BRAIN RESEARCH 1608 (2015) 167–176

Available online at www.sciencedirect.com

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Brain Research

Research Report

Three-dimensional culture conditions differentially affect astrocyte modulation of brain endothelial barrier function in response to transforming growth factor β1

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ARTICLE INFO

Article history:
Accepted 6 February 2015
Available online 23 February 2015

Keywords:
Blood–brain barrier
Astrocytes
Gliosis
In vitro
Three-dimensional cell culture

ABSTRACT

Blood–brain barrier (BBB) function is regulated by dynamic interactions among cell types within the neurovascular unit, including astrocytes and endothelial cells. Co-culture models of the BBB typically involve astrocytes seeded on two-dimensional (2D) surfaces, which recent studies indicate cause astrocytes to express a phenotype similar to that of reactive astrocytes in situ. We hypothesized that the culture conditions of astrocytes would differentially affect their ability to modulate BBB function in vitro. Brain endothelial cells were grown alone or in co-culture with astrocytes. Astrocytes were grown either as conventional (2D) monolayers, or in a collagen-based gel which allows them to grow in a three-dimensional (3D) construct. Astrocytes were viable in 3D conditions, and displayed a marked reduction in their expression of glial fibrillary acidic protein (GFAP), suggesting reduced activation. Stimulation of astrocytes with transforming growth factor (TGF)β1 decreased transendothelial electrical resistance (TEER) and reduced expression of claudin-5 in co-cultures, whereas treatment of endothelial cells in the absence of astrocytes was without effect. The effect of TGFβ1 on TEER was significantly more pronounced in endothelial cells cultured with 3D astrocytes compared to 2D astrocytes. These results demonstrate that astrocyte culture conditions differentially affect their ability to modulate brain endothelial barrier function, and suggest a direct relationship between reactive gliosis and BBB permeability. Moreover, these studies demonstrate the potential importance of physiologically relevant culture conditions to in vitro modeling of disease processes that affect the neurovascular unit.

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http://dx.doi.org/10.1016/j.brainres.2015.02.025
0006-8993/

1. Introduction

Passage of substances from the bloodstream into the central nervous system is limited by the blood–brain barrier (BBB), formed by the endothelium of brain capillaries. Brain capillary endothelial cells are distinguished from other endothelial cells by high electrical resistance, epithelial-like tight junctions, enriched expression of transport proteins that facilitate uptake of essential nutrients and efflux of xenobiotics, a paucity of pinocytotic vesicles, and an enrichment of mitochondria that support the considerable energetic demands of this system (Hawkins and Davis, 2005). This specialized phenotype is only partially recapitulated in cultured brain endothelial cells (Calabria and Shusta, 2008), implying that the cellular and extracellular milieu of the brain provides important cues for the differentiation and function of the microvascular endothelium (Stewart and Wiley, 1981).

Nonetheless, in vitro models of the BBB are valuable for CNS drug development and toxicity testing, given the expense of studies in animal models. "Tighter" endothelial barriers have been obtained by a number of approaches, including co-culture with astrocytes (Janzer and Raff, 1987; Hayashi et al., 1997; Haseloff et al., 2005; Abbott et al., 2006; Al Ahmad et al., 2010; Cantrill et al., 2012) pericytes (Al Ahmad et al., 2010; Daneman et al., 2010), and neural progenitor cells (Weidenfeller et al., 2007; Lippmann et al., 2011), application of steroids (Hoheisel et al., 1998; Weidenfeller et al., 2005), and the application of fluid shear stress (Colgan et al., 2007; Cucullo et al., 2011). Of these, co-culture with astrocytes is the best-studied and most widely used approach, and many studies have implicated astrocytes as the chief cell type responsible for BBB induction and/or maintenance (Janzer and Raff, 1987; Hayashi et al., 1997; Willis et al., 2004; Haseloff et al., 2005; Abbott et al., 2006), though other cell types (e.g., pericytes) are likely involved as well, particularly in development (Al Ahmad et al., 2010; Daneman et al., 2010).

Astrocytes are the most abundant cell type in the brain, but the full range of their biological functions remains poorly understood (Barres, 2008). A common feature of traumatic, ischemic, and inflammatory insults to neural tissue is "reactive gliosis", a process that involves hypertrophy of astrocytes, formation of stress fiber-like structures, migration to and/or proliferation at the site of injury, and secretion of extracellular matrix proteins that form a glial scar (Pekny and Nilsson, 2005). Interestingly, astrocytes grown under standard, two-dimensional (2D) culture conditions display hallmarks of reactive gliosis, specifically the expression of glial fibrillary acidic protein (GFAP) and secretion of chondroitin sulfate proteoglycans at much higher levels than observed in uninjured brain in situ (East et al., 2009). Thus, the spatial constraints imposed on astrocytes by a 2D surface cause them to behave as though injured. The baseline reactivity of astrocytes is greatly reduced when the cells are grown in a three-dimensional (3D) matrix, such as a collagen hydrogel, alginate-laminin constructs, or 3D nanofibers coated with extracellular matrix proteins (East et al., 2009, 2012; Frampton et al., 2011; Puschmann et al., 2013). Though BBB disruption can initiate reactive gliosis, the impact of reactive gliosis on endothelial barrier function is not well understood; however, given the substantial influence of astrocytes on brain endothelial cell function, we hypothesized that the reactivity of astrocytes impacts barrier function, and particularly the response of the BBB to injury. Here, we tested this hypothesis using immortalized murine brain endothelial cells and astrocytes in a co-culture system.

2. Results

2.1. *Optimization of bEnd.3 and C8 D1A culture conditions*

A series of preliminary experiments were conducted to determine the best growth conditions for bEnd.3 and C8 D1A cells in co-culture, since we were aware of only one published report addressing the utility of the C8 D1A cell line for in vitro BBB modeling (Booth and Kim, 2012). Moreover, there are some discrepancies in the literature regarding the extent to which astrocytes and/or astrocyte-derived factors can enhance the barrier properties of bEnd.3 cells (Brown et al., 2007; Li et al., 2010; Booth and Kim, 2012). In these studies, Brown et al. found no statistically significant increase in TEER of bEnd.3 cells with astrocyte (C6) conditioned media, and Li et al. found a modest increase in TEER with co-culture that was completely accounted for by the physical contribution of the astrocytes themselves to TEER. However, Booth and Kim's work (using the same combination of cell lines we report here) showed an increase in TEER with co-culture, which was most pronounced when the cells were grown under shear stress. Consistent with the optimization study recently published by Wuest et al. (2013), we found that bEnd.3 cells grown on polyethylene terephthalate (PET) consistently outperformed those grown on polycarbonate (PC) membranes in terms of TEER, regardless of initial cell seeding density or the presence of C8 D1A cells in co-culture (Supplemental Fig. 1). PET inserts were therefore used for all subsequent experiments. Comparison of bEnd.3 monolayers to bEnd.3/C8 D1A co-cultures indicated that co-cultures tended to have higher TEER values than monolayers on days 1–3 in culture, but these differences generally disappeared by days 4–5 in culture (Figs. S1B and 2B). Thus, C8 D1A cells had a modest "BBB-inducing effect" on bEnd.3 cells in our hands, decreasing the time required for bEnd.3 cells to reach their maximum TEER, but not increasing the maximum value of TEER, which typically ranged from 36 to 44 $\Omega\,cm^2$ and never exceeded 48 $\Omega\,cm^2$ in any of our experiments.

To accommodate the need for structural support for astrocytes grown in collagen hydrogels, bEnd.3 cells were seeded to the bottom surface of PET inserts prior to addition of C8 D1A cells to the top surface (Fig. 2A, refer to Section 4.5 for details). This protocol produced identical maximum TEERs to endothelial cells grown on the top surface, and was used for all subsequent experiments. TEER measurements were negligible in inserts containing cell-free collagen hydrogels without endothelial cells ($-0.5 \pm 1.5\,\Omega\,cm^2$, $n=4$) and in inserts with astrocyte-containing hydrogels without endothelial cells ($1.5 \pm 0.4\,\Omega\,cm^2$, $n=3$).

2.2. *C8 D1A cells in 2D and 3D culture*

To test whether 2D and 3D culture conditions differentially affect C8 D1A cell viability, cells were seeded at a variety of