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Establishing a Culture System that Supports *in Vitro* Expansion of Adult Microglia

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ABSTRACT

Introduction: The vast majority of *in vitro* research on microglia are based on cells isolated from neonatal animals (3-5 days of age). Studying microglia of adults has been limited by the lack of a suitable culture system that supports their growth. In this study, we describe a protocol for growing microglia of adults based on modifications of the technique for culturing microglia isolated from neonatal rats. **Methods:** Mixed glia isolated from adult rats (age range of 1 month to 3 years old) were seeded in culture flasks coated with poly-L-lysine. Cells were maintained in DMEM media supplemented with insulin-transferrin-selenium (ITS) and recombinant human macrophage colony-stimulating factor (M-CSF). Mild trypsinisation was carried out to isolate microglia from mixed glia culture. **Results:** Microglia cells of adult rats were successfully grown *in vitro*. For the expansion of adult microglia, it was observed that coating the cell culture flasks with poly-L-lysine was crucial to encourage cell adherence. The substitution of insulin in culture media with ITS was found to improve cell yield and reduced the number of days required for culture from 28 days to 14 days. Addition of M-CSF to cell culture medium, along with the improvisations described above provided the best adult microglia cell yield ($2.91 \pm 0.56 \times 10^6$ cells) compared to the technique of replating cells ($0.91 \pm 0.65 \times 10^6$ cells; $p<0.05$). **Conclusion:** Optimisation of primary cell culture technique by coating culture flasks with poly-L-lysine, supplementation of culture medium with ITS and M-CSF allowed microglia of adult rats to be successfully cultured *in vitro*.

Keywords: Adult microglia, culture, M-CSF

INTRODUCTION

Microglia are the resident macrophages of the central nervous system (CNS)[1]. The primary function of microglia is to survey the CNS microenvironment - monitoring extracellular chemical changes, debris accumulation and pathogen invasion[2]. Under normal, unstressed conditions, microglia assume a ramified morphology associated with the 'resting' state of microglia. Following activation by stress or injury, microglia acquire an inflammatory phenotype[3]. This includes a shift to amoeboidal morphology, upregulation of surface receptors and production of reactive oxygen species and inflammatory cytokines[4-5].

Microglia are subject to aging and aging-related deterioration of cell function[2]. It has been observed that with increasing age, microglia cells assume a state of continuous latent activation[6-9] that leads to an exaggerated glial response to injury[10-15]. Reactive microglia are known to be closely associated with locations of pathology in various neurodegenerative diseases[11, 12, 16-21]. These *in vivo* studies on microglia, although not without merit, are compounded by the complexity of the CNS microenvironment. An efficient cell culture system would be ideal for scrutiny of age-related changes in microglia. However, *in vitro* culture of microglia of adult animals has been limited by the lack of a suitable culture system that supports their growth.

To enhance yield of microglia in culture, growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) have both been shown to promote *in vitro* proliferation of microglia[3, 8-13]. However, as microglia are activated upon stimulus and have differentiation capabilities, it is important to ensure that any addition of culture supplement or change to cell culture protocol has minimal effect on microglia phenotype. Accordingly, reports have indicated that GM-CSF induces microglia to assume a dendritic cell-like phenotype, possibly affecting their immune properties[7, 14]. Although Esen and colleagues demonstrated that a dose of 0.5 ng/ml GM-CSF encouraged microglial expansion without significantly altering microglial responses[8], they only tested the effects of GM-CSF on resting microglia and did not examine whether the growth factor affects the responses of activated microglia.

Ponomarev and colleagues[22] developed a culture system that supports growth of 4-5 week old adult microglia in

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culture with the use of M-CSF. They showed that adult microglia supplemented with M-CSF proliferated in culture and retained their function. However, it remains to be explored whether use of M-CSF introduces activation artifacts for microglia. Previously, we have shown that 20 ng/ml M-CSF improved cell yield of neonatal microglia in culture without overt phenotypic changes[23]. The establishment of a cell culture system that supports growth of adult microglia would help address our future need to elucidate the effects of aging on microglia responses.

MATERIALS AND METHODS

Generation of primary microglia cell cultures

All animal procedures were approved by the Animal Care and Use Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Microglia were isolated from whole brains of Sprague Dawley rats. Rats within the age range of 1 month to 3 years were euthanised using an overdose of carbon dioxide (CO_2) to obtain mixed glia cultures. Briefly, meninges were removed and tissues minced and placed in a 75 cm^2 flask, resuspended in 0.25 % trypsin-EDTA and 100 U DNase. Cells were then placed in a shaker for 30 minutes at 100 rpm. Supernatant was collected and centrifuged at 1200 rpm for 4 minutes. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated FBS, 1 % Penicillin-Streptomycin, 250 µg/ml Fungizone (all from Invitrogen), 1 X insulin-transferrin-selenium (ITS; Sigma–Aldrich) and 20 ng/ml recombinant human M-CSF (R&D Systems). Culture medium was changed after 48 hours and subsequently changed every 3 days.

Mild trypsinisation

Mild trypsinisation was performed as described[24]. Three days following cell confluency (90-100 %), culture medium was removed from the flask and stored as 'conditioned medium'. Cells were washed with PBS and trypsin-EDTA solution (0.25 % trypsin, 1 mM EDTA) was added to serum free DMEM in a ratio of 1:3 (v/v). Cells were incubated at 37°C for 1 hour. Detached cells were washed away and remaining adherent cells maintained in DMEM media supplemented with ITS, M-CSF and 'conditioned medium'. Cell counts were performed following 60-100 % confluency of 75 cm^2 cell culture flasks. All cell counts were performed with trypan blue staining as a measure of cell viability.

Coating culture flasks with poly-L-lysine or collagen

1 ml of 1 mg/ml poly-L-lysine (Sigma Aldrich) or collagen (extracted from rat tail tendon) was added to T75 culture flasks and stored at 4°C overnight. Poly-L-lysine/collagen was then aspirated and flasks were left in a 37°C incubator overnight to dry completely. Flasks were then washed with phosphate buffered saline (PBS) before cell seeding.

Replating non-adherent cells

Cells isolated from adult rats remained afloat although cells had been in culture for several days. Therefore, on the second day of culture, media was collected and centrifuged at 1200 rpm for 4 minutes and cells were re-seeded in a new poly-L-lysine coated flask.

Lectin staining

Lectin staining was carried out to determine purity of microglia. Briefly, 3.5 x 10^4 cells were seeded on a 12 mm coverslip coated with poly-L-lysine in 24 well plates and left overnight. Media was then removed and the cells were fixed with 4 % paraformaldehyde for 20 minutes. Cells were then permeabilised with 0.2 % Triton-X for 5 minutes at room temperature and incubated with lectin (1:100 dilution with 1 x PBS, Sigma) for 1 hour at 4°C. Cells were counter-stained with DAPI (1: 3000 dilution with ddH_2O; Invitrogen) and coverslips were mounted on glass slides for observation under fluorescence microscope (Olympus BX51).

RESULTS

Preparation of primary microglia cell cultures is essentially based on disaggregation of whole brain tissue and the culture of the resulting mixed glia culture in a suitable medium. A mild trypsinisation step previously described[24] is then performed to isolate microglia from mixed cultures for further propagation.

We supplement adult microglia cells with M-CSF to promote their *in vitro* growth. Together with the coating of cell culture flasks with a poly-L-lysine matrix and addition of insulin-transferrin-selenium (ITS) to our culture medium, we have demonstrated here a culture system that supports *in vitro* expansion of adult microglia cells. An increase in cell yield and decrease in days required for culture were observed when using the devised protocol.

Cultures consistently demonstrated viability of ≥ 95 %. Although growth of adult cells in culture were made possible with the use of poly-L-lysine, cell yield remained low even after 28 days of culture in media supplemented with insulin, with a confluency capacity of 60 % (0.36 x 10^6 cells; Figure 1). Replating non-adherent adult cells into a second flask also led to microglia growth after 22 days in culture with a yield of 0.91 ± 0.65 x 10^6 cells. However, data for replated cells were highly variable, indicating inconsistency in this optimisation. Substituting insulin in our cell culture media with ITS allowed primary adult microglia cultures to reach confluency faster (17 days) and improved cell yield (1.12 ± 0.23 x 10^6 cells). Supplementation of culture media with both ITS and M-CSF gave the highest cell yield ($p<.05$) with the lowest number of days in culture: 14 days to reach 100 % confluency with a cell yield of 2.91 ± 0.56 x 10^6.

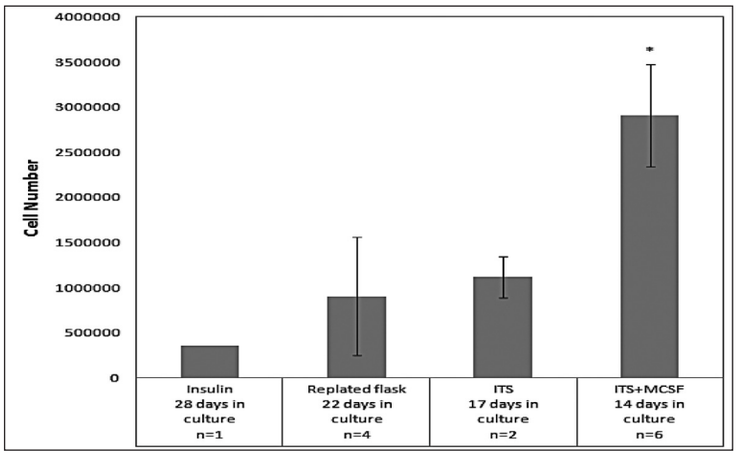

Figure 1. Cell yield of adult microglia cultures following various technical improvisations. Cell counts were performed following 60-100 % confluency. Cells were trypsinised, harvested and counted using a haemocytometer. Error bars depict ± SD values. Supplementing microglia with both ITS and M-CSF significantly increased cell yield compared to replated cells (*$p<0.05$; t-test)

With these optimised conditions, microglia isolated from adult animals can be routinely cultured utilising culture flasks coated with poly-L-lysine and culture media supplemented with both ITS and M-CSF. For instance, we were able to culture microglia from rats of up to 3 years of age, yielding a mean of 1.95 ± 0.44 x 106 cells per 75 cm^2 culture flask (Table 1). Lectin staining was performed to allow observation of microglia morphology. Interestingly, adult microglia appeared slightly deramified compared to microglia isolated from neonatal rats (Figure 2).

Table 1. Modified protocol allows for culture of adult microglia up to 3 years of age. Coating culture flasks with poly-L-lysine and supplementing culture media with both ITS and M-CSF supports growth of adult microglia

Age of rats (months)	Days in culture	Cell Yield
1-3	14	2.1 x 10^6
2-4	17	6.4 x 10^5
2-4	17	7.0 x 10^5
36	16	7.4 x 10^5
36	14	3.5 x 10^6
36	14	3.0 x 10^6
36	14	3.0 x 10^6
	Mean	1.95 ± 0.44 x 10^6

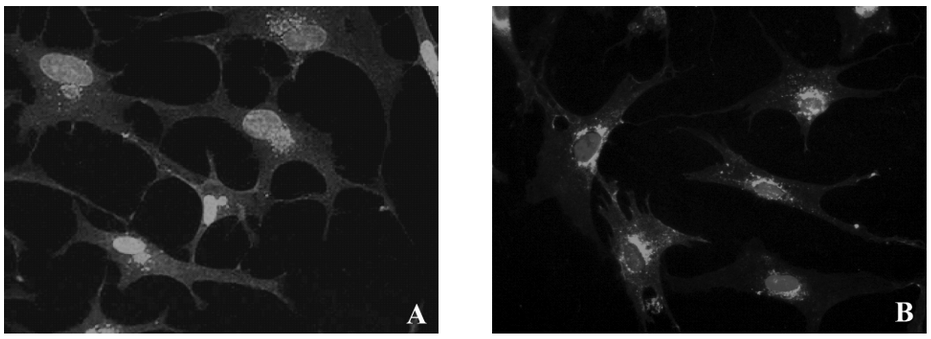

Figure 2. Morphology of cultured microglia cells. Neonatal (A) and adult (B) microglia cells were stained with FITC-tagged tomato lectin and DAPI and observed under fluorescent microscope (200 X magnification)

DISCUSSION

Although the activation of microglia following injury appears highly coordinated, there is increasing evidence that this organised response is lost with age[25, 26]. Aging microglia are unable to degrade proteins[27], more vulnerable to stress[14] and have increased proinflammatory cytokine production[15]. This phenomenon of 'microglia aging' is often linked to the pathophysiology of various neurological and neurodegenerative diseases[11-14, 17-20]. The lack of a suitable culture system that encourages growth of adult microglia has significantly inhibited research in this area. Neonatal microglia cells are functionally different from adult, and failure of adult microglia to grow in culture limits the study of aging on microglia responses. In this study, we described a method to support *in vitro* growth of adult microglia.

The basic principle of preparing primary microglia cultures involves mechanical and enzymatic digestion (with trypsin) of whole brain tissue. This results in a mixed glia culture which is grown to confluency. To isolate microglia from this mixed glia culture, a mild trypsinisation method is used[24]. Briefly, a weak solution of trypsin is added to culture flasks to detach astrocytes from the more adherent microglia. The enriched microglia cultures are then further propagated in a mixture of DMEM conditioned by mixed glia cultures (termed as 'conditioned medium' in Materials and Methods). This method is simple, reproducible, and allows preparation of microglia cultures of high purity (> 98 %) with higher cell yield[24].

The protocol for growth of neonatal microglia failed to work for adult cells. In our experience, culture of microglia cells isolated from adult animals was previously restricted by their limited mitotic capacity and poor adherence to culture flasks. In this study, improvised cell culture techniques were used to support adult microglia cell growth *in vitro*. We have demonstrated that optimisation of the neonatal microglia cell culture technique by coating culture flasks with poly-L-lysine and supplementing culture medium with ITS and M-CSF supported adult microglia cell growth *in vitro*. This study was performed with batch-controls, i.e primary microglia cultures, and the BV2 microglia cell line were cultivated with/without M-CSF and activated with lipopolysaccharide (LPS), and the active fragment of beta amyloid ($A\beta_{25-35}$).

An initial observation was that adult microglia failed to attach to cell culture flasks, therefore severely inhibiting cell proliferation. To encourage adherence, cell culture flasks were coated with poly-L-lysine or collagen (1 mg/ml) for 2 hours to overnight at 4°C. However, only poly-L-lysine and not collagen encouraged cell attachment. All adult cells were subsequently cultured on poly-L-lysine-coated flasks. Poly-L-lysine is composed of positively charged amino acid polymer which enhances electrostatic interaction between negatively charged ions of the cell membrane with the culture surface. Although this allows growth of adult cells, cell yield remained low even after many days in culture with some cells still remaining unattached to culture flasks. We then decided to re-seed the unattached cells into a new culture flask where the cell yield was improved albeit with high variation from flask to flask. Substituting insulin in our cell culture media with ITS, further improved cell yield and reduced culture period. ITS is a mixture of bovine insulin, human transferrin and sodium selenite. Insulin is a polypeptide hormone that promotes uptake of glucose and amino acids by cells. Transferrin is an iron-transport protein and selenium is an essential trace element that enhances cell growth[28].

The growth factor M-CSF has been known to improve *in vitro* growth of microglia[9] and is involved in microglia proliferation and development[10-12]. Previously we have shown that addition of M-CSF to culture medium enhances neonatal microglia proliferation without overtly altering microglia phenotype[23]. Interestingly, proliferation of adult microglia was not M-CSF-dependent but poly-L-lysine dependent, i.e. adult cells failed to grow without use of poly-L-lysine (personal observation). Nonetheless, addition of M-CSF significantly improved cell yield and presented the shortest time in culture from all previous modifications described. Interestingly, adult microglia displayed a deramified

morphology compared to neonatal microglia. This has been linked to age-related morphological changes in microglia as adult microglia have a slightly activated phenotype[2].

In conclusion, our study has demonstrated that optimisation of a microglia cell culture technique for neonatal cells can support growth of adult microglia in vitro. This includes coating culture flasks with poly-L-lysine and supplementation of culture media with ITS and M-CSF.

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