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You build me up, you break me down

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1

INTRODUCTION AND
SCOPE OF THE THESIS

INTRODUCTION

Diabetes mellitus is a global epidemic, and it is predicted that the prevalence of diabetic patients increases from 382 million in 2013 to an estimated 592 million by 2035[1]. Diabetic patients suffer from many complications, including macrovascular pathology such as ischemic heart disease and stroke, and microvascular pathology such as neuropathy, nephropathy, and diabetic retinopathy (DR). Over one-third of diabetic persons has some form of DR, and approximately 5-10% develop vision-threatening complications such as proliferative DR and macular edema[2].

The earliest clinical changes in the diabetic retina occur in the microvasculature and, as such, DR has traditionally been considered to be a vascular disease. Pathological clinical hallmarks of DR include intraretinal hemorrhages, pericyte loss, microaneurysms, lipid exudates, thickening of the lamina basalis, capillary nonperfusion, macular edema and, in the case of proliferative DR, retinal neovascularization and vitreous hemorrhage[3]. Proliferative DR prevails in patients with type 1 diabetes, but diabetic macular edema (DME) is the primary cause of vision loss in patients with type 2 diabetes. Given the high prevalence of type 2 diabetes, DME is the leading cause of vision loss in the working-age population[4].

The blood-retinal barrier (BRB)

The retina is part of the central nervous system and is metabolically highly active due to the presence of the photoreceptors – in fact, it has a higher oxygen consumption per unit tissue weight than any other human tissue[5]. To ensure sufficient blood supply to provide oxygen and nutrients to the retina in combination with its functions in vision, it has two distinct vascular beds, *i.e.*, behind the retina the choriocapillaris to feed the retinal pigment epithelium and photoreceptors in the outer retina, and the inner retinal vasculature, emerging from the central retinal artery[6]. The choriocapillaris is a single layer of densely arranged capillaries and consists of fenestrated (leaky) endothelium, facilitating rapid supply of nutrients to the outer retina. In contrast, the retinal circulation that supplies the inner retina is formed by 3 interconnected vascular plexi that have a continuous barrier-type endothelium. This barrier-type endothelium of the retina is analogous to the endothelium that forms the blood-brain barrier. It has no fenestrations and is characterized by a high number of tight and adherens junctions that limit paracellular transport between the endothelial cells, and a paucity of intracellular pinocytotic vesicles to keep transcytosis across the endothelium to a minimum[7]. Together with perivascular cell types like pericytes and glial cells, the endothelial cells of the retina form the neurovascular unit and provide the inner blood-retinal barrier (BRB). This BRB ensures maintenance of homeostasis of the neural retina and protects the retina against potentially harmful substances that are present in the circulation. Although disruption of the BRB is an essential step in the development of retinal disease such as DME, its mechanisms are poorly understood. It is known that focal and diffuse BRB disruption, associated with retinal ischemia caused by capillary non-perfusion[8], leads to vascular leakage and the development of DME. To date, there are

2 mechanistic explanations at the molecular level for BRB disruption, namely increased paracellular leakage, caused by impaired functioning of tight junctions, or increased transcellular leakage, caused by increased vesicle-mediated transcytosis. Extravasation of plasma proteins such as albumin is crucial in the development of DME[9], whereas proteins of this size are unlikely to cross the endothelium via paracellular transport[7]. Therefore, increased transcytosis may well be the most important mechanism of the development of DME.

Transcytosis and the role of plasmalemma vesicle-associated protein (PLVAP)

Plasmalemma vesicle-associated protein (PLVAP, also known as PV-1 or FELS) is associated with endothelial transcellular transport. It is an endothelial cell-specific protein that is absent in mature barrier endothelium of the BRB, the blood-brain barrier and the testis[10, 11]. In pathological conditions, such as DR[12] and glioblastoma[13-15], PLVAP is upregulated and is associated with loss of barrier function. PLVAP is a structural component of fenestral and stomatal diaphragms of fenestrae, caveolae and trans-endothelial channels[16]. In non-barrier endothelium, it is constitutively expressed in capillaries, venules and small- and medium-sized veins[17] and prevents excessive protein leakage into tissues[18, 19], a function that is apparently not needed in a properly functioning blood-brain barrier or BRB. Vascular endothelial growth factor (VEGF) is a major inducer of BRB breakdown[20, 21] and also a driver of PLVAP expression[20, 22]. However, the exact function of PLVAP in vascular permeability and angiogenesis, specifically in the context of barrier-forming endothelium, is still unclear.

Inflammation as a cause of BRB disruption?

Hypoxia-induced VEGF is one of the main drivers of BRB disruption, but a percentage of patients does not respond sufficiently to anti-VEGF therapies[23]. It has been suggested that, in addition to VEGF, proinflammatory cytokines such as tumor necrosis factor (TNFα) and interleukins (IL1β, IL6, IL8) are also involved in BRB disruption[24, 25], because elevated levels of these cytokines have been detected in the vitreous of patients with DR[26-29]. Moreover, VEGF, TNFα and IL1β affect BRB function in *in vitro* models of the BRB[30-32] and in (diabetic) animal models[30, 33, 34] by reducing expression of tight and adherens junctions. In diabetic animal models, leukostasis in BRB capillaries has been associated with the development of sequelae characteristic of preclinical DR[25, 35]. Add the successful use of glucocorticoid therapy in the treatment of DME into this equation, and the foundation for the assumption that human DR and DME are caused by low-grade chronic inflammation is laid.

On the other hand, small clinical trials of anti-TNFα antibodies or soluble TNFα receptors in patients with ocular diseases such as DME have had limited success to date[24, 36-39]. Moreover, leukostasis as a causative factor in the development of human DR remains speculative, since *in vivo* assessment of leukostasis in the human retina is not yet possible. Lastly, whereas the anti-inflammatory effects of glucocorticoids are well-known and suggested to be responsible for resolving DME, the beneficial effects on retinal swelling are fast (sometimes within 24 h). This timeframe advocates for direct effects on

the endothelium, rather than for counteracting inflammatory events in the retina, which usually involves transcriptional and translational regulation. Taken together, the relevance and contribution of inflammation as an inducer of BRB disruption and subsequent DME remains controversial.

SCOPE OF THE THESIS

The aim of this thesis is to:
- describe the formation of the BRB during early development at cellular and molecular levels, because understanding of physiological BRB formation may give us insights in the mechanisms of pathological BRB disruption,
- elucidate the role of PLVAP in BRB formation and disruption, and
- critically evaluate the contribution and possible mechanisms of inflammatory conditions in the development of DME and DR.

In part I of the thesis, a detailed overview of the formation of the BRB and the role of PLVAP in barrier endothelium is given. The retinal vasculature develops postnatally in mice, and thus neonatal mice are an excellent animal model to study BRB development. In **chapter 2**, the temporal and spatial recruitment of the neurovascular unit in the neonatal mouse retina is described, using retinal wholemounts of mice from postnatal day (P) 3 to P25. We apply fluorescence immunohistochemistry to stain the retinal vasculature, pericytes and astrocytes and assess expression of different markers of polarized astrocytic end-feet and pericytes. In **chapter 3**, BRB formation in neonatal mouse retinas is studied at the molecular level, with a specific focus on PLVAP and proteins involved in paracellular transport, transcellular transport and VEGF signaling. Transgenic heterozygous *Plvap* mice have been used as an animal model to determine the role of PLVAP in formation of the BRB and retinal vasculature. **Chapter 4** describes the role of PLVAP in VEGF-induced and hypoxia-induced retinal permeability by knocking down PLVAP expression in an *in vitro* BRB model and in the mouse oxygen-induced retinopathy model.

In part II of this thesis, the contribution of inflammation to BRB disruption is studied. **Chapter 5** is a critical review of the current literature on the involvement of leukostasis in the development of human DR. In **chapter 6**, the role of TNFα in the induction of endothelial permeability and its mechanism is investigated in an *in vitro* BRB model. In **chapter 7**, the effects of glucocorticoids that are used in the clinic and mechanisms of action of glucocorticoid-induced barrier enhancement are studied in an *in vitro* BRB model.

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