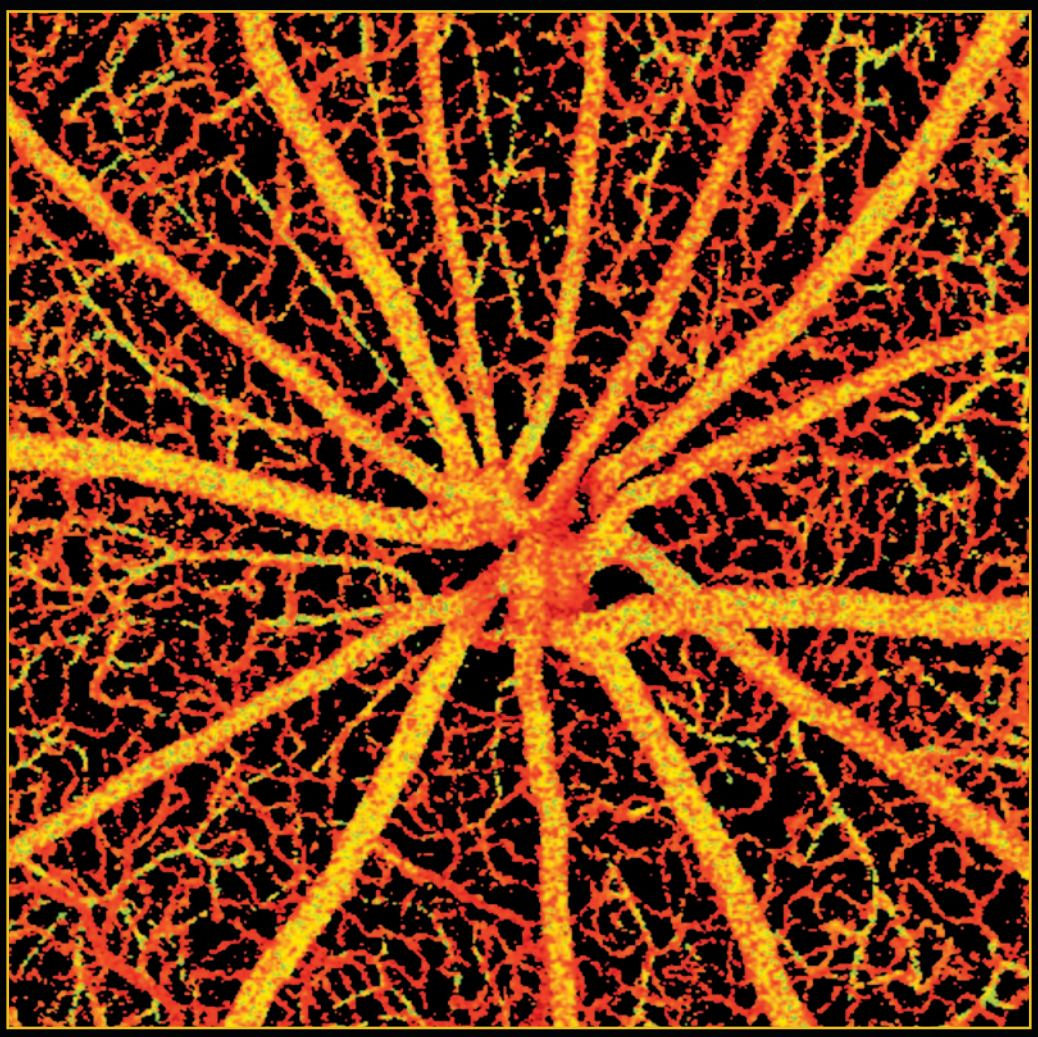
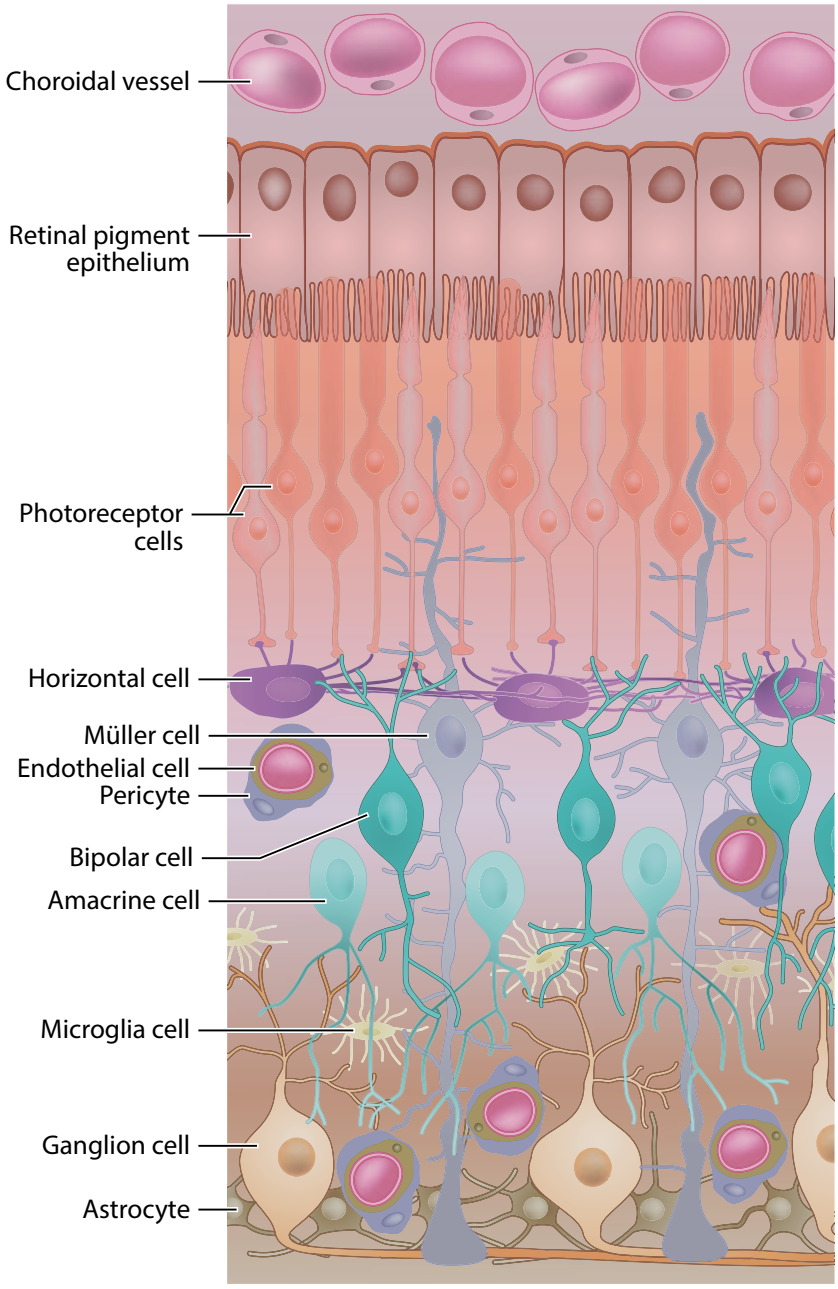
Diabetic Retinopathy
Where We Are and A Path to Progress



A Report by the Lasker/IRRF Initiative for Innovation in Vision Science



Cover: Doppler OCT imaging of capillary structure in rat. Imaging performed with high speed, ultrahigh resolution OCT system. Doppler volume is summed in the axial direction to create an en face image. Color coded to identify vessels in different layers. Yellow corresponds to shallower and red corresponds to deeper depths in the images. (Image courtesy of James Fujimoto, M.I.T.)

Frontispiece: Cells of the retina. Neurons - photoreceptors, horizontal, bipolar, amacrine and ganglion cells; Glia – Müller cells, microglia and astrocytes; Vasculature – endothelial cells and pericytes. (Image courtesy of Thomas Gardner, University of Michigan)

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Project background and acknowledgements

The Albert and Mary Lasker Foundation (Lasker) and the International Retinal Research Foundation (IRRF) entered into a ten-year collaborative research program on July 15, 2008 entitled the Initiative for Innovation in Vision Science (Initiative). The Initiative is designed to identify knowledge gaps in vision research and develop innovative strategies to advance research on retinal disease. The Initiative's long-term goal is accelerating discovery of sight-saving treatments and preventing retinal degenerative diseases using novel scientific, engineering and technological approaches.

John E. Dowling Ph.D., Gordon and Llura Gund Professor of Neurosciences at Harvard University, chairs the Initiative with the guidance of a Lasker/IRRF Joint Advisory Board. Individual studies conducted by the Initiative are undertaken using a Steering Committee of bench and clinical scientists with expertise in interdisciplinary fields and the combined skills, knowledge and experience necessary to identify key issues and hurdles confronting vision scientists. The Steering Committee also identifies leaders in diverse fields to participate in workshops at which they refine the issues, identify additional issues and begin to develop innovative approaches to the significant hurdles that impede scientific progress. These workshops are followed by a plenary session of all workshop participants at which small groups focus on specific targeted areas and develop a framework of innovative multidisciplinary approaches to accelerate discovery and its translation to clinical application for retinal degenerative diseases. The results of these sessions are published by the Initiative for wide distribution within the research community and to potential funders and other organizations interested in advancing research in retinal degenerative diseases. The Initiative's first study focused on the role of astrocytes in glaucomatous neurodegeneration, and a summary report of this study was published in November 2010.

In 2011, the Initiative chose to explore diabetic retinopathy because it is one of the leading causes of visual impairment and blindness in the world. With the rapid increase in obesity and the close link between diabetes and obesity, complications of diabetes, including retinopathy, are likely to be even more prevalent in the future. Two workshops were held during the summer of 2011 followed by a plenary session in March, 2012, at which participants identified key unsolved issues and important opportunities in diabetic retinopathy which, by using modern day experimental tools, may now be experimentally addressed. This report is the product of these sessions.

The Initiative thanks the Boards of Directors of the Albert and Mary Lasker Foundation and the International Retinal Research Foundation for their support; the Initiative's Joint Advisory Board and Steering Committee, for their guidance; the workshop and targeted session leaders who guided the development of the key issues discussed in this report and the scribes who recorded the discussions

and participated in preparing summary reports; and all participants, for their energy, expertise and lively discourse. Special thanks go to Karen M. Wright, Project Administrator, Kate Chapman, Project Manager, and Sandra Blackwood, Executive Director of the IRRF, for their diligent and essential administrative direction and logistical support.

We were particularly honored to have Lloyd M. Aiello, M.D., a pioneer in panretinal photocoagulation for the treatment of diabetic retinopathy and a leader in diabetes-related health care delivery, give a keynote address at the plenary session entitled "The road traveled with Airlie colleagues and patients: Beyond blindness—our memories are our realities." Dr. Aiello was an architect of the groundbreaking Airlie House conference in 1968 that launched a seminal series of multi-center controlled clinical trials in diabetic retinopathy, starting with the Diabetic Retinopathy Study that validated his laser-based treatment. Dr. Aiello captivated the group with his chronicle of research innovation in the diagnosis and treatment of diabetic retinopathy since the Airlie House meeting.

The Initiative gratefully acknowledges the Howard Hughes Medical Institute for its generous in-kind contribution by hosting the Initiative's plenary workshop at its Janelia Farm Research Campus in Ashburn, Virginia, and the gracious hospitality of the staff of the National Academy of Sciences' Erik Jonsson Center in Woods Hole, Massachusetts, where the two summer workshops were held.

For further information about the Initiative or for additional copies of this report, please contact: Karen M. Wright, Project Administrator at kwright@laskerfoundation.org or Kate Chapman, Project Manager at kchapman@laskerfoundation.org.

Photos Courtesy of Larry Donoso

Lasker/IRRF Initiative for Innovation in Vision Science

Summary Report on Diabetic Retinopathy
November 2012

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List of Abbreviations

ADPase – adenosine diphosphatase
AGE – advanced glycation endproduct
AMD – age-related macular degeneration
AO – adaptive optics
ASMase – acid sphingomyelinase
bFGF – basic fibroblast growth factor
BMI – body mass index
COX – cyclooxygenase
CSME – clinically significant macular edema
DARC – detection of apoptosing retinal cells
DHA – docosahexaenoic acid
DM – diabetes mellitus
DME – diabetic macular edema
DR – diabetic retinopathy
EPC – endothelial cell progenitor cells
ERG – electroretinography
FA – fatty acids
FDT – frequency-doubling technology
GFR – glomerular filtration rate
HbA1C – glycated hemoglobin
HDL – high-density lipoprotein
ICAM-1 – intercellular adhesion molecule 1
IL-1 – interleukin-1
IL-6 – interleukin- 6
IND – investigational new drug
iNOS – nitric oxide synthase
IRMA – intraretinal microvascular abnormalities
LDL – low-density lipoprotein
LFA-1 – lymphocyte function-associated antigen 1
LOX – lipoxygenase
MCP-1 – monocyte chemotactic protein-1
MEMRI – manganese enhanced magnetic resonance imaging
mfERG – multifocal electroretinography
MOX – methanol oxidase
MRI – magnetic resonance imaging
NPDR – nonproliferative diabetic retinopathy
NSAIDs – nonsteroidal anti-inflammatory drugs
OCT – optical coherence tomography
ON – optic nerve
PDGF – platelet-derived growth factor
PDR – proliferative diabetic retinopathy
PEDF – pigment epithelium-derived factor
PKCα – protein kinase C-alpha
PO_2 – partial oxygen pressure
PPARα – peroxisome proliferator-activated receptor alpha
PRP – panretinal photocoagulation
PSC – pluripotent stem cells
PUFA – polyunsaturated fatty acid
RAGE – receptor for advanced glycation end-products
RPE – retinal pigment epithelial
SLO – scanning laser ophthalmoscopy
SOD2 – superoxide dismutase 2
T1DM – type 1 diabetes mellitus
T2DM – type 2 diabetes mellitus
TNFα – tumor necrosis factor-alpha
VEGF – vascular endothelial growth factor
VLDL – very low density lipoprotein

List of Clinical Trials and Studies

DCCT - Diabetes Control and Complications Trial (DCCT) for type 1 diabetes in the United States and Canada

The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. DCCT Group (1993) (Introduction, Chapter 5)

UKPDS - UK Prospective Diabetes Study for type 2 diabetes in the United Kingdom

Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with newly diagnosed type 2 diabetes (UKPDS 33). UKPDS Group (1998) (Introduction, Chapters 2, 5)

EDIC - Epidemiology of Diabetes Interventions and Complications; the long-term follow-up of subjects originally enrolled in the DCCT (Chapter 5)

DRS - Diabetic Retinopathy Study

Photocoagulation treatment of proliferative diabetic retinopathy: The second report of diabetic retinopathy study findings (1978) (Chapter 5)

ETDRS - Early Treatment Diabetic Retinopathy Study

Photocoagulation for diabetic macular edema. ETDRS report number 1(1985) (Chapter 5)

Early photocoagulation for diabetic retinopathy. ETDRS report number 9 (1991) (Chapters 5, 8)

Effects of aspirin treatment on diabetic retinopathy. ETDRS report number 8 (1991) (Chapters 6, 8)

Association of elevated serum lipid levels with retinal hard exudate in diabetic retinopathy. Early Treatment Diabetic Retinopathy Study (ETDRS) report 22 (1996) (Chapter 2)

DRVS - Diabetic Retinopathy Vitrectomy Study

Early vitrectomy for severe proliferative diabetic retinopathy in eyes with useful vision. Clinical application of results of a randomized trial. DRVS report 4 (1988) (Chapter 5)

Early vitrectomy for severe vitreous hemorrhage in diabetic retinopathy. Two-year results of a randomized trial. DRVS report 2 (1985) (Chapter 5)

DRCRnet - Diabetic Retinopathy Clinical Research Network Study

Rationale for the diabetic retinopathy clinical research network treatment protocol for center-involved diabetic macular edema (2011) (Chapters 5, 6, 8)

Diabetic Retinopathy Clinical Research N, Beck RW, Edwards AR, Aiello LP, Bressler NM, Ferris F, Glassman AR, Hartnett E, Ip MS, Kim JE, Kollman C. Three-year follow-up of a randomized trial comparing focal/grid photocoagulation and intravitreal triamcinolone for diabetic macular edema. Arch Ophthalmol. 2009;127:245-251. (Chapters 5, 6, 8)

Diabetic Retinopathy Clinical Research N, Elman MJ, Aiello LP, Beck RW, Bressler NM, Bressler SB, Edwards AR, Ferris FL, 3rd, Friedman SM, Glassman AR, Miller KM, Scott IU, Stockdale CR, Sun JK. Randomized trial evaluating ranibizumab plus prompt or deferred laser or triamcinolone plus prompt laser for diabetic macular edema. Ophthalmology. 2010;117:1064-1077 e1035. (Chapters 5, 6, 8)

WESDR - Wisconsin Epidemiologic Study of Diabetic Retinopathy (Chapter 7)

WDRS - Wisconsin Diabetes Registry Study (Chapter 7)

AGES - Age, Gene/Environment Susceptibility Study (Chapter 7)

ARIC - Atherosclerosis Risk in Communities Study (Chapter 7)

CHS - Cardiovascular Health Study (Chapter 7)

LALES - Los Angeles Latino Eye Study (Chapter 7)

MESA - Multi-Ethnic Study of Atherosclerosis (Chapter 7)

NHANES - National Health and Nutrition Examination Survey (Chapter 7)

REMOVAL - Reduction with Metformin Vascular Adverse Lesions in Type 1 Diabetes Study (Chapter 7)

ACCORD-EYE - Action to Control Cardiovascular Risk in Diabetes Trial (Chapter 7)

SRPDM - State Registry of Patients with Diabetes Mellitus (Chapter 7)

Introduction

Robert N. Frank

Diabetic retinopathy (DR) is the most common and most serious of the ocular complications of diabetes mellitus. In the U.S. alone, the most recent (2007) estimate from the Centers for Disease Control and Prevention indicated that some 23.6 million individuals, 7.8 percent of the entire U.S. population, have diabetes. Of these, nearly 30 percent have retinopathy, and an estimated 4.4 percent, or about one million individuals, have advanced, vision-threatening retinopathy (Diabetic Retinopathy, 2012; Zhang et al., 2010). A very large proportion of these are from minority populations. Although the best incidence and prevalence data come from the U.S., the problem is widespread, as evidenced by publications from other parts of the world (Ding and Wong, 2012; Sivaprasad et al., 2012).

Type 1 diabetes, which worldwide affects approximately 5 percent of all individuals with diabetes, usually has its onset before age 30. It is believed to be an autoimmune disease that specifically destroys the insulin-producing beta cells of the pancreas, and persons with type 1 diabetes require injected insulin to maintain normal blood glucose levels (Eisenbarth, 2012). Type 2 diabetes affects approximately 95 percent of individuals with diabetes. Though individuals with type 2 diabetes produce their own insulin, complex mechanisms increase resistance to insulin-mediated transport of glucose into cells and decrease insulin release by the pancreas. Blood glucose levels in type 2 diabetes may be controlled by life style changes and oral medications, although the need for exogenously injected insulin may increase with greater duration of the disease, which often begins beyond the age of 30, but it is increasingly being recognized in individuals as early as the adolescent years. Individuals with type 2 diabetes are often obese and the epidemic of obesity may contribute to the increasing incidence and earlier onset of type 2 diabetes in many parts of the world. Weight loss has been found to be highly beneficial for improving glycemic control (McCulloch, 2012).

Since it was first described in the nineteenth century, during the early days of ophthalmoscopy, DR has been considered a disease of the retinal blood vessels. However, a variety of highly sensitive techniques that have been developed much more recently suggest that much earlier anatomic, biochemical and physiologic abnormalities in the neuronal and glial cells of the retina precede these vascular lesions, and lead to the question of whether these are true predecessors of the vascular disorder and may be appropriately called "preclinical" diabetic retinopathy. These include focal alterations of the amplitude and latency of the multifocal electroretinogram (mfERG) that presage the location of future vascular lesions (Harrison, 2011); histologic preparations from human and animal eyes demonstrating apoptosis of retinal neurons (Hammes, 1995; Barber, 1998); and immunocytochemical preparations from human and animal eyes with no visible vascular lesions, showing upregulation of vascular endothelial growth factor (Amin, 1997). Although there is a "chicken-and-egg" debate among clinical and basic researchers in the field as to whether the first abnormalities of DR occur in the retinal blood vessels or in the neuronal and glial cells of the retina, there is little doubt that the first clinically observable signs of the disease are microaneurysms, small outpouchings of the retinal capillaries caused by localized proliferations of capillary endothelial cells, together with tiny "dot-and-blot" hemorrhages that occur when the normal barrier function of the retinal blood vessels begins to break down (Figure 1).

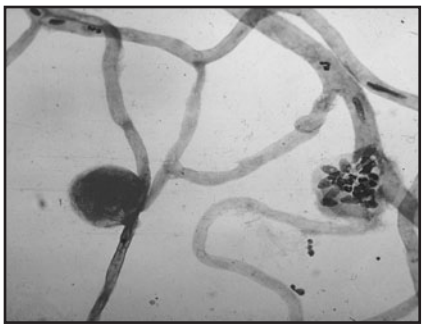

Figure 1: Digest preparation of retinal capillaries from a diabetic patient showing acellular capillaries and a cellular and an acellular microaneurysm (Kuwabara and Cogan, 1963).

Over time, the breakdown of the "blood-retinal barrier" also leads to the deposition, usually in the central portion of the human retina, of yellowish-white lipid deposits. Occlusion of small arterioles leads to focal areas of ischemic retina, which are clinically apparent as white, cotton wool spots. Later, the size and number of hemorrhages may increase, the retinal veins become dilated, irregular in caliber, and retinal arterioles appear as white, opacified tubes that no longer carry blood (Figure 2b, 2c). Collectively, these abnormalities have been termed "nonproliferative" DR (NPDR).

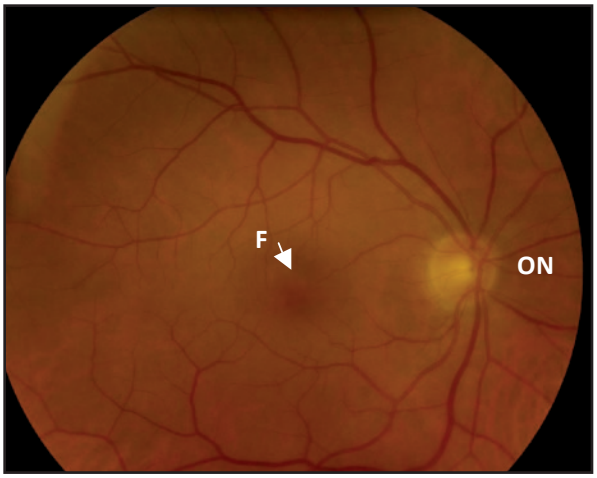

Figure 2a: Photograph of the optic nerve head (ON) and macular retina of a normal right eye. The fovea (F), the center of the macula in the human eye, with the highest visual acuity, is at the center of the darker area in the center of the photograph. The finer caliber, lighter red blood vessels are retinal arteries, while the darker red vessels with larger diameter are retinal veins.

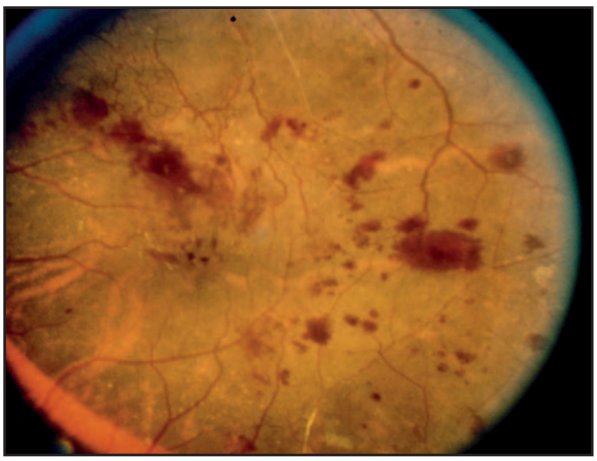

Figure 2b: Severe nonproliferative diabetic retinopathy. Note the extensive blot hemorrhages and the arteriolar abnormalities (white, vertically oriented vessels at the top and bottom of the picture).

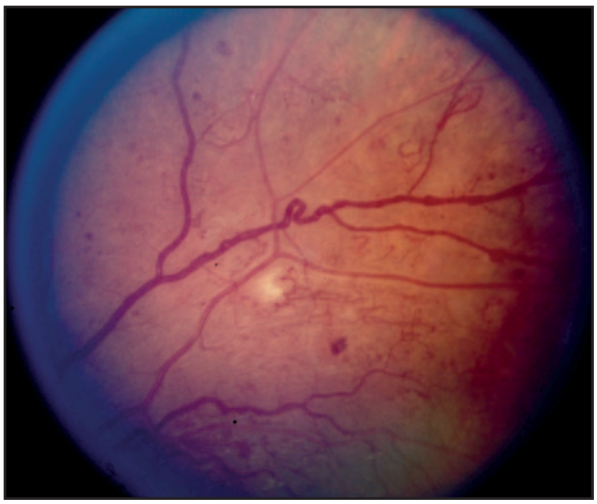

Figure 2c: More severe nonproliferative ("pre-proliferative") retinopathy. Venous irregularities (dilation, irregularity in caliber) and venous reduplication (the inverted "U" shaped vessel just above the center of the picture). There are frequent "intraretinal microvascular abnormalities" ("IRMA"). There is also a "cotton wool spot" at the center of the photograph. Patients with this picture will have an approximately 75% risk of developing "high risk" proliferative retinopathy within a year according to Early Treatment Diabetic Retinopathy (ETDRS) results.

Gradually, cellular components of the retinal capillaries are lost, and surrounding, ischemic retinal tissue elaborates signals to stimulate new vessel proliferation. This growth is always abnormal, with the newly formed vessels prone to leakage of intravascular fluid and hemorrhage. The earliest new vessels proliferate only just within the retinal tissue but, subsequently, the new vessels grow inward toward the vitreous, their endothelial cells elaborate collagenases and other proteases, which digest the inner limiting membrane of the retina, and they then grow into the vitreous cavity where they are prone to rupture. These intravitreal vessels are usually accompanied by fibroglial proliferations that can contract, producing traction on the retina, leading to retinal detachments. This later stage of the disease is termed "proliferative" DR (PDR). Without treatment, its end stage is marked by proliferation of new blood vessels on the anterior surface of the iris, growth of vessels and fibrous tissue into the anterior chamber angle, and occlusion of the outflow channels for aqueous humor. With complete blockage of aqueous outflow, a devastating rise in intraocular pressure called "neovascular glaucoma" can occur.

Macular edema, another disabling form of DR, can occur with, or in the absence of, other signs of retinopathy. Breakdown of the "blood-retinal barrier" in the central portion of the retina (macula) leads to accumulation of fluid, producing swelling of the macular tissue. Commonly, partial resorption of the intraretinal fluid leads to deposition of the relatively insoluble lipoprotein components of plasma, readily visible on ophthalmoscopic examination.

Research over nearly a half-century has established several undisputed facts. Two major clinical trials, the Diabetes Control and Complications Trial (DCCT) (1993), and the United Kingdom Prospective Diabetes Study (UKPDS) (1998) demonstrated unequivocally that control of blood glucose to near-normal levels reduces the incidence and progression of retinopathy and other complications in both types of diabetes. Prolonged hyperglycemia is clearly a condition *sine qua non* for the development of the microvascular complications of diabetes. In type 1 diabetes, whose time of onset is usually acute and easy to document, it is evident that retinopathy does not develop for at least three to five years after the diagnosis of diabetes. Conversely, the DCCT demonstrated that, after the initiation of "tight" blood glucose control, the beneficial effects of this regimen did not become evident for 2-3 years. This delay in the beneficial effect of a reduced average blood glucose level was true both in the "primary prevention" cohort of the DCCT, which had no evidence of retinopathy at the beginning of the study,

and in the "secondary intervention" cohort, which had mild to moderate retinopathy present at the study's outset. Furthermore, the DCCT results showed that the risk for development and progression of retinopathy formed a continuum, from those with the lowest average blood glucose to those with the highest. Data from that study showed no evidence that the risk of retinopathy has a "threshold" blood glucose level, below which there is no risk and above which the risk is greatly increased. However, later epidemiologic studies from ethnically diverse populations indicate that there is a glycemic level (6.5%, just above the upper limit of normal) above which the prevalence of diabetic retinopathy begins to rise sharply above background (International Expert Committee, 2009). With these data, and the standardization of HbA1c test methodology and values, a level of >/= 6.5% has become a diagnostic criterion for diabetes (American Diabetes Association, 2012).

Evidence to date indicates that mechanisms other than hyperglycemia also affect the development of DR. Some factors are known. Individuals who develop diabetes shortly after birth do not develop retinopathy until the time of puberty (Frank et al., 1982; Palmberg et al., 1981; Rogers et al., 1987). Adolescents with Mauriac's syndrome, a rare disorder causing difficult-to-control type 1 diabetes, delayed growth and sexual maturation, and enlarged fatty liver, even when brought into "tight" blood glucose control, may rapidly develop severe proliferative retinopathy (Daneman et al., 1981). These observations suggest that hormones, such as growth hormone and insulin-like growth factors, or other mechanisms may interact with hyperglycemia to produce delay in the onset of retinopathy. Anatomic or metabolic factors within the retina may also govern the development and progression of retinopathy. For example, the vascular lesions of proliferative DR develop preferentially at the optic nerve head, next most frequently along the superotemporal vessels followed by the inferotemporal and nasal vessels and, finally, elsewhere in the retina (Taylor and Dobree, 1970).

A phenomenon of great interest observed in long-term diabetic dogs, and in non-diabetic dogs fed a diet enriched in D-galactose (Engerman and Kern, 1987, 1995), has been termed "metabolic memory." This same phenomenon was seen in the human subjects who participated in the DCCT and its follow-up study, the Epidemiology of Diabetes Interventions and Complications (EDIC) (White et al., 2008). In dogs that had poorly controlled diabetes or had been fed high-galactose diets for 2 – 2 ½ years, and then had one eye removed and examined, no, or equivocal, retinopathy was observed. But if the diabetic dogs were kept on tight glucose control or if the galactose diet was withdrawn, the remaining eye showed typical diabetic retinopathy after five years (Engerman and Kern, 1987, 1995). In the DCCT, after the conclusion of the formal trial, subjects who had been in either the "tight" or "standard" control groups were instructed to maintain their blood glucose levels as they chose. Over a 10-year follow-up, when average blood glucose and hemoglobin A1c (a measure of average blood glucose levels over the preceding 3 months) in the two treatment groups had become equivalent, retinopathy in the original "tight" control group continued to progress more slowly than those in the original "standard" group (White et al., 2008). It appeared as though the original period of "tight" or "standard" control in both humans and diabetic or galactosemic dogs had triggered a "switch" that governed the progression of retinopathy for at least several years thereafter. It is plausible to consider that the nature of that "switch" is an important factor in the pathogenesis of DR.

There is also evidence for classical genetic influences on diabetic retinopathy. A subsequent DCCT report (1997) showed that close relatives of diabetic subjects who developed proliferative or severe nonproliferative retinopathy had significantly greater risk for developing retinopathy of similar severity

themselves. Attempts to find responsible genetic determinants have thus far not been successful (Arar et al., 2008).

An early histologic lesion of diabetic retinopathy is the loss of pericytes from the retinal capillary vessels (Kuwabara and Cogan, 1963). One mechanism of this loss is apoptosis (Mizutani et al., 1996) which, as noted earlier, also occurs in the neurons and glia of the retina in diabetic humans and animals (Hammes et al., 1995, Barber et al., 1998). The mechanism(s) of this apoptotic cell loss are unclear. Although the retina is an embryologic outgrowth of the brain, the capillary pericyte loss does not occur elsewhere in the brains of diabetic individuals (de Oliveira, 1966). Interestingly, the ratio of pericytes to endothelial cells in rats and rhesus monkeys (Frank et al., 1990) is significantly higher in the retina than in the cerebral cortex, for reasons that are unclear.

A highly active area of research in DR over the past 20 years has been the study of peptide growth factors, in particular vascular endothelial growth factor-A (VEGF) (Aiello et al., 1994). VEGF is clearly a major player in the development of proliferative DR and macular edema but, as noted previously, VEGF upregulation occurs early in the disease (Amin et al., 1997), while proliferative retinopathy and macular edema are late occurrences. Also, some eyes in human diabetic patients develop proliferative retinopathy but no macular edema; some develop macular edema but no proliferative retinopathy, and some develop both. What intervening steps, and what molecules in addition to the various isoforms of VEGF are necessary to produce one or the other form of DR, are not known.

Finally, two unique features of the retina that may be responsible for its high susceptibility to complications of diabetes are its layered neuronal structure and the fact that its outermost, photoreceptor layer has enormous metabolic activity in terms of its consumption of glucose and oxygen – in fact, the highest metabolic activity per unit weight of any tissue in the body, as first documented by Otto Warburg in the 1920s. Because of this enormous metabolic activity and the resultant great production of waste products of oxidation, the photoreceptors and the retinal pigment epithelium of the vertebrate retina have a generous blood supply from the underlying choroidal capillaries, while the inner neuronal layers of the retina have a sparse blood circulation, with the central retinal artery in humans and higher primates being an end-arterial supply with no collateral vessels. It is unclear how this anatomical arrangement may affect the pathogenesis of DR. To date, little attention has been paid to the blood supply of the choroid and the metabolism of the photoreceptors or retinal pigment epithelium as potential elements in the pathogenesis of diabetic retinopathy (but see Cao, et al., 1998 and Saidi, et al., 2011). This would seem to be a promising area for further research.

These observations from past basic and clinical investigations in DR serve as a backdrop for the discussions that took place in the several meetings that comprised the Lasker/IRRF Initiative on Diabetic Retinopathy. At the final conference, held at the HHMI Janelia Farm Research Campus in Ashburn, Virginia in March, 2012, discussion groups focused on the following topics, which are the subject of the individual sections in this volume:

1. Early Signs of Diabetic Retinopathy

2. Role of Glucose, Lipids and Oxygen in Diabetic Retinopathy

3. Diagnostic Methods

4. Genetic and Environmental Susceptibility

5. Present and Proposed Approaches to Therapeutics

6. Pathogenesis of Diabetic Retinopathy

7. Epidemiology and Unusual cohorts

8. Vascular and Neuronal Repair

9. Animal Models

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Chapter 1
Early Signs of Diabetic Retinopathy

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Introduction

The ophthalmic manifestations of diabetes mellitus were first described in 1855 by the Austrian ophthalmologist Eduard Jaeger (Kalantzis et al., 2006). The transparency of the neural retina precluded direct clinical observation of neuroretinal damage in diabetes, but the changes to the vasculature in early diabetes were readily apparent on ophthalmoscopy. Thus, diabetic retinopathy has been traditionally viewed as a microvascular complication of diabetes, and a clinical grading system based on vascular changes has been effectively used for decades to assess the progression and severity of diabetic retinopathy in patients with diabetes (Aiello, 2003). Nevertheless, the advent of new technologies that allow for a more comprehensive evaluation of the structure and function of the neurovascular retina has revealed that neurons and glia are also damaged early in the course of diabetes. A better understanding of the relationship between the early neural and vascular changes in diabetic retinopathy will be integral to the development of future therapies for this sight-threatening condition.

Early vascular changes

Without question, diabetes mellitus exerts deleterious effects on the retinal and surrounding vasculature early in the course of the disease. While not technically considered to be part of the neuroretina, the choriocapillaris plays an important role in providing oxygen and other nutrients to retinal photoreceptors. This network of capillaries has been shown to drop out (Cao et al., 1998) and lose its fenestrations (Fitzgerald et al., 1989) in early diabetes. The consequences of these changes require further exploration, though it is interesting to speculate on how the structural changes in these capillaries might affect photoreceptor function.

Another early histopathologic finding in DR is pericyte drop out (Kuwabara et al., 1963 and Hammes et al., 2004). Pericytes are modified smooth muscle cells that encircle blood vessels within the retina and central nervous system. They play an important role in regulating capillary blood flow and in preserving the blood-retinal barrier. Loss of pericytes in diabetes is associated with the development of microaneurysms (focal dilations of blood vessels), which are the earliest clinical sign of DR directly observable by ophthalmoscopy.

While the formation of microaneurysms is certainly an early manifestation of diabetic eye disease, there is accumulating evidence that other vascular changes precede the development of microaneurysms. For instance, patients with diabetes and no or mild retinopathy exhibit reduced arterial and venous vaso-

dilation in response to flickering light, which may reflect underlying endothelial or astrocyte dysfunction (Garhofer et al., 2004). Patients with type 1 diabetes and no clinical retinopathy also show absent constriction or paradoxical dilation of retinal arterioles in response to increased perfusion pressure, an indication that the myogenic response and its protective effects toward the downstream capillaries and neural parenchyma are jeopardized before the appearance of clinical retinopathy (Lorenzi et al., 2010). Furthermore, adolescent patients with type 2 diabetes and no clinical evidence of retinopathy have significantly larger retinal venule diameters than individuals without diabetes (Bronson-Castain et al., 2012). These changes in blood vessel diameter could potentially be linked to alterations in basal blood flow early in diabetes, but this relationship requires further exploration. In light of new understanding of the neurovascular unit, it is likely these early changes in vascular function are linked to alterations of the neurosensory retina (Kur et al., 2012).

In addition to the vascular alterations occurring outside the vessel lumen in the diabetic retina, diabetes also promotes leukostasis within the vasculature early in the disease course. Intercellular Adhesion Molecule 1 (ICAM-1) and P-selectin expression by endothelial cells are upregulated in diabetes, causing leukocytes to accumulate within the choroid and other retinal blood vessels even before the overt clinical signs of DR (e.g. microaneurysms) develop (McLeod et al., 1995). Leukostasis may be an early event in the subsequent activation of an inflammatory cascade in the diabetic retina, leading to diapedesis and disruption of the blood retinal barrier (Kern 2007). The inciting metabolic and molecular factors that trigger such an inflammatory response in diabetes remain to be elucidated.

Early neural changes

When Dr. Jaeger first described the characteristic retinal lesions of diabetes using ophthalmoscopy, he did not have at his disposal the wide array of clinical tools and molecular techniques that are today a part of the clinicians' and scientists' armamentarium. The development and use of electroretinography (ERG), optical coherence tomography (OCT), automated perimetry, and the techniques of molecular biology have allowed us to gain a deeper understanding of how diabetes affects the neural retina.

Outer retina

The blood retinal barrier is maintained by two distinct networks of cells. The retinal pigment epithelial (RPE) cells, bound together by tight junctions, constitute the outer blood retinal barrier. Additionally, non-fenestrated capillaries, whose endothelial cells are also connected to each other via tight junctions, form the inner blood retinal barrier (Gardner and Antonetti, 2008). Evidence for disruption of one or both components of the blood retinal barrier in early diabetes comes from an experiment using ocular fluorophotometry in diabetic monkeys who had no evidence of DR by ophthalmoscopy or fluorescein angiography. In this study, insulin dependent diabetic monkeys who were given intravenous injections of fluorescein were found to have significantly higher posterior vitreous levels of fluorescence than their control counterparts, suggesting that the blood retinal barrier's integrity was compromised (Jones et al., 1986).

Photoreceptor function also appears to be altered early in the course of diabetes. Specifically, diabetes is associated with dysfunction (Yamamoto et al., 1996) and perhaps even death (Cho et al., 2000) of blue sensitive S-cones in humans, which may explain the tritan-like color vision defect observed in

diabetic patients. While inhalation of 100 percent oxygen seems to improve the color vision defects associated with diabetes, the underlying cause of these defects remains unknown (Dean et al., 1997). Other tests of photoreceptor function, such as dark adaptation (Jackson et al., 2012) and the multifocal electroretinogram (mfERG) (Harrison et al., 2011), have yielded abnormal results in diabetic patients without clinically evident retinopathy. More precisely, those with diabetes and no or mild retinopathy require more time to adapt to the dark after a light flash than those without diabetes. Similar to abnormalities in color vision, dark adaptation appears to improve when diabetic patients inhale 100 percent oxygen (Kurtenbach et al., 2006 and Frost-Larsen 1991). Persons with diabetes and clinically absent retinopathy also exhibit prolongations in the implicit time of the mfERG (defined as the time between stimulus onset and the first positive peak in the ERG waveform). Importantly, the retinal areas that exhibit this increase in implicit time often develop vascular lesions characteristic of DR (e.g. microaneurysms, hemorrhages, etc.), suggesting that the neural dysfunction in the outer retina may precede clinically observable vascular anomalies in DR.

Inner retina

Studies in both human and animal models of diabetes have revealed that retinal ganglion cells die early in the disease course (Barber et al., 1998 and Hammes et al., 1995) and that their function may be altered before the onset of the classic vascular lesions associated with DR (Parravano et al., 2008). Ganglion cells play an important role in integrating the electrochemical information that they receive from bipolar cells and amacrine cells before such messages are relayed to the rest of the brain. In this way, ganglion cells help us to perceive edges and the contrast between light and dark. In fact, contrast sensitivity is reduced in patients with diabetes who have no overt evidence of retinopathy on clinical examination (Gualtieri et al., 2011). An impairment of ganglion cell function during diabetes has also been detected with other tests. The amplitude of the pattern ERG (Prager et al., 1990) and the amplitude of the scotopic threshold response of a full field ERG (Aylward, 1989) are both reduced in patients with diabetes and no or minimal retinopathy. Furthermore, mean sensitivity of the visual field test known as frequency-doubling technology (FDT) perimetry is also diminished early in the course of DR (Parravano et al., 2008 and Jackson et al., 2012). Finally, OCT measurements of retinal thickness show that diabetic patients have thinner ganglion cell layers and nerve fiber layers than their non-diabetic counterparts, even in the early stages of the disease (van Dijk et al., 2010) and especially around the macula (Park et al., 2011). One clinical trial is currently investigating whether or not the neuroprotective agents somatostatin and brimonidine can prevent or slow the neurodegenerative changes associated with diabetic retinopathy (Cunha-Vaz 2012).

The neurovascular retina in early diabetes

Glia

Glial cells are the “glue” that hold the neurovascular retina together. They protect neurons, maintain homeostasis, and mediate interactions between blood vessels and neurons. They play a vital role in maintaining the integrity of the neurovascular retina, and substantial work indicates that they are affected early in the course of diabetes.

Müller cells span the entire retina, from external limiting membrane to internal limiting membrane,

and play an important role in potassium and neurotransmitter regulation as well as the maintenance of the blood retinal barrier (Fletcher et al., 2007). Müller cells participate in the process of gliosis in diabetes, which refers to the cytologic changes observed in glial cells that typically occur as a response to neuronal injury. However, the temporal and spatial relationship between gliosis, vascular compromise, and neuronal dysfunction in diabetes is a puzzle that has yet to be solved.

Like Müller cells, astrocytes are macroglia that mediate the interactions between neurons and the vasculature. Astrocytes are found in the nerve fiber layer of the retina and have processes that wrap around inner retinal blood vessels. Thus, astrocytes (along with pericytes) may play a role in regulating blood flow to and from the neural retina.

Microglia are the resident macrophages of the central nervous system and retina. They phagocytose cellular debris in the non-inflamed state but become activated during diabetes. In their activated state, microglia release proinflammatory and cytotoxic substances that can damage the retina (Zeng et al., 2008). It will be important to identify the mechanisms through which microglia are activated in diabetes, as inhibiting these pathways may reduce retinal inflammation and the neuronal dysfunction associated with DR.

Concomitant measures of neural and vascular alterations

"No [cell] is an island entire of itself…."

As John Donne tried to impress upon us in Meditation XVII, we are each a part of a larger whole that is likely greater than the sum of its parts. Similarly, the retina is an exquisite collection of neuronal, glial, and vascular tissue, and each cell within the retina has important spatial and physiological relationships with its neighbors (Figure 1-1). While studies of specific cell types in DR have provided us with important clues about how the disease affects vision and the retina, the way forward will likely involve simultaneous assessments of retinal neurovascular structure and function. Unraveling the relationship between the retinal vascular and neural abnormalities induced by diabetes may eventually allow us to develop therapies that will address both the vascular and neuronal dysfunction characteristic of DR, thus providing diabetic patients with a more comprehensive means of combating vision loss from this dreadful affliction.

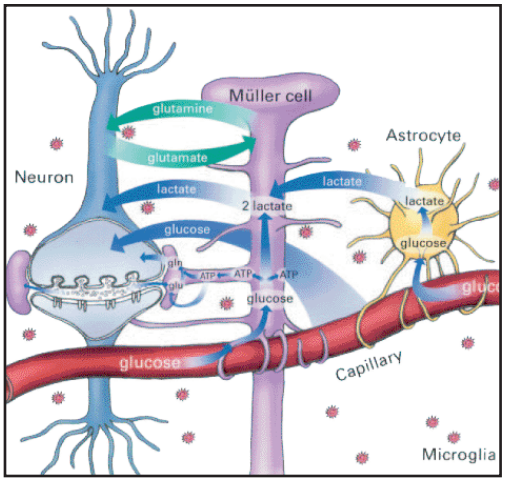

Figure 1-1: The neurovascular retina is composed of neuronal, glial, and vascular cells, and each cell within the retina is intimately connected (anatomically and physiologically) to its neighbors. The myriad connections and interactions among these cells create the visual experience. (Figure courtesy of Thomas Gardner, taken from Diabetes 2006:55:240 (used with permission).

Proposed studies

1) There is a need for better characterization of the relationship between the neural and vascular abnormalities that are occurring in early diabetes (in both animals and humans). For example, future studies might evaluate whether choriocapillaris dropout precedes dysfunction and death of S cones and whether there is a spatial relationship between these phenomena.

2) What are the earliest retinal changes that are induced by diabetes? By modulating these changes, can we slow the progression of retinopathy? For example, if basal blood flow is reduced early in the course of DR, can we give a drug to increase blood flow and thus reduce the burden of disease?

3) Do any of the early signs of DR worsen in a predictable fashion that would make them amenable to use as sensitive markers of disease progression? For example, are there specific cytokines, serum markers, or white blood cell counts that correlate with disease severity and that might be used to predict the progression of retinopathy?

4) Is retinal dysfunction occurring as a result of multiple insults happening in parallel or is retinal dysfunction in diabetes occurring as a result of a single insult that drives the other deleterious pathways?

5) What are the early adaptive responses seen in animals or patients with diabetes and how can we prolong these responses to slow the progression of DR?

6) In order to better characterize the entire phenotype of DR, researchers will need to conduct natural history studies of young patients with diabetes who do not yet have any comorbidities (e.g. hypertension) that could confound the measurements of retinal neurovascular structure and function.

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Chapter 2
Role of Glucose, Lipids and Oxygen in Diabetic Retinopathy

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Prior to discussing the role of glucose and oxygen in DR, it was necessary to define what symptoms constitute DR. This generated substantial discussion, since retinopathy currently is defined clinically by vascular lesions (microaneurysms, increased vascular permeability, retinal edema, and neovascularization), whereas impaired visual function (which undoubtedly involves neural cells of the retina) is a major reason for clinical interest in retinopathy. The spectrum of lesions is not unique to diabetes, as these same lesions develop also in nondiabetic patients, although much fewer in number than in persons with diabetes. The lesions of DR are morphologically similar in type 1 diabetes and type 2 diabetes, but some of the risk factors for developing the retinopathy differ between these disease types.

Role of glucose and glycemic control

Clinical trials and animal studies have established that hyperglycemia is strongly associated with the severity of retinopathy, and that improved metabolic control of diabetes significantly inhibits the development and progression of diabetic retinopathy (at least in the background stages of the retinopathy) (Diabetes Control and Complications Trial Research Group, 1993; Klein et al., 1988; United Kingdom Prospective Diabetes Study, 1998), indicating that glycemia is at least strongly associated with abnormalities that are critical in the pathogenesis of the nonproliferative stages of the retinopathy. Beneficial effects of improved metabolic control (also called improved glycemic control) in diabetes, however, are not adequate to establish that hyperglycemia is the sole cause of the retinopathy. In addition to the well-recognized hyperglycemia present in poorly controlled diabetes, there are also other alterations that occur secondary to insulin deficiency or resistance, for example, in lipid metabolism and amino acid levels, which might contribute to the retinopathy. Reports indicate that variations in glucose levels might cause more abnormalities of cellular structure or function than does chronic hyperglycemia, and there is speculation that such variability (which is common in many diabetic patients) might accelerate development of diabetic complications, including retinopathy (Monnier et al., 2007).

The evidence that most clearly implicates sugar *per se* in initiating the pathogenesis of the retinopathy is the demonstration that nondiabetic animals (dogs, rats and mice) made experimentally hyperhexosemic by feeding a galactose-rich diet develop retinal vascular lesions that are morphologically identical to those seen in diabetes, yet do so in the absence of the other metabolic sequelae that are characteristic of diabetes (Engerman and Kern, 1984). It remains unknown, however, whether the experimental galactosemia reproduces all early changes seen in the retina in diabetes (biochemical abnormalities inducing upregulation of VEGF and inflammatory mediators, alterations in retinal blood flow and oxygen tension, etc.).

It has been demonstrated in diabetic patients and animals that vascular lesions occur more frequently in the superior and temporal regions of the retina than in other quadrants of the same retina (Cunha-Vaz, 1972; Kern and Engerman, 1995; Taylor and Dobree, 1970). Since the entire retina presumably is exposed to the same blood glucose concentration, this finding suggests that elevated glucose is not by itself adequate to account totally for the severity of vascular pathology. It is unclear what accounts for the variation among regions of the retina.

Metabolic memory

In both patient and animal studies, DR can be better inhibited with improved glycemic control if started from the onset of diabetes (prevention) as opposed to after prolonged hyperglycemia (intervention) (Diabetes Control and Complications Trial Research Group, 1993; Engerman and Kern, 1987; Hammes et al., 1993; Holman et al., 2008). This resistance to intervention has been referred to as "metabolic memory." Intervention with improved glycemic control in early DR does eventually have beneficial effects to slow down progression of the retinal disease, but such intervention has not been found to inhibit the proliferative phase of the retinopathy. Whether or not retinal edema is influenced by metabolic memory has not been established. Besides the retina, metabolic memory occurs in other tissues developing pathology in diabetes. The molecular mechanism for metabolic memory and the types of cells mediating metabolic memory are under investigation by several labs (El-Osta et al., 2008; Madsen-Bouterse et al., 2010).

Molecular sequelae of diabetes or hyperglycemia

Numerous biochemical pathways activated in hyperglycemia have been linked to the development of at least some lesions characteristic of DR. Taken together, these findings suggest that hyperglycemia produces deleterious changes to retinal cells, leading to vascular and neuroglial dysfunction. Identification of molecular steps by which diabetes or hyperglycemia cause many of these abnormalities, however, needs additional research. Molecular sequelae of diabetes and hyperglycemia that have been postulated to contribute to DR include:

1) Advanced Glycation Endproduct (AGE) formation is increased in diabetes. AGEs are formed by non-enzymatic reactions between aldehydes (like glucose and other carbohydrates in the open chain form) or reactive dicarbonyls (methylglyoxal, glyoxal) and free amino groups on proteins, lipids, and nucleic acids. AGEs develop extracellularly and intracellularly. Extracellular AGEs interact with AGE receptors (notably RAGE), and evoke vascular inflammation, macrophage activation, and prothrombotic endothelial activation. AGE-RAGE activation also has been linked to reduced survival signals and oxidative stress. Inhibition of RAGE signaling blocks diabetes-induced defects in electroretinogram function and capillary degeneration (Barile and Schmidt, 2007; Li et al., 2011). Inhibition of AGE formation or signaling via RAGE is a potential therapeutic approach currently investigated for human trials. Work in retinal Müller cells suggested that inhibition of AGEs with pyridoxamine reduced oxidative stress and glial fibrillary acidic protein levels (Curtis et al., 2010). Methylglyoxal is generated intracellularly in all mammalian cells via enzymatic and non-enzymatic pathways, with the majority of MG production occurring via the glycolytic pathway.

2) Diabetes is known to cause oxidative stress in the retina, and this abnormality results from increased generation of reactive oxygen species (via mitochondria and NADPH oxidase) and decreased activity of antioxidant enzymes. Mitochondrial-generated superoxide can impair glucose metabolism in a variety of cell types, potentially leading to activation of several molecular pathways that have been linked to diabetic complications (Du et al., 2003; Nishikawa et al., 2000). Recent studies suggest that the oxidative stress in hyperglycemia might come also from uncoupled nitric oxide synthases or arginase I or NADPH oxidase. Inhibition of the oxidative stress with oral antioxidants or over-expression of antioxidant enzymes *in vivo* has been shown to inhibit early stages of the retinopathy in animals (Berkowitz et al., 2009; Kanwar et al., 2007; Kowluru et al., 2001). Antioxidants have not been found clinically to have strong effects to inhibit DR, although the retinopathy has not been the primary focus of those studies. The ability of newer or stronger antioxidants to inhibit diabetic retinopathy merits future study.

3) A number of studies have demonstrated that diabetes causes numerous pro-inflammatory changes in the retina, and inhibition of these local abnormalities by a variety of approaches has been found to inhibit the retinopathy in animal models. VEGF, which is known to be an important therapeutic target in DR, is one of the pro-inflammatory molecules induced in the retina by diabetes. Circulating white blood cells, which are important in inflammatory process throughout the body, also are being recognized as important contributors to the development of DR (Li et al., 2012).

4) Growth factors and protein kinases. Evidence is strong that VEGF plays an important role in retinal neovascularization in diabetes, but clinical administration of VEGF inhibitors in diabetes have had less dramatic effects on retinal edema. The contribution of other growth factors to DR is still unclear. Alterations in growth factors likely are not direct sequelae of diabetes or hyperglycemia, but are caused by alterations of other pathways, such as involving protein kinases. Effects of PEDF in the retina have seemed to be opposite of VEGF. Inhibition of several different protein kinases is being found to have important effects on metabolic abnormalities in the retina of diabetic animals, and clinical studies of diabetic patients have shown beneficial effects of PKC inhibition on visual acuity, but not other lesions of the retinopathy.

5) Data are accumulating indicating that diabetes alters *wnt* pathway signaling and decreases β-adrenergic receptor signaling in the retina. Metabolic causes of these changes and how they contribute to the retinal disease are not yet clear. Therapies have been devised for both of these abnormalities with these therapies showing beneficial effects on early retinopathy in animals.

Role of lipids

Previously, studies of the pathogenesis of DR focused almost solely on hyperglycemia. Recent studies suggest that lipids can contribute to the retinopathy and therapeutic manipulation of lipids might offer effective therapies to inhibit the retinopathy. The severity of retinopathy in individuals with type 1 diabetes has been found to correlate with serum lipids, even within the normal range (Lyons et al., 2004). Treatment of individuals having type 2 diabetes mellitus with the lipid-lowering agent, fenofibrate, reduced the need for laser treatment for DR, although the mechanism of this effect seemed not to be related to plasma concentrations of lipids (Keech et al., 2007).

Diabetes has been shown to decrease key retinal fatty acids docosahexaenoic acid (DHA), very-long-chain polyunsaturated fatty acids (PUFAs), and retinal fatty acid elongases, while increasing the retinal plasma membrane lipid degradation enzyme, acid sphingomyelinase. These changes in the fatty acid status of the diabetic retina were associated with increased levels of inflammatory markers, interleukin-6, VEGF, and ICAM-1 (Tikhonenko et al., 2010). Dietary supplementation with DHA has been reported to inhibit diabetes-induced abnormalities in the retina (including vascular inflammation and capillary degeneration in type 1 diabetic animals (Opreanu et al., 2011)), but a higher dose accelerated retinal disease (Hammes et al., 1996). Much work needs to be done to better address the contribution of lipids to the development of diabetic retinopathy.

Role of oxygen

A prevalent belief among clinicians and researchers has been that progressive vascular disease results in decreased oxygen tension in the inner retina, leading to local hypoxia, and subsequent induction of VEGF with its vaso-proliferative and vaso-permeability effects. Laser photocoagulation is known to significantly inhibit progression of advanced retinopathy to neovascularization and vision loss, and might do so in part by increasing oxygen availability (due to killing of large numbers of photoreceptors and pigment epithelium). Diabetes-induced decreases in retinal oxygen tension and dilation of retinal blood vessels are reversed following laser photocoagulation (Stefansson, 2006). Oxygen therapy has been demonstrated in a small number of patients to have a beneficial effect on diabetes-induced retinal edema, suggesting that hypoxia contributes to retinal edema in diabetes (Nguyen et al., 2004). Low levels of light during sleeping to prevent complete dark adaptation (thereby reducing oxygen consumption by rods) have been reported to improve visual acuity (Arden and Sivaprasad, 2012). Evidence in diabetic patients and animals suggest that retinal photoreceptors might be contributing to the development of retinal hypoxia in diabetes. Photoreceptors utilize large amounts of oxygen to maintain their normal function, and loss of some or all of those photoreceptors in rhodopsin-deficient mice (de Gooyer et al., 2006) or in patients with retinitis pigmentosa (Arden, 2001) inhibited the diabetes-induced loss of retinal capillaries.

There has been disagreement relating to how soon retinal hypoxia develops in DR. Tools to measure retinal oxygen tension noninvasively have been under development, but those methods are not yet capable of focusing on the small vessels that deliver the bulk of O_2 to tissues.

Questions and needs for the future

1) Why do retinal complications of diabetes require years to develop, and why are they resistant to arrest? How can these observations be explained on a molecular basis? Which cell types show metabolic memory in the retina?

2) Is there a relationship between diabetes-induced abnormalities in neural retina and retinal vasculature in diabetes? To what extent do alterations in neural cell function and viability affect the vasculature, and vice versa? Are the early neuronal or permeability changes relevant in the development or progression of long term complications?

3) What is the relationship between diabetic macular edema and altered vascular permeability in diabetes, and what are the molecular mechanisms for these abnormalities?

4) Biomarkers to predict which diabetic patients will progress to sight-threatening DR would have benefits both in clinical care and design of clinical trials.

5) Banking and distribution of both patient and animal tissues needs to be improved. Human eyes are routinely collected hours post mortem, during which time ischemia likely leads to altered cellular levels of mRNA and proteins (in particular, cytokines). With respect to laboratory-based research, unused tissues from animal experiments in laboratories willing to share could be offered to others. Open access to unused tissues and organs could expand the amount of information gained from a given set of animals.

6) Diabetes or elevated glucose has been found to cause numerous abnormalities in signaling or survival in retinal endothelial cells, pericytes, glial and ganglion cells. Recent evidence is beginning to implicate also bone marrow-derived cells and photoreceptors in alterations of the retina in diabetes, but the full picture of which cell types and how they interact to cause the various lesions of DR is not yet known.

7) Future experimental studies need to test effects of intervention on DR (instead of conducting only prevention studies), and to the extent possible, clinical studies should test effects of therapies from the onset of diabetes (although it is difficult to quickly identify onset of type 2 diabetes patients). Does the concept of metabolic memory pertain also to type 2 diabetes, and are there therapies that inhibit metabolic memory?

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Chapter 3
Diagnostic Methods

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What diagnostic methods have been developed for diabetic retinopathy?

The invention of the direct ophthalmoscope in 1851 by Hermann von Helmholtz (Helmholtz, 1851) gave ophthalmologists unprecedented visual access to the inner eye, and advances in clinical ophthalmoscopy since that time have allowed physicians to characterize many of the retinal changes associated with type 1 and type 2 diabetes mellitus. The introduction of the Snellen visual acuity chart in 1862 gave physicians a more uniform method for assessing visual function in patients with DR and other vision disorders (Snellen, 1862). Subsequent advances in technology, such as magnetic resonance imaging (MRI), multifocal electroretinography (mfERG), laser Doppler flowmetry, optical coherence tomography (OCT) and adaptive optics (AO) have revealed that diabetes affects the entire visual system, from microscopic vascular and neural changes in the retina at the level of a single cell, to subtle alterations in visual function. Nevertheless, there remains a need for better assessments of retinal structure and function that can be used to precisely and accurately document the earliest changes in DR, track the progression of the disease and evaluate response to therapy.

What structural and functional imaging modalities are available for clinical use in patients with diabetes?

Diabetes affects the entire neurovascular retina, and the earliest observable changes in DR are still under investigation. Currently, fundus photography and clinical ophthalmoscopy are used to monitor the progression of DR (from loss of autoregulation to vascular histopathology) and response to treatment. Techniques such as laser Doppler blood flowmetry have demonstrated early changes in retinal hemodynamics in patients with diabetes (Pournaras et al., 2008). Quantitative fundus imaging techniques such as the retinal vessel analyzer detected defects in retinal vessel constriction and dilation response in diabetes which may precede loss of autoregulation and the classic clinical manifestations of DR (Garhofer et al., 2004; Lorenzi et al., 2010). Imaging techniques such as OCT, AO, and MRI can detect retinal neurovascular abnormalities in diabetic patients before the onset of clinically observable vascular lesions, suggesting that these imaging methods could be helpful in assessing early DR in humans as well as small animals (Parravano et al., 2008; Tam et al., 2011; Trick et al., 2008).

The field of OCT has been advancing rapidly since its invention in 1991, (Drexler and Fujimoto, 2008; Huang et al., 1991) and many studies have used OCT to assess the effect of diabetes on retinal structure. Investigations have shown that diabetes is associated with an early thinning of the ganglion cell and nerve fiber layers of the retina (Parravano et al., 2008; van Dijk et al., 2010), a finding which

parallels OCT measurements in glaucoma patients. These structural abnormalities appear to precede the development of the classic microvascular lesions associated with diabetes. The next generation OCT instruments will be capable of scanning the retina at rates approaching several hundred thousand axial scans/second, more than ten times faster than current clinical instruments (Figure 3-1) (Srinivasan et al., 2008). This increase in speed will facilitate the acquisition of 3D volumetric data which contains additional information about retinal structure and pathology.

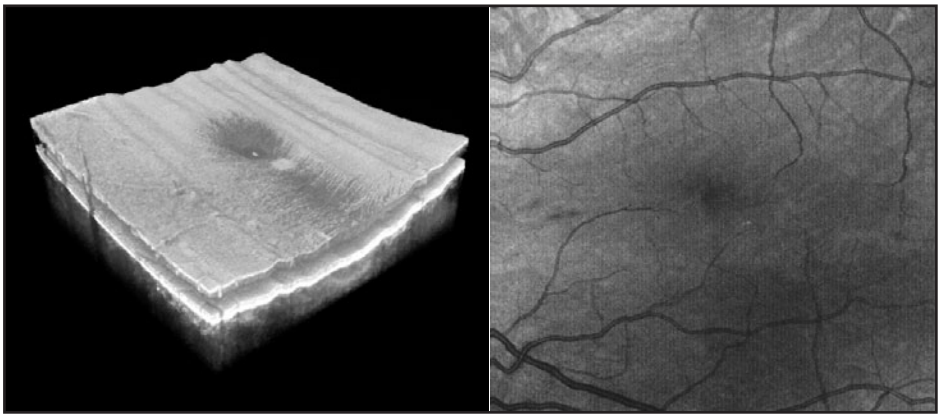

Figure 3-1: Advances in optical coherence tomography (OCT) enable comprehensive 3D imaging of retinal structure and pathology. Example shows next generation swept source / Fourier domain OCT at 580,000 A-scans per second, 10-20x faster than commercial systems. Left panel. 3D-OCT of the macula consisting of 1,700 x 700 A-scans acquired in 1.8 seconds. Right panel. OCT fundus projection image obtained from 3D-OCT data. Volumetric data sets will permit sensitive measurement of changes in focal pathologies with disease progression. (Courtesy of I. Grulkowski and J. Fujimoto, M.I.T.)

In addition to providing information about structure, Doppler OCT can measure total blood flow as well as changes in blood flow within specific areas of the retina (Chen et al.,1997). Preliminary studies using circumpapillary OCT imaging of the central retinal vessels demonstrate that total retinal blood flow is decreased in patients with treated proliferative DR compared to control subjects (Wang et al., 2011). With greater imaging speed and the ability to assess hemodynamic functional parameters in the retina, next generation OCT instruments promise to enable the detection of early retinal neurovascular changes occurring during diabetes.

AO is an emerging optical imaging method for achieving ultrahigh resolution retinal imaging which has been adapted from astronomy to correct for optical aberrations and has the potential to achieve diffraction-limited resolutions. In ophthalmology, AO can compensate for the inherent optical aberrations of the eye that limit the transverse resolution of traditional retinal imaging methods, thereby achieving diffraction limited resolutions (Liang et al., 1997; Williams, 2011). The use of AO along with other imaging tools, such as scanning laser ophthalmoscopy (SLO) and OCT, has enabled the *in vivo* assessment of individual photoreceptors as well as capillary level blood flow within the retina (Liang et al., 1997; Martin and Roorda, 2005; Zhong et al., 2011) (Figure 3-2). In adults with type 2 diabetes, AO-SLO imaging demonstrated that arteriovenous channels are disrupted even before the onset of clinically evident retinopathy (Tam et al., 2011). The unprecedented resolution AO images,

approaching the level of individual cells, will aid in the identification of early, previously undetectable retinal changes developing during diabetes.

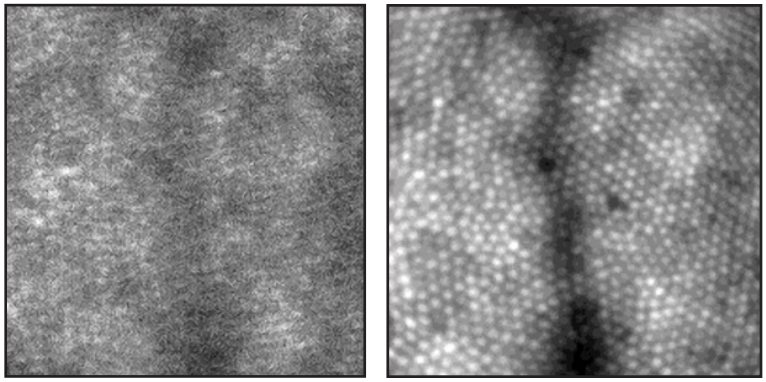

Figure 3-2: Adaptive optics (AO) corrects for optical aberrations in the eye, enabling the acquisition of much higher resolution images. Left panel: Image of the fovea without adaptive optics. Right panel: Image obtained using adaptive optics showing dramatic improvement in resolution. Individual cone photoreceptors are visible, with an overlying shadow from an anterior blood vessel. (Photos adapted from cfao.ucolick.org/pgallery/vision/php (The UC Center for Adaptive Optics)).

Magnetic resonance imaging (MRI) is another important technology that is a mainstay of neurology and cancer research. In ophthalmology it provides important information about retinal and visual brain structure as well as key neuroretinal functions of L-type voltage gated calcium channel activity which cannot be obtained using optical imaging (Bissig and Berkowitz, 2011; Trick et al., 2008). The throughput of MRI remains a limitation for routine clinical studies; however it is readily translatable from small animal preclinical to clinical phase II drug studies and is expected to be useful as a significant research imaging modality.

What psychophysical assessments and measures of visual function are available for use in patients with diabetic retinopathy?

Since alterations in visual function from diabetes tend to precede structural changes (as measured by current clinical imaging technologies), psychophysical tests that measure visual function are especially important. Examples of promising tests for the diagnosis and treatment monitoring of DR include the multifocal electroretinogram (mfERG), frequency doubling technology (FDT) perimetry and flavoprotein autofluorescence.

Prior studies have shown that the mfERG implicit time (defined as the time between stimulus onset and the first positive peak in the ERG waveform) is prolonged in patients with diabetes (Bronson-Castain et al., 2012; Harrison et al., 2011). Furthermore, retinal areas that exhibit implicit time prolongation are more likely to develop retinal vascular lesions compared to other retinal areas with normal implicit times (Harrison et al., 2011) (Figure 3-3). Indeed, retinal neural dysfunction in diabetic patients is

useful in predicting the sites at which vascular abnormalities will develop in the future. Thus, markers such as mfERG implicit time are a powerful early indicator of DR.

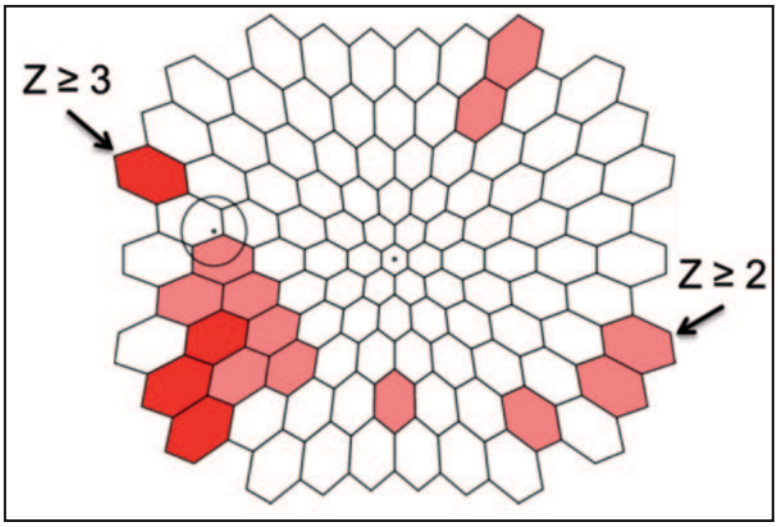

Figure 3-3: The multifocal electroretinogram detects delays in implicit time in subjects with diabetes who have no or minimal retinopathy. The functional map above shows that specific regions of the diabetic retina (red and pink hexagons) had abnormally long (≥ 2 standard deviations from the control mean) implicit times. These areas of neuronal dysfunction often developed vascular lesions later in the course of diabetes. Photo courtesy of Marcus Bearse Jr, PhD, University of California, Berkeley).

FDT is a visual field test where the stimulus consists of vertically oriented bars that alternate in color from white to black and black to white. The name for the test is derived from the observation that the number of bars appears to double above a specific rate of black to white and white to black transitions. Cross sectional studies using FDT in patients with no or mild retinopathy revealed abnormal visual field patterns compared to controls (Jackson et al., 2011; Parravano et al., 2008), and in one study the test demonstrated an 83 percent sensitivity for detecting patients with non-proliferative DR (Jackson et al., 2011). FDT is currently being evaluated in longitudinal studies to determine if the visual fields worsen in a predictable manner in patients with diabetes. If this hypothesis is correct, FDT might serve as a clinical trial endpoint to evaluate novel therapies for DR.

Other forms of functional imaging can assess visual function at the molecular level. In diabetes, hyperglycemia increases oxidative stress and induces apoptosis of cells within the retina (Du et al., 2003). Before apoptosis, mitochondrial flavoproteins are oxidized and can absorb blue light and emit green fluorescent light (Benson et al., 1979). The oxidation of flavoproteins is a marker of metabolic stress, and the relative level of fluorescence that results from shining blue light on these proteins has been shown to be abnormally high in people with diabetes compared to healthy controls (Field et al., 2008). Therefore, flavoprotein autofluorescence is another example of a promising technique to assess early DR.

What structural and functional imaging modalities are available for preclinical (animal) use?

SLO, OCT, AO, and MRI can be used in both preclinical and clinical applications, which facilitates the translation of research findings from animals to humans. However, imaging in animal models has powerful advantages because exogenous contrast agents can be used to investigate the molecular and cellular pathways of disease.

By using a functional contrast agent, manganese enhanced MRI (MEMRI) can analytically characterize neuroretinal dysfunction in diabetes as well as retinal water content and thickness and breakdown of the blood retinal barrier to study retinal edema, as well as changes in retinal oxygenation (Figure 3-4) (Bissig and Berkowitz, 2011; Trick et al., 2008).

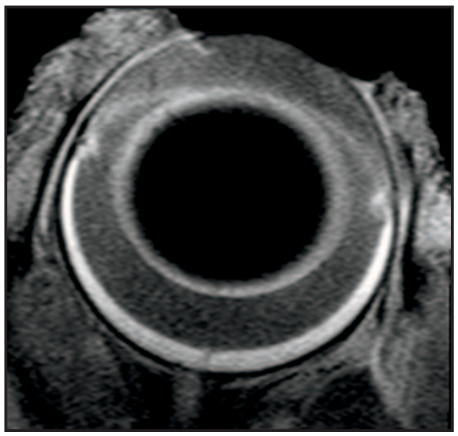

Figure 3-4: Manganese enhanced MRI (MEMRI) cross-sectional view of the mouse eye. Mn(2+) uptake in the retina can be used to measure functional neural response *in vivo*. (Photo courtesy of Bruce Berkowitz, PhD, Wayne State University School of Medicine).

The combination of exogenous fluorophores with molecular probes is a powerful approach for preclinical studies. One example, the detection of apoptosing retinal cells (DARC) which can be used to optically assess cellular dysfunction in animal models of diabetes, uses an exogenous contrast agent (Figure 3-5). Fluorescently labeled annexin V has a high affinity for phosphatidylserine, a molecule which manifests in the outer plasma membrane of a cell during apoptosis, but not during necrosis (Coxon et al., 2011). Since annexin V is approved for human use, these studies can also be translated from animal to human.

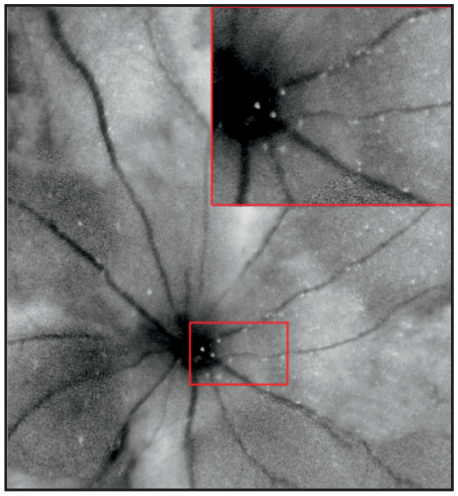

Figure 3-5: Detection of apoptosing retinal cells (DARC) is an *in vivo* method of assessing cellular function in animal models of diabetes. The fluorescent points in the above fundus photo indicate areas of active ganglion cell apoptosis. (Photo courtesy of M. Francesca Cordeiro, MD, PhD, UCL Institute of Ophthalmology).

In addition to the use of molecular contrast, other imaging techniques can be applied in animal models of diabetes. For example, OCT can assess changes in retinal structure and can quantify retinal edema in diabetic rodent models (Gao et al., 2007; Srinivasan et al., 2006). The combination of AO and multiphoton microscopy imaging enables *in vivo* resolution at the cellular level in animal eyes (Bueno et al., 2011; Palczewska et al., 2010). Multiphoton microscopy uses the light emitted from endogenous or exogenous fluorophores to perform high-resolution three dimensional imaging (Denk et al., 1990). By using femtosecond laser pulses to excite fluorescence localized only at the laser focus, multiphoton microscopy enables micron scale optical sectioning to visualize different cellular layers in the intact animal retina. AO is required for multiphoton imaging in the rodent eye in order to overcome optical aberrations and achieve a diffraction limited laser focus. Despite the potential of multiphoton imaging, it currently requires laser pulse intensities which are too high for use in humans. However, the combination of exogenous functional contrast agents with high resolution imaging in the animal eye is extremely powerful because it will facilitate the investigation of molecular, genomic and proteomic pathways of disease *in vivo*.

Small animal optical imaging achieves resolutions approaching that of histopathology, but without the need to sacrifice animals. In contrast to histological approaches, these techniques will enable the study of the dynamics of cellular responses as well as repeated longitudinal assessments to investigate disease progression and response to therapy. Therefore, developments in small animal imaging techniques are greatly accelerating fundamental research.

What methods are available to assess retinal oxygenation in humans or animals?

Hypoxia was implicated in the pathogenesis of DR decades ago (Ashton, 1963; Ditzel and Standl, 1975), and it is now known that hypoxia is a potent trigger of vascular endothelial growth factor (VEGF) synthesis (Shweiki et al., 1992). The success of anti-VEGF therapies in the treatment of diabetic macular edema and proliferative DR argues strongly for a role of hypoxia in the progression of diabetic eye disease. However, the progression of retinal hypoxia in humans with diabetes is currently unclear, as is the role that hypoxia plays in causing some of the early manifestations of DR.

Current methods to assess retinal oxygenation include intraretinal microelectrodes, MRI and retinal oximetry. Microelectrodes are the most direct means of measuring oxygenation within the retina, but are invasive and their use is restricted to animal models. MRI can be performed in both animals and humans and provides an indirect assessment of retinal oxygen tension by measuring vitreous oxygen tension in response to hyperoxic provocation. MRI studies demonstrated that vessel autoregulation is impaired early in diabetes (Trick et al., 2008). There are several optical imaging methods for retinal oximetry which can be applied in both animals and humans. These methods provide an indirect assessment of retinal oxy-genation by spectroscopically measuring oxy- and deoxy-hemoglobin absorption in the retinal vasculature (Delori, 1988; Hardarson et al., 2006; Khoobehi et al., 2004). However, calibration of spectroscopic information to obtain accurate and reproducible retinal oxygenation remains challenging because light scattering from the RPE causes spectroscopic signals to be obscured. Techniques such as the retinal functional imager (Nelson et al., 2005) or spectroscopic OCT (Faber et al., 2003) use time or depth resolved spectroscopic imaging in an effort to improve accuracy. Continued research and advances in non-invasive techniques that can accurately measure the partial pressure of oxygen across the entire retina may contribute to our understanding of the pathogenesis of DR.

How can we integrate visual function testing with structural/functional imaging to improve our understanding of diabetic retinopathy?

The combination of imaging methods such as OCT and AO with measures of visual function such as FDT, mfERG and MRI can provide researchers with an unprecedented means of assessing how diabetes affects retinal structure and function. For visual function, emphasis should be placed on developing tests that go beyond the classic measurement of visual acuity by providing maps of retinal response at different fundus locations. Structural/functional imaging modalities can then be used in conjunction with psychophysical tests of visual function to correlate abnormalities in retinal structure and visual function across the fundus. This approach could be an effective means of establishing the relationship between morphologic retinal abnormalities and retinal dysfunction during diabetes. Additionally, longitudinal analyses of retinal structure and function in patients with diabetes could lead to better ways of diagnosing DR, tracking disease progression, and monitoring response to therapy.

Future directions for diagnostic methods in diabetic retinopathy

1) Priority. The rationale for developing new imaging techniques (or refining existing ones) is that the detection of early neurovascular retinal abnormalities in diabetes could lead to the identification of predictors of clinical retinopathy. In turn, these predictors could serve as surrogate clinical trial endpoints for the development of adjunct interventions, thus shortening the time required to conduct clinical trials. Toward such goals, an immediate priority is to investigate and validate the predictive potential of techniques and measurements that are already available and have yielded promising information, such as OCT, FDT, mfERG and MRI. With regard to the development of newer tests, measures of photoreceptor, ganglion cell, and glial cell morphology and function would be especially useful as researchers attempt to unravel the connection between vascular and neural pathology in DR.

2) *In vivo* Functionality. Since many analyses of retinal structure and function in animal models of diabetes require sacrificing the animal, the development of *in vivo* assessments of the visual system is vitally important for testing novel therapies and improving our understanding of the natural history of DR. As previously noted, fluorescently labeled Annexin-5 has been used to measure ganglion cell apoptosis *in vivo* in diabetic rats and is an example of a technique which assesses molecular and cellular pathways of disease *in vivo*. The combination of developing novel *in vivo* imaging modalities and functional contrast agents would dramatically accelerate fundamental research.

3) Testing and Resources. It could be useful to identify 2 to 5 clinical research centers to investigate the natural history of DR using combinations of state-of-the-art technologies such as OCT, AO, MRI, FDT, mfERG, etc. in a consistent manner. These natural history studies would ideally involve young, otherwise healthy patients with type 1 diabetes mellitus to eliminate the potential for confounders (such as hypertension) to influence the outcome of the retinal tests. By using multiple tests of neural and vascular morphology and function that are performed using the same types of instruments in homogeneous cohorts of patients followed longitudinally to the development of clinical retinopathy, researchers would be likely to capture one or more robust markers of retinopathy risk and /or treatment efficacy. At the same time, knowledge of the relationships among early abnormalities would be gained.

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Chapter 4
Genetic and Environmental Susceptibility

Discussion Leaders: Usha Chakravarthy and Susanne Mohr

Scribe: George S. P. Murphy

Session Participants: A Bird, J Busik, L Donoso, P Dore-Duffy, R Frank, M Grant, S Iyengar, R Klein, D Puro, D Shima, A Swaroop

Five questions were addressed in this breakout session:

1. What is the evidence that genetic susceptibility to retinopathy exists?
2. Are there differences in genetic susceptibility in type 1 and 2 DR?
3. Have VEGF studies provided additional knowledge in terms of pathways?
4. Can changes in other tissues be linked to the development of DR? and,
5. What role do epigenetic changes have in DR?

For each question, the group was asked to comment on what we presently know, what we need to know and how we get there.

What is the evidence for genetic susceptibility in diabetic retinopathy?

Participants initially noted that the microvascular complications involving the retina in people with diabetes, typically known as DR, are mainly driven by duration of diabetes and hyperglycemia. The well recognized risk factors for susceptibility to DR include high HbA1C (which reflects chronic hyperglycemia), longer duration of diabetes, presence of hypertension, and nephropathy. Although these factors are important in explaining why some individuals develop more severe DR, a proportion of the variance remains unexplained. Some patients with good glycemic control develop DR and others with poor control are spared this complication.

The question of whether or not genetic predisposition modifies the onset of DR or influences the severity of DR was discussed. Group participants noted that there is strong familial clustering that has been identified in DR. In particular the group drew attention to the finding in the DCCT, which observed that among patients enrolled in the trial who developed severe, progressive retinopathy (reaching a score of level 47 or greater on the Early Treatment Diabetic Retinopathy grading Scale), there was a statistically significant increased risk of clustering of retinopathy of similar severity of clinically significant macular edema (CSME) and or laser treatment in either eye for PDR or CSME within families where multiple members had diabetes. Heritability has been subsequently shown to be a feature of DR in both type 1 and type 2 diabetes. The panel discussed whether different genetic pathways for susceptibility in the two types of diabetes were in existence or whether the same pathways led to DR in both types of diabetes.

The group consensus was that genetic susceptibility exists in DR, and that different pathways to susceptibility are possible within the two types of DR, in part, reflecting differences in dyslipidemia, hyperten-

sion, obesity and other factors between those with type 1 and 2 diabetes. The group also concluded that robust genetic markers for DR that would explain a significant proportion of the variance in susceptibility regardless of racial and geographical origin have not yet been detected. The observation was made that current research classification, merely on the basis of the severity of the vasculopathy as observed on color fundus photography, does not account for the entire range of phenotypes and may confound associations with genetic risk factors. Discussions ranged from single marker candidate gene studies to the value and better coverage of the recent genome-wide association studies. Several small to medium sized cohorts have been subjected to genome-wide association studies; however, no validated genes/markers have surfaced as definitive genes for DR. Thus, although there are potential novel candidates of interest, large scale replication studies are needed.

Are there differences between genetic susceptibility for DR in type 1 and type 2?

There are established differences between type 1 and 2 diabetes. After living with diabetes for 40 years, more than 80 percent of persons with type 1 diabetes will have retinopathy and almost half will have severe retinopathy. Notably, very long survivors with type 1 diabetes may exhibit little or no DR despite variable glycemic control; thus, genetic susceptibility has been proposed as a reason for the resistance to microvascular complications. Furthermore, as the type 1 diabetes phenotype is less variable than the type 2, the group felt that type 1 diabetes was better suited to understanding genetic risk as a contributory factor in the disease state.

The discussion was then channeled toward the role of genetics in type 2 diabetes. Although this has a more variable phenotype, some group participants expressed the view that the heterogeneity of the phenotypic spectrum may permit the association of genetic risk to specific phenotypic identities (e.g., dyslipidemia and DME). In addition, studies in Asian-Indians and Mexican-Americans suggest a possible role of heritability in the development of DR. Critical review of the existing data also confirms the absence of large scale studies on associations with DR, the failure to take into account the role of haplotype diversity at candidate gene loci and adjustment for important covariates such as diabetes duration. The group agreed that current knowledge does not permit definitive conclusions on whether genetic risk in DR is different in the two types of diabetes.

Have the therapeutic responses observed after VEGF inhibition provided additional knowledge in terms of pathogenetic pathways?

The group agreed that the present classification of DR may not be optimal for studies of the genetics of DME, in that it is based primarily on generalized retinal microvascular abnormalities, non-perfusion and proliferative retinopathy extent and severity and excludes the macula. DME can occur with early stages of DR (e.g., ETDRS Level 35), and its presence or absence correlates modestly with severity of DR. The frequency of DME also varies by type of diabetes. Notably, for any given severity level of DR the frequency of ME is higher in patients with type 2 DM (Table 4-1). Thus, it is important that DME extent and severity should also be graded.

Frequency Macular Edema (ME) and Clinically Significant Macular Edema (CSME) by Diabetic Retinopathy Severity Level (DR Level) at Baseline in the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) 1980-1982						
	Type I DM			Type II DM		
	No ME	Any ME	CSME	No ME	Any ME	CSME
DR Level	% (N)	% (N)				
10	100 (626)	0 (0)	0 (0)	100 (1267)	0 (0)	0 (0)
21-31	100 (508)	0 (0)	0 (0)	98.8 (590)	1.2 (7)	0.67 (4)
37-43	92.4 (269)	7.6 (22)	5.8 (17)	74.7 (233)	25.3 (79)	19.9 (62)
47-53	81.6 (62)	18.4 (14)	18.4 (14)	55.6 (40)	44.4 (32)	38.9 (28)
60+ (or ≥60)	54.7 (93)	45.3 (77)	20.6 (35)	40.2 (39)	59.8 (58)	27.8 (27)
DR= Diabetic Retinopathy ME=Macular Edema CSME=Clinically Significant Macular Edema Information is presented by eye						

Table 4-1. (courtesy of R. and B. Klein)

The discussion then focused on scientific facts underpinning responsiveness to VEGF inhibitors. Interestingly, there is heterogeneity to the response to anti-VEGF therapy in DME management. The group consensus was that this might indicate the presence of pathways mediated by factors other than VEGF in the pathogenesis of DME. DME is the most common presentation of DR in many countries; thus, addressing the limitations of the existing classification which does not take into account the various manifestations of DME is particularly important. Eyes with marked DR/PDR and DME appear to respond better to anti-VEGF therapy, signifying that VEGF may be the driver for both PDR and DME (Figures 4a, b). Where DME is the predominant phenotype with minimal or no DR, responsiveness to VEGF appears to be modest at best. The variability in treatment responsiveness therefore suggests that there may be alternative pathways for development of DME.

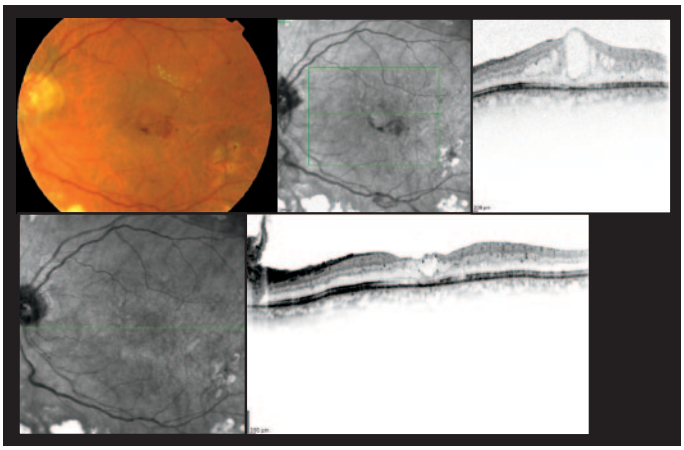

Figure 4a shows a fundus with DR (PRP laser burns visible) with DME. Four weeks post treatment with VEGF shows marked reduction of intraretinal fluid on OCT scans.

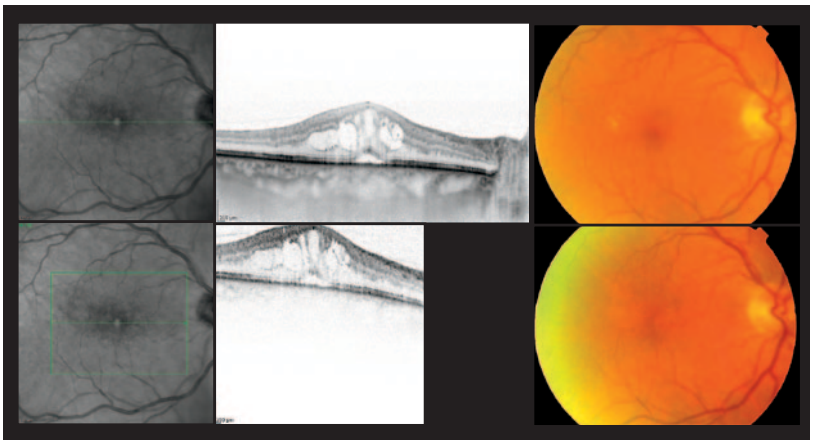

Figure 4b shows a case with DME with no other evidence of DR. 4 weeks after treatment with an anti-VEGF there is persistent intraretinal edema even though the lipid exudate has reduced.

In this context, recent research has demonstrated a link between dyslipidemia and DME. Additionally, inflammatory cytokines have been implicated as mediators of DME. Taken together, the body of evidence indicates multiple molecular pathways that lead to DR and susceptibility to DME and PDR. Future studies will require segregation of samples into specific DR phenotypes, some reflecting nonvascular changes affecting the retina (see below) so that the role of genetic risk can be appropriately investigated within these phenotypic groups.

Are there changes that occur in other tissues that can be linked to the development of diabetic retinopathy?

Retinopathy is only one of the manifestations of diabetes. There was discussion on how research on other organ systems might lead to a better understanding of the effect of diabetes on the eye. It has already been shown that the level of retinopathy correlates with presence of and severity of nephropathy. In addition, signs of renal damage such as microalbuminuria (which is an early measure of kidney malfunction) can predate manifest retinopathy (Christensen et al., 2000, Dalla Vestra et al., 2000). This pathology has been linked to hypertension in patients with type 2 DM and to pre-existing renal disease in both type 1 and type 2 DM. Photoreceptors in the eye and the primary cilia of the kidney express highly similar proteins and appear to share common lineage. Thus, one hypothesis is that retinopathy and nephropathy may share common genetic antecedents. Interestingly, many ciliopathies which include polycystic kidney, certain lung disorders and retinal degenerations have genetic antecedents. Alternatively, functional consequences of renal damage may drive the development of retinopathy, perhaps through shared factors in affecting the vasculature and/or blood supply. Taken together, the foregoing suggests a need for ophthalmologists and nephrologists to work more closely to develop better understanding of the pathogenesis of microvascular disease in diabetes.

There is less known about disordered metabolism of retinal neurons and glia as a feature of DR. As such for future research, information on retinal neural status is important with the expanding knowledge that the neural components of the retina, including photoreceptors, may be primarily involved in the pathogenesis of DR. Using any genetic markers identified for nephropathy, neuropathy or diabetic vascular disease may yield a potential research opportunity for an association with retinopathy.

What role do epigenetic changes have in diabetic retinopathy?

The group noted that complex interactions between genes and environment would influence the development of DR. Modification of the histone tails of chromatin by the biochemical abnormalities of diabetes is now known to occur and, thus, there was consensus that hyperglycemia, lipidemia and the other biochemical perturbations of the diabetic state were potential modifiers of DNA with consequent epigenetic changes. While information on the direct effects of epigenetic changes in DR is presently lacking, animal data suggest that methylation of histone H4 lysine 20 occurs in hyperglycemia and results in the down-regulation of retinal SOD2 and that this type of epigenetic change could result in increased susceptibility to DR.

It was postulated that genetic susceptibility may only manifest when combined with epigenetic changes. This could prove difficult to account for in future research due to the specificity that would be required in the cohorts. The consensus reached during this discussion was that these studies may be extremely expensive, time consuming and will require access to tissues rather than peripheral blood samples.

General conclusions

Genetic susceptibility exists in DR but may be difficult to detect due to behavioral differences in controlling hyperglycemia and other risk factors. Functional retinal deficits and retinal candidate genes need to be considered in examining genetic variants. While the natural history may appear similar, there are established differences between type 1 and 2 DR. Factors other than glycemic and blood pressure control and VEGF may be implicated in diabetic macular edema. Other tissues, such as the kidney, may share common genetic antecedents. Complex gene/environmental interactions may influence development of DR. And finally, epigenetics as a tool is not recommended at this time because of ethical considerations (requiring access to retinal and choroidal tissue) as well as being expensive and time consuming.

Proposed studies

Genetic susceptibility in DR

1) Studies with new cohorts should include more extensive phenocopying including function and morphology.

2) New cohorts should aim to recruit patients into contrasting groups; for example, fast progressors with good glycemic control versus slow progressors or non-progressors with poor glycemic control.

3) Information on duration of disease and biochemical parameters such as HbA1C should be captured. There was concern, however, that over correction/adjustment could obscure shared genetic risk in factors such as BMI/obesity or hypertension.

Genetic susceptibility for DR in type 1 and 2 diabetes

1) Studies with large sample sizes are needed.

2) Attention should be given to measurements of important covariates that have been previously shown to modify significantly the risk of DR such as HbA1C, duration of diabetes, hypertension, and nephropathy.

3) Results of the genetic studies being undertaken on the Joslin 50 Year Medalists should be revealing and carefully studied.

Therapeutic responses after VEGF treatment

1) Classification of persons as responders or non-responders to anti-VEGF therapy, followed by subsequent genetic analyses may yield valuable information. Acquisition and analysis of vitreous/ocular fluid samples prior to initiation of treatment and linking this information to the phenotype would be useful. Standardization in the acquisition of data and a simple clinically revised phenotypic classification is critical here.

2) OCT and newer imaging techniques should be included and will provide better and more accurate phenotyping than color fundus photographs alone. Grading techniques and validation studies of OCT concurrently with the established color fundus DR grading should be undertaken to allow improved categorization of persons with different DR phenotypes.

Other tissues and DR

1) New studies should include protocols that can distinguish morphological changes and functional deficits that are primarily driven by the neural retina from those that develop as a consequence of vascular disease. For example, phenotyping protocols for the kidney are based on function (micro and macro-albuminuria; 40 percent of patients developing neuropathy have impaired GFR rather than albuminuria), whereas phenotyping for DR is based on morphological classification. Studies should obtain data using both approaches to allow improved correlations and a better understanding of the relationships between the two organs in patients with DR.

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Chapter 5
Present and Proposed Approaches to Therapeutics

Discussion Leaders: Lloyd Paul Aiello and Jennifer Sun

Scribe: John Lillvis

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Despite recent advances in the treatment of DR and diabetic macular edema (DME), visual loss from DR and DME remains a common and significant problem for diabetic patients. This session reviewed current therapies for DR and DME, identified the major limitations of available therapeutics and prioritized approaches to overcome these limitations, with an emphasis on unmet needs in DR clinical and translational research.

What are the primary current therapies for DR and DME?

Systemic control remains the primary method of preventing diabetic eye disease and other vascular complications of diabetes. Hyperglycemia, hypertension, and hyperlipidemia each confer an increased risk of DR and/or DME development and progression; improved control of each of these decreases this risk. As demonstrated by the DCCT (1993), intensive blood glucose control reduces the risk of developing DR by 76 percent and slows progression of preexisting DR by 54 percent. The follow-up EDIC study further confirmed the long term benefits of early glycemic control in reducing retinopathy progression through the phenomenon of metabolic memory (White et al., 2010). Several trials, including the UKPDS (Matthews et al., 2004) have also demonstrated beneficial effects of hypertension management on DR progression, although these positive results have not been consistently repeated with different degrees of blood pressure control and different hypertensive agents (Antonetti et al., 2012). With regard to hyperlipidemia, high serum total cholesterol levels are associated with increases in hard exudates associated with DME and may lead to worse visual acuity results in patients with DME (Chew et al., 1996). In addition, trials using the PPAR-α antagonist fenofibrate demonstrate that it reduces the risk of DR progression in type 2 diabetic individuals with ocular benefits apart from its lipid-lowering effects (Group et al., 2010; Keech et al., 2007). Central to the benefit obtained with better control of systemic parameters is patient education emphasizing compliance and follow-up, as either DR progression or improvement may occur independently of perceptible changes in visual function.

Several eye-specific therapies are available to prevent the development and progression of DR and DME. Laser photocoagulation of the retina has been the primary treatment for severe proliferative DR (PDR) since the 1970's when the Diabetic Retinopathy Study (DRS) demonstrated that treatment with panretinal photocoagulation (PRP) results in a significant reduction in severe vision loss due to PDR (The second report of diabetic retinopathy study findings, 1978). Subsequently, the Early Treatment Diabetic Retinopathy Study (ETDRS) also demonstrated that focal/grid macular laser treatment could prevent and in some cases reverse central vision loss resulting from DME (ETDRS 1985), as

well as suggested that early panretinal photocoagulation should be considered in patients with type 2 diabetes with severe NPDR or early PDR (ETDRS report number 9 (1991). Vitreoretinal surgery is another therapeutic option for patients with PDR associated with vitreous hemorrhage or fibrovascular complications (DRVS study report 4 (1988); DRVS study report 2 (1985).

More recently, intravitreal administration of anti-vascular endothelial growth factor (anti-VEGF) agents either with prompt or deferred focal/grid macular laser has replaced focal/grid macular laser alone as first line therapy in treating eyes with visual impairment from center involved DME. Multiple phase 3 trials, including the Diabetic Retinopathy Clinical Research Network (DRCRnet) study, have demonstrated that intravitreal ranibizumab (an anti-VEGF agent) with either prompt or deferred laser is more effective than laser alone for the treatment of DME especially when the center of the macula has edema and vision is reduced. Similarly, when compared with laser photocoagulation for DME, the anti-VEGF antibody bevacizumab also leads to significantly greater improvements in best corrected visual acuity (Rajendram et al., 2012). Studies with pegaptanib, a VEGF binding aptamer, and aflibercept also suggest efficacy of these anti-VEGF agents for DME treatment (Do et al., 2011; Sultan et al., 2011). Anti-VEGF agents are highly effective for regressing retinal neovascularization from proliferative DR (PDR) (Avery et al., 2006), although phase 3 clinical trials directly comparing outcomes with anti-VEGF to those achieved with laser in PDR have only recently begun.

Therapy with intravitreal corticosteroids is an additional option for treatment of DME, although overall, visual acuity results with steroids appear inferior to those with anti-VEGF (DRCRnet, 2010) and steroid monotherapy is inferior to laser monotherapy for DME (DRCRnet, 2009). Intravitreal steroid therapy is limited by associated adverse events including the development of cataract and glaucoma, but may have a role in selected patients, especially pseudophakic patients or those who have not completely responded to prior anti-VEGF therapy.

What are the goals for future therapies?

Despite the advances in the treatment of DR and DME, there are still patients who lose vision from these conditions and, therefore, there remains a need for additional therapeutic options. During this session, multiple goals to improve future therapies were identified. These fell into the three general categories of improving safety, efficacy and treatment delivery; developing and evaluating targeted therapies; and allowing wider access to effective treatments.

Although current treatments for DR and DME clearly improve visual outcomes over the natural history of the disease, approximately 40-50 percent of patients with DME do not respond completely to anti-VEGF therapy and some patients still experience recurrent or worsening ocular neovascularization despite having undergone full PRP. Thus, future therapies should add expanded efficacy to the current treatment armamentarium. Ideally, these treatments would be minimally or non-invasive in order to reduce adverse events including endophthalmitis, and would be safe and well-tolerated from both an ocular and systemic perspective. Additionally, ideal future therapies would have an adequate duration of action to reduce treatment burden in comparison to current therapies, especially with anti-VEGF agents that currently require frequent intravitreal injections, initially monthly and then tapering based upon response.

Development of novel therapies targeted more specifically to fundamental mechanisms of DR and DME based upon our growing knowledge of the molecular pathways and pathophysiology of DR was also emphasized. Rationally designed therapies targeted to VEGF-independent pathways offer the promise of expanded treatment efficacy. It has also become increasingly clear that proliferative DR and DME have both shared and distinct underlying mechanisms; specific targeting of pathways for each of these manifestations of diabetic eye disease may allow for more effective therapies or treatment protocols. New methods to improve speed and prediction of clinical validation of new therapies, including the development of valid and reliable biomarkers of disease progression will also be vital in improving the process of therapeutic development and clinical trial evaluation.

Finally, expanding access to care on a global basis is an important long-term goal in the development of novel therapies. Although this should not come at the expense of innovation in the short-term, this will be an increasingly critical aspiration as the numbers of patients with diabetes worldwide increase dramatically over the next few decades. Many patients in underserved populations both in the United States and worldwide experience significant barriers to care that prevent them from receiving much needed treatments for their diabetic eye complications. Decreasing treatment costs and creating easily administered therapies that are scalable to large populations might substantially increase accessibility and thereby help to preserve vision in patients across the globe. Another long-term goal will be to create not just therapies for advanced DR, but also to develop preventive agents that target early disease progression and that might be given safely and easily to patients who may or may not have easy access to specialized diabetic eye care.

What are the obstacles we face in achieving these goals and what tools are necessary to overcome these obstacles?

Going forward, a significant investment of resources will be necessary to improve therapeutics and access to care for DR. To most efficiently utilize these resources, this session identified specific barriers to the development of new treatments and some of the tools that will be needed to overcome them. Although continued basic science research into identifying new therapeutic targets for DR and DME is also a priority, this session focused primarily on challenges in the translational and clinical research necessary to deliver new treatments that will directly impact patients. From the discussion, five priority research areas were identified: patient identification and phenotyping, surrogate markers and predictive biomarkers, biobanking, translation of basic findings into early phase clinical trials, and effective allocation of societal resources.

Patient identification and phenotyping:

Identifying and recruiting patients for clinical research, particularly studies with highly specific inclusion and exclusion criteria, can be a challenging undertaking. Collaborative research initiatives such as the DRCRnet have helped increase the efficiency of patient recruitment through creating and then leveraging multicenter clinical trial networks that can be quickly mobilized for acquisition of large study cohorts. In addition, the DRCRnet benefits from rigorous and standardized phenotyping of its study participants, potentially allowing for subsequent pooling of clinical data as well as rapid identification of potentially eligible patients for future study participation in non-competing protocols. In this vein, it is proposed to establish detailed, standardized phenotyping protocols for characterization of DR

and DME status as well as diabetes-relevant characteristics such as glycemic control, diabetes duration and presence or absence of other systemic diabetic complications that can be adopted across multiple clinical sites. Through these protocols, phenotyping could be accomplished in a prospective manner as patients present to clinics for routine care and then utilized to rapidly assess patient eligibility for new treatments and clinical research participation. Furthermore, widespread use of standardized phenotyping methods would enable the creation of a data-sharing network that would allow investigators to search a common database of patients to make multicenter recruitment more efficient. The pooling of data across sites and the creation of a large database of rigorously characterized patient outcomes might also improve our ability to perform natural history validation of proposed biomarkers, especially for early disease progression. Finally, the identification and utilization of unique and/or highly phenotyped cohorts, such as the Joslin 50 Year Medalists (Sun et al., 2011)), DR patients from the Age, Gene/Environment Susceptibility—Reykjavik Study (AGES-R) (Gunnlaugsdottir et al., 2012), or participants in the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) (Klein R et al., 2008) should be encouraged; such cohorts have the potential to ascertain protective/risk factors, novel mechanisms or predictive markers of DR using decreased numbers of patients.

Surrogate markers and predictive biomarkers:

Another major obstacle in the current therapeutics development process is our inability to predict which patients are at higher risk of DR progression and how individual patients will respond to available treatments. Current FDA-approved endpoints for DR trials generally require at least 2-3 years of follow-up, resulting in long duration and highly expensive studies (Csaky et al., 2008). The ability to develop predictive biomarkers of disease progression would allow much greater efficiency in early clinical trial evaluation of proposed therapies. In addition, the identification of reliable surrogate markers for ocular anatomic and functional outcomes might allow faster, less invasive and/or more objective testing methods to supplement those required for currently accepted primary outcomes.

Dilated funduscopic examination, fundus photography and visual acuity measurements have been the primary tools for clinical diagnosis and staging DR for many decades and are also acceptable clinical trial endpoints for registration of novel therapies for DR and DME. The non-invasive imaging technique of optical coherence tomography (OCT) has also become a widespread method for clinical assessment of DME, although it is not currently acceptable as a registration endpoint due to its only modest correlation with visual acuity. Parameters from these standard methods of clinical evaluation, however, have not yet been demonstrated as reliably predictive of DR onset or progression and are also not sufficient to assess early molecular, microscopic and physiologic changes that occur throughout the retina shortly after diabetes onset and before clinically visible lesions such as hemorrhages and microaneurysms are detected.

Thus, there is a need to extend recent advances in imaging technologies that might allow more sensitive assessment of the diabetic retina and thus potentially identify new biomarkers for DR onset, progression or treatment response. Advancements in and combination use of several imaging technologies, such as photoacoustic ophthalmoscopy, confocal scanning laser ophthalmoscopy, ultrahigh resolution OCT, and adaptive optics, have pushed the boundaries of visualization of the retina such that *in vivo* imaging of single cells or individual retinal cell layers is now possible (Song et al., 2011; Williams, 2011; Zhang et al., 2011). Novel imaging technologies also allow for the assessment of as-

pects of retinal function and physiology. Characterization of flow dynamics, including autoregulation, has been performed with high resolution doppler OCT and MRI (Trick et al., 2008, Yazdanfar et al., 2003). New generation OCT angiography techniques and MRI also give insight into localization and characterization of blood retina barrier defects (Trick et al., 2008, Bernardes et al., 2011). Hyperspectral imaging and MRI of the retina also allow for assessment of metabolic and physiologic information such as retinal oxygenation and L-type voltage gated calcium channel activity (Berkowitz et al. 2006, Johnson et al., 2007). Finally, cellular and molecular imaging, with techniques such as annexin V labeling (Cordeiro et al., 2011), offers the potential to better understand processes that may lead to cellular apoptosis in the diabetic retina.

Functional tests may lead to additional useful measures of retinal changes prior to the onset or progression of DR. Several tests of visual function are abnormal early in DR, including decreased color vision, contrast sensitivity, and dark adaptation, even with normal visual acuity (Jackson et al., 2012). Frequency doubling technology (FDT) response, which is indicative of ganglion cell function, may be a highly sensitive measure for discriminating between patients with and without early DR (Jackson et al., 2012). Studies of multifocal electroretinography (mfERG) have also shown that delays in mfERG response can predict future sites of DR as well as predict progression to retinopathy within the next year (Han et al., 2004; Harrison et al., 2011).

Finally, biochemical markers isolated from body fluids may yield additional insight into pathways that may serve as biomarkers or surrogate markers for DR and DME. Protein analysis of ocular fluids (Aiello et al., 1994) led directly to the development of anti-VEGF therapies that are used widely for treatment of diabetic eye disease today. Multiple cytokines and other signaling molecules show altered expression in the aqueous humor of eyes with diabetic complications (Hartnett et al., 2009; Sohn et al., 2011). A novel VEGF independent pathway associated with DME has also recently been identified through proteomics analysis of vitreous fluids, confirming the utility of this approach (Gao et al., 2007). Additional potential biomarkers for DR, including advanced glycation endproduct-low density lipoprotein (LDL) and oxidized LDL have also been assessed (Lopez-Virella et al., 2012) and if confirmed in future studies could allow assessment of DR progression risk from peripheral blood sampling.

In the future, as potential biomarkers are proposed, direct comparison of these biomarkers against each other and against the gold standards should be performed to determine their relative value in clinical research. It is proposed that this validation of novel biomarkers be accomplished by piggybacking evaluations whenever possible onto existing clinical trials in order to reduce costs and enhance efficiency. Visual function and DR severity measures should be evaluated in conjunction to provide insight into the pathways that link structure and function of the eye. Finally, cross-talk and collaboration between individuals with mechanistic, clinical and technological expertise should be encouraged in order to promote innovation through cross-fertilization of ideas from varying disciplines.

Biobanking:

Rapid innovation in gene expression profiling, next-generation sequencing, mass-spectrometry for proteomics and metabolomics, and other systems biology technologies offers great promise for high throughput identification of novel biomarkers, therapeutic targets and genetic risk loci. As these tech-

nologies become more affordable, one of the greatest challenges posed will be the accumulation of enough high-quality, well phenotyped samples to have sufficiently powered studies to use such tools effectively. To meet these future needs, it is recommended that biobanking initiatives be encouraged and expanded. For studies of DR, biobanking can include blood samples, DNA, pathologic tissue specimens, and vitreous or aqueous humor if appropriate. Standardized protocols should be developed and implemented for sample acquisition, storage, analysis and sharing of results. Also of critical importance is to coordinate biobanking efforts with standardized phenotyping as described above, which will allow for more effective pooling of samples and improved ability to perform subsequent validation studies.

Translation of basic findings into early phase clinical trials:

Translation of basic science research findings into clinically applicable new therapies is a complex, time-consuming but critically needed process. Many basic scientists are not adequately equipped with knowledge to design effective clinical trials or to efficiently navigate the FDA regulatory requirements. Even with a trial design in hand, building of the infrastructure necessary for study implementation, including site identification, contracting and monitoring can be daunting. Although private consulting firms specializing in trial design and implementation and Investigational New Drug (IND) packaging do exist, the costs associated can be prohibitive.

The creation of a consultancy for basic scientists looking to bring compounds into early phase clinical trials would be a valuable addition to the field of DR research. This consultancy, composed of national experts on clinical trials in DR from government, academia, private practices and industry, could greatly facilitate the translation of basic findings into clinical investigation by providing basic scientists in the field of DR and DME expertise in several areas:

1. Data needs in order to justify efficacy of proposed novel therapies and feasibility for clinical trials;

2. Effective and efficient trial design;

3. Access to expertise in running and analyzing clinical trials as well as an infrastructure for patient recruitment; and

4. Help in navigating FDA regulatory requirements associated with submitting and IND packaging.

Effective allocation of societal resources:

To provide the best possible care for the maximum number of individuals, it is critical that the finite set of societal resources (funding, personnel, expertise) are allocated in a manner that most efficiently allows for both innovation in therapeutic development and adequate access to care for patients who are at risk for vision loss from diabetes. Approximately 50 percent of patients with diabetes do not receive appropriate yearly dilated eye examinations that could identify treatable pathology.

To reach new populations, additional research should be undertaken to identify patients at risk for DR and the current barriers to care that prevent some patients from receiving vision preserving treatments. These efforts may include new screening strategies to identify at risk patients including greater

coordination of efforts between eye care and primary diabetes care providers as well as telemedicine technology that allows for remote diagnosis and triage (Li et al., 2011).

Equally important to the allocation of resources is finding ways to streamline the entire system of developing and approving new therapeutics. The identification of predictive biomarkers would greatly help to facilitate translational research leading to the identification of new therapeutic targets. Increasing efforts towards coordination and collaboration of research efforts with pooling of expertise is also important. Promoting innovations in clinical trial design can hopefully reduce study participant numbers, costs and timelines needed to develop effective new therapies.

Conclusion

Patients with diabetes have benefited from recent advances in treatments for diabetic eye disease, most notably the advent of anti-VEGF therapy for DME, as well as well-established therapies including laser photocoagulation for both proliferative DR and DME. There is an ongoing need, however, for new treatments that are safer, more efficacious, scalable to large, underserved populations, and that target early stage (i.e., prevascular) functional lesions. Priority research recommendations to overcome current obstacles that prevent the development of such treatments include projects that target patient identification and phenotyping, develop effective surrogate markers and biomarkers of DR-associated outcomes, create biobanking initiatives, more efficiently translate basic findings into early phase clinical research, and more effectively allocate societal resources for both treatment of and research into DR.

Summary of priority recommendations for future research

1. Patient identification and phenotyping
    A. Prospective categorization of patients for possible intervention or trial participation
    B. Standardized, detailed phenotyping and data sharing infrastructure
    C. Identify and utilize unique and highly phenotyped cohorts

2. Surrogate markers and predictive biomarkers: Imaging and outcomes
    A. Develop and apply improved functional assessments
    B. Extend advances in imaging technologies and promote cross talk between areas of expertise (clinical, technological, mechanistic)
    C. Piggyback evaluation/validation of surrogate endpoints and biomarkers on ongoing clinical trials

3. Biobanking
    A. Biobanking of tissue and fluid samples to support 'omics' analysis as well as candidate marker evaluation

4. Translation of basic findings into early phase clinical trials
    A. Consultancy for basic scientists looking to bring compounds into early phase trials, including data needs, trial infrastructure, trial design and IND packaging

5. Effective allocation of societal resources
    A. Identification and entry into the health system of diabetic patients
    B. Promote innovation in trial design to reduce patient number, costs and timelines

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Chapter 6
Pathogenesis of Diabetic Retinopathy and VEGF Therapy

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Introduction

The clinical use of anti-VEGF drugs has significantly reduced the debilitating vision loss due to DR (Diabetic Retinopathy Clinical Research et al., 2011; Elman et al., 2011; Nguyen et al., 2012). However, approximately 50 percent of patients treated with anti-VEGF drugs have persistent edema despite intervention. Discontinuation of anti-VEGF therapy in these patients often results in exacerbation of vision-threatening edema and progression of the proliferative stage of disease. There is a clear need to develop alternative and/or combination pharmacotherapies. There is also a growing need to be able to identify patients that will not respond significantly to anti-VEGF therapy, so that they can be informed of their likely outcome and, importantly, alternative therapies in development can be considered before serious visual decline. The objective for this session was to establish a framework for accelerated exploration of the pathology associated with DR, using clinical observations, patient samples and animal models in the hope of accelerating the development of diagnostic and therapeutic options for patients.

Edema

During the discussion it was agreed that although DME is the most frequent reason for vision loss, there was a lack of consensus about the mechanism(s) and source(s) of fluid accumulation. It is unclear why edema does not occur in most cases of PDR, where VEGF levels are presumably high. It is also unclear if fluid accumulates within cells, in the extracellular space or both. Furthermore, the source of fluid could be from retinal capillary permeability, failure of the RPE to control fluid influx from the choriocapillaris, and/or a failure to transport fluid out of the retina. Fluid migration along the neuroglial fibers, in the absence of a defined lymphatic system, was suggested as a possible mechanism to explain the increased incidence of edema at the macula. The observed differences in the resolution of DME upon anti-VEGF therapy may reflect patient variation in the mechanisms of fluid accumulation.

Inflammation in diabetic retinopathy

Many of the hallmarks associated with inflammation including increased retinal vascular expression of adhesion molecules, blood vessel dilation, edema, leukostasis, and inflammation-associated cytokines (including IL-6, IL-1, TNFα, VEGF and MCP-1), which are increased in both patients and animal models of DR are summarized in the literature (Adamis and Berman, 2008; Antonetti et al., 2012). Systemic anti-inflammatory therapies including NSAIDs ([controlled clinical trial of the effect of as-

pirin and aspirin + dipyridamole on the development of DR. I. General protocol. From the DAMAD study group (author's transl)], 1982; Zheng et al., 2007) and anti TNFα (Sfikakis et al., 2005) have shown promise in reducing diabetes-induced retinal vascular lesions and macular swelling in small pilot studies. However, systemic delivery of anti-inflammatory medications is often associated with side effects. Local delivery of antibodies formulated for ocular use would reduce the systemic exposure and systemic side effects. The group identified several targets for which there are drugs already clinically approved (specifically IL-1, IL-6, TNFα, LFA-1) for possible testing in clinical trials. It was agreed that these drugs represent a rapid and cost-effective way of increasing the therapeutic options for DR patients. The group recommended the development of a strategy for clinical testing, and further safety and animal model assessment of these drugs, both alone and in combination with anti-VEGFs.

Neuroprotection

Pilot data was presented on the effect of persistent edema on neuronal loss suggesting that earlier intervention reduced neuronal loss. Since up to 50 percent of ganglion cells may be lost prior to noticeable vision loss, it was suggested that there should be more focus on neuroprotection in combination with agents modulating the vascular dysfunction. Moreover, the fact that VEGF antagonists have been shown to reduce neuronal survival in animal models of retinal disease suggests that the coupling of anti-VEGFs with neuroprotectants could have additional value.

Animal model limitations

While animal models have been useful in understanding the relationship between hyperglycemia and non-proliferative diabetes-induced vascular lesions, there are no experimental animal models that develop PDR. Furthermore, evidence for edema in diabetic animals has only recently been described (Berkowitz et al., 2012). There is a need for further technology development as spatial visual acuity or adaptive optics in small animals (rodents) is currently restricted due to size restrictions of the eye and the resolution of current imaging equipment. Also, there needs to be more of an emphasis on functional assessment in animal models at earlier time points that would better clarify the early contribution of neural dysfunction in the disease. Finally, given the limitations of animal models, increased access to clinical samples and patient histories has become paramount in identifying the relevance of various biological pathways involved and risk factors based on genetic polymorphisms.

Databases

The group identified the lack of accessible clinical data as a hurdle in identifying risk factors associated with patient response to anti-VEGF treatment. Of interest were the history of previous or coincidental diseases or medications (including over the counter medications) that might correlate with the prevalence or severity of edema, vascular lesions, or neovascularization. It was noted that data from recent clinical trials of anti-VEGF therapy Lucentis®, PPARα agonist fenofibrate, and PKCα inhibitor ruboxistaurin would be useful in this regard; however a group would need to be established to assemble the data and establish working relationships with the pharmaceutical companies. Similar to the DRCRnet, this group could also organize the collection, storage and sharing of biological samples for future proteomic or genetic studies.

Genetic susceptibility

Several studies have already found a correlation between polymorphisms in the VEGF receptor and the incidence of DR. While a complete genetic screen for risk factors between responders and non-responders to VEGF treatment would be desirable in the long term, a smaller targeted subset of genes associated with particular inflammation pathways or the known biological mechanisms of action of drugs that already have been shown to have clinical relevance in DR would be less costly, in particular the clinical trials mentioned previously as well as AMD risk variants in DR population.

Diagnostic tools

There was general agreement that there is a significant need for new biomarkers and diagnostics to both assist in treatment decisions, but also to increase the ability to probe the pathology. Monitoring local inflammation and immune cell behavior and detecting apoptotic retinal neurons were both mentioned as area of future focus.

Proposed studies

Immediate goals

1) Establish a database of information about diabetic patients that have developed or may develop retinal complications. To do this, a group needs to be organized to assemble data and ensure controlled, but widespread access. Pharmaceutical companies need to be contacted to gain access to existing clinical datasets containing diabetic patients (specifically, those treated with anti-VEGF antibodies, the PPARα agonist fenofibrate, and PKCα inhibitor ruboxistaurin. Finally, the DRCRnet needs to be contacted to identify what information and tissue they would be willing to share.

Mid-term goals

2) Investigate and compare inflammation pathway genes. For example, Age-Related Macular Degeneration (AMD) risk variants in the DR population need to be probed as well as direct sequencing of a small number of genes involved in the mechanism of action of clinically evaluated compounds for DR (VEGF, PKCα and PPARα).

3) Identify additional anti-inflammatory medications for patients that could serve as combination therapies or treat VEGF therapy non-responders and identify drugs already clinically approved that target inflammation (specifically IL-1, IL-6, TNFα, and LFA-1) for possible testing.

4) Test the safety of co-injecting anti-inflammatory compounds with and without anti-VEGF into the vitreous of animal models, and conduct a cautious dosing program in patients. Use the data from clinical studies and directed pathway interrogation to probe mechanisms in animal models.

Long-term goals

5) Establish animal models and measurement techniques which enable studying the earliest stages of disease, prior to obvious microvascular dysfunction. Evaluate if retinal neural dysfunction/death precedes, follows or develops in parallel with vascular lesions or vascular permeability. Establish animal models and measurement techniques which enable the study of late stage DR (edema and angiogenesis).

6) Develop diagnostic tests that identify patients at risk for developing edema or PDR. Identify patients who are unlikely to respond to anti-VEGF therapy.

7) Inject and track fluorescently-labeled leukocytes to evaluate inflammatory status of retina in early and late DR.

8) Develop techniques that can be used in both patients and animal models (especially small animals) to evaluate neural survival and function after neuroprotective drug dosing. Test the usefulness of adaptive optics as well image and tracking of retinal neural cell apoptosis *in vivo* (detection of apoptosing retinal cells, DARC).

9) Evaluate clinical trial FA/OCT datasets for clues on source of edema fluid and thinning of retina. Determine impact of persistent edema in spite of anti-VEGF treatment on neuron loss. Determine impact of anti-VEGFs on long-term neuron survival.

10) Develop better tissue/fluid banking as a community.

11) Undertake whole genome sequencing of patient samples.

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Chapter 7
Epidemiology and Unusual Cohorts

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Overview

The premise upon which discussion was based is that there are insights concerning the risk factors for retinopathy and other complications of diabetes for which epidemiologic studies of groups (cohorts) of persons provide important information as well as to inform the searches for new avenues of preventive and treatment regimens. It is also clear that new cohorts will need to be followed as the course of diabetic retinopathy will continue to be influenced by therapies that are only now appearing and for risk factors and protective factors that are just being discovered. We begin with discussion of existing cohorts to understand the study designs as well as to understand some of the information that they have already gathered. We then describe some newly developed cohorts and contrast findings from the newer and more established cohorts.

Established cohorts

Wisconsin Epidemiologic Study Of Diabetic Retinopathy (WESDR)

This long term study of both type 1 (Klein et al., 1984a) and type 2 diabetes (Klein et al.,1984b) has provided long term rates of incidence and progression of DR, other vascular complications of diabetes as well as determining risk factors for them. Persons with long-term type 1 diabetes are not only at higher risk of DR but for cognitive dysfunction, and it has been hypothesized that a marker for cognitive dysfunction may be severity of DR. The WESDR is determining the relationships between modifiable risk factors (e.g., markers of advanced glycation end products [AGEs], receptors for AGEs [RAGEs], markers of antioxidant stress, hematocrit, clotting factors, and atherosclerosis) as well as macular edema and other long-term vascular complications of type 1 diabetes to assess associations of these with cognition. Another avenue of research that is currently being pursued is whether retinal vessel diameters measured by computer-assisted grading of digitized images of ETDRS Field 1 (Klein et al., 2012) (Figure7-1) are useful 'predictors' for diabetic retinopathy as well as for neuropathy and decreased cognition. Retinal vessel diameters may also be associated with thinning of the nerve fiber/ganglion cell layer of the retina as revealed by OCT and, if so, this may substantiate the hypothesis that retinal vasculature is causally related to neurodegeneration in diabetes. This study will determine whether retinal vessel diameters have value as a clinical tool for early detection of complications of diabetes and, consequently, serve as an early marker for preventive interventions of DR. Already the WESDR has shown that the number of microaneurysms in the retina is a significant indicator for the progression of retinopathy (Klein et al.,1995).

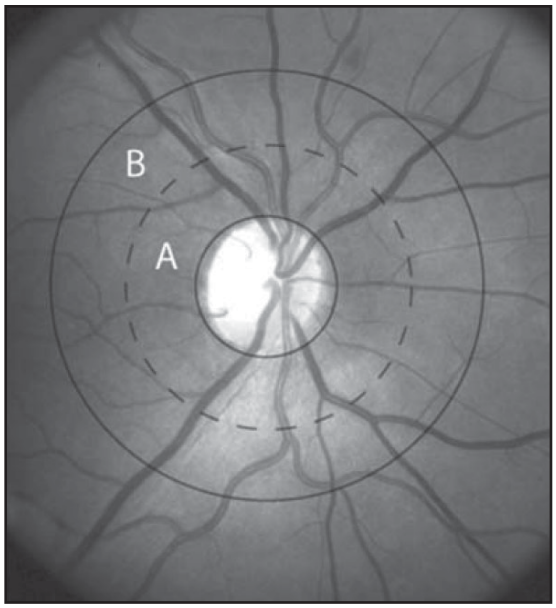

Figure 7-1: Digitized retinal photograph. Zone A is a half-disc diameter from the optic disc margin and Zone B is a half-disc to one and half-disc diameter from the optic disc margin. Retinal vessel diameter measurements are performed in Zone B (Knudtson et al., 2003) (used with permission).

Wisconsin Diabetes Registry Study (WDRS)

This is a 20 year study of type 1 diabetes of all residents younger than 30 years of age who live in central and southern Wisconsin and have been newly diagnosed with type 1 diabetes. Follow-up during the 20 years post-diabetes onset includes biannual or annual questionnaires for diabetes management, and periodic clinical examinations including blood samples and fundus photographs (Palta and LeCaire, 2009). This study provides the opportunity to examine the effects of temporal changes on DR and other complications of diabetes from the onset of disease.

There are many other cohorts that are being or have been studied to investigate risk factors for vascular disease or atherosclerosis, including the Age, Gene/Environment Susceptibility study (AGES) (Gunnlaugsdottir et al., 2012); Atherosclerosis Risk in Communities study (ARIC) (Klein et al., 2002); Cardiovascular Health Study (CHS) (Klein 2002a); Los Angeles Latino Eye Study (LALES) (Varma et al., 2004); Multi-Ethnic Study of Atherosclerosis (MESA) (Wong et al., 2006), and the National Health and Nutrition Examination Survey (NHANES) (Zhang et al., 2010).

Many of the subjects in these studies have type 2 diabetes. These cohorts also have provided the opportunity to investigate whether environmental risk factors such as air pollution and environmental toxins lead to the development of various complications of diabetes including DR.

Search for Diabetes in Youth (SEARCH)

The SEARCH study enrolled individuals below the age of 20 years, diagnosed with either type 1 or type 2 diabetes and had substantial racial and ethnic diversity (Writing Group for the SFDIYSG, 2007). It provided the opportunity to examine risk factors in the rapidly growing group of young people with type 2 diabetes who may have a different risk profile for complications from those with

type 1 disease. The prevalence for DR was found to be lower in non-Hispanic white people but higher in all other races/ethnicities (Mayer-Davis et al., 2012). It is possible that this is due to the better level of care that the non-Hispanic white people may experience. This may especially be the case for type 2 diabetes in youngsters who are overweight.

Reduction with Metformin Vascular Adverse Lesions in Type 1 Diabetes (REMOVAL) Study

REMOVAL is a clinical trial involving multiple collaborators in Australia, Canada, Denmark, and the Netherlands and led from the United Kingdom (Glasgow). Metformin is an oral anti-diabetic drug. It is in the biguanide class of drugs and is usually used for the treatment of type 2 diabetes. It works through regulation of AMP activated protein kinase (AMPK) leading to, among other effects, suppression of glucose production by the liver.

It is in start-up phase and will test whether metformin in those with type 1 diabetes has a protective effect on carotid intima-media thickness. A secondary endpoint is to test whether the drug regimen has a protective effect on DR.

Diabetes Control and Complications Trial (DCCT) and the Role of Dyslipidemia

The main focus of the DCCT was to evaluate glycemic control in diabetes. However, in addition to hyperglycemia, diabetes affects lipid control. Although dyslipidemia is mainly considered a feature of type 2 diabetes, recent studies demonstrate a strong link between lipid control and DR in type 1 diabetes. Type 2 diabetes is characterized by an elevation of blood levels of cholesterol and of esterified and nonesterified fatty acids. In type 1 diabetes, the overall cholesterol, triglyceride and nonesterified fatty acid levels do not significantly differ from the control values (Brown 2000, Brenner 2000, Decsi 2002); however, there is a substantial change in the fatty acid profile of these pools, oxidized lipids and sphingolipids pools. As a first crude measure of lipid metabolic profile, DCCT samples were analyzed for the size of the particles of serum lipoproteins. There was a strong association between severity of retinopathy in type 1 diabetes and the size of the particles of serum lipoproteins, VLDL, LDL, and HDL as well as LDL concentration in DCCT samples (Lyons et al., 2004). Further, oxidized LDL was demonstrated to be increased in diabetic patients. Auto-antibodies to oxidized LDL were linked to the development of DR. COX (COX-1 and -2), LOX (5-, 12- or 15-LOX), or MOX catalyze conversion of unsaturated fatty acids into a range of biologically active compounds that could have a pro- or anti-inflammatory role depending on the initial fatty acid substrates (Hwang 2000). The activity of these enzymes and fatty acid profiles are modified in diabetes. Lipid oxidation and fatty acid profile changes occur in both type 1 and type 2 diabetes, and are likely to play a role in the pathogenesis of retinopathy.

Action to Control Cardiovascular Risk in Diabetes Trial (ACCORD-EYE)

This study was aimed at evaluating the effect of blood pressure and triglyceride levels in the context of good glycemic control on the incidence of DR (Group et al., 2010). The effect of fenofibrate, the agent used for triglyceride control, was highly significant in decreasing the incidence of retinopathy. The effects of blood pressure medications on the retinal outcomes in this short trial were not significant. The fenofibrate effect was also given in the context of statin therapy. The effects of triglyceride control and more generally in lipid control bears further investigation as past studies have been equivocal. If the

effect found in ACCORD is confirmed, then a more consistent and rigorous control of blood lipids should be considered for preventing and/or delaying retinopathy.

Diabetes Registries in Russia

In 1996-1997, the Government of the Russian Federation adopted a Federal Target Programme named "Diabetes Mellitus." As part of the program, the State Registry of Patients with Diabetes Mellitus (SRPDM) was created. The registry records diabetes mellitus prevalence, incidence, disability, and mortality rates, direct causes of death among patients with diabetes, prevalence of diabetes complications, and provision of medication and instruments for self-monitoring. At present, the SRPDM includes 84 regional centers across the country, which submit their data to the SRPDM for detailed annual analysis. Analysis of SRPDM data and completed monitoring of epidemiological screening studies has demonstrated that the real prevalence of diabetes complications significantly exceeds the officially registered rate. In particular, a detailed epidemiological analysis conducted in 20 regions of the Russian Federation has revealed that the real prevalence of DR is 38 percent, which is 1.5 times more than the officially registered level (Dedov II).

In addition to SRPDM, there are several regional registries in Russia. For example, an automated computerized register of DR was created in the city of Ufa, Republic Bashkortostan (Valiullina 2011).

Joslin 50-Year Medalist Study

The Joslin 50-Year Medalists are a select group of persons with type 1 diabetes from around the country who have survived with insulin-dependent diabetes for at least 50 years (Sun et al., 2011). The aim of this study is to characterize genetic, environmental, psychological and physiologic factors that might contribute to long term survival in persons with diabetes. In general, this group has good current glycemic control. More than 60 percent of the Medalists produce some insulin, suggesting that they may have some protection against beta-cell destruction. Within the range of their hemoglobin A1c levels the correlation of glycemic control with complications of diabetes is not obvious. There does not appear to be a relationship between complication status and residual beta-cell function. While inferences from this group are not necessarily generalizable to all persons with type 1 diabetes, since the Medalist study participants are all relatively healthy volunteers and are able to travel to the Joslin clinic, insights from this unique cohort may allow mechanistic insight into why some patients are able to survive many decades of diabetes without significant vascular complications.

Proposed studies

1) Dark adaptation increases the oxygen consumption of rods in the eye substantially, and this has led to the hypothesis that hypoxia is an early event in the development of DR (Arden et al., 1998, 2010). In animals without rods, there is no increase in VEGF in the dark as occurs in normal animals, suggesting that rod metabolism may contribute to the stimulus for new vessel growth in the retina as is found in diabetes. Further, evidence from small human studies suggests that dark deprivation is associated with regression of diabetic macular edema (Arden et al., 1998, 2011). It is clearly worthwhile to explore the effects of dim light on the prevention, delay, or regression of DR and macular edema.

2) Bariatric surgery has recently been employed with some success to treat type 2 diabetes in adults (Dixon et al., 2012; Varadhan et al., 2012). It is becoming clear that diabetes will recur in a proportion of patients who have had this procedure. Extreme weight loss from other treatment modalities can also have long-term effects on regression or disappearance of the diabetes phenotype. Effects of these procedures on long-term microvascular complications of diabetes have not been reported and need study. The possibility of long-term dietary intervention on the onset and complications of type 1 diabetes from early in childhood will be addressed in The Environmental Determinants of Diabetes in the Young (TEDDY) study (Group, 2008; Hagopian et al., 2006).

3) While ongoing and previous studies provide the opportunity for new studies to use specimens that have been 'banked' with regard to etiology of diabetes (e.g., new genetic markers, geneXgene and geneXenvironment interactions, serum and plasma factors that may predict complications coupled with past examination and history data), it is clear that new studies incorporating new hypotheses and more current technology are needed. It seems likely that, especially for type 1 diabetes, inception cohort studies would be ideal. The advantage of this approach is that it is designed to identify type 1 diabetes early in the disease process, possibly permitting reversal of the disease itself or at least delaying the onset of need for medication as well as being able to institute preventive treatments to forestall complications. These studies could be family based where parents or siblings with type 1 diabetes indicate high risk families which are under 'surveillance' for new cases. Another avenue could be physician based where physicians report the first diagnosis of disease. If one were certain that electronic records were complete and that patient privacy guidelines were not impinged upon, then new diagnosis of type 1 diabetes could be routinely accessed through larger healthcare systems and this might yield a greater number of patients that could be studied.

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Chapter 8
Vascular and Neuronal Repair

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The nature of the problem

Dysfunction and eventual loss of cells from retinal microvessels is a characteristic lesion in diabetic retinopathy. Because of their anatomy, with long processes enveloping the endothelial lining of the capillary lumen and their content of smooth muscle actin, the initial loss of pericytes (Kuwabara and Cogan, 1963) (Figure 8-1) leads to abnormal regulation of capillary blood flow. Loss of the normal interaction between pericytes and endothelial cells via cytokines produced by pericytes with receptors on the endothelial cell membrane appears to release a brake on endothelial cell proliferation (Orlidge and D'Amore, 1987).

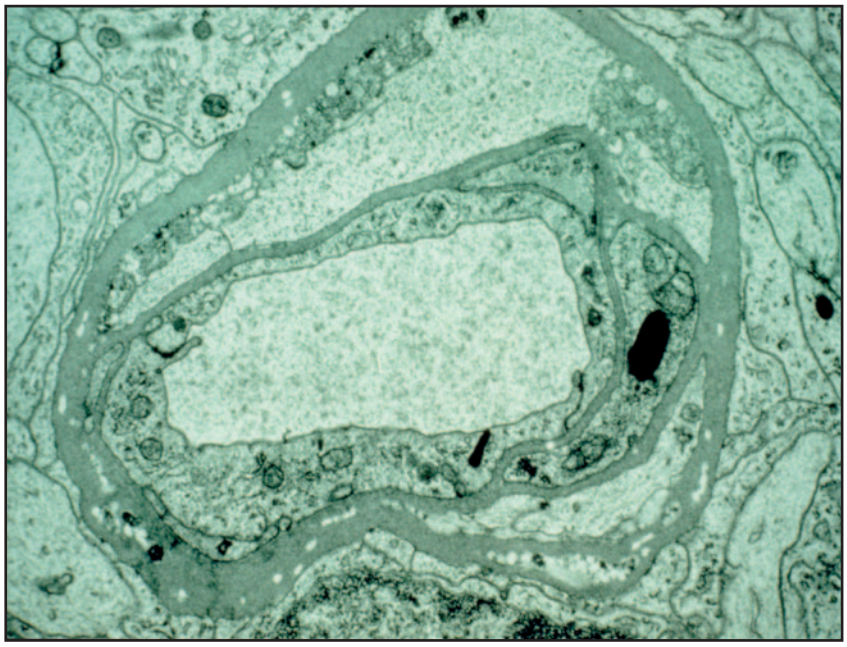


Figure 8-1: Electron micrograph showing a cross-section of a retinal capillary prepared from the eye of a diabetic human subject. Note the thickened capillary basement membranes with frequent vacuolizations (said to resemble Swiss cheese), the intact endothelial lining of the capillary lumen, and the vacant spaces with some residual, necrotic cellular debris, that was formerly filled by a viable pericyte. In addition to the larger empty space at the top of the capillary profile, note the several smaller spaces at the bottom of the profile, reflecting the fact that pericytes extend long processes that envelop the capillary endothelial tube. (Kuwabara and Cogan, 1963).

Eventually, endothelial cells are also lost, and the resultant acellular basement membrane tubes can no longer carry blood, leaving substantial regions of the retina ischemic. Although not widely recognized because the choroidal vasculature is less readily visible by present clinical methods, this capillary loss also occurs in the diabetic choroid (Figure 8-2).

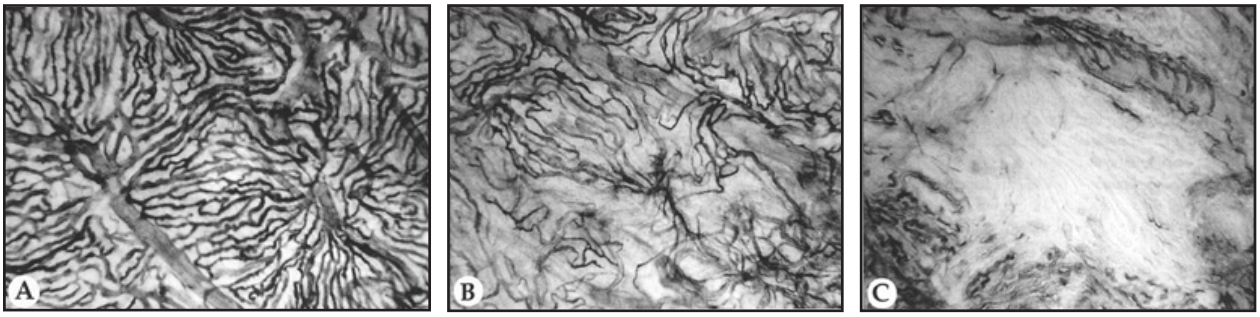


Figure 8-2: Flat mounts of human choroid that has been fixed and then stained by the alkaline phosphatase method. This montage shows varieties of choriocapillaris in subjects with longstanding diabetes shows several patterns of degeneration. (A) Normal choroid; (B) Diffuse degeneration; and (C) Focal degeneration. (Cao et al., 1998).

In the retina, which is the most metabolically active tissue in the body, ischemic neurons and glia will eventually malfunction and die, but may initially secrete angiogenic molecules such as VEGF which, in the postnatal vertebrate retina, stimulates neovascularization (Amin et al., 1997). Postnatal neovascularization in the mammalian retina does not serve a useful physiological function. Rather, these new vessels tend to grow inward from the neural retina into the vitreous cavity. Their endothelium does not have the barrier properties of normal retinal vascular endothelium. The new vessels are prone to hemorrhage into the vitreous cavity with loss of vision, and ultimately with fibroglial tissue that is contractile, producing detachment of the neural retina.

Secretion of VEGF and, most likely, other growth factors and cytokines can also break down the barrier function of normal retinal microvasculature. In the human macula, the leakage of intravascular contents into the extravascular neural retina produces edema. Chronic macular edema may also result in retinal neuronal damage even with little clinically apparent ischemia. In particular, subretinal fluid, common in the severely edematous retina, may cause detachment of the photoreceptor layer from the underlying retinal pigment epithelium (RPE) with loss of the physiologically necessary interactions between the photoreceptor cells and the RPE.

While current therapeutic modalities, including “panretinal” laser photocoagulation to reduce retinal and optic nerve head neovascularization, focal photocoagulation and, more recently, the intraocular injection of anti-VEGF drugs, have been impressive gains in the treatment of PDR and diabetic macular edema, these complications of diabetes still represent enormous causes of vision loss in the United States and worldwide. The following sections will summarize currently ongoing research, and future plans to preserve retinal neuronal function and to prevent retinal ischemia by repopulating the cellular components of the damaged microvasculature in the diabetic retinal circulation, and to restore the structure and functioning of retinal neurons.

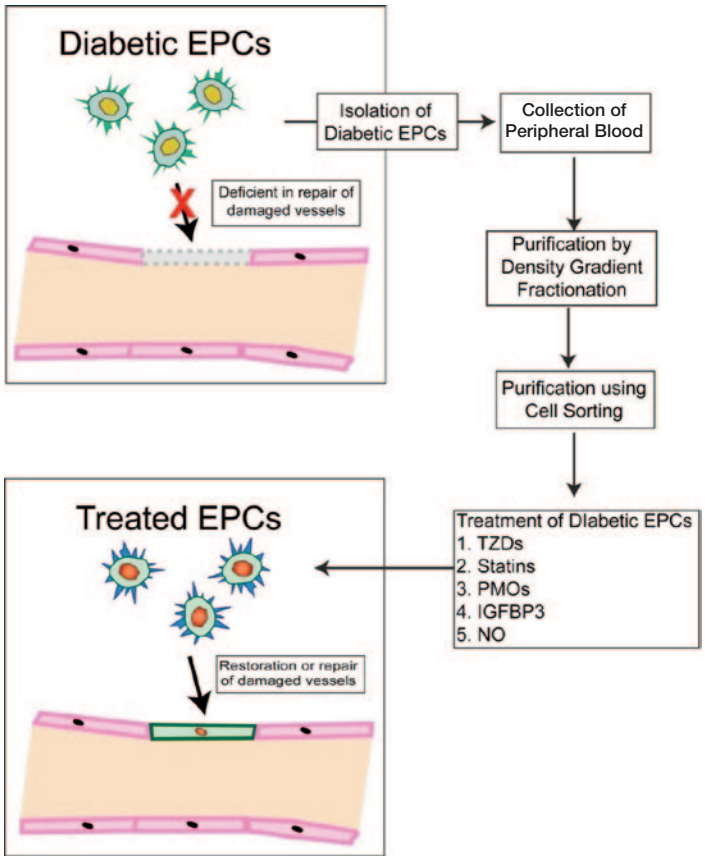
Vascular cell renewal in the normal retina and in diabetes

The cellular population of the normal postnatal retinal vasculature is quite stable. Mitoses in the retinal microvascular endothelium are rare (Engerman et al., 1967). While replacement of senescent endothelium or pericytes may occur by mitosis of cells resident in the extant vessel, replacement may also occur by recruitment of circulating, relatively undifferentiated endothelial cell progenitor cells (EPCs) or perhaps, pluripotent stem cells (PSCs). These are rare components of the normal blood circulation (Stitt et al., 2011), but, in the normal human or laboratory rodent, they have the potential to increase their numbers in appropriate circumstances. In diabetes, however, the release of these cells from the bone marrow is depressed and the normal circadian rhythmicity of this release is lost by inflammatory processes and the degeneration of nerve terminals within the bone marrow (Busik et al., 2009). The use of trophic factors to stimulate production of EPCs *in vivo* is a tantalizing possibility but is potentially hazardous because such trophic factors may also cause substantial proliferation of inflammatory cells. As a treatment for DR and other ischemic retinal vascular diseases, current attempts are being made to mobilize either bone marrow-derived or other endothelial progenitor cells or pluripotent stem cells and to stimulate them to differentiate into pericytes or endothelial cells that can then "home" to the empty basement membrane tubes of ischemic, diabetic retinas, thereby regenerating functional capillaries (Friedlander et al., 2007). One possible approach to treatment would be the selection of appropriate anti-inflammatory agents that can both enter the bone marrow and can also cross the blood-retinal barrier. A previous controlled clinical trial, in which patients with moderate to severe nonproliferative DR and/or macular edema were randomized to 650 mg/day of aspirin, or to placebo, showed no apparent benefit for the aspirin regimen [ETDRS report number 8 (1991)]. The tetracycline antibiotic minocycline has been suggested as another possible anti-inflammatory molecule, because it does cross the blood-retinal and blood-brain barriers.

Experimental studies of retinal vascular cell renewal

Bone marrow is the major source of stem cells that repair microvessels in the retina and elsewhere *in vivo*, but experimentally, hematopoietic stem cells can be obtained as well from adipose tissue or umbilical cord. Nevertheless, for experiments on retinal vascular repopulation, bone marrow remains the preferred source. EPCs were first identified from bone marrow by Asahara et al (1997) and characterized by the presence of the CD34 cell surface antigen. Subsets of these cells can be selected by culturing with different substrates and growth factors, and characterized by their possession of additional antigens (Li Calzi et al., 2010). Substantial controversy exists as to just which of many different cell markers characterize true endothelial progenitors (Li Calzi et al., 2010; Stitt et al., 2011).

Many difficulties are apparent in attempts to increase the production of EPCs and pluripotent stem cells (PSCs) in diabetic animals and humans, to stimulate these cells to differentiate into functional endothelial cells and pericytes, and to make these cells home to damaged sites in the retinal vasculature and to form intact and functional blood vessels with all of the properties of normal retinal vessels (Figure 8-3).



8-3: Healthy endothelial progenitor cells (EPCs) have proven ability to repair damaged endothelium by homing to sites of vascular damage and tissue ischemia. EPCs from diabetic donors lack this reparative ability due to inherent cytoskeletal defects and/or impaired signalling within defined transduction pathways. *Ex vivo* pharmacological manipulation of diabetic EPCs can restore this diabetic defects improving their reparative ability prior to autologous therapeutic transfer. (Neu et al., 2011).

Various chemoattractant and chemorepulsive signaling molecules have described as having roles in the homing process, among which are the semaphorins, which have been investigated most for their role in the developing nervous system and for their repulsant activity in directing growth away from certain areas.

Grant et al., (2002) showed that hematopoietic stem cells could revascularize ischemic regions of adult mouse retina, and Dar, et al., (2012) reported that PSCs could differentiate into pericytes that were incorporated into revascularization in the previously ischemic hind limb of mice. Stitt et al.,(2011) demonstrated a case in which a human patient with macular ischemia from radiation retinopathy demonstrated spontaneous remodeling and apparent revascularization (Figure 8-4).

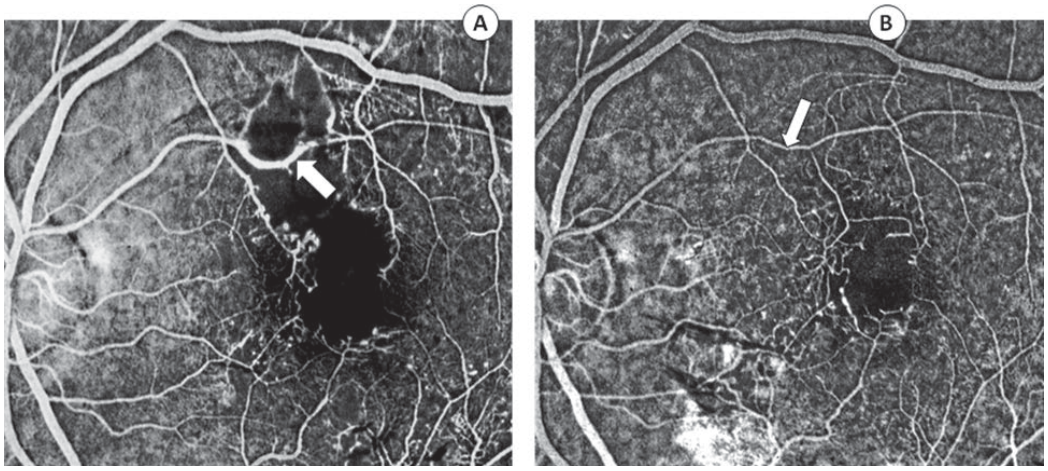


Figure 8-4: Clinical evidence for retinal vascular remodelling after ischemia. A. Radiation retinopathy in a patient's left eye. The acute-phase angiogram shows focal ischemia and microvascular disorganization in the posterior fundus. Note the presence of a dilated incompetent arteriole crossing an ischemia area (arrow). This patient's vision at this time was 3/60. B. One year later. The angiogram shows evidence of revascularization of the ischemia superior macula. The superior macular arteriole has normal caliber and has also regained competence (arrow). The patient's vision at this time was 6/6. (Stitt et al., 2011)

Ensuring revascularization in the ischemic retinas of diabetic patients may be a much greater challenge, not only to repopulate larger areas of acellular microvascular tubes, but also to ensure that the repopulated microvessels function like normal retinal blood vessels.

A critical problem in efforts to repopulate ischemic areas of the diabetic retina with a normally functioning microvasculature is to ensure that the progenitor cells that are introduced differentiate into mature endothelial cells and pericytes. Microvascular tubes consisting solely of endothelial cells will not function normally, and may also have decreased viability. Experimentally stimulating the differentiation of stem cells into retinal microvascular pericytes or endothelial cells has depended on the use of various trophic factors. It was reported several years ago that transgenic mice with an absent gene for the B-chain of platelet-derived growth factor (PDGF-B-/-) died *in utero* because of an inability to form pericytes and, thus, to be unable to develop a fully differentiated vascular system (Lindahl et al., 1997). Edwards, et al., (2012) have given a preliminary report of a study in which retinal angioblasts were cultured from neonatal dog retinas and incubated either with basic fibroblast growth factor (bFGF) or with PDGF-BB. The former growth factor promoted differentiation into an endothelial morphology and formation of capillary-like tubes in culture on an extracellular matrix gel, while the latter promoted differentiation into a pericyte-like cell that localized to the outside of the endothelial tube. Injection of the cultured cells into the vitreous of neonatal dogs, however, always produced a pericyte morphology and localization to the outside of the vascular tubes (Edwards 2012; Lutty et al., 2006). Injection of angioblasts into the vitreous of neonatal dogs that had been exposed to high oxygen atmospheres and were developing retinal neovascularization, however, led to localization in an endothelial position. Work in this area to date indicates both the therapeutic promise of retinal revascularization and the difficulty of the task ahead.

Retinal neuronal preservation and repair

The repair or restoration of cells in the neural retina is an even more challenging problem. Most cells in the CNS, including the retina, do not replicate. But restoration of function is clearly possible, as demonstrated by the dramatic improvement in visual acuity seen in many patients in a controlled clinical trial of macular laser treatment, intraocular injection of the steroid triamcinolone or the anti-VEGF monoclonal antibody ranibizumab in patients with diabetic macular edema (DRCRnet, 2010). Some years ago, Fitzgerald et al., (1980) described slow restoration of visual function over the course of about one year in a group of patients who had undergone surgery for macula-off retinal detachments. Because their recovery of visual acuity corresponded with restoration of the Stiles-Crawford effect, which measures the directional sensitivity of the retinal cones, these authors assumed that their results implied simply reorientation of the misaligned photoreceptor outer segments. But they may equally have been due to elongation of the outer segments (Forooghian et al., 2010), once they had resumed their normal apposition to the retinal pigment epithelium. Although repopulation of the retina when neurons have been lost, as in the apoptosis that occurs in the retinas of diabetic patients (Barber et al., 1998), appears to be a distant goal, some restoration of lost neuronal function is clearly possible. The duration of macular detachment or edema is clearly an important prognostic factor, but other influences on the preservation and restoration of retinal neuronal function remain to be determined.

Proposals for future investigation

1. Further characterize endothelial progenitor cells and pluripotential stem cells and determine the most efficient and safest way to stimulate their release from the bone marrow of diabetic individuals.
2. Explore whether one can effectively promote differentiation of these cells into pericyte and endothelial cell phenotypes, or whether pericyte progenitor cells are a separate, mesenchyme-derived population of stems cells that, in attempts to repopulate the ischemic retinal vasculature, may need to be introduced separately from endothelial progenitor cells.
3. Determine the most effective way to enable these cells to repopulate acellular or paucicellular retinal capillary tubes and to restore normal capillary function in ischemic retina. What signals stimulate the "homing" of precursor cells to damaged capillaries, and what signals repel these cells?
4. Determine the optimal means for prevention of neural and glial apoptosis, for protection of neural function and for its restoration when it is impaired in the diabetic retina.

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Chapter 9
Animal Models

Discussion Leaders: Renu Kowluru and Robert N. Frank

Scribe: John Lillvis

Session Participants: P D'Amore, P Dore-Duffy, E Duh, M Friedlander, M Grant, HP Hammes, G Lutty, K Palczewski, D Puro, J Steinle, A Stitt, L Zhuo

The members of the Vascular and Neuronal Repair session also devoted time to a discussion of appropriate animal models, which can be of great benefit in studying the pathogenesis and potential treatments for this sight-threatening disease. The session participants agreed that the existing animal models, especially rodent models, have been very useful, but the field remains open to new and potentially better animal models.

What is an optimal animal model of diabetic retinopathy?

The panel discussed in depth the characteristics that an animal model should have to be considered optimal. The development of DR is duration dependent; with nearly 40 percent of patients who have had diabetes for 5 years showing some signs of DR, and nearly all having some evidence of the disease after 20 years of diabetes (Frank, 2004). The optimal model should be able to reproduce within a short period of time (months to a maximum of about 2 years) anatomic lesions characteristic of DR in patients. Furthermore, since various functional abnormalities, such as changes in the gross electroretinogram (ERG) and the more finely detailed multifocal ERG may precede the development of histopathology, the optimal animal model should be suitable to measure such parameters.

What is the importance of a good animal model?

A good animal model is an integral part of a successful research strategy as it would allow closer approximation to a human response and can provide tissue for study at all stages of the disease. For example, experimentally diabetic or galactosemic dogs (of 3-5 years duration) develop retinal lesions similar to those observed in diabetic humans, including loss of capillary pericytes and the appearance of capillary microaneurysms, hemorrhages, and acellular capillaries, while diabetic rats develop pericyte loss and capillary acellularity, lesions that are consistent with the early stages of DR (Figure 9-1) (Engerman, 1989; Engerman and Kern, 1987, 1995). Such animal models can serve as platforms to test specific hypotheses and the results of an animal model study can help characterize a system or predict the outcome of a therapy. Ethically, studies in animal models are prerequisite to clinical trials of new therapies in humans.

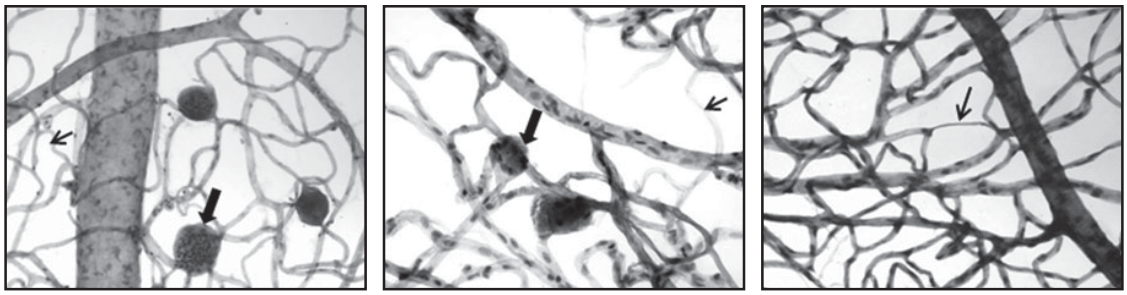

Figure 9-1: Trypsin digest preparations of retinal blood vessels from (a) human donor with diabetic retinopathy, (b) dog with alloxan-induced diabetes for ~5 years, and (c) Wistar rat with streptozotocin-induced diabetes for ~12 months. In this technique, the formalin-fixed retina was digested with a 1% solution of crude trypsin. After gently brushing away the neuroretinal tissue, the isolated vascular tree was air dried onto a glass microscope slide, and stained with periodic acid-Schiff and hematoxylin for histologic evaluation. Microaneurysms are indicated with heavy arrows and acellular capillaries with thin arrows.

What are the good animal models of diabetic retinopathy?

A number of animal species have been examined over the years for their ability to develop histopathology characteristic of DR, especially the early lesions of the disease. The panel discussed the advantages and disadvantages of various animal models of DR.

a. Experimentally-induced diabetic dogs develop lesions that are characteristics of background retinopathy in diabetic patients, including capillary microaneurysms, pericyte ghosts and intraretinal hemorrhages (Figure 1b). However, the time lag between the induction of diabetes and the manifestation of retinal microvascular pathology is long, and it takes over 3-5 years to see these lesions (Engerman, 1989; Engerman and Kern, 1987, 1995).

b. Rodent models: (i) Rat: Chemically-induced diabetic rats show some lesions typical of early DR in humans and dogs. Rats kept diabetic for about 10-12 months develop accelerated apoptosis of retinal microvascular cells. Their retinal microvasculature develops basement membrane thickening and vacuolization, loss of pericytes and subsequently endothelial cells with the resultant appearance of acellular capillary tubes that are incapable of carrying blood (Figure 1c) (Engerman and Kern, 1995; Kern and Engerman, 1994). Diabetic rats also show functional abnormalities, their retinal vasculature becomes leaky and ERG show impairments (Hancock and Kraft, 2004). (ii) Mouse: Streptozotocin-induced diabetic mice have shown features of early microvascular damage associated with DR including the appearance of pericyte ghosts, the formation of degenerated capillaries and capillary cell apoptosis. In addition, neuronal changes and ERG abnormalities are also consistent with the human disease (Feit-Leichman et al., 2005; Kern and Engerman, 1996; Kowluru, 2002). However, streptozotocin-induced diabetic mice do not show consistent ganglion cell apoptosis or signs of Müller cell reactivity (Kern et al., 2010), which are instead reproducibly found in streptozotocin-induced diabetic rats. It is of note that several biochemical changes occurring in the retina of diabetic rats do not occur in the retina of diabetic mice (Asnaghi V et al, 2003; Obrosova et al., 2006); and these differences could profitably be further investigated in relation to the pathogenesis of the lesions of retinopathy.

c. Large animals have also been studied. These larger animal models are highly valuable because they can bridge the gap from the results of small animal studies to clinical applications in humans. (i) The rhesus monkey is an attractive model of DR because of the similarities in the anatomy and physiology of the retina with those of the human retina. But, even after 6-15 years of diabetes in these monkeys (streptozotocin-induced or total pancreatectomy, or spontaneously insulin-dependent diabetic animals), very little retinopathy develops unless the monkeys are also hypertensive. In aged monkeys (25 years) with spontaneous diabetes, intraretinal hemorrhages and large areas of retinal capillary nonperfusion have been observed (Kim et al., 2004). (ii) Cats made diabetic by partial pancreatectomy alone or by pancreatectomy and alloxan have served as good experimental model to investigate DR. This animal model has the advantage of providing large eyes that can be readily used for surgical and other experimental manipulations. Diabetic cats do not develop cataracts, and this is a great advantage in the experimental study of DR, since other diabetic animals (such as rats, but not mice) readily develop cataracts, preventing visualization of the ocular fundus to monitor the progression of the disease (Hatchell et al., 1995). Furthermore, the maintenance of cats is less expensive than dogs or monkeys. (iii) The eyes of another model, the Yorkshire pig, have many morphological and physiological similarities with human eyes. Streptozotocin-induced diabetic pigs develop retinal capillary basement membrane thickening and other ultrastructural features of the human disease, occurring within a relatively short duration of diabetes (18-32 weeks), thus making it another good model of DR (Lee et al., 2010).

Other novel models

Cone-rich sand rats, found in the North African desert, become hyperglycemic when maintained on standard rat chow. This is a paradigm remarkably similar to that of many human populations, who develop a marked increase in the incidence of type 2 diabetes when improved economic conditions lead to their adoption of a more highly caloric diet. These animals show many characteristics of DR including pericyte loss, vasodilation and blood-retinal barrier breakdown. Importantly, the cone photoreceptors of these diabetic animals show reduced expression of short- and mid-/long-wavelength opsins (Saidi et al., 2011), suggesting the use of this experimental model to screen neural-targeted therapies that are difficult to study experimentally in human patients.

Zebrafish present another interesting vertebrate model for vision research, as they possess a retina with the same major cell types arranged in the identical laminar pattern as the human retina. Zebrafish have been shown to possess early signs of DR, suggesting their potential for use in this important area of research. Soaking fish in a 2 percent glucose solution on alternating days significantly increases blood glucose levels for 30 days, and results in neurodegenerative and vascular lesions resembling those of early non-proliferative DR (Gleeson et al., 2007). These initial studies have suggested that zebrafish may be a useful model for DR.

Marmoset monkeys: Initial studies using this non-human primate model have shown that hyperhexosemia (galactose-feeding) can induce many features of DR including acellular capillaries, microaneurysms, mild retinal edema and retinal vascular leakage (Chronopoulos A, 2011). This model presents the advantage of being genetically and physiologically close to humans, but the phylogenetic divergences among groups of nonhuman primates is an issue that needs to be considered. The panel reached an overall consensus that mice and rats are good animal models of human DR. They have the major advantage that the time lag between the induction of diabetes and the appearance of vascular histopa-

thology characteristics of diabetic retinopathy is relatively short (8-10 months) compared to ~5 years in dogs; however, other changes, particularly to glia appear much earlier (Kumar et al., 2010; Salido et al., 2012). Additionally, a large number of rodents can be used for individual experiments. The larger size of a rat compared to a mouse has advantages, including a great deal more retinal tissue, and the ease of performing some surgical procedures, as well as experimental measurements such as ERG. Rats are also considered better models for pharmacokinetics. However, the mouse genome is much better understood, which makes the mouse a good model to manipulate specific genes of interest to better understand their role(s) in the development of DR.

What are the limitations with the animal models?

An ideal animal model is expected to provide better understanding the disease, and the etiology of the disease should be similar to that in humans. Although animal models of DR have helped us to advance our knowledge of this disease, these models have some limitations. Since DR is a slowly progressing disease, all animal models to date show only the signs of background retinopathy. Retinal neovascularization has not yet been observed in any diabetic animals. Because they lack a fovea, rodents cannot serve as an adequate model of diabetic macular edema. It was recommended that investigators planning experiments on retinopathy in diabetic animals should consider any variations between the strains of animals and the vendors utilized to obtain them.

Cell culture

Capillary cells isolated from the retina (Figure 9-3) that have been grown in high glucose media have served as a useful tool to determine biochemical pathways associated with the development of DR. Cell culture has an advantage over animal models because specific pathways can be more readily isolated, and targeted, and it is easier to manipulate the targeted pathway using pharmacological/genetic means. However, removal of the cells from a tissue could induce changes in the genome, and the cellular environment in artificial culture media might not reflect conditions *in vivo*. The discussion concluded that despite the limitations with the *in vitro* cell culture systems, the complex *in vivo* system in the intact animal does not allow us to tease out the mechanistic details. Overall, the cell culture model is useful to investigate metabolic pathways, but the results obtained from cell culture studies should be validated using animal models.

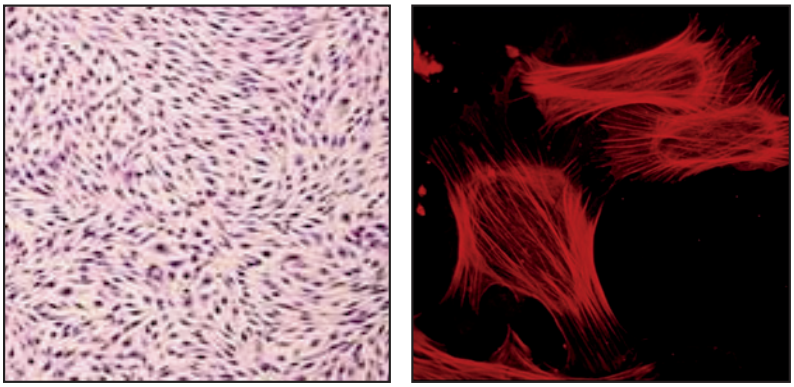

Figure 9-3: Histologic preparations of cell cultures of (a) pericytes and (b) endothelial cells, isolated from bovine retina and plated in modified Eagle's culture medium with 10% fetal bovine serum. The pericytes are immunostained for smooth muscle actin, which appears as numerous parallel muscle fibers. The cells have an irregular shape and are not confluent in this culture. However, the endothelial cells, here stained with hematoxylin-eosin, form a tighly-packed, confluent layer with a typical cobblestone-like morphology.

Proposals for future investigation

Although the overall consensus was that the rodent models have helped the DR field move forward, the limitations noted above seeks for better animal models. Future investigations should look for developing:

1. Animal models that can be made diabetic without the use of any chemicals; e.g., taking advantage of zebrafish, which become chronically hyperglycemic after incubating them in high glucose solutions.

2. Models which are in better proximity to the human diseases, e.g., small non human primates.

The main guidelines for a new model for this slowly progressing complication of diabetes should consider (i) the time frame required to observe retinal histopathology characteristic of DR (months not years), (ii) the cost involved in the purchase and the maintenance, and (iii) number of animals per treatment. Animal models with macula would be of immense advantage, and these models will allow us to investigate both DR and macular edema.

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Concluding Remarks

John E. Dowling

The primary event that leads to the development of diabetic retinopathy (DR) is clearly a rise in blood glucose. No one disputes this, and that tight glucose control in diabetic patients reduces the incidence and progression of retinopathy is unequivocal. Beyond that, things are murky. Whereas the first clinical signs of the disease are vascular in nature – microaneurysms and small hemorrhages in the retina – there is increasing evidence that neuronal and glial changes predate the vascular changes. What might connect the rise in blood glucose levels to changes in neuronal/glial function and to the vascular changes that eventually can destroy vision in an eye? Understanding that link is likely to be crucial for developing effective therapies for preventing the disease.

Do we have some clues? Many were noted during the workshops and plenary session, but nowhere are they gathered together into a coherent picture. This I will attempt to do here. A first clue comes from the fact that although the retina is a true part of the brain, pushed out into the eye during embryological development, and has all the features of the rest of the brain including a blood retinal barrier, vascular changes do not usually occur elsewhere in the brain and when present are less severe than those that occur in the retina (Cosentino et al., 2009, Selvarajah 2011, Strachan 2011). It is true that there appear to be more pericytes associated with the retinal blood vessels as compared to blood vessels in other brain regions (Frank et al., 1990), and pericyte loss is seen early in DR. However, this seems unlikely to be the underlying reason the retina is so vulnerable to the changes that occur in DR.

What, then, is different about the retina relative to the rest of the brain? First, there are few blood vessels in the outer retina and none in close association with the photoreceptors. The photoreceptors and much of the outer retina are supplied by the choroidal circulation beneath the pigment epithelium. Even in the inner retina the blood vessels are relatively sparse. Perhaps of more significance is the fact that the retina is very active metabolically. Indeed, parts of the retina (photoreceptor inner segments) may be more metabolically active than any other part of the brain or even any other tissue in the body.

Why is the retina so metabolically active? Primarily because of the photoreceptors, which behave as if darkness is the stimulus for them (reviewed in Dowling, 2012). That is, in the dark the outer segments of the photoreceptors are leaky to Na^+ and $Ca2^+$ and what light does is to decrease this leak. This is the light signal generated by the photoreceptors – the brighter the light, the less leak. But in both light and dark, the photoreceptors must be continually pumping out Na^+ and $Ca2^+$ from the cells, which requires much energy, and undoubtedly the pumps are most active in the dark. A single mouse rod, for example, uses 10^8 ATP molecules per second when dark adapted (Okawa et al., 2008) and in humans about 95 percent of the photoreceptors are rods. Thus, especially in the dark, the O_2 demand in the retina is substantial, especially around the photoreceptor inner segments, and this has been demonstrated by measurements of O_2 tension through the thickness of the retina (Figure 1). Especially in the dark, O_2 tension is very low around the photoreceptor inner segments, and whereas it is higher in this region in the light, it is still lower than elsewhere in the retina (Birol et al., 2007).

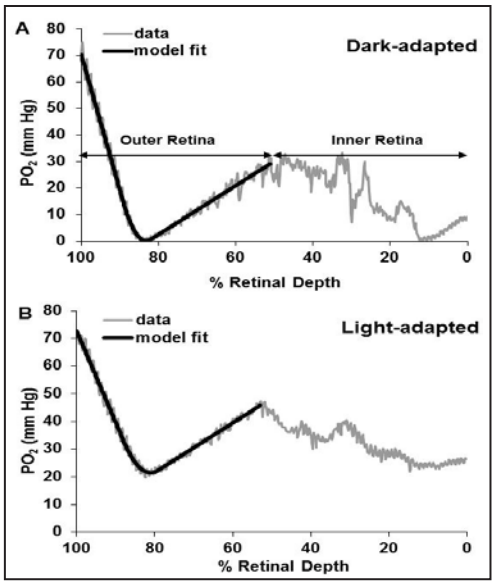

Figure 1. Profiles of PO_2 as a function of % retinal depth from the periforeal retina of a macaque (cynomologues) monkey in dark adaptation (A) and light adaptation (B) the solid lines are fits of a diffusion model to the data. The minimal PO_2 at ~ 80% retinal depth corresponds to the inner segment region of the photoreceptors where the Na^+ pumps are located. (from Birol et al., 2007) (used with permission)

These data raise the question of whether retinal hypoxia, especially around the photoreceptors, could be the precipitating cause of the changes that take place in DR. There are now several pieces of information that suggest this may be so. First, diabetic patients who have lost substantial numbers of photoreceptors because of diseases such as retinitis pigmentosa do not develop observable DR lesions, perhaps because the energy demand of the retina is reduced by the photoreceptor loss. Also, it has long been known that extensive photocoagulation which destroys both photoreceptors and retinal neurons is protective against DR, and has been used as treatment for the disease since the 1970's.

Other observations are pertinent as well. As noted earlier, before there are any clinically detectable retinal vascular changes in diabetes, there are signs of abnormal retinal function (see Chapter 1). Dark adaptation is slowed and incomplete, there is loss of color (blue) discrimination, and contrast sensitivity is reduced. These can be rapidly reversed (at least partially) by O_2 inhalation, suggesting that retinal hypoxia could be an early event leading to DR and perhaps its initiator (Harris et al., 1996, Dean et al., 1997). Finally, Arden and his coworkers (2011) have shown in preliminary studies that by partially suppressing rod activity with dim light at night in human patients, some amelioration of visual function deficits and diabetic macular edema occurs (see also Arden et al., 1998).

It needs to be stated, however, that at present there is no direct evidence for hypoxia in diabetic retinas. On the other hand, Figure 1 clearly shows that there are very low oxygen levels around the photorecep-

tor inner segments in the *normal* dark-adapted retina. Thus, quite small decreases in O_2 levels in this region of the retina in diabetes could affect the retinal Müller cells whose distal (fiber basket) processes envelop the inner segments (see Dowling, 2012), and this might be an initiating event in DR.

Can hyperglycemia and hypoxia in the retina be related? In the presence of high glucose, Müller cells (and probably other retinal cells) increase production of nitric oxide (NO) as a result of increased expression of the enzyme iNOS. Significantly, NO reduces light-evoked vasodilation, and probably this occurs also in complete darkness when the rod and cone photoreceptors are maximally active. In other parts of the brain, blood vessels ordinarily dilate when oxygen needs are increased, and this holds for the normal rat retina. In the diabetic rat retina, on the other hand, the blood vessels do not ordinarily dilate when the retina is light activated, which could lead to the hypoxia (Misha and Newman, 2010, 2012). Several studies have also shown that light evoked vasodilation is reduced in diabetic patients, findings consistent with the rat studies (Gartofer et al., 2004, Mandecka et al., 2007, Pemp et al., 2009).

With a reduction in blood vessel dilation, especially when the retina is highly metabolically active, hypoxia is likely to occur, and this could be the trigger for the neuronal and blood vessel abnormalities that occur in DR. Hypoxia is known to result in the production of substances such as VEGF, known to be crucial in the proliferation of blood vessels in DR, and hypoxia can result in the production of more NO, reducing further any vasodilation of retinal vessels in response to increased O_2 demand.

In addition to a decrease in light-evoked vasodilation, there are changes in basal vessel diameter in DR. Although results are somewhat conflicting, a decrease in vessel diameter has been observed in both diabetic patients and diabetic animals in early stages of DR. This vasoconstriction might also make the retina hypoxic. Indeed, a decrease in vessel diameter could be a more important factor in rendering the retina hypoxic than the loss of light-evoked vasodilation (Kern et al., 2000, 2001, Kowluru et al., 2000, Bolton et al., 2004).

An obvious way to counter these effects would be to inhibit iNOS and decrease NO production. This has been done in diabetic rats, and the results show no reduction in light-evoked vasodilation. A possible therapy for DR in humans is a potent inhibitor of iNOS coupled, perhaps, with a vasodilating agent. Indeed, aminoguanidine and other iNOS inhibitors have been shown to be effective in slowing the progression of DR in animal models and in one human study (Kern et al. 2000, 2001, Kowluru et al., 2000, Bolton et al, 2004).

The above scenario fits together many pieces of information but, of course, leaves out many other pieces of the puzzle. There is evidence, for example, that ganglion cells die early in DR and so the focus in early DR has long been on the inner retina and the role of hypoxia there. But a link between inner retinal hypoxia and DR has never been unequivocally established. The newer realizations, reviewed here, suggest rather that more focus should be on the outer retina and the photoreceptors and the possibility that hypoxia there could be the precipitating cause. Although the outer retinal hypoxia hypothesis is a candidate for initiating DR, others are certainly possible. Clearly, many changes occur in the diabetic retina including inflammation, oxidative stress, lipid alterations, reduction in glutamate uptake, and so forth, all of which likely contribute to DR. But what initiates these changes? If we can get some notion of the early initiating events that underlie DR, we will have moved forward substantially. We hope that this report generates new ideas and experiments that will provide new insights on this question and on this devastating disease.

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