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GFAP-positive astrocytes are rare or absent in primary adult human brain tissue cultures

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Abstract: Traditionally, astrocytes are divided into fibrous and protoplasmic types based on their morphologic appearance. Here the cultures were prepared separately from the adult human cortical gray and white matter of brain biopsies. Both cultures differed only in the number of glial fibrillary acidic protein (GFAP)-positive cells. In the gray matter these were absent or rare, whereas in confluent cultures from the white matter they reached 0.1% of all cells. Three main morphologic types of GFAP-positive cells were found in this study: stellate, bipolar and large flat cells. GFAP-positive cells with two or three long processes mimic a neuron-like morphology. We did not find process-bearing cells expressing neuronal markers (MAP-2, NF, and N-CAM). The conflicting reports concerning GFAP immunostaining and the study dealing with the presence of putative neurons in adult human brain cultures are discussed with respect to these findings. The latter classification of astrocytes into type 1 and type 2 is based on immunostaining to A2B5 antigen: type 1 (GFAP+/A2B5–) and type 2 (GFAP+/A2B5+) astrocytes are proposed to be analogous to protoplasmic and fibrous astrocytes, respectively. In adult human brain cultures we found only small amount of A2B5-positive cells. Double immunofluorescence revealed that astroglial cells of similar fibrous or bipolar shape grown on one coverslip were either GFAP+/A2B5+ or GFAP+/A2B5–. On the other hand, the A2B5+/GFAP– immunophenotype was not observed. These results indicate that in general the cell phenotype from adult human brain tissue is not well established when they are in culture.

Key words: adult human astrocytes; neuronal markers; GFAP; A2B5 antigen; O4 antigen; GalC antigen.

Abbreviations: CNS, central nervous system; DIV, days *in vitro*; GFAP, glial fibrillary acidic protein; PBC, process-bearing cells.

Introduction

Astrocytes are star-shaped subtype of glial cells distributed through the central nervous system. Initially they were considered as connective cells in the central nervous system (CNS) which provide support to the neurons. Currently astrocytes have been reconsidered and now thought to have a wide range of functions in the brain and spinal cord tissue (Kimelberg 1995; Ridet et al. 1997; Temburni & Jacob 2001; Barres 2008). In the past astrocytes were identified by the gold chloride sublimate method, now they are mainly identified by their expression of glial marker proteins. Glial fibrillary acidic protein (GFAP) is intermediate filament protein which is considered to be the most specific marker for astroglial cells both *in vivo* and *in vitro* (Bignami et al. 1972; Antanitus et al. 1975; Eng & Lee 1995).

The majority of glial studies have been performed on rodent brain and/or cultures. Although interest in adult human brain cultures has increased recently (Brunet et al. 2001, Holley et al. 2005, Oberheim et al. 2006), numerous studies have failed to resolve the basic question about glial cells behaviour in adult human brain cultures. For example GFAP-positive cells are rare or absent from these cultures which consist mainly of GFAP-negative flat cells, often termed as "glia-like" cells (Ponten & Westermark 1978; Rutka et al. 1986). Previously, we have shown that initially GFAP-negative flat or spindle shaped "glia-like" cells became GFAP-positive during the period of spontaneously decelerated growth (Perzelova & Mares 1993). We supposed that "glia-like" cells which predominate in adult human brain cultures are glial precursor cells (Perzelova et al. 2007). On the other hand, significant discrepancies have been observed in the expression of GFAP, fibronectin, vimentin and neuronal markers in adult human brain culture (Rutka et al. 1986; Estes et al. 1990; Van der Laan et al. 1997).

In this study we examined a small population of cultured process-bearing cells in relation to traditional and revised astrocyte classifications.

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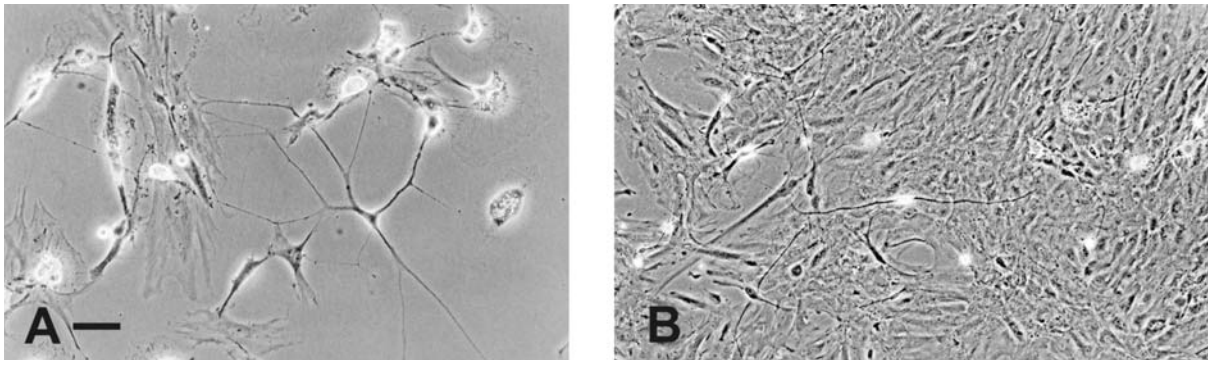

Fig. 1. Morphological features of primary adult human brain tissue cultures. Phase-contrast microscopy of living cells. (A) Three cell types: ameboid cells with grainy cytoplasm and strong bright effect (microglia/macrophages), flat polygonal "glia-like" cells and star-shaped astrocyte, 7 DIV. (B) Bipolar astrocyte overlaying flat confluent layer formed from "glia-like" cells, 28 DIV. Scale bar = 50 μm.

Material and methods

Cell cultures

Brain samples were kindly provided by the Department of Neurosurgery, Derer's Hospital, Bratislava. For this study we used 15 macroscopically normal brain biopsies from adult patients (23–55 years old) undergoing neurosurgical intervention for brain trauma, stroke or arteriovenous malformations. The samples were obtained from temporal or frontal lobes. Tissue cultures were prepared separately from cortical gray and white matter. Samples were cut into small pieces, less than 1 mm^3 (4–6 mm^3 of brain tissue per 1 cm^2) and seeded into plastic uncoated Petri dishes in medium consisting of MEM enriched with 10% fetal calf serum, glutamine, and non-essential amino acids. Simultaneously, cells used for immunocytochemical staining were grown under the same conditions on uncoated glass coverslips. We prepared 4 coverslips (24 × 32 mm) and 2 plastic dishes (25 cm^2) from each biopsy from cortical gray and white matter, respectively. Coverslips with confluent cell layers were cut and 2 cm^2 pieces were used for each immunostaining. Experiments with human brain biopsies were performed according the Slovak Laws 272/1994 and 576/2004.

Antibodies and immunofluorescence staining

Cells grown on glass uncoated coverslips were used for indirect and double immunofluorescence staining. Two types of astrocytes were identified using antibodies against GFAP: clone GF-01, 1:100 (Exbio, Prague); clone G-A-5, 1:100 (Sigma); polyclonal sera to GFAP, 1:100 (Dako) and to A2B5 antigen (a gift from Drs. S. Fedoroff and O. Berezovska, University of Saskatchewan, Canada, and from Dr. R. Asher, University of Cambridge, UK). Oligodendroglial cells were characterized by antibodies to GalC (clone mGalC, 1:10, Boehringer-Mannheim, Wien) and to O4 antigen (clone 81, 1:10, Boehringer-Mannheim, Wien). Neuronal cells were identified with antibodies to MAP2 (clone HM-2, 1:50, Sigma), N-CAM (clone NCAM-OB11, 1:50, Sigma), and NF (clone NF-01, 1:100, Exbio, Prague). The secondary fluorescein- and rhodamine-conjugated antibodies were purchased from Sigma and Sevapharma, Prague.

A2B5, O4, and GalC antigens were examined on live cells or paraformaldehyde-fixed cells. Staining with neuronal marker antibodies such as MAP2 and N-CAM was performed on cells fixed with cold methanol or following the method of Kirschenbaum et al. (1994) using 4% paraformaldehyde and permeabilization. The intermediate filament protein GFAP was detected on cells fixed in methanol-acetone (1:1) solution for 15 min at −15 °C. Cells for indirect immunofluorescence were incubated 1 h with primary antibodies and 30 min with 1:50 diluted secondary antibodies as appropriate. Co-expression of A2B5/GFAP, O4/GFAP was examined by double immunofluorescence. Cells were first incubated for 1 h with Mab to A2B5, GalC or O4 and 30 min with fluorescein-conjugated secondary antibodies. The cells were then fixed with cold methanol for 15 min and incubated 1 h with polyclonal sera to GFAP. Secondary rhodamine-conjugated antibodies were applied for 30 min. Staining intensities and percentage of glial cell types were determined in confluent cultures. To determine the percentages of glial marker positive cells, 70 fields were enumerated at 400× magnification, equally distributed over all coverslips processed. Fluorescence microscopy was performed with an Olympus IMT-2 microscope.

Results

Morphology of glial cells

The morphological features of primary cultures were described previously (Perzelova et al. 2007). Briefly, in primary cultures we found three main morphologically distinct cell types: ameboid cells with grainy cytoplasm, process-bearing cells (PBC) and flat or spindle shaped cells (Fig. 1A,B). Morphological features of PBC were observed on living cells using phase-contrast microscopy within 3 to 40 days *in vitro* (DIV). First PBC were observed as single cells at 5 to 7 DIV (Fig. 1A), they were rarely observed as outgrowing cells from brain explants. The majority of these cells possessed two or several thin and long processes. Evidently PBC were found in low percentage in comparison with rapidly proliferating flat polygonal "glia-like" cells which formed confluent layer within 3 to 6 weeks. PBC were often seen above this layer (Fig. 1B). The number of PBC was 0.1% of all cells in confluent layer in cultures derived from white matter. In cultures from cortical gray matter the PBC were absent or found rarely. Morphlogical features and counting of PBC was performed on plastic dishes and on glass coverslips, on 500 fields per one bioptic sample.

Immunofluorescence characterization of PBC

Quantitative analysis of immunostaining on PBC which

create 0.1% of all cells in confluent adult human brain cultures is summarized in Table 1.

Astrocytes

Astroglial cells were identified by GFAP immunostaining. Immunofluorescence was carried out using two MAb and polyclonal sera against GFAP. In confluent cultures derived from the white matter GFAP-positive cells reached 0.1% of the total and only a few scattered GFAP-positive cells were found in several cultures from the cortical gray matter. The three main morphologi-

Table 1. Quantitative analysis of immunostaining on PBC.[a]

Cell types	Percentage	Immunostaining
Astrocytes	99.99	GFAP+
	10	A2B5+/GFAP+
Oligodendrocytes	0.01	O4+ or GalC+
	>0.007	O4+/GFAP+
Neurons	0	MAP2–, NF–

[a] The PBC which create 0.1% of all cells in confluent adult human brain cultures.

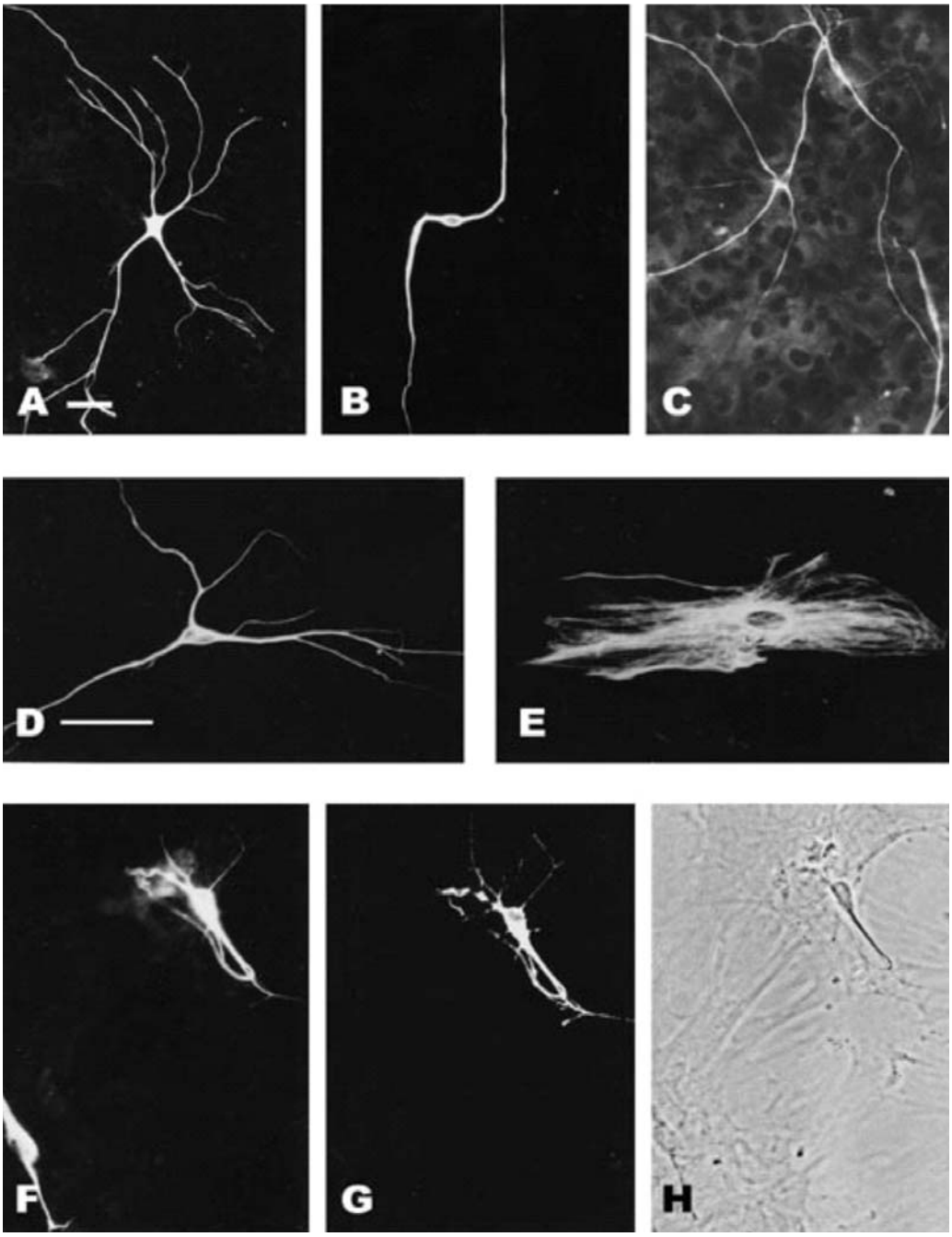

Fig. 2. Indirect immunofluorescence staining for GFAP (A-E) and double labelling for GFAP/A2B5 (F-H) in human brain cultures. Stellate (A,D), bipolar (B) and large flat (E) GFAP-positive astrocytes. (C) A layer of GFAP-negative flat cells above which are GFAP-positive astrocytes. Two GFAP positively stained cells (F), an A2B5–positive cell (G) and corresponding phase-contrast microscopy (H). Scale bars = 50 μm.

cal types of GFAP-positive astrocytes were found in all primary cultures: stellate, bipolar and large cytoplasmic cells. Stellate astrocytes reached approximately 60% of all GFAP-positive cells. These were cytoplasm-depleted cells with long and thin, frequently branched processes (Fig. 2A,D). Bipolar cells were present at 30% (Fig. 2B). The large flat cells with abundant cytoplasm and poorly developed processes reached only 10% of all GFAP-positive cells (Fig. 2E). The majority of cells in primary adult human brain cultures were flat GFAP-negative cells. These formed a confluent layer above which the GFAP-positive astrocytes were often seen (Fig. 2C).

In order to distinguish the two types of astrocytes, immunostaining was performed with MAb against A2B5. The number of A2B5-positive cells reached 0.01% of total cells in cultures from the white matter. Double staining for GFAP and A2B5 revealed the presence of A2B5 antigen in GFAP-positive cells. However, only 10% of bipolar or stellate GFAP-positive cells were positively stained for A2B5 (Fig. 2F-H).

Oligodendrocytes

Immunostaining of oligodendrocytes in primary adult human brain cultures was performed using antibodies against GalC and O4. Positively stained cells were found only in several cultures prepared mainly from the white matter. Oligodendrocytes reached 0.01% of all cells in confluent cultures. Over 30% of GalC- or O4-positively stained cells had morphologies typical of differentiated oligodendrocytes (Fig. 3A,B). The remaining oligodendrocytes were round with poorly developed processes (Fig. 3C) or small bipolar cells (Fig. 3D,E). Double labelling with O4 and GFAP antibodies showed that morphologically similar bipolar cells might be: O4+/GFAP+ or O4+/GFAP–, as well as O4–/GFAP+ (Fig. 4F-H). The multipolar and small round oligodendrocytes were always negatively stained for GFAP. The oligodendrocytes were often found upon a layer of flat cells (Fig. 3D,E).

Neurons

PBC in adult human brain cultures positively stained with neuronal antibodies (MAP-2, MAP-5, NF, N-CAM) and negatively for GFAP are considered to be neurons (Kirschenbaum et al. 1994). In our study, we found morphologically similar cells but these stellate or bipolar cells were positively stained for GFAP. All PBC in our cultures were negatively stained with neuronal markers such as MAP-2, NF or N-CAM.

Discussion

Two major classes of astrocytes have been described in histological brain sections based on their morphology and brain distribution (Miller & Raff 1984; Montgomery 1994). Fibrous astrocytes are common in the white matter and protoplasmic astrocytes in the gray matter. In this study the cultures were prepared separately from the cortical gray and white matter. We expected to find morphologically distinct protoplasmic and fibrous astrocytes under culture conditions. Both cultures, however, only differed in the number of GFAP-positive cells. In the gray matter they were absent or were found rarely, reaching 0.1% of all cells in confluent cultures from the white matter. The three main morphologic types of GFAP-positive cells found in this study were either stellate or bipolar; large flat cells were rare. 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. In the literature there are significant discrepancies in the occurrence of GFAP-positive cells in adult human brain cultures. Table 2 shows discrepant results obtained by immunocytochemical staining of adult human brain cultures. Although minor differences in the percentage of GFAP-positive cells may be attributed to random cell counting errors, it remains unclear why GFAP-positive cells were rare or even absent in adult human brain cultures reported by Osborn et al. (1981), Rutka et al. (1986) and Perzelova et al. (2007). In contrast, Estes et al. (1990) and Van der Laan et al. (1997) showed a high percentage (more than 80%) of GFAP-positive cells in explanted or dissociated adult human brain cultures. However, if adult human brain explants or dissociated cultures are mostly comprised of flat GFAP+ cells (Estes et al. 1990; Van der Laan et al. 1997), some questions remain open, such as: What is the histological origin of these cells?; Do they originate from fibrous or protoplasmic astrocytes or from a precursor cells present in adult brain?; How could long process-bearing astrocytes transform during the time of tissue dissociation or inside the explants and became flat astrocytes?

A2B5 antigen was described as a complex ganglioside in plazma membranes of retina neuron cell bodies

Table 2. Conflicting reports concerning GFAP, vimentin and fibronectin immunostaining in adult human brain tissue cultures.[a]

Reference	No. of patients	Age	TC	GFAP	Vimentin	Fibronectin
Osborn et al. (1981)	9 B	14–67	Diss.	3–14	>98	NA
Rutka et al. (1986)	8 B	?	Diss.	0–0.1	100	100
Estes et al. (1990)	4 B	?	Exp.	75–90	80	0
Van der Laan et al. (1997)	3 A	73–85	Diss.	>97	NA	97
Perzelova et al. (2007)	70 B	23–75	Exp.	0.1	100	95–98

[a] A, autopsy; B, biopsy, TC, tissue cultures – Diss., dissociate cultures; Exp., explant cultures; NA, not analysed. The values for GFAP, vimentin and fibronectin are in percentages.

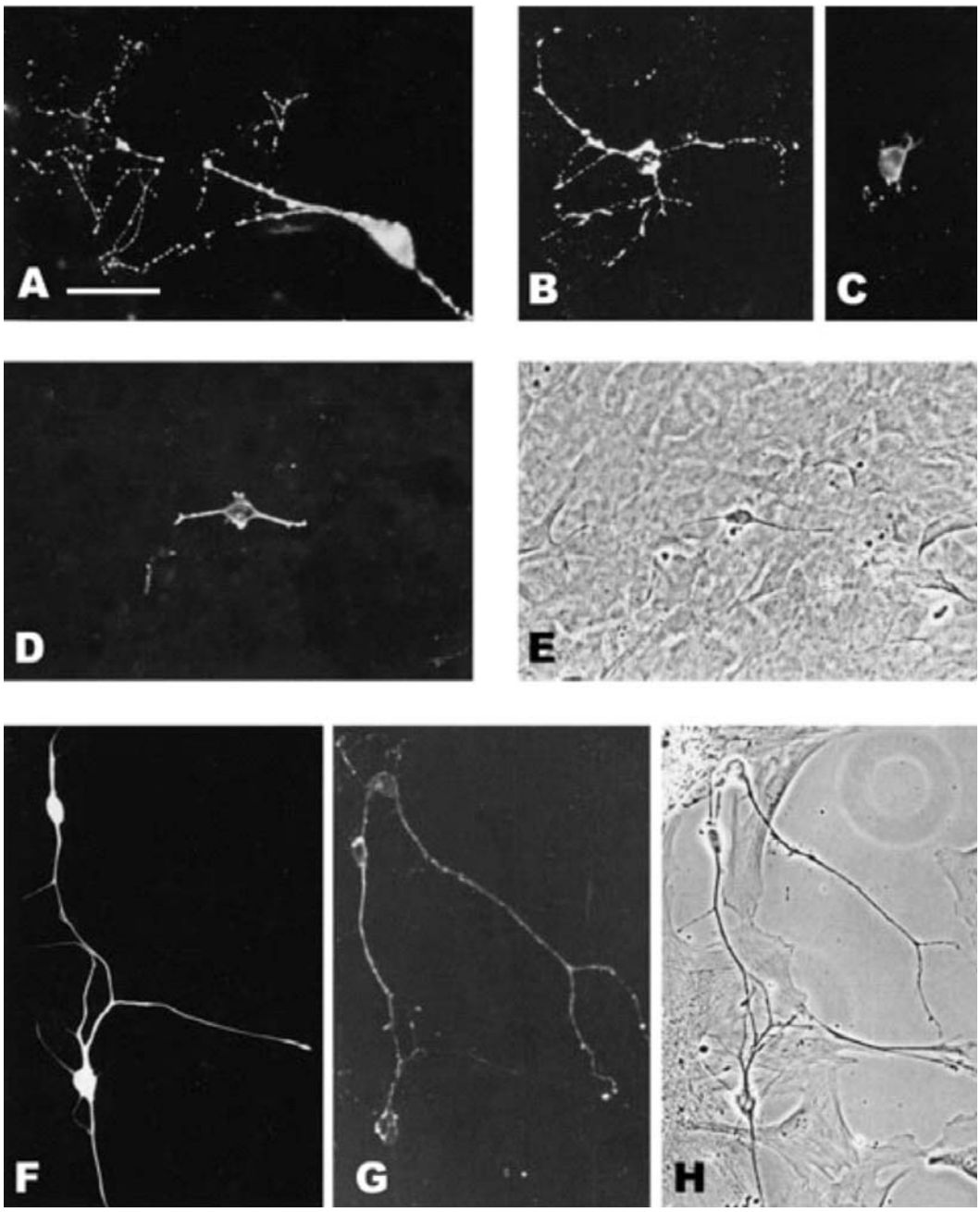

Fig. 3. Immunoidentification of oligodendrocytes in human brain cultures. An O4-positive cell (A), GalC-positive stained cells (B,C), an O4-positive bipolar cell above a layer of flat cells (D) and phase-contrast microscopy (E). Double labelling for GFAP/O4 (F-H): GFAP-positive cells (F), O4-positive cells (G) and phase contrast microscopy (H). Note the differential co-expression of both antigens in morphologically similar bipolar cells. Scale bar = 50 μm.

(Eisenbarth et al. 1979). Raff et al. (1983) described two types of astrocytes in cultures of developing rat brain. In cultures, type I astrocytes have a fibroblast-like morphology and do not bind A2B5 antibodies. Type II are process-bearing astrocytes binding A2B5 antibodies. In adult rats, A2B5 antibody binds to the great majority of fibrous astrocytes in optic nerve but not to protoplasmic astrocytes in cerebral cortex. Type 1 (GFAP+/A2B5–) and type 2 (GFAP+/A2B5+) astrocytes are proposed to be analogous to protoplasmic and fibrous astrocytes, respectively (Miller & Raff 1984). In adult human brain cultures we found only small amount of A2B5-positive cells. Double immunofluorescence revealed that all A2B5-positive cells were also positively stained for GFAP. However, it is striking that astrocytes of similar fibrous or bipolar shape grown on one coverslip were either GFAP+/A2B5+ or GFAP+/A2B5–. Consistent with our (A2B5) data, the study of Newcombe et al. (1988) revealed that only about 10% of long processes-bearing astrocytes were A2B5-positive. On the other hand, the staining for A2B5 was not found *in situ* in the nervous system of vertebrates (Privat et al. 1995). These results indicate that A2B5 antigen is expressed under culture condition in small subpopulation of GFAP-positive human astrocytes.

Previously, we have tried to answer the question about the rare appearance of GFAP-positive cells in adult human brain cultures (Perzelova & Mares, 1993). The presence of astrocytes was examined by immunohistochemical methods in adult human brain biopsies. It was surprising that GFAP-positive cells were located in the subpial area and in the white matter. No GFAP-positive cells were detected in the cortical gray matter. In the brain cortex (layer II–VI), however, the GFAP-positive cells have been found in damaged areas (Perzelova et al. 1997). We propose that the absence of GFAP-positive cells in adult human brain cultures derived from the cortical gray matter is due to the lack of GFAP-positive cells in the bioptic cortex. The rarity of GFAP-positive cells might arise from the subpial area. We do not suppose it is possible to obtain the cultures with high percentage of GFAP-positive cells from GFAP-negative apparently normal brain cortex. The presence of GFAP-positive cells in cultures from the white matter is proportional to GFAP-positive cells in this brain area. However, the "glia-like" cells growing out from the explants proliferate rapidly in 3 to 4 weeks and they overgrew all glial cells. Thus at confluence GFAP-positive astrocytes constitute only small proportion of all cells.

We observed that cultured GFAP-positive astrocytes with two or three long processes mimic the neuron-like morphology and the report by Kirschenbaum et al. (1994) demonstrated the presence of neuronal precursor cells in cultures derived from adult human brain biopsies. Their explanted and dissociated tissue cultures were prepared from cortical, subcortical and periventricular brain samples. The process-bearing neurons (MAP-2+, MAP-5+, N-CAM+, or NF+) were found in cultures from periventricular and subcortical tissue but were not observed in the cortical cultures. However, the morphological features of cultured neurons showed by Kirschenbaum et al. (1994) are very similar to our GFAP-positive adult human astrocytes. But in our cultures the PBC were negatively stained with neuronal marker antibodies. Adult human stellate or bipolar GFAP-positive astrocytes (but negatively stained for neuronal markers) were also described by Davies et al. (2000). In addition, the occurrence of neurons in adult human brain cultures corresponds to the astrocyte distribution in our cultures. It is remarkable that neurons were absent in cortical cultures, similarly as our GFAP-positive astrocytes that were rare or absent in cultures from the cortical gray matter. At higher DIV we observed astrocytes above the "glia-like" cell layer. Similar photos with neurons overlaying flat substrate cells were presented by Kirschenbaum et al. (1994).

These results demonstrate that immunocytochemically defined astrocytes in adult human cultures do not correspond to classical or revised astrocyte classification. In addition, comparative analysis of published studies showed large discrepancies in morphological and immunocytochemical identification of neuronal and astroglial cell types under culture conditions.

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