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BRAIN RESEARCH

Research Report

Neural precursor cell influences on blood–brain barrier characteristics in rat brain endothelial cells

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ARTICLE INFO

Article history:
Accepted 1 May 2007
Available online 26 May 2007

Keywords:
Neural precursor cell
Brain endothelial cell
P-Glycoprotein
MCM7 staining
Transendothelial resistance
Astrocyte

ABSTRACT

This study explores the effects of neural precursor cells (NPCs) on barrier characteristics in brain vasculature. Primary rat brain endothelial cells were exposed to conditioned medium from NPCs isolated from day 14 embryonic rat brains and maintained as free-floating undifferentiated neurospheres. Such exposure increased brain endothelial transcript levels of the *mdr1a* but not *mdr1b* gene encoding P-glycoprotein (Pgp) and reduced proliferation but did not alter transendothelial resistance (TER). These effects were compared to those seen following co-culture with differentiating NPCs or with primary astrocytes. NPCs, if grown adherent, differentiate into glial and neuronal cells as assessed by immunocytochemical and mRNA analysis. Brain endothelial cells when co-cultured with these cells also showed reduced proliferation and enhanced *mdr1a* expression, but in addition increased TER. Similar increases were observed in co-culture with astrocytes. These results suggest that undifferentiated NPCs produce factors that influence Pgp expression whereas their progeny also affect tight junction integrity.

1. Introduction

There is good evidence that a functional blood–brain barrier exists early during embryonic development. It is thought the neural microenvironment plays a key role in inducing barrier properties in the endothelial cells lining the capillaries (Bauer and Bauer, 2000; Engelhardt, 2003). Mature astrocytes are known to have an influence (Abbott, 2002) but cannot account for the presence of barrier markers in the developing brain before differentiated astrocytes appear relatively late in embryonic development. Thus mature astrocytes cannot be involved in initial blood–brain barrier formation, though they may assist in subsequent maintenance of the mature blood–brain barrier. The idea has gained ground that neural precursor cells (NPCs) present both early in development and in the adult brain might influence barrier properties of the microvascular endothelium (Bauer and Bauer, 2000).

Neural stem cells are known to concentrate around blood vessels where it is believed coordinated interactions between vascular and nervous systems take place, i.e. in the stem cell niche (Doetsch, 2003). The existence of this niche is of special interest since stem cells have remarkable powers of self-renewal and differentiation into various cell types with obvious implications for tissue regeneration and repair after brain injury. The vasculature, which includes endothelial cells and pericytes, forms an integral part of the stem cell niche.

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doi:10.1016/j.brainres.2007.05.032

Such close proximity makes possible cross-talk between neural stem cell and brain endothelial cells. Such cross talk can be investigated in vitro using either co-culture of endothelial cells with neural stem cells or culture in the presence of conditioned medium. Shen et al. (2004) have already reported that endothelial cells release soluble factors that stimulate proliferation of neural stem cells while inhibiting their differentiation. However, in their study, the possible influence of neural stem cells on the endothelial cells was not investigated.

In the present study, a rat syngenic in vitro system has been used to explore some interactions between NPCs and brain capillary endothelial cells (RBECs) that affect blood–brain barrier properties, including cell proliferation, transendothelial resistance (TER), and particularly expression of P-glycoprotein (Pgp). This multidrug efflux transporter has been shown to play an important role at the blood–brain barrier in preventing access of unwanted substances to the brain (Schinkel, 1999). Thus the signals responsible for inducing and maintaining its expression on brain endothelium are of some clinical relevance. We show here that factors released from undifferentiated NPCs increase Pgp expression but not tight junction integrity. By contrast differentiating progeny increase both Pgp expression and TER. This suggests that different factors are involved in the induction and maintenance of these separate barrier characteristics.

2. Results

2.1. *Characterization of undifferentiated neurospheres and of differentiating NPCs*

As observed previously (Caldwell and Svendsen, 1998), when NPCs are isolated from embryonic brains, they proliferate in the presence of specific growth factors and B27 supplement but absence of serum (see Experimental procedures) to form neurospheres (Fig. 1A). The cells within these spheres stain positively for nestin (Fig. 1B), a marker for neuroepithelial stem cells. Medium from around these spheres was harvested for use as neurosphere-conditioned media (NsCM) in the experiments described below that explore the effects of undifferentiated NPCs.

If these NPCs are dissociated, plated onto a poly-L-lysine-coated surface and maintained in a medium lacking added growth factors, they start to differentiate, losing their nestin expression until none is detectable within 5 days (Figs. 1C, D). By then, the cells are present mainly as GFAP-positive astrocytes (Fig. 1E) with some β-tubulin III-positive neurons interspersed (data not shown). The presence of FCS affects the extent of their proliferation (cell number per viewing frame after 9 days being 49.3±6.1, 148±18.8, and 210±17.7 in the presence of 0%, 1% and 10% FCS, respectively, $n=15$ for each).

Such differentiating NPCs were used for co-culture to determine the effects of NPC progeny. The extent of their differentiation after 9 days was assessed by measuring the transcript levels of early and late markers of glial cell differentiation (i.e. *CD44* and *GFAP*, respectively) (Liu et al., 2004), the levels being compared to those found in the primary cultured astrocytes. Expression of the late glial marker, *GFAP*, in the differentiating NPCs was found to be around 51% of that seen in mature astrocytes (values relative to *GAPDH* of 0.406±0.028, $n=5$ and 0.800±0.126, $n=3$, respectively) whilst expression of the early glial marker, *CD44* was similar to that of mature astrocytes (values relative to *GAPDH* of 0.126±0.016, $n=5$ and 0.130±0.003, $n=3$, respectively).

2.2. *Characterization of rat brain endothelial cell cultures*

Rat brain endothelial cell cultures were prepared both with (ptRBEC) and without (RBEC) puromycin treatment. The purity of the latter was assessed using morphological criteria and RT–PCR analysis to detect the expression of pericyte or astrocyte marker genes (*CD11b* and *GFAP*, respectively) (Dolman et al., 2005). The levels of expression of both pericyte marker, *CD11b*, and of astrocyte marker, *GFAP*, were found to be negligible. Ct values of samples were similar or more than those of non-template controls, i.e. ≥33 and ≥35 cycles for *CD11b* and *GFAP*, respectively, indicating non-specific amplification (the usual Ct value for *CD11b* with comparable amount of cDNA from whole rat brain is 29 cycles and similarly the usual Ct value for *GFAP* in mature astrocytes is 16 cycles). The TER values obtained from these preparations were however low (~42 Ω cm^2) suggesting the presence of defects in the monolayer structure occurring as a result of a few contaminating cells not detectable by morphological examination. Thus experiments were also conducted using cultures prepared with puromycin treatment (Perriere et al., 2005). These produced populations of ptRBECs that were purer as judged from the higher TER values obtained (~120 Ω cm^2).

2.3. *Effect of conditioned medium or co-culture on proliferation of rat brain endothelial cells*

The degree of cell proliferation in any given population was assessed by staining for the cell proliferation marker, MCM7, which is present in early G1 phase in nuclei about to replicate (Tye, 1999). Staining was performed on RBECs from the different co-culture systems (Figs. 2A, B). Their nuclei were stained simultaneously using DAPI which binds to DNA and labels all nuclei (Figs. 2C, D). The proportions of MCM7-positive nuclei relative to DAPI-stained nuclei were then estimated at 9 days with RBECs grown in monoculture (ctrl), in neurosphere-conditioned medium (NsCM), in co-culture with differentiating NPCs (DNPC) or with astrocytes (A), or in astrocyte-conditioned medium (ACM). As shown in Fig. 2E, each of these culture or co-culture conditions produced percentages of MCM7 positively stained cells significantly lower than that seen in RBECs grown in monoculture.

2.4. *Effects of conditioned medium or co-culture on relative expression of mdr1a and mdr1b in rat brain endothelial cells*

The expression at the mRNA level of the *mdr1a* and *mdr1b* genes, i.e. two of the genes that encode P-glycoprotein in the rat, was determined in RBECs using real-time qPCR. Values were estimated relative to expression of *GAPDH*.

Conditioned medium was obtained every second day from undifferentiated neurosphere cultures and applied to the lower chamber of wells containing RBECs on inserts. After