumoniae* affects the processing of βAPP into 1-42 β-amyloid higher molecular weight species presumably representing Amyloid beta derived diffussible ligands (ADDLS). Therefore, these studies and previous research support the implication of *C. pneumoniae* as a pathogenic agent that can perpetuate the production of neurotoxic amyloid found in Alzheimer's disease.

Methodology

Tissue Culture

SK-N-MC, neuroblastoma cells (ATCC HTB-10) were cultured in minimum essential medium (MEM) (ATCC 30-2003) containing 10% FBS and maintained at 37°C in 5% CO_2.

Infection of Cells/Chamber Slides

Chamber slides (4 wells/slide Culture Slides)(BD Falcon cat# 354114) were seeded with 1 x 10^5 cells/well. Within 24 hours of seeding cells, were infected in 200μL growth media with *C. pneumoniae* AR-39 (ATCC cat # 53592) at an MOI of 1. The cells were centrifuged in a Sorvall Legend RT at 750Xg for 30 minutes at 20°C. The cells were incubated for 1 hour at 37°C in 5% CO_2. An additional 300μL of growth media was added to the cells and then incubated for 24, 48 and 72 hours at 37°C in 5% CO_2 prior to processing for immunocytochemistry.

Infection of Cells/Flasks

T25 flasks were seeded with 1 x 10^5 cells/mL. The cells were infected at the time of seeding with *C. pneumoniae* AR-39 at an MOI of 1. Neuronal cells were centrifuged in 3 mls of growth media with *C. pneumoniae* in a Sorvall Legend RT at 750X g for 30 minutes at 20°C. An additional 7 mls of growth media was added. The cells were then incubated at 37°C in 5% CO2 for 24, 48, and 72 hours.

Immunocytochemistry

Cells were rinsed with Hanks buffered salt solution (HBSS) and then fixed with cytofix/cytoperm (BD Bioscience, San Jose, CA cat # 554722) for 30 min. The cells were washed in phosphate-buffered saline (PBS) three times and then blocked in Perm Wash (BD Bioscience, San Jose, CA cat #554723) for 30 minutes at 20°C. The slides were incubated with primary antibodies in a 37∞C water bath for 1 hr, and then washed in PBS three times. For those antibodies not directly conjugated, secondary antibodies were added to the slides and incubated for 1 hour in a 37∞C water bath. Following incubation, the slides were washed in PBS and rinsed in distilled water two times and then incubated at 20°C for 5 minutes with Bis-benzimide or Hoechst stain (Dapi) (Sigma-Aldrich, St. Louis, MO B2261). The slides were washed in distilled water and cover slipped with anti-fading aqueous mounting media (Biomeda cat# M01).

Image Capture

Slides were viewed on a Nikon E800 epifluorescence microscope. Images were captured with a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using Image Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD).

Western Analysis

Cells were homogenized in lysis buffer (Sigma mammalian cells lysis kit; MCL-1) or Ripa Buffer (Thermo Scientific cat# 89901) and electrophoresed into a 10-20% gradient Tris-Tricine gel (Cat# 161-1160 BioRad) and transferred onto 0.45um nitrocellulose (Schleicher & Schuell). The transfers were blocked in 1% casein (Pierce cat #37352) for 30 minutes, incubated in primary antibody diluted in PBS for 2hrs at RT and then further blocked in 1% casein for 15 minutes at RT followed by incubation in secondary antibody diluted in 1% casein overnight at 4°C. The transfers were rinsed in PBS for 1hr and then water. They were developed in ECL solutions (Santa Cruz) and exposed to radiographic film.

ELISA Assay

The β-Amyloid (1-42) Elisa Protocol kit (Covance-Beta mark x-42, sig-38952) was utilized for quantitation of 1-42 amyloid in cell lysates. The standard curve, samples, working incubation buffer and wash buffer were prepared following a modified manufacturer's protocol. The samples were diluted up to 100ul in working incubation buffer. 300μL of wash buffer was added to each well and the fluid was decanted and the ELISA plate was patted dry. 100ul of each standard and 100ul of sample was added to the plate. The plate was covered and incubated at 4°C over night. The contents were then removed from the plate and the wells were washed by adding 300ul of wash buffer to each well. This was repeated for a total of 5 times. The detection reagent was modified for a HRP reaction and the HRP detection antibody was added to the working incubation buffer and incubated at 4°C over night. The wells were then rinsed with PBS 3 times. 50ul of tetramethylbenzidine (TMB) ELISA reagent was added to each well and incubated for 30 minutes at room temperature. 50ul/well of stop reagent was added to the plate. The solutions were pipetted into a clear bottom plate and read on a BioRad ELISA Reader at 450nm.

Results

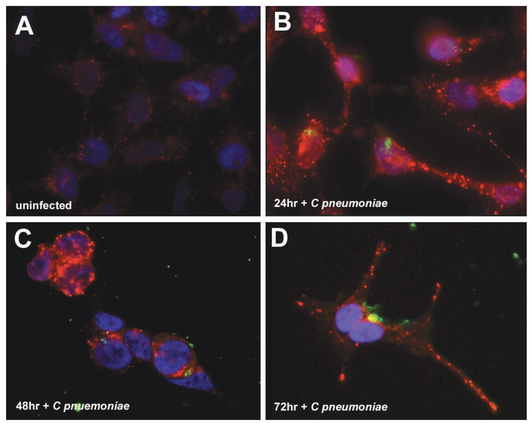

Figure 1: Neuronal cells infected with C. pneumoniae AR39 and immunolabeled for 1-42 β-amyloid using monoclonal antibody 6E10. Infected neuronal cells at 24 hrs (panel B), 48 hrs (panel C), 72 hrs (panel D) displayed similar fluorescence intensity in cytoplasmic labeling of 1-42 β-amyloid. The fluorescence intensity of 1-42 β-amyloid cytoplasmic labeling was diminished in the uninfected neuronal cells (panel A) relative to the infected neuronal cells. 1-42 β-amyloid polyclonal primary antibody (6E10) was labeled with secondary anti-mouse rhodamine (alexa fluor 594) (red) chlamydial inclusions were labeled with a monoclonal 60C19 Chlamydia FITC-conjugated antibody (green). Nuclei were labeled with Dapi stain (blue)

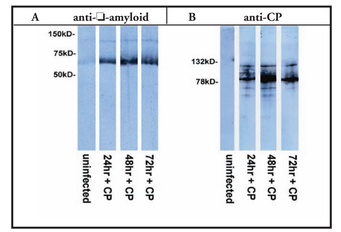

Figure 2:
A. Western blot analysis of neuronal cell lysates labeled with 1-42 β-amyloid antibody 75B. 1-42 β-amyloid is present in the uninfected and the infected neuronal cells. There is an increase of 1-42 β-amyloid in the infected AR39 neuronal cells compared to the uninfected neuronal cells.

B. Western blot of neuronal cell lysates labeled with CP antibody 10C27. 10C27 established the presence of CP in neuronal cells at 24, 48 and 72 hours that have been infected at an MOI of 1.

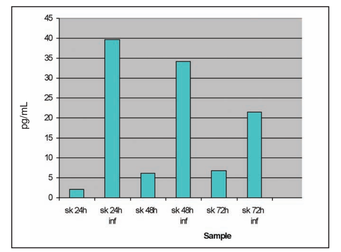

Figure 3: Quantitation of β-amyloid protein in infected/uninfected neuronal and astrocytes cells. The quantity of 1-42 β-amyloid is greater in the infected neuronal cells than in the uninfected cells. 1-42 β-amyloid is highest at 24 hour post infection in the neuronal cells, decreasing at 48 and 72 hours however remaining increased compared to time-matched uninfected neuronal cells.

Conclusions

- C. pneumoniae infection of neuronal cells was associated with increased cytoplasmic labeling of β1-42 amyloid
- Western blot analysis confirmed an increase in higher molecular weight (>50kDa) 1-42 β-amyloid oligomers in the infected neuronal cell lysates at 24 to 72 hrs post-infection
- The ELISA assay revealed in the infected cells an increase of 3 to 16-fold of 1-42 β-amyloid over the uninfected cells from 24 to 72 hrs post-infection

Increase of β1-42 amyloid following C. pneumoniae infection implicates this organism as a microbial trigger in the pathogenisis of AD.

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