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The effect of hyperglycaemia on permeability and the expression of junctional complex molecules in human retinal and choroidal endothelial cells

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ABSTRACT

Diabetic retinopathy is the leading cause of preventable blindness in the working population and its prevalence continues to increase as the worldwide prevalence of diabetes grows. Diabetic choroidopathy is less well studied and occurs in the late stages of diabetic eye disease. The main cause of visual loss in diabetic eye disease is diabetic macular oedema caused by an increase in microvascular endothelial permeability. Endothelial cell permeability is influenced by multiple factors which have not been fully elucidated, particularly in human models. In addition, the gene and protein expression between retinal and choroidal endothelial cells, even in humans, has been shown to be heterogeneous. The aim of this project was to determine, *in vitro*, the effect of high glucose (25 mM) on human paracellular permeability in retinal and choroidal endothelial cells. The expression of selected tight junction molecules (Occludin, Claudin-5, JAM-A and JAM-C) and adheren junction (VE-Cadherin) molecules was also compared between retinal and choroidal endothelial cells and with high glucose. High glucose conditions significantly increased the permeability in both retinal and choroidal endothelial cells monolayers although the increase was higher in retinal endothelial cells. Under normal glucose culture conditions microarray analysis determined that occludin and claudin-5 gene expression was higher in retinal endothelial cells than choroidal endothelial cells, and western blotting indicated that claudin-5 protein expression was also higher in retinal endothelial cells whilst JAM-A, and C and VE-Cadherin levels were similar. In retinal endothelial cells exposed to high glucose claudin-5, occludin and JAM-A was found to be reduced, whereas the expression of VE-Cadherin and JAM-C was unchanged when evaluated with western blotting, immunofluorescence and qPCR. None of the proteins were significantly decreased by high glucose in choroidal endothelial cells. The increase in retinal endothelial cell permeability is likely caused by a decrease in selective tight junction protein expression, leading to increased paracellular permeability. This may indicate differences in junctional molecule regulation of permeability in retinal compared to choroidal endothelial cells.

1. Introduction

Diabetic retinopathy (DR) has been extensively studied over the years, and its incidence correlates with poor glycaemic control and hyperlipidaemia (Ferris et al., 1996; Klein et al., 2002). Diabetic choroidopathy is a less well studied entity, and is thought to occur in the advanced stages of diabetic eye disease (Bischoff and Flower, 1985; Fryczkowski et al., 1989; Hidayat and Fine, 1985; Ishibashi et al., 1999). Diabetes and hyperglycaemia have obvious effects on intraocular vascular endothelial cell (EC) permeability, adhesion to leukocytes, as well as proliferation (Ciulla et al., 2003; Miyamoto and Ogura, 1999; Mohr, 2004). These alterations result in

Abbreviations: JAM, junctional adhesion molecule; DR, diabetic retinopathy; EC, endothelial cell; BRB, blood retinal barrier; TJ, tight junctions; AJ, adherens junctions; REC, retinal endothelial cells; CEC, choroidal endothelial cells; CNV, choroidal neovascularisation; RPE, retinal pigment epithelium.

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increased vascular leakage (increased permeability), vascular occlusions, ischaemia, and angiogenesis (Bhavsar, 2006; Bhavsar and Tornambe, 2006).

Clinical evidence indicates that diabetic macular oedema results from breakdown of the inner blood retinal barrier (BRB) (Vitale et al., 1995) which is maintained by EC tight junction (TJ) complexes (Mitic and Anderson, 1998). TJ proteins include occludins, claudins, and junctional adhesion molecules (JAMs), transmembrane molecules which interact with intracellular molecules including zonula occludens (ZO) and cingulin (Mitic and Anderson, 1998). Adherens junctions (AJ) are mediated by VE-Cadherin which promotes calcium dependent homophilic cell to cell contacts, and interact intracellularly with actin through catenins. Dejana et al. (2001) have reported that the principal TJ proteins found in retinal endothelial cells (REC) are occludin and claudin-5. Phosphorylation of occludin and ZO1 contribute to the dysregulation of EC paracellular permeability (Abbott et al., 2006; Thorin and Shreeve, 1998).

JAMs also signal to cytoskeleton associated proteins, as well as recruit cellular polarity proteins. As opposed to JAM-A, JAM-C increases paracellular EC permeability (Aurrand-Lions et al., 2001b; Orlova et al., 2006). It is suggested that the preferential expression of different junctional molecules in different vascular beds may explain their specific physiologic functions (Aurrand-Lions et al., 2001b), for example the high expression of JAM-A in the blood-brain-barrier (Orlova et al., 2006), the relative high expression of JAM B in high endothelial venules (Aurrand-Lions et al., 2001a; Palmeri et al., 2000), and JAM-A and C in the liver compared to its minimal expression of JAM-B (Geraud et al., 2012; Palmeri et al., 2000). Molecular crosstalk, substitution, or redundancy in the JAM system may also play a part in different tissues where all the different JAMs are expressed (Weber et al., 2007). JAM-C expression in retinal endothelial cells (REC) (Daniele et al., 2007; Klaassen et al., 2009) and the retinal pigment epithelium (RPE) (Economopoulou et al., 2009) has been described. It is possible that preferential expression of different adhesion or TJ molecules (occludin, claudins, JAM-A, B and C) as well as AJ molecules (VE-Cadherin) and associated integrins in different vascular beds may contribute to their relative susceptibility to diabetes and hyperglycaemia. Different JAMs are reportedly altered in the retina in diabetes (Antonetti et al., 1998; Harhaj et al., 2006; Klaassen et al., 2009). It is also known that different capillary beds may respond differently to different stimulatory or inhibitory factors (Aird, 2003). In addition, the effects of JAMs are specific to cell type (Li et al., 2009). This is supported by the observation that although diabetic retinopathy is a microvascular disease, microangiopathy does not seem to play a major pathogenic role in the cerebral complications of diabetes, and the blood-brain barrier is not as susceptible to hyperglycaemia as the retinal microvasculature (Mooradian, 1997).

The exact roles of these different molecules in diabetic eye disease are not fully understood. Although there are studies describing the adhesion and tight junction molecules in different intraocular vasculature (Daniele et al., 2007; Klaassen et al., 2009; Lamagna et al., 2005; McLeod et al., 1995; Penfold et al., 2002) and the RPE (Economopoulou et al., 2009) as well as experimental choroidal neovascularisation (CNV) (Hou et al., 2012), we are unaware of any studies comparing the expression of these important junctional molecules in human choroidal and retinal vascular EC and their relative expression in retinal and choroidal vasculature in diabetes. Furthermore, the role of JAM-C, in particular, in the retinal and choroidal EC permeability in diabetes (hyperglycaemia) requires elucidation. We have investigated the permeability of cell monolayers of human REC and choroidal endothelial cells (CEC) in normal glucose (NG) and high glucose conditions (HG) *in vitro*. We used microarray analysis to determine the relative gene expression for selected junctional molecules in REC and CEC in NG, as well as compared the protein expression profiles of selective junctional proteins in REC and CEC in HG with western blots and qPCR.

2. Materials and methods

2.1. Endothelial cell culture

REC and CEC were isolated and cultured separately for each donor as previously described (Stewart et al., 2011). Paired eyes from each donor were used to make one primary REC line and one primary CEC line.

2.2. Microarray analysis

Microarray analysis of cellular mRNAs was performed as previously described (Browning et al., 2012). Affymetrix CEL files were imported into GeneSpring GX 11.0.1 and processed with the MAS5 algorithm. Data was then normalised with GC-RMA to provide expression values. To identify differentially expressed genes between cell groups, ANOVA was performed with Tukey-HSD post-hoc testing and Benjamini–Hochberg false discovery rate control. A difference in expression between probe sets with a corrected *p*-value of <0.05 and a fold change of greater than 2 in all samples from a particular tissue bed were considered to be statistically significant.

2.3. Trans-endothelial permeability assay

To assess transendothelial permeability of REC and CEC, cells were initially cultured on a 35 mm tissue culture dish coated for >2 h with 5 μg/mL fibronectin in EGM2-MV medium with hydrocortisone omitted and containing 5 mM glucose, classed as NG (Lonza, Tewkesbury, UK). Confluent EC were transferred into 6 wells of a 6 well cell culture cluster coated with fibronectin as above and maintained in NG medium. Once 80–90% confluent EC were removed and allowed to adhere to 6 × 0.4 μm pore size transwell inserts (Becton Dickinson, USA) in 12 well format. EC on transwell inserts were cultured to confluence in NG medium, before being washed once with sterile phosphate-buffered saline (PBS) with antibiotics and the culture medium was then changed to either NG medium, or EGM2-MV with added glucose to give a final concentration of 25 mM glucose (HG) for 72 h. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and the medium was changed every day throughout the 3 days. The permeability was the assessed by the diffusion of Evan's blue-labelled albumin. Briefly, confluent inserts were transferred to fresh 12-well cell culture clusters containing 2 mL Hank's balanced salt solution (HBSS) in the basolateral chambers. In the apical compartments, culture medium was replaced by 500 μL of HBSS containing Evan's blue-labelled albumin (165 μg/mL; molecular weight, 67 kDa). Both luminal and abluminal sample absorbance was measured at 650 nm after 30, 60 and 120 min, using a microplate reader (THERMOmax). The coefficient of permeability was calculated as μL of luminal compartment volume from which the tracer is completely cleared. The experiments were repeated on 5 independent occasions, with 5 different unmatched donor REC and CEC samples in NG and HG conditions.

2.4. Western blotting

Confluent cells were allowed to attach in standard growth medium for 72 h and then the culture medium was changed to either NG or HG conditions (as in Section 2.2), for three days. Cultures