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MORPHOLOGICAL AND PROLIFERATIVE RESPONSES OF RAT MICROGLIAL CELLS TO LIPOPOLYSACCHARIDE AND BETA AMYLOID

By

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June 2008

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Microglia are the residential macrophages of the central nervous system (CNS) and are sensitive to any changes in their environment. There is rapid microglial activation in most pathological conditions in CNS. The association of activated microglia with various inflammatory and neurodegenerative diseases are well documented. In this study we evaluated the *in vitro* responses of microglia to lipopolysaccharide (LPS) or beta amyloid (Aβ) in an attempt to further define the activation of microglia. LPS is a potent activator of monocytes and macrophages and Aβ is used to activate microglia *in vitro* into a neurotoxic phenotype distinguished by secretion of proinflammatory molecules. Microglia were cultured from Sprague-Dawley neonatal rats and purity of culture determined using a common marker for microglia, lectin. Morphological changes following LPS or Aβ treatment were evaluated using fluorescent and confocal microscope. Treated microglia assumed a

deramified shape with a condensed cytoplasm, typical of activated microglia. Stimulation of microglia with LPS or Aβ also resulted in significant ultrastructural changes. Transmission electron microscopy revealed an increased number of enlarged, elongated and swollen mitochondria. There were also some changes in the appearance of the endoplasmic reticulum. Untreated microglia displayed mainly smooth endoplasmic reticulum (SER) whereas LPS-treated cells displayed polyribosomes and rough endoplasmic reticulum (RER). It is therefore assumed that LPS-treated microglia have an increased ability in synthesising protein, some of which may be secretory molecules necessary for inflammation. Aβ-treated microglia also displayed more RER compared to control, but lesser compared to LPS. The nucleus in treated microglia appeared enlarged in comparison to untreated cells. The number of cells following treatment revealed that microglia are more viable following LPS treatment compared to Aβ. Immunophenotyping assays demonstrated upregulation of MHC II and CD40 by microglia following treatment. Both MHC II and CD40 are implicated in antigen-presenting and this result indicates that in comparison to LPS, Aβ may have more ability to induce antigen-presenting capabilities in microglia. As shown by viability counts, carboxy fluorescein succinimidyl ester (CFSE) staining also revealed no microglia proliferation following treatment with LPS and Aβ. In conclusion, our study illustrates activation-induced alterations in the morphology, ultrastructure and cell surface phenotype of microglia, which were not accompanied by proliferation of microglia. These changes represent the range of effects that occur in microglia following activation. In an attempt to culture microglia from adult rats for the purpose of determining the effects of

aging on microglia responses, modifications were made on the established protocol for neonatal microglia cultures. This includes growing adult cells on poly-L-lysine-coated tissue culture flasks and substituting insulin in cell culture media with insulin-transferrin-selenium (ITS). We were able to successfully support the *in vitro* growth of adult microglia, which was also enhanced with the addition of the growth factor M-CSF.

Key words: microglia, lipopolysacharide, beta amyloid, morphology, proliferation, CD40/MHC II

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

Respon morfologi dan pembahagian pada mikroglial sel tikus kepada lipopolisakarida dan amyloid beta

Oleh

ALIREZA BADIEI

Jun 2008

Pengerusi : Sharmili Vidyadaran, PhD

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Mikroglia merupakan makrofaj residen dalam sistem saraf pusat. Mikroglia dirangsang dengan cepat dalam kebanyakan keadaan patologi. Banyak penulisan saintifik yang membuktikan hubungan di antara mikroglia dengan inflamasi dan penyakit neurodegenerasi. Dalam kajian ini, kami menilai gerakbalas mikiroglia ke atas rawatan dengan *lipopolysaccharide* (LPS) dan *beta amyloid* (Aβ) secara *in vitro* untuk mengenalpasti cara pengaktifan mikroglia dengan lebih lanjut lagi. Mikroglia dikultur daripada tikus neonat Sprague- Dawley dan tahap ketulenan kultur ditentukan melalui lektin yang merupakan satu molekul penunjuk umum pada sel mikroglial. Perubahan morfologi mikroglia selepas rawatan dengan LPS dan Aβ ditentukan dengan menggunakan mikroskop fluoresen dan konfokal. Mikroglia yang dirawat dikenal sebagai sel yang kurang bercabang (deramified) dengan sitoplasma yang kondens. Ini adalah keadaan lazim bagi sel mikroglia yang telah menjadi aktif. Rangsangan dengan LPS dan Aβ juga menyebabkan

perubahan ultra-struktur dan rupabentuk yang ketara. Mikroskop elektron transmisi menunjukkan penambahan bilangan mitokondria yang membengkak, memanjang dan membesar. Terdapat juga perubahan rupabentuk pada reticulum endoplasma. Sel mikroglia yang tidak menerima sebarang rawatan mempunyai kebanyakannya retikulum endoplama yang licin (smooth endoplasmic reticulum, SER) dan apabila dirawat dengan LPS, sel-sel mempamerkan poliribisom (polyribosome) dan reticulum endoplasma kasar (rough reticulum endoplasmic, RER). Sel yang dirawat dengan Aβ juga mempamerkan lebih banyak reticulum endoplasma kasar berbanding dengan sel kawalan. Akhirnya, sel yang dirawat juga mempamerkan pembesaran nukleus berbanding dengan sel yang tidak dirawat. Asei "immunophenotyping" menunjukkan penambahan ekpresi molekul MHC kelas dua dan CD40 pada permukan sel mikroglial sejurus menerima rawatan. Tiada proliferasi diperhatikan selepas rawatan sel dengan LPS dan Aβ, seperti yang telah ditunjukan melalui pewarnaan CSFE. Ringkasnya, kajian kami telah menunjukkan perubahan ultrastruktur dan rupabentuk serta "fenotip" permukaan sel-sel mikroglial sejurus selepas sel-sel tersebut dirangsang. Perubahan-perubahan ini bukanlah disebabkan proliferasi sel. Semua perubahan ini mewakili pelbagai easan yang terjadi kepada sel mikroglial selepas diaktifkan. Untuk pengkulturan sel mikroglia dewasa bagi tujuan kajian ke atas kesan penuaan kepada mikroglia, beberapa modifikasi telah dijalankan kepada protokol pengkulturan mikroglia daripada tikus muda. Pada amnya, ini termasuk pengkulturan mikroglia dewasa di dalam kelalang kultur tisu yang terlebih dahulu disaluti dengan poly-L-lysine dan menggantikan insulin di dalam media kultur sel dengan insulin-transferrin-

selenium (ITS). Kami berjaya mengkultur sel mikroglia dewasa, dan ini dibaiki lagi dengan penambahan faktor pertumbuhan, M-CSF.

Kata kunci: mikroglia, lipopolisakarida, amyloid beta, morfologi, proliferasi, CD40/MHC II

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I certify that an examination Committee has met on 26/11/2008 to conduct the final examination of ALIREZA BADIEI on his Master of Science thesis entitled “MORPHOLOGICAL AND PROLIFERATIVE RESPONSES OF RAT MICROGLIAL CELLS TO LIPOPOLYSACCHARIDE AND BETA AMYLOID” in accordance with Universiti Pertanian Malaysia (higher Degree) Regulation 1981. The Committee recommends that thestudent be awarded the Master of Science.

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This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the supervisory Committee were as follows:

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Date: 12 February 2009

DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

ALIREZA BADIEI

Date:

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LIST OF ABBREVIATIONS

A	Alpha
Γ	Gamma
CNS	Central nervous system
Aβ	Beta amyloid
LPS	Lipopolysaccharide
AD	Alzheimer's disease
PD	Parkinson's Disease
MS	Multiple sclerosis
EM	Electron microscopy
APC	Antigen presenting cells
RER	Rough endoplasmic reticulum
SER	Smooth endoplasmic reticulum
TEM	Transmission electron microscopy
GM-CSF	Granulocyte macrophage stimulating factor
M-CSF	Macrophage colony stimulating factor
TLR	Toll like receptor
Gp	Glycoprotein
IL	Interleukin
IFN	Interferon
°C	Degrees centigrade
µl	Microlitre
BSA	Bovine serum albumin

EDTA	Ethylene-diamine-tetra-acetic acid
g	Gram
H	Hours(s)
Min	Minute(s)
Ml	Millilitre
NO	Nitric oxide
ROS	Reactive oxygen species
CFSE	Carboxyfluorescein diacetate succinimidyl ester
FACS	Fluorescence activated cell sorting
MHC	Major histocompatibility complex
CD	Cluster of differentiation
FITC	Fluorescein isothiocyanate
%	Percentage
µg	Microgram
Th	T helper
U	International unit
DC	Dendritic cell
DNA	Deoxyribonucleic acid
NFkB	nuclear factor kappa beta

CHAPTER 1

INTRODUCTION

1.1. Background

Microglia are the residential macrophages of the central nervous system (CNS). Their immunological roles include phagocytosis of invading microorganisms and removal of dead cells within the CNS. In the normal CNS, microglia are in a resting state, however they quickly become activated following any stress or injury to the CNS.

Ramified microglia, represent the resting state of the cells, are found throughout the healthy CNS. They are composed of long branching processes and a small cellular body. While the branches of resting ramified microglia are constantly moving and surveying the surrounding area, the cell body remains fairly motionless. The branches are very sensitive to small changes in physiological condition. Unlike activated or amoeboid microglia, ramified microglia are unable to phagocytose cells (Aloisi, 2001; Christensen *et al.*, 2006). In addition to being very sensitive to small changes in their environment, each microglial cell also physically surveys its domain on a regular basis. While moving through CNS regions, the microglial cell scavenges for foreign material, damaged cells, apoptotic cells, neural tangles, DNA fragments, or plaques. It will then become activated and phagocytose the material or launch inflammation if necessary. In this manner microglial cells also act as “housekeepers”, cleaning up random cellular debris (Aloisi, 2001).

Following activation, microglia proliferate, express MHC class I and II antigens, cell adhesion molecules, release inflammatory cytokines and become phagocytotic cells (Gehrmann *et al.*, 1995). Injury to neurones leads to activation of microglia, which causes cellular hypertrophy, decrease in ramification and cellular proliferation (Olesen *et al.*, 2007).

It is believed that hyperactivation of microglia may however be counter-productive in its effort to limit CNS damage. Activated microglia is detected brains of patients with Alzheimer's disease (AD) and Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), human immunodeficiency virus (HIV) infection, and prion-related diseases (Liu & Hong, 2003). Previously, the inflammatory activities of microglia were conceived as only a reactive response to neuronal damage. However, increasing reports demonstrate that inflammatory activities of microglia worsen neuronal damage, which then fuels a self-propelling cycle of neuronal death. Proinflammatory mediators such as the cytokines IL-6, IL-1β, and TNF-α are secreted by activated microglia and can induce cell death in neurodegeneration (Glezer *et al.*, 2007). Thus, while the triggers of various neurodegenerative diseases are different, inflammation may be a basic mechanism driving the progressive nature of these diseases. Although astrocytes and infiltrating lymphocytes have also been listed as contributors to inflammation-mediated neurodegeneration, microglia are concerned as critical components of neuroinflammation (Aloisi *et al.*, 1998; Stoll & Jander, 1999; Miljkovic *et al.*, 2007).

It is of great scientific benefit to further study the process of microglia activation and its role in degeneration. Limiting the inflammatory actions of microglia may prove to be a suitable therapeutic approach for neurodegenerative and neuroinflammatory diseases. In this study *in vitro* responses of microglia to two activators, LPS or Aβ were determined. Although *in vivo* studies are equally important for dissecting the mechanisms of microglia activation, *in vitro* systems have the advantage of being a more controlled environment for the study of cell form and function.

Microglia were cultured from neonatal rats and activated with lipopolysaccharide (LPS) or the active fragment of beta-amyloid (Aβ). LPS is commonly used to induce microglia activation both *in vitro* and *in vivo* and Aβ is the misfolded protein found in senile plaques of AD patients. Morphological changes to microglia following activation include deramification, with microglia assuming an amoeboidal shape with a condensed cytoplasm. We further explored morphological changes by examining changes at the ultrastructure level. Stimulation of monocytes and macrophages by LPS is reported (Meng & Lowell, 1997) and Aβ is used to activate microglia *in vitro* into a neurotoxic phenotype distinguished by secretion of excess of proinflammatory molecules (Floden *et al.*, 2005).

We also investigated whether activation of microglia affects expression of the cell surface molecules, MHC II and CD40. MHC II is expressed on antigen presenting cells and is reportedly upregulated following microglia activation. CD40 is a co-stimulatory molecule required for microglia activation, shown to

be upregulated in IFN-γ-activated microglia (Nguyen *et al.*, 1998). Strategies to attenuate microglia inflammatory responses by inhibiting the activation of microglia (by suppression of CD40 expression) may be of benefit for several neuroinflammatory diseases.

In vivo studies suggest that microglia number expand following insult via (i) resident microglia division, and/or (ii) recruitment of circulating monocytes and differentiation to microglia (Lawson *et al.*, 1992; Streit, 2006; Remington *et al.*, 2007). Details of this proliferation wave in activated microglia are limited. In this study, we will evaluate the *in vitro* proliferation cycle of microglia following treatment with LPS or Aβ.

Majority of *in vitro* studies utilise microglia isolated from neonatal rodents, as these cells have high proliferative capacity in culture. However, as neonatal microglia are expected to respond differently from adult microglial cells (Conde & Streit, 2006), the need for a suitable culture system for adult microglia is also apparent. It is assumed that neonatal microglial cells are functionally immature. Compared to the amoeboidal neonatal microglia, adult microglia demonstrate a ramified morphology. Several studies have used *ex vivo* isolated adult microglia to scrutinise the activities of these cells. The difficulty with using adult microglial cells that have been isolated *ex vivo* is their affinity to undergo cell death a few days after culture.