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Synergistic effects of neurons and astrocytes on the differentiation of brain capillary endothelial cells in culture

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Abstract

Brain capillary endothelial cells form a functional barrier between blood and brain, based on the existence of tight junctions that limit paracellular permeability. Occludin is one of the major transmembrane proteins of tight junctions and its peripheral localization gives indication of tight junction formation. We previously reported that RBE4.B cells (brain capillary endothelial cells), cultured on collagen IV, synthesize occludin and correctly localize it at the cell periphery only when cocultured with neurons. In the present study, we describe a three-cell type-culture system that allowed us to analyze the combined effects of neurons and astrocytes on differentiation of brain capillary endothelial cells in culture. In particular, we found that, in the presence of astrocytes, the neuron-induced synthesis and localization of occludin is precocious as compared to cells cocultured with neurons only.

Keywords: astrocytes - blood-brain barrier - brain capillary endothelial cells - cortical neurons - cocultures - occludin - tight junctions

Introduction

The brain capillary endothelial cells form tight junctions (TJs) that are responsible for formation of the physical and metabolic barrier between systemic circulation and CNS, known as blood-brain barrier (BBB) (for review, see: [1–5]). Changes in the physiological function of this structure have been described in neurological diseases such as stroke, Alzheimer's disease and multiple sclerosis [2]. Moreover, malignant brain tumors cause brain edema because they have leaky TJs that allows passage of plasma fluid into the brain [6–8].

TJs, or *zonulae occludentes*, contain integral membrane proteins of fundamental importance for the properties of BBB, among which junctional-associated molecules (JAM) [9], occludin [10-12] and claudins [13-16].

Several lines of evidence suggest that occludin

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plays a crucial role in the physiology of TJs and regulation of vascular permeability, and indeed its tissue expression and localization correlates with barrier properties of cells and the extent of transendothelial electrical resistance [17–20].

We previously found that extracellular matrix and neurons modulate the expression of occludin by rat endothelial cells RBE4.B [21] and that occludin is correctly localized at the cell periphery only if endothelial cells are cocultured with neurons for at least one week [21]. In these conditions, RBE4.B form a barrier with BBB permeability properties [22]. As also astrocytes are known to induce and maintain BBB properties in endothelial cells [23–25], we set a three-cell type-culture system that includes RBE4.B brain capillary endothelial cells, astrocytes and neurons.

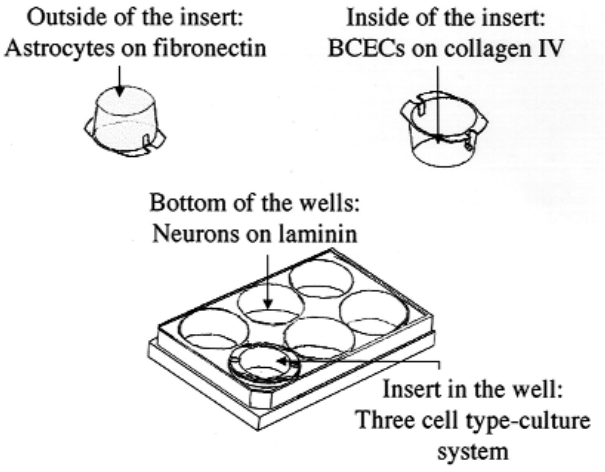

Fig. 1 Schematic drawing of the three-cell type culture system used as *in vitro* BBB model. Details of cell culture preparation are given in the text.

Materials and methods

Animals

Sprague-Dawley rats (Stefano Morini, San Polo d'Enza, Italy) were housed in our institutional animal care facility under direction of a licensed veterinary. Procedures involving animals were conducted according to the European Community Council Directive 86/609, OJL 358 1, 12 December 1987.

Cell cultures

RBE4.B immortalized rat brain microvessel endothelial cells were kindly donated by Dr F. Roux, under the permission of Neurotech SA (Orsay, France). Cell were plated on collagen I (6 μg/ml) and maintained in DME/Ham's F12 (2:1), supplemented with 10% heat-inactivated fetal calf serum, in humidified 5% CO_2/95% air at 37°C, till half-confluence. Cells were then progressively adapted to serum-free Maat medium (MM) [23], as described elsewhere [21].

Neurons were purified from fetal rat cortices at the 16th day of gestation and cultured in MM, on laminin (2.5 μg/cm^2), as already described [24].

Astrocytes were purified from brain cortices of 2 day old rats, as described by Cole and de Vellis [25], exploiting the differences in adhesion of the various brain cell types, and cultured in DME/Ham's F12 (2:1), supplemented with 10% heat-inactivated fetal calf serum in humidified 5% CO_2/95% air at 37 °C, till half-confluence. Astrocytes to be used for the three cell type-system were then progressively adapted to MM as in the case of RBE4.B cells. In some cases, confluent astrocytes were detached with trypsin-EDTA, diluted in 93% fetal calf serum, 7% DMSO and frozen. Astrocytes, frozen as soon as purified from brain cortices, successfully recovered and started proliferating, when thawed in fresh medium

Cocultures

Astrocytes adapted to MM were plated on the outside of fibronectin-coated (2.5 μg/ml) inserts (transparent, 23 mm diameter, 0.4 mm pore size, Falcon), as shown in Fig. 1. After 4 hours, the inserts were placed in the companion wells where neurons had been already cultured for at least 2 days (Fig. 1). After additional 48 hours, RBE4.B cells adapted to MM were plated inside the inserts, previously coated with collagen IV (3.5 μg/ml). As controls, RBE4.B cells were also cultured with neurons alone, with astrocytes alone and without other brain cells. All cultures and cocultures were fed for 5-8 days with daily changes of MM in the insert chamber, before harvesting cells for further analyses.

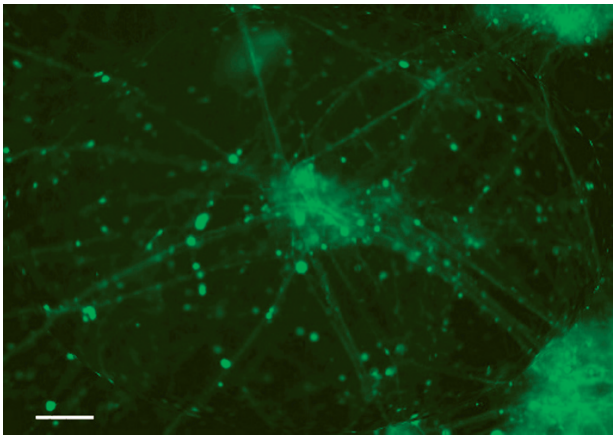

Fig. 2 Cortical neurons purified from rat brain cortices at the 16th day of embryonal development (E16) and cocultured for one week, in Maat Medium, with astrocytes and RBE4.B cells. Neurons were immunostained with anti-NF-68 antibodies. Bar= 50 μm.

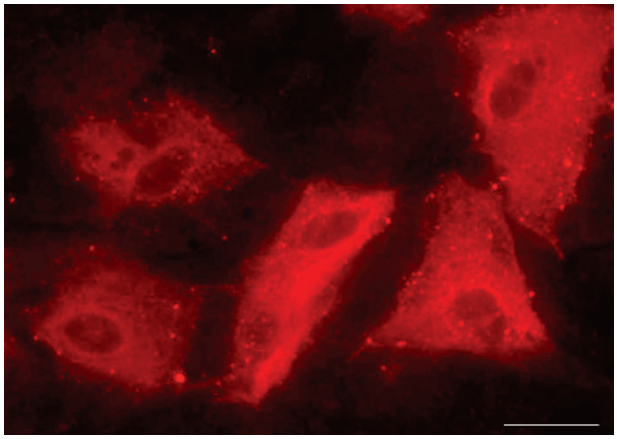

Fig. 3 Astrocytes purified from rat brain cortices at the 2nd day of postnatal development (P2) and cocultured for one week, in Maat Medium, with neurons and RBE4.B cells. Astrocytes were immunostained with anti-GFAP antibodies. Bar=50 μm.

Immunofluorescence

All procedures were performed as already described [21]. To stain neurons mouse monoclonal antineurofilament (NF), 68 kDa component, antibodies (Boehringer) were used, while astrocytes were stained with rabbit polyclonal fibrillary acidic protein (GFAP) antibodies (Sigma). Occludin was stained with rabbit polyclonal anti-occludin antibodies (Santa Cruz Biotechnology, Inc), at a dilution of 1:400. Pictures of the same field were taken under both visible light and fluorescence, in order to calculate the average number of occludin-expressing cells/field. The number of cells showing peripheral localization of occludin was also determined respect to the total of fluorescent cells. Up to 10 fields/sample were analyzed in each experiment.

Results

Three cell type culture system

We previously observed that neurons are able to induce RBE4.B endothelial cells to synthesize and segregate occludin at the cell periphery [21]. As other authors reported the importance of astrocytes

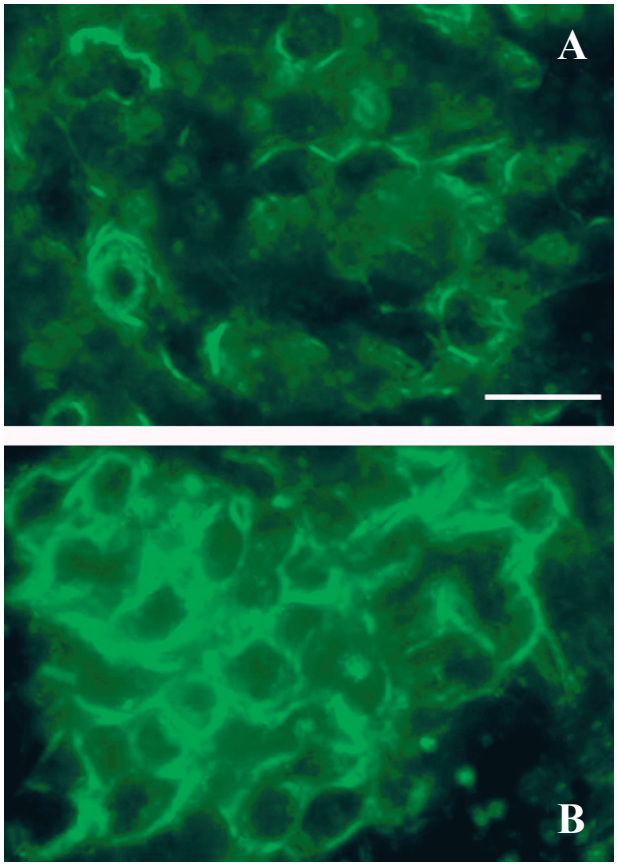

Fig. 4 Localization of occludin in RBE4.B cells cultured for 5 days with astrocytes (A) or with both astrocytes and neurons (B). Endothelial cells were stained with rabbit polyclonal anti-occludin antibodies. Bar= 20 μm

in the establishment and maintenance of BBB, we decided to set a culture system that included all three-cell types. The system used is schematically shown in Fig.1. It contains: i) cortical neurons, purified from rat brain cortices at the 16th day of embryonal development (E16) and cultured on laminin, at the bottom of the wells of the Transwell system; ii) astrocytes, purified from rat brain cortices at the 2nd day after birth (P2) and plated on fibronectin, on the outer side of the inserts of the Transwell system, and iii) RBE4.B endothelial cells, cultured on collagen IV, inside the inserts of the Transwell system. We found that astrocytes must be plated on the outside of inserts at a concentration of 1.0-1.5 x 10^5 cells/cm^2, at least 4 hours before inverting the inserts and placing them into the companion wells. Moreover for better results it is important to leave astrocytes with neurons for 24-48 hrs, before adding RBE4.B endothelial cells (at a plating concentration of 2.5 x 10^5 cells/cm^2) to the system. We found that using frozen astrocytes (see Materials and methods section) allows easier establishment of the three-cell type culture system, leaving neurons as the only cells to be freshly purified from fetal brain.

Neurons and astrocytes at the end of one week of culture are shown in Fig. 2 and Fig. 3, respectively: both cell types acquired and maintained well-differentiated phenotypes, and adhered stably to substrata.

Effects of astrocytes and neurons on localization of occludin in RBE4.B cells

Our previous results showed that endothelial cells cultured on collagen IV synthesize significant amounts of occludin only when cultured with cortical neurons [21]. However, the cells must be cocultured with neurons for at least one week in order to segregate occludin to cell periphery. We asked then whether astrocytes can have any effect on the timing of occludin expression and localization. Fig.4 shows immunostaining with anti-occludin antibodies of RBE4.B cells, cocultured with either astrocytes alone (Fig. 4A) or with both astrocytes and neurons (Fig. 4B) for 5 days. The results clearly demonstrated that RBE4.B cells, cocultured with both astrocytes and neurons (Fig. 4B) already synthesize and sort occludin at the cell periphery after 5 days in culture. Interestingly, endothelial cells synthesize significant amounts of immunoreactive occludin also when cultured for 5 days with astrocytes alone (Fig. 4A); However, in this case, less than 10% of RBE4.B cells showed peripheral immunostaining with anti-occludin antibodies.

Discussion

Different *in vitro* models of BBB have been developed in order to explore the molecular mechanisms that underline formation and maintenance of this complex structure, as well as to understand the influence of many pathological events on BBB functional polarity and permability (for review, see [27]). The most recent *in vitro* models of BBB rely on the use of coculture systems in which endothelial cells are cultured in porous inserts, then placed into companion wells in which either astrocytes [18, 27–28] or neurons [21] are cultured. The present study aimed at setting a more complex *in vitro* system in which endothelial cells could be cultured at the same time with both neurons and astrocytes. The system should have a geometrical organization resembling at most the situation of brain vessels, also allowing each cell type to settle on its preferred substrate.

Several lines of evidence suggest that occludin plays a fundamental role in the physiology of TJs [10–12, 17–20]. Therefore it is likely that modulation of its synthesis, and localization at the plasma membrane is of crucial importance in the establishment and maintenance of a functional BBB [22-27].

Previously we reported that specific molecules of the extracellular matrix as well as soluble factors released by neurons modulate the expression of occludin by rat brain endothelial cells RBE4.B cells [21].

In the present study, we demonstrate that occludin synthesis and sorting to the cell periphery is precocious if the cells are cocultured with both neurons and astrocytes. In the case of coculture with astrocytes only, astrocytes are able *per se* to induce synthesis of significant amounts of occludin in RBE4.B endothelial cells. However, the protein appears not to be sorted in the same amounts at the plasma membrane. These results suggest that both neurons and astrocytes have a key effect on synthe-

sis and sorting of occludin by RBE4.B endothelial cells. Moreover, it seems that the two cells types have independent effects, possibly by inducing post-transcriptional and/or post-translational events that modulate occludin dynamics in the cell. In the presence of both cell types, these effects might be added up, leading to synergistic stabilization of TJs and formation of a functional BBB. The culture system described might be a versatile model of BBB and might allow the analysis of the molecular mechanisms underlining both physiological and pathological regulation of BBB permeability, as well as studies aimed at delivering drugs of medical importance to brain.

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