·Basic Research·

Morphological and immunocytochemical analysis of human retinal glia subtypes *in vitro*

Shao-Fen Lin[1], *Yu-Xiang Mao*[1], *Bin Li*[2], *Wei Sun*[1], *Shi-Bo Tang*[1]

Foundation item: National Nature Science Foundation of China (No.81170865)

[1]Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou 510060, Guangdong Province, China
[2]Department of Ophthalmology, the Affiliated Wuhan Tongji Hospital of Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Co-first authors: Shao-Fen Lin and Yu-Xiang Mao
Correspondence to: Shi-Bo Tang. Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou 510060, Guangdong Province, China. Tangsb@mail.sysu.edu.cn

Received: 2013-01-11 Accepted: 2013-07-18

Abstract

• AIM: To examine the morphological characteristics and antigen expression patterns of cultured human retinal glia to define novel subtypes.

• METHODS: Morphologic characteristics and marker expression were examined during cultivation using hematoxylin and eosin (HE) and immunostaining for glial fibrillary acidic protein (GFAP) and vimentin.

• RESULTS: A subtype of human retinal glia distinct from radial glia (Müller cells) was successfully isolated by digesting the retina first in diastase vera (pancreatin) and then in clostridiopeptidase, followed by culture on fibronectin substrate in human endothelial cell medium (supplemented with 10% fetal bovine serum, growth factors, and heparin sodium). Adherence was detected at 72h and cell-cell coupling at 9d-10d after seeding. These cells were extensively and strongly immunopositive for GFAP and vimentin, consistent with glial expression patterns in the human retina, but were morphologically and immunohistochemically distinct from previously reported cultured retinal glia, including GFAP -positive and glutamine synthetase (GS)-positive Müller cells.

• CONCLUSION: A unique human retinal glial cell type can be isolated using diastase vera and clostridiopeptidase and then maintained *in vitro*. Further studies are required to characterize the physiological and pathological functions of these cells.

• KEYWORDS: retina; glia; morphology

DOI:10.3980/j.issn.2222-3959.2013.05.01

Lin SF, Mao YX, Li B, Sun W, Tang SB. Morphological and immunocytochemical analysis of human retinal glia subtypes *in vitro*. *Int J Ophthalmol* 2013;6(5):559-563

INTRODUCTION

Retinal glia helps establish and maintain the normal cytoarchitecture of the retina, regulate extracellular potassium, and act to support neuronal metabolism. Retinal glia also participates in multiple pathological events, such as proliferative vitreoretinopathy (PVR), proliferative retinal diseases, and idiopathic macular pucker. Methods for *in vitro* cultivation of various retinal glia subtypes may facilitate studies on the pathophysiology of glia-related retinal diseases. In a previous study, we successfully isolated and purified retinal glia with morphological characteristics and antigen expression patterns distinct from all previously described retinal glia [1]. In this study, we examined the morphological and immunohistochemical properties of these cells in detail and compared them to retinal radial glial (Müller cells). And introduce a unique human retinal glial cell type which can be isolated using diastase vera and clostridiopeptidase and then maintained *in vitro*.

MATERIALS AND METHODS

Primary Cell Culture Cells were cultured as described eyeballs were soaked in media for 15min (Invitrogen-Gibco)[1]. The cornea with scleral ring was cut 6mm from the corneoscleral junction. The crystalline humor in the anterior segment of the eyeball between the retina and optic disc were removed. From left to right, the retinal tissue was stripped from the surface of the corporis vitrei. The tissues sections were placed into sterilized culture capsules with dissection media and shaken to remove any associated tissue (including retinal pigment epithelium). The isolated retinal tissue was then cut into pieces with scissors. Pieces of retina were added to 1-2mL of 2% diastase vera (pancreatin, Sigma) in centrifuge tubes and incubated for 25min at 37℃. Digestion was terminated by addition of 1-2mL DMEM/F12 culture media supplemented with 10% fetal bovine serum (Gibco). The digested tissue was centrifuged for 10min at

1 200r/min and the supernatant removed. To the remaining cell pellet, 1-2mL 0.133% type IV collagenase (Gibco) was added, mixed well, and incubated at 37℃ for 30min. The second digestion was also terminated by adding 1-2mL DMEM/F12 with 10% fetal bovine serum. The mixture was centrifuged for 10min at 1 200r/min and the supernatant removed. Finally, 2mL heparin sodium and β-ECGF endothelial cell culture media supplemented with 10% fetal bovine serum (Gibco) was added to the culture capsules, mixed well, and the cell suspension seeded on fibronectin (FN)-coated cover slips (0.5mg/mL FN for 2h at 37℃). After 72h, the media was changed and cell morphology examined by phase contrast microscopy. At confluence, cultures were split at 1:2 using 0.25% diastase vera. We used Fibronectin (FN) at.

Characterization of Human Retinal Glia

Morphology Cell morphology was examined using an inverted microscope. For histological examination, monolayer cultures were rinsed twice with D-Hank's solution (WHIGA, Guangzhou, China), fixed in ethanol, stained with hematoxylin and eosin (HE), and mounted with neutral gummi.

Immunohistochemistry Confluent cover slips were fixed in acetone and ethanol (1:1), rinsed, and permeablized using 5% Triton X-100 (WHIGA, Guangzhou, China). Endogenous peroxidase activity was quenched using 3% hydrogen peroxide. Fixed cultures were incubated for 60min with the primary antibody or PBS (negative control group). Immunolabeling was visualized by adding 50μL polymer accentuator for 10min at 37℃ (Reagent A, Maixin, China), followed by horse radish peroxidase (HRP)-labeled anti-rat/rabbit secondary Ig (Reagent B, Maixin, China) for 20min at 37℃. Stained slides were washed three times in phosphate belanced solution (PBS) and then stained with diaminobenzidine (DAB) for 3-10min. Positive coloration is buffy.

Immunofluorescence staining Confluent cultures were rinsed three times with D-Hank's solution, fixed with 4% paraformaldehyde for 20min, rinsed three times in PBS (3min/wash), permeablized with 50μL for 30min at room temperature, blocked in 50μL of 1% bovine serum albumin (BSA) for 30min at room temperature, and incubated in a 50μL solution of the primary antibody (Maxin, China) for 1h at room temperature. Cultures were rinsed three times in PBS (5min/wash), and the labeled with 50μL of FITC-conjugated secondary antibody (1:30,) for 5min in the dark at room temperature. Following a final two rinses in PBS (3min/rinse), fixed and stained slides were mounted in antifade medium and photographed under a fluorescence microscope. Living cells dynamic observation system, ordinary fluorescent biomicroscopy (German Zeiss Company).

RESULTS

Morphological Observations Isolated cells adhered to the fibronectin substrate within 72h. Cells were ellipsoid, round, irregular, or polygonal. Single nuclei were star-shaped and some cells exhibited two nuclei, indicative of active proliferation. Orbicular membrane formed among the synapses like pygmy water lily flowers (Figure 1A, B). After 9-10d, the cells spread throughout the culture slide (Figure 1B, C, D,). In HE-stained cover slips, cells were ellipsoid, round, irregular, or polygonal, and the nuclei stained blue with clear boundaries, while the endochylema (orbiculus membrane) was abundant and salmon pink (Figure 1E).

Immunohistochemical Staining Cultivated retinal glia were positive for glial fibrillary acidic protein (GFAP) and vimentin (Figure 1H, I) while no NSE, S100, CD34, or Ⅷ factor immunoreactivity was observed. In contrast, Müller cells were GFAP-, glutamine synthetase-, and buffy positive (Figure 2A, 2B). Immunofluorescence staining of these newly isolated non-Müller glia confirmed GFAP and vimentin expression (green). Nuclei were counterstained blue (Figure 1F, G).

Morphological Comparison to Müller Glia We cultivated Müller cells as described and directly compared the marker expression pattern and morphology characteristics to those of the newly isolated non-Müller glia [2]. The newly detected cells resembled pygmy water lily flowers (Figure 1B) when stained with HE, with multiple cirri extending around a cardinal red nucleus. The cytolymph formed a lightly colored disc (Figure 1E). In contrast, the Müller cells were bipolar, slender in the middle, and with abundant endochylema. Some cell bodies projected axis cylinders. Polygonal cells with large cell bodies had abundant cytoplasm and round or ellipsoid nuclei but showed significant morphological differences to the newly isolated glia (Figure 2F, G,). The Müller cells nuclei were round and blue with clear circumscriptions when stained with HE, while the endochylema was abundant and salmon pink (Figure 2C). Müller cells were positive for GFAP and glutamine synthetase (GS) (Figure 2A, B). After 9-10d, both cell types formed confluent monolayers (Figure 2H). Müller cell bodies were slender with abundant endochylema. Confluent cells are sometimes arranged in parallel, sometimes forming a daisy-shaped structure. After 2-3 passages, About 95% were GFAP-positive and GS-positive. Expression of GFAP and GS was confirmed by immunofluorescence staining (Figure 2D, E).

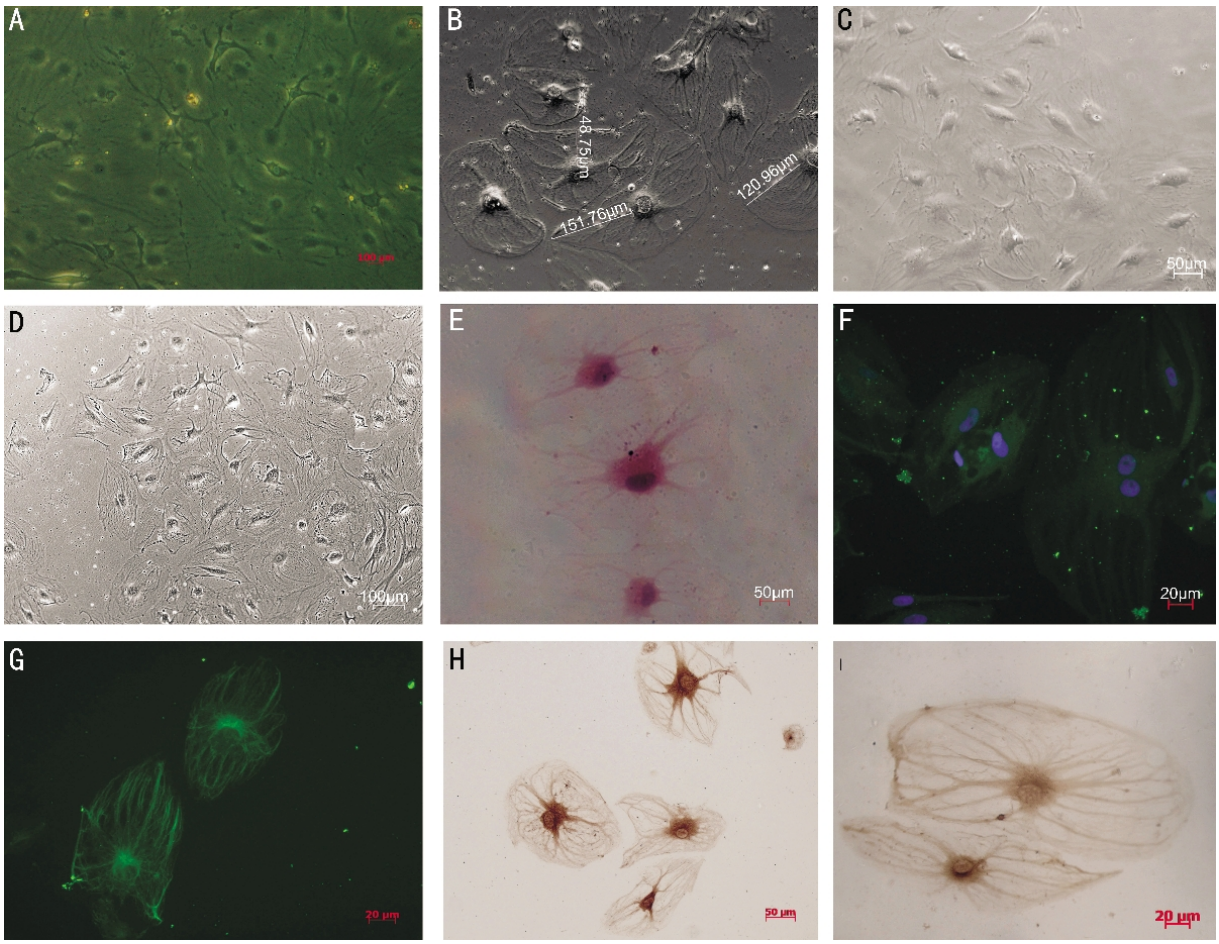

Figure 1 Human retinal glial cells under optical microscope A: Vigorous growth of human retinal glial cells under inverted microscope ×100; B: Human retinal glial cells under Miriam dynamic system of living cells ×200; C: Human retinal glial cells under Miriam dynamic system of living cells ×200; D: Fusion of human retinal glial cells under Miriam dynamic system of living cells ×100; E: HE staining of human retinal glial cells in the retina (cultured human glial cell nuclei were star and blue , cytoplasm was rich of pale red ×200; F: Positive expression in cultured human retinal glial cells immunofluorescent GFAP ×200; G: Positive expression in cultured human retinal glial cells by immunofluorescence VIMETIN ×200; H: Positive expression in cultured human retinal glial cells immunohistochemistry GFAP ×200; I: Positive expression in cultured human retinal glial cells by immunohistochemistry VIMETIN ×200.

DISCUSSION

The glial cell population in the human retina is composed mainly of Müller cells, astrocytes, small gliocytes, and other as yet uncharacterized cells. The Müller cells are by far the largest in size and best described [3]. The cultivation of human retinal glia *in vitro* is the foundation for studies on proliferative lesions such as proliferative vitreoretinopathy, proliferative diabetic retinopathy, and idiopathic macular pucker. Many glial subtypes have been successfully cultivated but none resemble those isolated and described here. The endochylema contained large numbers of intermediate filaments of 8-10nm. Cells were both GFAP-positive and vimentin-positive as revealed by immunohistochemical and immunofluorescence labeling. All of the above are received characteristics of RGC [4,5]. These novel glial cells were isolated using techniques originally developed for the isolation and cultivation of endothelial cells but exhibited several hallmarks of glia, particularly GFAP expression.

The cell bodies of Müller cells are found in the inner nuclear layer and extend processes that terminate in end-feet at both the internal and external limiting membrane. Müller glia provides structural support through cell-cell adhesion. In addition, Müller cells directly participate in the regulation of the retinal microcirculation, act to insulate retinal synapses to maintain the precision of synaptic transmission, synthesize and secrete neurotrophic factors and metabolites, and maintain the blood-retina barrier [6,7]. Finally, Müller glia control neuronal excitability by regulating external potassium and by clearing aminoglutaminic acids from the synaptic cleft (and subsequently transforming into glutamine) [8,9]. Increased GFAP expression by Müller cells is an indicator of retinal injury.

Our cultivated Müller cells had typical morphological features, including projections that grew broader towards the ends, large polygonal cell bodies with abundant cytoplasm,

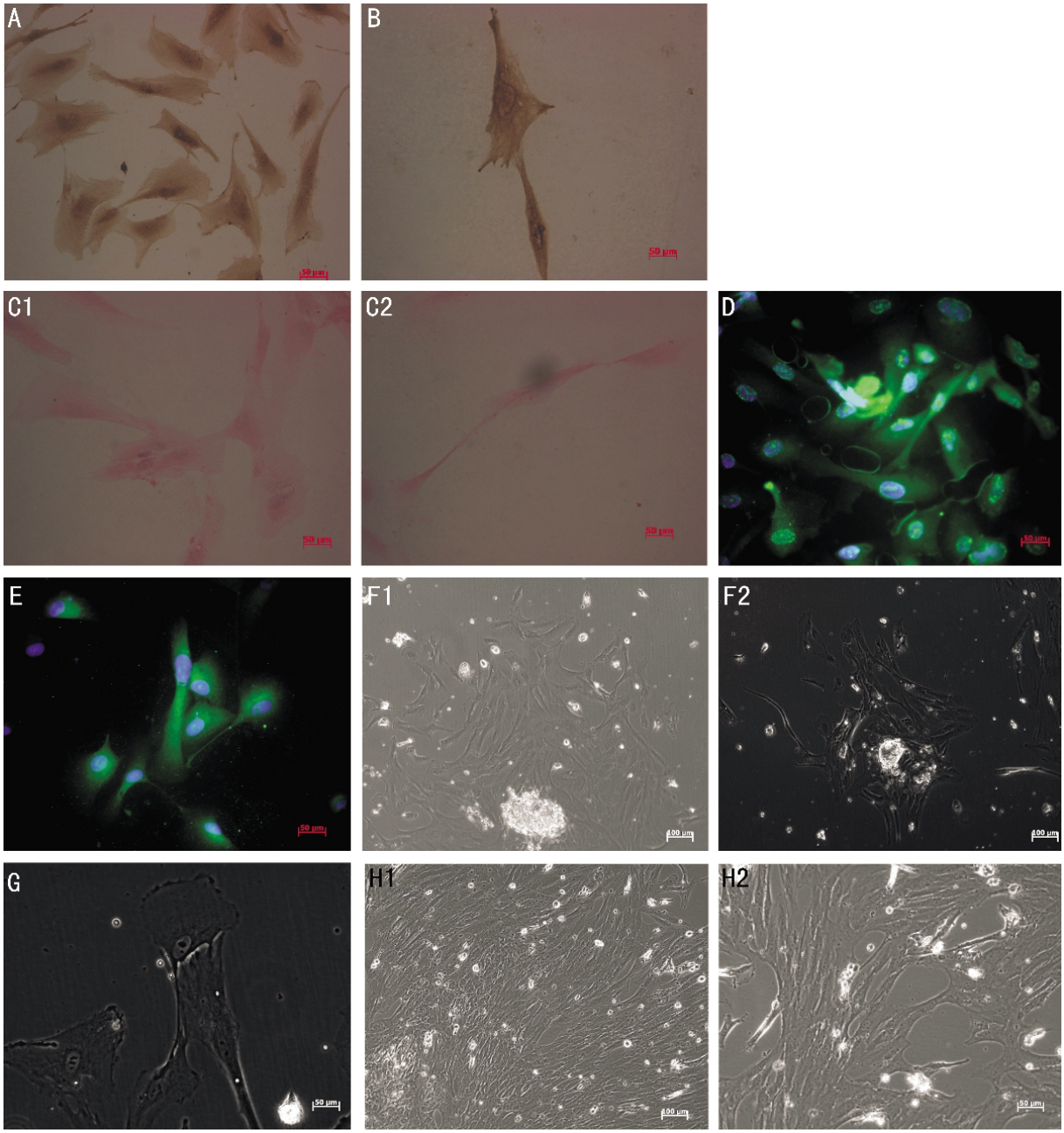

Figure 2 Human retinal mullers under optical microscope A: Positive expression in cultured human retinal mullers immunohistochemistry GS ×200; B: Positive expression in cultured human retinal mullers immunohistochemistry GFAP ×200; $C_{1,2}$: HE Staining of human retinal mullers in retina (the cultured human muiier nuclei were round and blue, cytoplasm was rich of pale red ×200); D: Positive expression in cultured human retinal mullers immunofluorescence GS ×200; E: Positive expression in cultured human retinal mullers immunofluorescence GFAP ×200; $F_{1,2}$: Vigorous growth of primary retinal mullers of cells under the observation of the living cells instrument dynamic system; G, I: Vigorous growth of the first generation of the retina mullers cells under the observation of the living cells instrument dynamic system; $H_{1,2}$: Fusion of human retinal mullers cells under the observation of live cytometry dynamic system ×100.

and round or ellipsoid nuclei [10]. The unique glial subtype reported here was isolated by a two-step digestion process whilst Müller cells were isolated using brief (30min) digestion in alidase and diastase vera. Glial fibrillary acid protein is a ubiquitous marker for glia and an important cytoskeletal component determining cell morphology. It is often present in the cytolymph and cytoplasm and is the most marker of glial cytoplasm. Indeed, GFAP expression is often used to identify Müller cells, but the present study indicates that GFAP is not a specific Müller cell marker. In contrast, an antibody against glutamine synthetase (GS) stained virtually 100% of Müller cells.

In contrast to the glia described here, many smaller gliocytes have been described *in vitro* with slender or ellipsoid cell bodies from which extend many small spiny processes[11]. No previously described retinal glial cell matches the morphology of that described here. To successfully cultivate these cells, several steps appear necessary [12]. 1) When we

were stripping the retina, we used forceps to strip from the left side to the right side to completely remove any residual corporis vitrei because once embedded in this gel, cells could not be easily precipitated during centrifugation. This in turn necessitated longer centrifugation, which can degrade cell viability. 2) After the cells were seeded, the cultures were kept still for 72h to allow adhesion and growth. 3) After 72h, the media was changed carefully so as not to disrupted adhesion. 4) The initial separation procedure was performed quickly as this promoted better adherence, survival, and growth. 5) We used diastase vera (pancreatin) to first separate the cells in the retina and hydrolyze the proteins. Then we used collagenase for complete cell separation with relatively little injury to the processes.

A unique human retinal glial cell type can be isolated using diastase vera and clostridiopeptidase and then maintained *in vitro*. There are many issues to be further studied, such as the function of the new type human retinal glial cells in the retina; Does it provide trophic and anti-oxidative support of photoreceptors and neurons and regulate the tightness of the blood-retinal barrier? And also as soft substrate required for neurite growth and neuronal plasticity? Further studies are required to characterize the physiological and pathological functions of these cells.

REFERENCES

1 Luo XM, Jiang DY, Nie A. Human retinal glial cells in primary culture. *Chin J Ocul Fundus Dis* 1995;(4):247-249

2 Wu YF, Li Y. Practical medical cell culture-techniques. Guangzhou: Sun Yat-sen University, 2010:373-374

3 Rodieck RW. The Vertebrate Retina: principles of structure and function. San Francisco: Wh Freeman, 1973:213

4 Scherer J, Schnitzer J. Growth factor effects on the proliferation of different retinal glial cells *in vitro*. *Dev Brain Res*1994;80(1-2):209-221

5 Vinores SA, Campochiaro PA, Conway BP. Ultrastructral and electron-immunocytochemical characterization of cells in epiretinal membranes. *Invest Ophthalmol Vis Sci* 1990;31(1):14-28

6 Ogden TE. Nerve fiber layer astrocytes of the primate retina: morphology, distribution, and density. *Invest Ophthalmol Vis Sci* 1978;17 (6):499-510

7 Ramírez JM, Triviño A, Ramírez AI, Salazar JJ, García-Sánchez J. Structural specializations of human retinal glial cells. *Vision Res* 1996;36 (14):2029-2036

8 Zhang HR. Ocular microcirculation and related diseases. Peking: Joint publishing house of Peking medical college and Peking union medical college, 1993;153

9 Garner A. Histopathology of diabetic retinopathy in man. *Eye (Lond)* 1993;7(Pt 2):250-253

10 Li SN, Wang H. Müller cells in the retina. *Chin Ophthalmol Res* 2005; 23(1):104-107

11 Chen X, Zhang P, Cao AM. SD rat retinal microglial cells in primary culture method improvement. *Zhongguo Shiyan Dongwu Xuebao* 2009;17 (4):50-55

12 Lin SF, Mao YX, Li B, Tang S. Human retinal glial cells in primary culture and identification. *Chin Ophthalmol Res* 2012;30(1):17-19