A new astrocytic cell line which is able to induce a blood-brain barrier property in cultured brain capillary endothelial cells

Carsten T. Beuckmann, Katja Dernbach, Ansgar Hakvoort & Hans-Joachim Galla*
Institut für Biochemie, Westfälische Wilhelms-Universität, Wilhelm-Klemm-Straße 2, D-48149 Münster, F.R. Germany

Received 2 August 1994; accepted 27 February 1996

Key words: astrocytes, blood-brain barrier, clone, continuous cell line

Abstract

Astrocytes, a member of the glial cell family in the central nervous system, are assumed to play a crucial role in the formation of the blood-brain barrier (BBB) in vertebrates. It was shown that astrocytes induce BBB-properties in brain capillary endothelial cells (BCEC) *in vitro*. We now established an astroglial cell line of non-tumoral origin. The cloned cell line (A7) shows a highly increased proliferation rate and expresses the astrocytic marker glial fibrillary acidic protein. Furthermore, the clone A7 expresses S-100-protein and vimentin, which are also expressed by primary cultured astrocytes. This cell line therefore shows general astrocytic features. In addition, we were able to show that A7 cells re-induce the BBB-related marker enzyme alkaline phosphatase in BCEC, when these two cell types are co-cultured. Thus we have a cell line which can be readily cultured in large quantities, shows common astrocyte properties and is able to influence BCEC with respect to a BBB-related feature.

Abbreviations: AB – antibody; ALP – alkaline phosphatase; BBB – blood-brain barrier; BCEC – brain capillary endothelial cells; CNS – central nervous system; DMEM – Dulbecco's modified minimal essential medium; GABA – γ -aminobutyric acid; GFAP – glial fibrillary acidic protein; MDCK – Madin-Darby Canine-Kidney; MeOH/a.a. – methanol/acetic acid; SMA – smooth muscle actin; vWF – von Willebrand-factor.

Introduction

Astrocytes are a member of the glial cell family in vertebrates. They can be found in the central nervous system (CNS) and got their name according to their characteristic star-like morphology.

In vivo, two major classes of astrocytes can be distinguished: protoplasmatic and fibrous (fibrillary) astrocytes. Protoplasmatic astrocytes are found mainly in grey matter, whereas fibrous astrocytes predominate in white matter (Roots, 1986). *In vivo* as well as *in vitro* astrocytes can be readily identified by their expression of glial fibrillary acidic protein (GFAP), which is an intermediate filament protein of the cytoskeleton only in astrocytes (Bignami *et al.*, 1972). Other character-

istic proteins of astrocytes are glutamine synthetase, carboanhydrase, and S-100-protein (DeVellis *et al.*, 1986).

In vitro astrocytes express vimentin, another intermediate filament protein, which is normally not expressed in mature astrocytes *in vivo* (Chiu *et al.*, 1981). In the brain it only occurs during gliogenesis and reactive astrogliosis (Eng, 1988). The distinction of astrocytes *in vitro* is twofold, too: type I and type II astrocytes (Raff, 1990). Both types of astrocytes express GFAP in culture but can be discriminated by the expression of ganglioside GQ1_b on type II astrocytes which can be detected by A2B5-antibody (Fredman *et al.*, 1984).

The functions of astrocytes in the CNS are numerous and yet not fully understood. One task is the main-

* To whom correspondence should be addressed.

tenance of homeostasis in the brain (in addition to the blood-brain barrier [BBB]) (Kimelberg and Norenberg, 1989; Hertz, 1989). Astrocytes also play a role in reactive astrogliosis during brain injuries (Eng, 1988) and are assumed to fulfill major functions in signal transduction in brain (Roeder *et al.*, 1985; Cornell-Bell *et al.*, 1990; reviews by Hansson, 1988 and Martin, 1992).

In addition to all these properties, astrocytes are probably involved in the formation of the BBB, which is constituted by capillary endothelial cells of the brain (BCEC) and controls the flow of hydrophilic substances from blood to brain in order to preserve brain homeostasis. Tight junctions between BCEC are the structural basis for the prevention of uncontrolled efflux of blood-borne substances. Brain capillaries are ensheathed almost without exception (99%) by astroglial endfeet (Crone, 1986) forming the *membrana gliae limitans perivascularis*. The direct contact of capillaries to astrocytes seems to be a prerequisite for endothelial cells to differentiate into the specialized BBB phenotype (Janzer and Raff, 1987; Bouchaud *et al.*, 1989). The molecular basis of this interaction has not been clarified yet.

In vitro models of the BBB are commonly based on cultures of BCEC. In addition to the general endothelial marker protein von Willebrand-factor (vWF), brain capillary endothelial cells express the BBB-related enzyme alkaline phosphatase (ALP; Vorbodt, 1988). BCEC rapidly lose their ALP activity during cultivation (Rauh *et al.*, 1992). ALP activity can be re-induced by coculturing BCEC together with astrocytes (Meyer *et al.*, 1991). Another effect which can be observed *in vitro* is the loss of tight junction complexity in cultured BCEC. The complexity can be restored up to *in vivo* values by coculturing BCEC together with astrocytes or astrocyte-conditioned medium (Tao-Cheng *et al.*, 1987; Wolburg *et al.*, 1994).

In our laboratory we co-culture porcine primary BCEC together with rat astrocytes. Our interest is focused on the interactions of these two cell types in order to establish a functioning *in vitro* model of the BBB. Here we report the establishment of a new astrocytic cell line. This cell line has been characterized with respect to astrocytic phenotype and cocultured with BCEC.

Materials and methods

All chemicals used were supplied from Sigma, Deisenhofen, F.R.G. unless otherwise noted.

Isolation and culture of astrocytes from neonatal rats

We isolated astrocytes from neonatal rats (PN 2–5) according to McCarthy and DeVellis (1980). Shortly, the rats were anaesthetised with diethylether and decapitated. *Cerebri* were isolated, meninges removed, and the remaining tissue mechanically disrupted by trituration with syringes of decreasing diameter until apparent homogenization was reached. Cells were seeded into uncoated culture flasks and nourished with Dulbecco's modified minimal essential medium (DMEM; Biochrom, Berlin, F.R.G.). After the cells had reached confluency (2–3 weeks), they were shaken overnight (200 rpm) at 37 °C. Detached cells were removed and the remaining adherent cells were treated for 48 h with 20 μ M cytosine arabinoside. After 24 h, cytosine arabinoside treatment was repeated. Finally, 5 mM leucine-methylester was added to the cultures in order to remove microglia (Thiele *et al.*, 1983).

Astrocytes were routinely characterized by immunofluorescence detection of GFAP. They were passaged by trypsinization and stored in serum containing 10% (v/v) dimethylsulfoxide in liquid nitrogen.

Cloning

Astrocytes were trypsinized with 0.25% (w/v) trypsin/1 mM EDTA-solution (Seromed, Berlin, F.R.G.) and counted. They were seeded into collagen-coated 24 well-plates with a statistical density of one cell per well. DMEM containing 20% (v/v) ox serum was used for cultivation. After 4 weeks the cloned cells were passaged with a split ratio of 1:4 into uncoated culture flasks and immunologically characterized.

Culture of MDCK II-cells

Madin-Darby Canine-Kidney (MDCK) II-cells were cultured in Earle's Minimal Essential Medium containing 4 mM glutamine and 10% (v/v) ox serum. For coculture experiments MDCK II-cells of passage no. 13 were used.

Isolation and culture of cerebral capillary endothelial cells

Cells were isolated from adult porcine brains according to a slightly modified enzymatic procedure from Bowman *et al.* (1983) and Mischeck *et al.* (1989). The endothelial cells were grown in medium M199 (Biochrom, Berlin, F.R.G.) containing 700 μ M glutamine (Biochrom, Berlin, F.R.G.) and 10% (v/v) ox serum (PAA, Linz, Austria). The cultured cells were characterized by their expression of vWF. For co-culture, we grew the BCEC for 4 days before adding A7 astrocytes or MDCK II-cells at a density of 2000/cm². Control cultures of BCEC were kept in serum free medium from day 4 on without adding further cells.

Immunocytochemistry

Indirect immunocytochemical stainings were performed as follows: cells were fixed either with 4% (v/v) formaldehyde and 0.1% glutardialdehyde in phosphate buffered saline containing Ca²⁺ and Mg²⁺ at room temperature for 10 min or by applying methanol containing 3% (v/v) acetic acid (MeOH/a.a.) at -20 °C for 10 min. For detection of GFAP, cells were fixed with absolute ethanol at room temperature for 10 min. Fixation at room temperature led to identical results as compared to fixation at -20 °C. After fixation cells were incubated with primary antibody (AB) at 37 °C in humidified atmosphere for 1 h. Incubation with secondary AB was performed identically. Control stainings were made by omission of primary AB and incubation with 3% (w/v) bovine serum albumine in phosphate buffered saline instead. The primary and secondary AB and their concentrations used are listed in Tables 1 and 2:

Histochemistry

Histochemical ALP activity detection was performed according to Burstone (1961) with the substrates fast red violet (diazotated 5-benzamido-4-chloro-2-toluidine) and naphthol-AS-BI (6-bromo-hydroxy-3-naphthaloate-2-methoxyanilidephosphate). The chemicals were included in a commercially available kit for detection of leukocyte alkaline phosphatase activity (86-R).

Results and discussion

In addition to many other tasks, astrocytes seem to be an essential part of the perivascular space in brain, where they form close contacts to capillaries. A growing number of indications are reported that astrocytes influence BCEC to express their differentiated BBB phenotype (Janzer and Raff, 1987; Bouchaud *et al.*, 1989). Therefore, we prepared astrocytes according to McCarthy and DeVellis (1980) and co-cultured them together with BCEC in order to study BBB-properties. By passaging primary cultures of astrocytes we established cell lines. One of these cell lines spontaneously developed from passage no. 11 on into a continuous cell line of non-tumoral origin. The cells were neither chemically nor virally transformed. From passage no. 15 on proliferation rate seemed to be constant. At this stage astrocytes needed 5 days to reach confluency at a split ratio of 1:5.

In passages no. 15 and 16 we performed an immunological characterization of the continuous cell line. In addition to type I astrocytes, the following cell types could have been isolated by using the described procedure: type II astrocytes, oligodendrocytes, neuroectodermal precursor cells, pericytes, and smooth muscle cells. Therefore we used AB directed against GFAP (which is expressed in type I and type II astrocytes), ganglioside G_{Q1b} (displayed by type II astrocytes, A2B5-AB detects this ganglioside [Fredman *et al.*, 1984]), smooth muscle myosin (in pericytes and smooth muscle cells) and G_{D3} (a disialoganglioside found on neuroectodermal precursor cells [Goldman *et al.*, 1984] and on cultured human brain oligodendrocytes [Kim *et al.*, 1986]). Furthermore we tested for the presence of smooth muscle actin (SMA), which is a general protein of the cytoskeleton. In our continuous cell line we could not detect G_{Q1b}, G_{D3} or smooth muscle myosin (data not shown). Therefore it did not consist of type II astrocytes, oligodendrocytes, neuroectodermal precursor cells, pericytes, or smooth muscle cells. Double immunofluorescence staining of GFAP and SMA showed the continuous cell line to be heterogeneous with regard to these two proteins. As seen in Figure 1, the cultures contained cells expressing GFAP and SMA, other cells did express either GFAP (Figure 1B) or SMA (Figure 1C).

In another astrocyte cell line (passage no. 4), we were able to detect some contaminating cells, which were GFAP-negative and SMA-positive (data not shown). Therefore it was not surprising that these not further defined contaminating cells existed also in

Table 1. Primary antibodies utilized for indirect immunofluorescence

Antigen	AB from species	Fixation	Concentration	Supplier
GFAP	rabbit	ethanol	100 μ g/ml	Dakopatts, Glostrup, Danmark
A2B5	mouse	aldehydes	10 μ g/ml	Boehringer, Mannheim, F.R.G.
G _{D3}	mouse	aldehydes	1 : 300 (solute)	Sigma
vimentin	mouse	MeOH/a.a.	1 : 4 (solute)	Boehringer, Mannheim, F.R.G.
S-100-protein	rabbit	MeOH/a.a.	20 μ g/ml	Sigma
vWF	rabbit	aldehydes	10 μ g/ml	Sigma
smooth muscle actin	mouse	MeOH/a.a.	1 : 400 (solute)	BioMakor, Rehovot, Israel
smooth muscle myosin	mouse	MeOH/a.a.	1 : 200 (solute)	Sigma

Table 2. Secondary antibodies utilized for indirect immunofluorescence

Antigen	AB from species	Concentration	Fluorophore	Supplier
Mouse-Ig	sheep, F(ab') ₂ -fragment	20 μ g/ml	fluoresceine	Boehringer, Mannheim, F.R.G.
Rabbit-IgG	sheep, F(ab') ₂ -fragment	20 μ g/ml	rhodamine	Boehringer, Mannheim, F.R.G.

the continuous cell line. Their immunological appearance fully coincided with astrocyte cultures of low passages. Nevertheless, this result from double immunostaining showed the necessity of cloning the continuous cell line in order to obtain immunologically homogeneous cultures.

Cloning resulted in 7 clones (A1-A7) which were characterized immunologically and tested for their ability to re-induce ALP activity in BCEC. The latter ability was displayed by clone A7 only.

A7 cells showed the presence of astrocytic marker protein GFAP as shown in Figure 2A and 2B. Furthermore, the glia specific S-100-protein could be detected (Figure 2C and 2D). Astrocytes express the cytoskeletal protein vimentin *in vitro* and we found a positive immuno-reaction in A7 cells (Figure 2E and 2F). SMA could not be detected (data not shown). Whether this indicates an instability of our cell line cannot be decided at present.

Thus A7 possesses features of cultured type I astrocytes. Cell size and morphology seem to be dependent on the proliferative state. When A7 cells reached confluency they stopped to proliferate and seemed to reduce their size, displaying a more elongated morphology as the comparison of Figure 2A, 2C and 2E shows. In a subconfluent state, the proliferation rate of A7 was estimated. Cell number increased by a factor of 2.4 in 10 days.

Furthermore, we were interested to clarify if A7 cells were able to re-induce ALP activity in BCEC. This has already been shown for astrocytes of low pas-

sage numbers (Meyer *et al.*, 1991). BCEC were cultured for 4 days (and identified by detection of vWF) before A7 cells were added to form a mixed coculture which allowed the cell types to get into direct plasma membrane contact with each other. The histochemical ALP activity stainings are shown in Figure 1. BCEC cocultured with A7 cells showed a more intense red-dish staining (Figure 1G) than control cultures (Figure 1I). This indicates a higher ALP activity in cocultured BCEC. Additionally, proliferation of BCEC was inhibited by A7 cells like it is the case with astrocytes of low passages.

In order to examine whether re-induction of ALP in BCEC is a glia-specific property we cocultured BCEC together with MDCK II-cells, an established and well-characterized cell line of epithelial origin. By means of histochemical ALP activity staining we could not detect any re-induction of ALP in BCEC during coculture with MDCK II-cells (Figure 1H and 1I). Comparably to astrocytes, MDCK II-cells inhibited proliferation of BCEC. On the other hand it has already been demonstrated that BCEC can be re-induced to display ALP activity without being inhibited in their proliferation. This effect has been observed in mixed cocultures of BCEC with epithelial cells of the choroid plexus, which also belong to the glial cell family (Beuckmann *et al.*, 1994).

We therefore conclude that ALP activity induction in BCEC co-cultured with cells of neuroectodermal origin is caused by a glia-specific property and is not due to the inhibition of proliferation.

Figure 1. Immunological characterization of an astrocytic cell line by double immunofluorescence and histochemical ALP stainings of cultured BCEC. Bar = 100 μ m; A-F: A7 cell line; A: Passage no. 15, phase contrast micrograph, two cells are marked with arrows; B: Immunofluorescence against GFAP with rhodamine, exposure time 15 sec, only the left cell is positive for GFAP (arrow); C: Immunofluorescence against SMA with fluoresceine, exposure time 15 sec, only the right cell is positive for SMA (arrow); D: Passage no. 15, phase micrograph; E: Control of secondary AB labelled with rhodamine, exposure time 30 sec; F: Control of secondary AB labelled with fluoresceine; exposure time 30 sec; G-I: BCEC cultures; G: 6 day old coculture with A7 cells (passage no. 30) since day 4; EN = endothelial cells, A7 = A7 cells; H: 6 day old coculture with MDCK II-cells (passage no. 13) since day 4; EN = endothelial cells, EP = MDCK II epithelial cells; I: 6 day old monoculture without serum since day 4.

In future studies, it would be very interesting to investigate whether our newly established cell line is also capable of increasing junction tightness between BCEC *in vitro* as is the case for primary cultures or low passage astrocytes (Tao-Cheng *et al.*, 1987; Wolburg *et al.*, 1994). This could be achieved by means of electron microscopy or permeability measurements. Moreover, we intend to examine whether A7 cells pos-

sess the same metabolic pathways as primary cultured astrocytes.

Conclusions

A7 is a continuous cell line of non-tumoral origin with typical immunological features of type I astrocytes but

Figure 2. Immunological stainings of A7 cells. Bar = 100 μ m; A: Passage no. 27, phase contrast micrograph; B: Immunofluorescence against GFAP with rhodamine, exposure time 15 sec; C: Passage no. 29, phase contrast micrograph; D: Immunofluorescence against S-100-protein with rhodamine, exposure time 15 sec; E: Passage no. 29, phase contrast micrograph; F: Immunofluorescence against vimentin with fluoresceine, exposure time 15 sec; G: Passage no. 29, phase contrast micrograph; H: Control of secondary AB labelled with rhodamine, exposure time 30 sec; I: Passage no. 29, phase micrograph; K: Control of secondary AB labelled with fluoresceine; exposure time 30 sec.

with a much higher proliferation rate. Because A7 re-induces ALP activity in BCEC, we now have the opportunity to culture astrocytes in large quantities, which induce a BBB-property. With regard to isolation and characterization of the inducing components of astrocytes, this could be of great benefit for BBB research.

Acknowledgements

We would like to thank Prof. Simons (EMBL, Heidelberg, F.R.G.) for providing cultured MDCK II-cells and diploma chemist T. Tilling for proofreading the manuscript. Financial support was obtained from Deutsche Forschungs-Gemeinschaft (DFG) under grant no. SFB 310/B13.

References

- Beuckmann C, Gath U, Rauh J, Heitkamp B and Galla H-J (1994) 'Induction of the blood-brain barrier-related enzyme alkaline phosphatase in cerebral capillary endothelial cells *in vitro*', Materials Science and Engineering C2, 31–35
- Bignami A, Eng LF, Dahl D and Uyeda CT (1972) 'Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence', Brain Res. 43, 429–435
- Bouchaud C, Le Bert M and Dupouey P (1989) 'Are close contacts between astrocytes and endothelial cells a prerequisite condition of a blood-brain barrier? The rat subfornical organ as an example', Biol. Cell 67, 156–159
- Bowman PD, Ennis SR, Rarey KE, Betz AL and Goldstein GW (1983) 'Brain microvessel endothelial cells in tissue culture: a model for study of blood-brain barrier permeability', Ann. Neurol. 14, 396–402
- Burstone MS (1961) 'Histochemical demonstration of phosphatases in frozen sections with naphthol-AS-phosphates', J. Histochem. Cytochem. 9, 146–153
- Chiu F-C, Norton WT and Fields KL (1981) 'The cytoskeleton of primary astrocytes in culture contains actin, glial fibrillary acidic protein, and the fibroblast-type filament protein, vimentin', J. Neurochem. 37 (1), 147–155
- Cornell-Bell AH, Finkbeiner SM, Cooper MS and Smith SJ (1990) 'Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling', Science 247, 470–473
- Crone C (1986) 'The blood-brain barrier: a modified tight epithelium', in Suckling AJ, Rumsby MG and Bradbury MWB (eds.) The blood-brain barrier in health and disease, Ellis Horwood Ltd., Chichester, 17–40
- DeVellis J, Wu DK and Kumar S (1986) 'Enzyme induction and regulation of protein synthesis', in Fedoroff S and Vernadakis A (eds.) Astrocytes (Vol. 2), Academic Press Inc., Orlando
- Eng LF (1988) 'Regulation of glial intermediate filaments in astrogliosis', in Norenberg MD, Hertz L and Shousboe A (eds.) The biochemical pathology of astrocytes, Liss, New York, 79–90
- Fredman P, Magnani JL, Nirenberg M and Ginsburg V (1984) 'Monoclonal antibody A2B5 reacts with many gangliosides in neuronal tissue', Arch. Biochem. Biophys. 233, 661–666
- Goldman JE, Hirano M, Yu RK and Seyfried TM (1984) 'G_{D3} ganglioside is a glycolipid characteristic of immature neuroectodermal cells', J. Neuroimmunol. 7, 179–192
- Hansson E (1988) 'Astroglia from defined brain regions as studied with primary cultures' Prog. Neurobiol. 30, 369–397
- Hertz L (1989) 'Regulation of potassium homeostasis by glial cells', in Levi G (ed.) Differentiation and function of glial cells, Wiley-Liss, New York, 225–234
- Janzer RC and Raff MC (1987) 'Astrocytes induce blood-brain barrier properties in endothelial cells', Nature 325, 253–256
- Kim SU, Moretto V, Lee V and Yu RK (1986) 'Neuroimmunology of gangliosides in human neurons and glial cells in culture', J. Neurosci. Res. 15, 303–321
- Kimelberg HK and Norenberg MD (1989) 'Astrocyten und Hirnfunktion' Spektrum der Wissenschaft 6, 52–60
- Martin L (1992) 'Synthesis and release of neuroactive substances by glial cells', Glia 5, 81–94
- McCarthy KD and DeVellis J (1980) 'Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue', J. Cell Biol. 85, 890–902
- Meyer J, Rauh J and Galla H-J (1991) 'The susceptibility of cerebral endothelial cells to astroglial induction of blood-brain barrier enzymes depends on their proliferative state', J. Neurochem. 57 (6), 1971–1977
- Mischeck U, Meyer J and Galla H-J (1989) 'Characterization of γ -glutamyl transpeptidase activity of cultured endothelial cells from porcine brain capillaries' Cell Tissue Res. 256, 221–226
- Raff MC (1990) 'Subclasses of astrocytes in culture: what should we call them?', in Levi G (ed.) Differentiation and function of glial cells Wiley-Liss, New York, 17–23
- Rauh J, Meyer J, Beuckmann C and Galla H-J (1992) 'Development of an *in vitro* cell culture system to mimic the blood-brain barrier', Prog. Brain Res. 91, 117–121
- Roeder LM, Williams IB and Tildon JT (1985) 'Glucose transport in astrocytes: Regulation by thyroid hormone', J. Neurochem. 45 (5), 1653–1657
- Roots B (1986) 'Phylogenetic development of astrocytes' in Fedoroff S and Vernadakis A (eds.) Astrocytes (Vol. 1) Academic Press, Orlando, 1–34
- Tao-Cheng J-H, Nagy Z and Brightman MW (1987) 'Tight junctions of brain endothelium *in vitro* are enhanced by astroglia', J. Neurosci. 7 (10), 3293–3299
- Thiele DL, Kurosaka M and Lipsky PE (1983) 'Phenotype of the accessory cell necessary for the mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester', J. Immunol. 131 (5), 2282–2290
- Vorbrodt AW (1988) 'Ultrastructural cytochemistry of enzymatic activities in brain microvasculature', in Vorbrodt AW (ed.) Ultrastructural Cytochemistry of Blood-Brain Barrier Endothelium, G. Fischer, Stuttgart, 12–18
- Wolburg H, Neuhaus J, Kniesel U, Krauß B, Schmid E-M, Öcalan M, Farrell C and Risau W (1994) 'Modulation of tight junction structure in blood-brain barrier endothelial cells', J. Cell Sci. 107, 1347–1357

Address for correspondence: Hans-Joachim Galla, Institut für Biochemie, Westfälische Wilhelms-Universität, Wilhelm-Klemm-Straße 2, D-48149 Münster, F.R. Germany.