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Original article

Novel insights in the dysfunction of human blood-brain barrier after glycation

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ABSTRACT

The blood-brain barrier (BBB) provides a dynamic and complex interface consisting of endothelial cells, pericytes and astrocytes, which are embedded in a collagen and fibronectin-rich basement membrane. This complex structure restricts the diffusion of small hydrophilic solutes and macromolecules as well as the transmigration of leukocytes into the brain. It has been shown that carbonyl stress followed by the formation of advanced glycation endproducts (AGE = glycation) interfere with the BBB integrity and function. Here, we present data that carbonyl stress induced by methylglyoxal leads to glycation of endothelial cells and the basement membrane, which interferes with the barrier-function and with the expression of RAGE, occludin and ZO-1. Furthermore, methylglyoxal induced carbonyl stress promotes the expression of the pro-inflammatory interleukins IL-6 and IL-8. In summary, this study provides new insights into the relationship between AGE formation by carbonyl stress and brain microvascular endothelial barrier dysfunction.

1. Introduction

The essential function of the blood-brain barrier (BBB) is to protect the brain from uncontrolled cross of agents. Alongside its protective function, the BBB constantly supplies not only the brain with nutrients by specific transport systems but also organises removal of metabolites (Bernacki et al., 2008; Hawkins et al., 2006). Different types of cells define the complex structure of the BBB: microvascular endothelial cells, pericytes and astrocytes. Endothelial cells (Choi and Kim, 2008) and pericytes (Allt and Lawrenson, 2001) are embedded in a basement membrane consisting mainly of collagen IV, fibronectin and laminin (Baeten and Akassoglou, 2011). The basement membrane, which serves as a scaffold, is also crucial for the BBB functionality. It establishes contact sites for the surrounding neuronal cells (Carvey et al., 2009). Endothelial cells embody the main mediators between blood and the brain (Calabria and Shusta, 2008). They function as regulators for the selective transport and metabolism of substances from the luminal to abluminal side as well as in the opposite direction (Zheng et al., 2003). As a consequence, the apical and basolateral plasma membranes have different protein compositions establishing a cellular polarity (Wolburg et al., 2009).

Tight junctions represent protein complexes between endothelial cells, which restrict the passive diffusion of molecules toward a concentration gradient. These complexes include transmembrane and membrane-associated proteins, such as claudins or occludins, which tightly interact with each other (Begley and Brightman, 2003). The result of extensive tight junction formation is the reason for a high electrical resistance of brain capillaries of about $2000\,\Omega \times cm^2$ and a low permeability (Stamatovic et al., 2008).

The central nervous system is described as an immune-privileged region (Wekerle, 2006). The BBB plays an important role in maintaining the separation of central nervous system from the adaptive and the innate immune system (Carson et al., 2006; Francis et al., 2003). Pathological events, such as viral infections, severe brain injuries or chronic-inflammatory diseases promote transmigration of immune cells through the BBB, leading to inflammation of the brain (Persidsky et al., 2006). Brain inflammation is characterised by activation of microglia, astrocytes and endothelial cells, which leads to expression of different

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cytokines and prostaglandins (Phillips et al., 2014; Takeda et al., 2014). In turn, these mediators can up-regulate the expression of several endothelial adhesion molecules, such as selectins, vascular cell adhesion molecule-1 (V-CAM-1) and intracellular adhesion molecule-1 (ICAM-1; Simi et al., 2007). Most importantly, the expression pattern of matrix metalloproteinases (MMPs) can be altered by pro-inflammatory cytokines. MMPs degrade not only the extracellular matrix components of the basement membrane but also tight junctions of the BBB (Lukes et al., 1999). The disruption of the basement membrane alters the cytoskeleton of endothelial cells, which in turn affects tight junctions proteins and barrier integrity (Rosenberg et al., 1993). Thus, during several pathological conditions the destruction of the basement membrane has been strongly associated with increased BBB permeability (Tilling et al., 2002). A breakdown of the BBB is also accompanied with cardiac surgery. Post-surgical breakdown of the BBB especially in elderly patients often leads to neurological dysfunction, such as delirium (Cavaglia et al., 2004; Merino et al., 2013; Okamura et al., 2010; Rinder, 2006). However, little is known about the mechanism leading to impaired BBB function in elderly patients that occurs after cardiac surgery, and its mediators.

Formation of advanced glycation endproducts play a major role in ageing and also in impaired BBB function. Advanced glycation endproducts (AGE) represent posttranslational modifications generated by irreversible non-enzymatic crosslinking reactions between carbonyl-containing molecules (e.g. sugars) and amino groups of proteins-a reaction referred to as glycation. Glycation is one feature of ageing and leads to the formation of non-degradable and less functional proteins and enzymes and can additionally induce inflammation and further pathophysiological processes. AGEs are involved in the pathogenesis of many diseases, such as diabetes, cataracts, atherosclerosis, diabetic nephropathy, and neurodegenerative diseases, including Alzheimerís disease and multiple sclerosis (Basta et al., 2004; Bierhaus et al., 1998; Chappey et al., 1997; Lassmann, 2007). It has been shown that patients suffering from diabetes mellitus and coronary heart disease have higher serum levels of AGEs than those without heart disease (Hofmann et al., 2013). Furthermore, AGEs are associated with many other cardiovascular complications, such as carotid stenosis, peripheral artery occlusive disease, and increased pulse pressure (Furth, 1997; Giacco and Brownlee, 2010; Stitt et al., 2002). In this study, we analysed the impact of age-formation caused by elevated carbonyl stress on endothelial cells of the BBB in a transfected human brain microvascular endothelial cell-based model. As agent for glycation (carbonyl-containing molecule) we used methylglyoxal (MGO). MGO is a natural side product of the glycolysis, which under physiological conditions is cleared by the detoxifying glyoxylase system. We could demonstrate that glycation of the endothelial cells interfere with barrier integrity. The expression of tight junctions proteins occludin, ZO-1 was down-regulated, whereas the expression of the receptor of advanced glycation end products (RAGE) was up-regulated after glycation. A dysfunction of the barrier integrity was also observed after glycation of the underlying extracellular matrix components. Furthermore, glycation (via MGO-treatment) of the endothelial cells leads to secretion of IL-6 and IL-8. Our findings suggest that glycation of endothelial cells and the underlying extracellular matrix damage the BBB directly and promote further damage of the BBB by inflammation.

2. Material and methods

2.1. Cell culture

Transfected human brain microvascular endothelial cells (THBMECs; Stins et al., 2001) were cultured as described recently (Labus et al., 2014). Briefly, cells were grown in DMEM:F12 = 1:1 medium at 37 °C in a humidified cell culture incubator (Hera cell – Heraeus Holding GmbH, Hanau, Germany). Medium was supplemented with penicillin/streptomycin (100 U/ml), L-glutamine (440 mg/l) and heat-inactivated fetal bovine serum (10%). Cells were passaged every 3–4 days. THBMECs were detached with Trypsin/ETDA and pelleted at 11,000*g* for 5 min.

2.2. Transwell cell culture setup

12 mm transwell inserts with 3 μm pore size (Greiner Bio, Germany) were coated with 10 μg/ml fibronectin and collagen IV each for 2 h at 37 °C in a humidified incubator. Remaining solution was aspirated and the inserts were rinsed in PBS to remove the collagen IV and fibronectin solution. Inserts were ready for use or for storage at 4 °C for 1 week. 2×10^5 THBMECs were seeded onto inserts. Cells were grown to confluence for 10–15 days. Medium was changed every 2–3 days. For cytokine stimulation, growth medium containing 10 ng/ml of the proinflammatory cytokine IL-1β (Immunotools, Friesoythe, Germany) was added to the upper chamber.

2.3. Permeability measurement

Permeability of the confluent THBMEC monolayers to sodium fluorescein was measured by adding 10 μg/ml sodium fluorescein to the upper chamber in serum-free medium. After 60 min incubation in a humidified incubator at 37 °C an aliquot from the lower chamber was taken. The fluorescence intensity was measured (excitation 485 nm/emission 528 nm). By means of a standard curve the concentration of sodium fluorescein in the lower chamber was calculated. The apparent permeability coefficient P app (in cm/s) is based on the Fick's law, which can be calculated according to the following equation:

$$\mathrm{Papp(cm/min)} = \mathrm{VA}/(\mathrm{A} * [\mathrm{C}]\mathrm{luminal}) * \Delta[\mathrm{C}]\mathrm{abluminal}/\Delta\mathrm{t}$$

2.4. Glycation procedure

THMBECs were grown until confluence and incubated with different concentrations (0.1 mM, 0.3 mM, 1 mM) of MGO (Sigma-Aldrich, St. Louis, USA). MGO was added to serum-free culture media and incubated for 4 h or 24 h in a humidified incubator at 37 °C. Untreated THBMEC cultured in serum-free media served as control.

Inserts were coated with collagen IV and/or fibronectin (10 μg/ml) (ECM proteins) for 2 h at 37 °C in a humidified incubator. In order to induce AGE-formation on these ECM proteins, inserts were incubated with 1 mM MGO for 4 h at 37 °C.

2.5. MTT assay

MTT assay was performed to determine the cytotoxicity of MGO. 2×10^5 cell/ml were seeded into 96 well plates and treated with different concentrations of MGO for 4 h 10 μl MTT (Sigma, Saint Louis, USA) was added to each well after MGO treatment and incubated for further 3 h in a humidified incubator. After incubation formazan crystals were dissolved by adding 100 μl MTT solubilisation solution (Sigma, Saint Louis, USA) into the wells and gently mixed. The absorbance was measured spectrophotometrically at a wavelength of 560 nm on a microplate reader Multiskan EX (Thermo Fisher Scientific, Rockford, USA). Relative cell viability (mitochondrial activity as parameter for cell viability) was calculated and compared to the untreated control.