UNIVERSITI PUTRA MALAYSIA

A THREE-DIMENSIONAL CULTURE MODEL OF LIPOPOLYSACCHARIDE-ACTIVATED MICROGLIA

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FPSK(m) 2015 31

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By

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Thesis was submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

June 2015

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

A THREE-DIMENSIONAL CULTURE MODEL OF LIPOPOLYSACCHARIDE-ACTIVATED MICROGLIA

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June 2015

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In vitro studies utilising conventional two dimensional (2D) culture systems have been used regularly in countless research, although this approach has its own drawbacks. Cells loses their multi-layered histological organisation and interact only on one plane with a flat plastic surface. Furthermore, the competency of 2D culture has also become increasingly questionable when tackling complex 3D biological problems, such as response of the central nervous system (CNS) to injury or infection.

Microglia is a type of macrophage that is found in the brain and acts as the main line of defence in the CNS by evoking inflammatory responses. With an interest in modelling the mechanical state of microglia embedded in CNS parenchyma, this study explored the use of type I collagen as a matrix for growth of microglial cells in a three dimensional (3D) manner. For this, BV2 microglia or primary mouse microglia cell suspensions were prepared with type I collagen and cast into culture plates.

Keen to also determine whether microglia cultured in 3D were capable of shifting to an activated phenotype, cultures were treated with 1 µg/ml lipopolysaccharide (LPS) or co-stimulated with LPS and IFN-γ (for primary microglia). Concurrently, conventional 2D culture (monolayer and collagen coated-monolayer) were set-up for comparison. BV2 microglia cultured in 3D had a doubling time of 39.90 ± 2.86 hours. It was also determined by the lactate dehydrogenase (LDH) assay that LPS was not cytotoxic to BV2 microglia. The expression of NO was determined using the Griess Assay. At 48 hours, the expression of NO for unstimulated BV2 microglia (resting state) in 3D was 2.33 ± 0.56 µM. Upon LPS stimulation, the expression of NO by BV2 microglia in 3D significantly increased to 24.47 ± 2.14 µM. Using RT-qPCR, the expression of inflammatory cytokine mRNA (IL-6, IL-10, IL-1β, IL-12β, MCP-1 and TNF-α) of post-stimulated BV2 microglia in 3D culture were significantly upregulated. Additionally, a bead array was used to measure the level of cytokine protein expression (IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70) by post-stimulated BV2 microglia. Expression of IL-10, IFN-γ and IL-12p70 were negligible. At 48 hours after LPS stimulation, only the protein levels of IL-6, TNF-α and MCP-1 of BV2 microglia significantly increased from

0.7 ± 0.8 pg/ml, 8.1 ± 3.1 pg/ml and 284.0 ± 73.5 pg/ml to 1999.0 ± 685.2 pg/ml, 1744.0 ± 911.6 pg/ml and 5403.0 ± 517.6 pg/ml (*p<0.5, **p<0.1; Mann Whitney Test) respectively.

Primary microglia was obtained from brains of C57BL/6 mice. The viability of primary microglia in 3D was determined using DAPI/PI staining method. Primary microglia showed low PI staining (viable) after 72 hours of LPS and IFN-γ co-stimulation in 3D culture. The expression of NO by primary microglia cultured in 3D was in 3D 0.95 ± 1.01 µM to 39.37 ± 9.53 µM after a 72 hour co-stimulation. Using flow cytometry, CD40 expression of primary microglia cultured in 3D was determined. Percentage of CD40 expression increased from 59.0% and 39.3% to 85.7% and 90.9% after a 72 hour co-stimulation.

In summary, microglia cultured in 3D undergo a robust activation response when stimulated with LPS/LPS with IFN-γ. Importantly, the 3D culture is able to model this activation response with minimum cell death, and the availability of both culture supernatant and cells for analysis can be done with relative ease. This model could provide a platform for other research to be conducted on the pathophysiology of neuroinflammatory processes.

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

MODEL KULTUR TIGA DIMENSI MIKROGLIA YANG DIAKTIFKAN OLEH LIPOPOLYSACCHARIDE

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Kajian *in vitro* telah kerap menggunakan sistem kultur konensional dua dimensi (2D) dalam banyak penyelidikan, walaupun sistem ini mempunyai kelemahannya. Sel-sel akan kehilangan organisasi histologi berlapis-lapis dan hanya berinteraksi pada satu satah dengan permukaan plastik yang rata. Tambahan pula, kebolehpercayaan kultur 2D juga telah semakin dipersoalkan apabila menangani masalah biologi kompleks 3D, seperti tindak balas system saraf pusat (CNS) apabila mengalami kecederaan atau jangkitan.

Mikroglia adalah sejenis makrofaj yang terdapat di dalam otak dan sebagai pertahanan utama dalam CNS mempunyai peranan penting dalam tindak balas keradangan. Dengan minat untuk modelkan keadaan mekanikal mikroglia dalam parenkima CNS, kajian ini mempergunakan sejenis kolagen (type I collagen) sebagai matriks untuk mengkultur sel-sel mikroglia dalam cara tiga dimensi (3D). Oleh itu, sel-sel BV2 mikroglia atau mikroglia diisolasi daripada tikus, disediakan bersama dengan kolagen jenis I dan dimasukkan ke dalam plat kultur.

Dengan minat juga untuk menentukan sama ada mikroglia yang dikultur dalam 3D mampu menukar kepada phenotype aktif, kultur telah dirangsangkan dengan 1μg/ml lipopolysaccharide (LPS) atau bersama dirangsangkan dengan LPS dan IFN-γ (mikroglia tikus). Kultur 2D konvensional (sel monolayer dan monolayer bersalut kolagen) disediakan untuk membuat perbandingan dengan kultur 3D. Mikroglia BV2 yang dikultur dalam 3D mempunyai masa dua kali ganda sebanyak 39.90 ± 2.86 jam. LPS juga telah ditentukan tidak sitotoksik kepada mikroglia BV2 oleh ujian laktat dehydrogenase (LDH). Expresi NO ditentukan dengan ujian Griess. Selepas 48 jam, expresi NO untuk mikroglia BV2 yang tidak dirangsangkan (keadaan rehat) dalam kultur 3D adalah 2.33 ± 0.56 μM. Selepas rangsangan dengan LPS, expresi NO oleh mikroglia BV2 dalam kultur 3D meningkat dengan ketara kepada 24.47 ± 2.14 μM ($p<.05$). Dengan menggunakan RT-qPCR, expresi sitokin radang mRNA (IL-6, IL-10, IL-1β, IL-12β, MCP-1 dan TNF-α) oleh mikroglia BV2 dalam kultur 3D (telah dirangsangkan) didapati meningkat secara ketara.. Selain itu, tahap expesi protein sitokin (IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70) oleh mikroglia BV2 yang telah dirangsangkan telah diukur

dengan menggunakan manik array. Expresi protein IL-10, IFN-γ, dan IL-12p70menunjukkan perbezaan yang boleh diabaikan. Selepas rangsangan LPS selama 48 jam, expresi protein IL-6, TNF-α dan MCP-1 BV2 microglia meningkat dengan ketara dari 0.7 ± 0.8 pg/ml, 8.1 ± 3.1 pg/ml dan 284.0 ± 73.5 pg/ml kepada 1999.0 ± 685.2 pg/ml, 1744.0 ± 911.6 pg/ml dan 5403.0 ± 517.6 pg/ml (* p <0.5, ** p <0.1; Ujian Mann Whitney) secara masing-masing.

Mikroglia tikus diperolehi daripada otak tikus C57BL/6. Kebolehhidupan mikroglia tikus dalam kultur 3D telah ditentukan dengan kaedah DAPI/PISetelah melumur mikroglia tikus dengan DAPI/PI, mikroglia tikus yang telah dirangsangkan dengan LPS dan IFN-γ untuk 72 jam, menunjukan perlumuran yang rendah dengan PI. Oleh itu, kebolehhidupan mikroglia tikus dalam kultur 3D tidak terjejas. Expresi NO oleh mikroglia tikus telah ditentukan selepas 72 jam bersama rangsangan LPS dan IFN-γ. Rangsangan ini menyebabkan expresi NO mikroglia tikus dalam kultur 3D meningkat dari 0.95 ± 1.01 μM kepada 39.37 ± 9.53 μM. Dengan aliran sitometrii, expresi CD40 dikaji dalam mikroglia tikus yang dikultur dlam 3D. Peratusan expresi CD40 meningkat dari 59.0% dan 39.3% kepada 85.7% dan 90.9% selepas dirangsang dengan LPS dan IFN-γ selama 72 jam.

Kesimpulannya, mikroglia yang dikultur dalam 3D menjalni tindak balas pengakitfan yang teguh apabila dirangsang oleh LPS / LPS dengan IFN-γ. Lebih pentingnya ialah, kultur 3D boleh memodelkan tindak balas aktif dengan kematian sel minimum, dan ketersediaan kedua-dua pupernatan kultur dan sel-sel untuk analisis boleh dilakukan dengan agak mudah. Model ini boleh menyediakan platform untuk kajian lain yang dijalankan ke atas patofisiologi proses keradangan neuron.

ACKNOWLEDGEMENTS

First and foremost, I would like to take the opportunity to extend my utmost gratitude to my leading supervisor, Dr. Sharmili Vidyadaran whose guidance and encouragement throughout my Master degree, I will never forget. Next, special thanks to my co-supervisors, Prof. Seow Heng Fong and Dr. James B. Philips for the support and advice. My acknowledgement also extends to the Neuroinflammation Group, Tong Chih Kong, Tan Shi Wei, Shinsmon Jose, and fellow laboratory mates and staffs, all who had been patient and supportive in assisting me throughout my research project.

I would also like to say thank you to my family members who have given their love, care and support all this while. Last but not least, my thanks to Universiti Putra Malaysia (UPM) and the UPM Graduate Research Fellowship Scheme (GRF) for providing me the personal financial support.

I certify that a Thesis Examination Committee has met on 9 June 2015 to conduct the final examination of Haw Tatt Yhew on his thesis entitled "A Three-Dimensional Culture Model of Lipopolysaccharide-Activated Microglia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student 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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Name of Member of Supervisory Committee : Dr. James B. Phillips

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2D	two dimensional
3D	three dimensional
AD	Alzheimer's disease
APC	antigen presenting cells
Aβ	beta amyloid
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
CXCL4	chemokine C-X-C motif ligand 4
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
E7	embryonic day 7
ECM	extracellular matrix
EGF	epidermal growth factor
FBS	foetal bovine serum
HAPI	Highly Aggressive Proliferating Immortalised
IFN-γ	interferon gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MEM	minimum essential medium
MHC	major histocompatibility complex
MMPs	matrix metalloproteinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADPH	nicotinamide adenine dinucleotide phosphate

CHAPTER 1

INTRODUCTION

Microglia is a type of macrophage found in the brain and spinal cord that acts as the main line of defence in the CNS. It is the sole resident immune cell of the CNS. Although this defence mechanism provides beneficiary effects and protects against infection and injury, it does have its detrimental effects. Continuous activation of microglia causes the excess production of inflammatory mediators which can lead to severe neuronal damage. Consequently, microglia inflammation and activation has been observed in pathology of various neurological conditions.

In vitro studies are commonly used in neuroscience research. In cell culture systems, the original microenvironment (growth media and adherent surface) is mimicked as closely as possible to allow cells to grow. The usage of 3D culture is increasing as it is able to mimic the natural microenvironment of tissues and organs better. Cells can be cultured in a stratified manner along a matrix provided to allow cells to behave more like those *in vivo*. There are studies in *in vitro* three dimensional (3D) systems that serve as research models of neurodegeneration and astrogliosis using type I collagen gels (East et al., 2010; East et al., 2009; East et al., 2012)

The aim of the research project was to create a 3D culture model for microglia to mimic the *in vivo* experiment/microenvironment better. Being highly reactive cells, culturing microglia on a stiff plastic surface in 2D cultures most likely affects their phenotype. Our specific aim was to emulate the physical characteristic of microglia being embedded within CNS parenchyma. As we are a laboratory focused on studying the inflammatory reactions of microglia, the lipopolysaccharide (LPS) model of microglia activation was employed to determine whether microglia cultured in 3D are capable of transitioning into an activated and inflammed phenotype.

To approach this, the BV2 microglia cell line was cultured within a 3D matrix consisting of type I collagen. Type I collagen is a matrix material that is relatively simple, easy to manipulate and has previously been used to develop 3D cultures for astrocytes and neurons (East et al., 2010; East et al., 2009; East et al., 2012). Parallel monolayer and collagen-coated monolayer cultures was set-up to allow for comparisons. Cultures were then stimulated with LPS and assayed for various inflammatory mediators to determine the capacity of microglia cultured in 3D collagen to be activated with LPS. Finally, as an extension of this model, primary microglia were isolated from mouse brains and cultured in 3D collagen to assess the suitability of this culture system in the maintenance and activation of primary microglia. It was hypothesised that microglia can be maintained within the 3D microenvironment and demonstrate an inflammatory response when stimulated with LPS.

By characterising the 3D collagen culture model for microglia, it is possible to demonstrate the activation status of microglia in the brain more closely. This will also help facilitate the understanding of the activation pathway of microglia leading to an inflammatory response. Additionally, this model serves as a progression to the conventional monolayer microglia cultures routinely performed and studied by our research group (Neuroinflammation Group, UPM).

Objectives of the Study

The general objective of this study is to utilise a 3D collagen culture model to mimic the *in vivo* physical state of microglia embedded within the brain parenchyma and subsequently examine their phenotype in an LPS-activation model.

Whilst the specific objectives are:

1. To determine the cytotoxicity of BV2 microglia cultured in 3D.
2. To evaluate the expression of nitric oxide by LPS-activated BV2 microglia cultured in 3D.
3. To evaluate mRNA and protein expression of pro-inflammatory cytokines by LPS-activated BV2 microglia cultured in 3D.
4. To determine activation of primary microglia in the 3D collagen culture model by examining nitric oxide and CD40 expression.

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