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Original Article

A simple method of isolation, identification, and culturing retinal microglia

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Abstract: The present study aimed to investigate a simple method for isolating, culturing, and identifying growth conditions and the culturing environment of retinal microglia. Eyeballs were obtained from newborn Sprague-Dawley rats (postnatal day 1). The retinas were then collected aseptically. Retinal vessels were carefully removed from the retinas. After digestion and centrifugation of the retinas, the obtained mixed cell suspensions were cultured for 12 days. The culturing flask was placed on a 37°C constant temperature oscillation shaker at 100 rpm for 1 hour. The supernatant was then collected, transferred to uncoated dishes, andincubated for 30 minutes. The dishes were gently shaken andthe supernatant was removed. After three times, oscillating separation was performed for purification. The cells were purified byoscillating separation and were identified by double immunofluorescence staining withmicroglia-specific marker CD11b and Isolectin-B4. This modified method was easily carried out.

Keywords: Retinal microglia, mixed culture, CD11b, IB4

Introduction

The neural retina mainly consists of ordered neurons and surrounding glial cells. Retinal glial cells are further divided into large glial cells (astrocytes or Müller cells) and microglia. In 1919, Del Rio-Hortega used a silver carbonate staining method to distinguish microglia from neurons and other glial cells. The microglia is the smallest group of glial cells, accounting for about 10-20% of all glial cells. They are specific in terms of structure, characteristics, and cell lineage. They belong to a separate group of nervous system migratory macrophages characterized by very active function [1]. Microglia are immune cells in the nervous retinal tissue which help to comprise the central nervous system (CNS) [2-4].

Because of the close relationship between the retina and CNS, more and more attention has been paid to the potential roles of retinal microglia in the pathogenesis of multiple types of retinopathy [5]. However, separation and purification of microglia has remained very difficult [2]. Therefore, establishment of a simple *in vitro* purification and culturing system for primary retinal microglia is an important step in studying the function of these cells.

In 1932, Rio-Hortega first recognized that microglia were a special group of cells in the CNS [6, 7]. It was found that when the brain was damaged, the microgliareactively migrated to the injured region, going through morphological changes and proliferation. Previously, it was considered that the retina is an immune-privileged organ. However, with more in-depth research, it has gradually become recognized that the microglia are, in fact, the only antigen-presenting cells in the retinaand are quite active. Therefore, application of cell culture technology to obtain high quality retinal microglia at a large quantity would be an important step for further research.

At present, the culturing method of retinal microglia is based on the standard culturing method of microglia. In 1986, through simultaneously separating, staining, and identifying immune cells, Giulian et al. [8] established a high-yield isolating method for microglia by oscillation. The principle was based on the growing features of large glial cells (astrocytes)

and microglia. Oscillating separation was performed after the formation of growing layers. This method resulted in obtaining microglia and oligodendrocytes with relatively high purity. Oligodendrocytes could then be removed according to differences in the ability and speed of oligodendrocytes and microglia to attach to the surfaces of culturing containers.

The present study aimed to introduce a simple method by refining the steps and improving specific steps.

Materials and methods

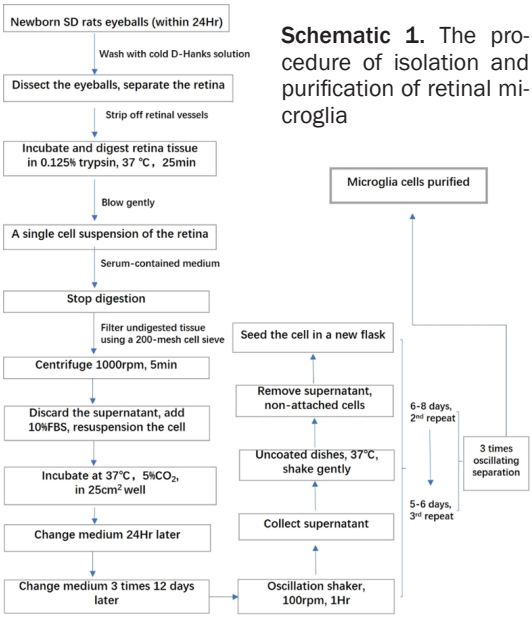
Materials

Premium fetal bovine serum and Dulbecco's modified Eagle medium/F12 were obtained from Gibco. Trypsin, bovine insulin, glutamine, and fluorescence secondary antibodies were purchased from Sigma. Mouse anti-CD11b antibodies were obtained from Abcam. Mouse IB4 antibodies were purchased from Sigma. Penicillin and streptomycin were obtained from North China Pharmaceutical Factory. Petri dishes, centrifuge tubes, and 25-cm^2 flasks were acquired from Corning-Costar. Newborn Sprague-Dawley (SD) rats were provided by the Animal Laboratory of the Second Xiangya Hospital of Central South University within 24 hours of birth.

Culturing methods

Mixed culture of glial cells: In a sterile environment, eyeballs were obtained from newborn SD rats (within 24 hours of birth) and washed with cold D-Hanks solution (containing 1% tobramycin). The sclera of the eyeballs was cut open along the limbus and the retina was obtained aseptically, with the retinal vessels carefully stripped off. Retinal tissue was incubated and digested in 0.125% trypsin at 37°C for 25 minutes, then gently blown into a single cell suspension. The same amount of pre-cooled serum-contained medium was added into the reaction system to stop digestion. Undigested tissue mass was then filtered out using a 200-mesh cell sieve. The filtrate was placed in a sterile centrifuge tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, then 10% fetal bovine serum was added, generating a cell suspension. The cell suspension was placed into a 25-cm^2 culturing flaskand incubated at 37°C in an incubator with 5% CO_2. The next day, the medium was refreshed once with an equal volume to remove the dead cell debris. The medium was replaced every 4 days. The cells were observed for growth and survival over time under a microscope.

Isolation and purification of retinal microglia: After culturing for 12 days, the cells were observed to be growing in a complete layer. The culturing flask was then placed on a 37°C constant temperature oscillation shaker and shaken at 100 rpm for 1 hour. The cells in the supernatant were collected, transferred to uncoated dishes, and incubated in a 37°C incubator for about 30 minutes. The dishes were gently shakenand the supernatant was aspirated to remove any non-attached cells and cells that were not tightly attached. The cells were then seeded in a new flask. After the first oscillating separation, fresh medium was added to the flask with the original mixed cell culture to continue cultivating the mixture for 6-8 days, when the second oscillating separation would take place. Cells were then cultured for another 5-6 days, after which the third oscillating separation was performed for purification (**Schematic 1**).



Schematic 1. The procedure of isolation and purification of retinal microglia

Immunocytochemical staining of retina microglia: Coverslips with the cell culture were washed thoroughly with normal saline, fixed in 4% para-

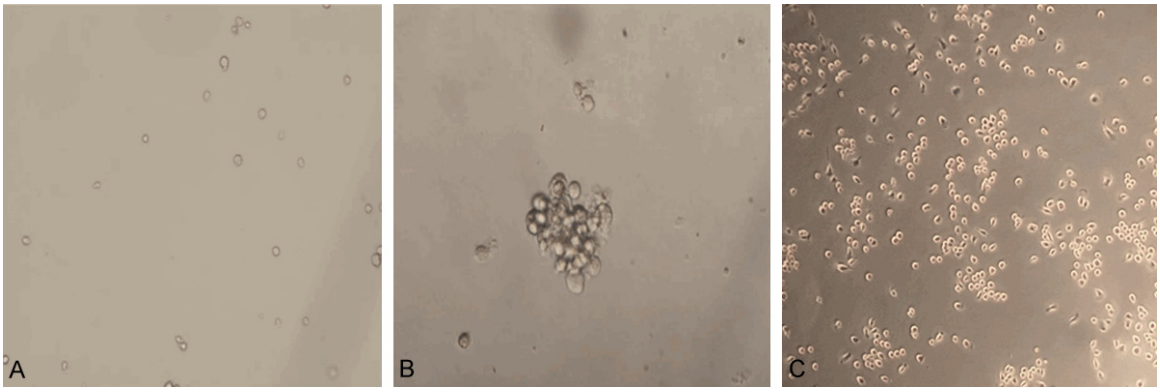


Figure 1. A. The first generation of retinal microglia was small in volume, round in shape, and was growing in a scattered manner; B. The second passage of the retinal microglia formed a cluster; C. The third passage become.

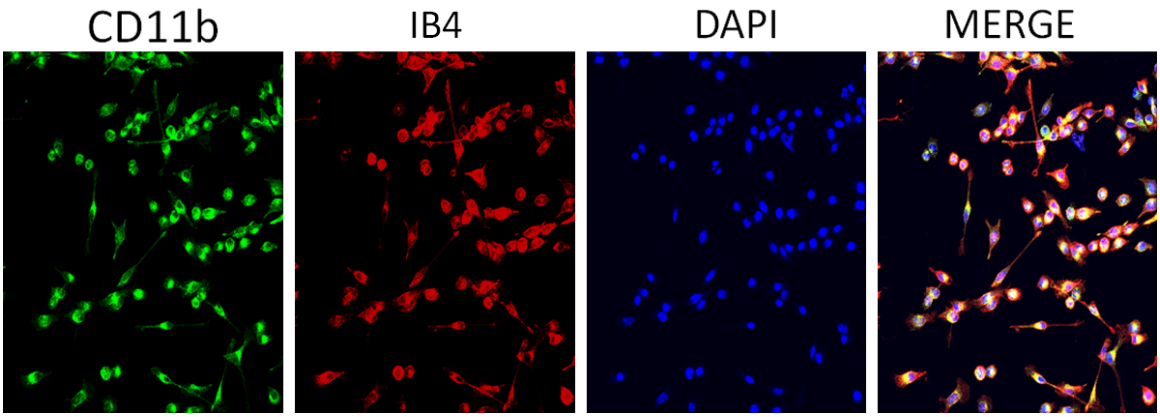


Figure 2. Immunofluorescence staining of retinal microglia by different markers, CD11b and IB4, nuclear marked by DAPI.

formaldehyde for 20 minutes, and washed with 0.01 M phosphate-buffered saline (PBS, pH 7.4) three times for 5 minutes each. Standard steps for immunocytochemical staining were carried out as follows. First, 10% goat serum + 0.25% Triton X-100 (diluted with 0.01 M PBS) was dropped onto the coverslips with the cell culture and the coverslips were incubated in a wet box at 37°C for 30 minutes. Next, mouse anti-CD11b monoclonal antibodies (1:100) were added onto the coverslips and the coverslips were kept at 4°C in a wet box overnight. PBS, instead of the primary antibodies, was used for negative control. The coverslips were washed with 0.01 M PBS (pH 7.4) three times for 5 minutes each. Secondary antibodies (1:100; Sigma) were added and the coverslips were incubated at 37°C for 1 hour. The coverslips were then washed again with 0.01 M PBS (pH 7.4) three times for 5 minutes each. Finally, the coverslips were sealed, then observed and photographed under a fluorescence microscope (**Figure 2**).

Results

Morphology of the purified and cultured retina microglia

The mixed cultures grew in layers after being cultured for 7 days. The layers became clearer and more distinct at days 11-13. Cells in the upper layer were microglia while cells in the bottom layer were astrocytes.

The first and second generations of retinal microglia after separation by oscillation were small in volume, round, scattered, floating, and showed strong refraction (**Figure 1A**, **1B**).

After the third cell passage, the retinal microglia showed attachment and began to aggregate (**Figure 1C**).

Identification of retinal microglia

After successful cell passages, microglia, marked with CD11b and IB4 monoclonal antibodies, showed strong fluorescence staining. Some of the cells were elongated. CD11b-positive cells and CD11b-negative cells were counted in five randomly sampled fields of view under a 20× microscope. The percentage of CD11b-positive cells was calculated for each field of view (**Figure 2**).

Discussion

The present study observed that, during the first 1-2 days of the mixed culturing stage, the number of neurons decreased gradually. At days 3-4, the death rate of neurons accelerated, the number of large glial cells increased, and layers gradually formed. At days 5-6, the astrocytes formed an apparent bottom-layer growing pattern, the number of microglia increased significantly although scattered, and the morphology of retinal microglia kept changing. Present results were in accord with findings reported previously [9].

The method developed in this study was based on the method established by Giulian et al., with an improvement of some specific steps. First, the centrifugation of cells when processing the retinal tissue was minimized by reducing centrifugation speed and time. This effectively reduced cell damage and improved the density of cells in initial seeding. Second, retinal blood vessels were removed as much as possible. All separation operations were carried out under a microscope since immunohistochemical identification cannot distinguish microglia from macrophages. Third, multiple steps of oscillating separation were performed to obtain retinal microglia with higher purity (as high as 95%).

At the same time, through experiments, it was discovered that trypsin digestion was not required for the separation of microglia, in contrast to the requirement for general cell separation. Trypsin digestion may easily cause damage to the microglia and lead to the simultaneous shedding of large blocks of astrocytes and microglia, resulting in low product purity.

Microglia belongs to monocytes. Therefore, immunofluorescence was applied using CD11b (OX42) as a microglia-specific marker.

In conclusion, the present study improved the classic microglia culturing methods with an objective of obtaining retinal microglia of high purity and high quantity, aiming to provide a foundation for further investigation into their role and function.

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Disclosure of conflict of interest

None.

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