

Impaired Induction of Blood-Brain Barrier Properties in Aortic Endothelial Cells by Astrocytes From GFAP-Deficient Mice

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ABSTRACT Cell culture models have been extensively used for studies of blood-brain barrier (BBB) function. However, most in vitro models fail to reproduce the peculiar physiological and morphological properties of in situ brain microvascular endothelial cells. A recently developed, tridimensional and dynamic model of the BBB has permitted studies of glial-endothelial interactions in hollow fibers exposed to intraluminal flow. We have taken advantage of this technique and have investigated the ability of glial fibrillary acidic protein (GFAP)-deficient (GFAP^{-/-}) astrocytes to induce BBB properties in aortic endothelial cells (BAEC) cultured in vitro. BAEC exposed to flow were seeded intraluminally in hollow fibers and co-cultured with extraluminally seeded mouse astrocytes. Under these conditions, astrocytes have been shown to induce blood-brain barrier properties in non-brain endothelial cells. We followed induction of a BBB phenotype by measuring the transendothelial resistance, as well as endothelial permeability to potassium, theophylline, 8-sulphophenyl-theophylline (8-SPT), sucrose, and Evans blue. Wild-type mouse astrocytes induced BBB properties in aortic endothelial cells following 3–4 weeks of co-culturing. Thus, these endothelial cells restricted passage of K⁺ ions into the extracapillary space and selectively excluded hydrophilic molecules, such as 8-SPT and ¹⁴C-sucrose. GFAP^{-/-} astrocytes failed to induce a significant restriction to the passage of potassium and hydrophilic drugs (sucrose, 8-SPT), failed to induce transendothelial resistance values comparable to control co-cultures, but were capable of inducing exclusion of Evans blue by endothelial cells. These results suggest that GFAP (and intermediate filaments) may play a role in the induction of BBB properties in non-BBB endothelial cells. *GLIA* 22:390–400, 1998. © 1998 Wiley-Liss, Inc.

INTRODUCTION

The blood-brain barrier (BBB) shields the brain from molecules and ions while simultaneously providing access to nutrients and amino acids to the brain parenchyma. In mammals, the BBB is comprised of highly specialized endothelial cells (EC) characterized by 1) interendothelial tight junctions (zonulae occludentes); 2) dramatically reduced or absent pinocytosis; and 3) presence of specific transport mechanisms for various molecules. This anatomic and functional specialization

of brain microvascular EC is responsible for the exclusion properties of the BBB and constitutes the morphological substrate of the high transendothelial resistance (TER) characteristic of brain capillaries.

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BBB properties are bestowed on brain EC by neighboring astrocytes (Davson and Oldendorf, 1967; De Bault and Cancilla, 1980; Stewart and Wiley, 1981). When transplanted into ectopic sites of host embryos of a different species, embryonic brain tissue has been shown to induce both angiogenesis in the host and BBB characteristics in the invaded blood vessels (Stewart and Wiley, 1981). Conversely, peripheral tissue transplanted into the brain displays angiogenesis characterized by fenestrated EC. A specific role for brain astrocytes in the induction of BBB properties in EC has recently been confirmed (De Bault and Cancilla, 1980; Janigro et al., 1995, 1996). Thus, it is not surprising that experimental attempts to reconstitute the BBB in vitro have aimed at co-culturing EC and astrocytes (Joo, 1993; Stanness et al., 1996b); alternatively, astrocyte-conditioned media have been used to maintain (or induce; Tontsch and Bauer, 1991) BBB properties in EC (Davson and Oldendorf, 1967; Stewart and Wiley, 1981; De Bault and Cancilla, 1980). Stanness and co-workers Janigro et al., 1995; Stanness et al., 1996a,b; Stanness et al., 1997) have developed a tridimensional model of the BBB aiming at replicating several crucial features of the in vivo BBB. This was achieved by co-culturing astrocytes and EC on opposite sides of hollow fibers, the latter cells being exposed to flow (Stanness et al., 1996b). This dynamic model of the BBB (DIV-BBB) is characterized by segregated luminal/abluminal transporters, high transendothelial resistance (>1,000 Ohm/cm²), and low permeability to intraluminal potassium (Stanness et al., 1996a,b).

While several lines of evidence support the hypothesis that BBB EC properties depend on astrocytic influences, it is not clear whether direct contact between astrocytes and endothelium is necessary. Furthermore, the role of glia in the induction of BBB properties in non-brain endothelium is still poorly understood. Interestingly, a variety of brain tumors become vascularized by "leaky" capillaries, suggesting that some neoplasms may lack expression of BBB-inducing (or permissive) factors (e.g., Guerin et al., 1990).

Mature astrocytes in situ are characterized by the expression of an astrocyte-specific intermediate filament (IF) protein, glial fibrillary acidic protein (GFAP). Recently generated mice deficient for GFAP (Gomi et al., 1995; Liedtke et al., 1996; McCall et al., 1996; Pekny et al., 1995) made it possible to study GFAP-deficient (GFAP^{-/-}) astrocytes both in vivo and in vitro, and evidence has been provided supporting a role for GFAP in glial proliferation (Pekny et al., 1997). Consistent with the finding that some astrocyte-derived brain tumors lack GFAP immunoreactivity, Pekny et al. (1997) have shown that GFAP^{-/-} astrocytes displayed an increased rate of proliferation. GFAP^{-/-} mice, nevertheless, developed a grossly normal BBB to Evans blue (Pekny et al., 1995) or peroxidase (Gomi et al., 1995) even though subtle and localized changes in the spinal/blood barrier have been reported for old GFAP^{-/-} animals (Liedtke et al., 1996). This, along with the fact that GFAP expression occurs during or after establish-

ment of a BBB, speaks against a direct role for GFAP in the ontogenesis of the mammalian BBB. However, since GFAP expression is increased following brain injury, it is possible that GFAP (and/or other IF proteins) is required for BBB repair. It is also possible that in vitro induction of BBB may resemble restoration of an intact BBB and that GFAP and other IF proteins are required for this process.

We have taken advantage of the GFAP^{-/-} mice and the existing dynamic model of the BBB to investigate astrocyte-endothelial interactions in vitro. The specific question we attempted to address is: Can astrocytes from GFAP^{-/-} mice induce the same BBB properties in peripheral endothelial cells as normal astrocytes? To this end, we compared the physiological properties of BAEC co-cultured in hollow fiber apparatus with either GFAP^{+/+} or GFAP^{-/-} astrocytes or rat glioma (C6) cells.

MATERIALS AND METHODS

Preparation of GFAP^{-/-} Astrocyte-Enriched and C6/BAEC Cultures

The GFAP^{-/-} mice were generated as previously described (Pekny et al. 1995). Mice of postnatal days 1–2 were killed by decapitation and primary astrocyte-enriched cultures were prepared as described previously (Hansson and Rönnbäck, 1989; Pekny et al., 1997). For the experiments described herein, we used litters obtained from GFAP^{-/-} or GFAP^{+/+} crossings. Both the GFAP-deficient and the control mice were hybrids between C57BL/6 and 129 SV mouse strains. Briefly, whole brains were dissected, freed of meninges, and placed into 37°C pre-warmed and 5% CO₂ equilibrated medium, which consisted of 80% Dulbecco's modified Eagle's medium (DMEM) (Sigma, D5671), 20% fetal calf serum (GIBCO) and was complemented with L-glutamine (2 mM) and antibiotics (GIBCO penicillin/streptomycin supplement). The brains were placed on a 80 µm nylon mesh, which was positioned over and partially submerged in the dish with medium. Using a Teflon-covered rod, the brain tissue was mechanically disintegrated and forced through the nylon tissue into the medium underneath. The resulting suspension was plated in 25 cm² tissue culture flasks (Falcon, Plymouth, England) and on Lab-Tek Chamber Slide culture chambers (Nunc, IL). The same amount of plating material per bottle was used from each brain. The cultures were maintained at 37°C with 100% humidity and 5% CO₂. Medium (same as above) was changed every 3 days. The cultures used for these experiments were prepared from four GFAP^{+/+} and four GFAP^{-/-} mice.

Bovine aortic endothelial cells (BAEC) were obtained from Dr. H. Sage at the University of Washington and grown in DMEM + 10% fetal bovine serum (FBS) supplemented with 1.8 g/L glucose, 2 mM glutamine, 10 mM HEPES, MEM essential vitamin mixture, non-essential amino acids 100 µM each, 1 mM Na-pyruvate, and Pen/Strep Fungizone Mix. C6 (a rat glial tumor

line) was purchased from ATCC (Rockville, Maryland) and grown under the same conditions.

Immunocytochemical Staining of Astrocyte-Enriched Cultures

Cultures were fixed for 15 min in cold methanol at 20°C and rinsed 3 times in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) prior to staining. Endogenous peroxidase was blocked by incubation with 0.3% H₂O₂ in PBS for 30 min followed by incubation with 5% bovine serum albumin (BSA; Sigma, St. Louis, MO) in PBS for 30 min to reduce nonspecific background. The mouse monoclonal antibody to GFAP (clone G-A-5) and the vimentin monoclonal antibody (IgM fraction; clone 13.2) were obtained from Sigma. The cells were incubated with the respective primary antibody (GFAP, dilution 1:100; vimentin, dilution 1:250) for 2 h at room temperature. The cultures were then rinsed with PBS and incubated for 1 h either with horseradish peroxidase (HRP)-conjugated goat antiserum against mouse immunoglobulins (Dako A/S, Glostrup, Denmark) for GFAP detection, or with biotinylated rabbit antibody against mouse IgM (Dako A/S) and HRP-conjugated streptavidin (Dako A/S) for vimentin detection. Following the final incubation, sections were rinsed in PBS and stained with 3'-diaminobenzidine tetrahydrochloride (DAB) (Dako A/S) according to the manufacturer's recommendations. The reaction was stopped by rinsing in water and the sections were counterstained with 50× diluted hematoxylin and erythrosin, dehydrated, and mounted.

Characterization of Endothelial Cells

Endothelial cells were characterized by specific uptake of Ac-LDL (DiI-Ac-LDL). For immunocytochemical staining the cells were fixed in 4% paraformaldehyde for 1 h (at 4°C). After several washes the cells were placed in a blocking buffer containing 3% goat serum, 1.5, 3% BSA, and 0.1% Triton in 0.1 M triethanolamine-buffered saline (TBS), pH 7.4, for 1 h to prevent nonspecific binding. Antibodies were diluted in the same buffer and allowed to react from 1 h to overnight. MRC OX-42 antibody (Bioproducts-Harlan) and GFAP (Dako) were used to visualize microglia and astrocytes, respectively. After several washes cells were placed in a fluorescent secondary antibody (anti-mouse IgG_{2a} for OX-42 and anti-rabbit IgG for GFAP) for 1–3 h.

Hollow Fiber Apparatus

Cells were co-cultured using hollow fiber tubes (the "capillary vessels") inside a sealed chamber (the "extraluminal space") accessible by ports (CELLMAX QUAD, Cellco, Germantown, MD; (Knazek et al., 1972; Ott et

al., 1995; Stanness et al., 1996b)). The cartridge/hollow fiber culturing system consists of artificial capillaries made from polypropylene and coated with ProNectin F (Protein Polymer Technologies) in a clear plastic chamber connected by gas-permeable tubing to a source of growth medium allowing exchange of O₂ and CO₂. A pulsatile pump forces medium through the lumen of the artificial capillaries allowing diffusion of nutrients out to the extraluminal space through the 0.5 μm transcapillary pores at a controllable rate. Metabolic products are similarly removed from the cartridge. The entire apparatus resided in a water-jacketed incubator with 5% CO₂ and could be sterilely sampled by moving it inside a laminar flow hood.

EC were seeded intraluminally and allowed to establish themselves for 0–15 days before C6 or astrocytes were introduced into the ECS surrounding the capillaries. EC were grown to confluence in 75 cm² flasks, removed with trypsin, and resuspended in DMEM containing 1% FBS and PSF. Amounts seeded ranged from 10 × 10⁶ to 20 × 10⁶ in two loadings. Flow rate was adjusted to 1 dyne/cm². For microscopic examination, cells were fixed by intracapillary perfusion with PBS + 4% paraformaldehyde, at room temperature. Capillaries were then dissected free from the cartridge plastic support and postfixed at 4°C for 24 h. Samples were subsequently cryoprotected and frozen on dry ice.

Electrical Resistance

A significant hardware modification of the existing Cellco system was performed. A metal electrode was inserted in the ECS and sealed to the plastic support by dental cement. A second electrode was placed within the inflow path for intraluminal perfusion. Thus, the extraluminal electrode (V₁) is electrically continuous with the ECS, while the luminal electrode (V₂) is immersed in the intraluminal portion of the (DIV-BBB) apparatus. The theoretical assumptions used for the development of the system dynamic in vitro BBB used for transendothelial resistance measurements are similar to those described elsewhere (Crone and Olesen, 1982). Briefly, a voltage waveform is applied to V₁ and the corresponding voltage deflections are measured in V₂. The parameters we measured represent the capillary resistance to current flowing perpendicular to the capillary wall, R_m (Ohm/cm²). The electrical signals collected by V₂, as well as the analog output to V₁, are delivered, acquired, timed, analyzed, and by a DA/AD board interfaced to a PC.

Permeability Measurements

A known concentration of the drug under investigation was dissolved directly into the media bottle and perfused intraluminally at a rate of 4 ml/min. Samples were taken from the extracapillary space or the lumen as described (Stanness et al., 1997). Alternatively, a

concentrated bolus of the drug was injected directly into the lumen and the diffusion into the extracapillary space was monitored over time while maintaining a 4 ml/min intraluminal perfusion rate. After appropriate dilution factors were taken into account, no significant differences in the permeability values were obtained when using these different approaches. ¹⁴C-sucrose was detected by standard detection techniques; 8 sulphophenyl theophylline (8-SPT) and theophylline (both from RBI) were detected by high-performance liquid chromatography (HPLC). The permeability/surface product was calculated by graphical integration of the concentration of the drug in the lumen and in the extracapillary space (ecs) over variable time periods (30–100 min). Permeability for a compound x was calculated by integrating the area under the ecs and lumen data points according to the following formula describing P (permeability):

$$K * \frac{[x]_{\text{ecs,final}} - [x]_{\text{ecs,t=0}}}{\int_{0-t} [x]_{\text{lumen}} - \int_{0-t} [x]_{\text{ecs}}}$$

where *K* is a constant used to normalize rate of efflux flux for luminal surface and lumen/ecs volume ratios; $[x]_{\text{ecs, final}}$ and $[x]_{\text{lumen}}$ are the ecs and lumen concentration of x.

RESULTS

Immunocytochemistry for GFAP and Vimentin

The GFAP^{+/+} and GFAP^{-/-} primary astrocyte-enriched cultures have been characterized and consist of about 90% astrocytes (Pekny et al., 1997). Both types of cultures are positive for nestin and vimentin, the other two intermediate filament proteins expressed in cultured astrocytes, but the amount of intermediate filaments in GFAP^{-/-} astrocytes has been shown to be decreased due to the absence of GFAP (Pekny et al., 1997). As demonstrated in Figure 1, 7 days following plating, the primary GFAP^{+/+} astrocyte-enriched cultures exhibited strong staining with GFAP antibodies (a), while GFAP staining was absent in GFAP^{-/-} cultures (b); both GFAP^{+/+} and GFAP^{-/-} cultures were positive for vimentin (c and d, respectively).

Patterns of Cellular Growth

The inherent structural characteristics of the hollow fiber apparatus do not allow for direct visualization of cellular growth (Stanness et al., 1996b). Thus, an indirect measure is used to assess the patterns of cellular expansion (Ott et al., 1995; Stanness et al., 1996b). For the experiments presented herein, we measured the amounts of glucose consumed by the cells: this is roughly proportional to the number of cells growing in the system (Ott et al., 1995; Stanness et al.,

1996b). As shown in Figure 2, addition of glia to intraluminally seeded EC resulted in an augmented consumption of glucose in cultures consisting of either BAEC + GFAP^{+/+} or BAEC + GFAP^{-/-} astrocytes. However, a significantly higher rate of glucose consumption was observed in the GFAP-deficient culture (open symbols, n = 6; the asterisk refers to *P* < 0.02). Note that the initial jump in glucose consumption following addition of glia was identical for both GFAP^{+/+} and GFAP^{-/-} cultures.

Immunocytochemistry of 5 μm thick sections revealed the typical appearance of endothelial/glia interactions in these cultures (Stanness et al., 1997). The microscopic appearance of the sections revealed that intraluminally seeded EC formed a monolayer structure (Fig. 3), as previously reported by Ott et al. (1995). Extraluminal glia, in contrast, developed extensive multilayer growth (Stanness et al., 1996a,b, 1997). We consistently observed that glia formed cellular elongations within the capillary wall, suggesting that actual contiguity between extra- and intraluminal cells may be established. This is also evident in Figure 3, where a cross section of a capillary stained for GFAP immunocytochemistry is shown. Note the dark band of GFAP-positive staining present at the abluminal surface of the EC. While direct contacts between EC and GFAP-positive elongations were beyond the detection limits of light microscopic techniques used, a close proximity between these two cell populations was invariably present. Sections from GFAP^{-/-} cultures were devoid of GFAP immunostaining (inset in Fig. 3).

Extravasation of Evans Blue

We have investigated whether a barrier to intraluminally applied dyes could be induced in BAEC co-cultured with either GFAP^{+/+} or GFAP-deficient astrocytes. These results were obtained from two GFAP^{+/+} and two GFAP^{-/-} cultures co-cultured with intraluminal BAEC for up to 4 weeks. In control (wild-type) cultures, a barrier to Evans blue became evident 2 weeks following co-culture of glia and BAEC. Thus, minimal extravasation of the dye was detected. In contrast, GFAP^{-/-} astrocytes failed, under identical conditions, to induce an appreciable barrier to the dye. However, following 1 month of culturing, GFAP^{-/-}/BAEC co-cultures effectively excluded Evans blue from the extracellular space.

C6 rat glioma cells readily induced in BAEC selective exclusion of Evans blue. This was evident 3–4 weeks following co-culture. Since visual determination of Evans blue does not allow for rigorous or quantitative testing of permeability, we further investigated the permeability of these endothelial monolayers to potassium and other molecules normally excluded by the BBB.

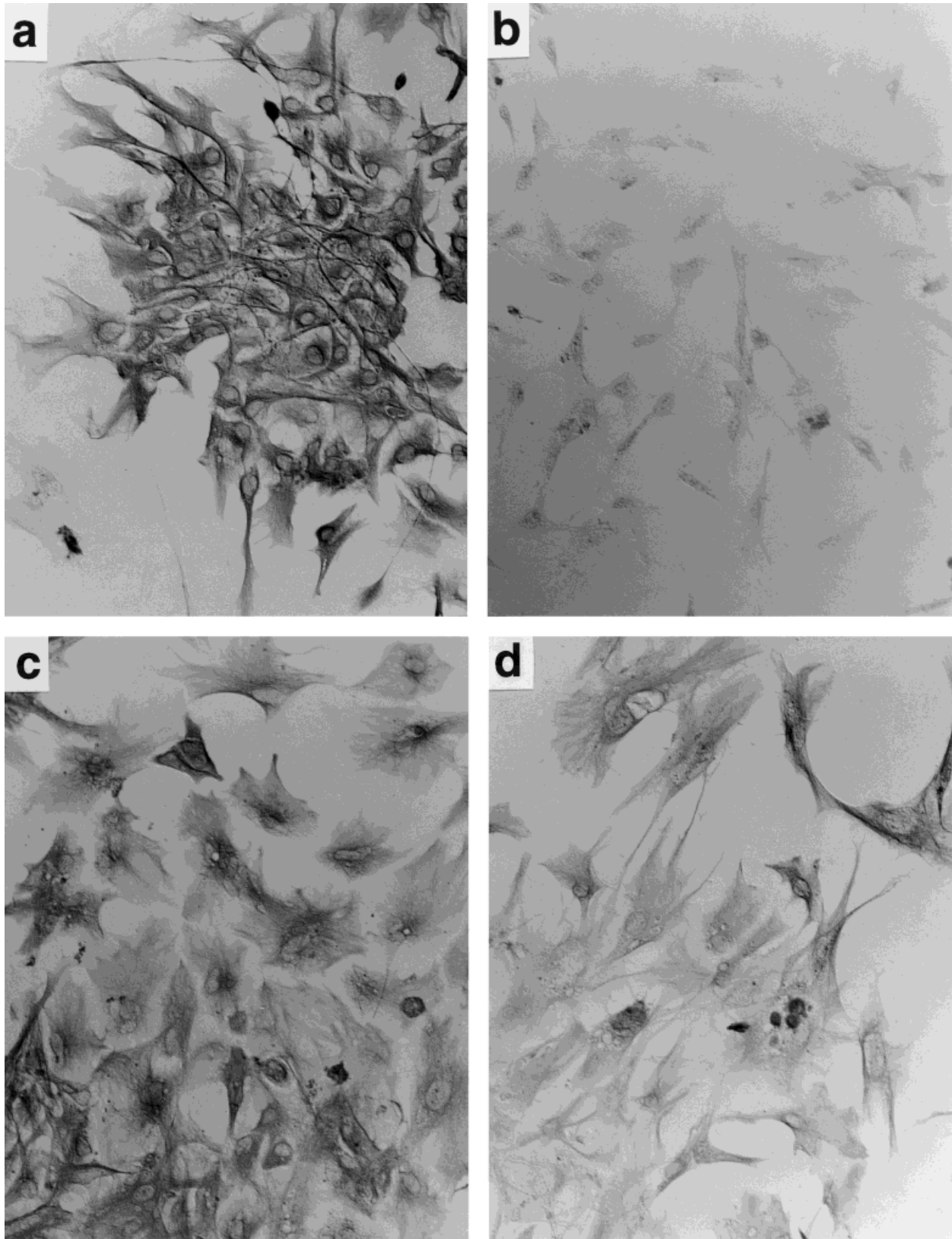


Fig. 1. Primary astrocyte-enriched cultures prepared from 1–2-day-old GFAP^{+/+} (a,c) and GFAP^{-/-} (b,d) mice. Seven days following plating, the overwhelming majority of cells in GFAP^{+/+} cultures were positive for both GFAP (a) and vimentin (c); GFAP^{-/-} cultures were negative for GFAP (b; counterstained with 50× diluted hematoxylin/erythrosin), but exhibited normal vimentin immunostaining (d).

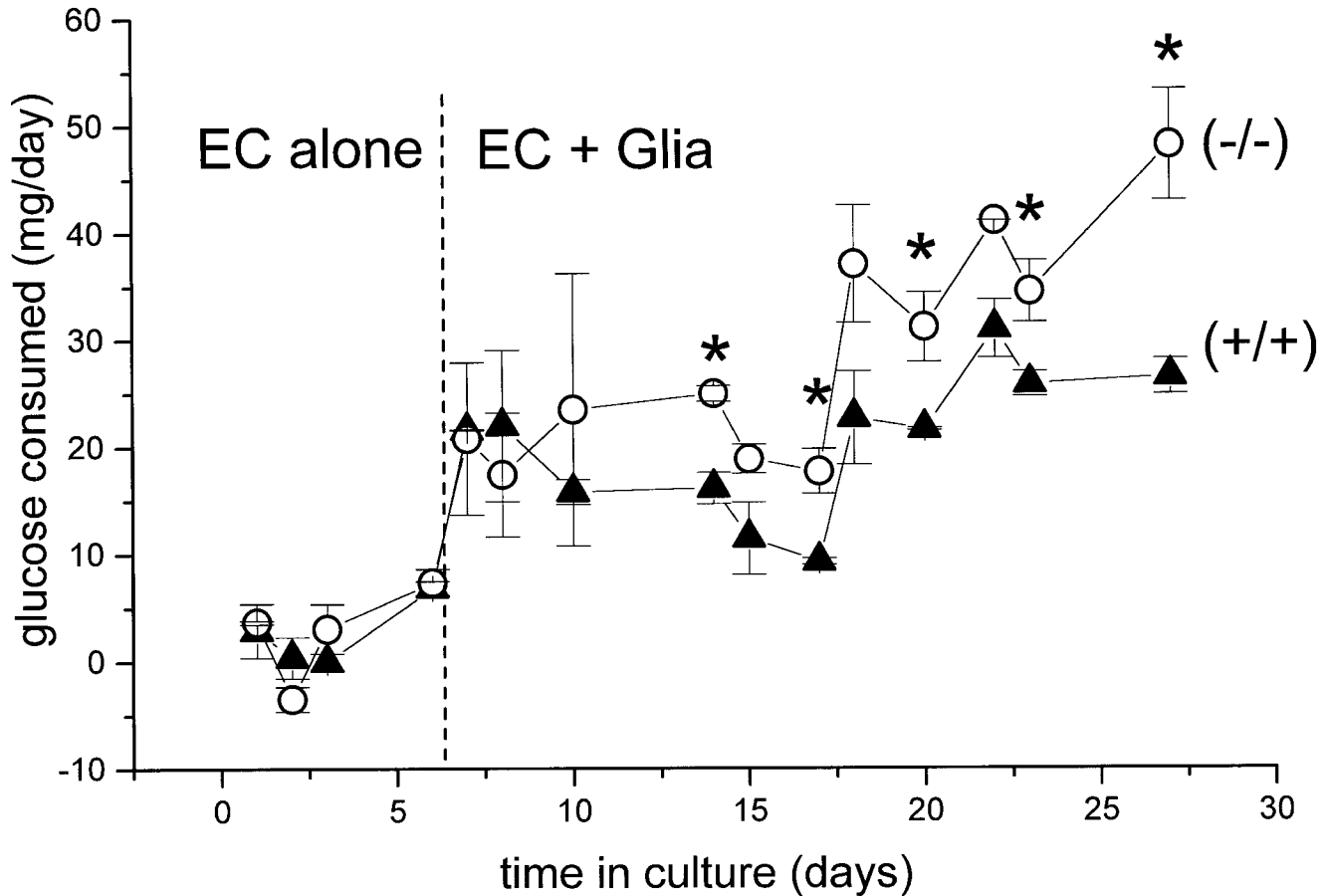


Fig. 2. Cellular growth in hollow fibers can be monitored by measurements of glucose consumption. Note that prior to addition of astrocytes to the extraluminal compartment (vertical dashed line), aortic endothelial cells (EC) consumed low levels of glucose. Following co-culturing with glia, metabolic activity increased dramatically in

both GFAP^{+/+} and GFAP^{-/-} cultures. The latter, however, were characterized by a higher consumption of glucose. The results show the means (\pm SEM) of four experiments for each experimental group; the asterisks refer to $P < 0.02$ (ANOVA).

Development of a Barrier to Intraluminal Potassium

In a previous study, we have shown that following co-culturing with glia, intraluminally seeded BAEC develop an asymmetric barrier to potassium (Stanness et al. 1996b). Both primary cultures of brain astrocytes and rat glioma cells were effective (e.g., Fig. 4C). We thus performed experiments to determine whether GFAP^{-/-} astrocytes may similarly induce this BBB phenotype in non-BBB endothelial cells (Fig. 4). Intraluminal endothelium (BAEC) was grown for 1–2 weeks and exposed to constant flow (Stanness et al. 1996b; Stanness et al. 1997). Under these conditions, no barrier to $[K]_{\text{lumen}}$ was detected. Thus, following application of 18 mM $[K]_{\text{lumen}}$, significant accumulation of potassium in the ecs occurred within 45 min of intraluminal perfusion. However, following co-culture with extracapillary astrocytes for 1–4 weeks, a progressive decrease of potassium extravasation became apparent. Thus, addition of GFAP^{+/+} astrocytes induced a significant improvement of the BBB to $[K]_{\text{lumen}}$ after 3 weeks in culture (Fig. 4A; compare open circles and open

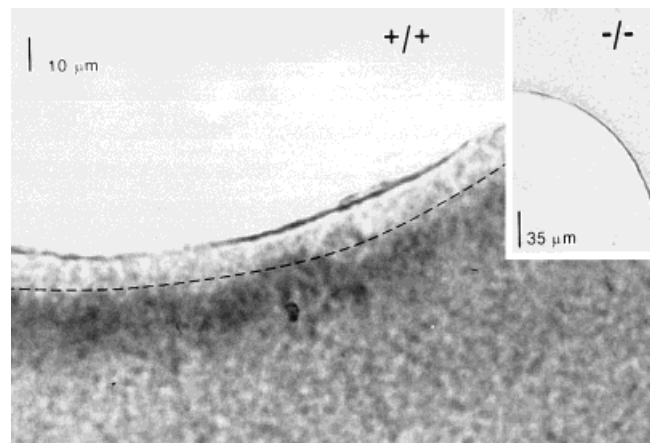


Fig. 3. Morphological appearance of the GFAP positive plexus in a transverse section of the hollow fiber capillary. Note the distinct band of immunopositive growth towards the intraluminal layer of endothelial cells. Intraluminally seeded endothelial cells formed a monolayer structure when grown in proximity to either GFAP^{+/+} or GFAP^{-/-} astrocytes. No immunostaining for GFAP was observed in GFAP^{-/-} co-cultures (inset).

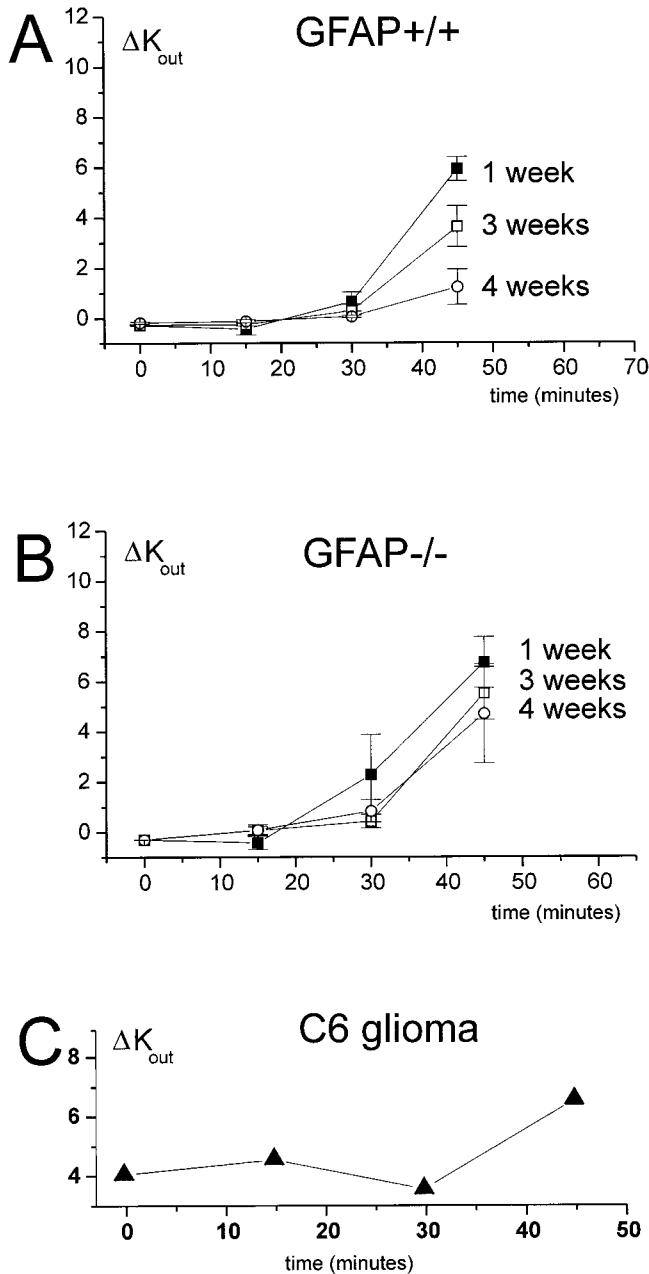


Fig. 4. Development of a barrier to intraluminally applied potassium. **A:** Endothelial cells were grown intraluminally for 1–2 weeks. Under these conditions no significant improvement of a barrier to potassium was observed. However, following addition of glia to the extracapillary space, GFAP^{+/+} astrocytes caused a significant increase of the resistance of the endothelial layer to potassium extravasation into the extracapillary space. **C:** Similar results were obtained when co-culturing endothelial cells with extraluminal rat glioma cells for 2 weeks. **B:** In contrast, GFAP-deficient cultures failed to induce any significant change in potassium extravasation from the lumen to the ecs. Intraluminal potassium = 18 mM; extraluminal potassium at the beginning of the experiment = 5 mM. The ordinate values in A and B refer to accumulation of potassium above baseline values. In C the absolute potassium levels are shown.

squares). Permeability to $[K]_{\text{lumen}}$ was further reduced after 4 weeks of co-culturing (dark squares). This improvement in BBB function was statistically significant ($P < 0.03$, $n = 6$); following 4 weeks in co-culture;

these BAEC monolayers grown in proximity to GFAP positive astrocytes allowed only 0.308 mM/min extravasation of potassium in the ecs.

In contrast, GFAP^{-/-} cultures failed to induce a selective exclusion to potassium (Fig. 4B). In fact, following 2 weeks of co-culturing, the DIV-BBB was more permeant to $[K]_{\text{lumen}}$ than the same culture at 1 week of co-culture. Following 4 weeks of co-culture, BAEC co-cultured with GFAP^{-/-} astrocytes allowed 1.066 mM/min of potassium extravasation, a value 80% higher than that measured in wild-type co-cultures.

Electrical Resistance

We have shown that under dynamic conditions, and when cultured in hollow fibers exposed to intraluminal flow, peripheral EC develop transendothelial resistance properties that are similar to those reported from in vivo experiments (Stanness et al., 1996a, 1997). Both rat glioma and primary cultures of either rat brain or human fetal astrocytes were effective. We repeated these experiments but co-cultured BAEC with either GFAP^{-/-} or GFAP^{+/+} astrocytes. Following 1 month in co-culture, BAEC monolayers from GFAP^{+/+} cultures had a mean resistance of $850 \pm 292 \text{ Ohm/cm}^2$ ($n = 4$, range 650–1,200; Fig. 5); under identical culturing conditions, GFAP^{-/-} glia induced in intraluminal BAEC a resistance of $520 \pm 120 \text{ Ohm/cm}^2$ ($n = 4$, range 200–900; $P < 0.05$ vs. GFAP^{+/+}). BAEC monolayers grown in the absence of astrocytes developed a resistance of only $50 \pm 15 \text{ Ohm/cm}^2$ ($n = 6$).

Permeability to Drugs

Under dynamic conditions, BAEC co-cultured with glia (C6 glioma or primary astrocytes) develop a barrier to intraluminally applied drugs that closely resembles the permeability properties of the BBB in vivo (Stanness et al., 1997). We tested the permeability properties of BAEC monolayers co-cultured with GFAP^{+/+} or GFAP^{-/-} astrocytes (Figs. 6,7). To this end, we applied two adenosine receptor antagonists (theophylline and 8-SPT) intraluminally and measured the passage into the ecs of these two compounds over 1 h of intraluminal perfusion. We choose these two adenosine analogues for their pharmacodistribution properties: previous in vivo studies have shown that while theophylline can readily diffuse from the blood to the brain by virtue of its lipophilic chemical nature, the hydrophilic 8-SPT is selectively excluded by the BBB (Ngai and Winn, 1993). Thus, the lipophilic compound theophylline ($0.5 \mu\text{M}$) readily traversed the BAEC monolayer independently of the presence of extraluminal glia. In fact, the permeability of theophylline was largely independent of the presence of a barrier to potassium. Accordingly, theophylline was equally excluded from the lumen in both GFAP^{+/+} and GFAP^{-/-} cartridges.

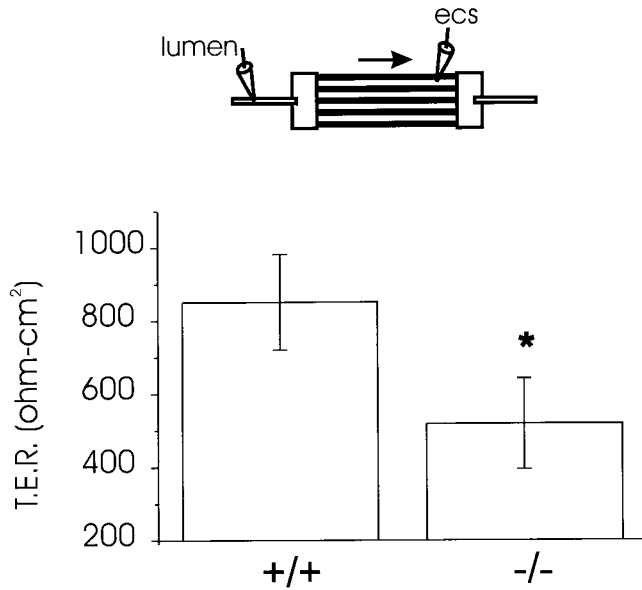


Fig. 5. Electrical resistance across endothelial monolayers is decreased in GFAP^{-/-}/BAEC cultures. The upper panel shows a diagrammatic representation of the placement of the recording (ecs) and waveform electrode (lumen). The lower panel shows the comparison of the mean TER (\pm SEM) values recorded from GFAP^{+/+} and GFAP^{-/-} co-cultures. (The asterisk indicates a statistical difference at $P < 0.05$; values were adjusted for the resistances measured with intraluminally seeded BAEC alone.)

In contrast, the membrane- (and BBB-) impermeant 8-SPT (0.7 μ M) did not gain access to the extracapillary space when BAEC were co-cultured with GFAP^{+/+} astrocytes. GFAP^{-/-} astrocytes, however, failed to induce a selective exclusion mechanism; thus, both theophylline and 8-SPT readily leaked into the extracapillary space regardless of the presence of glia in the ecs (asterisk indicates statistical difference at $P < 0.03$).

We also tested the permeability to intraluminally applied ¹⁴C-sucrose and obtained similar results: GFAP astrocytes effectively induced a barrier to sucrose extravasation into neighboring BAEC while GFAP^{-/-} astrocytes did not significantly induce drug permeation selectivity in these endothelial cells. Quantitative analysis of the permeation coefficients gave P_{sucrose} values of $3.29 \cdot 10^{-7} \pm 1.71$ and $3.39 \cdot 10^{-5} \pm 1.35$ cm/s for the GFAP^{+/+} and GFAP^{-/-} cultures, respectively (Fig. 7; $P < 0.02$).

DISCUSSION

The use of mice generated by gene targeting has brought extensive knowledge to mechanisms as diverse as neuronal function and genetic diseases. We have taken advantage of mice deficient for GFAP (GFAP^{-/-} mice), which we had previously generated (Pekny et al., 1995) to study the role of this IF protein in the development of the BBB. Using the in vitro model for BBB (Janigro et al., 1995; Stanness et al., 1996b, 1997), we have found a significant decrease in BBB induction

by GFAP^{-/-} astrocytes. While our results are confined to in vitro experiments, they suggest the involvement of GFAP (and thus IF) in the induction of normal BBB properties in non-BBB EC.

The morphological and growth characteristics of GFAP^{-/-} astrocytes in vitro as well as the presence of IF and IF proteins in these cells have been investigated by Pekny et al. (1997). These studies have revealed that GFAP^{-/-} astrocytes in vitro contained decreased amount of IF composed of vimentin and nestin while the levels of these two IF proteins were not affected by the absence of GFAP. Vimentin and nestin form (normally together with GFAP) IF in both cultured astrocytes and reactive astrocytes in vivo, in which the amount of IF is up-regulated. GFAP^{-/-} mice exhibited a normal BBB to Evans blue (Pekny et al., 1995) and peroxidase (Gomi et al., 1995), even though a study focusing on old GFAP^{-/-} mice suggested subtle changes in BBB permeability to albumin confined to the lumbar portion of the spinal cord (Liedtke et al. 1996). Thus, the absence of GFAP, which leads to reduction in the density of IF in astrocytes (Pekny et al., 1997), may exert quantitative or selective effects on the formation of BBB, which may become enhanced after a suitable challenge, such as the in vitro reconstitution of BBB used in this study.

Similarly to the in vivo data (Pekny et al., 1995), our results have shown that following prolonged coculturing, GFAP^{-/-} astrocytes can induce an endothelial barrier to Evans blue/albumin complexes, even though the formation of this barrier was delayed. In contrast, we have demonstrated that other BBB-specific features were not found in BAEC exposed to GFAP^{-/-} astrocytes. This apparent contradiction (presence of a barrier to Evans blue vs. lack of a barrier to highly diffusible ions, such as K⁺) suggests that there may be separate mechanisms responsible for the regulation of transendothelial permeability of BBB. This would certainly be an interesting direction to follow since the BBB is characterized by three distinct features that are present together in endothelial cells in the central nervous system: 1) a mechanical barrier formed by tight junctions between endothelial cells; 2) absence of pinocytosis (a transport mechanism of limited selectivity); and 3) presence of stereospecific transport mechanisms. It is possible that these three features of the BBB have been differentially affected by GFAP^{-/-} astrocytes. However, none of the in vivo studies so far published (Gomi et al., 1995; Liedtke et al., 1996; Pekny et al., 1995) addressed the question of whether the same functional deficiencies observed in our study (permeability to potassium and hydrophilic drugs) were also present in vivo. Due to the fundamental role that the BBB plays in the regulation of the brain environment, it appears unlikely that mice lacking BBB-mediated exclusion mechanisms could behave normally. It is possible that the GFAP^{-/-} mice exhibit a quantitative difference in their BBB that could become more prominent following a suitable challenge or in the old animals in which different functional capacities are consumed. This idea of quantitative and selective de-

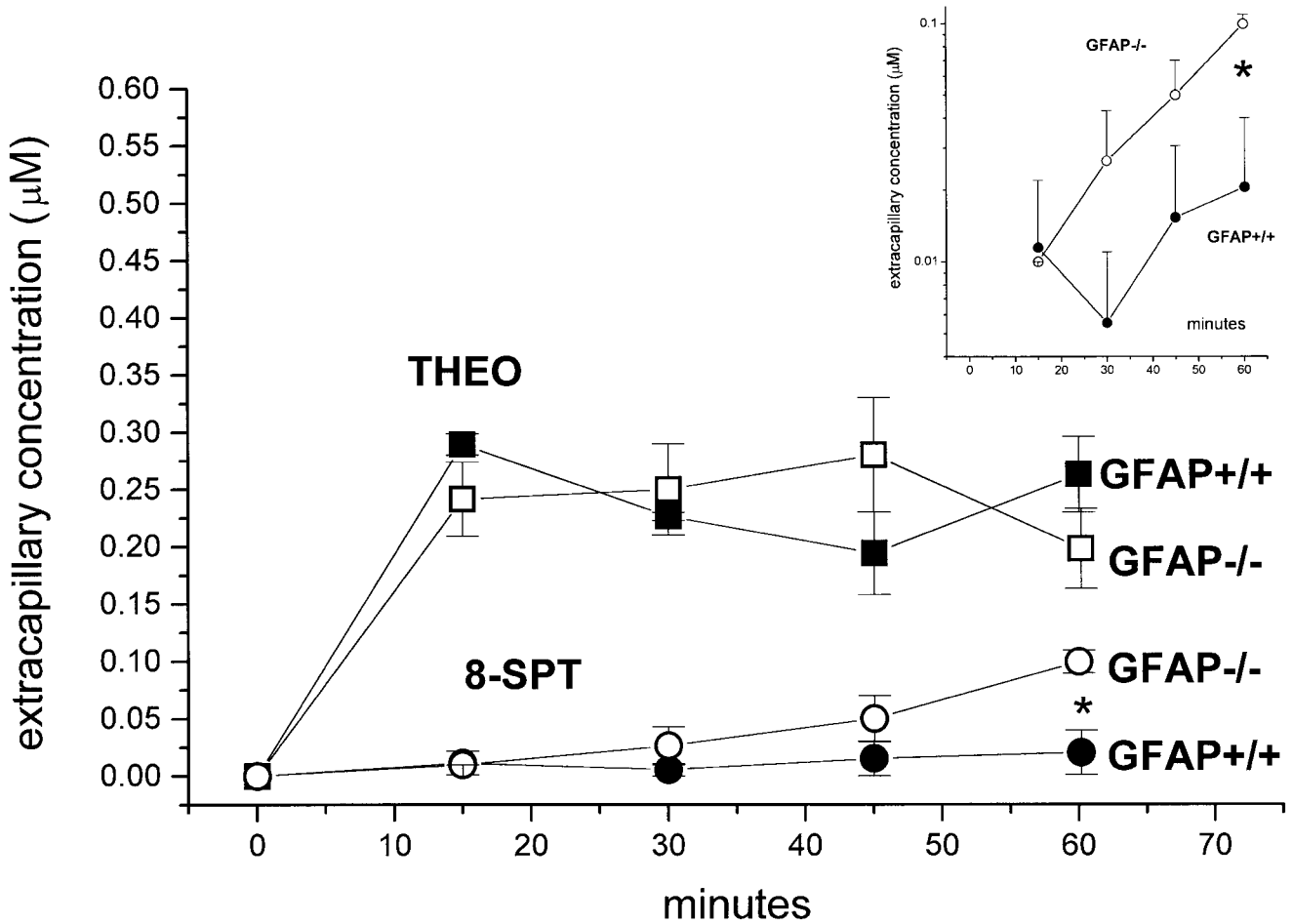


Fig. 6. Comparison of the permeability properties of GFAP^{+/+}/BAEC and GFAP^{-/-}/BAEC cultures. BAEC were exposed intraluminally to 8 sulphophenyl theophylline (8-SPT) or theophylline. Note that in contrast to Figure 7, for these experiments drugs were added to the reservoir and the cultures were thus continuously perfused intraluminally for the whole duration of the experiment. Only the profile of ecs concentration changes are shown for clarity. While

extravasation of theophylline occurred in both GFAP^{+/+}/BAEC and GFAP^{-/-}/BAEC cultures, the latter showed a higher permeability to the membrane-impermeant adenosine receptor antagonist 8-SPT. The results shown represent mean \pm SEM of three experiments. The inset shows the data concerning extraluminal accumulation of 8-SPT plotted on a log scale. The asterisk refers to a statistically significant difference ($P < 0.05$).

fects of the BBB in GFAP^{-/-} mice would be quite compatible with the increased permeability to albumin observed regionally in the spinal cord of old, but not young, GFAP^{-/-} mice (Liedtke et al., 1996).

Alternatively, our study compared with the results obtained in vivo may suggest that induction of a BBB phenotype and functional properties in non-BBB EC (such as BAEC) differs in its mechanisms from the normal development of the BBB in vivo. It is possible that induction of BBB in non-brain EC may mimic the mechanisms of cellular repair, such as those observed following trauma, injury, or, more generally, neurotoxicity. This is of importance in view of the fact that GFAP expression increases following injury and may therefore participate in the mechanisms of vascular/BBB repair. Further in vivo experiments may shed more light on the mechanisms of BBB repair following injury to the brain in normal and GFAP-deficient mice.

In addition to the advances in our understanding of the mechanism involved in BBB ontogenesis and re-

pair, the present in vitro study may also be viewed as an approach toward the understanding of the subcellular mechanisms responsible for the induction of the physiological BBB phenotype in BAEC cultured in the hollow fiber apparatus, or more generally, in culture. Our previous studies (Stanness et al., 1996b, 1997) have shown that proximity of glial terminals and the abluminal membrane of EC occurs in the DIV-BBB. These glial elongations grow within the narrow porosities of the artificial capillary support and travel for distances greater than 100 μ m toward the lumen. Herein we confirmed these findings by immunocytochemical detection of GFAP positivity 10–50 μ m beyond the albumin membrane of BAEC. A possible explanation of the lack of BBB induction in BAEC by GFAP^{-/-} astrocytes may reside in the fact that intermediate filaments may play a role in determining the capacity of glia in forming specialized areas morphologically described as "astrocyte feet." In this respect, it is interesting to point out the difference in the number of layers of astrocytic

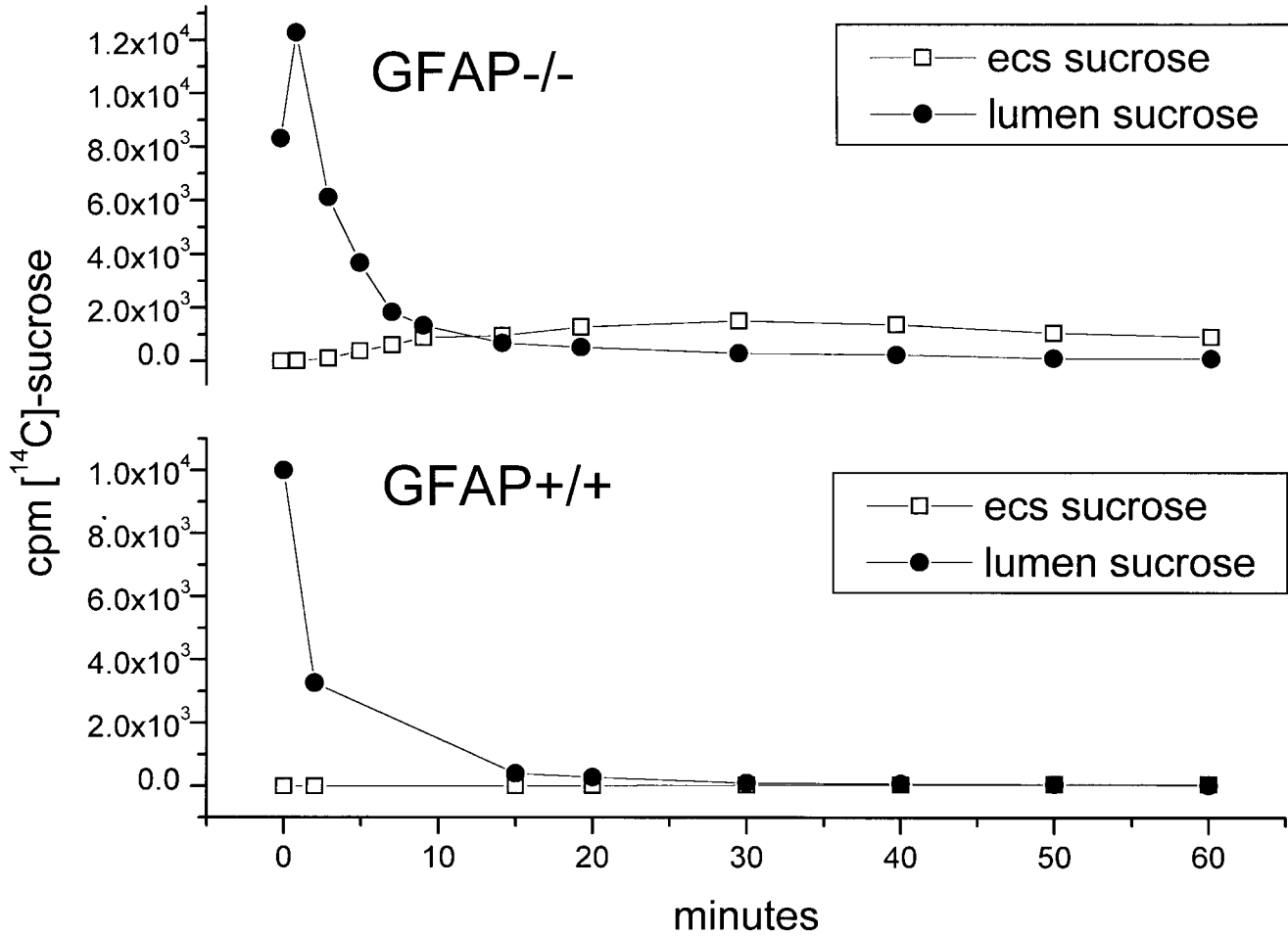


Fig. 7. Determination of the permeability to ¹⁴C-sucrose in the DIV-BBB. A bolus of concentrated ¹⁴C-sucrose was injected in the lumen (diluted in 1 ml of medium). Perfusion rate was kept constant throughout the experiments. The extracapillary space (ecs) and the lumen contents were sampled at the time points indicated in abscissa. Permeability values were calculated by graphic integration of two areas underlying the changes of [¹⁴C_{sucrose}]_{ecs} and [¹⁴C_{sucrose}]_{lumen} according to the following equation:

$$P = \frac{([\sup{14}\text{C}_{\text{sucrose}}]_{\text{ecs},t=60} - [\sup{14}\text{C}_{\text{sucrose}}]_{\text{ecs},t=0})}{([\sup{14}\text{C}_{\text{sucrose}}]_{\text{lumen}}) - ([\sup{14}\text{C}_{\text{sucrose}}]_{\text{ecs}})}$$

Note that a significantly higher extravasation of ¹⁴C-sucrose occurred in the cartridge where GFAP-deficient astrocytes were used to induce BBB properties in BAEC.

processes around EC (multiple layers in GFAP^{+/+} mice compared with a single layer in GFAP^{-/-} mice) reported in the spinal cord of older animals (Liedtke et al., 1996). Alternatively, the capacity of GFAP^{-/-} astrocytes to send long processes through the cartridge wall (150 μm thick) to the vicinity of BAEC may have been compromised. A requirement for GFAP in the development of processes that U251 astrocytoma cells send when co-cultured with neurons has previously been proposed (Chen and Liem, 1994; Weinstein et al., 1991). However, when the same co-culture experiments were performed with GFAP^{-/-} astrocytes, their ability to form processes was not compromised (Pekny et al., 1997). Further morphological studies are clearly required to elucidate fully the role of GFAP in determining the BBB properties of this BBB model.

The comparison of the results obtained with highly proliferative GFAP-deficient astrocytes or GFAP^{+/+} or C6 rat glioma cells strongly suggests that increased

cellular proliferation does not per se facilitate induction of BBB properties in BAEC. Thus, while rat glioma cells grew at a faster rate (as judged by glucose consumption measures), these glia were capable of inducing in aortic endothelial cells a barrier to potassium and hydrophilic drugs. In contrast, GFAP-deficient astrocytes, while growing at a similar rate in the ecs, failed to differentiate BAEC fully into a BBB phenotype. Brain astrocytomas/gliomas grow at exceedingly fast rates and are known to impact BBB permeability negatively. The results presented here suggest that lack of GFAP, as a result of astrocytic dedifferentiation or by the absence of a functional gene, is indeed responsible for the failure of BBB induction or maintenance.

In conclusion, we have demonstrated that GFAP^{-/-} astrocytes have a compromised ability to induce a BBB phenotype in aortic EC. Our results also rule out the hypothesis that abnormal cell proliferation compro-

mises the BBB. The exact role for GFAP in determining BBB properties needs further elucidation: nevertheless, our data strongly suggest a role for this intermediate protein in determining organ-specific phenotypic features in vascular EC.

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