DOI: 10.4149/BLL_2022_100

Bratisl Med J 2022; 123 (9)
625 – 630

EXPERIMENTAL STUDY

Uncertain histological origin of "glia-like" cells in adult human brain tissue cultures

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ABSTRACT
OBJECTIVES: Most brain cells studies come from cultured rodent brain tissue, so basic questions about the behaviour of cultured adult human glial cells may remain unanswered.
BAGROUND: Cells cultured from adult human brain have been poorly defined until now and are often termed "glia-like" based on some morphological similarities with astrocytes. However, the cells in question fail to express glial markers and may be well be of non-glial origin.
METHODS: We examined adult human brain and cultures from 10 patients with non-malignant diagnoses. Immunofluorescence methods were used for glial and non-glial cell type identifications.
RESULTS: Confluent cultures contained the following: 0.1 % astrocytes, ≤ 0.01 % oligodendrocytes, 2–5 % microglial and 95–98 % "glia-like" cells. Astrocytes tested as followed: GFAP+/Vim+, microglia: Ferr+Vim+, "glia-like" cells: Vim+/Fn+/CK- or CK+. In the brain tissue, astrocytes were GFAP+/Vim+, microglia Ferr+/Vim-, fibronectin expression was restricted to brain vessels.
CONCLUSION: This report demonstrates considerable morphological and cytoskelatal dedifferentiation of cultured brain cells. Cytokeratins, specific markers for epithelial cell differentiation, were absent in the brain tissue. However, they were expressed in "glia-like" cells. This finding could be considered glial dedifferentiation given the ectodermal origin of the brain tissue. We suggest that "glia-like" cells come from currently unknown glial progenitor cells scattered through the brain tissue *(Tab. 1, Fig. 4, Ref. 19)*. Text in PDF *www.elis.sk*
KEY WORDS: "glia-like" cells, human brain cultures, GFAP, cytokeratins, fibronectin.

Introduction

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein considered to be the best astroglial marker. Most brain cell studies have been performed hitherto on embryonic or new-born rodent cultures, which contain up to 95 % of GFAP-positive cells, which became GFAP-negative by the fourth passage (1). Primary cultures derived from foetal human brain consist of a heterogeneous neuronal, glial and non-glial cell population (2, 3). However, the predominant cell population in adult human brain cultures does not express GFAP. These spindle and flat cells without, or with poorly developed processes are often termed "glia-like" cells, a term introduced by Ponten in 1978 (4). In literature, there are significant discrepancies in the occurrence of GFAP-positive cells in adult human brain cultures. GFAP-positive cells were rare or even absent in dissociated (2, 5) or explant cultures (6). In contrast, other authors showed a high percentage (more than 80 %) of GFAP-positive cells in adult human brain cultures (7, 8). Moreover, according to conflicting reports, GFAP+ cells were stained either positively (8) or negatively (7) for fibronectin.

How could long process-bearing astrocytes transform during tissue dissociation or inside explants into flat astrocytes? What is the histological origin of cells in these different cultures? To help resolve these conflicting reports, we compared the morphological and immunochemical properties of glial and non-glial cells in the brain tissue and during early culturing.

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Materials and methods

Tissue cultures and brain specimens

Brain samples were kindly provided by the Department of Neurosurgery, Derer's Hospital, Bratislava. This study reports the results performed on brain biopsies collected between 1989 to 2000. Experiments with human brain biopsies were performed according to Slovak laws 272/1994, 76/2004 and approved by the Ethical Committee of UNB Bratislava. We chose ten brain biopsies from adult donors undergoing neurosurgical intervention for brain trauma, stroke, or arteriovenous malformations for this study. Samples were obtained from temporal or frontal lobes. Brain biopsies were used for tissue culturing and adjacent samples

for immunohistochemistry. Part of brain biopsies were covered with cryo-embedding media (OCT), cryopreserved in liquid nitrogen, and used for cryosections. Brain samples were fixed in 4 % neutral formalin and embedded in paraffin for immunohistochemistry. Tissue cultures were prepared by an explant method, separately from cortical grey and white matter. Samples were cut into small pieces and seeded in uncoated plastic dishes (25 cm^2). The culture medium consisted of MEM with glutamine, nonessential amino acids and 10 % foetal calf serum. Simultaneously, the cells used for immunocytochemical staining were grown under the same conditions on uncoated glass coverslips.

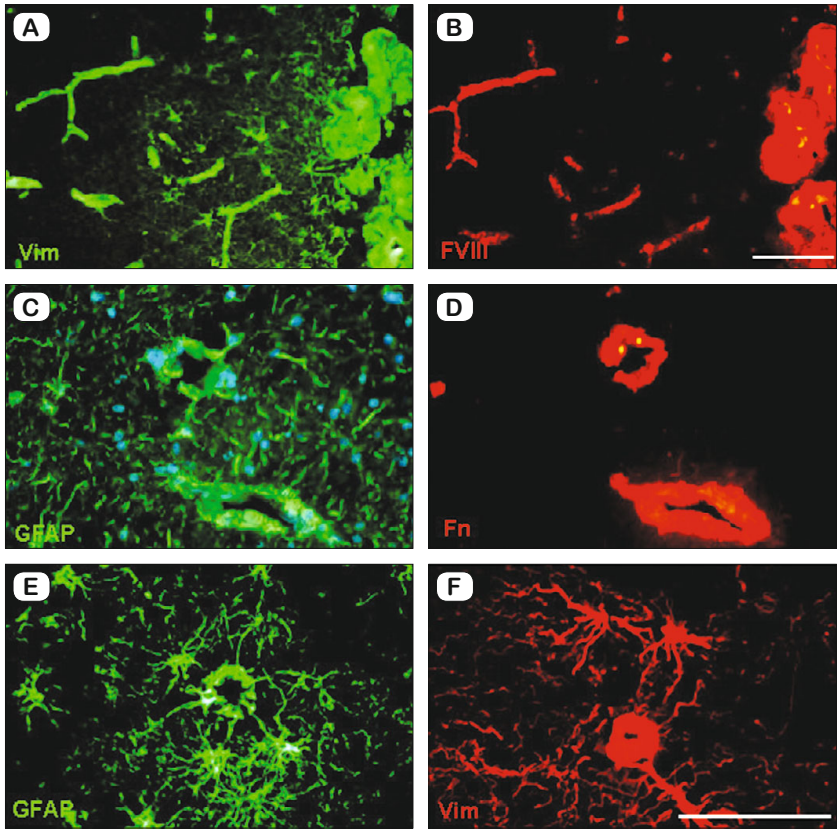

Fig. 1. Immunofluorescence staining of brain cryosections. Double labelling (A-D): Vimentin positive staining of astrocytes and brain vessels in the subpial area (A), FVIII positive staining in brain vessels (B). GFAP-positive astrocytes (C), fibronectin positive brain vessels (D). Indirect immunofluorescence white matter (E, F). GFAP-positive astrocytes, endfeet surrounding brain vessels (E), vimentin positive astrocytes and brain vessels (F).

Antibodies

To identify astroglia cell types, we used antibodies against GFAP (clone GF-01, 1:100, Exbio, Prague), and polyclonal sera to GFAP (1:100, Dako). Oligodendroglia cells were detected by antibodies to GalC (clone mGalC, 1:10, Boehringer-Mannheim, Vienna) and to O4 antigen (clone 81, 1:10, Boehringer-Mannheim, Vienna). Microglial cells were defined as immunoreactive with antibodies to CD11c (clone BU15, 1:50, Immunotech, France), and polyclonal sera to ferritin (1:50, Sigma). Neuronal cells were identified with antibodies to MAP2 (clone HM-2, 1:50, Sigma), and NF (clone NF-01, 1:100, Exbio, Prague). The endothelial cells were detected by polyclonal sera against Von Willebrand Factor-VIII-related antigen (1:100, Dako). The antibodies to vimentin (clone V9, 1:100, Sigma), to cytokeratins monoclonal anti-pan CK (types: 1,4,5,6,8,10,13,18,19, 1:100, Sigma), and polyclonal sera against fibronectin (1:100, Sigma) were used for further characterization of human brain cells. The secondary fluorescein- and rhodamine-conjugated antibodies were purchased from Sigma and Sevapharma (Prague).

Immunofluorescence

Cells grown on uncoated glass coverslips were used for indirect and double immunofluorescence staining. The intermediate filament proteins (GFAP, vimentin, neurofilaments), MAP2, ferritin, fibronectin and F VIII were detected on cells fixed in methanol-acetone (1:1) solution for 15 min. at (–15 °C). The surface antigen CD11c was examined on cells fixed in 4 % paraformaldehyde for 15 min at room temperature.

Cells for indirect immunofluorescence were incubated 1 h with primary antibodies and 30 min with 1:50 diluted appropriate secondary antibodies. Double-labelling was performed with primary, and afterwards with appropriate mixtures of secondary antibodies for 1 h and 30 min, respectively. Nuclei were stained with Hoechst 33258 fluorochrome (5 µg/ml in PBS, Sigma) for 1 min. To determine the percentage of immunoreactive glial cell types, 20 fields were enumerated at 200x magnification on confluent cultures.

Staining of brain cryosections (10 um thick) was performed by the same immunofluorescence methods as described above. Formalin-fixed, paraffin-embedded sections were stained for ferritin, 1 h incubation with peroxidase-conjugated antirabbit secondary antibodies (Dako). The diaminobenzidine reagent was used as the chromogen. Fluorescence microscopy was performed using an Olympus BX51 microscope (Hamburg, Germany).

Results

Immunostaining of brain tissue.

Cryosections were examined with antibodies to vimentin, GFAP, FVIII and fibronectin. Immunostaining revealed the presence of vimentin in astrocytes and brain vessels (Figs 1A, F). GFAP expression was observed in astrocytes and their endfeet

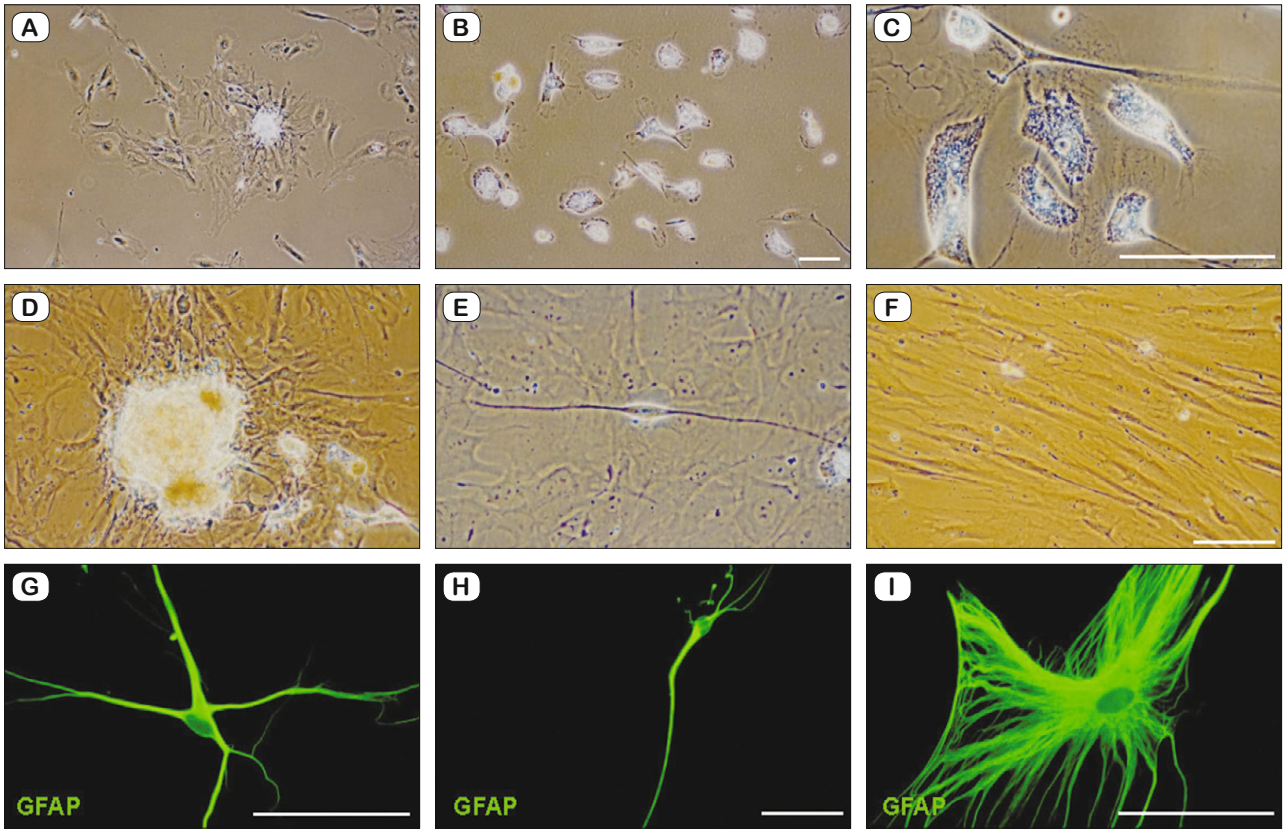

Fig. 2. Morphology of living cells in adult human brain cultures. Phase-contrast microscopy (A-F). An explant with outgrowing flat cells DIV 7 (A), an area of amoeboid cells DIV, 5 (B), microglial cells with grainy cytoplasm and strong bright effect, DIV 9 (C). An explant with outgrowing glia-like cells, DIV 21 (D). Bipolar astrocyte overlaying confluent layer of flat cells, DIV 21 (E). Confluent layer of spindle shaped cells, DIV 21 (F). Indirect immunofluorescence for GFAP (G-I). GFAP-positive stained stellate (G), bipolar (H) and large plasmatic (I) astrocytes.

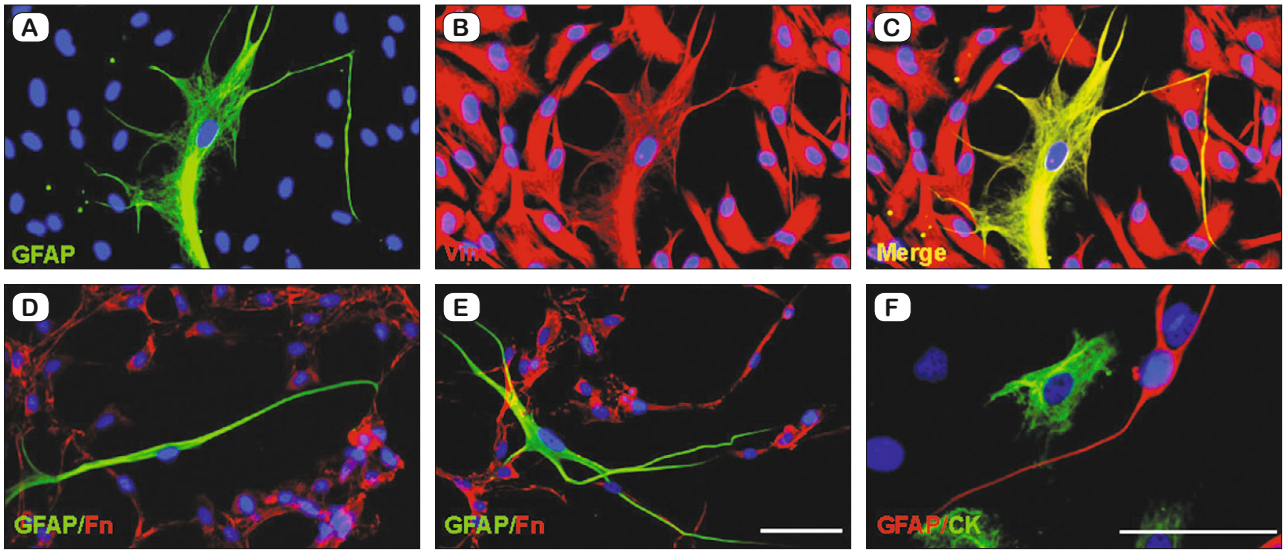

Fig. 3. Double labelling of tissue cultures. GFAP/Vim (A-D), GFAP/ Fn (D-E) and GFAP/CK (F). GFAP+ astrocyte and GFAP- "glia-like" cells (A), Vim + astrocyte and "glia-like" cells (B), merge (C). GFAP+/Fn- astrocyte and GFAP-/Fn+ "glia- like" cells (E-F). GFAP+/CK- astrocyte and GFAP-/CK+ or GFAP-/CK- "glia-like" cells.

Tab. 1. Immunostaining of adult human brain cultures and brain tissue.

Brain cells	Immunostaining					
Brain tissue	GFAP	Vim	CK	Fer	FN	FVIII
Astrocytes	+++	+++	–	–	–	–
Microglia	–	–	–	+++	–	–
Vessels	–	+++	–	–	+++	+++
Tissue cultures						
Astrocytes	+++	+++	–	–	–	–
Microglia	–	+++	–	+++	–	–
"Glia-like" cells	–	+++	–, ++	–	+++	–

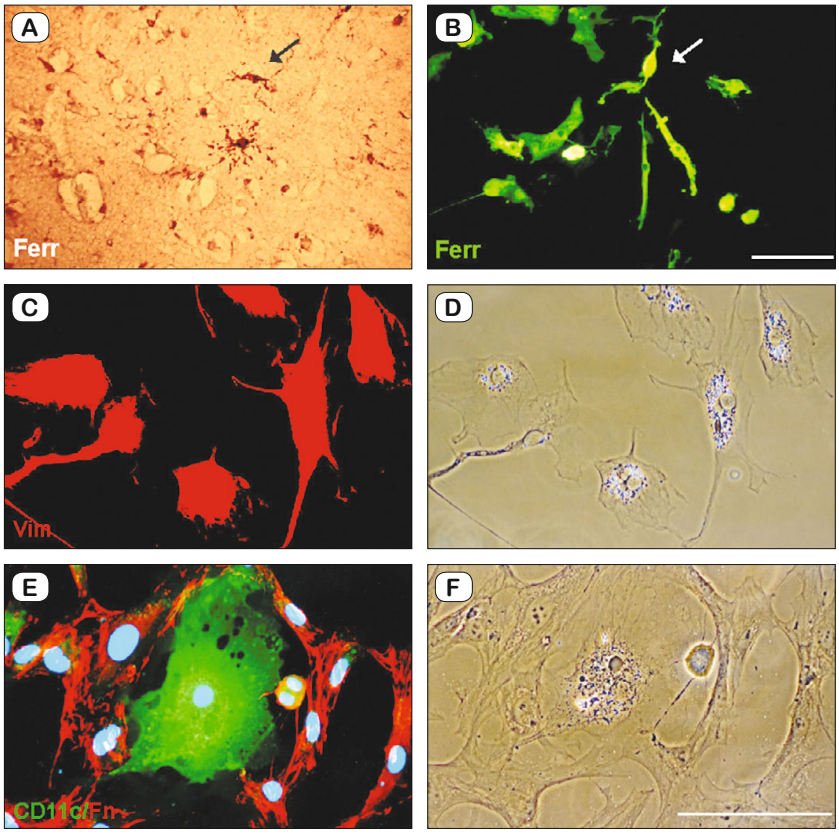

Fig. 4. Immunostaining of microglial cells. Indirect immune-peroxidase staining for ferritin in the brain tissue, positively stained ramified and activated microglia (A). Indirect immunofluorescence of brain cultures: ferritin positive (B) and vimentin (C) positive microglial cells, phase contrast microscopy (D). Double labelling: CD11c+/Fn- microglia and CD11c-/Fn+ "glial-like" cells (E), phase-contrast microscopy (F).

surrounding the brain vessels (Figs 1C, E). Positive staining for FVIII and fibronectin was restricted only to brain vasculature (Figs 1B, D). The indirect immunoperoxidase technique and ferritin antibodies were used for detection of microglial cells. Positive staining revealed ramified and activated microglia (Fig. 4A).

Morphology and immunostaining of brain cultures

Morphological features of living cells were evaluated using phase-contrast microscopy. The first single attached cells were microglia, strongly phase-bright cells with granular, vacuolated cytoplasm (Figs 2 B, C). Within 5 to 10 days in vitro (DIV) we observed outgrowing cells from small brain explant, which were mostly flat or spindle shaped "glia-like" cells (Fig. 2A). Confluent cell layers in all the cultures were formed over 3–6 weeks, where "glia-like" cells constituted more than 95 % of all cells (Figs 2D, E, F), plus microglia at 2–5 % (Fig. 2D). Long thin process-bearing cells occurred rarely and were often found as overlaying the confluent layer of "glia-like" cells (Fig. 2E).

Glial and non-glial cells in adult human brain cultures were identified by indirect immunofluorescence using specific marker antibodies and, the results summarized in Table 1. Astrocytes were detected by GFAP antibodies. The three main morphological types of GFAP-positive cells were found in all the cultures: stellate, bipolar and large cytoplasmic cells (Figs 2G, H, I). In confluent cultures derived from the white matter, GFAP-positive cells reached about 0.1 % of all the cells. However, only a few scattered GFAP-positive cells were seen in cultures from the cortical grey matter. The majority (95–98 %) of the cells in primary adult human brain cultures were GFAP-negative "glia-like" cells, which were positively stained for vimentin (Figs 3B, C) and fibronectin (Figs 2D, E). Subpopulation of cytokeratin positive "glia-like" cells at widely different percentages (3–70 %) were found in all the cultures (Fig. 3F). Double labelling showed that GFAP positive astrocytes stained positively for vimentin (Figs 3A, B, C), negatively for both fibronectin (Figs 3D, E) and cytokeratins (Fig. 3F). Oligodendrocytes were identified by marker antibodies to GalC and O4. Because of the rare appearance of oligodendrocytes (≤ 0.01 % at confluent cultures) they were not further analysed. All the phase-bright morphologically distinct cells stained positively with microglial marker antibodies to ferritin (Fig. 4B) and CD11c (Fig. 4E). Double labelling for CD11c and fibronectin revealed negative staining for fibronectin in microglial cells and positively stained "glia-like" cells (Figs 4 E,F). Vimentin was expressed in all microglia (Figs 4C, D). Cells similar to activated microglia were found extremely rarely (Fig. 4B). Microglia occurred mainly as amoeboid cells with vacuolated or granular cytoplasm (Fig. 4D). Staining with antibodies against FVIII did not detect endothelial cells and staining with neuronal marker antibodies to MAP2 and NF did not reveal neuronal cell population in our cultures.

Discussion

In this report, we compared the morphology and immunophenotypes of glial and non-glial cells in adult human brain tissue and cultures, as an attempt to illuminate the histological origin of "glia-like" cells. Cells in adult human brain cultures formed confluent layer within 3–6 weeks and at confluency comprised the following: 0.1 % astrocytes, ≤ 0.01 % oligodendrocytes, 2–5 % microglial cells and 95–98 % "glia-like" cells. Astrocytes in brain tissue and cultures showed the same immunophenotypes, staining positively for GFAP and vimentin. However, three main morphologic types of GFAP-positive cells were found in these cultures: stellate, bipolar and large flat cells. Stellate astrocytes *in vitro* may correspond to fibrous astrocytes *in vivo*. However, large flat and bipolar GFAP-positive astrocytes were not observed in adult human brain tissue. Bipolar GFAP-positive astrocytes negatively staining with neuronal markers were also described by Davies, 2000 (9). On the other hand, the cells with two or three long processes described here mimic a neuron-like morphology. A low number of processes bearing cells in adult human brain cultures positively stained with neuronal marker antibodies and negatively for GFAP were considered to be neurons (10). However, we described here morphologically similar cells as astrocytes because under our culture condition, they were positively stained for GFAP and negatively with neuronal markers. False positive staining may be due to non-specific antibodies. However, neuron specific markers can also react with astrocytes and/or oligodendrocytes (11, 12). An identity crisis of neurons using immunostaining was reviewed by Rakic (13).

Microglia, the brain tissue parenchymal resident macrophages occur as ramified, activated (less branched), bipolar/rod shaped and ameboid (14, 15). In the brain tissue, we detected ramified and activated microglia positively stained for ferritin and negatively for vimentin. In cultures, microglia appeared rarely as activated or bipolar/rod shaped but ameboid morphologies were abundant. They retained ferritin expression and became vimentin positive.

Cytokeratins are specific for normal and neoplastic epithelial cell differentiation. Cryosections from brain tissue stained negatively with pan-CK antibodies while "glia-like" cells expressed cytokeratins at various percentage. The unexpected presence of cytokeratins may be caused by the fact that epithelial and brain tissue share a common ectodermal origin. Fibronectin is one of the major glycoproteins of the interstitial matrix. Although fibronectin is produced by variety of mesenchymal and neoplastic cell types, it is often considered to be specific for fibroblasts. It is remarkable that fibronectin was co-expressed with GFAP in astrocyte cultures from embryonic and early postnatal rat brain (16) or in adult human brain cultures (8, 16). In brain tissue, we found fibronectin restricted to blood vessels. While in cultures the "glia-like" cells stained positively, cells expressing glial marker stained negatively for fibronectin.

Some authors suggest that adult human "glia-like" cells might be of non-glial origin (2, 17). The study by Rutka (2) demonstrated the similarities between normal human brain and leptomeningeal cultures. Both cultures were morphologically similar stained positively for fibronectin, vimentin, laminin and collagen IV. These authors suggest that brain cultures predominantly contain leptomeningeal cells, which envelope and only follow cortical arteries penetrating into brain parenchyma. However, different culture conditions may induce changed morphological and/or immunochemical properties of so-called "glia-like" cells (18) Previously we have shown that GFAP-negative "glia-like" cells became GFAP-positive during the period of spontaneously decelerated growth at higher passages (19).

Our comparative analysis of brain cells *in vivo* and *in vitro* did not identify the histological origin of so called "glia-like" cells. However, we present evidence that culture conditions may induce cell dedifferentiation in relation to positive expression of vimentin in microglial and cytokeratins in "glial-like" cells. We suggest that "glia-like" cells come from currently unknown progenitor cells scattered through the brain tissue.

References

1. Rutka JT, Giblin JR, Balkissoon R, Wen D, Myatt CA, McCulloch JR, Rosenblum ML. Characterization of fetal human brain cultures. Dev Neurosci. 1986; 9: 154–173.

2. Rutka JT, Kleppe-Hoiford H, Emma DA, Giblin JR, Dougherty DV, McCulloch JR, DeArmond SJ, Rosenblum ML. Characterization of normal human brain cultures. Lab Invest 1986; 55 (1): 71–85.

3. Balmiki Ray, Nipun Chopra, Justin M. Long and Debomoy K Lahiri. Human primary mixed brain cultures: preparation, differentiation, charecterization and application to neuroscience research. 2014; 7:63.

4. Ponten J, Westermark B. Properties of human malignant glioma cells in vitro. Med Biol 1978; 56 (4): 184–193.

5. Osborn M., Ludwig-Festl M., Weber K., Bignami A., Dahl D. & Bayreuther K. Expression of glial and vimentin type intermediate filaments in cultures derived from human glial material. Differentiation 1981; 19: 161–167.

6. Perzelova A, Macikova I, Tardy M, Mraz P, Bizik I. Subpopulation of nestin positive glial precursor cells occur in primary adult human brain cultures. Biologia 2007; 62: 633–40.

7. Estes ML, Ransohoff RM, McMahon JT, Jacobs BS, Barna BP. Characterization of adult human astrocytes derived from explant culture. J Neurosci Res 1990; 27: 697–705.

8. Van der Laan LJW, De Groot CJA, Elices MJ, Dijkstra CD. Extracellular matrix proteins expressed by human adult astrocytes in vitro and in vivo: An astrocyte surface protein containing the CS1 domain contributes to binding of lymphoblasts. J Neurosci Res 1997; 50: 539–548.

9. Davies DL, Niesman IR, Boop FA, Phelan KD. Heterogeneity of astroglia cultured from adult human temporal lobe. Int J Devl Neuroscience 2000; 18: 151–160.

10. Kirschenbaum B, Nedergaard M, Preuss A, Barami K, Fraser RAR, Goldman SA. In vitro neuronal production and differentiation by precursor cells derived from the adult human forebrain. Cereb Cortex 1994; 6: 576–589.

11. Draberová E, Del Valle L, Gordon J, Marková V, Smejkalová B, Bertrand L, de Chadarévian JP, Agamanolis DP, Legido A, Khalili

K, Dráber P, Katsetos CD. Class III beta-tubulin is constitutively co-expressed with glial fibrillary acidic protein and nestin in midgestational human fetal astrocytes: implications for phenotypic identity. J Neuropathol Exp Neurol 2008; 67: 341–354.

12. Geisert EE, Johnson HG, Binder LI. Expression of microtubule-associated protein 2 by reactive astrocytes. Proc Natl Acad Sci 1990; 87: 3967–3971.

13. Rakic Pasco. Adult neurogenesis in mammals: An identity crisis. J. Neurosci. 2002; 22 (3): 614–618.

14. Williams K, Bar-Or A, Ulvestad E, Olivier A, Antel JP, Yong, VW. Biology of adult human microglia in culture: Comparisons with peripheral blood monocytes and astrocytes. J Neuropathol Exp Neurol 1992; 51: 538–549.

15. Ngan Pan Bennett AU, Chi Him E Ma. Recent advances in the study of bipolar/rod-shaped microglia and their roles in neurodegeneration. Front Aging Neurosci 2017; 9: 128. DOI: 10.3389/fnagi.2017.00128.

16. Liesi P, Kirkwood T, Vaheri A. Fibronectin is expressed by astrocytes cultured from embryonic and early postnatal rat brain. Exp Cell Res 1986; 163: 175–185

17. Gibbons HM, Hughes SM, Van Roon-Mom W, Greenwood JM, Narayan PJ, Teoh HH, Bergin PM, Mee EW. Wood PC, Faull RLM, Dragunow M. Cellular composition of human glial cultures from adult biopsy brain tissue. J Neurosci Met 2007; 166: 89–98.

18. Zhang Ye, Steven A Sloan, Laura E Clarke, Gerald A Grant, Melanie G Hayden Gephart, Ben A Barres. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. Neuron 2016; 89: 37–53.

19. Perzelova A, Mares V. Appearance of GFAP-positive cells in adult human brain cultures spontaneously decelerated in growth. Glia 1993; 7: 237–244.

Received April 1, 2022.
Accepted April 21, 2022.