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Research Report

An immortalised astrocyte cell line maintains the *in vivo* phenotype of a primary porcine *in vitro* blood–brain barrier model

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ABSTRACT

Whilst it is well documented that all components of the neurovascular unit contribute to the restrictive nature of the blood–brain barrier (BBB), astrocytes have been identified as the cellular component most likely to play an essential role in maintaining the barrier properties. The aim of this study was to examine the impact of the rat astrocyte cell line, CTX-TNA2, on the structural and functional characteristics of an *in vitro* BBB and determine the capacity of this astrocyte cell line to maintain the BBB phenotype. Co-culture of the CTX-TNA2 cells with primary porcine brain endothelial cells produced an *in vitro* BBB model which retains key features of the *in vivo* BBB. High transendothelial electrical resistances, comparable to those reported *in vivo*, were obtained. Ultrastructural analysis revealed distinct intercellular tight junction protein complexes and immunocytochemistry confirmed expression of the tight junction proteins ZO-1 and occludin. Western blotting and fluorescent tracer assays confirmed expression and functional activity of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) efflux transporters. Studies employing Alexa-fluor 555-conjugated human transferrin revealed temperature-sensitive internalisation indicating the BBB model retains functional receptor-mediated transferrin uptake. The findings of this study indicate that a robust BBB model has been produced and this is the first report of the inductive capacity of the CTX-TNA2 cell line. Since this *in vitro* BBB model possesses many key characteristics of the BBB *in vivo* it has the potential to be a valuable tool for the study of biochemical and physiological processes associated with the BBB.

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1. Introduction

The blood–brain barrier (BBB) represents a critical interface between the central nervous system (CNS) and the systemic circulation. Its unique characteristics help maintain homeostasis within the CNS by restricting the passage of blood borne substances, both endogenous and exogenous, from blood into brain tissue. The BBB consists of specialised brain endothelial cells which, along with astrocytes, neurons and pericytes form the neurovascular unit (NVU). The specialised microvascular

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endothelial cells that form the BBB are connected by tight junction complexes serving as physical barriers to restrict paracellular passage and which contribute to the high transendothelial electrical resistance (TEER) associated with the BBB (Butt, 1995; Crone and Olesen, 1982).

Whilst it is well documented that all components of the NVU contribute to the restrictive nature of the BBB, astrocytes have been the cellular component of the NVU most widely investigated and are thought to play a role in maintaining the barrier properties of the BBB (Abbott et al., 2006; Bauer and Bauer, 2000; Janzer and Raff, 1987; Wolburg et al., 1994, 2009). Still little is known about the precise molecular mechanisms that regulate cross-talk between astrocytes and brain endothelial cells and which determine the restrictive nature of the BBB although recent studies have highlighted the importance of the canonical signalling pathway, Wnt, in the maintenance of the BBB phenotype (for review see Liebner and Plate, 2010). Early studies investigating the inductive nature of astrocytes demonstrated that primary rat astrocytes induced chick endothelial cells of non-nervous system origin to form non-leaky vessels. This is strongly suggestive that astrocytes were responsible for not only inducing endothelial cells of non-neural origin to form a BBB phenotype, but was also an indication that the signalling molecules that mediated this transformation must be highly conserved since induction was observed across species (Janzer and Raff, 1987). To date, numerous studies have supported this theory (Hayashi et al., 1997; Kuchler-Bopp et al., 1999; Laterra et al., 1990; Tio et al., 1990).

In order to generate a stable and reproducible *in vitro* BBB model, one of the primary aims of this study was to examine the effect of maintaining primary porcine brain microvascular endothelial cells in culture with a rat astrocyte cell line. For any cell culture model to be considered as a valid tool, specific phenotypic characteristics must be expressed and continuously maintained without down-regulation or complete loss throughout the time period of culture. An *in vitro* BBB model must accurately reflect the *in vivo* BBB, *i.e.,* be comprised of cells with physiologically convincing morphology which form a highly restrictive barrier and express key functional transport mechanisms, in addition the cell model should be relatively straightforward to establish and maintain and permit generation of reproducible results and high throughput analysis (Gumbleton and Audus, 2001). Various cell co-culture approaches have been employed in an attempt to maintain the physiological properties of *in vitro* BBB models, since such properties are often reported to be down-regulated, or even lost, following isolation of the cells from the primary tissue. The ability of astrocytes to induce the BBB phenotype has been well-documented by a number of different methods, including co-culture with brain endothelial cells (Gaillard et al., 2001; Megard et al., 2002) and maintenance of endothelial cells in astrocyte-conditioned medium (Lauer et al., 2004; Sobue et al., 1999; Zhang et al., 2006). The use of supplemented growth medium or serum-free medium has also been employed in *in vitro* BBB models to help maintain model functionality (Hoheisel et al., 1998; Nitz et al., 2003). These approaches have been reported to increase BBB-associated γ-glutamyl transpeptidase and alkaline phosphatase activities and formation of tight junctions (El Hafny et al., 1996; Gaillard et al., 2001; Haseloff et al., 2005). A large increase in the expression of the *P*-glycoprotein (*P*-gp, ABCB1) efflux transporter has also been observed in brain endothelial cells co-cultured with astrocytes compared to endothelial cells maintained in mono-culture (Cecchelli et al., 1999).

The aim of this study was to determine the impact of the immortalised rat astrocyte cell line, CTX-TNA2, on the structural and functional characteristics of an *in vitro* BBB comprised of primary porcine brain microvascular endothelial cells. In particular, the capacity of this astrocyte cell line to maintain the BBB phenotype with the potential to provide a robust, reproducible *in vitro* model possessing key features of the *in vivo* BBB.

2. Results

2.1. *Puromycin pre-treatment increases transendothelial electrical resistance of primary porcine brain endothelial cell monolayers.*

In vitro BBB model integrity is often disrupted due to the presence of contaminating cells such as astrocytes and pericytes. Purity of isolated cell preparations is likely to affect the integrity of the *in vitro* BBB model and thus influence reproducibility (Perriere et al., 2005). Based on the assumption that *P*-glycoprotein expression is known to be much higher in brain endothelial cells than in contaminating cells, primary cell cultures were treated with puromycin as described by Perriere et al., 2005. Monolayers of PBECs pre-treated with puromycin and co-cultured with CTX-TNA2 astrocytes demonstrated significantly higher ($p<0.001$) transendothelial electrical resistance (TEER) ($1778\pm252\ \Omega/cm^2$) compared to co-cultures in which PBECs were not pre-treated with puromycin ($182\pm20\ \Omega/cm^2$, Fig. 1). There was no significant difference in TEER between PBECs that were not pre-treated with puromycin and co-cultured with CTX-TNA2 astrocytes ($126\pm57\ \Omega/cm^2$) and TEER of PBECs subjected to puromycin pre-treatment in mono-culture ($193\pm39\ \Omega/cm^2$), Fig. 1. These results suggest contaminating cells significantly affect the integrity of the cell monolayer, even when PBECs are maintained in co-culture with astrocytes.

2.2. *Astrocytes increase transendothelial electrical resistance of primary porcine brain endothelial cell monolayers*

To examine the effect of astrocyte cell type on the TEER of PBEC monolayers, PBECs seeded onto Transwell® inserts were maintained in mono-culture or in co-culture with either primary rat astrocytes, isolated from 2-day old rat pups, or with the CTX-TNA2 rat astrocyte cell line. PBEC monolayers co-cultured with astrocytes, irrespective of type, demonstrated significantly higher TEER than were observed in PBECs maintained in mono-culture. Likely exposure to soluble glial factors produced by primary rat astrocytes increased TEER significantly from $115\pm22\ \Omega/cm^2$ to $1693\pm315\ \Omega/cm^2$, in total demonstrating a 14.7-fold increase in TEER within 2 day

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