You build me up, you break me down

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GENERAL DISCUSSION AND CONCLUSIONS

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The blood-retinal barrier (BRB) is formed by the retinal vascular endothelium and is regulated by the neurovascular unit, which is a cellular complex of endothelial cells, glial cells and pericytes. It protects the neural retina against disturbances of homeostasis, and from potentially harmful substances in the circulation. Disruption of the BRB results in vascular leakage and as a consequence edema formation and retinal damage. In this thesis, molecular and cellular mechanisms of BRB disruption are investigated in the context of DME and DR.

The first part of this thesis is focused on the development of the BRB and the role of PLVAP in BRB development and disruption. After a general introduction in **chapter 1**, the temporal and spatial recruitment of the neurovascular unit in the neonatal mouse retina is described in detail in **chapter 2**. It was observed that astrocytes preceded the vascular sprouting front, and that pericytes and tip cells invaded the retina together as vascular sprouting front, suggesting that in the retina, recruitment of perivascular cell types is a prerequisite for retinal vascularization and BRB formation.

Chapter 3 is a comprehensive study of the formation of the BRB in neonatal mice, showing that expression of tight and adherens junctions in the retina is temporally regulated, and that immature (and still leaky) vessels already have tight junctions. In addition, PLVAP expression decreased during BRB development, and the absence of PLVAP is a prerequisite for a functional BRB. With the use of heterozygous *Plvap* mice, it was shown that reduced PLVAP levels affect expression of proteins involved in both paracellular and transcellular transport, but without functional consequences for the BRB. Moreover, VEGF signaling was disturbed and these mice had a delay in retinal vascularization during early development, indicating a role for PLVAP in early developmental angiogenesis.

Chapter 4 describes the role of PLVAP in BRB permeability, both *in vitro* and *in vivo*. In an *in vitro* BRB model, knockdown of PLVAP resulted in significantly diminished VEGF-induced permeability for a 70 kDa molecular tracer, in both monocultures of BRECs and triple co-cultures of BRECs, pericytes and astrocytes. In human retinal explants, PLVAP inhibition prevented caveolae formation induced by VEGF stimulation, a phenomenon which may be involved in the reduction of endothelial permeability by PLVAP inhibition. Knockdown of PLVAP reduced hypoxia-induced retinal permeability in the mouse oxygen-induced retinopathy model, showing that PLVAP is involved in both VEGF-mediated and hypoxia-mediated BRB disruption.

The second part of this thesis consists of a literature review and *in vitro* studies of the BRB to establish the role of inflammation in the pathogenesis of DR or DME (**chapter 5 and 6**), and the effects of glucocorticoids on the BRB (**chapter 7**). In **chapter 5**, the prevailing paradigm that leukostasis and subsequent low-grade inflammation are central causes of early DR is questioned because of conflicting evidence in the literature on the causal role of leukostasis in DR.

In **chapter 6**, the controversy on the role of pro-inflammatory cytokines in BRB disruption in the context of DME was studied *in vitro*. TNFα induced permeability for small molecular sodium fluorescein, but not for larger tracers, whereas TNFα in combination

with IL1β and VEGF induced permeability for the larger molecules 70 kDa FITC-dextran and 66 kDa albumin-FITC. Both permeability effects were mediated by a second messenger molecule, cyclic AMP. However, the downstream pathway activated by cyclic AMP appeared to be different in barrier endothelium in comparison to non-barrier endothelium. This study shows the relevance of TNFα in BRB disruption and DME as part of its complex pathogenesis.

The effects of glucocorticoids on retinal endothelial cells are described in **chapter 7**. Glucocorticoids directly affected barrier properties of BRECs. Hydrocortisone, dexamethasone and triamcinolone acetonide induced improved tight junction expression and/or localization. Triamcinolone acetonide, but not hydrocortisone and dexamethasone reduced endothelial permeability for small (766 Da) and larger (70 kDa-FITC and 66 kDa albumin-FITC) molecular tracers. The 3 glucocorticoids also increased expression of caveolin-1, and triamcinolone acetonide prevented VEGF-induced upregulation of *Plvap* expression. We identified triamcinolone acetonide as the most potent glucocorticoid in retinal endothelium, and show that most, but not all, effects of triamcinolone acetonide are mediated via the glucocorticoid receptor in BRECs.

CONCLUDING REMARKS

The studies described in this thesis unraveled cellular and molecular mechanisms of BRB formation and BRB disruption. We have shed light on the function of PLVAP in barrier endothelium and thereby raised new hypotheses for the unique role of PLVAP in the central nervous system (CNS), *i.e.*, that the absence of PLVAP is imperative for a functional BRB and BBB, and that acquisition of PLVAP is necessary for loss of the CNS barriers. Therefore, lack of PLVAP may provide continuous endothelium with a more barrier-like endothelium status, whereas outside the CNS loss of PLVAP in fenestrated endothelium causes excessive extravasation of serum proteins from the circulation.

In the light of a possible contribution of inflammation in the development of DME and DR, it is concluded here that 1) retinal leukostasis is more likely an epiphenomenon of the diabetic retinal milieu than a crucial and specific step in human DR development, 2) the interplay of 3 cytokines (VEGF, TNFα and IL1β) induces increased BRB permeability for larger tracers but, *e.g.*, TNFα alone is not potent enough to induce such changes, and 3) the mechanism of action of glucocorticoids on BRB function encompasses more than anti-inflammatory effects. On the basis of these conclusions, it can be stated that DR is primarily not an inflammatory disease, although para-inflammation may enhance the progression and severity of DR and DME. Ultimately, all roads seem to lead to VEGF, as hypoxia-induced VEGF is the main driver of PLVAP expression – which is key in BRB permeability as observed in DR, and VEGF is needed *in vitro* to induce permeability changes for large tracers. Finally, the glucocorticoid triamcinolone acetonide directly enhances barrier properties of BRECs but may also prevent VEGF-induced upregulation of *Plvap* expression, which is highly likely to be important in the successful resolution of DME.