Endothelial dysfunction in experimental models of preclinical diabetic retinopathy
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Chapter 1

Introduction
General introduction

Diabetic retinopathy (DR) is known to be a leading cause of blindness in individuals of working age living in western countries.¹ In patients with type 1 diabetes, 97% develop some form of DR. Patients with type 2 diabetes who are not insulin dependent have a 57% risk of developing DR after 15 years of diabetes. This risk is increased to 84% for type 2 patients who are insulin dependent.² With the ever increasing incidence of type 2 diabetes and the average age of onset steadily decreasing,³ DR presents a growing dilemma for diabetic patients and ophthalmologists alike. The lack of an effective therapy for DR only adds to this problem.

As the earliest clinically observable changes in the diabetic retina are vascular related, DR is traditionally considered to be a vascular disease. Clinically, DR is divided into three stages: non-proliferative diabetic retinopathy (NPDR), pre-proliferative DR and proliferative diabetic retinopathy (PDR). Fundoscopic findings characteristic for NPDR are venous dilation, microaneurysms, intraretinal hemorrhaging, hard exudates, and edema. In the pre-proliferative stage, soft exudates, venous tortuosity, extensive intraretinal hemorrhaging, areas of capillary occlusion and capillary shunt formation are observed. Finally, retinal neovascularization signals the advent of PDR in which intravitreal hemorrhaging and retinal ablation, as a result of retinal fibrosis, can lead to acute loss of vision.

DR is most often diagnosed after many years of diabetes, but it is important to realize that clinical DR is preceded by a period, characterized by various microscopic retinal pathologies, commonly referred to as preclinical DR (PCDR). Hallmark retinal pathologies of preclinical DR are pericyte loss, endothelial cell proliferation, thickening of the vascular lamina basalis (LB), breakdown of the blood-retina barrier (BRB), and acellular capillaries. It is not known when in the course of diabetes these retinal pathologies first appear, though animal research suggests that it could be as soon as several weeks to months after the onset of hyperglycemia.⁴⁻⁶ The exact role of these early retinal pathologies in the development of the clinical stages of DR remains unclear.

Currently there are only two forms of therapy available which have been proven effective in preventing or slowing DR-associated vision loss; tight glycemic control and laser coagulation therapy.⁷ Tight glycemic control can reduce the incidence as well as the progression of DR. Laser therapy can be applied in both NPDR, in which local areas of vascular leakage are photocoagulated, and in PDR, in which the entire peripheral retinal is obliterated. It reduces the net metabolic needs of the retina and thus decreases the expression of angiogenic growth factors produced by hypoxic retinal tissue. In spite of these two therapies, up to 50% of patients continue to have progressive vision loss.⁷ Newer therapies using anti-inflammatory agents and angiogenesis inhibitors have initially shown promising results in limiting PDR-induced retinal damage. However, conclusive evidence of their long-term benefits is still lacking.⁸ It is through a better understanding of the pathological processes leading to DR, that new and more effective treatment modalities can be discovered.
Diabetes-induced hyperglycemia is associated with systemic vascular pathology which over time can lead to organ failure. Other complications besides DR are nephropathy, cardiovascular disease, stroke, and peripheral limb vasculopathy. Endothelial cells form the inner lining of the vasculature and are directly exposed to the hyperglycemic environment. Hyperglycemia induces several pathogenic biochemical processes including the formation of advanced glycation end products (AGEs), protein kinase C activation, increased oxidative stress and increased flux through the polyol pathway which lead to impaired functioning of the vascular endothelium. It is therefore of no surprise that endothelial dysfunction is believed to play an important role in the development of DR.

The endothelium plays a crucial role in many physiological functions, including metabolic homeostasis, the control of vasomotor tone, blood cell trafficking, haemostatic balance, permeability, proliferation, survival, wound healing and innate and adaptive immunity. It interacts and influences its surroundings through autocrine, paracrine and endocrine pathways. Dysfunction of the endothelium can therefore have dire consequences for the proper functioning of its associated organs. Endothelial phenotype and function is known to vary in order to suit the specific needs of the surrounding tissue. In a number tissues, the endothelium is fenestrated allowing free diffusion of nutrients, metabolites and (macro)molecules from plasma to tissue fluids and vice versa. Retinal endothelium, however, lacks fenestrations. Rather, it contains specialized intercellular adhesion proteins which form so-called tight junctions between the retinal endothelial cells. These tight junctions are an important component of the blood retinal barrier (BRB) of the inner retina. The BRB effectively isolates the retinal neural tissue from the blood, thus protecting it from potentially damaging components of plasma and from immune reactions. Proper retinal function is dependent on the ability of the retinal endothelium to transport the proper nutrients into and waste products and potentially toxic metabolites out of the retina through well-regulated specific permeability mediated by tight junctions and vesicular transcytosis.

Many cytokines, growth factors, signaling molecules and bioactive molecules have been implicated in the pathogenesis of DR. In the present thesis, we examine the effects of AGEs, vascular endothelial growth factor (VEGF), and connective tissue growth factor (CTGF) on the diabetic retina and their role in DR-related endothelial dysfunction. The role of AGEs and VEGF in the development of DR-related vascular pathology has been well established, but the importance of CTGF has only recently begun to become apparent.

AGEs

AGEs are the result of a non-enzymatic glycation reaction between glucose and the free amino group of lysine residues in proteins. The reaction starts with the formation of an unstable Schiff base between glucose and the ε-amino group of lysine. Increasing amounts of these Schiff bases slowly rearrange to form amadori adducts which have a half life of several months. These amadori adducts then undergo further modification.
through which the glycosylation of the proteins becomes permanent, ultimately resulting in AGEs. AGEs can also be formed by the direct reaction between proteins and reactive dicarbonyl compounds, the levels of which are elevated during hyperglycemia.\textsuperscript{19}

AGEs can directly cause cellular pathology through their ability to chemically modify, cross-link, and increase pigmentation and fluorescence of proteins. AGEs also indirectly affect cell function by binding to AGE-specific receptors initiating intracellular signalling cascades.\textsuperscript{19} Two receptors of AGEs are known to be expressed in the retinal vasculature: receptor for AGEs (RAGE)\textsuperscript{20} and AGE-receptor complex (AGE-RC),\textsuperscript{21} consisting of the three subunits AGE-R1, AGE-R2 and AGE-R3.

In diabetic rats, retinal AGE accumulation first appears in the vascular LB where AGEs form crosslinks between the various extracellular matrix (ECM) molecules. This alters the LB, making it more rigid and possibly thicker as inhibition of AGE formation prevents LB thickening in diabetic rats, through an, as yet, unknown mechanism.\textsuperscript{21} As the disease progresses, AGEs accumulate intracellularly. This likely occurs via interactions with AGE receptors located in caveolin-rich membrane domains.\textsuperscript{22} Intracellular accumulation of AGEs is thought to be cytotoxic and leads to increased pericyte and neural cell apoptosis in the diabetic retina.\textsuperscript{23-26} AGEs are also capable of inducing BRB leakage. The mechanism through which this occurs is not yet completely understood, but direct AGE formation on tight junction proteins between retinal vascular endothelial cells could compromise the BRB and lead to paracellular leakage. AGE-induced upregulation of VEGF, a well-characterized inducer of vascular permeability in the diabetic retina, is also likely to contribute to BRB loss.\textsuperscript{27} AGEs are cytotoxic for pericytes, but they induce endothelial cell proliferation and tube formation, which are essential steps in angiogenesis.\textsuperscript{28} Additionally, AGEs are capable of inducing increased leukostasis in the diabetic retina.\textsuperscript{29} The importance of AGEs in the development of DR is best exemplified by the lack of characteristic PCDR vascular pathology in diabetic rats receiving anti-AGE treatment.\textsuperscript{30}

**VEGF**

VEGF is a growth factor which plays an important role in many physiological and pathological processes. In the embryo, it is crucial for normal development of the systemic vascular system.\textsuperscript{31} In the adult, it plays an important role in wound healing, the female reproductive cycle and physiological angiogenesis.\textsuperscript{32} Endothelial cells are the main target of VEGF. It stimulates EC proliferation, survival and migration. It also leads to increased endothelial permeability and vasodilation. In the retina, VEGF and its receptors VEGFR-1, VEGFR-2 and VEGFR-3 are expressed constitutively at low levels in many cell types in the eye including retinal pigment epithelial cells, pericytes, endothelial cells, astrocytes, Müller cells and photoreceptors.\textsuperscript{32} VEGF also has a neuroprotective effect and can induce neural growth.\textsuperscript{33,34} It is therefore possible that VEGF plays an important role in retinal neural cell survival in normal physiological conditions. Under pathological conditions, VEGF expression can be greatly increased in these cells.\textsuperscript{32} Hypoxia, inflammatory mediators, hyperglycemia and AGEs are all
capable of increasing VEGF expression in retinal cells.\textsuperscript{32}

Increased VEGF expression has been demonstrated in patients with NPDR\textsuperscript{35} as well as in rodent models of DR within weeks of induction of diabetes.\textsuperscript{36} This suggests that VEGF plays a crucial role in the early development of DR. In early DR, VEGF acts as a permeability factor, with its increased expression leading to BRB leakage\textsuperscript{36} through increased vesicular transport\textsuperscript{37} and/or alterations in inter-endothelial tight junction proteins.\textsuperscript{38} VEGF is also responsible for the increased leukostasis observed in rodent models of DR through upregulation of the leukocyte adhesion protein ICAM-1 in the retinal endothelium.\textsuperscript{39} Furthermore, administration of exogenous VEGF in the eye yields retinal vascular abnormalities similar to those observed in NPDR including vascular tortuosity and microaneurysms.\textsuperscript{40,41} VEGF also plays an important role in the pathological neovascularization that is characteristic of PDR as it is increased in the vitreous and in proliferative vitreoretinopathy (PVR) membranes in patients with PDR.\textsuperscript{42,43}

\textbf{CTGF}

CTGF is a member of the CYR61/CTGF/NOV (CCN) family of proteins. It plays an important role in many physiological processes including skeletal growth, wound healing, and embryonic angiogenesis.\textsuperscript{44} Similar to VEGF, it has been shown to promote endothelial cell proliferation, migration, adhesion and survival.\textsuperscript{45} It also has the ability to modulate VEGF activity through the inhibitive binding of VEGF.\textsuperscript{46} Additionally, CTGF is a well-characterized downstream regulator of TGF-β and regulates ECM production.\textsuperscript{47} In diabetic patients, CTGF expression is increased in the arteries, heart and kidneys where the resulting increase in ECM production leads to pathological fibrosis and organ damage.\textsuperscript{44} Recently, NH\textsubscript{2}-terminal fragments of CTGF were found in increased levels in the vitreous of patients with PDR.\textsuperscript{18,48} Expression of CTGF has also been found in PVR membranes of patients with PDR.\textsuperscript{49} Moreover, the ratio of vitreal CTGF and VEGF levels strongly correlate with the degree of fibrosis indicating that CTGF may drive an angio-fibrotic shift and subsequent fibrosis in PDR.\textsuperscript{16} Furthermore, increased CTGF expression has been detected in rat retinas after merely 12 weeks of diabetes\textsuperscript{50} and is at least partly responsible for DR-related LB thickening.\textsuperscript{17} These findings indicate that CTGF may play a role in the vascular pathology of PDR as well as PCDR.

\textbf{CHAPTER SUMMARY}

As previously mentioned, pericyte loss, thickening of the vascular LB, breakdown of the BRB, and acellular capillaries are preclinical phenomena regarded as hallmarks of the onset of DR. The pathogenesis of these retinal sequelae and their exact role in the development of DR remain unclear. Through the experiments described in the present thesis, we attempt to further elucidate the role of endothelial cell dysfunction in the development of a number of these vascular defects with specific attention paid to the effects of AGEs, VEGF and CTGF on the retinal vascular endothelium.

In \textbf{chapter 2}, a review of the role of leukostasis in the development of DR is
presented. We take a critical look at the role of leukostasis in the development of DR sequelae in animal models of early clinical DR and examine its relevance in the development of DR in humans. In chapter 3, we report the expression of several vascular adhesion molecules considered to be markers of endothelial cell dysfunction in the vasculature of the human diabetic retina. Chapter 4 reviews the current strategies of normalization of quantitative real-time PCR data. We discuss which strategies are most relevant to the study of DR and propose a novel method of normalizing gene expression levels in heterogeneous tissue samples obtained in models of systemic disease. In chapter 5, we examine the role of AGEs in LB thickening. This is performed by examining the effects of AGEs on the CCN family of proteins, including CTGF, which are known to induce the expression of ECM molecules which comprise the LB. The pathogenesis of LB thickening is further studied in chapter 6 by examining the role of VEGF in inducing CCN protein and ECM protein expression in vivo in the rat retina and in vitro in cultured retinal endothelial cells. In chapter 7, we attempt to elucidate which pathways of cellular transport contribute to BRB leakage by studying the gene expression patterns of molecules involved in transcellular and paracellular transport in retinal endothelium. Finally, in chapter 8 we present a general discussion of our findings and their relevance to the current understanding of the pathogenesis of DR.

Reference List

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