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Research Report

Regulation of the blood–brain barrier integrity by pericytes via matrix metalloproteinases mediated activation of vascular endothelial growth factor in vitro

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ABSTRACT

The blood–brain barrier consists of the cerebral microvascular endothelium, pericytes, astrocytes, and neurons. In this study, we analyzed the influence of primary porcine brain capillary pericytes on the barrier integrity of primary porcine brain capillary endothelial cells in a species-consistent in vitro coculture model. We were able to show a barrier integrity-decreasing impact of pericytes by transendothelial electrical resistance (TEER) and ^{14}C-sucrose permeability measurements. The morphology analysis revealed serrated cell borders and a shift of the endothelial morphology towards a cobblestone shape under the influence of pericytes. The analysis of the two major barrier integrity modulators vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) displayed higher MMP activity and higher levels VEGF, MMP-2, and MMP-9 in the coculture, whereas VEGF levels were decreased by the MMP inhibitor GM6001, indicating a complex interplay of both. Inhibition experiments with neutralizing VEGF antibody and GM6001 increased the TEER, which proves the involvement of VEGF and MMPs in the barrier-decreasing process. Analysis of occludin yielded decreased protein content and discontinuous expression at the endothelial cell borders under the influence of pericytes. These results together reveal the potential of pericytes to regulate the endothelial barrier integrity via MMPs and VEGF.

1. Introduction

The blood–brain barrier (BBB) is a unique barrier which plays a crucial role in the maintenance of the central nervous system homeostasis by restricting the passage of soluble compounds, toxic substances, and xenobiotics from the blood to the brain parenchyma. It is constituted of capillary endothelial cells, pericytes, astrocytes, and neurons which together form the neurovascular unit. The fundamental basis for the barrier function is the formation of complex tight junctions which seal the paracellular pathway between adjacent brain capillary endothelial cells (Abbott et al., 2010). Main tight junction proteins are occludin, claudins, and zonula occludens 1 (ZO-1). While the induction of the BBB

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Abbreviations: BBB, blood–brain barrier; PBCP, porcine brain capillary pericyte; PBCEC, porcine brain capillary endothelial cell; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; TEER, transendothelial electrical resistance; ZO-1, zonula occludens-1; α-SMA, α-smooth muscle actin; GFAP, glial fibrillary acidic protein; TGFβ, transforming growth factor β; DIV, days in vitro

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phenotype by astrocytes is well described (Abbott et al., 2006; Arthur et al., 1987), less is known about the role of pericytes within this cellular network. Recent studies revealed a contribution of pericytes to angiogenesis (Shibuya, 2009) and endothelial barrier properties (Hayashi et al., 2004). Moreover, the expression of the tight junction protein occludin was regulated by pericytes (Hori et al., 2004). Under pathological conditions like brain tumor genesis, multiple sclerosis, Alzheimer's disease, diabetic retinopathy, and prolonged oxygen deprivation (Al Ahmad et al., 2009; Argaw et al., 2006; Hellstrom et al., 2001; Iadecola, 2004; Winkler et al., 2004; Joussen et al., 2004), the impairment of pericyte–endothelial cell interaction seems to play an important role. Nevertheless, it is still not fully clarified how pericytes regulate the BBB integrity. In the present study, we concentrated on two possible regulators within the endothelial cell/pericyte interaction with impact on the barrier properties, the vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs).

The VEGF family consists of six members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF). VEGF-A (commonly referred to as VEGF) is the most prominent in brain tissue because of its key role in vasculogenesis, angiogenesis, formation of blood vessels, and regulation of endothelial permeability (Kim and Lee, 2009; Shibuya, 2009; Ferrara, 2000). Furthermore, it is involved in disease pathogenesis like brain tumor growth (Machein and Plate, 2000), stroke (Shibuya, 2009), and diabetic retinopathy (Aiello et al., 1997). VEGF can be released as a soluble molecule and can be sequestered into the extracellular matrix (ECM) where it remains inactive until it is released by proteases (Bergers et al., 2000). Capillary endothelial cells and pericytes express both VEGF and the corresponding receptor VEGFR-1 (Flt-1) while VEGFR-2 (KDR/Flk-1) is expressed exclusively by endothelial cells (Witmer et al., 2004).

MMPs are zinc- and calcium-dependent proteolytic enzymes which are secreted by endothelial cells as well as pericytes. Their activity is controlled not only transcriptionally and posttranscriptionally but also on the level of secretion, zymogen activation, and by endogenous tissue inhibitors of metalloproteinases (TIMPs). MMPs regulate many intercellular signaling mechanisms under physiological and pathological conditions by remodeling ECM components (Agrawal et al., 2008) and controlling the activation of incorporated proteins like growth factors and other signaling molecules (Rosenberg, 2009). Moreover, MMP-2 and MMP-9 were identified as major contributors to BBB disruption under various conditions. One key feature of MMP-9 is the ability to release and thereby activate matrix bound VEGF (Bergers et al., 2000).

Our aim was to study the impact of pericytes on the barrier properties of endothelial cells in vitro. For this purpose, transendothelial electrical resistance (TEER) measurement, ^{14}C-sucrose permeability measurement, and immunocytochemistry were combined to quantify the barrier integrity and morphological changes of primary porcine brain capillary endothelial cells (PBCEC) under the influence of primary porcine brain capillary pericytes (PBCP). In addition, we analyzed the expression of occludin and ZO-1 by Western blot analysis. To clarify how pericytes regulate the barrier integrity, we investigated the expression patterns of VEGF and MMPs. We followed the effect of the inhibition of VEGF and MMPs in order to understand the cellular interaction at the BBB and to gain new insights about the role of pericytes.

2. Results

2.1. *Impact of pericytes on the endothelial barrier integrity*

As a quantitative measure for the impact of pericytes on the endothelial barrier integrity we monitored the TEER of the PBCEC monoculture and PBCP monoculture in comparison to the coculture of PBCEC and PBCP on microporous Transwell® filter insert (Fig. 1A). Fig. 1B demonstrates a strong effect of cocultivated PBCP by decreasing the endothelial barrier integrity at all time points measured. The difference between PBCEC monoculture (361±9 Ωcm^2) and the coculture (188±9 Ωcm^2) is mainly visible on DIV 6. On this day, the endothelial cell barrier reaches its maximal tightness as well. Therefore, DIV 6 was set as the relevant time point for the following experiments to analyze the influence of PBCP on the PBCEC barrier functions. A significant TEER decrease to 66±10% was observed under coculture conditions (Fig 1C). PBCP alone does not build up a confluent monolayer with barrier properties and therefore displays extremely low TEER values (22±2 Ωcm^2). To verify these results, we measured the ^{14}C-sucrose permeability. Fig. 1D displays low permeability values of the PBCEC monoculture ($5.6\times10^{-7}\pm1.1\times10^{-7}$ cm/s) and higher permeability values of the cocultured cells ($25.4\times10^{-7}\pm3.0\times10^{-7}$ cm/s) on DIV 6. The observed increased sucrose permeability in the coculture compared to the PBCEC monoculture is consistent with the reduced TEER value.

The integrity of the endothelial barrier depends critically on the cell morphology which can be analyzed by visualization of tight junction protein expression patterns at the cell borders (Kroll et al., 2009; Calabria et al., 2006). Therefore, we performed immunocytochemical studies to check the pericytic impact on the morphology of endothelial cells. Immunostaining of the tight junction protein occludin (red) in PBCEC monocultures (Fig. 2A) shows the endothelial cell typical spindle-like morphology and expression of occludin at the cell borders. In contrast, cocultured PBCEC (Fig. 2B) displayed serrated cell borders and increased intracellular occludin localization. In addition, a shift of the PBCEC morphology towards a cobblestone shape was observed.

2.2. *Expression and function of VEGF in the coculture model*

Since VEGF is reported to be a permeability-inducing factor secreted mainly by pericytes, we analyzed the secretion levels in cell culture supernatants. In the apical compartment of cocultured PBCEC (0.68±0.11-fold), a significantly decreased amount of VEGF compared to the PBCEC monoculture was detected (Fig. 3A). In contrast, VEGF levels were found to be significantly higher in the basolateral side of the coculture (3.21±0.49-fold, Fig. 3B). A higher expression of VEGF was observed in the PBCP monoculture (2.44±0.79-fold) compared to the PBCEC monoculture, indicating PBCP as the major source of VEGF. Since the apical VEGF expression is decreased in the coculture while the basolateral VEGF expression is