Endothelial dysfunction in experimental models of preclinical diabetic retinopathy

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Chapter 8
General discussion
General discussion and summary

Diabetic retinopathy (DR) is a leading cause of blindness in persons of working age in western countries. The majority of patients with diabetes develop some form of DR, with the incidence increasing with duration of diabetes and dependence on insulin. With the ever increasing incidence of type 2 diabetes and the average age of onset steadily decreasing, DR presents a growing dilemma for diabetic patients and ophthalmologists alike. The continued lack of an effective therapy for DR only adds to this problem.

The earliest manifestations of DR occur long before it can be clinically diagnosed. This earliest stage of DR, preclinical diabetic retinopathy (PCDR), is characterized by pericyte loss, endothelial cell (EC) proliferation, thickening of the vascular lamina basalis (LB), diffuse breakdown of the blood-retinal barrier (BRB), formation of acellular capillaries and loss of neural cells. After years of diabetes, clinically-observable retinal pathology such as venous dilation, microaneurysms, intraretinal hemorrhaging, hard exudates, and edema begins to develop in association with areas of capillary non-perfusion. This stage of DR is referred to as non-proliferative diabetic retinopathy (NPDR). As these pathologies progress, vascular leakage in NPDR may lead to macular edema with hard exudates and vision loss (diabetic macular edema (DME)), or to extensive intraretinal hemorrhaging, whereas areas of capillary non-perfusion increase significantly. This latter stage of DR is often referred to as pre-proliferative DR. Proliferative diabetic retinopathy (PDR) is the most severe form of DR. It is characterized by retinal neovascularization followed by intravitreal hemorrhaging, retinal ablation, and eventually retinal fibrosis, which can lead to permanent vision loss.

Currently, no treatment exists with which DR can be prevented or cured. In order to find new modalities of treatment of DR, it is essential to gain a better understanding of the processes which lead to its development. As the earliest clinically observable changes in the diabetic retina are vascular-related, DR is traditionally seen as a vascular disease. Dysfunction of the endothelial lining of the retinal vasculature is considered to play an important role in the development of DR. In this thesis, we attempt to further elucidate the role of EC dysfunction in the development of DR with a special interest in leukostasis and the involvement of advanced glycation end products (AGEs), vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF).

Chapter 1 sets the stage for this thesis by introducing several concepts of DR and presenting the reader with the central question of this thesis: what is the role of endothelial dysfunction in the development of DR?

Chapter 2 is a critical review of the current literature regarding leukostasis in the diabetic retinal vasculature and its role in the development of DR. The current data accumulated from various animal models of DR is conflicting, as the role which leukostasis plays in the development of DR appears to vary considerably depending on the type of animal model used. In streptozotocin-induced diabetic rat and mouse models, leukostasis is induced 2- to 4-fold, the inhibition of which was able to prevent
many early vascular changes which are associated with DR. On the other hand, in a rat model of spontaneous diabetes, increased leukostasis was not observed, and yet the characteristic vascular pathologies of DR still occurred. A major pitfall of animal models of DR is that they are not known to progress to the non-proliferative or PDR stages. This prevents researchers from studying the direct effects of leukostasis on DR pathology such as DME and neovascularization, which ultimately lead to loss of sight. This raises questions about the specificity and relevance of leukostasis data obtained in animal models of DR. A wide variety of growth factors, cytokines and cell signaling molecules as well as environmental factors such as increased shear stress and hyperosmolarity all lead to a similar 2- to 4-fold increase in leukostasis. Furthermore, rat models of hypertension and hyperlipidemia also demonstrate increased leukostasis without retinal vascular pathology characteristic for DR. Taken together, these findings indicate that increased leukostasis is likely the result of aspecific EC dysfunction and, therefore, an epiphenomenon of the retinal diabetic milieu rather than instrumental in further progression of the disease.

In chapter 3, we describe expression of vascular adhesion molecules considered to be markers of EC dysfunction in the vasculature of the human diabetic retina. Expression of the adhesion molecule ICAM-1 is increased on the retinal endothelium in diabetic rats and has been shown to be responsible for increased leukostasis observed in these rats. In our immunohistochemical study of the human retina, we did not observe an increase in the vascular expression of ICAM-1 or three other well-characterized leukocyte-endothelial adhesion molecules. This indicates that increased endothelial adhesion is not likely to contribute to the development of DR in patients.

A large part of the work presented in the second part of this thesis was based on the determination of mRNA levels in tissue samples of retina obtained from animal models employing quantitative real-time PCR. Chapter 4 reviews the current strategies of normalizing gene expression data obtained with the use of quantitative real-time PCR. Normalization is an important step in the analysis of gene expression data as it serves to limit artificial differences in gene transcript numbers which can be introduced during the collection and processing of tissue samples. We propose a new method of normalization and critically compare it with several widely used methods using a set of data obtained from whole retinas of diabetic rats. This comparison reveals that commonly used methods of normalization which rely on the use of single reference gene or a set of these genes (genes which are believed to be universally expressed in all cells at more or less constant levels) provide inadequate normalization and can skew whole data sets. This is mainly due to the fact that many of the widely-accepted reference genes are involved in processes such as cellular metabolism which are greatly affected in metabolic diseases such as diabetes. Our novel method of data normalization uses three parameters, the wet weight of RNA, total amount of RNA and the relative total amount of transcript RNA in each sample to normalize the data. This method is the most appropriate method for normalizing data obtained in heterogeneous tissue samples. In chapter 5, we employed this normalization strategy, and demonstrate that AGEs are
necessary and sufficient for inducing the increased expression of CTGF observed in the diabetic retina. We have previously demonstrated that diabetic mice lacking one functional allele of CTGF are protected against LB thickening in PCDR, implicating CTGF as having a causal role in diabetes-induced LB thickening.\textsuperscript{6} AGE inhibition has also been shown to prevent diabetes-induced LB thickening.\textsuperscript{7} Taken together, these findings indicate that the thickening of the retinal microvascular LB observed in diabetes is the result of AGE-induced CTGF modulation of extracellular matrix (ECM) components which comprise the LB (Fig. 1).

The pathogenesis of LB thickening was further studied in chapter 6 by examining the role of VEGF in inducing expression of CTGF and ECM molecules in vivo in the rat retina and in vitro in cultured retinal ECs and pericytes. When injected into the rat vitreous, VEGF induced both mRNA and protein expression of CTGF, fibronectin (an ECM protein and component of the LB) and TIMP-1 (a protein which inhibits breakdown of ECM including the LB). Similar results were obtained when cultured retinal ECs and pericytes were exposed to VEGF, implicating a role of these vascular cells in the pathological process of LB thickening. On the basis of results described in chapter 5 and 6, we propose that AGEs, known to increase VEGF expression in the diabetic retina,\textsuperscript{8} induce LB thickening using VEGF as a downstream regulator. VEGF, in turn, induces expression of CTGF in EC and pericytes, which in turn leads to LB thickening.
through increased ECM protein production in pericytes and increased TIMP-1 expression that inhibits enzymatic breakdown of the LB (Fig. 1). As the inhibition of LB thickening can prevent retinal vascular abnormalities such as apoptosis of pericytes and ECs and vascular leakage which are hallmarks of DR, the molecular pathway which leads to its occurrence can lead to new, possibly more effective treatments of DR.

Finally in chapter 7, now focusing on another aspect of PCDR, diffuse loss of the blood-retinal barrier, we demonstrate that expression of EC tight junction genes and particularly that of occludin and claudin-5 is transiently reduced in the diabetic retina and in bovine retinal ECs (BRECs) after exposure to VEGF. Expression of the vesicular transport-related genes plasmalemma vesicle-associated protein (PV-1) and caveolin-1 is increased in the diabetic retina and in BRECs exposed to VEGF. VEGF is responsible for the breakdown of the BRB as observed in DR, both in the early diffuse vascular leakage in NPDR, and in focal profuse leakage in diabetic macular edema in NPDR. The mechanisms of these two forms of leakage remain unclear, but two mechanisms have been proposed: 1) it is the result of paracellular leakage between ECs due to a decrease in the inter-endothelial tight-junction bonds; and 2) it is due to increased transcellular transport through increased pinocytotic vesicular transport from the luminal membrane to the abluminal membrane of the endothelium. Our data suggest, at least for the diffuse leakage of PCDR, a transient induction of the paracellular pathway and prolonged involvement of transcellular endothelial transport in the increased permeability of retinal capillaries in PCDR indicating that the transcellular pathway is the main mechanism of BRB leakage.

In conclusion, the findings in this thesis help to further define the role of the dysfunctional endothelium in the development of DR. We have shown that increased leukocyte-endothelial adhesion can lead to capillary non-perfusion in the rat retina, but that this is not likely the case in humans as the retinal endothelium shows no increased expression of leukocyte-endothelium adhesion molecules. Furthermore, we have provided new insights into the pathway and molecular players involved in the characteristic vascular LB thickening observed in the earliest stages of DR and the potential involvement of the endothelium. Lastly, we present new evidence that the transcellular pathway of retinal vascular leakage via pinocytotic vesicular transport in the endothelium is a major long-term contributor to BRB breakdown in DR.

Reference List


