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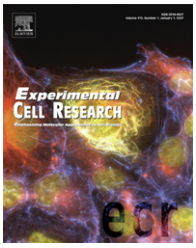

Research Article

The impact of glia-derived extracellular matrices on the barrier function of cerebral endothelial cells: An *in vitro* study

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ABSTRACT

The blood–brain barrier (BBB) is composed of the cerebral microvascular endothelium, which, together with astrocytes, pericytes, and the extracellular matrix (ECM), contributes to a "neurovascular unit". It was our objective to clarify the impact of endogenous extracellular matrices on the barrier function of BBB microvascular endothelial cells cultured *in vitro*. The study was performed in two consecutive steps: (i) The ECM-donating cells (astrocytes, pericytes, endothelial cells) were grown to confluence and then removed from the growth substrate by a protocol that leaves the ECM behind. (ii) Suspensions of cerebral endothelial cells were seeded on the endogenous matrices and barrier formation was followed with time. In order to quantify the tightness of the cell junctions, all experiments were performed on planar gold-film electrodes that can be used to read the electrical resistance of the cell layers as a direct measure for endothelial barrier function (electric cell–substrate impedance sensing, ECIS). We observed that endogenously isolated ECM from both, astrocytes and pericytes, improved the tightness of cerebral endothelial cells significantly compared to ECM that was derived from the endothelial cells themselves as a control. Moreover, when cerebral endothelial cells were grown on extracellular matrices produced by non-brain endothelial cells (aorta), the electrical resistances were markedly reduced. Our observations indicate that glia-derived ECM – as an essential part of the BBB – is required to ensure proper barrier formation of cerebral endothelial cells.

Introduction

The central nervous system (CNS) is separated from the circulating blood flow by highly specialized endothelial cells that line the cerebral capillaries. This diffusion barrier is referred to as the blood–brain barrier (BBB) [1,2]. Together with neighboring astrocytic, pericytic and neuronal cells, as well as the extracellular matrix (ECM) between them, the cerebral endothelial cells constitute a "neurovascular unit" [3] that is essential for protecting the brain from fluctuations in blood composition. Hence, it guarantees an undisturbed homeostasis of the CNS that is prerequisite for neuronal function.

The expression of the BBB-phenotype depends critically on the local microenvironment. Exposition of brain capillary endothelial cells (BCECs) to glia cells promoted the expression of P-glycoprotein [4] and of γ-glutamyl-transpeptidase (γ-GT) [5], both considered as marker proteins of the BBB. A pericyte-induced increase of occludin and MRP-6 mRNA expression in

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Abbreviations: BBB, blood–brain barrier; ECM, extracellular matrix; PBCEC, porcine brain capillary endothelial cells; ECIS, electric cell–substrate impedance sensing

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BCEC has been demonstrated recently [6,7]. In addition, BCECs showed elevated transendothelial electrical resistances (TEER) as a direct measure of barrier efficiency when co-cultured with astrocytes or pericytes [8,9].

Nonetheless, the nature of the underlying cell–cell communication is still under controversial discussion. One communication pathway between the BCECs and the cerebrum is assigned to soluble molecules such as pericyte-secreted TGF-β [10] or astrocyte-secreted bFGF [11]. However, the induction of BBB features is generally more effective in co-cultures, where glial processes are allowed to contact the basal surface of the endothelium or its basal lamina [12]. The brain ECM shared by endothelium and surrounding neuroglia cells is commonly considered to be an important part of the BBB. It has already been demonstrated that individual components of BCEC–ECM (fibronectin, laminin 1, and collagen type IV) are able to elevate the TEER of endothelial cell monolayers [13,14].

Taken together, the influence of the cellular microenvironment and certain ECM components on the development of the BBB-phenotype has already been demonstrated. Thus, it was the objective of this work to investigate the capacity of endogenous ECM derived from glia or non-glia cells to modulate endothelial differentiation *in vitro*. Macrovascular endothelial cells isolated from porcine aorta were used as a non-glia (negative) control.

The experiments presented here were performed with the well-established *in vitro* cell culture model of the BBB based on primary BCECs isolated from porcine brain microvessels [15]. We tested the impact of different endogenous ECMs on endothelial barrier function via an experimental technique referred to as electric cell–substrate impedance sensing or short ECIS [16–18]. With this technique it is possible to follow the formation of both, cell–cell (tight junctions) and cell–matrix (adhesion and spreading) interactions in parallel as a function of time. In ECIS, cells are grown on small gold-film electrodes that are used to measure the transendothelial electrical resistance as a direct measure for endothelial barrier function. The electrodes can be used and coated like ordinary cell culture dishes. In order to study endogenous ECM we have cultured astrocytes, pericytes, macrovascular endothelial cells, or BCECs on these electrodes and after they had established their individual ECM, the cell bodies were removed. After seeding a suspension of BCECs on these precoated electrodes, we followed the electrical resistance of the establishing endothelial cell layer as a sensitive indicator for the formation of an endothelial barrier phenotype.

Our data show that endogenous ECM secreted by brain-derived microvascular endothelial cells improves the development of BBB properties of BCECs *in vitro* compared to ECM provided by non-brain, aorta endothelial cells. Moreover, BCECs grown on pericyte and astrocyte ECM form a tighter barrier than BCECs grown on their own matrix.

Materials and methods

Materials

All cell culture media were obtained from Biochrom (Berlin, Germany) except for Dulbecco's modified Eagle's medium Ham's F-12, which was purchased from Sigma (Deisenhofen, Germany). Biochrom was also the supplier of L-glutamine, antibiotics, trypsin, and collagen G. Newborn calf serum (NCS) was obtained from PAA (Linz, Austria). All other chemicals were obtained from Sigma (Deisenhofen, Germany). All cells were grown on ECIS-arrays (model 8W10E, Applied BioPhysics, Troy, NY), which consist of 8 wells with 10 electrodes deposited on the bottom of each well (Applied-Biophysics, Inc. Troy, NY). ECIS-measurements were performed via the *ECIS™ Model 1600R* (Applied BioPhysics, Troy, NY). Both data acquisition and processing were performed using the *ECIS Data Analysis Software* supplied by Applied Biophysics (Troy, NY).

Cell culture

All cell types were kept in an ordinary humidified cell culture incubator at 37 °C and 5% CO_2 (v/v).

- Porcine brain capillary endothelial cells (PBCEC) were isolated and cultured according to Franke et al. [19]. In brief, the cerebra of freshly slaughtered adult pigs were freed from the meninges and homogenized mechanically. The brain homogenate was digested enzymatically using 0.3% (w/v) protease/dispase II from *B. polymyxa* in *preparation medium* (Medium 199 Earle supplemented with 0.7 mM L-glutamine, 100 μg/ml gentamicin, 100 μg/ml penicillin, and 100 μg/ml streptomycin). After incubating for 2 h at 37 °C, brain capillaries were separated from myelin and cell debris by dextrane density centrifugation. The capillary pellet was triturated using a glass pipette. A second enzymatic digestion with 1% (w/v) collagenase/dispase II in *plating medium* (*preparation medium* supplemented with 10% (v/v) NCS) for 45 min at 37 °C served to remove the capillary basement membrane. Released endothelial cells were further purified using a discontinuous Percoll density gradient and were seeded on collagen G-coated culture flasks. Contaminating pericytes within the endothelial cultures were removed according to Perriere et al. [20] by adding puromycin (2.5 μg/ml) to the culture medium.

  Twenty-four hours after initial plating, cells were washed with phosphate buffered saline (PBS) containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} and supplied with fresh *culture medium* (plating medium with puromycin but without gentamicin). Primary cultures of PBCEC were subcultured by gentle trypsinization at RT on day 3 *in vitro* (day in vitro=DIV) in order to reduce contamination by other cell-types. On DIV 5 PBCEC were frozen and stored in liquid nitrogen. PBCECs were characterized as endothelial cells by their expression of factor-VIII-related antigen [21,22]. Their microvascular origin has been verified by the elevated activity of the marker enzymes' alkaline phosphatase (ALP) [22,23] and of γ-glutamyl-transpeptidase (γ-GT) [24,25] shortly after isolation and when co-cultured with astrocytes.
- Pericytes were obtained from PBCEC primary cultures that were not treated with puromycin. Since pericytes attach more firmly to the culture substrate than PBCEC, selective trypsinization of PBCEC yielded pure pericyte cultures that were grown to confluence in *plating medium*. Pericytes were checked for their expression of alpha-smooth-muscle actin and desmin [26,27]. As reported by other authors [28]