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Microglia increase tight-junction permeability in coordination with Müller cells under hypoxic condition in an *in vitro* model of inner blood-retinal barrier

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Abbreviations: AMD, age-related macular degeneration; BBB, blood-brain barrier; CNS, central nervous system; DM, diabetes mellitus; DMEM, Dulbecco's modified Eagle's medium; DR, diabetic retinopathy; EB, Evans blue; ECs, endothelial cells; ELISA, enzyme-linked immunosorbent assay; EVOM, epithelial volt-ohm meter; FCS, fetal calf serum; FITC, fluorescein-5-isothiocyanate; GFAP, glial fibrillary acidic protein; HIF, hypoxic inducible factor; iBRB, inner blood-retinal barrier; IL, interleukin; LDH, lactate dehydrogenase; MCs, Müller cells; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TEER, trans-endothelial electrical resistance; TNF, tumor necrosis factor; TJ, tight junction; VEGF, vascular endothelial growth factor; ZO-1, zonula occludens-1

Abstract

Microglia and Müller cells (MCs) are believed to be critically involved in hypoxia-induced blood-retinal barrier (BRB) disruption, which is a major pathogenic factor of various retinopathies. However, the underlying mechanism remains poorly defined. The inner BRB (iBRB) is primarily formed of microvascular endothelial cells (ECs) with tight junction (TJ), which are surrounded and supported by retinal glial cells. We developed a novel in vitro iBRB model sheet by sandwiching Transwell membrane with layered mouse brain microvascular ECs (bEnd.3) and mouse retinal MCs (QMMuC-1) on each side of the membrane. Using this model, we tested the hypothesis that under hypoxic condition, activated microglia produce inflammatory cytokines such as interleukin (IL)-1 β , which may promote vascular endothelial growth factor (VEGF) production from MCs, leading to TJ disruption. The iBRB model cell sheets were exposed to 1% oxygen for 6 h with or without mouse brain microglia (BV2) or IL-1 β . TJ structure and function were examined by zonula occludens (ZO)-1 immunostaining and fluorescein isothiocyanate permeability assay, respectively. Relative gene expression of IL-1 β in BV2 under normoxic and hypoxic conditions was examined by real-time reverse transcription-polymerase chain reaction. VEGF protein concentration in QMMuC-1 supernatants was measured by enzyme-linked immunosorbent assay. The bEnd.3 cell sheet incubated with BV2 in hypoxic condition or with IL-1 β in normoxic condition showed abnormal localization of ZO-1 and aberrated barrier function. Under normoxic condition, EC-MC iBRB model cell sheet showed lower permeability than bEnd.3 cell sheet. Under hypoxic conditions, the barrier function of EC-MC iBRB model cell sheet was more deteriorated compared to bEnd.3 cell sheet. Under hypoxic condition, incubation of EC-MC iBRB model cell sheet with BV2 cells or IL-1 β significantly increased barrier permeability, and hypoxia-treated BV2 cells expressed significantly higher levels of IL-1 β mRNA. Incubation of QMMuC-1 with IL-1 β increased VEGF production. These results suggest that under hypoxic condition, microglia are activated to release proinflammatory cytokines such as IL-1 β that promote VEGF production from MCs, leading to

disruption of iBRB function. Modulating microglia and MCs function may be a novel approach to treat hypoxia-induced retinal BRB dysfunction.

Keywords: Hypoxia; Blood retinal barrier; Tight-junction; Microglia; Müller cell; IL-18

1. Introduction

The retina is one of the most metabolically active tissues and consumes a large amount of energy (Wong-Riley, 2010). Insufficient oxygen supply to the retina leads to impaired cellular oxygen balance. In hypoxic tissue, vascular endothelial growth factor (VEGF) and pro-inflammatory cytokines are induced in a hypoxia inducible factor (HIF)-1-dependent manner (Jung et al., 2003; Ramakrishnan et al., 2014), causing disruption of the inner blood-retinal barrier (iBRB) and increasing vascular permeability (Inada et al., 2018; Kaur et al., 2006).

Hypoxia-induced VEGF is a key factor in many sight-threatening retinal diseases, including age-related macular degeneration (AMD) (Ferrara et al., 2007) and diabetic retinopathy (DR) (Duh et al., 2004). In neovascular AMD, increased VEGF expression leads to abnormal growth of choroidal vessels into the sub-retinal pigment epithelial (RPE) or subretinal space (Ambati et al., 2003). In proliferative DR, the neovascularization that arises from the inner retina is mediated by hypoxia-induced VEGF (Tilton et al., 1997). Anti-VEGF agents are now widely used for the treatment of ischemic retinal disorders. On the other hand, VEGF is an essential growth factor for retinal vasculature and neurons (Stalmans et al., 2002), and the use of anti-VEGF agents may interfere with endogenous survival signals, causing unexpected neural toxicity (Saint-Geniez et al., 2008). Furthermore, clinical applications of those agents require multiple injections, which may increase the risk of infections as well as social and economic burden (Mintz-Hittner et al., 2011). Therefore, identification of more effective and safe therapeutic strategies based on the underlying mechanism of hypoxia-induced iBRB disruption are inevitably advocated to overcome these problems.

The iBRB is primarily formed by the tight junction (TJ) of retinal vascular endothelial cells (ECs) and supported by pericytes, astrocytes, Müller cells (MCs), microglia, neurons, and extracellular matrix proteins (Daneman, 2012; Hawkins and Davis, 2005). Microvascular ECs control transport of substances between the blood and retinal tissue via TJ complexes (Wolburg and Lippoldt,

2002). At least three different types of transmembrane proteins; the claudin family, occludin and junctional adhesion molecules, are located at the TJ (Wolburg and Lippoldt, 2002). Integral TJ proteins are linked to each other as well as to the cytoskeleton by cytoplasmic adaptor proteins, such as zonula occludens (ZO), that provide structural stability for the TJ (Gonzalez-Mariscal et al., 2003).

The detailed mechanisms of hypoxia-induced TJ disruption have not been fully elucidated, although some reports related to this field have been published. These reports show that following exposure to hypoxia, the TJ of cultured ECs is destroyed accompanied by decreased ZO-1 and claudin-5 protein expression (Koto et al., 2007), leading to decline in transepithelial electrical resistance (TEER) and increase in permeability (Hu et al., 2017).

Microglia, the main resident immune cells of the central nervous system (CNS), are a key player of the innate immune response of the CNS, including the retina (Ransohoff and Brown, 2012). In hypoxic condition, the resting microglia with a ramified morphology are transformed into activated microglia (Kettenmann et al., 2011), and subsequently, produce proinflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , which provoke the increase in TJ permeability (Dudvarski Stankovic et al., 2016). Due to multiple effects on vessels and neurons, microglia are considered as a new therapeutic target for CNS diseases (Seki et al., 2013; Wes et al., 2016).

Müller cells (MCs), the most common type of glial cell found in the retina, possess a wide range of functions and are vital for the maintenance of TJ (Bai et al., 2009; Pierce et al., 1995; Reichenbach and Bringmann, 2013; Vecino et al., 2016). MCs are the major source of VEGF in the retina (Watkins et al., 2013), although VEGF can also be produced by other retinal cells such as retinal pigmented epithelial cells, pericytes, astrocytes, and ECs (Gupta et al., 2013). MC-derived VEGF production can be further enhanced by microglia (Nurnberg et al., 2018). We previously showed that microglia and MCs were activated, accompanied by upregulation of IL-1 β gene expression in a mouse model of hypoxia-induced retinal edema (Inada et al., 2018). In this study, using an *in vitro* model of iBRB, we show that active microglia produce inflammatory

cytokines such as IL-1 β , which promote VEGF production from MCs leading to TJ disruption under hypoxic conditions.

2. Materials and Methods

2.1. Single Cell cultures

The immortalized mouse brain microvascular EC line bEnd.3 was purchased from American Type Culture Collection (ATCC® CRL-2299™, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 450 mg/dl glucose, 10% fetal calf serum (FCS), at 37°C in a humidified atmosphere of 95% air and 5% CO₂, and the medium was changed every three days. The cells were detached using 0.25% trypsin/EDTA when they reached 80% confluence and passaged at 1:5.

The immortalized mouse microglial cell line BV2 was purchased from AcceGen Biotechnology (ABC-TC212S, New Jersey, USA). BV2 cells exhibit both the phenotypic and functional properties of reactive microglia and are a suitable inflammation model as described previously (Kacimi et al., 2011). Cells were grown in DMEM with 10% FCS at 37°C in a humidified atmosphere of 95% air and 5% CO₂, and cells were detached using 0.1% trypsin/EDTA twice a week and passaged at 1:10.

Queen's University Murine Müller glia Clone-1 (QMMuC-1), the immortalized mouse retina MCs, have morphologic features resembling primary MCs and remain stable (Augustine et al., 2018). Cells were grown in DMEM with 10% FCS at 37°C in a humidified atmosphere of 95% air and 5% CO₂, and the medium was changed every three days. The cells were detached using 0.25% trypsin/EDTA when they reached 80% confluence and passaged at 1:6.

2.2 *In vitro* iBRB model cell sheet

The *in vitro* EC-MC iBRB model cell sheets were produced by layering bEnd.3 on the inner side and the QMMuC-1 on the outer side of a Transwell membrane insert™ (Fig. 1, 0.4 μ m pore size, Corning Inc., NY, USA). In order to form a dual-layer iBRB, QMMuC-1 cells (5.0×10^4 cells/cm²) were seeded when

the insert was inverted (Fig. 1) and left for 2h to allow cell attachment. The insert with QMMuC-1 cells was then placed to a 24-well receiver plate, and bEnd.3 cells (1.0×10^5 cells/cm²) were seeded on the inner side of the insert. The QMMuC-1 and bEnd.3 cells can communicate with each other through the pores of the membrane. After 3-4 days of culture in DMEM with 10% FCS, both cells were fully confluent, and the EC-MC iBRB model cell sheets were ready for further experiments (Fig. 1).

2.3. Co-culture with BV2 cells

The bEnd.3 cell sheet or EC-MC iBRB model cell sheet cultured in Transwell membrane insert was transferred to a new 24-well receiver plate with BV2 cells grown to 80% confluency in each well. These co-cultures were incubated for 24 hs before subjecting to further assessments (Fig. 1).

2.4. Barrier function assessment

The bEnd.3 cell sheets and EC-MC iBRB model cell sheets were grown in Transwell membrane inserts, and barrier functions were measured by three methods as follows (n = 3 or more per culture condition).

Evans Blue (EB) (MW, 960.8, Sigma-Aldrich, MO, USA), a non-permeating dye that binds to albumin (Wolman et al., 1981), was used to assess barrier function over time. As EB is more resistant to fading than fluorescein-5-isothiocyanate (FITC) under ambient light, it is suitable for time-course experiments. Immediately after establishing bEnd.3 cell sheets in Transwell inserts, EB (0.67 mg/mL) was loaded to the upper compartment and the cell sheets were exposed to hypoxia for 0, 2 and 6 hs. Thereafter, 100 μ L of medium from the lower compartment was transferred to a 96-well microplate, and EB that passed through the bEnd.3 cell sheet was measured spectrophotometrically at 620 nm.

Sodium fluorescence assays were performed to assess changes of barrier function more precisely than EB assays due to its smaller molecular weight. Confluent bEnd.3 cell sheets and EC-MC iBRB cell sheets were incubated with sodium fluorescence (MW 376.27, Sigma-Aldrich) (100 μ g/mL) in DMEM without

FCS for 6 hs. Thereafter, as with EB assay, the fluorescence intensity of sodium fluorescence that passed through the cell sheet was measured at 492/520 nm (absorption/emission). All measurements were normalized to blank inserts.

TEER was measured using an epithelial volt-ohm meter (EVOM; LMS Co., Tokyo, Japan) every 24 hs until cells reach confluence and every 2 hs during hypoxia exposure. TEER value was calculated by subtracting the resistance of the blank insert from that of the sample at each time point.

2.5. Lactate dehydrogenase assay

For analysis of cytotoxicity of IL-1 β , the release of lactate dehydrogenase (LDH) into the medium was determined using Cytotoxicity Detection Kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. All measurements were normalized to control samples.

2.6. Hypoxia cell culture

Cells were exposed to hypoxia by incubating in a humidified, 37°C, 1% O₂ chamber (Coy Laboratories, MI, USA). Culture medium and all reagents were preconditioned by exposing to 1% O₂ before use.

2.7. Immunocytochemistry

When bEnd.3 cells reach confluence, the cell sheets were fixed with 1.5% (w/v) formaldehyde (10 min), and then permeabilized by 0.1% (v/v) Triton X-100 (10 min) and blocked by 1% (w/v) bovine serum albumin (30 min) at room temperature. The samples were incubated overnight at 4°C with the following primary antibodies: anti-HIF-1 α (1:100 dilution), anti-ZO-1 (1:250 dilution) and anti-claudin-5 (1:100 dilution) (all from Invitrogen, CA, USA). After washing thoroughly with phosphate-buffered saline (PBS), the samples were then stained with Alexa-Fluor 488- or 546-conjugated donkey anti-rabbit antibodies (Invitrogen) (1:200 dilution) and incubated at 37°C for 1 h. Samples were mounted using Vectashield medium containing DAPI (Vector Laboratories, CA, USA) and examined under a confocal laser microscope (Leica SP8, Wetzlar, Germany).

2.8. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

bEnd.3 cells were grown in 24-well receiver plate as target cells, and BV2 cells were cultured in Transwell inserts as stimulant cells. After the target cells reached confluence, the Transwell inserts with stimulant cells were transferred to the 24-well receiver plate with target cells and co-cultured for 24 hs before hypoxia exposure. Co-cultures of other combinations of cells were performed by the same method.

Total RNA of target cells was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and transcribed to cDNA using SuperScript II Reverse Transcriptase Kit (Thermo Fisher Scientific, Massachusetts, U.S.), according to manufacturers' instructions. The following validated TaqMan probes were purchased from Thermo Fisher Scientific: VEGF (Mm00437304), ZO-1 (Mm00493699_m1), claudin-5 (Mm00727012_s1), glial fibrillary acidic protein (GFAP) (Mm01253033), IL-1 β (Mm00434228), TNF α (Mm00443258_m1), and Rn18s (Mm03928990_g1). Rn18s was used as an endogenous control. Real-time RT-PCR was performed by the $\Delta\Delta$ CT method using the LightCycler 480 System (Roche, Basel, Switzerland), which provides normalized expression values. All reactions were performed in technical triplicates.

2.9. IL-1 β treatment

After reaching confluence, bEnd.3 cells were treated with IL-1 β (10 ng/ml) which was added both to the inserts and bottom chamber for 6 hs. Then, FITC assay, TEER, and immunocytochemical staining for ZO-1 were performed as described above. To calculate the ratio of ZO-1 positive area, 5 images of immunocytochemical staining for ZO-1 acquired by fluorescence microscope (5 magnifications) (Keyence BZ-8000, Osaka, Japan) in each group were analyzed by ImageJ software (National Institutes of Health, MD, USA).

2.10. Enzyme-linked immunosorbent assay (ELISA)

IL-1 β and VEGF protein concentrations in culture medium were measured using IL-1 β ELISA kit (ELISA MAX Deluxe Set, BioLegend, CA, USA) and VEGF

ELISA kit (DuoSet, R&D Systems, Minnesota, USA), respectively, according to manufacturers' instructions. For IL-1 β ELISA assay, confluent bEnd.3 cell sheets and EC-MC iBRB model cell sheets were transferred to a new 24-well receiver plate with or without BV2 and incubated for 24 hs. The cell sheets were then washed and replaced with fresh media before exposing to either normoxia or hypoxia for 6 hs. The supernatants were then collected for cytokine measurement. For VEGF ELISA assay, confluent QMMuC-1 cells with fresh culture medium were exposed to either normoxia or hypoxia for 6 hs with or without IL- β . The supernatants were then collected for VEGF measurement.

2.11. Statistical analysis.

Quantitative data were expressed as mean $\pm$ standard deviation (SD) of a minimum of three biological replicates. Variance homogeneity was evaluated by F tests. The difference between two groups was compared using the two-tailed Student's t-test. One-way ANOVA or two-way ANOVA followed by Tukey's post-hoc tests were used for multiple group comparisons. All statistical analysis was performed with JMP Pro 14 (SAS Institute Inc., NC, USA). A p-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of hypoxia on bEnd.3 cell sheet

To assess the effects of hypoxia on TJ structure and function, confluent bEnd.3 cell sheets were exposed to either normoxia or hypoxia for 2 or 6 h (Fig. 2). The cells were then subject to below investigations.

First, bEnd.3 cells were immunostained for HIF-1 α . Cells under normoxic condition exhibited no HIF-1 α immunoreactivity. Upon hypoxia exposure, cells showed intense HIF-1 α immunoreactivity within 2 h, which were maintained after 6 h (Fig. 2A).

Next, to investigate whether hypoxia alters the TJ complex, bEnd.3 cell sheets were immunostained for ZO-1 and claudin-5. Under normoxic condition, linear immunoreactivities of ZO-1 and claudin-5 were localized along the cell

boundary. Under hypoxic condition, however, the expression pattern changed over time, from the linear pattern at 2 h to a granular appearance (arrows) with some isolated granules (arrowheads) at 6 h (Fig. 2B).

To evaluate TJ function in hypoxic condition, permeability for EB was measured. The amount of EB passing through blank inserts increased over time in both normoxic and hypoxic conditions. However, the amount of EB passing through bEnd.3 cell sheet remained unchanged over time under both conditions (Fig. 2C).

TEER in bEnd.3 cell sheet was measured before and during hypoxia exposure and the data was expressed as a relative change compared to blank insert. TEER was not affected by hypoxia exposure at all time points (Fig. 2D).

3.2. Effects of hypoxia on bEnd.3 cell sheet co-cultured with BV2

To assess the effects of BV2 cells on bEnd.3 cell sheet under hypoxic condition, inserts containing bEnd.3 cell sheets were transferred to 24-well receiver plate with BV2 (co-culture group) or without BV2 (mono-culture group) cells and incubated for 24 h. Subsequently, the cultures were exposed to either normoxia or hypoxia for 6 h (Fig. 3).

The bEnd.3 cells were immunostained for ZO-1. In normoxic condition, TJ structure appeared normal in both mono- and co-cultures. In hypoxic condition, the ZO-1 immunoreactivity pattern changed from linear to granular appearance in both mono- and co-cultures. The changes were more marked in co-cultures than in mono-cultures; loss of boundary immunoreactivity (arrows) in patches was detected in co-cultures under hypoxic condition (Fig. 3A).

After exposure to either normoxia or hypoxia for 6 h, the permeability of bEnd.3 cell sheet with or without co-culturing with BV2 cells was measured using sodium fluorescence. In normoxic condition, permeability for sodium fluorescence was not significantly different between the mono- and co-culture groups. In hypoxic condition, however, the permeability increased significantly in the co-culture group compared to the mono-culture group in hypoxia (1.77 ± 0.19 times, $p < 0.01$) (Fig. 3B). The permeability increased significantly in hypoxic condition compared to normoxic condition in the mono-culture group

(1.22 ± 0.16 times, $p < 0.01$)

3.3. Gene expression in bEnd.3 and BV2 cells

Messenger RNA expression in bEnd.3 cells was evaluated after incubation with or without BV2 cells for 24 h. Relative gene expression of VEGF was significantly higher under hypoxic condition in both mono-culture (3.67 ± 0.38 times, $p < 0.05$) and co-culture groups (2.09 ± 0.26 times, $p < 0.05$). Although relative gene expressions of ZO-1 and claudin-5 did not change by hypoxia in the mono-culture group, both expressions were significantly reduced in the co-culture group (ZO-1: 0.42 ± 0.10 times, claudin-5: 0.41 ± 0.05 times, $p < 0.05$) under hypoxic condition (Fig. 4A).

Messenger RNA expression in BV2 cells was evaluated after incubating with bEnd.3 cells for 24 h. The expression of IL-1 β mRNA was significantly increased (1.87 ± 0.41 times, $p < 0.05$) in BV2 cells in hypoxic condition, while TNF α expression did not change (Fig. 4B).

3.4. Effects of IL-1 β on bEnd.3 cell sheet

To investigate whether TJ of bEnd.3 cell sheet is altered by IL-1 β , the cell sheets were exposed to increasing concentrations of IL-1 β for 6 h in normoxic condition. FITC assay showed a maximum increase in permeability in bEnd.3 cell sheet at IL-1 β concentration of 10 ng/ml (1.13 ± 0.01 times, $p < 0.05$) (Fig. 5A). The data obtained from LDH assay indicated that IL-1 β had no significant cytotoxicity on bEnd.3 cells for the concentration range of 1 to 100 ng/ml (Fig. 5B). Measurement of TEER indicated that IL-1 β (10 ng/ml) significantly suppressed the barrier function of bEnd.3 cell sheet at all time points up to 6 h (Fig. 5C). After incubation with or without IL-1 β (10 ng/ml) for 6 hs, bEnd.3 cells were immunostained for ZO-1 and claudin-5. Linear ZO-1 and claudin-5 immunoreactivity was localized along cell boundary in control cells but changed to granular appearance with loss of immunoreactivity in some areas after incubation with IL-1 β (Fig. 5D, arrowheads).

3.5. Effect of hypoxia on iBRB model cell sheet

To better mimic the iBRB, the QMMuC-1 and bEnd.3 cells co-culture sheet was developed (Fig. 1). The iBRB model cell sheets were exposed to either normoxia or hypoxia for 6 h and then subjected to permeability or TEER assay (Fig. 6).

The permeability of QMMuC-1 cell sheets was the same as blank inserts in both normoxic and hypoxic conditions, indicating that QMMuC-1 cell sheet had no barrier function. In normoxic condition, the relative permeability of iBRB model cell sheet (0.82 ± 0.00 times, $p < 0.05$) was significantly lower than that of bEnd.3 cell sheet (0.86 ± 0.01 times, $p < 0.05$). On the other hand, in hypoxic condition, the relative permeability of iBRB model cell sheet (0.87 ± 0.01 times, $p < 0.05$) was significantly higher than that of bEnd.3 cell sheet (0.84 ± 0.00 times, $p < 0.05$) (Fig. 6A).

TEER was measured before and during hypoxia exposure and the data was expressed as a relative change compared to blank inserts. In normoxic condition, TEER of iBRB model cell sheet was significantly higher than that of bEnd.3 cell sheet, consistently over 6 h. On the contrary, in hypoxic condition, TEER of iBRB model cell sheet decreased significantly over time, while that of bEnd.3 cell sheet remained unchanged (Fig. 6B).

Taken together, our results suggest that the QMMuC-1 and bEnd.3 cells co-culture sheet formed strong barriers than bEnd.3 cell alone, and the barriers have a good response to hypoxia. The QMMuC-1 and bEnd.3 cells co-culture sheet, therefore, is a model suitable to study the mechanism of iBRB leakage related to retinal diseases.

3.6. Effects of BV2 and IL-1 β on iBRB model cell sheet

To investigate the causal role of microglial activation and its related cytokines in iBRB leakage, the iBRB model cell sheets were incubated with BV2 cells or IL-1 β in either normoxic or hypoxic condition for 6 h.

In normoxic condition, the permeability of iBRB model cell sheet did not change by co-culturing with BV2 cells but increased significantly by the addition of IL-1 β (Fig. 7A). In hypoxic condition, the permeability of iBRB model cell sheet increased significantly by co-culturing with BV2 cells or by addition of

IL-1 β (Fig. 7A). Further analysis showed that the supernatants of the iBRB model cell sheet co-cultured with BV2 cells in hypoxic condition contained a significantly higher level of IL-1 β than other groups (Fig. 7B). Our results suggest that BV2 cells may enhance iBRB permeability through the release of IL-1 β in hypoxic conditions.

3.7. Activation of QMMuC-1 by BV2 and IL-1 β

To understand the role of Müller cells in BV2-induced iBRB leakage in hypoxic condition in our model, the mRNA expression of GFAP was measured as an indicator for MC activation. The expression of GFAP mRNA in QMMuC-1 cells was significantly downregulated (0.08 ± 0.06 times, $p < 0.05$) by hypoxia treatment when cultured alone, but was significantly upregulated (9.35 ± 2.96 times, $p < 0.05$) when co-cultured with BV2 under hypoxic condition (Fig. 8A).

VEGF protein concentration in the culture medium of QMMuC-1 cells incubated with or without IL-1 β was measured by ELISA. The VEGF concentration increased significantly when incubated with IL-1 β under both normal and hypoxic conditions (Fig. 8B).

4. Discussion

In this study, we developed an iBRB model system composed of ECs (bEnd.3) and MCs (QMMuC-1) on a permeable membrane in the Transwell system. Using this iBRB model system, we show that microglia can induce iBRB leakage in hypoxic condition via the release of inflammatory cytokine IL-1 β , which can not only directly affect TJ permeability, but also indirectly by activating MC and enhanced VEGF production. Our results suggest that microglia and MCs are critically involved in hypoxia-induced iBRB disruption.

Müller cells are an important component of the retinal neurovascular unit and provide critical supports to endothelial cells. Retinal vasculatures are ensheathed by the foot processes of MCs (Bringmann et al., 2006). In physiological conditions, MCs enhance the barrier function of ECs (Tout et al., 1993) by secreting factors such as pigment epithelium-derived factor, thrombospondin-1, and glial cell line-derived neurotrophic factor (Abukawa et

al., 2009; Eichler et al., 2004a; Eichler et al., 2004b; Igarashi et al., 2000). In our EC-MC Transwell co-culturing system, the MC on the outer side of the membrane can communicate with EC in the inner side of the membrane through the 0.4 μm pores leading to a better barrier function. Comparing to the EC (bEnd.3) monolayer cultured in the Transwell membrane, the EC-MC iBRB model cell sheets had higher TEER, lower barrier permeability and were more sensitive to inflammatory stimuli under hypoxic conditions. Interestingly, the barrier function was not affected by the addition of BV2 microglia under normoxic conditions (the permeability was neither enhanced nor reduced, Fig. 7A), suggesting that microglia are unlikely to play a role in iBRB integrity in physiological conditions. This EC-MC Transwell cell sheet, therefore, models closely the *in vivo* iBRB and is an ideal tool to investigate factors affecting iBRB stability.

Retinal ischemia is a major cause of macular edema although the underlying mechanism remains to be fully elucidated. Whether the BRB is damaged directly by ischemia related hypoxia or indirectly through inflammation or its related inflammatory and angiogenic growth factor production is still on debate. Using bEnd.3 monolayer sheets, we observed a mild change of TJ protein localization (Fig. 2B), but no significant changes in barrier function (Fig. 2C, D) and TJ gene expression (Fig. 4A) 6 h after 1% oxygen treatment. A previous study by Koto et al., 2007 showed that 24 h hypoxia treatment reduced barrier function in bEnd.3 cells but did not affect claudin-5 mRNA expression (Koto et al., 2007). However, Ma et al., 2013 reported downregulation of ZO-1 and claudin-5 gene expression as well as reduced barrier function in bEnd.3 cells after 24 h 1% oxygen treatment (Ma et al., 2013) and the results was confirmed by Hu et al., 2017 in mouse retinal microvascular EC (Hu et al., 2017). Hao et al., 2016 also reported a reduction of TJ protein expression and TEER in hypoxia (2% oxygen, 24 h) treated human cerebral microvascular ECs (Hao et al., 2016). On the other hand, Mark and Davis, 2002 observed no changes in TEER and TJ (claudin-1, occludin, ZO-1 and ZO-2) protein expression (by Western blot) but significant disruptions in the localization of occludin and ZO-2 in bovine brain microvascular ECs after 24 h 1% oxygen treatment (Mark and Davis, 2002). The inconsistent and

sometimes controversial observations in the literature and our study may be related by the differences in the type and origin of cells and the culture methods used in different studies, which also highlights the needs for a better and stable model of iBRB for mechanistic investigations. The short (6 h) hypoxia duration in our study may also explain the discrepancy particularly the barrier permeability, between our study and those of others in bEnd.3 cells.

Using our EC-MC iBRB cell sheet model, we found that hypoxia (1% oxygen) significantly increased the permeability and time-dependently decreased TEER. The reduction in TEER reached statistical significance as early as 2 h (Fig. 6B). Our results suggest that hypoxia can damage the iBRB integrity quickly in the presence of Müller cells. The role of MC on iBRB integrity in retinal disease (e.g., DR) has been well-documented in many studies (Abbott et al., 2010; Cardoso et al., 2010). In response to hypoxia, inflammation, or glucose deprivation, MCs produce VEGF and increase vascular permeability (Pierce et al., 1995; Reichenbach and Bringmann, 2013). It is also reported that astrocyte-derived VEGF was increased by IL-1 β in CNS inflammatory diseases (Argaw et al., 2012) and SSeCKS is involved in the endothelial hyperpermeability induced by IL-18 in inflammatory process (You et al., 2010). The cause of abnormal VEGF production in Müller cells remains elusive. Our EC-MC iBRB cell sheet model offers a unique opportunity to investigate the underlying mechanism of MC-induced iBRB damage. Apart from hypoxia, additional factors can be applied to the bottom chamber or inside the Transwell, mimicking changes in retinal extravascular microenvironment or circulation. For example, we show that VEGF production by MC is increased under hypoxic condition or upon IL-18 treatment (Fig. 8B) and MC-derived VEGF can impair iBRB barrier function in our EC-MC iBRB cell sheet model (Fig. 7A). The results highlight the importance of extravascular or retina-derived IL-18 or VEGF on iBRB leakage in disease conditions.

GFAP has been widely used as a marker of Müller cell activation and increased GFAP expression has been observed in various models of retinal ischemia, such as oxygen-induced retinopathy (Liu et al., 2020) and retinal detachment (Augustine et al., 2019). In this study, we found that 6 h hypoxia

treatment significantly decreased GFAP mRNA expression in QMMuC-1 Müller cells (although VEGF production was increased) and this was observed in two independent experiments that we repeated. The results suggest that GFAP expression in hypoxic retina may be regulated by both inflammatory mediators from other retinal cells (e.g., endothelial cells, microglia etc.) and the tissue levels of oxygen. Although GFAP has been widely used as a marker of Müller cell activation, the expression of GFAP, VEGF and inflammatory mediators (e.g., CCL2) in Müller cells do not always match. The effect of hypoxia on Müller cell GFAP expression in vitro warrant further investigation.

Another novel observation of this study is the indirect role of microglia in iBRB damage in hypoxic conditions. Although microglia did not affect iBRB under normoxic conditions, they significantly increased iBRB permeability under hypoxic condition. We further found that the supernatants from the EC-MC iBRB cell sheet + microglia (BV2) group contained three times higher levels of IL-1 β than those in other groups (Fig. 7B) and IL-1 β significantly enhanced hypoxia-induced VEGF production in MC (Fig. 8B). Since the IL-1 β productions in EC-MC iBRB cultured under normoxic and hypoxic conditions were comparable, the higher level of IL-1 β in the EC-MC iBRB + BV2 in hypoxia condition is likely to originate from BV2 cells. This is further supported by the higher levels of IL-1 β mRNA expression in hypoxia treated BV2 cells (Fig. 4B). Our results suggest that microglia can indirectly impair iBRB integrity by releasing IL-1 β under hypoxia conditions. Hypoxia-induced IL-1 β production in microglial cells has been reported by others (Heng et al., 2019; Kaur et al., 2013; Martiney et al., 1990). It is possible, that when the retina suffers from ischemia, such as in DR, microglia are activated and produce higher levels of IL-1 β , which can directly damage TJ of EC and impair barrier function (Fig. 5). The microglia-derived IL-1 β can also active Müller cells leading to higher levels of VEGF production and iBRB impairment. Higher levels of IL-1 β and TNF α mRNA expression have been detected in hypoxic retina (Inada et al., 2018; Sivakumar et al., 2011). The increased IL-1 β expression in hypoxic retina may attribute to active microglia. Interestingly, hypoxia did not upregulate TNF α expression in BV2 cells in our study. The source of TNF α in hypoxic retina remains to be

elucidated. The effect of exogenous IL-1 β on iBRB leakage was significantly weaker than activated microglia (Figure 7B), indicating that, in addition to IL-1 β , microglia may produce other cytokines and chemokines that can also affect the integrity of iBRB in hypoxic condition.

Although our EC-MC iBRB cell sheet model offers a unique opportunity to dissect the role of Müller cells in iBRB integrity, the results should be interpreted with caution, as the three types of cells used in this study were all immortalized cell lines. Further validations using primary cells or in vivo models will be necessary. It should also be noted that the model does not fully recapitulate the neurovascular unit in vivo. Apart from ECs, MCs and microglia, the iBRB is also supported by pericytes, astrocytes, and neurons in the neurovascular unit. A more sophisticated model will be needed to investigate the role of other cellular components in iBRB damage in disease conditions.

Nevertheless, our findings suggest that although microglia may not be critically involved in iBRB integrity under normal physiological conditions, in the ischemic retina, microglia are activated to release inflammatory cytokines such as IL-1 β that can promote VEGF secretion from MCs, leading to the disruption of iBRB. Targeting microglia and/or Müller cell activation may be an effective approach to manage hypoxia-induced retinal disorders.

Declaration of competing interest

All authors have no conflict of interest to disclose.

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Figure Legends

Fig. 1. Schematic diagrams of *in vitro* inner blood-retinal barrier (iBRB) model cell sheets with or without BV2 cells (mouse brain microglia). The *in vitro* EC-MC iBRB model cell sheets are produced by layering bEnd.3 cells (mouse brain microvascular endothelial cells) and QMMuC-1 (murine Müller glia clone-1) cells separated on each side of the Transwell membrane with 0.4- μ m pore size. QMMuC-1 cells are seeded on the outside of the membrane when the Transwell insert is inverted. Two hours later when the QMMuC-1 cells adhere to the membrane, the insert with QMMuC-1 cells is placed in well of a 24-well plate, and bEnd.3 are seeded on the inner side of the membrane. The culture system allows QMMuC-1 and bEnd.3 cells to communicate through the pores of the membrane. After 3-4 days, the EC-MC iBRB model cell sheets are subjected to further analysis including hypoxia, treatment with IL-1 β or BV2 cells. For co-culturing with BV2 cells, the EC-MC iBRB model cell sheets were transferred to a new 24-well receiver plate containing BV2 cells grown to 80% confluency in each well. This assembly is incubated for 24 h before being used in each experimental protocol.

Fig. 2. The effect of hypoxia (1% oxygen) on the structure and functions of bEnd.3 cell sheet. (A & B) The bEnd.3 cells were cultured in 24-well plate. After reaching confluence, cells were exposed to either normoxia or hypoxia for 2 or 6 h. (A) Immunocytochemistry for HIF-1 α (red). (B) Immunocytochemistry for ZO-1 (red) and claudin-5 (red). Arrows - granular aggradations of TJ proteins in the junction area; Arrowheads - isolated granular TJ protein inside the EC. (C & D) The bEnd.3 cells were cultured in the Transwell membrane insert in a 24-well plate. After confluency (day 6), cells were exposed to either normoxia or hypoxia for 2 or 6 h. (C) Barrier function assessed by Evans Blue (EB). (D) Barrier function quantified by TEER. $n \geq 3$. The experiments were conducted 2 or 3 times.

Fig. 3. The effect of BV2 cells in ZO-1 expression and barrier permeability of

bEnd.3 cell sheets. The bEnd.3 cells were grown in 24-well receiver plate, and BV2 cells were cultured in Transwell inserts. After the bEnd.3 cells reached confluence, the Transwell inserts with or without BV2 cells were transferred to the 24-well receiver plate with bEnd.3 cells and incubated for 24 h. After that, they were exposed to either normoxia or hypoxia for 6 h. The bEnd.3 cells were then subject to immunocytochemistry for ZO-1 (A) or (B) Barrier permeability measured by FITC assay. Arrows - patchy loss of boundary immunoreactivity. Mean $\pm$ SD, $n = 3$, $\dagger$: $p < 0.01$ (Student t test). The experiments were conducted twice.

Fig. 4. The effect of hypoxia on VEGF, tight junction and inflammatory cytokine gene expressions in bEnd.3 and BV2 cells. The target cells (bEnd.3 cells in (A), BV2 cells in (B)) were grown in 24-well receiver plate, and stimulant cells (BV2 cells in (A), bEnd.3 cells in (B)) were cultured in Transwell inserts. After the target cells reached confluence, the Transwell inserts with stimulant cells were transferred to the 24-well receiver plate with target cells and incubated for 24 h. After that, they were exposed to either normoxia or hypoxia for 6 h. Total RNA was extracted from target cells and qPCR were conducted. (A) Gene expression of VEGF, ZO-1, and claudin-5 in bEnd.3 mono-culture and bEnd.3 co-cultured with BV2. (B) IL-1 β and TNF α gene expressions in BV2. Mean $\pm$ SD, $n = 3$, *: $p < 0.05$ (Student t-test). The experiments were conducted 3 times.

Fig. 5. The effect of IL-1 β on bEnd.3 cell sheet. (A and C) the bEnd.3 cells were cultured in the Transwell insert in a 24-well plate. After confluency, bEnd.3 cells were treated with IL-1 β which was added both upper and lower compartment for 6 h. The cell barrier function, viability and TJ were then analyzed. (A) Barrier function assessed by FITC permeability assay (A). Mean $\pm$ SD, $n = 4$ *: $p < 0.05$ (one-way ANOVA with Tukey's post-hoc tests). The experiments were conducted once. (B) LDH assay to quantify cell viability. (C) Barrier function quantified by TEER. Mean $\pm$ SD, $n = 3$, *: $p < 0.05$ (Student t-test). The experiments were conducted twice. (D) Immunocytochemistry for ZO-1 and claudin-5 (green) in bEnd.3 in control and IL-1 β (10 ng/ml) treated

groups. Arrowheads - loss of immunoreactivity. Arrows - isolated granular TJ protein inside the EC. (E) Ratio of ZO-1 positive area. Mean $\pm$ SD, n = 5.

Fig. 6. The effect of hypoxia on the barrier function in different models. The bEnd.3 cells, the QMMuC-1 cells, and iBRB cell sheet were cultured in the Transwell membrane inserts and exposed to either normoxia or hypoxia for 6 h. (A) Barrier function assessed by FITC permeability assay. Mean $\pm$ SD, n = 4, *: p < 0.05 (one-way ANOVA with Tukey's post-hoc tests). The experiments were conducted 3 times. (B) Barrier function quantified by TEER. Mean $\pm$ SD, n = 3, *: p < 0.05 (t-test) (bEnd.3 vs iBRB), †: p < 0.05 (t-test) (normoxia vs hypoxia). The experiments were conducted twice.

Fig. 7. The effect of BV2 and IL-1 β on EC-MC inner brain retinal barrier (iBRB) model cell sheet. The EC-MC iBRB cell sheets with/without BV2 cells or with/without IL-1 β were exposed to either normoxia or hypoxia for 6 h. (A) Barrier function assessed by FITC permeability assay. Mean $\pm$ SD, n = 4, *: p < 0.05 (one-way ANOVA with Tukey's post-hoc tests). The experiments were conducted twice. (B) IL-1 β protein concentration in culture medium measured by ELISA. Mean $\pm$ SD, n = 3, †: p < 0.01 vs. all other groups (Two-way ANOVA with Tukey's post-hoc tests). The experiments were conducted once.

Fig. 8. The effect of hypoxia on QMMuC-1 cells with or without BV2 or IL-1 β . The QMMuC-1 cells grown in Transwell inserts cultured with or without BV2 cells in the bottom chamber of 24-well plates were exposed to either normoxia or hypoxia for 6 h. The mRNA was extracted from QMMuC-1 cells and qPCR was conducted for GFAP expression (A). Mean $\pm$ SD, n = 3, *: p < 0.05 (Student t-test). The experiments were conducted twice. (B) The QMMuC-1 cells cultured in 24-well plate with or without IL-1 β treatment were exposed to either normoxia or hypoxia for 6 h. Supernatants were collected and VEGF production was measured by ELISA. Mean $\pm$ SD, n = 4, *: p < 0.05 (Two-way ANOVA with Tukey's post-hoc tests). The experiments were conducted once.

A

B

A

B

A

B

Highlights

- Barrier function of endothelial cells is enhanced by co-culturing with Müller cells
- Endothelial-Müller cell co-culture is a unique model of the inner blood retina barrier (iBRB)
- Microglia do not affect iBRB integrity in normal culture condition but impair the barrier under hypoxic condition
- Microglia release excessive amount of IL-1 β under hypoxic condition
- IL-1 β induces Müller cell VEGF production and damages the iBRB