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EXPERIMENTAL STUDY

Astroglial differentiation of fibronectin-positive human "glia-like" cells

Ivana SIVAKOVA, Peter MRAZ, Anna PERZELOVA

Department of Anatomy, Faculty of Medicine, Comenius University, Bratislava, Slovakia.
Anna.perzelova@fmed.uniba.sk

ABSTRACT
OBJECTIVES: Fibronectin (Fn) is a glycoprotein of extracellular matrix produced by a variety of mesenchymal and neoplastic cell types.
BACKGROUND: In adult brain tissue, Fn is restricted to blood vessels. However, adult human brain cultures are almost entirely comprised of flat or spindle-shaped Fn-positive cells usually referred to as "glia-like" cells. Since Fn is primarily present in fibroblasts, these cultures may be considered to be of non-glial origin.
METHODS: Cells gained by long-term culturing of adult human brain tissue derived from brain biopsies obtained from 12 patients with non-malignant diagnoses were examined by immunofluorescence methods.
RESULTS: Primary cultures contained GFAP–/Vim+/Fn+ "glia-like" cells (95–98 %) and GFAP+/Vim+/Fn- astrocytes (0.1 %) which disappeared by passage number 3. The formation of cell processes and enlargement of cell bodies was observed in 9 of 12 cultures with decreased cell growth during passages 12 to 17. It is remarkable that during this period, all "glia-like" cells became GFAP+/Vim+/Fn+.
CONCLUSION: Herein, we confirm our previously published hypothesis about the origin of adult human "glia-like" cells, which we consider to be precursor cells scattered through the brain cortex and subcortical white matter. Cultures were comprised entirely of GFAP–/Fn+ "glia-like" cells and showed morphological and immunochemical astroglial differentiation with spontaneously decelerated growth during prolonged passaging. We propose that the adult human brain tissue contains a "dormant population" of undefined glial precursor cells. Under culture, these cells show to have a high proliferative capacity and different stages of cell dedifferentiation *(Fig. 2, Ref. 21)*. Text in PDF *www.elis.sk*
KEY WORDS: astrocytes, GFAP, fibronectin, glial differentiation, glia-like cells.

Introduction

Traditionally, astrocytes are divided into fibrous and protoplasmic types based on their morphologic features and brain distribution. Fibrous and protoplasmic astrocytes are common in the white and gray matter, respectively (1). Over the last three decades we have performed cultures of adult human brain biopsies in order to address the basic questions about the identification and behavior of glial and non-glial cells under culture conditions (2). To find fibrous and protoplasmic astrocytes, cultures were prepared separately from the cortical gray and white matter. Both cultures, however, differed only in the number of GFAP-positive cells, which were absent or rare in the gray matter cultures.

In all cultures, we observed mainly stellate GFAP-positive cells which may correspond to fibrous astrocytes, however, no morphologies of protoplasmic astrocytes. On the other hand, large flat and bipolar GFAP-positive cells occurred in cultures that were not observed in adult human brain tissue (3). Bipolar GFAP-positive and neuronal marker-negative stained cells were also present, as described by Davies (4). These cells with two or three long thin processes mimic neuronal morphology and appear as being neurons. The report by Kirschenbaum (5) described neuronal precursor cells in cultures derived from adult human brain biopsies. It is remarkable that the morphology of these neurons is also similar to that of our astrocytes as they occurred in the white matter. Kirschenbaum et al (5) addressed the numeration of processes-bearing cells present as a minority population, and cultures described as mixtures of flat uncharacterized cells, small stellate astrocytes, capillary endothelial cells, and microglia. They found 393 GFAP+ astrocytes and 56 MAP-2+ neurons from which only six neurons were deemed proliferative having incorporated 3H-thymidine. On the other hand, these authors highlighted the MAP-2 neuronal marker observed in reactive white matter astrocytes (6).Another neuronal marker, beta III-tubulin used for identification of neuronal cells (7) was found in fetal astrocytes (8). Falsely positive identified neurons were described by Rakic who portrayed them as being in an "identity crisis" (9).

Many reports deal with the function of astrocytes. However, they resulted in a mixture of findings that have been obtained on various mainly embryonic or newborn rodent cultures. These cul-

Department of Anatomy, Faculty of Medicine, Comenius University, Bratislava, Slovakia

Address for correspondence: Anna PERZELOVA, Department of Anatomy, Faculty of Medicine, Comenius University, Sasinkova 2, SK-813 72 Bratislava, Slovakia.

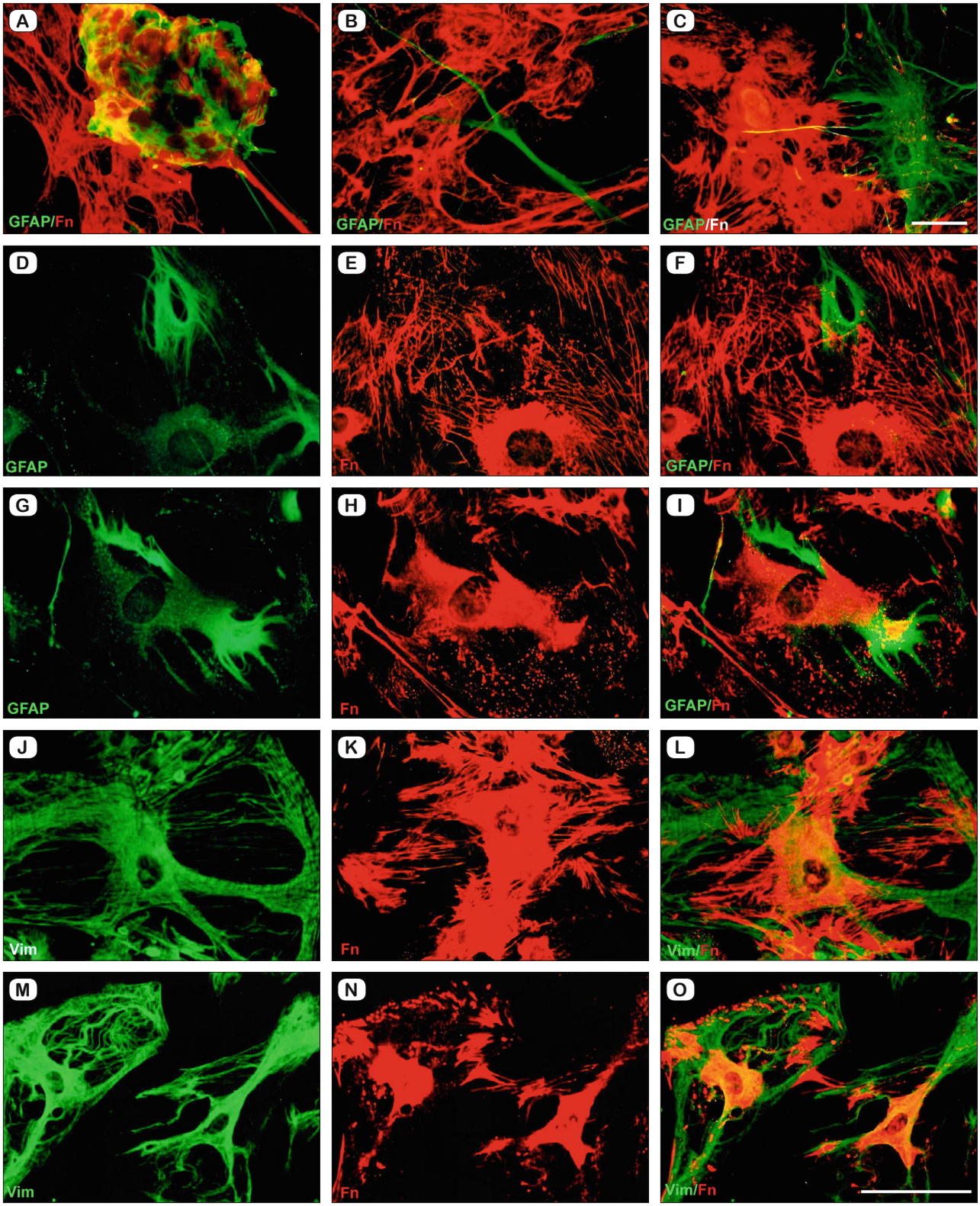

Fig. 1. Double labelling of adult human brain cultures. GFAP/Fn (A–I) and Vim/Fn (J–O). Primary cultures, DIV 10 (A–C), brain explant with outgrowing of Fn+/GFAP– "glia-like" cells GFAP+/Fn– astrocyte processes (A), GFAP+/Fn– astrocyte and GFAP–/Fn+ cells (B, C). The culture of non-proliferating cells at passage 15 became GFAP+/Fn+ (D–I) and Vim+/Fn+ (J–O). Scale bars: 100 µm.

tures in early passages may be comprised almost entirely of GFAP-positive cells. On the other hand, the cultures from adult human brain tissue remain undefined because they are comprised of cells without glial morphology and immunophenotypes (2, 10). They are often termed "glia-like" cells (11) and considered to be non-glial in origin (10, 12). In addition, a meta-analysis of published studies has shown large discrepancies in cells after immunostaining of adult human brain cultures (2, 10, 13, 14).

In this study we pursue further characterization of "glia-like" cells using long-term culturing which allows to compare the morphology and immunophenotypes of rapidly and slowly proliferating cells in human brain cultures.

Materials and methods

Tissue cultures and brain specimens

Adult human brain biopsies were kindly provided by the Department of Neurosurgery, Derer's Hospital, Bratislava. This study reports the results performed on brain samples collected during the period of 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.

For this study, we chose 12 brain biopsies from adult donors undergoing neurosurgical intervention after non-malignant diagnoses. Tissue cultures were prepared by an explant method, separately from cortical gray and subcortical white matter. Samples obtained from temporal or frontal lobes were cut into small pieces and seeded in uncoated plastic dishes. The culture medium consisted of MEM with glutamine, nonessential amino acids and 10 % fetal calf serum. Cultures were passaged using 0.2 % EDTA and 0.25 % trypsin. Simultaneously, the cells used for immunocytochemical staining were grown under the same conditions on uncoated glass coverslips.

Immunofluorescence

To identify astroglial cells, we used antibodies against GFAP (clone GF-01, 1:100, Exbio, Prague). Neuronal cells were identified with antibodies to MAP2 (clone HM-2, 1:50, Sigma), and NF (clone NF-01, 1:100, Exbio). The antibodies to vimentin (clone V9, 1:100, Sigma) and polyclonal sera against fibronectin (1:100, Sigma) were used for further characterization of human "glia-like" cells. The secondary fluorescein- and rhodamine-conjugated antibodies were purchased from Sigma and Sevapharma (Prague).

Cells grown on uncoated glass coverslips were used for indirect and double immunofluorescence staining. The intermediate filament proteins (GFAP, vimentin, neurofilaments), MAP2 and fibronectin were detected on cells fixed in methanol-acetone (1:1) solution for 15 min. at (–5 °C). Cells for indirect immunofluorescence were incubated for 1h with primary antibodies and 30 min with 1:50 diluted appropriate secondary antibodies. Double labeling was performed with primary, and afterwards with appropriate mixtures of secondary antibodies for 1 h and 30 min, respectively. Immunofluorescence microscopy was performed using an Olympus BX51 microscope (Hamburg, Germany).

Results

Morphology and cell growth in brain cultures

Morphological features of nonpassaged primary cultures were described previously (2, 3). Briefly, cells outgrowing from a small brain explant were mostly flat or spindle-shaped "glia-like" cells.

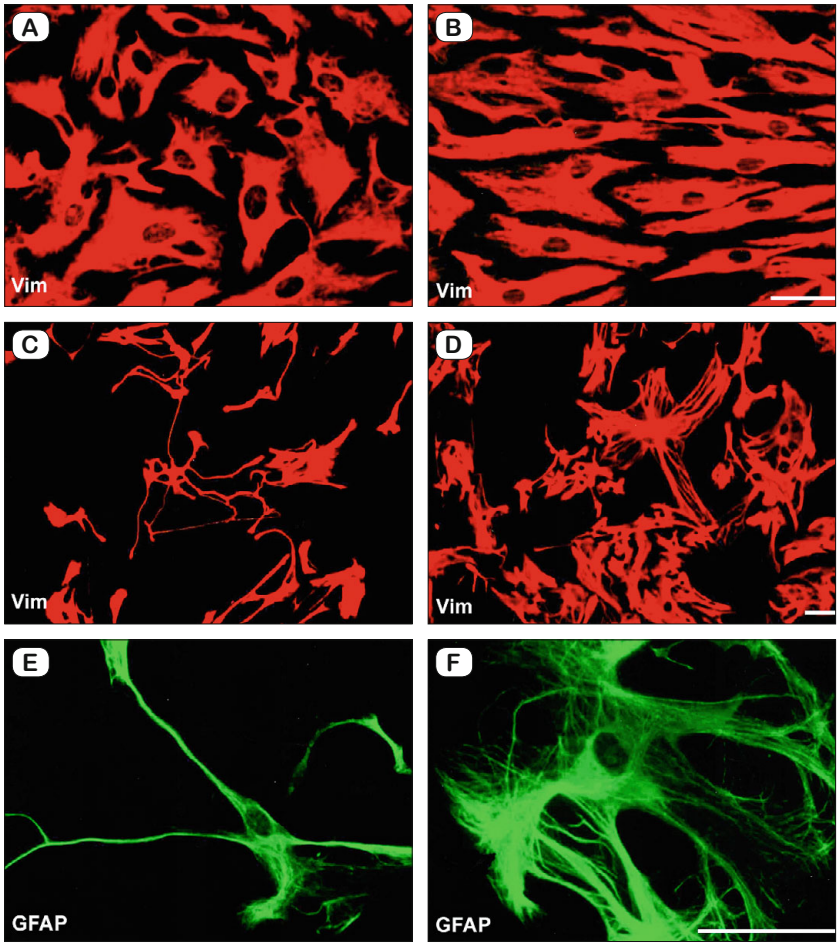

Fig. 2. Indirect immunofluorescence staining for vimentin (A–D), and GFAP (E–F). Flat and spindle-shaped well-proliferating Vim+ cells at passage 2 (A–B) and cultures with decreased cell growth at passage 10 (C, D). Please note the initial formation of cell processes (C) and enlargement of cell bodies (D). GFAP+ staining of differentiated cell in non-proliferating cultures (E, F). Scale bars: 100 µm.

Confluent cell layers were formed over 3–6 weeks, where "glia-like" cells constitute 95 to 98 % of all cells, while remaining cells were morphologically mainly microglia alongside with rarely occurring long thin process-bearing cells of astroglial morphology (0.1 %).

In this study, we performed long-term modified passaging of cultures with decreased cell growth. In passages 1 to 7 of all cultures, the cells were mainly flat polygonal, or spindle-shaped (Fig. 2A, B). Process-bearing cells typical of fibrous astrocytes completely disappeared by passage number 3. Morphological changes appeared in 9 of 12 cultures with spontaneously decelerated cell growth in passages 12 to 17. This culture period was specific with formation of cell processes and enlargement of cell bodies (Fig. 2C, D). After passage 17, the cell growth successively ceased, cultures failed to reach confluency and the cells gradually disappeared after splitting 1:2 weekly. In nonpassaged cultures, the cells degenerated after the 3rd week. On the other hand, the maintenance of non-proliferating cells was successful during 8 to 10 weeks when the cultures were split 1:1 every second week. This period was specific with further enlargement of cell bodies and different formation of cell processes (Fig. 2E, F). The decrease in cell growth in 3 of 12 cultures were detected at passages 7 to 12. The cells in these cultures showed no astroglial cell differentiation.

Immunofluorescence

Indirect immunofluorescence with antibodies to GFAP, NF, MAP2, vimentin, and fibronectin was performed for the purpose of detecting glial, and neuronal cell types in primary cultures after long-term passaging. The three main morphological types of GFAP-positive cells were found in all nonpassaged cultures, i.e., stellate, bipolar and large cytoplasmic cells. In confluent cultures derived mainly from the white matter, GFAP-positive cells reached about 0.1 % of all cells. These results confirm the findings of our previous study (2, 3). GFAP-positive astrocytes persisted only in early passages and completely disappeared by passage number 3. Flat or spindle-shaped rapidly proliferating "glia-like" cells were GFAP-negative. However, the cell growth that decreased due to morphological changes of "glia-like"cells was accompanied by GFAP expression in 9 of 12 cultures. Staining for GFAP showed a fibrillary intracytoplasmic structure (Fig. 2E, F). The GFAP expression was absent in the remaining 3 cultures. The staining for neuronal markers remained negative during the whole culturing process. Immunostaining with antibodies to vimentin and fibronectin was positive for all "glia-like" cells. However, in early passages, GFAP-positive astrocytes showed a positive staining for vimentin but remained negative for fibronectin.

Double labeling with antibodies to GFAP and fibronectin revealed that even the "glia-like" cells outgrowing from brain explant were Fn+/GFAP– (Fig. 1A) while GFAP-positive astrocytes were GFAP+/Fn– (Fig. 1B, C). During the period of decreased cells growth, cultures became GFAP-positive, but retained their Fn expression (Fig. 1D–I). Long-term cultivation as well as the decreased and arrested cell growth has no influence on vimentin expression, which was present in all cells from all cultures throughout cultivation. Vimentin was co-expressed with Fn (Fig. 1J–O).

Discussion

Recently, we tried to clarify the origin of human cultured "glia-like" cells that are often considered to be non-glial in origin. Although we did not ascertain the histological origin of these cells, we demonstrated strong dedifferentiation of cells in adult human brain cultures (15). Previously, we have shown that initially GFAP-negative "glia-like" cells became GFAP-positive at higher passages during the period of spontaneously decelerated growth (16). This finding was demonstrated on three human brain cultures. Here we studied 12 tissue cultures to investigate astroglial differentiation in more detail. The new findings are as follows:

1. Not all cultures became GFAP-positive at higher passages, as 3/12 cultures showed decreased proliferation at lower passages without evident morphological changes as well as GFAP expression. We did not find any explanation for this observation.
2. GFAP and fibronectin were co-expresssed in "glia-like" cells with spontaneously decelerated growth.

Fibronectin is an important extracellular matrix protein, produced by variety of mesenchymal cells. In brain tissue, fibronectin is restricted to blood vessels (17, 18). Co-expression of GFAP and fibronectin has been described in embryonic and early postnatal rat brain (19). On the other hand, there are conflicting reports concerning GFAP and fibronectin in adult human brain cultures. Here, cells in early passages were GFAP–/Fn+ (2, 10), GFAP+/Fn+ (14) or even GFAP+/Fn– (13). It is remarkable that all these controversialy immunostained cells were of similar non-glial morphologies.

These findings confirm our hypothesis abou the glial origin of "glia-like" cells. These immunocytochemically undefined brain cells, outgrowing from brain explants, may react to changed environmental conditions with rapid growth due to changed cytoskeletal protein expression which can induce fibronectin.

Neurogenesis, considered as generation of mature neurons from neural progenitor cells has been described during embryonic and postnatal stages in mammals. Over the last several decades, numerous studies dealing about neurogenesis which continues throughout the animal and human life have been publlished (20). The investigations have mainly focused on the subventricular zone (SVZ), the outside wall of lateral ventricles in mammalian brain. This brain area is considered to be a source of dividing neural precursor cells during developing as well as in adult human brain. However, the idea of the therapeutic use of neuronal precursor cells in the treatment of brain diseases is far away from new therapeutic target because the problems with identification of neuronal precursors. The reasons are described in a recently published critical reviews (9, 20), and in study which demonstrates the nonrenewal of neurons in the cerebral cortex of adult monkey (21).

On the other hand, little attention has been paid to the phenomenon that culture from adult human brain contain rapidly proliferating cells with high differentiative capacity but unknow histological origin. Perhaps adult human brain tissue contains only glial precursor cells. It is difficult to imagine the replacement and renewal of cortical neurons from SVZ.

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