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Clinical and Experimental Approach to the Benefits and Risks of Laser Treatment for Diabetic Macular Edema

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Clinical and Experimental Approach to the Benefits and Risks of Laser Treatment for Diabetic Macular Edema

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Abstract

Diabetic retinopathy is an ophthalmic complication of diabetes that currently affects 93 million people worldwide. Diabetic macular edema is a subtype of diabetic retinopathy that is characterized by leaking blood vessels in the central part of the retina, and it is a major cause of vision loss in individuals with diabetes. Screening for retinopathy is performed at regular intervals to identify subjects in need of treatment. In Sweden, the recommended screening interval for type 2 diabetic subjects with no diabetic retinopathy is 3 years. Laser treatment for diabetic macular edema has been used for decades, but studies have reported conflicting results regarding possible clinical complications following such therapy. Furthermore, the molecular events involved in the clinical benefits of laser treatment have not been fully explored.

To determine the safety of the current screening interval, the 3-year incidence of diabetic retinopathy and macular edema was monitored in type 2 diabetic subjects with no retinopathy (Study I). To identify the long-term effects of laser treatment, the retinal sensitivity in 29 consecutively recruited subjects with diabetic macular edema was assessed by visual fields before and after laser treatment every 6 months during a period of 2 years (Study II). To investigate the cellular and molecular events that may be involved in the clinical benefits of laser treatment, a model of in vitro laser coagulation of the retinal pigment epithelium was established and used to evaluate cell death and cell repair (Study III). The same model was also used to explore the effects of laser treatment on mRNA and protein levels by analysis of microarray and proteomics data (Study IV).

Study I demonstrated a very low incidence of sight-threatening diabetic retinopathy, which supports the current use of a 3-year retinal screening interval for the subgroup in question. Study II showed no change in retinal sensitivity following laser treatment for diabetic macular edema during the 2-year follow-up compared to baseline. Study III revealed that laser-induced damage involved both necrosis and apoptosis, and the repair process entailed both proliferation and migration. In study IV, the downregulation of the protein Carbonic anhydrase 9 and the upregulation of heat shock proteins 1A and 1B were the most interesting findings and might explain some of the beneficial effects of laser treatment for diabetic macular edema.

Key words: Diabetic macular edema, laser treatment, retinal pigment epithelium, ARPE-19

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Till min livskamrat,
Line

Live as if you were living a second time, and as though you had acted wrongly the first time.

— *Viktor E. Frankl*

Contents

Abstract

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List of papers

I. Agardh E, **Tababat-Khani P**. Adopting 3-year Screening Intervals for Sight-Threatening Retinal Vascular Lesions in Type 2 Diabetic Subjects Without Retinopathy. Diabetes Care. 2011 Jun;34(6):1318–9. DOI: 10.2337/dc10-2308.

II. **Tababat-Khani P**, Bengtsson B, Elisabet A. Effects of focal/grid laser treatment on the central visual field in diabetic macular oedema: a 2-year follow-up study. Acta Ophthalmol. DOI: 10.1111/aos.12956. [Epub ahead of print]

III. **Tababat-Khani P**, Berglund LM, Agardh CD, Gomez MF, Agardh E. Photocoagulation of Human Retinal Pigment Epithelial Cells In Vitro: Evaluation of Necrosis, Apoptosis, Cell Migration, Cell Proliferation and Expression of Tissue Repairing and Cytoprotective Genes. PLoS One. 2013 Aug 1;8(8):e70465. DOI: 10.1371/journal.pone.0070465.

IV. **Tababat-Khani P**, de la Torre C, Canals F, Bennet H, Simo R, Hernandez C, Fex M, Agardh CD, Hansson O, Agardh E. Photocoagulation of human retinal pigment epithelium in vitro: unravelling the effects on ARPE-19 by transcriptomics and proteomics. Acta Ophthalmol. 2015 Jun;93(4):348–54. DOI: 10.1111/aos.12649.

The papers are referred to in the text as Studies I–IV and are reproduced here with permission from the publishers.

Abbreviations

AGE	advanced glycation end product
Anti-VEGF	anti-vascular endothelial growth factor
ARPE-19	human retinal pigment epithelial cell line
cDNA	complementary DNA
CSME	clinically significant macular edema
DMEM	Dulbecco's Modified Eagle's Medium
ETDRS	Early Treatment Diabetic Retinopathy Study
HbA1c	glycosylated hemoglobin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDH	lactate dehydrogenase
mRNA	messenger RNA
mfERG	multifocal electroretinography
Nd:YAG	neodymium-doped yttrium aluminum garnet
PCNA	proliferating cell nuclear antigen
RAGE	receptor for advanced glycation end product
RPE	retinal pigment epithelium
ROS	reactive oxygen species
RT-qPCR	reverse transcription quantitative PCR
SAP	standard automated perimetry
SWAP	short-wavelength automated perimetry
TD-OCT	time-domain optical coherence tomography
VEGF	vascular endothelial growth factor

Introduction

Diabetes mellitus is a chronic disease that is characterized by elevated blood glucose levels (hyperglycemia). The global prevalence of diabetes among adults was estimated to be 6.4% in 2010, and calculations indicate that the rate will rise to 7.7% by 2030, with expected increases of 69% and 20% in developing and developed countries, respectively [1]. Concurrently, the global cost of diabetes was at least 376 billion USD in 2010 and is projected to be approximately 490 billion USD by 2030 [2].

Hyperglycemia impacts both small and large blood vessels in the body, causing what are known as microvascular and macrovascular complications. The microvascular complications affect the eyes (retinopathy), kidneys (nephropathy), and the peripheral nerves (neuropathy), and the macrovascular complications affect the cardiovascular system. In general, these complications lead to increased morbidity, including visual impairment and blindness, renal failure, peripheral nerve damage, myocardial infarction, and stroke. Additional ocular complications of diabetes are cataract and keratopathy. Diabetic retinopathy is the focus of this thesis.

Diabetic retinopathy

Diabetic retinopathy affects 35% of the global diabetic population, representing approximately 93 million people, of which 28 million are estimated to have vision-threatening diabetic retinopathy [3]. This disease is considered to be a leading cause of blindness among working-age adults worldwide [4].

Diabetic retinopathy impacts the retina and is characterized by morphological vascular changes, initially microaneurysms and hemorrhages. Subsequent features—in no specific order—are exudates, retinal thickening, dilatation of venules, capillary dropout, and pathological connections between retinal arteries and veins. The advanced stages of retinopathy include neovascularization, fibrovascular growth, and finally retinal traction and detachment, which can result in permanent vision loss. Neovascularization, or angiogenesis, is defined as the formation of fragile, easy bleeding, pathological blood vessels, which exhibit high vascular permeability and hence leak fluids and substances that can damage the retina.

Pathophysiology of diabetic retinopathy and diabetic macular edema

Epidemiological studies have confirmed that the microvascular damage that occurs in diabetic retinopathy is linked to chronic hyperglycemia [5, 6], but the mechanisms behind the development of the retinal microvascular abnormalities have not yet

been thoroughly explained. The biochemical pathways and structural abnormalities that have been identified as key contributors in this context are summarized in Table 1. These pathways are interrelated and intertwined, and therefore none of them can be pinpointed, nor overlooked, as being at least partly responsible for the retinal vascular abnormalities that arise in diabetes.

Table 1.

Pathways involved in the development of diabetic retinopathy.

Name	Initiating process	Endpoints	References
Polyol pathway	Hyperglycemia	↑ Sorbitol (osmotic damage) ↑ AGEs and oxidative stress ↓ Anti-oxidative protection	[7–9]
Advanced glycation end products (AGEs)	Hyperglycemia Polyol pathway RAGE binding	↑ Vessel stiffness (cross-links lipids and proteins) ↑ Transcription of growth factors and inflammatory cytokines	[10,11]
Protein kinase C	Hyperglycemia	↑ Growth factors ↑ Pericyte loss	[12–14]
Oxidative stress	Hyperglycemia Polyol pathway AGEs Protein kinase C activation	↑ Reactive oxygen species (ROS) ↑ Growth factors	[15,16]

Additional processes involved in the development of diabetic retinopathy

Breakdown of the inner blood retinal barrier

The inner blood retinal barrier consists of tight junctions between the endothelial cells of the capillaries in the inner retina, which prevent leakage of molecules from the retinal capillaries [17]. In diabetes, dysfunction of the blood retinal barrier causes extravasation of fluids and accumulation of lipids and lipoproteins in the intraretinal space, which eventually leads to irreversible retinal damage. The mechanisms underlying the fluid leakage include increased paracellular permeability due to dysfunctional proteins in the tight junctions between the endothelial cells and

augmented transcellular permeability of caveolar vesicles within the endothelial cells (e.g., induced by VEGF and AGEs) [18].

Pericytes contribute to the integrity of the blood retinal barrier and, together with endothelial cells and the basal membrane, they make up the retinal capillaries [19]. There is evidence that detachment and apoptosis of pericytes are among the earliest events in the development of diabetic retinopathy, and may be triggered by intercellular alterations that weaken adhesion between the pericytes and endothelial cells [18].

An additional factor in breakdown of the blood retinal barrier is the modulation of aquaporin 4, a protein that is involved in the transport of water across cell membranes. In diabetes, it is possible that disturbance of aquaporin 4 causes swelling of the Müller cells, which are responsible for regulating the water homeostasis in the retina. Accordingly, such an effect may play a role in the development of retinal edema [20].

Thickening of the basal membrane also occurs in diabetes, probably due to an imbalance between synthesizing and degrading factors [21]. The retinal capillary basement membrane is 200–600 nm thick [22] and is composed mainly of type IV collagen, fibronectin, laminin, and heparan sulfate proteoglycans, and it is located between the pericytes and the endothelium of blood vessels. This membrane serves as a substratum for cell attachment, provides a selective permeability barrier, and regulates cell survival [23]. Events that promote basal membrane thickness include increased levels of growth factors, activation of protein kinase C, and accumulation of AGEs. Alteration of the basal membrane structure is believed to affect the permeability of the retinal capillaries.

Growth factors

The pathways presented in Table 1 are all linked to an upregulation of growth factors associated with the development of diabetic retinopathy, of which VEGF is the most extensively studied. Numerous other growth factors have been found to be involved in the developlemt of diabetic retinopathy (Table 2), in addition to downregulation of several anti-angiogenic factors.

Table 2.
Alphabetical list of growth factors reported to be involved in the development of diabetic retinopathy [24, 25].

Growth factor	Pro- or antiangiogenic
Angiopoietin 2	Pro
Angiostatin	Anti
Atrial Natriuretic Peptide	Anti
Chemokine ligand 12	Pro
Connective Tissue Growth Factor	Pro
Cystein-rich 61	Pro
Endostatin	Anti
Erythropoietin	Pro
Fibroblast Growth Factor	Pro
Hepatocyte Growth Factor	Pro
Insulin-like Growth Factor 1	Pro
Interferon-α 2a	Anti
Osteopontin	Pro
Pigment Epithelial Derived Growth Factor	Anti
Placental Growth Factor	Pro
Platelet Derived Growth Factor	Pro
Thrombospondin	Anti
Transforming Growth Factor-beta	Pro
Vascular Endothelial Growth Factor	Pro

Inflammation

Development of diabetic retinopathy is most likely influenced by inflammatory mechanisms, which in turn mediate structural and molecular alterations associated with the development of retinal vascular changes. Examples of this are the following: VEGF has been identified as a proangiogenic cytokine [26]; binding of AGEs to RAGE leads to leukostasis and tight junction dysfunction [27]; and static leukocytes produce ROS and inflammatory cytokines [28]. In addition, the cytokine IL-6 induces

endothelial cell permeability by increasing the gene expression of *VEGF* [29], and IL-8 promotes angiogenesis [30]. Furthermore, IL-1β activates transcriptional factor NF-κB, which is involved in the transcription of inflammatory cytokines [31] and acts together with TNF-α to stimulate production of IL-6 and IL-8 in retinal pigment epithelium (RPE) cells, and also promotes angiogenic activity.

Carbonic anhydrase

Carbonic anhydrases are enzymes that catalyze the conversion of carbon dioxide and water to bicarbonate and a proton, and they participate in physiological processes that involve electrolyte balance, pH regulation, water, and transport of CO_2 and HCO_3^- [32]. An observational study conducted in 1988 [33] suggested that the carbonic anhydrase inhibitor acetazolamide can have a beneficial effect on macular edema, probably by enhancing fluid resorption, improving retinal blood flow, and increasing retinal oxygen tension [34]. Carbonic anhydrase 9 is also a known marker of hypoxia in tumors [35].

Screening of diabetic retinopathy

Due to the complex characteristics of diabetic retinopathy, grading scales have been developed to standardize the classification of the severity of vascular changes [36,37]. In this context, monitoring in screening programs is crucial to identify subjects in need of treatment, because the retinal vascular changes in diabetic subjects can occur without affecting visual acuity. Screening is performed by trained medical staff or ophthalmologists using either fundus photography or fundus examination. Epidemiological studies have shown that the risk of progression of vascular changes increases with the severity of both the diabetes per se [38] and the retinal vascular changes [36].

The ever-increasing burden on health service providers that has accompanied the increasing number of people with diabetes has prompted a debate regarding appropriate screening intervals for diabetic retinopathy. Alternative and affordable screening methods with smart phone photography have been studied and found to show considerable agreement with clinical examination [39]. Assessments of fundus images by use of automated algorithms [40, 41] and by non-professionals [42] have also provided promising results and may help decrease the burden on the health care system in the future.

Recommended screening intervals for type 2 diabetic subjects with no retinopathy differ between countries. For example for this subgroup of patients, annual screening is advised in the United States [43] and the United Kingdom [44], whereas biennial screening is advocated in Iceland [45]. Biennial screening was also considered appropriate by the Swedish Board of Health and Welfare until 2010, when it was replaced by the currently applied recommendation of a 3-year interval [46].

Extended screening intervals are believed to reduce the workload of health care providers, and yet the screening interval applied in Sweden until 2010 for diabetic subjects with no retinopathy was based on expert opinion rather than epidemiologic investigations. A large cohort study of the incidence of diabetic retinopathy in England, which included more than 20 000 screening events, was published in *The Lancet* in 2003 [47], and the results that were reported suggested that it could be safe to extend the screening interval from 2 to 3 years in type 2 diabetic subjects without retinopathy. In accordance with that observation, the local guidelines in Malmö, Sweden, were changed in 2006 to recommend a 3-year interval. The aim of Study I included in this thesis was to determine whether a 3-year interval could be safe in a Swedish population by conducting prospective longitudinal monitoring of the incidence rate of diabetic retinopathy in this cohort.

Diabetic macular edema

Thickening of the retina in the macular region is considered to be a major cause of vision loss in diabetic subjects and is commonly referred to as diabetic macular edema. Such edema can occur at any stage of diabetic retinopathy and does not necessarily affect visual acuity. The term clinically significant macular edema (CSME, defined in Table 3) was introduced in the Early Treatment Diabetic Retinopathy Study (ETDRS) [48,49]. In that investigation, it was found that photocoagulation led to stable visual acuity in eyes with CSME but provided no benefit in eyes with retinal edema that did not fulfill the criteria for CSME. The term CSME has been considered the gold standard in terminology for sight-threatening macular edema in clinical research.

Table 3.
The definition of clinically significant macular edema includes one or more of the listed characteristics.

- Thickening of the retina at or within 500 µm of the center of the macula.
- Hard exudates at or within 500 µm of the center of the macula, if associated with thickening of adjacent retina.
- A zone or zones of retinal thickening one disc area or larger, any part of which is within one disc diameter of the center of the macula.

Measurement of retinal sensitivity

Standard automated perimetry (SAP) is based on the principles of the Goldmann perimeter using a white stimulus on a white background. What is known as selective perimetry was introduced as being more sensitive than SAP for detecting subtle visual field loss, and one such approach is called short-wavelength automated perimetry (SWAP). Initial investigations suggested that SWAP is particularly sensitive to

diabetic macular edema [50,51], although that finding was not confirmed in a later study [52].

Microperimetry is a technique in which fundus imaging and tracking are performed simultaneously during perimetric examination. This strategy enables direct testing of areas of the retina exhibiting morphological changes, and it may offer an advantage over SAP for evaluation of retinal sensitivity in subjects with impaired fixation. A drawback of microperimetry is that it has a limited dynamic range compared to SAP [53], and hence it may be less efficient in detecting subtle changes in retinal sensitivity.

SAP is the conventional method for monitoring subjects with suspected or established visual field loss, and this technique has not yet been proven to be inferior to any other type of perimetry for detection of visual field disturbances. Moreover, SAP is most widely employed to determine fitness for operating motor vehicles in the United Kingdom [54] and in Sweden [55], and also for classifying visual impairment and blindness [56]. Therefore, we chose to use SAP to study the long-term effects of laser treatment on diabetic macular edema.

Perimetry reflects the function of the entire visual pathway, whereas mfERG allows assessment of retinal sensitivity alone and also enables mapping of local and global retinal defects without involvement of the visual pathway. Lung et al. [57] performed visual field perimetry and mfERG to investigate retinal sensitivity in type 2 diabetic subjects with and without retinopathy and found that mfERG was superior in showing differences between the groups. Moreover, abnormal responses have been demonstrated in CSME patients by mfERG [58], and models using this method to predict diabetic macular edema have been developed [59]. Nevertheless, clinical application of mfERG is limited due to the need for trained staff and high equipment costs, as well as the negative influence of lens media opacity and the lack of age-matched normative data [60].

Focal and grid photocoagulation

Despite the increasing trend towards use of intravitreal anti-VEGF injections to treat diabetic macular edema, laser therapies still constitute an important part of the arsenal of treatment options. Since publication of the results of the ETDRS, focal and grid photocoagulation have been considered standard treatments for CSME. In focal photocoagulation, the laser beam is aimed at leaking microaneurysms, where the energy is absorbed by the hemoglobin in the blood, leading to thrombus formation and vessel wall contraction [61]. In grid photocoagulation, the laser beam is directed toward areas of leakage or retinal thickening originating from dilated leaking capillaries, and, in this case, the energy is absorbed by the melanin of the RPE [62]. Both focal and grid photocoagulation are often required and performed during the same treatment session.

The amount of energy delivered to the retina is determined not only by the settings of the laser machine, but also by the characteristics of the eye being treated,

such as the opacities of the cornea, lens, or vitreous, the amount of fluid between the retinal layers, and the concentration of retinal pigment. Lower energy settings are required for a pigment-rich than for a pigment-poor retina. Also, a highly edematous retina or an eye with cataract or other forms of decreased transparency can require higher energy settings compared to an eye with less edema or normal transparency [62].

The temperature rise in the retina can also disperse to adjacent retinal structures and injure the neuroretina, Bruch's membrane, or choriocapillaris. Such damage, can in turn impair retinal sensitivity, and even lead to choroidal neovascularization, epiretinal fibrosis, and long-term expansion of laser scars [63–65].

Change in retinal sensitivity after photocoagulation in patients with CSME has been assessed, but previous clinical investigations have been based on short follow-up time and/or have reported conflicting results. In Study II, we aimed to map changes in retinal sensitivity in diabetic subjects with CSME over a period of 2 years following laser photocoagulation.

Cellular and molecular effects of retinal photocoagulation

Despite the widespread awareness of the clinical benefits of photocoagulation, the cellular and molecular effects of this method remain unclear. It has been proposed that the positive therapeutic outcome of laser coagulation is due either to biochemical changes occurring on the RPE level during the healing process after treatment [66] or to improved retinal oxygenation as an effect of a reduction in retinal tissue [67,68]. The numerous roles of the RPE in sustaining retinal function are listed in Table 4.

The effects of a specific treatment can be investigated using human cell culture models in which experimental parameters and conditions can easily be altered. Three immortalized human RPE cell lines have been created: Davis et al. [70] produced the D407 cell line, but it was found to differ from RPE cells *in vivo* due to loss of enzymatic activity and cytoskeletal polarization [71]; Bodnar et al. [72] established the hTERT-RPE cell line by transfecting RPE cells with vectors that encode the human telomerase activity and thereby extend the cellular life span; Dunn et al. [73] generated the ARPE-19 cell line, which, because of its characteristics in terms of polarization and permeability [71], became the line used most often to study the outer blood–retinal barrier.

The objective of Study III was to use the ARPE-19 cell line to establish a model of *in vitro* photocoagulation and to investigate necrosis, apoptosis, migration, proliferation, and the expression of genes involved in proliferation, migration, repair, and cytoprotection. The aim of Study IV was to explore the effects of *in vitro* photocoagulation on mRNA and protein levels by microarray and proteomics analysis of the model developed in Study III.

Table 4.
Functions of the RPE [69].

Function	Description
Light absorption	Absorbs scattered light which enhances vision quality and protects against cell damage.
Nutrient and ion transport	Transports electrolytes and water from the subretinal space to the choroid, and nutrients from the choroid to the photoreceptors.
Spatial buffering of ions	Buffers ions in the subretinal space, compensates for fast changes in ion concentration, and maintains photoreceptor excitability.
Visual cycle	Achieves re-isomerization of all-trans-retinal to 11-cis-retinal.
Phagocytosis	Phagocytizes photoreceptor outer segments and redelivers important molecules to the photoreceptors.
Secretion of growth factors	Produces and secretes numerous growth factors.

Methods

Research design (Studies I and II)

All examinations and procedures were performed at Skåne University Hospital in Malmö, Sweden. Subjects who were younger than 30 years of age at the time they were diagnosed with diabetes and were being treated only with insulin treatment were considered to have type 1 diabetes. All other diabetic subjects were regarded as having type 2 diabetes.

In Study I, a total of 1,691 type 2 diabetic subjects with no retinopathy detected at a photographic screening session in a program for early detection of diabetic retinopathy were registered in a database and scheduled for a 3-year follow-up. Red-free images of one central and one nasal 50° field per eye were obtained by digital fundus photography. The International Diabetic Retinopathy and Macular Edema Severity Scales [37] were used for grading, and CSME was defined in cases involving macular edema.

In Study II, 29 diabetic subjects with untreated CSME were consecutively enrolled during a 2-year period. The macular edema was treated with focal/grid photocoagulation (Fig. 1), and the long-term effects of that treatment on two aspects of retinal sensitivity were tested every six months: visual acuity was assessed using ETDRS charts at a distance of four meters and was expressed as the logarithm of minimum angle of resolution (LogMAR scores), and the central 10° of visual fields was evaluated using the SITA Standard 10-2 program of the Humphrey Field Analyzer (Carl Zeiss Meditec Inc., Dublin, CA, USA). In addition to clinical examination, retinal thickness was measured by time-domain optical coherence tomography (TD-OCT; Carl Zeiss Meditec Inc.) at baseline and at each follow-up visit. Blood glucose and HbA1c measurements were also performed at each visit.

The Humphrey Field Analyzer provides tools for interpreting SAP, such as probability maps flagging test points that are significantly depressed below normal limits. We counted the number of significantly depressed points at the $p < 0.01$ level in the total deviation probability maps. Trends in visual fields over time were calculated by linear regression analysis of the number of significantly depressed points at the $p < 0.01$ level.

An in vitro model of photocoagulation (Studies III and IV)

The RPE cell line ARPE-19 was obtained from the American Type Culture Collection (Manassas, VA, USA) and used in all experiments. Cells were incubated in a

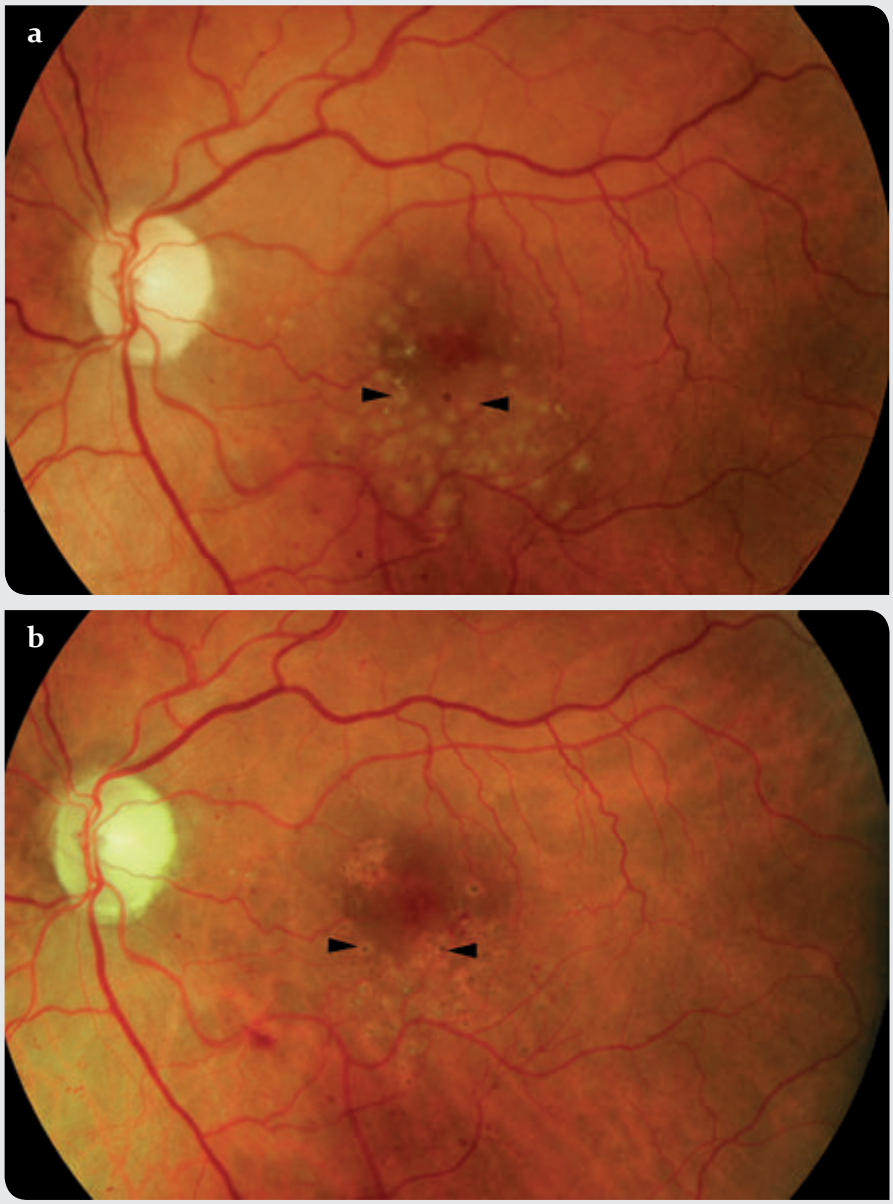

Figure 1.
Fundus images demonstrating focal/grid photocoagulation burns (black arrowheads) 15 minutes (a) and 24 months (b) after treatment (*figure 1a is reproduced with permission from Wiley [Study II]*).

humidified environment, and the medium was changed every third day. Prior to the laser treatment (referred to as time "zero"), the cells were plated at a density of 3 x 10^4 on glass cover slips placed in cell culture wells and incubated for seven days to reach confluence. In Study III, cells were cultured in DMEM (Invitrogen, Paisley, UK) containing 1000 mg/L D-Glucose; in Study IV, culturing was performed in DMEM containing either 1,000 or 4,000 mg/L D-Glucose to be able to differentiate the effects of hyperglycemia on ARPE-19 after photocoagulation.

Inasmuch as ARPE-19 cells lack pigment, a black paper was placed under the cover slips before starting the laser treatment to facilitate absorption of the laser beam. Photocoagulation was performed using a 532-nm frequency-doubled Nd:YAG laser (Visulas 532, Carl Zeiss, Oberkochen, Germany). Each sample was subjected to 50 uniformly spaced laser burns (Fig. 2). To determine which settings generated the highest reproducibility with respect to lesion size, we used spot sizes of 100, 200, and 300 µm and powers of 200 and 300 mW at a duration of 0.1 s.

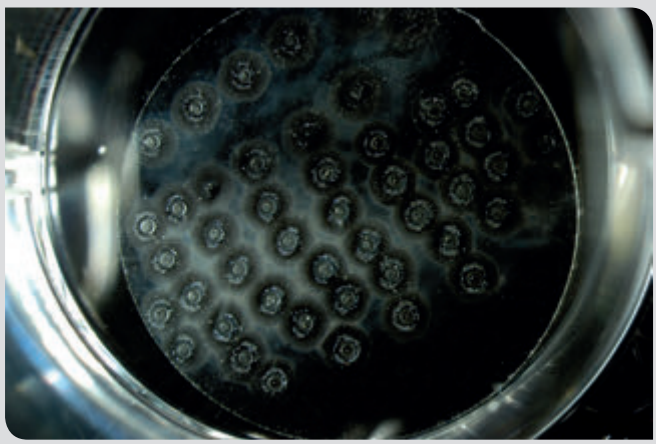

Figure 2.
Lesions observed within minutes of in vitro photocoagulation of ARPE-19 cells cultured on glass cover slips (spot size 200 µm). Small gas bubbles appeared between the paper and the glass coverslip after photocoagulation, forming the pale punctuated rings observed around the lesions. (*Figure also published in Study III; permission not required due to open access rights.*)

Morphology and cell death (Study III)

To monitor the morphological appearance of the laser lesions, ARPE-19 cells were fixed and stained at various time points between 0 and 168 h after laser treatment. The cells were examined with a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) and imaged using a Nikon DS-5Mc camera, a Nikon DS-U1 control unit, and NIS-Elements software (version 3.22, Nikon). Six to eight samples were analyzed for each time point.

To visually distinguish between living and dead ARPE-19 cells after photocoagulation, the cells were stained green with fluorescent Calcein AM (which stains living cells) and red with ethidium homodimer-1 (which stains dead cells), and subsequently imaged at various time points between 0 and 168 h after the laser treatment using the equipment described above. Appropriate filter sets were used to detect fluorescence. Cell death was also verified by quantification of LDH activity in the culture medium at various time points between 0 and 72 h after the treatment.

To identify apoptotic ARPE-19 cells after photocoagulation, cells were fixed at various time points between 2 and 168 h after the treatment and were visualized with an *In Situ* Cell Death Detection Kit, POD (Roche Applied Science, Mannheim, Germany). Apoptosis was also measured at various time points between 0 and 168 h after the laser treatment by using a Cell Death Detection ELISA^{plus} assay (Roche Applied Science, Mannheim, Germany) to quantify cytoplasmic histone-associated DNA fragments in cell homogenates and in the culture medium.

Cell proliferation and migration (Study III)

At various time points between 0 and 168 h after laser treatment, proliferation of ARPE-19 cells was visualized and quantified using a monoclonal antibody against PCNA and a secondary antibody. SYTOX Green (1:3,000, Molecular Probes) was used to identify nuclear regions and individual cells. Samples were examined in a Zeiss LSM 5 Pascal laser scanning confocal microscope (Carl Zeiss, Öberkochen, Germany), and the nuclear PCNA intensity was quantified using the Zeiss LSM 5 analysis software.

To determine the effects of mitomycin C and docetaxel on cell proliferation and migration, ARPE-19 cells were scratched with the tip of a sterile pipette and photographed at 0 and 24 h later after incubation with or without either of the two agents at various concentrations below a predetermined level of toxicity. The cells were inspected with a Nikon TMS microscope and imaged using a Nikon DS-Fi1 camera and NIS Elements F version 2.20 software (Nikon). Scratched areas were measured using ImageJ software [74]. The effects of mitomycin C and docetaxel on proliferation and migration during the repair process after photocoagulation were evaluated by measuring the size of the cell-free region within the laser lesions at 12,

24, 48, and 72 h after the laser treatment.

Real-time RT-qPCR (Studies III and IV)

RNA extraction was performed at 6 and 24 h after photocoagulation in Study III and at 2, 6, and 24 h in Study IV. The quality of RNA was assessed before cDNA was synthesized from RNA, and the mRNA levels were analyzed with a 7900HT real-time PCR system (Applied Biosystems, Foster City, CA, USA) using TaqMan Gene Expression Master Mix (Applied Biosystems), TaqMan Gene Expression Assay (Applied Biosystems), cDNA, and deionized diethylpyrocarbonate-treated water (Fermentas). Cyclophilin B was used as an endogenous control in Study III, and cyclophilin A, actin beta, and hypoxanthine guanine phosphoribosyl transferase 1 were the endogenous controls in Study IV.

Microarray hybridization and analysis of microarray results (Study IV)

Four control and four laser-treated normo- and hyperglycemic ARPE-19 RNA samples for each time point (2, 6, and 24 h after photocoagulation) were subjected to microarray hybridization using the Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA, USA). Raw data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository under accession number GSE41332. Basic chip quality control, probe summarization, and data normalization were performed by robust multi-array analysis using Expression Console Software v 1.1.2 (Affymetrix).

Protein extraction, trypsin digestion, iTRAQ labeling, and peptide fractionation (Study IV)

Four control and four laser-treated normo- and hyperglycemic ARPE-19 pellet samples (total 16 samples) were solubilized, sonicated, centrifuged, purified, and diluted to identical protein concentrations of 5.47 µg/µl. After reduction and alkylation, the protein samples were diluted with TEAB buffer and modified porcine trypsin (Promega, Madison, WI, USA), and the tryptic digests were purified by solid-phase extraction. The peptide chains in the samples were identified using isobaric tags for relative and absolute quantification (iTRAQ 8-plex; AB Sciex, Framingham, MA, USA). Samples were desalted and fractionated using an electrofocusing system (OFFGEL Fractionator; Agilent Technologies, Böblingen, Germany) with immobilized pH gradient gel strips.

Mass spectrometry, protein identification and quantification, and processing of KEGG data (Study IV)

The iTRAQ-labeled mixtures were analyzed on an LTQ Velos-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a nano-high-performance liquid chromatography (nano-HPLC) system (Proxeon, Hvidovre, Denmark). Proteins were identified using Mascot 2.3 (Matrix Science, London, UK) to search the Swiss-Prot 2010.11 database. For each MS/MS spectrum, the ratios between the intensity of each reporter ion and the internal standard were calculated.

Pathway information was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [75]. A hypergeometric test was employed to assess the probability of selected pathways showing more than the expected number of genes in the analysis group. The test was based on all genes and proteins queried by the microarray and mass spectrometry.

Data pooling (Study IV)

The group comparisons between the two concentrations of glucose showed no differentially expresed genes. Therefore, we assumed that the glucose concentration would not influence gene expression and thus increased the number of samples from four to eight by pooling the data on low and high glucose in both laser-treated and untreated cells.

Results

Three-year incidence of diabetic retinopathy (Study I)

Only one eye in one subject developed CSME during the 3-year follow-up period. This subject had moderate non-proliferative retinopathy in both eyes, was on diet treatment, and received no anti-hypertensive treatment at either baseline or follow-up. None of the subjects developed proliferative diabetic retinopathy. Baseline and follow-up characteristics are presented in Table 5. Baseline characteristics did not differ between dropouts and completers. Incidence data are given in Table 6.

Table 5.
Proportions (%) and median values of subject characteristics at baseline and follow-up.

	Baseline	Follow-up	CI (95%) for Δ*
Sex, % (no. males)	56.8 (961)	56.6 (748)	−9.25 to 8.85
Diabetes duration (yrs)	4.0 [1.0–4.0]	8.0 [5.0–11.0]	
Missing data	*2.0 (33)*		
Anti-diabetic treatment (%)			
Diet only	25.1 (424)	12.2 (161)	−15.65 to −10.15
Oral agents	51.7 (874)	61.0 (806)	5.71 to 12.89
Oral and insulin	7.3 (123)	14.1 (187)	4.53 to 9.07
Insulin only	12.4 (209)	11.7 (155)	−3.06 to 1.66
Missing data	*3.6 (61)*	*1.0 (13)*	
Anti-hypertensive treatment (%)			
Anti-hypertensive treatment	55.8	68.3	9.01 to 15.99
Missing data	*1.0*	*2.6*	

*Values in the right column indicate 95% confidence interval for the difference between the proportions (sex, anti-diabetic and anti-hypertensive treatments).
Values within parentheses indicate number of subjects. Values within brackets indicate interquartile range.

Table 6.
Three-year incidence of diabetic retinopathy in the worst eye (grading according to the International Clinical Diabetic Retinopathy and Diabetic Macular Edema Disease Severity Scales [37]).

Degree of retinopathy	%	Number of subjects
None	72.6	960
Any retinopathy	27.4	362
Mild	18.5	245
Moderate	8.9	117
Severe non-proliferative and proliferative	0	0
Clinically significant macular edema	0.07	1

Subjects in whom the grade of retinopathy progressed two steps (to moderate retinopathy) had significantly higher median HbA1c levels compared to subjects with one-step progression (to mild retinopathy). The proportion of subjects with progression to mild and moderate retinopathy was larger among those who required insulin treatment at baseline than among those on diet treatment or oral agents only at baseline. Duration of diabetes at baseline did not differ between subjects with one-step progression and those with two-step progression.

Human subjects (Study II)

None of the studied subjects developed laser-induced visual field damage during the 24-month follow-up. At baseline, visual acuity ranged from 0.32 to 1.58 Snellen (median 1.0), and the severity of macular edema varied with respect to localization, thickness, and extension. At follow-up, visual acuity improved or was stable in 12 subjects but on average declined four letters in the remaining subjects.

Comparing TD-OCT subfields above the 99th percentile at baseline with corresponding subfields at 24 months showed that thickness decreased by 49 µm in all subfields in 19 subjects and in some, albeit not all, subfields in two subjects. Visual fields improved or were stable in 20 subjects but declined five points on average in the remaining six subjects, which corresponded to the laser-treated area in only one case.

The mean of the visual field trend slopes for all subjects did not differ from 0 (mean = 0.5 significantly depressed test points per month; $p = 0.63$), and the standard deviation was small (0.5) (Fig. 3). There was no correlation between the number of significantly depressed points at any follow-up visit and the total number of laser shots. The number of significantly depressed points at the last visit did not correlate with the number of re-treatments over time.

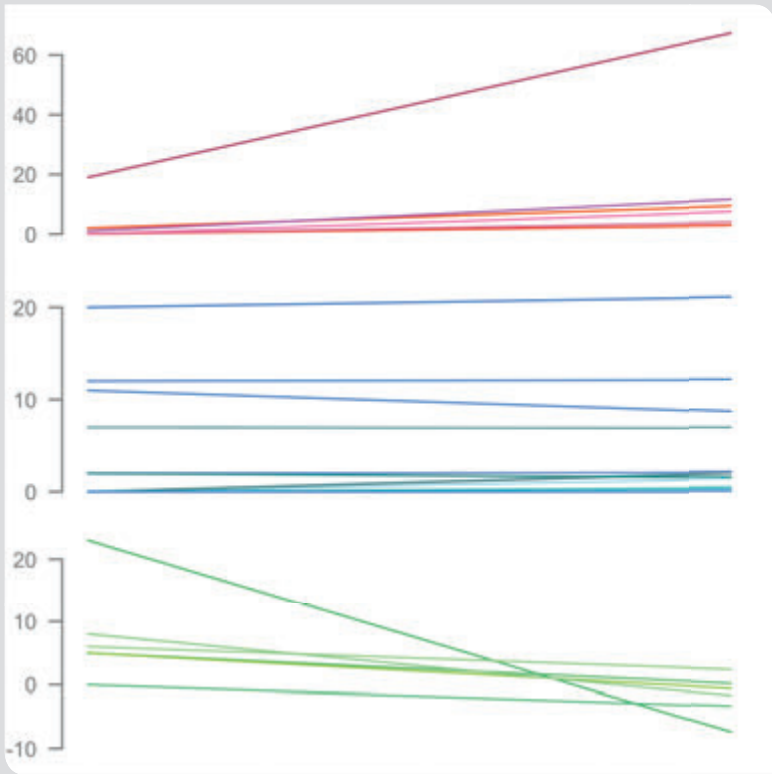

Figure 3.
Visual field trend slopes (number of significantly depressed points per month) for all subjects in Study II. Positive and negative slopes indicate deterioration and improvement, respectively. ***Top***: positive slopes (> 0.1 points per month) indicating minor increases in number of depressed points over time in all but one subject who developed proliferative retinopathy and required panretinal photocoagulation at 21 months. ***Middle***: Flat slopes (–0.1 to 0.1 points per month) indicating no change over time. ***Bottom***: Negative slopes (< –0.1) indicating minor decreases in number of depressed points over time in all but one subject who showed considerable improvement from 20 points at baseline to three at 12 months and zero at 18 and 24 months. This subject had six significantly depressed points at the training session, and hence it is unlikely that the baseline field test was actually representative of the baseline status.

The in vitro model of photocoagulation (Study III)

Photocoagulation caused a combination of necrotic and apoptotic cell death, and was followed by a healing process involving both migration and proliferation. Laser shots delivered at 300 mW and with a spot size of 200 µm and duration of 0.1 s provided the most reproducible lesions in the ARPE-19 cell culture and were therefore used throughout the experiments. Laser-irradiated areas could be identified after only 30 min. An empty rim appeared around the lesions at 2 h; ARPE-19 cells covered the rim at 24 h; the cells had started to cover the center of the lesion at 48 h; and the lesions were almost completely covered at 72 h.

Morphological evaluation of necrosis demonstrated that necrotic cells appeared after only 30 min, with a peak at 6 h, and quantification of necrosis showed a corresponding significant increase in necrotic activity at 30 min (peak) and 2 and 6 h. Morphological evaluation of apoptosis demonstrated apoptotic cells with a maximum intensity at 24 h (cf. 6 h for necrosis), and quantification of apoptosis revealed a corresponding significant increase in apoptotic activity, also with a peak at 24 h.

Proliferation activity was measured as expression of the S-phase marker PCNA at different time points and distances from the center of the lesions. The results showed that the activity was significantly reduced 2 to 6 h after photocoagulation at all distances, and this effect was most prominent within 200 µm of the center. Proliferation levels returned to normal after 24 h and continued to increase at all distances from the center of the lesions over the following days. To distinguish between proliferation and migration in the healing process after photocoagulation, we used docetaxel and mitomycin C to block migration and proliferation, respectively. Both these agents resulted in larger lesion diameter compared to untreated controls.

Changes in gene and protein expression after photocoagulation (Studies III and IV)

Real-time RT-qPCR showed upregulation of genes involved in cell proliferation, migration, tissue repair, and cytoprotection at 6 and 24 h after photocoagulation. Microarray analysis revealed 911 significantly down- or upregulated genes (out of a total of 28,869 genes), and 33, 713, and 165 of those 911 genes were altered at 2, 6, and 24 h after photocoagulation, respectively. Further downstream analysis of the microarray data was performed using the KEGG. Pathways and matched genes were found primarily in processes involved in the cell cycle, systemic lupus erythematosus, purine metabolism, and the DNA replication pathway (denomination according to KEGG). Mass spectrometry revealed a total of 242 significantly down- or upregulated proteins (out of a total of 1,537 proteins). KEGG analysis demonstrated only one significant pathway: the chemokine signaling pathway (denomination according to

KEGG).

The datasets from the microarray and mass spectrometry analyses were cross-referenced, and 24 gene–protein duplicates were found. After laser exposure, the vascular permeability factor Carbonic anhydrase 9 exhibited suppressed gene–protein expression, whereas the cytoprotective heat shock proteins 1A and 1B showed increased gene–protein expression. KEGG analysis of the 24 gene–protein pairs revealed three pathways (according to KEGG denomination: the glycine, serine, and threonine metabolism pathway; the nitrogen metabolism pathway; and the histidine metabolism pathway), each with two matched genes.

Discussion

The objectives of the studies underlying this thesis were to explore the effects that laser coagulation for diabetic macular edema has on retinal sensitivity and on cultured human RPE cells.

Screening intervals for type 2 diabetic subjects without retinopathy (Study I)

In the cohort of type 2 diabetic subjects with no retinopathy included in Study I, only one individual developed CSME and none developed proliferative diabetic retinopathy during a 3-year period, which corresponds to a 3-year incidence of 0.07%. This observation agrees with three investigations conducted in the United Kingdom comprising larger cohorts of subjects with no retinopathy at baseline in Liverpool (n = 3,743) [47], Wales (n = 49,763) [76], and Norfolk (n = 16,444) [77]. In Wales [76], the estimated cumulative 3-year incidence was 0.8% for referable retinopathy, defined as maculopathy or preproliferative or proliferative retinopathy that required assessment or treatment at a hospital eye service. In Norfolk [77], the calculated cumulative 3-year incidence was 0.24% for maculopathy and 0.27% for proliferative retinopathy, whereas the corresponding rates in Liverpool [47] were 1.1% and 1.6%, respectively.

In Study I, all images were graded at a hospital eye service, and subjects found to have sight-threatening vascular lesions were invited to undergo clinical evaluation. The use of slightly different definitions of retinopathy complicates comparison of the various assessments and may explain the higher incidence rates noted in the UK investigations cited above than in Study I. The term "referable retinopathy" was defined as the collection of maculopathy (exudates within one disc diameter of the fovea) or preproliferative or proliferative retinopathy in the Welsh study [76], whereas a simplified ETDRS classification was used in the Norfolk investigaiton [77], which unfortunately did not define the term "maculopathy". In Study I, the term referable was not used, because both the grading and the clinical evaluation of the subjects were performed at the same clinical center. In contrast, in two of the UK studies, subjects with eyes graded as referable retinopathy (Wales) or as maculopathy, preproliferative retinopathy, or worse (Norfolk) were referred to hospital eye services for further clinical assessment. Thus it is not known whether all the subjects who were referred for further evaluation actually had sight-threatening vascular lesions requiring treatment. If the results of Study I had been based solely on digital retinal image grading, two additional subjects would have been identified as having maculopathy (exudates within one disc diameter), which would have resulted in a total 3-year incidence of 0.2%.

Study I confirmed a low risk of progression to sight-threatening diabetic

retinopathy during a 3-year period in type 2 diabetic subjects without retinopathy, and hence it can be concluded that it is safe to retain the current 3-year screening guideline for this subgroup in Sweden. However, it is still a matter of debate whether other countries should prolong the screening interval from 1 to 3 years, considering that generalizations of results depend on similarities between study populations with regard to metabolic control, diabetes duration, and compliance. It might be argued that prolonged intervals can lead to a lower screening compliance rate, but this problem can be addressed by designing an organization to minimize the risk for drop out. Mehlsen and colleagues [78] investigated individualized intervals based on a calculated risk for each diabetic subject, and such an approach may prove suitable in the future.

Impact of focal/grid laser treatment on the visual field (Study II)

We investigated the long-term effects of focal/grid photocoagulation on eyes with CSME and found that this laser treatment did not affect the visual fields, even though it did reduce the thickness and extension of the retinal edema. The use of SAP to evaluate the effects of focal/grid photocoagulation on retinal sensitivity has previously been assessed in only a few investigations, which applied considerably shorter follow-up time than Study II and were inconclusive due to conflicting results. Indeed, it is difficult to compare these investigations due to differences in the study design and the methods used. Greenstein et al. [79] included only subjects with CSME in their study and used two types of modified SAP with thresholds measured at areas corresponding to a retinal area of either 28° by 22° or 47° by 39°, and those authors found very little change in the visual fields three months after focal/grid laser treatment. In two other studies, Striph et al. [80] and Shimura et al. [81] reported decreased sensitivity in the visual fields 4 to 6 months after laser treatment; both those investigations included only subjects with diffuse diabetic macular edema corresponding to an area of at least two or more disc diameters. Furthermore, SAP was used in both those studies, but Striph et al. selected the central 10° for perimetry, whereas Shimura et al. chose the central 30°. The results obtained by these two research groups may be explained by greater severity of the macular edema, and presumably also subsequent use of more intensive laser treatment, as compared to the corresponding aspects in Study II and in the investigation conducted by Greenstein et al. [79].

Thus, even though photocoagulation for CSME did not affect visual fields during a 2-year follow-up period, it cannot be excluded that impaired retinal sensitivity could have been detected by using other methods, such as microperimetry or ERG. In Study II, focal/grid laser coagulation did not cause decreased retinal sensitivity during the first 2 years after treatment. On average, visual acuity declined three letters (i.e., less than one Snellen line) in the entire cohort. It is possible that intravitreal therapy with

anti-VEGF would have resulted in better visual acuity at follow-up, but the outcome of retinal sensitivity after such treatment can only be speculated upon and must be addressed in future research.

Effects of laser irradiation on RPE cells in vitro (Studies III and IV)

To explore the cellular mechanisms that occur after photocoagulation, we investigated the morphological and cellular effects of laser irradiation on the immortal human cell line ARPE-19. Laser-induced damage involved both necrosis and apoptosis, and the repair process lasted for 3 to 7 days and involved both proliferation and migration. By cross-referencing the gene and protein datasets, 24 gene–protein pairs were found to be directionally consistent on both gene and protein levels.

The ARPE-19 cell line was chosen for the experiments, because it has been widely used in previous RPE cell culture studies. Using a single human cell line makes it possible to focus the evaluation of treatment effects on the characteristics of that specific line. However, in terms of resemblance to human tissue, employing only one human cell line will certainly not mirror all significant processes that occur in the RPE *in situ*. Therefore, the disadvantages associated with using a single human cell line should always be taken into consideration and weighed against the benefits of using that particular line. Other RPE cell lines that can be selected include well-differentiated fetal or human pigmented RPE or RPE derived from embryonic/pluripotent stem cells. Those options were not available in the present laboratory at the time Studies III and IV were conducted.

The morphological healing process demonstrated in Study III guided the selection of time points deemed to be relevant in the repair process in Studies III and IV. In Study III and in the microarray analyses performed in Study IV, attention was focused primarily on the first 24 h after treatment, because the major part of the healing process occurred during that time. The selection of the time point (48 h) for proteomics in Study IV was based on the morphological appearance of the healing process in addition to the findings regarding apoptosis, necrosis, and proliferation in Study III.

The poor correlation between mRNA and protein expression noted in Study IV is a known biological issue caused by parameters that influence mRNA–protein correlation [82].

Carbonic anhydrase 9 was suppressed in the laser-treated samples as compared to controls. Gao et al. [83] found that levels of carbonic anhydrase were higher in vitreous samples from diabetic subjects than in samples from non-diabetic controls. In the same investigaiton, it was also observed that intravitreal injections of carbonic anhydrase and erythrocyte lysis in rats increased both retinal vascular permeability and intraretinal edema to the same extent as VEGF did. Those findings suggest that

both carbonic anhydrase and erythrocyte lysis play a role in blood retinal barrier breakdown, and that retinal hemorrhages in diabetic retinopathy can potentiate an increase in vitreous carbonic anhydrase. Thus the laser-induced decreased levels of Carbonic anhydrase 9 may be one explanation for the beneficial effects of photocoagulation on macular edema.

In Study IV, the expressions of heat shock proteins 1A and 1B were enhanced in the laser-treated cells compared to untreated controls. The heat shock proteins expressed in the samples had a molecular weight of 70 kDa and thus belong to the Hsp70 family of stress-inducible proteins that enhance the ability of cells to manage and survive the accumulation of denatured proteins that occurs following stressful events. It has been shown that an elevated level of Hsp70 increases resistance to many inducers of apoptosis and/or necrosis, such as oxidative stress [84], which is involved in retinal ischemia. Accordingly, enhanced expression of Hsp70 may result in increased cellular tolerance to retinal ischemia in diseases such as diabetes. It remains to be determined whether the increased Hsp70 expression represents a first step in cellular activities aimed at inhibiting retinal vascular leakage or edema.

Conclusions

1. The current 3-year screening interval used in Sweden for subjects with type 2 diabetes but no retinopathy is safe to retain, considering the very low incidence rate of sight-threatening diabetic retinopathy in this group.

2. SAP showed that retinal sensitivity was preserved in diabetic macular edema during a period of 2 years after laser treatment.

3. Laser coagulation of cultured ARPE-19 cells caused a combination of necrosis and apoptosis, which was followed by a healing process involving both migration and proliferation.

4. The beneficial effects of laser treatment for diabetic retinopathy might be partly explained by the downregulation of Carbonic anhydrase 9 and upregulation of the heat shock proteins 1A and 1B in the retinal pigment epithelium.

Populärvetenskaplig sammanfattning

Diabetes är en välkänd folksjukdom som orsakar förhöjda blodsockernivåer. Runt 6,4 % av jordens befolkning har diabetes och till år 2030 förväntas den andelen öka till ca 7,7 %. Det förhöjda blodsockret kan långsiktigt förorsaka kärlskador och medföra nedsatt funktion i bl.a. ögon, njurar, nerver och hjärta. Kärlförändringar i ögonens näthinna, s.k. diabetesretinopati, är huvudämnet för denna avhandling. Den förekommer i ca 35 % av den globala diabetesbefolkningen vilket motsvarar ca 93 miljoner individer. En typ av diabetesretinopati kallas diabetiskt makulaödem och orsakas av att vätska läcker ut ur näthinnans små blodkärl och orsakar förtjockning av näthinnan. Denna åkomma kan leda till allvarlig synnedsättning om den inte behandlas i tid.

Då diabetesretinopati kan uppstå utan påverkan på synfunktionen, genomgår patienter med diabetes fotografering av ögonbotten eller undersökning hos ögonläkare med regelbundna mellanrum, s.k. screening. Hur ofta undersökningarna görs skiljer sig åt mellan olika länder. I USA rekommenderas ett-åriga intervall, på Island rekommenderas två-åriga intervall och i Sverige rekommenderar Socialstyrelsen tre-åriga intervall för typ 2 diabetiker utan retinopati.

I studie I undersöktes hur stor risk det nuvarande tre-åriga intervallet utgjorde för utvecklingen av diabetesretinopati hos typ 2 diabetiker. Drygt 1 600 patienter utan kärlförändringar registrerades i en databas och planerades för tre-årig uppföljning. Endast en person insjuknade i diabetiskt makulaödem och inga patienter utvecklade andra former av synhotande diabetesretinopati. Detta motsvarade 0,07 % insjuknandegrad för tre-årsperioden. Då denna siffra är mycket låg bekräftar den att det befintliga tre-åriga intervallet för typ 2 diabetiker utan kärlförändringar är säker.

Patienten som i studie I drabbades av diabetiskt makulaödem erhöll laserbehandling med syfte att få ödemet att tillbakabildas. Vid laserbehandling riktas effekterna mot det förtjockade området av näthinnan. Laserenergin omvandlas till värme i ett speciellt lager av näthinnan som kallas pigmentepitelet. En möjlig biverkan av laserbehandlingen är att värmen sprids från pigmentepitelet och gör åverkan på övriga lager av näthinnan som är viktiga för att ögat skall uppfatta ljus. Sådan eventuell skada kan upptäckas med hjälp av synfältsundersökning som kontrollerar funktionen i flera områden till skillnad från en vanlig synundersökning som endast kontrollerar funktionen inom ett centralt område av näthinnan.

I studie II undersöktes om laserbehandling av diabetiskt makulaödem kunde försämra näthinnans funktion. Trots att laserbehandling är en väl beprövad metod som har använts mot diabetiskt makulaödem sedan 80-talet, har de studier som hittills gjorts för att undersöka laserns eventuella skadeeffekter med hjälp av synfält varit kortsiktiga och resultaten motstridiga. I denna studie värvades tjugonio patienter med diabetiskt makulaödem. Synfälten undersöktes före laserbehandling och därefter med ett halvårs mellanrum under två år. Resultaten av synfält

jämfördes över tid och visade att inga patienter utvecklade laserorsakad skada under uppföljningstiden.

De förändringar som laserbehandlingen förmodligen orsakar på cellerna i pigmentepitelet, och som bidrar till ödemets tillbakabildning, är ännu inte kartlagda. En sådan kartläggning hade kunnat bidra till utvecklingen av nya läkemedel mot diabetiskt makulaödem.

I studie III undersöktes effekterna av laserbehandling på cellnivå. I denna studie etablerades en metod för laserbehandling av pigmentepitelceller i laboratoriemiljö. Med hjälp av metoden undersöktes vilken typ av celldöd som laser gav upphov till samt vilken typ av läkning som följde därefter. Det finns två typer av celldöd; plötslig och programmerad celldöd. Plötslig celldöd (även kallad nekros) har ett dramatiskt förlopp där cellens vägg går sönder och cellinnehåll svämmar ut. Den programmerade celldöden (även kallad apoptos) sker under mer kontrollerade förhållanden genom att cellen stegvis bryter ned sitt eget innehåll och begår självmord. Vår undersökning visade att laserbehandling orsakade plötslig celldöd, troligtvis p.g.a. den hastiga värmeutveckling som sker i cellerna, men den visade även att laser orsakade programmerad celldöd. Vad detta har för innebörd är svårt att tolka i nuläget, men programmerad celldöd kan tänkas spela en möjlig roll i tillbakabildningen av ödemet, antingen i sig självt genom att sjuka celler utraderas och nya friska växer till, eller genom att det parallellt gynnar händelser i cellerna som ökar deras motståndskraft. Vår undersökning visade också att den läkningsprocess som följde efter laserbehandling utgjordes dels av att cellerna växte i antal genom delning och dels genom att cellerna förflyttade sig och täckte laserskadan.

Pigmentepitelcellerna har många roller som är viktiga för ögats funktion. Bland annat tillverkar de proteiner som används av andra celler i näthinnans lager. Proteinerna tillverkas utifrån cellernas gener, som utgör ett slags mall för proteintillverkningen. Hur cellerna styr vilka proteiner som ska tillverkas är en komplicerad kedja av händelser. De kan sammanfattas med att cellen efter avläsning av en gen först tillverkar ett slags mellanprotein som i sin tur används som en mall till att tillverka ett protein. Typen och mängden av mellanprotein är alltså ett resultat av hur mycket respektive gen uttrycks i en cell, och kallas således för genuttryck. Typen och mängden av proteiner som cellen tillverkar i steget efter genuttrycket, kallas proteinuttryck.

I studie IV undersöktes hur pigmentepitelcellernas uttryck av gener respektive proteiner ändrades efter laserbehandling, med hjälp av metoden från studie III. Uttrycket ändrades hos 911 av ca 28 000 undersökta gener och hos 242 av ca 1 500 undersökta proteiner. För att undersöka om dessa gener och proteiner har några gemensamma roller, utfördes en datauträkning som jämförde respektive gens och proteins funktion. Denna analys visade reparationsmekanismer samt inflammatoriska mekanismer som främsta gemensamma nämnare.

Därutöver uppmärksammades att uttrycket av proteinet karbanhydras 9, som i tidigare studier påvisats vara förhöjd i ögonvävnad hos patienter med

diabetesretinopati, var sänkt i de laserbehandlade cellerna. Även uttrycket av heat shock proteinerna 1A och 1B, som i tidigare studier påvisats vara delaktiga i läknings- och skyddsmekanismer av celler som utsätts för stress, var förhöjda. Det är möjligt att dessa proteiner är delaktiga i den förmånliga process som följer efter laserbehandling av diabetiskt makulaödem.

Denna avhandling sammanfattar resultaten av fyra studier vars respektive slutsats är:

- Det befintliga tre-åriga intervallet för typ 2 diabetiker utan kärlförändringar är säker.
- Laserbehandling av diabetiskt makulaödem skadar inte näthinnans funktion.
- Laser orsakar plötslig celldöd samt programmerad celldöd och följs därefter av läkningsprocesser som innefattar både växt och förflyttning av celler.
- Det sänkta uttrycket av karbanhydras 9 och det höjda uttrycket av heat shock proteinerna 1A och 1B efter laserbehandling kan vara delaktiga i den förmånliga processen i näthinnan efter laserbehandling av diabetiskt makulaödem.

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