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KATI KINNUNEN

Vascular Endothelial Growth Factors In Eye Diseases

Pathophysiology and New Therapeutic Strategies for Retinal and Choroideal Angiogenesis

Doctoral dissertation

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Department of Biotechnology and Molecular Medicine
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ABSTRACT

Ocular neovascular diseases, including age related macular degeneration and diabetic retinopathy are major causes for blindness in the western world. The pathogenesis of these diseases remains partly unclear and currently there is no permanent cure for these diseases. In this study, we attempted to clarify the factors involved in the pathogenesis of diabetic retinopathy in type 1 and type 2 diabetes. We also experimentally evaluated the effects of overexpression of vascular endothelial growth factors (VEGF) -A and –D in the rabbit eye with adenovirus and baculovirus vectors. Finally, ocular phenotype of IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mouse was studied in order to develop a new animal model for age related eye diseases.

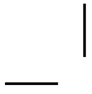
Diabetic retinopathy in type 1 and type 2 diabetes showed a different pattern of growth factor expression, which may explain the differences seen in these states. In diabetic patients, in addition to VEGF-A also other growth factors, particularly angiopoietin 2 were abundantly present. VEGF-D seemed to be important especially in type 2 diabetic retinopathy.

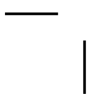
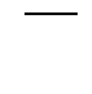
VEGF-A plays a major role in the pathogenesis of ocular neovascularization. Intravitreal injection of adenoviral VEGF-A$_{165}$ into the rabbit eye led to breakdown of the blood-retina barrier and ultimately neovessel formation. Furthermore, blocking the action of VEGF-A$_{165}$ prevented the progression of the angiogenic process. Intravitreal VEGF-D$^{\Delta N\Delta C}$ in our experimental series with rabbits led to a similar breakdown of the blood retina barrier as VEGF-A$_{165}$ and increased permeability but it was not capable to induce neovessel formation alone.

Administration of the therapeutic agent to the retina and choroidea can be difficult due to the tight barriers in the eye. Multiple injections into the eye may damage ocular structures and the risk of complications including endophthalmitis, cataract and haemorrhages increases. The optimal method for the delivery of therapeutic agents to the retina and choroidea has not yet been developed. Gene therapy offers an alternative in which the therapeutic protein or proteins are induced in the target tissue with a single injection for a prolonged time period. Eye is an ideal target for gene therapy because of its small size and tissue boundaries that prevent leakage of the therapeutic material to other tissues and systemic circulation. Viruses are widely used as vectors in gene transfer. In this study, intravitreal injection of adenovirus vector into the rabbit eye was efficient in delivering genes to the ganglion cell layer and inner retina. Baculoviruses showed transduction in the retinal pigment epithelium (RPE) and the photoreceptor layer even after the intravitreal injection into the rabbit eye. However, both vectors initiated an immune response in the target tissue, which limited the expression of the transducted gene to few weeks.

Diabetic IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice had moderately increased plasma glucose levels simulating early diabetes treated with diet therapy in humans. Aging transgenic mice showed changes especially in their retinal structures. Neovascularization was not seen in the retina. Instead, photoreceptor atrophy and dysregularities of normal retinal cell layers were found throughout the retina. This model might be useful for the evaluation of early diabetic changes and retinal degeneration.

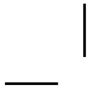
National Library of Medicine Classification: WW 270, WW 245, WK 835
Medical Subject Headings: Adenoviridae; Angiopoietins; Baculoviridae; Blood-Retinal Barrier; Choroidal Neovascularization; Diabetic Retinopathy; Disease Models, Animal; Eye/ blood supply; Gene Therapy; Gene Transfer Techniques; Neovascularization, Pathologic/ etiology; Retinal Degeneration; Retinal Neovascularization; Vascular Endothelial Growth Factor A; Vascular Endothelial Growth Factor D; Vascular Endothelial Growth Factors

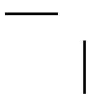
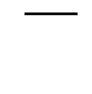


''What was that?'', asked Moomintroll, for a discovery (next to Mysterious Paths, Bathing, and Secrets) was what he liked most of all.

Tove Jansson



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Kuopio, March 2009

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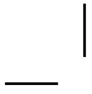
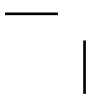
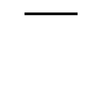
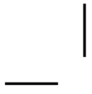
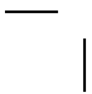


ABBREVIATIONS

Abbreviation	Meaning
AAV	Adeno associated virus
Ad	Adenovirus
AGEs	advanced glycation end products
α –SMA	alpha smooth muscle actin
AMD	age related macular degeneration
ANGs	angiopoietins
BlamD	basal laminar deposits
BlinD	basal linear deposits
Bv	baculovirus
CAR	coxsackie/adenovirus receptor
CFH	complement factor H
CMV	cytomegalovirus
CNV	choroideal neovascularization
DAG	diacylglycerol
DM	diabetes mellitus
DNA	deoxyribonucleic acid
EC	endothelial cell
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
EMEA	European Medicines Agency
ERG	electroretinogram
FAG	fluorescine angiography
FDA	Food and Drug Administration
FGF	fibroblast growth factor
Flt-1	vascular endothelial growth factor receptor 1
Flt-4	vascular endothelial growth factor receptor 3
GCL	ganglion cell layer
GFP	green fluorescent protein
GHbA1c	glycosylated haemoglobin
HIF-1α	hypoxia inducible factor
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGFR	insulin-like growth factor receptor
INL	inner nuclear layer
IRMA	intraretinal microvascular abnormality
ITR	inverted terminal repeat
IPL	inner plexiform layer
IU	infectious unit
KDR	vascular endothelial growth factor receptor 2
kb	kilobase
kDa	kilodalton
LacZ	β-galactosidase
mRNA	messenger RNA
NF-κB	nuclear factor κB
Nrp	neuropilin
OCT	ocular coherence tomography
OIR	oxygen induced retinopathy
ONL	outer nuclear layer
OPL	outer plexiform layer
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PDT	photodynamic therapy
PEDF	pigment epithelium derived factor
PKC	protein kinase C
PlGF	placental growth factor
PRL	photoreceptor layer
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RPE	retinal pigment epithelium
si-RNA	short interfering RNA
Tie-1	endothelial cell-specific receptor tyrosine kinase 1
Tie-2	endothelial cell-specific receptor tyrosine kinase 2
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor





LIST OF ORIGINAL PUBLICATIONS

This study is based on the following original articles, which are referred to in the text by the corresponding Roman numerals (I-IV):

I Kinnunen K, Puustjärvi T, Teräsvirta M, Nurmenniemi P, Heikura T, Laidinen S, Paavonen T, Uusitalo H, Ylä-Herttuala S. Differences between type 1 and type 2 diabetics in retinal neovascular tissue and vitreous humour.
British Journal of Ophthalmology *in press*

II Kinnunen K, Korpisalo P, Rissanen TT, Heikura T, Viita H, Uusitalo H, Ylä-Herttuala S. Overexpression of VEGF-A induces neovascularization and increased vascular leakage in rabbit eye after intravitreal adenoviral gene transfer.
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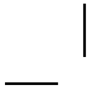
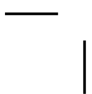
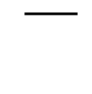
III Kinnunen K, Kalesnykas G, Mähönen AJ, Laidinen S, Holma L, Heikura T, Airenne K, Uusitalo H, Ylä-Herttuala S. Baculovirus is an efficient vector for the transduction of the eye. Comparison of Baculovirus- and Adenovirus-mediated intravitreal VEGF-D gene transfer in rabbit eye.
Journal of Gene Medicine *in press*

IV Kinnunen K*, Heinonen S*, Kalesnykas G, Laidinen S, Uusitalo-Järvinen H, Uusitalo H, Ylä-Herttuala S. Ocular phenotype of the diabetic IGF-II/LDLR$^{-/-}$ ApoB$^{100/100}$ mice reveals photoreceptor atrophy and altered morphology of the retina. Manuscript.

*Authors with equal contribution.



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1 INTRODUCTION

The human eye is a complex organ composed of many sections important to normal visual acuity. The cornea, lens, vitreous and outer retina are unique avascular structures allowing light to enter the eye. The light rays focused by the cornea and lens form an image to the retina, the light-sensing portion of the eye. The photoreceptor cells in the retina convert the image into an electrical signal that travels down the optic nerve to the brain (Figure 1).

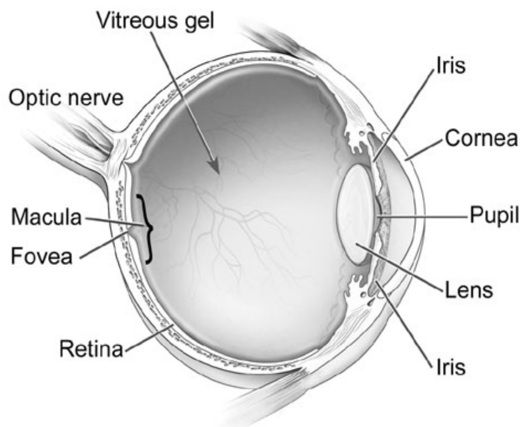

Figure 1. Normal anatomy of the eye. Image Source: National Eye Institute, National Institutes of Health.

Optical transparency of the eye is needed for normal visual function. On the other hand, the adult retina is a neural tissue with high metabolism and the highest oxygen consumption per unit weight of all human tissues. Therefore, the choroid, the most vascular portion of the eye also nourishing the retina, has one of the highest blood flow rates in the body, 800-1000 ml/100g tissue/min (Alm, 1992). In healthy adults this delicate ocular vascular system is maintained and controlled by the balance between the angiogenic factors and angiogenic inhibitors (Cao, 2001; Folkman and Ingber, 1992; Gao and Ma, 2002; Ma et al., 2005). Angiogenesis, or neovascularization, refers to the development of new vessels from preexisting vasculature. Most neovascular diseases in the eye begin with a shift of the balance between angiogenic and antiangiogenic factors towards angiogenesis. This phenomenon is called the angiogenic switch. The angiogenic switch occurs during neoplastic processes and various ocular diseases, including retinal and choroideal angiogenesis. Retinal and choroideal neovascularization leads to oedema, haemorrhages and fibrosis, causing visual impairment and blindness.

Diabetic retinopathy and age related macular degeneration (AMD) are the leading causes for visual impairment in developed countries (Table 1). Diabetes mellitus (DM) affects over 170 million people worldwide and the estimated amount of patients in the year 2030 is 366 million. The prevalence of diabetes is increasing particularly in low- and middle-income countries and in the working age population (WHO, 2005). 75% of both type 1 and type 2 diabetics will have diabetic retinopathy after 20 years of disease (Resnikoff et al., 2004).

The incidence, prevalence, and progression of AMD increase with advancing age and will rise as the population of older than 65 years increases (Mitchell et al., 2002a). Worldwide estimates indicate that the amount of AMD patients will double by the year 2020 (The Eye Diseases Prevalence Research Group, 2004). The prevalence of early signs of AMD is 18% in the population aged 65 to 74 years and 30% in the population older than 74 years (Klein et al., 1992). In a combined analysis of population-based eye disease prevalence data, AMD was present in 0.2% of the population aged 55 to 64 years, rising to 13% of the population older than 85 years (Smith et al., 2001). About 900 persons are visually impaired due to AMD in Finland each year (Finnish Federation of the Visually Impaired, 2007).

Table 1. Statistics of visual impairment in developed countries and the world.

Region		Western countries	World
No. of blind people (millions)		2.02	36.86
Prevalence of blindness, %		0.23	0.57
No. of blind people (millions) by age (years)	<15	0.05	1.37
	15-49	0.39	5.18
	>50	1.59	30.31
No. of people with low vision (millions)		11.35	124.25
Prevalence of low vision, %		1.23	2.00
No. of visually impaired (millions)		13.37	161.12
AMD as a cause of blindness, %		50.00	8.70
Diabetic retinopathy as a cause of blindness, %		17.00	4.80

Modified from WHO global data on visual impairment in the year 2002 (Resnikoff et al., 2004). Sample of western countries include Australia, Denmark, Finland, Iceland, Ireland, Italy, Netherlands, UK and USA.

In multiple studies, vascular endothelial growth factor (VEGF) has been shown to be the most important factor in ocular angiogenesis (Adamis et al., 1994; Aiello et al., 1994; Kvanta et al., 1996). Recently discovered anti-VEGF treatments have revolutionized the therapy of neovascular diseases in the eye. These agents have shown not just to stop the angiogenic process and maintain visual acuity but also improve vision in a great proportion of patients at least during the two year follow-up (Brown et al., 2009). However, there are also problems with these agents and their delivery regimens and new therapeutic strategies are needed.

2 REVIEW OF THE LITERATURE

2.1 DIABETIC RETINOPATHY

2.1.1 Clinical signs

Diabetic retinopathy begins as mild, nonproliferative abnormalities and progresses to moderate and severe nonproliferative diabetic retinopathy (Figure 3A) and proliferative diabetic retinopathy (Figure 3B). Macular edema can develop at any time in the progression of diabetic retinopathy (Ciulla et al., 2003). Clinical features of nonproliferative diabetic retinopathy include microaneurysms, intraretinal hemorrhages, soft and hard exudates, venous tortuosity or beading and intraretinal microvascular abnormalities (IRMA) (Davis, 1992). Microaneurysms, focal dilations of retinal capillaries, are early signs of diabetic retinopathy. Hypoxia caused by capillary dysregulation provoke nerve fibre layer infarctions creating soft exudates, which are visualized as gray or white lesions and are the result of stasis of axoplasmic flow. Growth factors expressed due to hypoxia cause induction of fenestrations in the vascular endothelium and dissolution of tight junctions leading to accumulations of intraretinal plasma proteins and hard exudates. Increased venous tortuosity and beading is caused by dysregulation of the capillaries. Prolonged occlusion of capillaries causes hypoxia, which leads to the development of IRMA, additional routes for blood through arteries to veins.

Diabetic retinopathy can be divided into stages depending on the severity of the disease. Microaneurysms in the retina refer to mild nonproliferative diabetic retinopathy. In moderate nonproliferative diabetic retinopathy there are more changes than microaneurysms including soft and hard exudates. Severe nonproliferative diabetic retinopathy is defined as more than 20 intraretinal hemorrhages/microaneurysms in each of four quadrants, definite venous beading in at least two quadrants or prominent IRMA with no signs of proliferative diabetic retinopathy in any quadrant. Proliferative disease is diagnosed in the case of neovascularization or preretinal or vitreous hemorrhage in the eye. New vessels traverse the internal limiting membrane and grow into the vitreous. Neovessels are fragile and tend to bleed causing vitreous hemorrhages. Fibrous scar formation can result with accompanying tractional retinal detachment, leading to sudden blindness if left untreated (Figure 2).

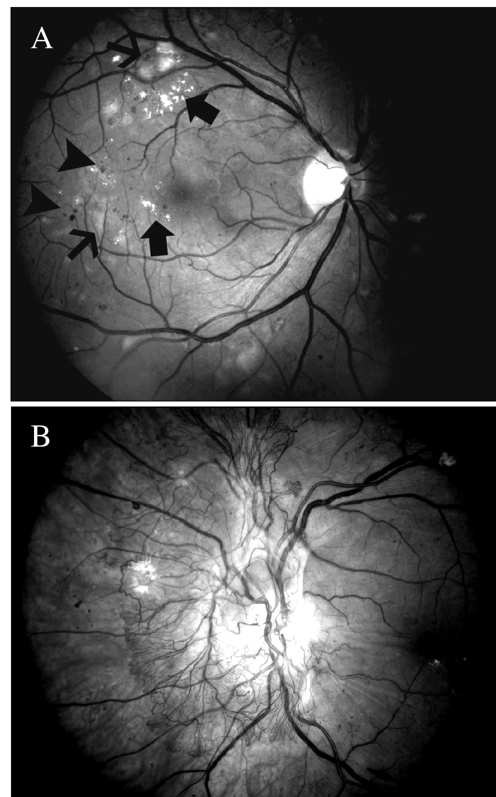

Figure 2. (A) Moderate nonproliferative diabetic retinopathy with hard exudates (large arrows), soft exudates (small arrows) and microaneurysms (arrowheads). (B) Proliferative diabetic retinopathy and neovascularization in the optic disc.

The foveal region is especially susceptible to hypoxia because of the lack of retinal vessels in this region. Diabetic maculopathy is characterized by an increase in vascular permeability, which results from a breakdown of the inner blood retinal barrier and leads to leakage of plasma component like proteins and lipids to the intaretinal space. Clinically significant macular oedema occurs if there is thickening of the retina involving the area within 500 µm of fovea, if there are hard exudates within 500 µm of the fovea with thickening of the adjacent retina, or if there is a zone of retinal thickening one disk area or larger in size, any part of which is within one disk diameter of the fovea (Early Treatment Diabetic Retinopathy Study Research Group, 1985). Changes in the macular area cause metamorphopsia, micropsia, decreased colour vision and contrast sensitivity and are the main reasons for moderate visual loss in diabetic patients (Daley et al., 1987). It is not clear why some diabetic patients develop severe macular oedema and others suffer from neovascularization and proliferative diabetic retinopathy.

2.1.2 Pathophysiology

Preretinal neovascularization and chronic retinal oedema in the macular area are the two major causes for visual loss in diabetic retinopathy (Fong et al., 1999). Hyperglycaemia induces alterations in the pericytes and vascular endothelial cells (EC) activating a cascade of signaling pathways leading to cellular dysfunction and eventually death. Histological lesions in diabetic retinopathy include capillary basement membrane thickening, EC dysfunction and loss, increased deposition of extracellular matrix (ECM) components, pericyte loss and leucocyte adhesion to the vessel wall (Ansari et al., 1998; Koya and King, 1998; Miyamoto and Ogura, 1999; Mogensen et al., 1979; Paget et al., 1998; Speiser et al., 1968). Endothelial dysfunction refers to a generalized alteration in EC phenotype and function, characterized by an abnormal vasodilator response, such as decreased nitric oxide or increased production of vasoconstrictors, such as endothelin-1 (Brownlee et al., 1984; Brownlee, 2001; Chen et al., 2004; Cukiernik et al., 2003; Deng et al., 1999; Dogra et al., 2001; Johnstone et al., 1993; Khan and Chakrabarti, 2003; Khan et al., 2006; McVeigh et al., 1992; van de Ree et al., 2001). EC dysfunction disrupts autoregulation of retinal blood flow, which maintains optimal nutrition and oxygenation of the retina in different situations of ocular perfusion and intraocular pressures.

Pericytes are essential in the regulation of retinal capillary perfusion, and damage to pericytes leads to altered retinal hemodynamics (Ciulla et al., 2002). Pericytes are responsible for the control of growth and survival of ECs, especially under stress conditions (Hammes et al., 2002). Damage to pericytes may lead to EC migration, growth and dysfunction. The absence of pericytes correlates with endothelial hyperplasia, increase capillary diameter and abnormal endothelial morphology (Hellstrom et al., 2001b). Unlike other organs, there is a high number of pericytes in the retinal microvasculature (Frank et al., 1990). The pericyte-EC ratio is 1:1 in the retina, as compared to a ratio of 1:10 in other microvasculature beds in the human body (Chakravarthy and Gardiner, 1999). Pericytes are very sensitive to disruptions of the extracellular environment. The level of apoptosis is much higher in pericytes than in ECs under the same amount of glucose concentration fluctuations and diabetic conditions (Li et al., 1996; Li et al., 1997).

Leukocytes possess large cell volume, high cytoplasmic rigidity, a tendency to adhere to the vascular endothelium, and a capacity to generate toxic superoxide radicals and proteolytic enzymes (Miyamoto and Ogura, 1999). In diabetes, there is increased retinal leukostasis, which affects retinal EC function, retinal perfusion, angiogenesis, and vascular permeability. In addition, leukocytes in diabetic patients are less deformable and may

be involved in capillary nonperfusion, EC damage, and vascular leakage (Miyamoto and Ogura, 1999).

Inflammation plays an important role in the degeneration of retinal capillaries in diabetic patients. The increased expression of many inflammatory proteins is regulated at the gene transcription level. NFκB is a widely expressed inducible transcription factor that is an important regulator of many genes involved in the inflammatory and immune responses, proliferation and apoptosis. NFκB is activated in retinal ECs or pericytes exposed to elevated glucose concentration and in retinas of diabetic rats (Kowluru et al., 2003; Zheng et al., 2004). In addition, inhibition of NFκB induced proteins inhibits diabetes-induced degeneration of retinal capillaries. Furthermore, compounds known to inhibit NFκB likewise inhibit the development of diabetic retinopathy (Kern, 2007). Increased levels of TNFα, IL-1β, and other inflammatory mediators have been found in the vitreous of diabetic patients (Demircan et al., 2006; Doganay et al., 2002; Mysliwiec et al., 2006; Yuuki et al., 2001). Activity of caspase-1, the enzyme responsible for production of IL-1β, increases in retinas of diabetic humans, and correlates with the distribution of lesions in the retina (Tang et al., 2003). In addition, deposition of C5b-9, the terminal product of complement activation, has been observed within retinal blood vessels of diabetic humans (Zhang et al., 2002).

Biochemical abnormalities related to diabetes include increased polyol pathway flux, which results in elevated levels of intracellular sorbitol (Gabbay, 1975). This disrupts the osmotic balance and results in cellular damage (Gabbay, 1975). In the presence of high glucose, carbohydrates interact with protein side chains in a nonenzymatic fashion to form advanced glycation end products (AGEs) (Brownlee et al., 1984; Friedman, 1999). AGEs may affect such functions as enzyme activity and susceptibility of proteins to proteolysis (Brownlee et al., 1984).

Experimental studies have shown that protein kinase C (PKC) activity and levels of an activator of PKC, diacylglycerol (DAG), are increased in hyperglycemia (Inoguchi et al., 1992; Xia et al., 1994). PKC activity is also increased after exposure of ECs to oxidative stress (Nishikawa et al., 2000; Taher et al., 1993). PKC-ß and –δ have been identified as the main isoforms of PKC activated in vascular tissues in response to hyperglycemia (Inoguchi et al., 1992; Koya and King, 1998). PKC-ß has been shown to have an important role in regulating EC permeability (Nagpala et al., 1996). It is also an important signaling component for angiogenic factors (Xia et al., 1996). The PKC-β inhibitor ruboxistaurin has been studied in animal and human clinical trials in patients with diabetic retinopathy. The trials have demonstrated a significant reduction in visual loss and need for laser treatment in patients with moderate to severe diabetic retinopathy over a 3-year period (Aiello et al., 2006).

Production of reactive oxygen species (ROS) has also been implicated in the development of diabetic complications. Diabetes may cause ROS production through glucose auto-oxidation and increased flux through the polyol pathway (Giugliano et al., 1996). ROS may activate aldose reductase and PKC and increase AGE production and DAG formation.

Besides vascular changes, chronic hyperglycemia also causes damages in retinal neural cells and glial cells (Barber, 2003; Rungger-Brandle et al., 2000). Retinal function is reduced in type 1 diabetics and in diabetic animal models (Hancock and Kraft, 2004; Phipps et al., 2004; Phipps et al., 2007; Shirao and Kawasaki, 1998; Simonsen, 1980). Electrophysiological changes occur before visible retinopathy and can be monitored by electroretinogram (ERG) (Simonsen, 1980). These changes often precede the onset of microvascular lesions and predict the worsening of retinopathy better than clinical characteristics (Bresnick and Palta, 1987; Parisi and Uccioli, 2001).

The optic nerve and visual pathway may also be affected as an early manifestation of diabetes. A progressive delay of the visual evoked potentials of diabetic patients has been demonstrated (Anastasi et al., 1985). Also nerve fibre layer defects have been detected by red-free photography in 20% of patients without microaneurysms and in 57% of patients with only microaneurysms (Chihara et al., 1993). The clinical findings on ganglion cell layer (GCL) have been confirmed in studies on rodents. Optic nerves of spontaneously diabetic rats have significantly smaller nerve fibres with increased atrophy and dystrophic changes in the nerve fibre layer (Sima et al., 1992).

Extensive loss of retinal microvasculature leads to retinal hypoperfusion, ischemia, and subsequent tissue hypoxia. Ocular neovascularization is strongly associated with retinal ischaemia, and multiple growth factors upregulated by hypoxia have been implicated in its pathogenesis. The ischaemic retina secretes growth factors that stimulate pre-existing vessels to proliferate. The most important of these factors are VEGF, basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), platelet-derived growth factor (PDGF) and angiopoietins (ANG) (Paques et al., 1997). Histologic studies have demonstrated the presence of growth factors and receptors in the preretinal membranes and the vitreous humour of patients with proliferative diabetic retinopathy (Adamis et al., 1994; Aiello et al., 1994). These factors have also been shown to correlate with the neovascular activity.

VEGF expression was found to be upregulated in rat vessels soon after the induction of experimental diabetes suggesting that VEGF is implicated in the vascular hyperpermeability that occurs early in the course of diabetic retinopathy (Vinores et al., 1997). Furthermore, intravitreal injection of VEGF induces vasodilation and microaneurysm formation before causing neovascularization, in a pattern similar to the initial stages of diabetic retinopathy (Miller et al., 1997). Several studies have been demonstrated an acute increase in serum levels of IGF-I preceding the onset of proliferative diabetic retinopathy in animal models (Grant et al., 1993; Hyer et al., 1988). Subsequently, increased IGF-I levels were measured in the vitreous of patients with proliferative diabetic retinopathy indicating that IGF-I may play a role in retinal neovascularization (Lee et al., 1994). Several *in vitro* studies have been shown that IGF-I can induce almost all steps of the angiogenic process including EC proliferation, migration and basement membrane degradation (King et al., 1985; Nakao-Hayashi et al., 1992; Nicosia et al., 1994). bFGF is stored at high concentration within the ECM as an inactive complex, and released when ECs dissolve ECM via the release of proteases (Bashkin et al., 1989; Globus et al., 1989; Presta et al., 1989). bFGF and hypoxia act synergistically to upregulate VEGF in ECs, resulting in retinal angiogenesis (Stavri et al., 1995). PDGF-B is known to induce a vascularized connective tissue stroma formation in many angiogenic and proliferative processes (Forsberg et al., 1993). Also in retinal neovascularization PDGF-B may be involved in the formation of fibrovascular retinal membranes. ANG-1 has been reported to induce sprouting in ECs *in vitro*, whereas ANG-2 appears to play a critical role in vascular remodeling (Hayes et al., 1999; Koblizek et al., 1998; Kukk et al., 1997; Stratmann et al., 1998). It has been shown that ANG-2 is upregulated by hypoxia during normal and pathologic angiogenesis (Hackett et al., 2000; Oh et al., 1999a).

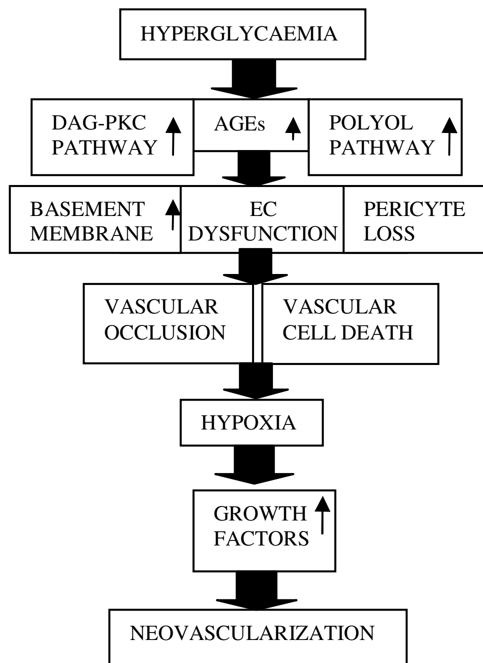

Figure 3. Consequences of hyperglycemia.

2.2 AGE RELATED MACULAR DEGENERATION

2.2.1 Clinical signs

AMD can develop to either the dry form with RPE and photoreceptor atrophy, or the wet form with choroideal neovascularization (CNV). In the dry AMD, RPE and therefore also photoreceptors in the macular area gradually disappear and finally large scars of retinal atrofy develop. Geographic atrophy leads to gradual progression of visual loss, most likely because photoreceptors overlying areas of RPE atrophy are metabolically dependent on RPE cells (Ambati et al., 2003). Marked apoptosis of the outer nuclear layer (ONL) and the inner nuclear layer (INL) of the retina is found near areas of RPE atrophy (Dunaief et al., 2002). The wet AMD is responsible for 90% of cases of severe visual loss in AMD patients (Bressler et al., 1988). In the wet AMD, CNV causes sub- and intraretinal accumulation of plasma and its components and hemorrhages (Figure 4). Vision loss occurs through the structural and metabolic damages caused by exudates and hemorrhages, and the secondary cell death and reactive gliosis. Metamorphopsia is a common symptom. Repeated and untreated leakage of blood, serum, and lipid stimulates fibroglial organization leading to a disciform scar (Ambati et al., 2003). The disciform scar represents an end-stage of AMD. It is usually vascularized, almost invariably from the choroidal circulation but sometimes with retinal contribution as well, and can have both subretinal and sub-RPE components (Green, 1999; Green and Enger, 2005). The degree of RPE and photoreceptor degeneration is proportional to the diameter and thickness of the disciform scar. A tear of the RPE can accompany a minority of disciform scars.

The disease starts with subretinal drusens. Drusens are classified morphologically either as hard or soft. Hard drusen are yellow-white lesions, typically less than 63 µm in diameter, appearing as window defects on fluorescine angiography (FAG). In the earliest stage, they may be visible ophthalmoscopically as semi-translucent punctate dots in retroillumination. Later in the disease, soft drusen, defined as being larger than 63 µm in diameter, may appear. (Ambati et al., 2003). Soft drusen have a tendency to become confluent, and multiple drusens are an independent risk factor for visual loss from AMD (Macular Photocoagulation Study Group, 1997). In addition, they can lead to deficits in macular function such as color contrast sensitivity and central visual field sensitivity (Frennesson et al., 1995; Midena et al., 1994; Midena et al., 1997; Stangos et al., 1995; Sunness et al., 1988; Tolentino et al., 1994). A prospective evaluation of patients with drusen in the fellow eyes of unilateral wet AMD suggests that the risk of developing CNV in the second eye peaks at 4 years, with an increasing incidence of geographic atrophy thereafter (Sarraf et al., 1999). Immunohistochemical analyses have shown several agents, such as apolipoproteins B and E, different

immunoglobulins, factor X, amyloid P component, complement C5 and C5b-9 terminal complexes, fibrinogen and vitronectin to be present in drusen (Anderson et al., 2001; Hageman and Mullins, 1999; Hageman et al., 1999). These agents suggest a role of immunological and inflammatory processes in drusen pathogenesis.

The diagnosis of AMD is made with FAG and ocular coherence tomography (OCT). In dry AMD there is no leakage or oedema but atrophy in the macula. The earliest signs of CNV are subretinal or sub-RPE oedema and/or haemorrhages appearing as a greenish gray subretinal tissue in ocular examination. FAG leakage patterns of CNV are classified either as classic or occult. The former refers to discrete areas that hyperfluoresce early and continue to exhibit progressive leakage with increasing intensity and extent during the examination. Occult CNV refers either to a fibrovascular RPE detachment with irregular elevation of the RPE with stippled hyperfluorescence and late leakage, or to late leakage of undetermined origin. However, subretinal blood or lipid exudates may block the angiographic hyperfluorescence. In addition, CNV may not exhibit fluorescein leakage if it has undergone involution or is enveloped by RPE proliferation (Ambati et al., 2003).

In an OCT image classic CNV may appear as a highly reflective, fusiform thickening between the retina and the hyperreflective external band that corresponds to the RPE/Bruch's membrane/choroid complex. In addition, intraretinal oedema is present. Occult CNV can appear as an elevation of the RPE or as an irregularity of the external hyperreflective band. In geographic atrophy associated with the dry form of AMD, soft drusen may be identified as elevations of the RPE. Furthermore, atrophy is seen as thinning of the retina with an increased reflectivity of the choroid caused by the lack of pigment in the RPE (Hee et al., 1996).

2.2.2 Pathophysiology

Vascular risk factors, smoking, age, race, and family history have been shown to be the most important pathogenetic factors for the development of AMD (Age-Related Eye Disease Study Research Group, 2000; Delcourt et al., 1998; Klein et al., 1998; McCarty et al., 2001; Mitchell et al., 2002b; Smith et al., 2001). Also excessive exposure to light can damage the retina and has been implicated in the development of AMD (Tomany et al., 2004). Genetic predisposition has been demonstrated by familial aggregation studies and twin studies. The chromosomes most commonly implicated are 1q25-31 and 10q26. In particular, variants in the gene for the complement factor H (CFH) and the genes PLEKHA1/LOC387715, Factor B (BF) and complement component 2 (C2) have been implicated as major risk or protective factors for the development of AMD (Edwards et al., 2005; Gold et al., 2006; Hageman et al., 2005; Haines et al., 2005; Jakobsdottir et al., 2005; Rivera et al., 2005; Seitsonen et al., 2008).

An early pathological change in AMD is the appearance of basal laminar deposits (BlamD) and basal linear deposits (BlinD) (Green and Enger, 2005; Green and Key, 2005). BlamD consist of membrano-granular material between the plasma membrane and basal lamina of the RPE. BlinD consist of vesicular material located in the inner collagenous zone of Bruch's membrane. Although BlamD persists in areas of geographic atrophy, BlinD disappears, which is consistent with the fact that BlinD arises mostly from the RPE-photoreceptor complex (Sarks et al., 1994). BlinD may be more specific to AMD than BlamD (Curcio and Millican, 1999). Soft drusen can represent focal accentuations of BlinD and a localized accumulation of BlamD (Bressler et al., 1994).

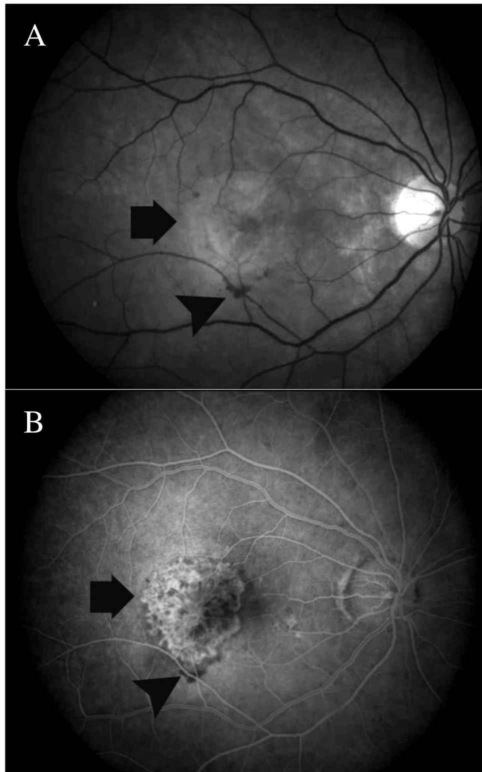

Figure 4. Wet form of age related macular degeneration with oedema, CNV (arrows) and haemorrhages (arrowheads). (A) Fundus photograph. (B) FAG image in the 30 sec time point.

The RPE serves a variety of metabolic and supportive functions that are of vital importance for retinal photoreceptors, including maintenance of the blood-retina barrier, participation in the visual cycle, and phagocytic uptake and degradation of constantly shed apical photoreceptor outer segments (Strauss, 2005). Furthermore, in addition to many other growth factors the RPE produces VEGF *in vivo* under physiologic conditions to maintain the fenestrated choriocapillaris endothelium (Kim et al., 1999). One of the reasons for the RPE dysfunction is an age-dependent phagocytic and metabolic insufficiency of postmitotic RPE cells. Impaired phagocytosis of photoreceptor outer segments by the RPE leads to a progressive accumulation of lipofuscin granules, a diverse group of autofluorescent lipid and protein aggregates, in the RPE (Kennedy et al., 1995; Sparrow and Boulton, 2005; Warburton et al., 2005). Lipofuscin accumulation reduces RPE phagocytic capacity (Sundelin et al., 1998), increases with age and is concentrated in the macula (Delori et al., 2001; Wing et al., 1978). Lipofuscin disrupts RPE function by mechanical distortion of cellular architecture and potentiating phototoxicity. RPE cells fed lipofuscin granules and exposed to short wavelength visible light (390–550 nm) undergo lipid peroxidation, suffer structural disintegrity and ultimately cell death (Davies et al., 2001; Rózanowska et al., 1995; Shamsi and Boulton 2001).

Oxidative stress has a significant role in the pathogenesis of AMD (Beatty et al., 2000). The phagocytosis of photoreceptor outer segments by RPE cells generates oxidative stress caused by ROS (Tate et al., 1995). H_2O_2 treatment of RPE cells results in marked mitochondrial DNA damage (Ballinger et al., 1999). Photoreceptor outer segments enriched in polyunsaturated fatty acids can undergo lipid peroxidation. In vitro studies show that RPE lipofuscin is a photoinducible generator of ROS that can compromise lysosomal integrity, induce lipid peroxidation, reduce phagocytic capacity and cause RPE cell death (Boulton et al., 1993; Holz et al., 1999; Sundelin et al., 1998). Lipofuscin granules are continuously exposed to visible light and high oxygen tension, which cause ROS production and possibly further oxidative damage to the RPE cell proteins and lipid membranes (Wassell et al., 1999; Winkler et al., 1999). In humans, lipid peroxidation is greatest in the macula, particularly with age (De La Paz and Anderson, 1992; Stone et al., 1979; van Kuijk and Buck, 1992). Furthermore, AGE products occur at sites of oxidant stress with hydroxyl radical formation. AGE products occur in soft drusen, in BlamD and BlinD, and in the cell cytoplasm of RPE associated with CNV (Crabb et al., 2002; Ishibashi et al., 1998).

It has been postulated that choroidal dendritic cells are activated and recruited by injured RPE and oxidized proteins and lipids in the Bruch's membrane (Hageman et al., 2001). The RPE cells respond to dendritic cell activation by secreting immune response modulators including vitronectin and apolipoprotein E (Johnson et al., 2001). Activation of choroidal dendritic cells might also initiate an autoimmune response to retinal or RPE antigens (Hageman et al., 2001). Antiretinal and anti–RPE antibodies have been detected in the serum of patients with AMD (Gurne et al., 1991; Niederkorn, 1990; Penfold et al., 1990). Inflammatory cells like multinucleated giant cells and leukocytes are involved in the later stages of AMD (Killingsworth et al., 1990; Penfold et al., 1985; Seregard et al., 1994). Macrophages near the Bruch’s membrane become more common when BlinD is present (Killingsworth et al., 1990). Activated macrophages and other inflammatory cells secrete enzymes that can damage cells and degrade the Bruch’s membrane. By releasing cytokines, inflammatory cells might promote the growth of CNV into the sub-RPE space (Oh et al., 1999b).

Wet AMD is characterized by the proliferation of CNV. CNV originates from the choroid and extends through a defect in Bruch's membrane and into a plane between BlamD and Bruch's membrane (Green and Enger, 2005). CNV can also extend through the RPE into the subretinal space. CNV arise as capillary-like structures with multiple points of origin (Green and Enger, 2005; Green and Key, 2005; Schneider et al., 1998). CNV can cause serous detachment of the RPE or retina, pigment modeling, RPE tears, haemorrhages and lipid exudation. Morphometric data indicate that the elastic lamina of Bruch's membrane in the macula is 3-6-fold thinner and 2-5-fold less dense relative to that in the mid-periphery in individuals of all ages rendering the macula more susceptible to the ingrowth of CNV than peripheral retina (Chong et al., 2005). Elastin fiber destruction in the macula could also play a role in the initiation of CNV because elastin degradation peptides are highly angiogenic (Kamisato et al., 1997; Nackman et al., 1997). High concentrations of VEGF and VEGF receptors are found in CNVs, surrounding tissue, and RPE cells (Kliffen et al., 1997; Kvanta et al., 1996; Lopez et al., 1996). Levels of VEGF are increased in cadaver AMD eyes, in the vitreous of patients with AMD, and in the plasma of patients with AMD (Kliffen et al., 1997; Lip et al., 2001; Wells et al., 1996). ANG-1, ANG-2, and Tie-2 immunoreactivity have been found in CNVs from AMD eyes (Otani et al., 1999). Cultured RPE cells also express ANG-1 and ANG-2 mRNA (Hangai et al., 2001).

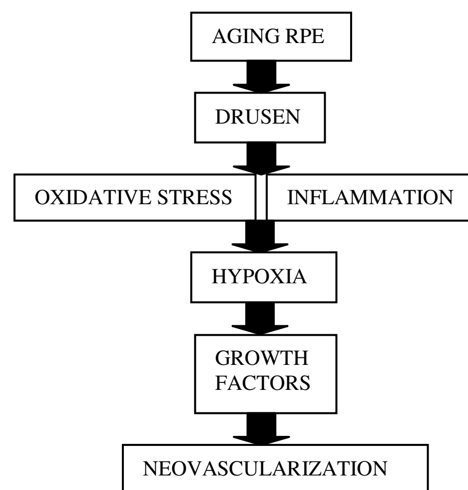

Figure 5. Consequences of wet AMD.

2.3 OTHER ANGIOPROLIFERATIVE DISEASES

The main pathogenesis is comprised of neovascularization and upregulation of VEGF also in several other diseases in the retina. These include retinal angiomatous proliferation, retinal telangiectasia, retinal vein occlusions and retinopathy of

prematurity (ROP). ROP is a disease in premature babies characterized by incomplete vascularization of the peripheral retina leading to retinal neovascularization (Terry, 1942). Angiogenesis can lead to both normal and abnormal vessel formation. In experimental oxygen induced retinopathy (OIR) model, VEGF levels have been shown to rise resulting in neovascularization (Pierce et al., 1995; Smith et al., 1994). This finding was confirmed with elevated VEGF levels in the vitreous of an ROP patient (Young et al., 1997). However, VEGF is also involved in normal vascular embryogenesis of the retina (Alon et al., 1995; Ozaki et al., 2000; Pierce et al., 1996; Stone et al., 1995). In addition to VEGF insulin-like growth factor I (IGF-I) is critical for normal retinal vascular development. A lack of IGF-I in the early neonatal period is associated with the proliferative ROP (Hellstrom et al., 2001a). Supplementation of IGF in preterm infants prior to the development of ROP could stimulate normal vessel development and prevent ROP. However, in the proliferative phase of ROP, IGF-I has been shown to stimulate neovascularization independent of VEGF levels as well as potentially regulate the effects of VEGF (Smith et al., 1999). Current therapy consists of monitoring oxygen supplementation, aggressive screening in children who are at risk of having ROP and laser treatment of the retina once threshold disease is reached (Palmer et al., 2005).

Pathological angiogenesis also occurs in tissues in the anterior segment of the eye. Neovascularization of the iris typically occurs within ischemic retinopathies like ischemic ocular syndrome and central retinal vein occlusion, and can cause loss of vision through the associated closure of the irido-corneal drainage angle, resulting in raised intraocular pressure and neovascular glaucoma. Neovascularization of the cornea can occur in response to a number of different insults, including trauma, infection or inflammation (Chang et al., 2001).

2.4 ANGIOGENIC FACTORS

2.4.1 Vascular endothelial growth factors

The first and the most potent member of VEGF-family, VEGF (also called VEGF-A), was first identified in highly vascularized tumors in 1983 (Ferrara and Henzel, 1989; Senger et al., 1983). After the discovery of VEGF, four other members in the human VEGF family have been identified: VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Achen et al., 1998; Joukov et al., 1997b; Maglione et al., 1991; Olofsson et al., 1996). In addition to these VEGFs, viral VEGF homologs (VEGF-E) and snake venom VEGFs (VEGF-F) have been found (Ogawa et al., 1998; Yamazaki et al., 2003). VEGF is a 46-kDa homodimeric glycoprotein with several isoforms including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ generated by alternative mRNA splicing from the same gene (Ferrara et al., 1991). It is a potent angiogenic stimulator, promoting proliferation, migration, proteolytic activity and capillary tube formation of ECs, thus playing a crucial role in both normal and pathological angiogenesis (Aiello and Wong, 2000; Dvorak et al., 1995; Ferrara and Henzel, 1989). Heterozygous deletion of the VEGF gene results in embryo death between days 8.5 and 9.5. The embryos are characterized by impaired angiogenesis (Carmeliet et al., 1996). VEGF increases vascular permeability with an efficacy 5000-fold higher than that of histamine (Senger et al., 1996). Injection of VEGF into the vitreous can induce preretinal and iris neovascularization (Tolentino et al., 1996; Tolentino and Adamis, 1998). In the retina, VEGF is produced by multiple cell types, including the RPE, pericytes, ECs, Müller cells and ganglion cells (Dorey et al., 1996; Lu et al., 1999; Pe'er et al., 1995). Among them, Müller cells and RPE are believed to be the major source of VEGF in the retina, and ECs to be the primary target of VEGF (Aiello et al., 1994; Dorey et al., 1996; Pierce et al., 1995). VEGF synthesis is strongly

upregulated by hypoxia (Aiello et al., 1995b; Marti and Risau, 1998).

VEGF levels in the ocular fluid are very low in the normal eye (Aiello et al., 1994; Ogata et al., 2002). In normal adult monkey and mouse eyes, the most abundantly expressed isoforms are $VEGF_{121}$ and $VEGF_{165}$ (Kim et al., 1999). In humans, intravitreal levels of VEGF were elevated in proliferative diabetic retinopathy (Adamis et al., 1994; Aiello et al., 1994). Therapeutic laser photocoagulation decreases VEGF levels in the vitreous humour by 75% in patients with proliferative diabetic retinopathy, suggesting that the development and regression of retinal neovascularization is associated with VEGF levels in the diabetic eye (Aiello et al., 1994). In addition, significantly elevated VEGF levels in the aqueous humor have been reported in diabetic patients with macular edema (Funatsu et al., 2002). High levels of VEGF are also found in CNV membranes excised from patients with AMD and in the vitreous humour of a ROP baby (Kvanta et al., 1996; Rakic et al., 2003; Young et al., 1997).

Different VEGF isoforms may have different functions in ocular diseases. $VEGF_{165}$ has been shown to be the most important isoform in the blood-retina barrier breakdown and pathologic intraocular neovascularization (Ferrara et al., 2003). $VEGF_{165}$ is the predominant isoform expressed at the time of maximal preretinal neovascularization in a neonatal rat model (McColm et al., 2004). It is also the primary isoform in the retina of diabetic rats (Ishida et al., 2003; Usui et al., 2004). Levels of both $VEGF_{121}$ and $VEGF_{165}$ are increased in monkeys after laser-induced retinal vein occlusion (Shima et al., 1996). $VEGF_{121}$ is the main isoform expressed in mouse CNV membranes, and inhibition of $VEGF_{121}$ results in reduction of CNV in mice (Akiyama et al., 2005; Rakic et al., 2003).

VEGF has also been shown to have neuroprotective properties in the retina (Jin et al., 2000). Intravitreal $VEGF_{165}$ and $VEGF_{121}$ displayed a protective effect on apoptotic retinal cells in a retinal ischemia–reperfusion rat model. This protective effect was inhibited by blockade of all VEGF isoforms but not by blockade of $VEGF_{165}$ alone (Shima et al., 2004). It seems that VEGF isoforms may differ in neuroprotection suggesting that specific VEGF isoform targeting would be beneficial in blocking vessel growth and sparing the neuroprotective effects. In addition, VEGF acts as a survival factor for newly formed blood vessels in the retina and also inhibits apoptosis induced by tumor necrosis factor (Alon et al., 1995; Spyridopoulos et al., 1997). Development of the choroideal circulation is dependent on VEGF produced by RPE (Yi et al., 1998). The RPE expresses VEGF and the ECs of the choriocapillaris express VEGFR-2 which supports the role for VEGF in the maintenance of the adult choriocapillaris (Kim et al., 1999). Long-term therapeutic neutralization of VEGF may lead to the unexpected degeneration of the choroidal circulation (Peters et al., 2007).

Also other members of the VEGF family stimulate neovascularization and excessive vascular permeability. PlGF, VEGF-B, VEGF-C and VEGF-D are EC mitogens *in vitro* and *in vivo*, but their role in ocular angiogenesis remains unclear (Achen et al., 1998; Olofsson et al., 1996). VEGF-B does not seem to play a role in retinal neovascularization, because mice deficient in VEGF-B have normal retinal vascular development and no difference in hypoxia-induced retinal neovascularization when compared to wild type mice (Reichelt et al., 2003). VEGF-C and VEGF-D are produced as prepropeptides and further processed to biologically fully active forms (Achen et al., 1998; Joukov et al., 1997a). VEGF-C and VEGF-D are involved primarily in lymphangiogenesis but also in angiogenesis (Cao et al., 1998; Jeltsch et al., 1997; Marconcini et al., 1999). VEGF-C and VEGF-D have been found in subretinal vascular membranes of AMD patients implicating a role also in ocular angiogenesis (Ikeda et al., 2006). PlGF has also been

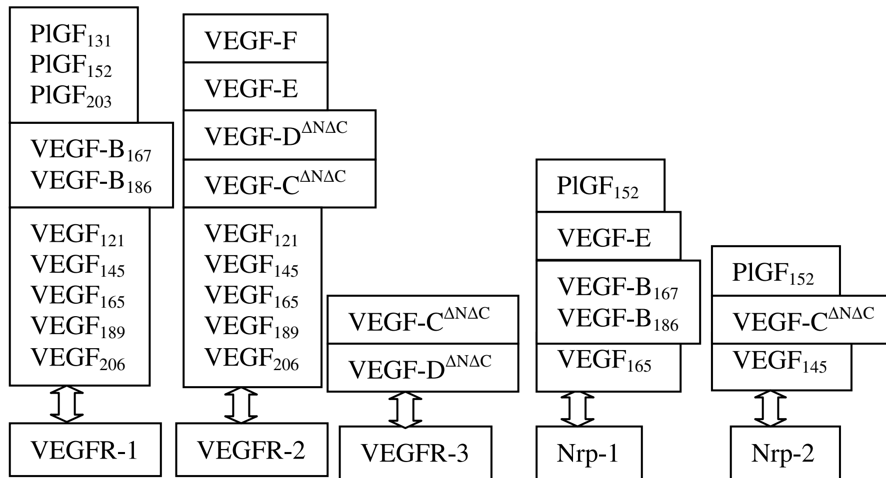

Figure 6. VEGF family and VEGF receptors.

directly implicated in proliferative retinopathy (Khaliq et al., 1998). Knockout of PlGF or neutralization with an anti-PlGF antibody substantially suppresses retinal neovascularization (Carmeliet et al., 2001; Luttun et al., 2002).

2.4.2 VEGF Receptors

VEGF exerts its biological activities through multiple receptors; VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4) which are expressed predominantly in ECs, and to a lesser extent on monocytes and macrophages (Ferrara et al., 2003; Terman et al., 1992). The binding of VEGF to its receptors initiates a signal transduction cascade mediating vascular permeability and EC proliferation and migration. In addition, two co-receptors for VEGF, neuropilin-1 and 2 (Nrp-1 and Nrp-2) has been described (Soker et al., 1998; Soker et al., 2002). VEGFR-1 binds VEGF, VEGF-B and PlGF. VEGFR-2 binds VEGF and proteolytically modified VEGF-C and –D. VEGFR-3 binds VEGF-C and -D and mediates lymphangiogenesis (Figure 6). The VEGF receptors are almost exclusively found on ECs. Homozygous VEGFR-1 deletion permits EC differentiation, but the vascular channels that form are grossly abnormal, and the animals die in utero (Fong et al., 1995). Homozygous deletion of VEGFR-2 leads to death of mice between embryonic days 8.5 and 9.5, with the embryos having no yolk–sac blood islands and ECs (Shalaby et al., 1995). In the histopathological analysis of a normal human retina and choroid, choriocapillaris shows an immunohistochemical positive reaction with VEGFR-1 and VEFGR-2. Furthermore, VEGFR-3 is present in the VEGFR-2 positive choriocapillaris endothelium that faces the RPE layer (Blaauwgeers et al., 1999). VEGFR-2 is the major mediator of mitogenesis of ECs (Gille et al., 2001). By activating VEGFR-1, VEGF promotes assembly of ECs into tubes. The role of VEGFR-1 is context dependent. In embryos and some adult tissues, it acts as a decoy receptor that modulates angiogenesis and in some adult tissues it mediates VEGF signaling and is proangiogenic (Fong et al., 1999; Luttun et al., 2002; Park et al., 1994). In the eye, VEGFR-1 is proangiogenic, and its inhibition can suppress retinal or choroideal neovascularization (Shen et al., 2006). VEGF also interacts with neuropilins (Klagsbrun et al., 2002; Neufeld et al., 2002). In early development, Nrp-1 is expressed in arteries and Nrp-2 is expressed in veins (Herzog et al., 2001). The absence or

blockade of either Nrp-1 or -2 suppresses ocular neovascularization (Oh et al., 2002; Shen et al., 2004). Neuropilins do not have kinase domains and must complex with VEGF receptors for intracellular signaling to occur. Nrp-1 is involved in retinal neovascularization in diabetic retinopathy and in ischemic animal models (Ishida et al., 2000; Ishihama et al., 2001). Coexpression of VEGFR-2 and Nrp-2 was suggested to facilitate fibrovascular proliferation in diabetic retinopathy (Ishida et al., 2000).

2.4.3 PDGFs

The platelet-derived growth factor (PDGF) was one of the first isolated and cloned angiogenic growth factors (Saint-Geniez and D'Amore, 2004). The PDGF family of growth factors is composed of four different polypeptide chains. The four PDGF chains assemble into disulphide-bonded dimers via homo- or heterodimerization, and five different dimeric isoforms have been described so far; PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD (Fredriksson et al., 2004; Li et al., 2005). PDGF-A and PDGF-B chains were discovered more than two decades ago, and only recently were PDGF-C and PDGF-D, also known as Iris-expressed growth factor, chains discovered (Bergsten et al., 2001; Heidaran et al., 1991; Hirst et al., 1996; LaRochelle et al., 2001; Li et al., 2000; Seifert et al., 1989). The overall sequence homology among the different growth factor domains of the PDGFs is around 25%, while PDGF-A and PDGF-B are approximately 50% identical. Similarly, the growth factor domains in PDGF-C and PDGF-D display approximately 50% homology (Fredriksson et al., 2004). There are two PDGF receptors: the PDGF α receptor (PDGFαR) and the PDGF ß receptor (PDGFßR). The PDGFαR binds both PDGF-A and -B and therefore PDGF-AA, -BB, and -AB can all activate PDGFαR. PDGFßR binds PDGF-B but not -A, and therefore PDGF-BB, but not -AA, can activate PDGFßR. The action of PDGF-AB is more complex, but it appears that at physiologically relevant concentrations, PDGF-AB can activate PDGFßR only in combination with PDGFαR (Seifert et al., 1993). Unlike PDGF-A and -B, PDGF-C and -D are secreted as inactive proteins that are activated by proteolytic cleavage, but similar to PDGF-A and -B, they signal through PDGFαR or PDGFßR (Mori et al., 2002a). In the retina PDGFs are generally considered as pericyte recruitment and survival factor (Hellstrom et al., 2001b; Leveen et al., 1994; Lindahl et al., 1997). PDGF-BB and PDGF-CC are more potent in stimulating angiogenesis than PDGF-AA (Li et al., 2005; Risau et al., 1992). Additionally, significantly elevated concentrations of PDGF-AB are found in the vitreous and preretinal membranes of patients with proliferative diabetic retinopathy (Freyberger et al., 2000; Robbins et al., 1994).

2.4.4 Angiopoietins

Angiopoietin-1 (ANG-1) and angiopoietin-2 (ANG-2) belong to a family of endothelial growth factors that function as ligands for the endothelial cell-specific receptor tyrosine kinase, Tie-2 (Nourhaghighi et al., 2003). Also Tie-1 has been identified but there is no ligand found for it. ANG-1 and ANG-2 share 60% amino acid homology (Davis et al., 1996). Tie-1 and Tie-2 receptors are selectively expressed on vascular ECs and are required for embryonic vascular development (Dumont et al., 1994; Sato et al., 1995). Activation of Tie-2 promotes maturation of leaky vascular tubes into competent blood vessels. Over-expression of ANG-1 in the retina significantly reduced VEGF-induced retinal vascular permeability, and also suppressed the development of retinal neovascularization in an OIR model and in laser-induced CNV (Nambu et al., 2004). ANG-2 acts as an endogenous antagonist of the action of ANG-1 by decreasing its binding to Tie-2 (Maisonpierre et al., 1997). ANG-2 is upregulated by hypoxia and angiogenic cytokines including VEGF. In addition, it is involved in pathologic angiogenesis associated with ischemia in the

retina in an animal model and in CNV associated with AMD (Mandriota and Pepper, 1998; Oh et al., 1999a; Otani et al., 1999). Mice deficient in ANG-2 showed delayed and incomplete development of the superficial vascular bed of the retina, and complete absence of the intermediate and deep vascular beds, suggesting that ANG-2 is a crucial factor in the maturation of retinal vasculature (Hackett et al., 2002). In patients with ROP, colocalization of Tie-2, VEGF and ANG-2, but not ANG-1 was observed in fibrovascular membrane, suggesting a pathological role of ANG-2 and Tie-2 in retinal neovascularization (Umeda et al., 2003). In OIR mice, ANG-2 expression was up-regulated in the retina during the period of angiogenesis and reached the peak at the maximal angiogenic response (Das et al., 2003). In addition, ANG-2 has also been shown to enhance the sensitivity of retinal blood vessels to VEGF (Oshima et al., 2004).

2.4.5 IGFs

Insulin-like growth factors (IGF-I and IGF-II) are growth-promoting peptides with multiple biological effects (LeRoith and Roberts, 1993). IGF-I was initially identified as a circulating factor that appeared to mediate the effects of growth hormone (Salmon and Daughaday, 1957). IGF exerts its effect on ECs via the IGF receptors (IGF-IR and IGF-IIR) (Miller et al., 1997). Also six well-characterized IGF binding proteins (IGFBP) have been found in circulation and extracellular fluids and can inhibit or potentiate IGF-I activity (LeRoith and Roberts, 1993). IGF-I is known to be present in small amounts in normal retina, and IGF-IRs are found in retinal vascular ECs (Grant and Guay, 1991). IGF-I mediates both physiological and pathological neovascularization by stimulating migration and proliferation of retinal ECs and RPE cells and tube formation of retinal ECs (Grant et al., 1990). In the course of diabetic retinopathy, serum IGF-I levels were elevated in patients with proliferative disease (Dills et al., 1991). Also intravitreal levels of IGF-I were found to be elevated during proliferative diabetic retinopathy (Grant et al., 1986; Meyer-Schwickerath et al., 1993).

Normoglycemic transgenic mice overexpressing IGF-I in the retina developed several vascular alterations characteristic for DM including pericyte loss, thickened capillary basement membrane, IRMA, proliferative changes and retinal detachment (Ruberte et al., 2004). IGF-I have not been found to have a causative role in diabetic retinopathy but may act as an aggravating factor (Miller et al., 1997). In IGF-I knockout mice, normal retinal vascular development was arrested, despite the presence of VEGF (Smith et al., 1999). In a study on premature infants, the development of ROP was strongly associated with a prolonged period of low levels of IGF-I (Hellstrom et al., 2001a). Infants with higher IGF-I levels earlier in life had more normal retinal vascular development, and did not have ROP. Thus, IGF-I has a critical role in normal retinal vascular development suggesting that the lack of IGF-I in the early neonatal period increases the risk of having ROP (Das and McGuire, 2003). Although the role of IGF-I in retinal neovascularization has been studied extensively, its role in CNV is still not clear.

2.4.6 FGFs

Acidic and basic fibroblast growth factors are prototypes of the fibroblast growth factor (FGF) family. Basic fibroblast growth factor (bFGF) is the best characterized of the FGFs (Miller et al., 1997). It belongs to a large family of growth factors consisted of over 23 proteins. bFGF lacks a signal sequence, remains cell-associated and is apparently not secreted (Miller et al., 1997). Therefore, the origin of its presence in the ECM is not clear. bFGF has been localized to the astrocytes of the ganglion cell layer, as well as to the cells of the inner nuclear layer (Gao and Hollyfield, 1992; Kostyk et al., 1994). bFGF has a high affinity for heparin which protects it from inactivation and degradation (Gospodarowicz and Cheng, 1986; Sommer and Rifkin, 1989). bFGF has been shown to

stimulate EC proliferation and migration. In addition, it induces capillary ECs to form capillary-like tubes (Mignatti et al., 1989; Montesano et al., 1986).

The role of FGF in ocular angiogenesis is controversial. In the OIR mouse model, FGF-like polypeptides were found to be elevated in the retinal tissues during neovascularization (Nyberg et al., 1990). bFGF injected into the subretinal space in rabbits induced subretinal neovascular membranes histologically similar to those seen in patients with AMD (Kimura et al., 1999). In an animal model of laser-induced subretinal neovascularization, RPE cells stained with both aFGF and bFGF after laser treatment, indicating the role of these factors in choroidal angiogenesis (Zhang et al., 1993). Some studies have reported elevated levels of bFGF in vitreous humour and retinal neovascular membranes in patients with proliferative diabetic retinopathy (Frank et al., 1996; Hanneken et al., 1991; Sivalingam et al., 1990). However, actively growing neovascular membranes show only minimal bFGF in the basement membranes of new vessels in spite of their capacity to bind exogenous bFGF (Hanneken et al., 1991). In the retina, overexpression of bFGF could not induce neovascularization (Ozaki et al., 1998). Transgenic mice deficient in bFGF developed the same amount of retinal or CNV as the wild-type mice in OIR or laser-induced subretinal neovascularization models, indicating bFGF expression may not be necessary for the development of choroidal or retinal new vessels (Ozaki et al., 1998; Tobe et al., 1998b). It has been hypothesized that increased retinal expression of bFGF is angiogenic only when there is accompanied cell injury unmasking control mechanisms that sequester bFGF (Yamada et al., 2000).

2.4.7 HIF-1

Hypoxia inducible factor (HIF)-1 is a transcription factor that regulates the response to both acute and chronic hypoxia. It is the primary hypoxic signaling protein in cells for regulating angiogenesis and is able to induce the transcription of more than 70 genes (Semenza, 2004). HIF-1 stimulates the transcription of genes whose promoters include a hypoxia-response element (HRE) (Semenza and Wang, 1992; Tian et al., 1997; Wiesener et al., 1998). Products of these genes mediate the restoration of tissue oxygenation and limit tissue damage. HIF-1 is a heterodimeric transcription factor that typically consists of an oxygen-regulated labile 120 kDa HIF-1α subunit and a constitutively expressed stable 92 kDa HIF-1β subunit (Wang et al., 1995). Two other HIF molecules, HIF-2α and HIF-3α, have also been described (Wiesener et al., 1998). Homozygous knockouts of HIF-1α are embryonic lethal, whereas HIF-2α knockouts have less severe phenotypes but still exhibit vascular and respiratory abnormalities as well as blindness in mice one month old (Ding et al., 2005; Iyer et al., 1998). Expression of the HIF-1α subunit is tightly regulated by the cellular oxygen concentration, but HIF-1β is oxygen insensitive. HIF-1α increases exponentially as oxygen concentration declines and determines the level of HIF-1 activity (Jiang et al., 1996; Semenza et al., 1996; Wang et al., 1995). Under normoxic conditions, HIF-1α is continuously synthesized and degraded proteosomally but in exposure to low oxygen tensions, it accumulates rapidly and begins to act as a transcriptor factor by moving into the nucleus and activating an array of genes (Huang et al., 1998; Kallio et al., 1999; Salceda and Caro, 1997). In developing retina and in OIR mouse model HIF-1α levels have shown to be significantly increased (Ozaki et al., 1999). HIF-1α levels are also high in the nerves of diabetic rats (Chavez et al., 2005). HIF-1α has been found in neovascular membranes from diabetic patients (Abu El-Asrar et al., 2007). Furthermore, the role of HIF in ROP has been implicated in experimental studies (Brafman et al., 2004; Morita et al., 2003).

2.5 GENE THERAPY

Gene therapy is a promising strategy for the treatment of several inherited and acquired diseases in the eye. Gene therapy means the delivering of genes into the target cells to treat a disease. The encoding gene may be designed to induce a useful gene expression, to block a harmful gene expression, or, like in hereditary diseases, a defective mutant allele is replaced with a functional one. For effective gene therapy it is necessary to deliver therapeutic genes to specific cells at high efficiency, express the gene for a prolonged period of time and ensure that the introduction of the therapeutic gene is not harmful to the target tissue.

There are several methods and vectors in use for the delivery of therapeutic nucleic acids into cells (Verma and Somia, 1997). These methods can be classified as viral and non-viral technologies, and a number of different vector systems for ocular gene transfer have been developed (Wright, 1997). Viral vectors are very efficient in transducing genes into cells. However, their use has been restricted because of immunological problems and risk of the insertional mutagenesis. In addition, producing viral vector can be difficult (Dobbelstein, 2003). Non-viral vectors are easier to engineer and manufacture, but significantly less efficient in gene delivery compared with virus-based vectors. In addition, the lack of their chromosomal integration precludes long-term therapeutic effects (Masuda et al., 1996).

The eye is an ideal organ for *in vivo* gene transfer. It is easily accessible by microsurgical techniques under direct visualization and allows local application of therapeutic vectors (Figure 7). Precise targeting of vector within the globe minimizes systemic dissemination and the possibility of unwanted systemic side effects. Furthermore, the optical transparency of the eye enables transgene expression within the retina and effects of treatments to be monitored by a variety of noninvasive examinations. The small size of the eye means that small volumes of vector suspensions can transduce an adequate proportion of cells in the target tissue. Also non-dividing cell populations may be efficiently transduced by a single dose. In addition, the potential risk of virus-mediated insertional mutagenesis is significantly lower in the eye than in systemic applications as the ocular cells targeted are relatively few in number.

Furthermore, immune responses following intraocular vector administration are typically attenuated compared to those following systemic administration; a relatively immune priviledged system protects from immune responses directed against vector antigens that might otherwise cause inflammation and limit transgene expression (Bennett et al., 1996). If only one eye is treated, the untreated eye may serve as a useful control for the evaluation of the efficacy of the treatment. The first clinical trials in ocular gene transfer strategy have been focused in gene replacement in inherited retinal degeneration, Leber's congenital amaurosis (Bainbridge et al., 2008; Maguire et al., 2008).

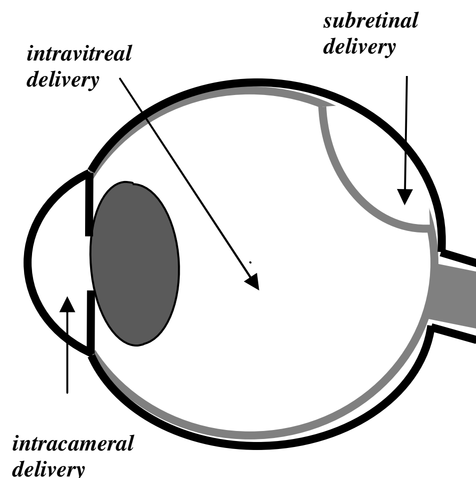

Figure 7. Delivery routes in ocular gene therapy.

2.5.1 Viral vectors

Viruses are intra-cellular parasites with specialized molecular mechanisms to efficiently transport their genomes to the cells. Viruses transfect their own DNA or RNA efficiently into the host cells which are then harnessed to produce new viral particles. By replacing genes that are needed for the replication phase of viruses life cycle with therapeutic genes, the recombinant viral vectors can transduce the cell type it would normally infect (Figure 8). The most studied viruses in ocular gene therapy field are adenoviruses (Ad), adeno-associated viruses (AAV) and lentiviruses. Adenoviral vectors efficiently target cells of the outer retina but their duration of expression is limited to a few weeks by immune responses to the vector (Ali et al., 1998a; Bennett et al., 1994; Bennett et al., 1996; Reichel et al., 1998).

AAV vectors are used for sustained transduction of photoreceptor cells (Ali et al., 1996; Bennett et al., 1999; Flannery et al., 1997). Lentiviral vectors stably transduce RPE cells but are less efficient than AAV in transducing photoreceptors (Bainbridge et al., 2001). Retroviral vectors specifically transduce dividing cells and have been developed for proliferative and neoplastic intraocular disorders (Hurwitz et al., 1999; Sakamoto et al., 1995). Ocular tissues are widely distributed, and therefore the capacity of the vector to transfect certain tissue is dependent on the site of its intraocular administration. Delivery of AAV vectors into the subretinal space results in the transduction of photoreceptors and RPE cells, whereas injection of the same vector into the vitreous humour targets only ganglion cells in the inner retina (Ali et al., 1998b).

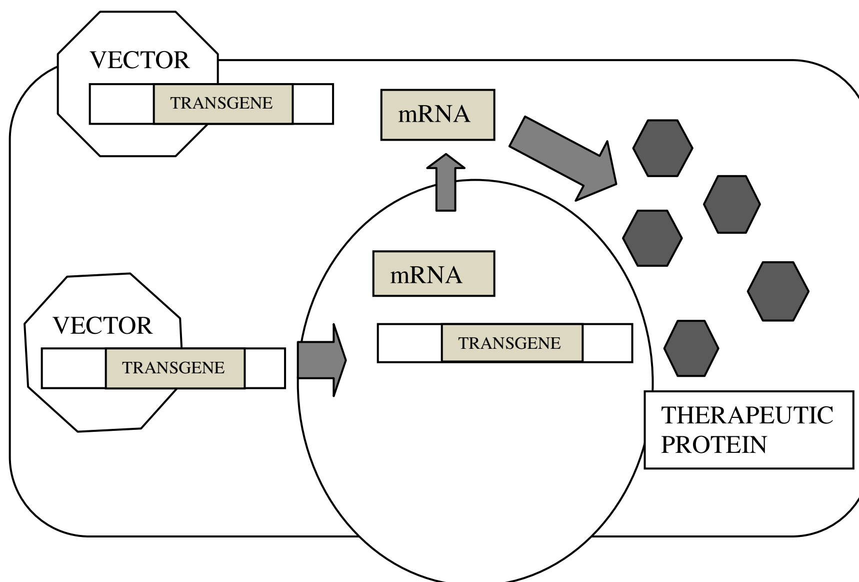

Figure 8. Basic principal of gene transfer with viral and nonviral vectors.

2.5.1.1 Adenovirus

Adenovirus is a non-enveloped double-stranded DNA virus with maximal carrying capacity of 30kb of foreign DNA (Gonçalves and Vries, 2006). It is a widely used vector in the gene therapy field due to many advantages. Ad vectors are relatively easy to produce, have good capacity, and with an appropriate promoter can mediate good expression levels in many types of cells (Campochiaro, 2007). The transduction efficiency for a certain cell type varies depending on the serotype of the Ad vector. The most studied serotypes are first or second generation type 2 and 5 (Campochiaro, 2007; Chuah et al., 2003). Adenoviruses enter the cell by CAR-receptor mediated endocytosis and stay in the nucleus as episomes (Kovesdi et al., 1997). Therefore, they lack the ability to integrate the transferred gene into chromosomal DNA and their presence in cells is short-lived typically limited to a few weeks (Jomary et al., 1994). Repeated vector administration would be required to enhance expression levels, but the induction of a humoral immune response against the capsid proteins precludes vector readministration (Kafri et al., 1998). The T-cell–mediated immune response of the host has been shown to play a role in limiting the duration of adenovirus-mediated transgene expression in the eye (Hoffman et al., 1997; Reichel et al., 1998; Tripathy et al., 1996).

The wild-type adenoviral genome can be divided into regions that are expressed either early or late after the infection of the target cell. The early region transcription units are E1-E4. The early gene products have different functions, such as initiation and activation of adenoviral replication, suppression of endogenous host gene expression and activation of late adenoviral gene expression (L1-L5), which encode most of the virion structural proteins (Chuah et al., 2003). The first generation adenoviral vectors have deletions in at least one of the early genes, which consequently impair viral gene expression and replication (Figure 9) (Kozarsky and Wilson, 1993; Krougliak and Graham, 1995). Whereas early generation adenoviral vectors still contain residual viral genes that contribute to inflammatory immune responses, the latest generation, so called gutless adenoviral vectors, do not contain any residual viral genes. Gutless vectors contain only inverted terminal repeat (ITR) parts and a packaging sequence of the viral genome. Gutless adenoviral vectors are capable of transducing also rods, and mediate much longer transgene expression (Kumar-Singh and Chamberlain, 1996). They have significantly improved safety and expression profile of adenovirus vectors (Ehrhardt and Kay, 2002; Schiedner et al., 1998). However, the immune system is still activated with gutless vectors due to their interaction with antigen-presenting cells (Chuah et al., 2003; Thorrez et al., 2004). However, in clinical trials second generation Ad vectors were well-tolerated (Campochiaro et al., 2006; Chevez-Barrios et al., 2005).

Intravitreous injections of Ad vectors resulted in transduction of corneal endothelium, trabecular meshwork, iris pigmented epithelium, ciliary epithelium, and GCL in the inner retina (Budenz et al., 1995; Mori et al., 2002c). Subretinal injections result in transduction of RPE cells and occasional Müller cells, but little or no transduction of retinal neurons (Bennett et al., 1994; Hoffman et al., 1997; Li et al., 1994). In eyes with proliferative retinopathy, there is strong transduction of cells participating in the disease processes, which could be a therapeutic advantage (Mori et al., 2002c).

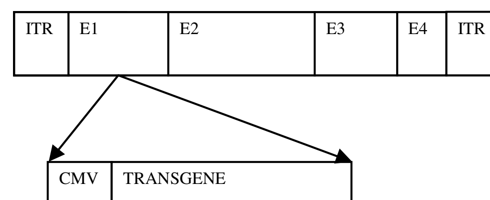

Figure 9. First generation adenovirus vector.

2.5.1.2 Baculovirus

Baculovirus (Bv) is a double-stranded DNA virus more seldom used in gene therapy. It is a large, approximately 130 kb sized virus with a transgene capacity up to 100 kb of foreign DNA. Over 500 different types of baculovirus have been found and the hosts are most usually insects (Hu, 2005). The most usually used and the best known serotype is *Autographa californica* multiple NPV (AcMNPV). Bv cannot replicate in vertebrate hosts and it is capable of transducing differentiated, nondividing cells (Hu, 2006; Mähönen et al., 2007). Bvs have a low cytotoxicity in mammalian cells even at a very high virus load and they can be easily produced in high titers (Airenne et al., 2003; Laitinen et al., 2005). Bv enters nondividing mammalian cells by endocytosis and loses its envelope passing from the endosome to the cytoplasm. The nucleocapsid is then transported to the nucleus through the nucleopore (van Loo et al., 2001). Recombinant Bvs have the capability of transducing a variety of mammalian cells *in vitro* (Bilello et al., 2001; Boyce and Bucher, 1996; Condreay et al., 1999; Ma et al., 2000; Merrihew et al., 2001; Sarkis et al., 2000; Shoji et al., 1997; van Loo et al., 2001). Despite the ability to transduce mammalian cells *in vitro*, only limited success has been reported *in vivo*. This is most likely due to viral inactivation by the complement system (Hofmann and Strauss, 1998). In the eye, the anterior chamber, the subretinal space, and to a lesser extent, the vitreous cavity, are relatively immune-privileged sites (Ferguson and Griffith, 1997; Kaplan et al., 1999; Niederkorn, 1990). Antigens in these areas are not subject to the complement pathway and therefore Bvs are potential vectors for ocular gene therapy. Intravitreal injections of Bv resulted in GFP expression in the corneal endothelium, lens, RPE, and photoreceptor cells (Haeseleer et al., 2001). GFP expression was observed for up to two weeks after injection. Subretinal injection of BvGFP results in transduction of RPE cells. No alteration in ERG responses was observed after injection of BvGFP (Haeseleer et al., 2001).

2.5.1.3 Other viruses

Adeno-associated viruses (AAVs) are nonenveloped parvoviruses with linear, single-stranded DNA genomes that have many characteristics that make them advantageous for use as viral vectors (Grimm and Kay, 2003). In humans AAV vectors appear to invoke little immune response and therefore have little toxicity and mediate prolonged transgene expression (Ali et al., 1998b; Bennett et al., 1997; Flannery et al., 1997). AAVs have the ability to transduce both dividing and non-dividing cells and recombinant adeno-associated viruses have been developed that integrate randomly in the host genome and have been shown to effect stable transduction of the retina for more than 1 year (Bennett et al., 1999; Hauswirth and Beaufrere, 2000). Limitations of AAV include difficulties to produce the virus, low maximal insert size of less than 5 kb and the induction of insertional mutagenesis (Miller et al., 2002). Moreover, AAV administration in patients has been associated with the induction of a possible cellular immune response directed against the processed AAV capsid antigens, leading to transient and acute hepatotoxicity (Manno et al., 2006; Zaiss and Muruve, 2005). There are currently six known serotypes of AAV, of which type 2 has been most extensively studied as a potential vector (Rabinowitz et al., 2002). Different AAV serotypes have different virion shell proteins and therefore they vary in their ability to bind to and transfect different host cell types (Martin et al., 2002). Recombinant vectors can be generated using both capsid proteins and genomes from the same serotype or the vector genome can be derived from one serotype and included in the capsid from an alternative AAV serotype (Chao et al., 2000; Hildinger et al., 2001).

Intravitreous injections of AAV2/2 vectors result in transduction of ganglion cells,

trabecular meshwork cells and various cells of the inner nuclear layer, including Müller cells (Ali et al., 1998b; Auricchio et al., 2001; Borrás et al., 2006; Flannery et al., 1997; Martin et al., 2002). Subretinal injections of this serotype results in the transduction of photoreceptors and RPE cells, with an onset of transgene expression that peaks at 4 weeks after vector administration (Ali et al., 1996; Bennett et al., 1997; Flannery et al., 1997). Subretinal administration of AAV5/5 or AAV2/5 also results in early RPE transduction, but more efficiently than that with AAV2/2 (Auricchio et al., 2001; Lotery et al., 2003; Yang et al., 2002).

Lentivirus-based vectors are attractive candidates for ocular gene transfer because they efficiently transduce a variety of nondividing cells with little or no host response resulting in long-term transgene expression (Naldini et al., 1996; Poznansky et al., 1991). Human immunodeficiency virus-1 (HIV-1) and feline immunodeficiency virus (FIV) based lentiviral vectors efficiently transduce the corneal endothelium and trabecular meshwork following delivery into the anterior chamber (Challa et al., 2005; Loewen et al., 2004). Subretinal injection in rodents led to stable transgene expression in the RPE cells for at least 2 years (Cheng et al., 2005; Loewen et al., 2004; Tschernutter et al., 2005). HIV-1 based lentiviral vector has been shown to mediate therapeutic effects in retinal degenerations where RPE is involved (Miyazaki et al., 2003; Tschernutter et al., 2005). Lentivirus-mediated transduction of photoreceptor cells appears to be less predictable than transduction of RPE cells, but is reported to occur under certain circumstances depending on retinal maturity, the used promoter and anatomical barriers. Photoreceptor transduction is evident following subretinal vector delivery in neonatal rodents, but the efficiency of photoreceptor transduction in adults is relatively low (Kostic et al., 2003). The efficiency of photoreceptor cell transduction in adults is improved in locally traumatized retinas suggesting that anatomical barriers to tissue penetration by vector particles may be a limiting factor (Bainbridge et al., 2001; Gruter et al., 2005; Kostic et al., 2003). Vectors based on nonprimate lentiviruses such as FIV and bovine immunodeficiency virus (BIV) have transduction efficiencies and durations of expression in ocular tissues that are comparable to HIV-1 based vectors and may provide alternatives with potential safety advantages (Bainbridge et al., 2001; Cheng et al., 2005; Loewen et al., 2004; Molina et al., 2004). BIV is a lentivirus that is not known to cause human disease. Subretinal injection of a BIVGFP vector resulted in a prolonged transduction of RPE cells with no inflammatory response (Takahashi et al., 2002).

2.5.2 Non-viral vectors

Problems associated with virus vectors have led to the development of non-viral methods (Abdallah et al., 1995; Glover et al., 2005; Niidome and Huang, 2002). These techniques are categorized into two general groups. Naked DNA delivery by a physical method, including electroporation, gene gun and ultrasound employ a physical force that permeates the cell membrane and facilitates intracellular gene transfer (Heller et al., 2005; Lawrie et al., 2000; Liu et al., 1999; Neumann et al., 1982; Wolff et al., 1990; Yang et al., 1990; Yang and Sun, 1995; Zhang et al., 1999). The chemical approaches use synthetic or naturally occurring compounds such as cationic polymer and lipid as carriers to deliver the transgene into cells (Liu et al., 2003; Neu et al., 2005; Niidome and Huang, 2002). Advantages of non-viral systems include their reduced immunogenicity, unlimited size of therapeutic expression cassette and improved safety profiles (Li and Huang, 2000). In addition, non-viral vectors are easier and less expensive to manufacture (Nabel et al., 1993; Stewart et al., 1992). However, non-viral approaches have been suffering from inefficient delivery resulting

in transient transgene expression (Dass, 2004). In addition, vitreous humour has been shown to limit nonviral gene delivery to RPE cells substantially *in vitro* (Peeters et al., 2005; Pitkanen et al., 2003).

2.5.2.1 Plasmids

Simple injection of plasmid DNA directly into a tissue without additional help from either a chemical agent or a physical force is able to transfect cells (Li and Huang, 2000). Specific or nonspecific receptors on the cell surface that bind and internalize DNA have been implicated as a mechanism (Gao et al., 2007). However, owing to rapid degradation by nucleases in the serum and clearance by the mononuclear phagocyte system, the expression level and the area after injection of naked DNA are generally limited (Li and Ma, 2001). Following intravitreous injection of naked plasmid in mice, expression of the reporter gene was observed in retinal ganglion cells for only one day (Hangai et al., 1998a). However, subretinal injection of a reporter gene encoding plasmid did not lead to gene expression (Kachi et al., 2005).

2.5.2.2 Physical methods

Various physical manipulations have been used to improve the efficiency of non-viral gene transfer. The transduction efficiency of non-viral vectors in the retina can be substantially improved by adjunctive electroporation. Electroporation is the use of electric fields to facilitate the penetration of macromolecules into cells; it is based upon the observation that electric fields can alter the structure and permeability of cell membranes (Coster, 1965). Following intravitreal injection of plasmid DNA electroporation results in short-lived but efficient transduction of retinal ganglion cells (Ishikawa et al., 2005). Electroporation of plasmid DNA delivered to the subretinal space in neonatal rodents leads to efficient reporter gene expression in photoreceptors, bipolar cells and Müller cells that is sustained for at least 50 days (Matsuda and Cepko, 2004). Electroporation can be associated with variable adverse effects on normal ocular structures resulting in inflammation, cataract, retinal degeneration and phthysis (Bainbridge et al., 2006).

Gene gun technique consists of "bombarding" a tissue with gold or tungsten bullets covered with DNA. It is potentially applicable for the treatment of ocular surface diseases and particularly for corneal diseases (Bloquel et al., 2006). Ultrasound can alter the permeability of plasma membrane transiently and thereby facilitate DNA uptake (Bloquel et al., 2006). A number of groups have demonstrated the utility of these various modalities of ultrasound in enhancing the delivery of plasmid DNA (Chen et al., 2003; Danialou et al., 2002; Sonoda et al., 2006; Taniyama et al., 2002).

2.5.2.3 Lipoplex

Lipofection reagents are molecules composed of phospholipids that contain both hydrophobic and hydrophilic domains. These reagents form complexes with DNA in a physicochemical manner by electrostatic interactions between the positively charged (cationic) lipid and the negatively charged DNA (Felgner and Ringold, 1989). The DNA condenses with the lipofection reagent in a non-enzymatic fashion to form lipid/ DNA complexes that can be used to deliver foreign DNA to cells *in vitro* and *in vivo* (Bebok et al., 1996; Caplen et al., 1995; Chaum et al., 2000; Chaum, 2001; Felgner et al., 1987; Gershon et al., 1993; Kukowska-Latallo et al., 1996; Masuda et al., 1996). Virus-coated liposomes have also been used to transfer genes to the anterior segment, retina, and choroidal neovascular tissue (Hangai et al., 1996; Hangai et al., 1998b; Otsuji et al., 2000). The lipid/DNA complexes enter the cell by endocytosis (Wrobel and Collins, 1995). After endocytosis, much of the DNA is degraded by fusion of the endosome with lysosomes (Friend et al., 1996). However, some of the DNA is released into the cytoplasm from the endosomes and makes its way to the nucleus

probably by a concentration-dependent manner (Friend et al., 1996; Xu and Szoka, 1996). Active transport of DNA to the nucleus following lipofection has not been demonstrated (Chaum and Hatton, 2002). Disadvantages of lipofection include poor target selectivity, reduced efficiency compared to viral vectors, and short duration of expression. Transient transgene expression results from the episomal status of the plasmid vector after it reaches the nucleus, with low frequency of integration into the host genome. However, a small percentage of transfected retinal cells do show transduction with sustained transgene expression *in vitro* (Chaum et al., 2000).

2.5.2.4 Transposons

Transposable elements are non-viral gene delivery vehicles found ubiquitously in nature. Transposon-based vectors have the capacity of stable genomic integration and long-lasting expression of transgene constructs in cells. Transposons are discrete segments of DNA that have the distinctive ability to move from one genetic location to another in a genome (Ivics and Izsvak, 2006). Transposon integration into chromosomes provides the basis for long term transgene expression in transgenic cells and organisms. The only known, naturally occurring, active transposable element of vertebrate origin is the Tol2 transposon isolated from the medakafish (Koga et al., 1996). In addition, Tc1/*mariner*-type, active elements from transposon fossils found in fish genome were reconstructed and named Sleeping Beauty and Frog Prince (Ivics et al., 1997; Miskey et al., 2003). Sleeping Beauty transposition is efficient in cells of different vertebrate classes in tissue culture (Huang et al., 2006; Izsvák et al., 2000). Transposons have not been used in ocular gene therapy.

Table 2. Vectors in gene therapy.

Vector	Advantages	Disadvantages	Gene expression after	
			Intravitreal injection	**Subretinal injection**
Plasmid DNA and complexes	Easy to produce Safe	Low transduction efficiency Transient expression	Ganglion cells	No gene expression
Adeno-virus	Easy to produce High transduction efficiency Broad host spectrum	Immunogenic Transient expression Repeated injections impossible	Corneal endothelium Trabecular meshwork Ciliary epithelium Ganglion cells	Occasional Müller cells RPE
AAV	Broad host spectrum Long expression Not associated with human diseases	Difficult to produce Limited DNA capacity	Trabecular meshwork Ganglion cells Müller cells	RPE Photoreceptors
Baculo-virus	Easy to produce Not associated with human diseases High DNA capacity	Inactivation by complement fractions Moderate transduction efficacy Transient expression	Corneal endothelium Lens Photoreceptor cells RPE	RPE
Lentivirus	Broad host spectrum Long expression Random integration of DNA	Difficult to produce Low transduction efficiency Limited DNA capacity	No gene expression	RPE Photoreceptors

2.6 ANTIANGIOGENIC AGENTS FOR OCULAR NEOVASCULARIZATION

2.6.1 PEDF

One of the most studied proteins that has been shown to inhibit ocular neovascularization is pigment epithelium derived factor (PEDF). It was first isolated from cultured RPE cells as a neurotrophic factor (Tombran-Tink et al., 1991). PEDF is a 50 kDa glycoprotein which belongs to the serine protease inhibitor (serpin) family in the plasminogen system, but it lacks the protease inhibitor activity normally seen in serpins (Becerra et al., 1995; Steele et al., 1993). PEDF has been shown to inhibit the migration of ECs *in vitro* in a dose-dependent manner more effectively than angiostatin or endostatin (Dawson et al., 1999). It also promotes survival of cultured neurons and protects photoreceptors from the effects of excessive light exposure (Araki et al., 1998; Bilak et al., 1999; Cao et al., 2001; Steele et al., 1993). In the eye, PEDF is normally found in high concentrations in the vitreous humour, the lens and the cornea and it could be partly responsible for the avascularity of these tissues (Dawson et al., 1999; Wu and Becerra, 1996). PEDF is produced by the RPE, cornea and ciliary epithelium (Tombran-Tink et al., 1995). Oxidative stress is known to induce a dose-dependent reduction in the expression of PEDF (Cao et al., 1999). The vitreous concentration of PEDF declines with increasing age and is more marked in patients with AMD (Holekamp et al., 2002). It is not clear whether this is due to decreased synthesis or increased proteolytic activity in the vitreous (Wu and Becerra, 1996). In addition, the levels of intraocular PEDF have been shown to decrease with advancing stages of diabetic retinopathy (Boehm et al., 2003; Ogata et al., 2001a; Ogata et al., 2002; Spranger et al., 2001). Systemic delivery of recombinant PEDF inhibited ischemia-induced retinopathy in animal models (Duh et al., 2002; Stellmach et al., 2001). Subretinal transplanation of autologous iris pigment epithelial cells expressing PEDF inhibited laser-induced CNV in rats (Semkova et al., 2002). In the rat model of ischemia-induced retinopathy, an increased VEGF/PEDF ratio correlated with the presence of retinal neovascularization (Gao et al., 2001). Intraocular AAV-mediated gene transfer of PEDF has also been shown to inhibit retinal and choroidal neovascularization (Auricchio et al., 2002; Mori et al., 2002d; Raisler et al., 2002). Furthermore, intravitreous or subretinal injection of adenoviral vector expressing human PEDF (AdPEDF) suppressed the development of retinal or choroidal neovascularization and also caused regression of established neovascularization (Mori et al., 2001b; Mori et al., 2002b). Injection of AdPEDF beneath the conjunctiva along the outer border of the sclera resulted in transduction of episcleral cells that produced PEDF on the outside of the eye (Gehlbach et al., 2003a). The PEDF penetrated the sclera resulting in high levels in the choroid that caused regression of CNV. Subconjunctival injection of AdPEDF also inhibited CNV in pigs (Saishin et al., 2003b). Also AdPEDF phase I clinical trial in patients with advanced CNV due to AMD have been done. In this study, there were no serious adverse events and a significant proportion of the patients had an improvement in lesion size from baseline (Campochiaro et al., 2006).

2.6.2 sFlt-1

Soluble (s)Flt-1 is a naturally occurring protein antagonist of VEGF formed by alternative splicing of the pre-mRNA for the full length VEGFR-1 (He et al., 1999; Kendall et al., 1996). The angiostatic activity of sFlt-1 results from inhibition of VEGF by two mechanisms. It causes both sequestration of VEGF to which it binds with high affinity and forms inactive heterodimers with VEGFR-1 and VEGFR-2 (Kendall et al., 1996). It is not clear whether sFlt-1 has a role in normal eyes, but several studies have tested the effect of overexpression of sFlt-1 in ocular neovascularization models (Gehlbach et al., 2003b; Honda et al., 2000; Lai et al.,

2001; Rota et al., 2004). Inhibition of VEGF by repeated intravitreal injections of recombinant sFlt-1 chimeric proteins and antisense oligodeoxynucleotides have been shown to reduce retinal neovascularisation in OIR mouse model (Aiello et al., 1995a; Robinson et al., 1996). Intraocular injection of AdsFlt-1 suppressed retinal or choroidal neovascularization (Bainbridge et al., 2002; Honda et al., 2000; Rota et al., 2004). Periocular injection of AdsFlt-1 resulted in transduction of episcleral cells, penetration of the sclera and high levels of AdsFlt-1 in the choroid, which markedly suppressed CNV (Gehlbach et al., 2003b). Long-term suppression of CNV was achieved with intraocular injection of AAVsFlt-1 in mice and monkeys (Bainbridge et al., 2002; Lai et al., 2005; Lai et al., 2002).

2.6.3 RNA interference

The production of growth hormones can be inhibited by RNA interference. It is a natural mechanism to inhibit the intracellular production by silencing gene coding for a specific protein (Elbashir et al., 2001). Small interfering RNA (siRNA) is a double stranded RNA, which consists of 21-22 nucleotides. After being further processed to a RNA-induced silencing complex (RISC) by intracellular enzymes, the fragment binds specifically to messenger RNA (mRNA), causing cleavage and further digestion of the mRNA. The RISC can then bind to other mRNA molecules and the process is repeated multiple times resulting in a very efficient overall inhibition of the production of the targeted protein (Schmidt-Erfurth and Pruente, 2007). Bevasiranib, siRNA targeting VEGF, has been shown to inhibit retinal neovascularization in a mouse model (Reich et al., 2003). In non-human primates the area of laser induced CNV was significantly decreased with intravitreal injection of bevasiranib (Tolentino et al., 2004). In the phase II randomized doubleblinded CARE (Cand5 Anti-VEGF RNAi evaluation) study designed to assess the safety and efficacy of bevasiranib, 129 patients with CNV due to AMD were randomized to receive three different intravitreal doses of bevasiranib at baseline and at 6 weeks. No local or systemic serious adverse events were found. However, there was evidence of continuing deterioration during the first three weeks of treatment. The phase III COBALT study is currently assessing the safety and efficacy of bevasiranib administered every 8 or 12 weeks as maintenance therapy following 3 monthly injections of ranibizumab in 330 patients with AMD.

Intravitreous or periocular injection of AGN211745 (Sirna-027), siRNA directed against VEGFR-1, resulted in significant reductions in the area of neovascularization in mouse models of retinal and choroidal neovascularization (Shen et al., 2006). A phase I dose-escalation study with AGN211745 in 26 patients with AMD showed single intravitreal injection of siRNA-027 to be safe and well tolerated. Visual acuity stabilization was achieved in 92% of patients at 3 months and decreased foveal thickness was seen in some patients. A phase II 24-month single blinded safety and efficacy study is currently in enrolment (Chappelow and Kaiser, 2008).

RTP801i-14 (PF-4523655) is a siRNA designed to inhibit the expression of the hypoxia-inducible gene RTP801. The RTP801 gene is upregulated in response to ischemia, hypoxia and oxidative stress both *in vitro* and *in vivo* (Shoshani et al., 2002). It has been shown to promote neuronal cell apoptosis and the generation of ROS *in vitro* by a mechanism that is independent of growth factors such as VEGF (Ellisen et al., 2002; Shoshani et al., 2002). In both RTP801-knockout and therapeutic mouse and primate models of laser-induced CNV, inhibition of RTP801 expression leads to inhibition or reduction of CNV and vessel leakage more efficiently than anti-VEGF drugs (Brafman et al., 2004; Nozaki et al., 2006). In addition, knock out of RTP801 ameliorates diabetes–induced retinal vascular permeability and ERG abnormalities in diabetic mice (Timothy et al., 2005). Because

it has anti-inflammatory and antiapoptotic properties it may also be useful for the treatment of dry AMD. Results from a Phase I/II trial showed that RTP801i-14 was safe and well tolerated in patients with wet-AMD who failed to respond to currently approved therapies. The phase II prospective, randomized, dose-ranging study is currently evaluating the safety and efficacy of RTP801i14 versus laser therapy in 160 patients with diabetic macular edema (ClinicalTrials, 2009b).

2.6.4 Aflibercept

Aflibercept, VEGF Trap, is a receptor decoy with a higher affinity for VEGF than native VEGF receptors or any of the currently available anti-VEGF drugs. It is a soluble protein, combining ligand–binding elements from the extracellular domains of VEGFR-1 and VEGFR-2 fused to the Fc constant region of immunoglobulin G_1. The molecule binds to all members of VEGF family with high affinity, but does not non-specifically attach to any components of the ECM. It has been shown to penetrate through all retinal layers. In a mouse model with laser induced CNV, aflibercept induced the regression of CNV by 85% within 10 days (Saishin et al., 2003a). Furthermore, the CNV completely disappeared in a large percentage of eyes. Intravitreal administration of aflibercept significantly reduced retinal vascular permeability in diabetic rat model (Cao et al., 2006). Data from a phase I, randomized, double-blind, placebo-controlled, ascending-dose trial of 25 patients with AMD showed a dose-dependent decrease in retinal thickness in patients who received intravenous aflibercept (Nguyen et al., 2006). However, dose-limiting toxicity (hypertension in one patient and proteinuria in another patient) was observed and the study and further clinical development of systemic aflibercept for ocular disease was halted (Nguyen et al., 2006). The safety, tolerability and bioactivity of intravitreal aflibercept for the treatment of neovascular AMD was evaluated in the two-part CLEAR-IT-1 (Clinical Evaluation of Anti-angiogenesis in the Retina Study). In part 1, 21 patients were randomized to receive one of six doses of aflibercept as a single intravitreal injection at baseline, then assessed at 1, 2, 4 and 6 weeks using ETDRS (Early Treatment Diabetic Retinopathy Study) best-corrected visual acuity, FAG and OCT. Aflibercept was well tolerated and there were no adverse events. At 6 weeks visual acuity remained stable or improved in 95% of patients and the total area of CNV decreased 35%. Reduction in central thickness in OCT was also seen (Benz et al., 2007).

A randomized, double-blind phase III trial of aflibercept in approximately 1200 patients with the neovascular form of wet AMD, VIEW 1 (VEGF Trap: Investigation of Efficacy and safety in Wet age-related macular degeneration) study will evaluate the safety and efficacy of intravitreal aflibercept at different doses administered at two different dosing intervals compared with 0.5 mg ranibizumab administered every 4 weeks (ClinicalTrials, 2009a).

Additionally, a phase I trial of aflibercept in five patients with diabetic macular edema has been done. Results indicated that a single 4 mg injection resulted in a marked decrease in mean central retinal thickness and mean macular volume. Aflibercept was well tolerated, and there were no drug-related serious adverse events (Do et al., 2007).

2.6.5 Endostatin

Endostatin is a cleavage product of collagen XVIII that inhibits EC adhesion, migration and proliferation, as well as the induction of apoptosis (O'Reilly et al., 1997). It has been studied most widely in cancer therapy. In the eye, endostatin has been found in the lens epithelium and in the ciliary body. In the retina, endostatin has been found in the inner limiting membrane. It has also been detected in the lens capsule and all border membranes lining the aqueous humor including the anterior surface of the iris. These findings suggest that there are specific endostatin expressing structures forming a barrier

around the anterior chamber and the vitreous, which may prevent ocular blood vessels from sprouting into these avascular compartments (Ohlmann et al., 2005). In collagen XVIII gene knockout mice, deficient endostatin production causes delayed regression of blood vessels in the vitreous and abnormal outgrowth of retinal vessels (Fukai et al., 2002). In addition, the age-dependent vision loss in these mutant mice is associated with pathological accumulation of deposits under the RPE, as seen in early stages of AMD in humans suggesting that endostatin is an important endogenous regulator of vasculature formation and tissue development in the eye (Marneros et al., 2004). Endostatin levels in the vitreous and aqueous humor are decreased in patients with diabetic retinopathy and negatively correlate with the severity of retinopathy and VEGF levels (Funatsu et al., 2001; Noma et al., 2002). Endostatin is also reduced in Bruch's membrane and choriocapillaris basement membrane in AMD subjects compared to aged normal human subjects (Bhutto et al., 2004). Intravenous injection of adenoviral vectors encoding endostatin increased the serum level of endostatin and inhibited laser-induced CNV (Mori et al., 2001a). In OIR mouse model intraocular injection of AAV endostatin inhibited ischemia-induced retinal neovascularization (Auricchio et al., 2002). Injection of endostatin into the eyes of the VEGF transgenic mice demonstrated that endostatin significantly reduced the VEGF-induced retinal vascular hyper-permeability and inhibited retinal neovascularization (Takahashi et al., 2003).

2.6.6 Angiostatin

Angiostatin, a 38-45 kDa internal fragment of plasminogen, is a potent inhibitor of angiogenesis, which selectively inhibits proliferation and induces apoptosis in ECs. It is a cleavage product of fibrinogen that inhibits tumor angiogenesis and downregulates VEGF (O'Reilly et al., 1994). As a naturally occurring peptide, it is not likely to stimulate an immunogenic response (Cao, 2001). In the patients with proliferative diabetic retinopathy, significant elevation of vitreal angiostatin levels and decrease of VEGF concentration in the vitreous was observed in those who had previous laser photocoagulation, suggesting that local release of angiostatin and down-regulation of VEGF mediate the therapeutic effects of retinal photocoagulation in proliferative diabetic retinopathy (Spranger et al., 2000). Intravitreal delivery of AAV encoding angiostatin reduced vascular leakage in a diabetic rat model (Shyong et al., 2007). In OIR mouse model, systemic and intravitreal injections of angiostatin before the appearance of retinal neovascularization resulted in significantly fewer pre-retinal vascular cells, suggesting a preventive effect of angiostatin on the retinal neovessel formation (Drixler et al., 2001; Meneses et al., 2001). Subretinal or intravitreous injection of AAV or lentiviral vector expressing angiostatin has been shown to suppress retinal and choroidal neovascularization (Igarashi et al., 2003; Lai et al., 2001; Raisler et al., 2002). However, in normal neonatal mice, angiostatin does not affect any physiological development of retinal vasculature, indicating no toxicities to normal vasculature with the angiostatin administration (Drixler et al., 2001).

2.6.7 Rapamycin

Rapamycin, also known as sirolimus, targets the protein kinase mammalian target of rapamycin (mTOR), which regulates cell growth and metabolism. In addition to possessing anti-inflammatory, anti-fibrotic and anti-proliferative properties, rapamycin acts as an antiangiogenic agent decreasing VEGF and transforming growth factor-β1 (Guba et al., 2002). Rapamycin also exerts an antiangiogenic effect by downregulating HIF-1α, which effectively decreases VEGF production and inhibits VEGF induced EC proliferation. In preclinical studies, rapamycin inhibited VEGF-induced hyperpermeability in mice and reduced CNV in a murine laser-induced model of CNV

(Dejneka et al., 2004; Kleinman et al., 2007). In the phase I dose escalation study in 30 patients with CNV and 50 patients with diabetic macular edema, single intravitreal injection of rapamycin was well tolerated and functional and anatomical improvements were seen (Blumenkranz et al., 2008; Dugel et al., 2008).

2.7 ANTI-VEGF THERAPY FOR OCULAR NEOVASCULAR DISEASES

VEGF is an attractive target in anti-CNV therapy because of its important role in ocular angiogenesis. Anti-VEGF treatments of neovascular AMD and other ocular neovascular diseases have beneficial effects on disease progression. However, intravitreal administration of current treatment molecules has also several risks including endophthalmitis, retinal detachment, cataract and uveitis. In patients treated for up to 2 years with pegaptanib the drug was well tolerated in the eye. Endophthalmitis occurred at a rate of 0.16% per injection, retinal detachment at 0.08% per injection, and traumatic cataract 0.07% per injection during the first year of study (D'Amico, 2006). Aseptic technique, including periocular and ocular surface preparation, lid speculum, and drape were found to be essential (D'Amico, 2006). With improved aseptic techniques, the incidence of endophthalmitis was decreased to 0.10% per injection within the second year of the study.
Intravenous bevacizumab in cancer therapy has shown systemic side-effects including hypertension, increased rate of thromboembolic events, gastrointestinal perforations, myocardial infarctions, and death (Hurwitz et al., 2004; Miller et al., 2005). Intravitreal injections lead to detectable but significantly lower serum levels of ranibizumab and pegaptanib than with intravenous administration (Gaudreault et al., 2005; Siddiqui and Keating, 2005). There was no evidence of an increase in deaths, hypertension or thromboembolic events in the 2-year safety data from the VISION trial (D'Amico, 2006). In the first year of the ANCHOR and MARINA trials the risk of myocardial infarction and stroke was slightly higher in the 0.5 mg ranibizumab compared to the control group.

Current anti-VEGF drugs have a relatively short half-live leading to repeated administrations (Cao, 2001). The long term effects of these multiple injections on the structure and function of eye are still largely unknown. The risk of complications is anyway increased with the number of injections, and safer and more sustained regimens need to be developed for intraocular antiangiogenic therapy.

2.7.1 Pegaptanib

Pegaptanib is a pegylated ribonucleic acid oligonucleotide aptamer approved as the first antiangiogenic aptamer for use of wet AMD by the FDA in 12/2004 followed by an approval by the EMEA in 1/2006. Aptamers are oligonucleotides designed to bind to specific molecules based on their three-dimensional structure. Aptamers rarely evoke immune responses (Eyetech Study Group, 2002). Pegaptanib binds specifically to VEGF-A_{165} and inhibits angiogenesis and pathologic leakage (Moshfeghi and Puliafito, 2005). The recommended dose of pegabtanib is 0.3 mg and the drug is administred intravitreally every six weeks as the molecule is rapidly degraded enzymatically by intraocular nucleases (Schmidt-Erfurth and Pruente, 2007). The VISION trial, designed as two parallel phase III double-masked, sham-controlled, dose-ranging studies included a total of 1186 AMD patients. Intravitreal injections were performed on a fixed regular schedule with six weeks intervals. After one year, 70% of pegabtanib treated eyes versus 55% of control eyes lost less than 15 letters in visual acuity (Gragoudas et al., 2004). At two years, 59% of eyes treated with a dose of 0.3 mg pegabtanib lost less than 15 letters compared to 45% of standard care treated eyes (D'Amico, 2006). Patients discontinuing pegabtanib treatment after the first year

experienced an additional mean loss of 15 letters in 27% of eyes. These results suggest that pegabtanib injections need to be continued over a period of at least two years in order to maintain a small benefit consisting of a mean loss of two lines.

Pegaptanib has also been studied in diabetic retinopathy. Safety and efficacy of pegaptanib were assessed in a randomized, sham-controlled, double-masked Phase II trial enrolling 172 diabetic subjects with diabetic macular edema. Intravitreous injections were administered every six weeks. At Week 36, 0.3 mg pegaptanib was significantly superior to sham injections. 34% of patients gained ≥10 letters and also change in mean central retinal thickness decreased significantly (Macugen Diabetic Retinopathy Study Group, 2005). In addition, a retrospective subgroup analysis revealed that pegaptanib treatment led to the regression of baseline retinal neovascularization in 8 of 13 patients with proliferative diabetic retinopathy (Macugen Diabetic Retinopathy Study Group, 2006).

2.7.2 Ranibizumab

Ranibizumab is a recombinant humanized fab fragment derived from a monoclonal antibody. It was approved by the FDA for the treatment of all lesion types in neovascular AMD at a dose of 0.5 mg in 7/2006 and by the EMEA in the first quarter of 2007. It has a rather small molecular size of 48 kDa which ables the drug to penetrate the inner limiting membrane and reach the subretinal space when injected intravitreally. It binds all biologically active isotypes of VEGF with high affinity (Chen et al., 1999). Intravitreal injections of ranibizumab prevented formation of CNV in animal models, and decreased leakage of already formed CNV with no significant toxic effects (Husain et al., 2005; Krzystolik et al., 2002). The half-life of ranibizumab is 2–4 days, resulting in a rapid systemic clearance and high systemic safety. A randomized, sham-controlled phase III MARINA study included 716 patients with minimally classic or occult CNV. Patients were randomized into two treatment and one sham group. Injections were given in monthly intervals over 24 months. At the 12 month visit, 95% of treated eyes had maintained stable vision within three lines compared to 62% of control eyes. After 24 months, 90% of eyes in the 0.5 mg group versus 53% in the control group demonstrated stable vision (Rosenfeld et al., 2006). The ANCHOR study was a prospective, randomized phase III trial including 423 patients with predominantly classic CNV. Fixed monthly injections of 0.3 or 0.5 mg ranibizumab were compared to the photodynamic therapy (PDT). At one year, 96% of all eyes treated with 0.5 mg ranibizumab had lost less than three lines versus 64% of PDT-treated eyes (Brown et al., 2006). The PIER study included 182 patients with all lesion subtypes of CNV. The aim of this study was to evaluate the efficacy and safety of ranibizumab initially administered monthly for three injections followed by a fixed regimen of re-treatments in three month intervals. Overall, patients treated with ranibizumab remained stable at baseline visual acuity for 12 months. However, the proportion of gainers with 3 lines was only 13% compared to 34% of three line gainers in MARINA and 40% in ANCHOR. For the majority of eyes, the fixed quarterly regimen was not sufficient and recurrence was not treated adequately. This observation highlights the importance of an individualized re-treatment regimen based on an individualized diagnostic monitoring (Schmidt-Erfurth and Pruente, 2007).

2.7.3 Bevacizumab

Bevacizumab is a full length recombinant, humanized antibody of a molecular weight of 149-kDa which binds to all VEGF isoforms. Like ranibizumab, the drug reduces angiogenesis and vascular permeability. The drug was originally developed to target pathologic angiogenesis in tumors and was approved by the FDA for the treatment of metastatic colorectal cancer. Due to its substantially larger molecular weight, local

and systemic clearance of bevacizumab may be delayed, resulting in an extended durability of the treatment, but associated with higher systemic toxicity (Schmidt-Erfurth and Pruente, 2007). Experimental studies showed no toxic effect of a 2.5 mg dose to the retina in a rabbit model, however, toxic effects were found following the application of a dose of 5 mg (Manzano et al., 2006). Using immunohistochemistry, full thickness penetration following intravitreal injection of 500 µg bevacizumab was demonstrated in the rabbit retina (Shahar et al., 2006). In a phase I SANA study, 15 AMD patients were given bevacizumab intravenously in 2-week intervals. This resulted in a significant improvement in visual acuity, OCT and angiographic outcomes at a follow-up of 12 weeks (Michels et al., 2005). A mild elevation in systolic blood pressure was seen as a systemic adverse event, which was reportedly controlled by anti-hypertensive medication. Promising results have been reported from a case series including 266 AMD patients treated with intravitreal injections of bevacizumab at a dose of 1.25 mg. Within three months, improvement in function and visual acuity was seen in 38% of treated patients and the mean central retinal thickness decreased significantly (Spaide et al., 2006). Also improvement in the multifocal-ERG responses consistent with photoreceptor recovery was seen (Shahar et al., 2006). Small studies with intravitreal bevacizumab have also been done in diabetic patients (Avery et al., 2006; Spaide and Fisher, 2006). Intravitreal bevacizumab was given in monthly intervals and functional benefit was seen with mean visual acuity improvement after two months (Avery et al., 2006). Also other neovascular diseases have been treated with bevacizumab with positive effects (Avery, 2006; Iturralde et al., 2006; Spaide and Fisher, 2006). Bevacizumab appears to have a beneficial effect in the off-label treatment of intraocular neovascularization at least based on retrospective case series.

3 AIMS OF THE STUDY

The aim of the study was to determine factors involved in the pathogenesis of proliferative diabetic retinopathy and retinal neovascularization. Furthermore, the differences between type 1 and type 2 diabetics were studied. From the basis of these results, we evaluated the effects of overexpression of vascular endothelial growth factors -A and –D in the rabbit eye. In addition, we evaluated adenovirus and baculovirus vectors for their efficacy, safety and distribution in ocular gene therapy in order to develop more sustain therapies for ocular neovascular diseases. Finally, ocular phenotype of IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice was studied.

The following questions were addressed:

-Which factors are involved in proliferative diabetic retinopathy? Are there differences between type 1 and type 2 diabetic patients? (I)

-What are the effects of VEGF-A$_{165}$ and VEGF-D$^{\Delta N\Delta C}$ in ocular tissues? (II-III)

-What is the efficacy, safety and distribution of adenoviral and baculoviral vector after intravitreal gene transfer? (II-III)

-What is the ocular phenotype of diabetic IGF-II/LDL$^{-/-}$ApoB$^{100/100}$mice? (IV)

4 MATERIALS AND METHODS

The following tables and figure contain the summary of the materials and methods used in this study. The materials and methods have been described in details in the original publications (I-III) and in the manuscript (IV).

4.1 Human samples

Table 3. Clinical characteristics of the patients.

	Dm type 1	Dm type 2	Controls
Number of patients	13	17	7
Age (years)	34.7±8.8	60.4±8.4	71.6±4.5
Duration of diabetes (years)	24.2±5.9	17.5±8.4	-
Gender (male/female)	5/3 62%/38%	6/4 53%/47%	4/3 57%/43%
GHbA1c (%)	10.2±1.9%	9.1±1.9%	-

The study was approved by the Ethical Committee of Kuopio University Hospital, Kuopio, Finland and the study was in accordance with the principles outlined in the Declaration of Helsinki.

Both vitreous samples and neovascular tissues were collected from vitreoretinal surgeries. Vitreous humour was injected into a manually adjusted 2 ml aspiration syringe with infusion disconnected at the beginning of the pars plana vitrectomy. The sample was moved into a Eppendorf tube (Eppendorf Nordic, Horsholm, Denmark), and immediately stored in liquid nitrogen.

Later during the surgical procedure, a fibrovascular membrane was isolated from the surrounding vitreous and from the optic nerve head or retina, grasped with vitreous forceps and pulled out through a sclerotomy. The sample was placed in a 2.5 ml polypropylene tube (Meka Mini, Mekalasi Oy, Helsinki, Finland) in 4% PFA-sucrose, kept 1 h per 0.5 cm of neovascular tissue and embedded in paraffin for later immunohistochemical analyses.

Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study.

4.2 Antibodies

Table 4. Antibodies used in this study.

Antibody	Specificity	Code/clone	Species	Dilution	Distributor	Used
CD-31	EC	JC/70A	mAb mouse anti-human	1:50	DAKO	I-III
PECAM-1		MEK13.3	mAb rat anti-mouse	1:50	BD	IV
CD-68	Macrophages	KP1	mAb mouse anti-human	1:50	DAKO	I
RAM11		RAM11	mAb mouse anti-rabbit	1:200	DAKO	II-III
mMQ		AIA31240	pAb rabbit anti-mouse	1:5000	Accurate	IV
VEGF-A	VEGF-A	sc-7269	mAb mouse anti-human	1:200	Santa Cruz	I-III
VEGF-B	VEGF-B	sc-1878	pAb goat anti-human	1:200	Santa Cruz	I
VEGF-C	VEGF-C	sc-1881	pAb goat anti-human	1:200	Santa Cruz	I
VEGF-D	VEGF-D	78923.11	mAb mouse anti-human	1:500	R&D	I,III
PDGF-B	PDGF-B		mAb mouse anti-human	1:100	R&D	I
PlGF	PlGF	sc-1880	pAb goat anti-human	1:200	Santa Cruz	I
anti-β-gal	LacZ	Z378	mAb mouse anti-β-gal	1:200	Promega	II-III
Ki-67	Proliferating cells	7B11	mAb mouse anti-human	1:100	Zymed	I
HIF-1α	Hypoxia inducible factor α	H1α67	mAb mouse anti-human	1:100	Neomarkers	I
NFκB	Nuclear factor κB	20	mAb mouse anti-human	1:250	BD	I
αSMA	α-smooth muscle actin	1A4	mAb mouse anti-human	1:200	Sigma	I-III
ANG-1	Angiopoietin 1	sc-6319	pAb goat anti-human	1:100	Santa Cruz	I
ANG-2	Angiopoietin 2	sc-7015	pAb goat anti-human	1:100	Santa Cruz	I
Flt-1	VEGFR-1	sc-316	pAb rabbit anti-human	1:200	Santa Cruz	I
KDR	VEGFR-2	sc-6251	mAb mouse anti-human	1:200	Santa Cruz	I
Flt-4	VEGFR-3	sc-321	pAb rabbit anti-human	1:200	Santa Cruz	I
Tie-1	Angiopoietin receptor 1	88016	mAb mouse anti-human	1:25	R&D	I
Tie-2	Angiopoietin receptor 2	83715	mAb mouse anti-human	1:25	R&D	I
BrDU	Proliferating cells	Bu20a	mAb mouse anti-human	1:100	DAKO	II-III
Caspase-3	Caspase-3	7481	pAb rabbit anti-human	1:250	Promega	IV
Calbindin	Calbindin D-28k	CB38-a	pAb rabbit anti-rat	1:1000	SWANT	IV
Calretinin	Calretinin	7699/4	pAb rabbit anti-human	1:1000	SWANT	IV
anti-Rhodopsin	Rhodopsin	9279	pAb rabbit anti-rhodopsin	1:500	Chemicon	IV

4.3 Gene transfer techniques

Table 5. Animal models and viruses.

Animal	n	Vector	Promoter	Transgenes	Used in
New Zealand White rabbit	43	Adeno serotype 5 1st generation	CMV	hVEGF-A_{165}	II
				hVEGF-$D^{\Delta N\Delta C}$	III
				KDR	II
				LacZ	II,III
	23	Baculo boost FGII-B2		hVEGF-$D^{\Delta N\Delta C}$	III
				GFP	
				LacZ	

Animal		Diet, n		IV
		Normal chow diet (R36, Lactamin, Sweden) for the whole time	Western diet (TD 88173, Harlan Teklad: 42 % of calories from fat and 0.15 % from cholesterol, no sodium cholate) for the last three months	
IGF-II/$LDLR^{-/-}ApoB^{100/100}$ mouse	12	7	5	
$LDLR^{-/-}ApoB^{100/100}$ mouse	11	6	5	

All animal procedures were approved by Institutional Animal Care and Use Committee of the University of Kuopio, Kuopio, Finland.

Replication-deficient human clinical grade first generation adenoviruses (serotype 5) encoding transgenes under CMV promoter were constructed by homologous recombination, and produced in 293 cells (Laitinen et al., 1998; Rissanen et al., 2003). Adenoviruses were analyzed to be free of endotoxin and microbiological contaminants (Hedman et al., 2003).

Recombinant viruses encoding transgenes under CMV promoter were constructed using pFASTBac1-plasmid (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and a Bac-To-Bac Baculovirus Expression (Gibco BRL) system (Airenne et al., 2000). Baculoviruses were titered on Sf9 insect cells. Virus preparations were tested for sterility and analyzed for the absence of endotoxin and mycoplasma contaminations. In order to demonstrate that the virus preparations contained equal amounts of viral particles per volume, purified viruses (107 pfu/lane) were subjected to immunoblotting using mouse anti-gp64 (Bioscience, San Jose, CA, USA) as a primary antibody and goat anti-mouse IgG-AP as a secondary antibody (Biorad Hercules, CA, USA).

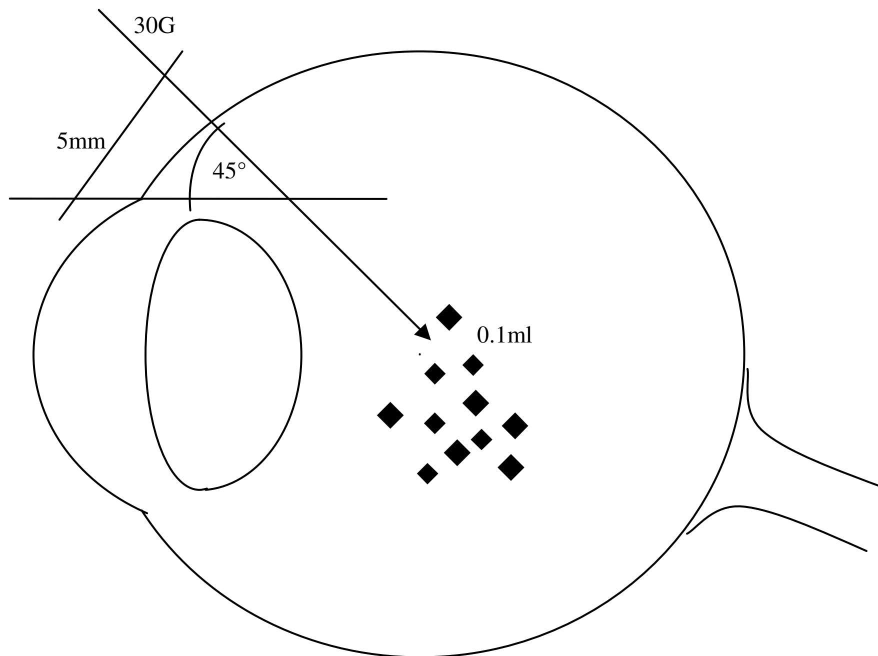

Figure 10. Illustration of the gene transfer technique used in studies II-III.

For the gene transfer, animals were anesthetized with subcutaneous injections of ketamin hydrochloride 0.3ml/kg (Ketalar, Pfizer, Espoo, Finland) and medetomidin hydrochloride 0.3ml/kg (Domitor, Espoo, Finland). Intravitreal injections were performed through pars plana 5mm from the limbus in the temporal side of the eye with a 30G needle (BD Microlance, Drogheda, Ireland). The site of the needle tip was ensured with a microscope and the solution was injected into the vitreous humour. Oxybuprocain (Obucain, Santen, Tampere, Finland) was used for topical anesthesia. Animals were given cefuroxim 125mg (Zinacef, GlaxoSmithKline, Research Triangle Park, NC, USA) intramuscularly to avoid postoperative infections.

4.4 In vivo methods

Table 6. In vivo methods used in this study.

Method		Description	Distributor	Used
Anesthesia	Ketamin hydrochloride 0.3ml/kg (Ketalar)	Intramuscular/ subcutaneous injection	Pfizer, Espoo, Finland	II-IV
	Medetomidin hydrochloride 0.3ml/kg (Domitor)	Intramuscular injection	Orion, Espoo, Finland	II-III
	Xylazine 10 mg/kg (Rompun)	Subcutaneous injection	Bayer	IV
Topical drugs	oxybuprocain (Obucain)	Topical anesthesia	Santen, Tampere, Finland	II-IV
	Tropicamid 5mg/ml and Phenylephrine hydrochloride 100mg/ml	Mydriasis		II-IV
Photographs		Anterior and posterior parts of the eye	Zeiss, FF450 PLUS IR, Jena, Germany; Canon, CF-60UVi, Latham & Phillips Ophthalmic Products, Grove City, Ohio, USA	II-III
			Nikon D70s, Nikon Corp., Tokyo, Japan	IV
FAG	0.3 ml of Fluorescein Sodium (AK-FLUOR 10%)	Intravenous injection, evaluation of the fundus in 2min time period	Akorn, Buffalo Grove, IL, USA	III
Biomicroscopy		Evaluation of of the eye	Haag-Streit, Bern, Switzerland	II-IV
Metabolic analyses	Blood glucose	Overnight fasting plasma samples	Glucometer Elite analyzer , Bayer	IV
	Plasma insulin levels		Rat/mouse Insulin ELISA Kit, Linco Research Inc.	
	Triglycerides		Ecoline S+, GPO-PAP method, Diagnostic Systems	
	Total cholesterol		Ecoline 25, CHOD-PAP method, Merck Diagnostica	

4.5 Analytical methods

Table 7. Analytical methods used in this study.

Method	Description				Distributor	Used
Tissue processing	Fixation		Embedding	Sectioning		I-IV
	Paraffin blocks	4% PFA for 5 h	paraffin	4-7 µm sections		
	Frozen sections	x-gal fix	33% optimal cutting temperature (OCT) compound		Sakura Finetek, Zoeterwoude, the Netherlands	II-III
	Flat mount	4% PFA for 1 h				IV
	Liquid nitrogen	Vitreous samples (0.3–0.4 mL) from each eye with a 1 mL syringe and a 24 G needle				I-III
Immunology	Immunohisto-chemistry	Paraffin-embedded samples				I-IV
	ELISA, Quantikine	Assays with vitreous samples	R&D Systems, Minneapolis, MN, USA			I-III
Histological analyses	Olympus AX70	Photographs and analyses of the histological sections	Olympus Optical, Tokyo, Japan			I-IV
	AnalySIS		GmBH, Munster, Germany			
	PhotoShop		Adobe, San Jose, CA, USA			
Other methods	Fluorescence microscopy for GFP expression		ECLIPSE E600, Nikon, Japan			III
	Retina flat mounts	4% PFA for 10 min. The cornea, lens, sclera, and vitreous excised and the retina isolated. Retina flat mounts fixed with 4% PFA for 1 hour	Confocal microscopy	Olympus IX70, Perkin Elmer		IV
			Fluorescence conjugated isolectin	GS lectin, I-21413; Molecular Probes		
Statistical analyses	mean± SEM or SD					I-IV
	one-way ANOVA					
	independent samples t-test					
	Kruskal-Wallis test					
	Mann-Whitney U-test					
	Spearman's correlation analysis					I

5 RESULTS

5.1 Angiogenic factors in vitreous humour and neovascular samples in type 1 and type 2 diabetic retinopathy

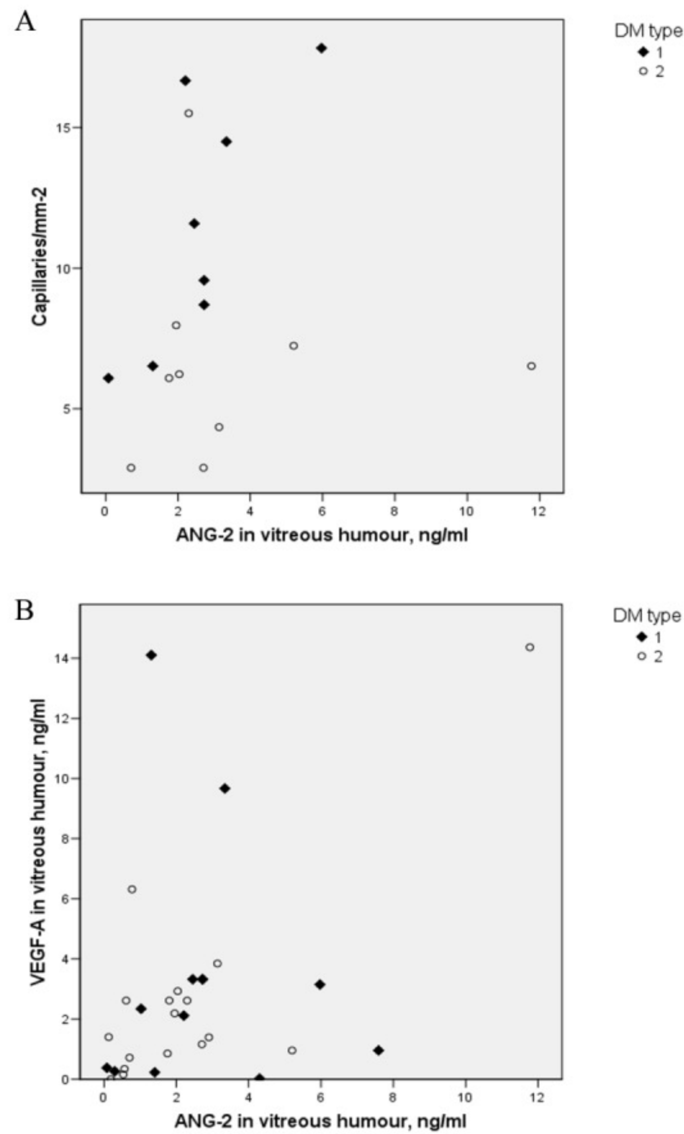

Figure 11. Scatter Plot analysis of type 1 and type 2 diabetic patients with proliferative retinopathy. (A) Correlation between the number of CD-31 positive endothelial cells in the tissue/mm^2 and ANG-2 in vitreous humour ($p<0.05$). (B) Correlation between ANG-2 and VEGF-A in vitreous humour ($p<0.01$).

VEGF-A, ANG-1 and ANG-2 were found in the vitreous samples in both types of diabetics but there was no difference between type 1 and type 2 diabetics in ELISA analysis in the amounts of measured growth factors. In addition, ANG-1 was found in much lower levels than VEGF-A or ANG-2.
The number of capillaries correlated positively with the amount of ANG-2 in the vitreous humour in type 1 ($p < 0.05$, $r = 0.77$) but not with type 2 diabetics. ANG-2 and VEGF-A in the vitreous humour correlated positively ($p < 0.01$, $r = 0.59$) with type 2 diabetics (Figure 11).

Numerous CD31-positive ECs and several CD68 positive macrophages were present in all neovascular samples. In type 1 diabetics, the number of ECs in fibrovascular tufts was significantly higher than in type 2 diabetics ($p < 0.05$). α-SMA positive pericytes were found in the neovascular tufts in the same areas as ECs, but the number of ECs was higher than activated pericytes in the tissues.

Transcription factor NFκB, which mediates inflammatory responses, was also present in the nuclei in the samples of all patients indicating an ongoing inflammatory process in the neovascular tissues. A positive staining for Ki-67 indicates a proliferative process in the tufts in all patients. Almost every sample had HIF-1α stained nuclei reflecting hypoxic conditions in the eye. There were no differences in inflammatory response, number of proliferating cells, immunostaining for HIF-1α or α-SMA stainings between type 1 and type 2 diabetics.

Duration of diabetes correlated positively with the age of the patient ($p < 0.05$) but had no effect on the number of ECs neither in type 1 nor type 2 diabetics. The number of ECs and age had a significant negative correlation ($p < 0.05$, $r = -0.74$) in type 1 diabetics. GHbA1c levels ($p < 0.01$, $r = 0.58$) had a positive correlation with the number of ECs in type 1 but not in type 2 diabetics (Figure 12).

All studied growth factors were found in the endothelial cells in neovascular tufts. There were staining differences in the presence of growth factors in the neovascular tufts between type 1 and type 2 diabetics. In type 1 diabetics, VEGF-A was most strongly present whereas over 50% of type 2 diabetics had either equal or more intense staining for VEGF-D than VEGF-A. ANG-2 were less abundant than VEGF-A or VEGF-D, but more abundant than the other measured factors. The overall order of the growth factors was **VEGF-A > ANG-2 > VEGF-D, ANG-1 > VEGF-C > VEGF-B, PlGF > PDGF-B** in type 1 diabetics and **VEGF-D, VEGF-A > ANG-2 > ANG-1 > PLGF > VEGF-B > VEGF-C > PDGF-B** in type 2 diabetics.

In type 1 diabetics, VEGF-C was more abundant than VEGF-B, and in type 2 diabetics, the situation was opposite. The presence of all VEGF receptors was significantly higher in type 1 diabetics than in type 2 diabetics ($p < 0.05$) (Figure 13). In addition, VEGFR-1 and VEGFR-3 were stronger than VEGFR-2 particularly in type 2 diabetics. Presence and location of PDGF-B, ANG-1, ANG-2, Tie-1 and Tie-2 in type 1 diabetics were similar to type 2 diabetics. ANG-1 and ANG-2 and their receptors Tie-1 and Tie-2 were mostly present in the same areas as pericytes. Immunostaining for PDGF-B was less abundant than VEGFs. VEGF-A and VEGFR-1 were also present in the control samples but much less abundantly than in the diabetic tissues. ECs and α-SMA positive pericytes were also present in non-diabetic eyes but in much less numbers than in diabetic fibrovascular tissues. There were no NFκB, Ki-67 or HIF-1α positive cells in the control retinas.

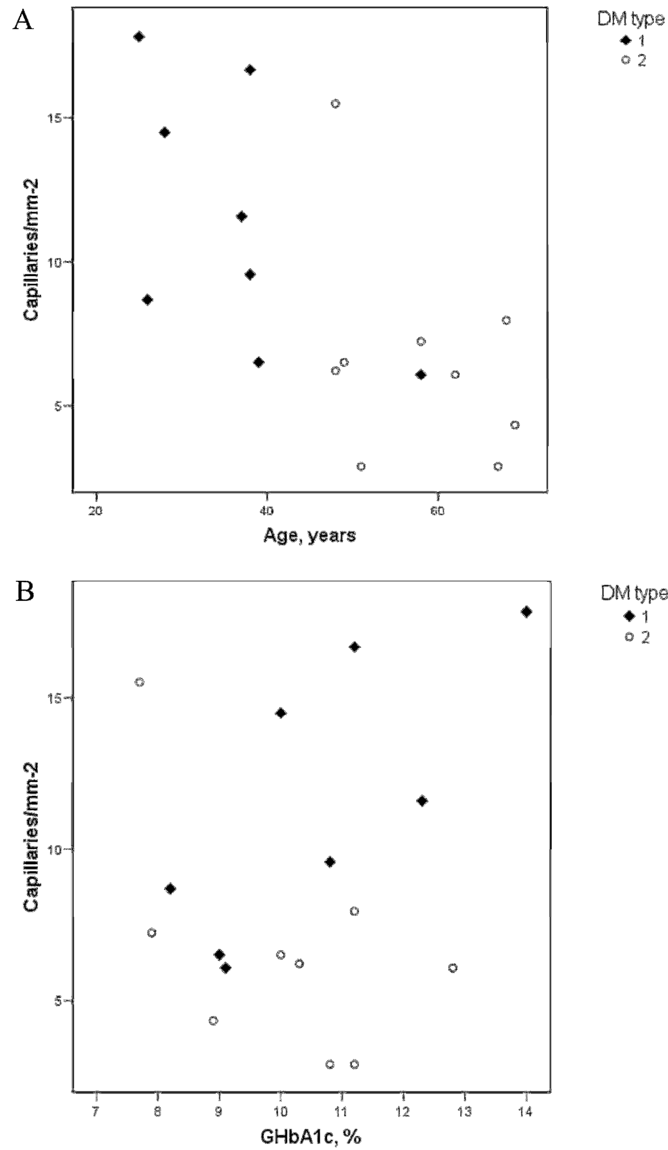

Figure 12. Scatter Plot analysis of type 1 and type 2 diabetic patients with proliferative retinopathy. (A) Correlation between the number of CD-31 positive endothelial cells/mm2 and age of patients ($p<0.05$). (B) Correlation between the number of CD-31 positive endothelial cells/mm^2 and glycosylated haemoglobin (GHbA1c, %) ($p<0.01$).

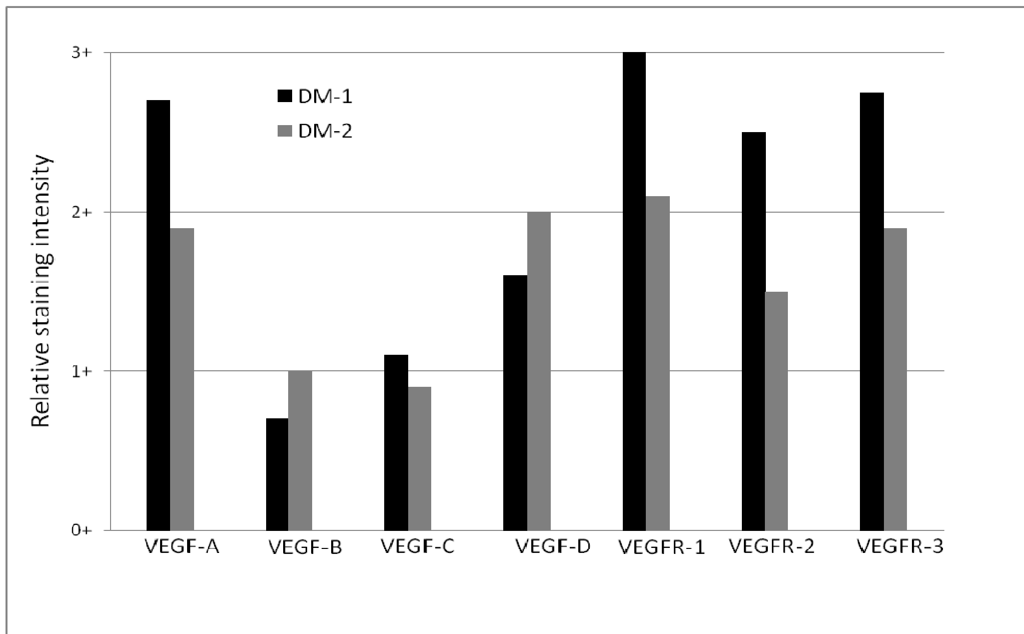

Figure 13. Vascular endothelial growth factors and their receptors in type 1 and type 2 diabetic retinopathy.

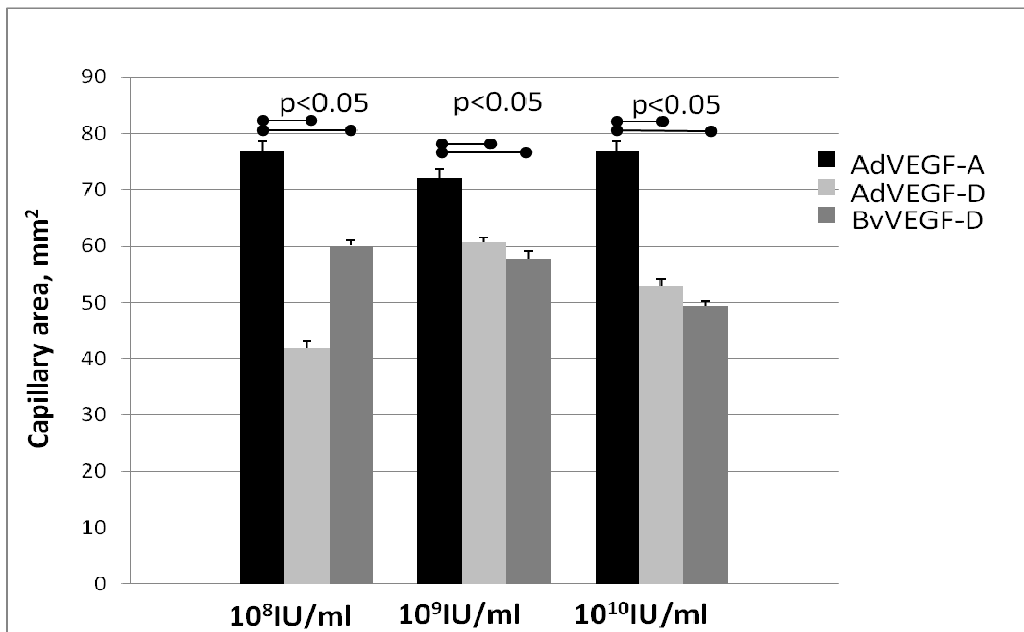

Figure 14. Capillary area in the retina after adenoviral and baculoviral intravitreal gene transfer encoding VEGF-A_{165} and VEGF–$D^{\Delta N \Delta C}$.

5.2 Intravitreous VEGF-A_{165} and VEGF-D $^{\Delta N \Delta C}$ gene transfer

5.2.1 Dose–response studies

The amount of human VEGF-A_{165} and VEGF-$D^{\Delta N \Delta C}$ in the vitreous humour showed a dose-dependent increase with the virus dose in the vitreous humour after intravitreous injection.

In the 10^8 IU/ml eyes, there was no significant difference in the amount of human VEGF-A_{165} or VEGF-$D^{\Delta N \Delta C}$ in vitreous humour when compared to control eyes. However, in the vascular structures a significant dilation and tortuosity of the vessels were seen in postoperative photographs and in the histological analyses 6 days after the gene transfer with both growth factors. The difference in capillary area between VEGF-A_{165} and VEGF-$D^{\Delta N \Delta C}$ gene transfer was significant ($p < 0.05$) (Figure 14).

In the 10^9 IU/ml eyes, clear changes were observed in the photographs taken after gene transfer. Moreover, the amount of human VEGF-A_{165} or VEGF-$D^{\Delta N \Delta C}$ in vitreous humour was significantly higher ($p < 0.05$) than in control eyes. Haemorrhages and microaneurysms were present near the optic disc. The optic nerve head was swollen indicating an increased permeability of the retinal vessels and breakdown of the blood-retina barrier. Retinal vessels were dilated and tortuous. Hyperaemia was seen in the anterior segments in the conjunctiva and the iris. Histological analyses revealed a significant difference ($p < 0.05$) between VEGF-A or VEGF-$D^{\Delta N \Delta C}$ 10^9 IU/ml eyes and control eyes in the average capillary area. Furthemore, VEGF-A_{165} transduced eyes had a significantly higher average capillary area than VEGF-$D^{\Delta N \Delta C}$ transduced eyes ($p<0.05$) (Figure 14).

In the VEGF-A_{165} $5x10^9$ IU/ml eyes, new vessels were seen in post-operative fundus photographs. In histological analyses, there were lots of new vessels both in the anterior segments and the retina.

A strong inflammatory reaction was seen in the VEGF-A_{165} and VEGF-$D^{\Delta N \Delta C}$ 10^{10} IU/ml transduced eyes. The fundus was not clearly visible any more due to the leaky retinal vessels. After VEGF-A_{165} 10^{10} IU/ml gene transfer, anterior segments were strongly hyperaemic and chemotic and the cornea was swollen and clouded. Also in histologic analysis anterior segments and normally avascular cornea were full of sprouting fragile new vessels. Microvascular density in the anterior segments of the VEGF-A_{165} 10^{10} IU/ml eyes was much higher than with other doses. In the AdVEGF-A_{165}/AdsKDR combination group, no new changes were seen in the vasculature in the fundus photographs or in histologic analyses confirming the significant role of VEGF-A_{165} in the pathogenesis of ocular neovascularization.

Also VEGF-$D^{\Delta N \Delta C}$ 10^{10} IU/ml caused an increase in the number of enlarged capillaries in the retina and optic nerve head ($p<0.05$) when compared to the control eyes. However, the difference in capillary area after VEGF-A_{165} and VEGF-$D^{\Delta N \Delta C}$ gene transfer was significant ($p < 0.05$) (Figure 14).

5.2.2 Time curve of the effects of adenoviral VEGF-A_{165} or baculoviral VEGF-$D^{\Delta N \Delta C}$ gene transfer

The amount of human VEGF-A_{165} in the vitreous humour was significantly increased ($p < 0.05$) 3 days after the adenoviral VEGF-A_{165} gene transfer when compared to AdLacZ injection (Figure 15). However, only slight changes were found in the vasculature of the eye in fundus photographs or in histological analyses.

The amount of human VEGF-A in the ELISA assay in vitreous humour was highest 6 days after the gene transfer (Figure 15). In fundus photographs 6 days after the AdVEGF-A_{165} injection blood vessels were swollen, leaky and tortuous. Hyperaemia was present in the anterior segments. In histological analyses, a clear response to the AdVEGF-A_{165} gene transfer was found especially in the retina and the optic nerve head. The increase in the average capillary area in the AdVEGF-A_{165} 10^9 IU/ml eyes

compared with the AdLacZ 10^9 IU/ml eyes was significant in the retina, the optic nerve head and the anterior segments ($p < 0.05$). Microvascular density was increased in the retina and the optic nerve head but not in the anterior segments.

Two weeks after the gene transfer, the amount of human VEGF-A_{165} in the vitreous humour had decreased (Figure 15). Vitreous haemorrhages were present in fundus photographs and it was very difficult to evaluate retinal vessels. The anterior parts were still slightly hyperaemic. In histological analyses, the response to the AdVEGF-A_{165} gene transfer was as strong as 6 days after the gene transfer in the retina and the optic nerve head and even stronger in the anterior segments. Microvascular density was increased in the retina, the anterior segments and the optic nerve head. The number of proliferating cells was at its highest 2 weeks after the gene transfer especially in the anterior segments.

Four weeks after the gene transfer, no human VEGF-A_{165} was found in the vitreous humour (Figure 15). The anterior parts had returned to normal. In fundus photographs, haemorrhages were resolved and fibrous scars were seen just above the optic nerve head. There were no changes in the control AdLacZ eyes. In histological analyses, there were no significant differences in the average capillary area in the anterior segments when compared to the AdLacZ 10^9 IU/ml eyes. However, in the optic nerve head and especially in the retina the difference was still significant ($p < 0.05$). Microvascular density in the retina and in the anterior segments was also increased. Double immunostaining with CD31 and alpha-actin revealed vessels with abnormal pericytes and vessels containing only ECs. The number of macrophages in RAM-11 staining was at its highest four weeks after the AdVEGF-A_{165} gene transfer.

The highest baculovirus-mediated VEGF-$D^{\Delta N \Delta C}$ expression in the vitreous humour was detected at 3 days after the intravitreal administration (Figure 15). Haemorrhages were seen near the optic nerve head both in the BacVEGF-$D^{\Delta N \Delta C}$ transduced eyes and in the BacLacZ control eyes. Furthermore, in the BacVEGF-$D^{\Delta N \Delta C}$ treated eyes capillaries were slightly dilated and the capillary density was significantly increased in the optic nerve head and in the anterior segments compared to the BacLacZ eyes ($p < 0.05$). However, there was no significant difference between BacVEGF-$D^{\Delta N \Delta C}$ and BacLacZ treated eyes in the dilatation of retinal vessels. Only a few macrophages were found both in the BacVEGF-$D^{\Delta N \Delta C}$ and BacLacZ treated eyes.

At 6 days after the BacVEGF-$D^{\Delta N \Delta C}$ intravitreal injection the amount of VEGF-$D^{\Delta N \Delta C}$ in the vitreous humour had diminished by half (Figure 15). The fundus was not clearly visible because of the breakdown of the blood retina barrier. There was an increased number of enlarged capillaries in the retina and optic nerve head ($p < 0.05$) in the BacVEGF-$D^{\Delta N \Delta C}$ eyes when compared to the control eyes. The number of RAM-11-immunopositive macrophages was significantly higher in the BacVEGF-$D^{\Delta N \Delta C}$ treated eyes at 6 days after intravitreal injection than at 3 days ($p < 0.05$).

Two weeks after the intravitreal injection, a 40-fold drop was detected in the amount of VEGF-$D^{\Delta N \Delta C}$ in vitreous humour compared to the highest level at 3 days (Figure 15). Both BacVEGF-$D^{\Delta N \Delta C}$ and BacLacZ eyes were calm and the fundus area was normalized. The capillaries in the optic nerve head and in the retina looked diminished and the number of RAM-11-immunoreactive macrophages was lower when compared to eyes of rabbits that were treated for 6 days.

The lowest level of VEGF-$D^{\Delta N \Delta C}$ expression was found in the 4-week treatment group (Figure 15). Four weeks after the intravitreal injection the eyes were calm. No leakages were found in the retinal vessels in FAG. Capillaries were not dilated and the average capillary area was not increased when compared to the control eyes. However, the number of RAM-11-immunoreactive cells in the transduced eyes was similar to that seen in the two weeks group.

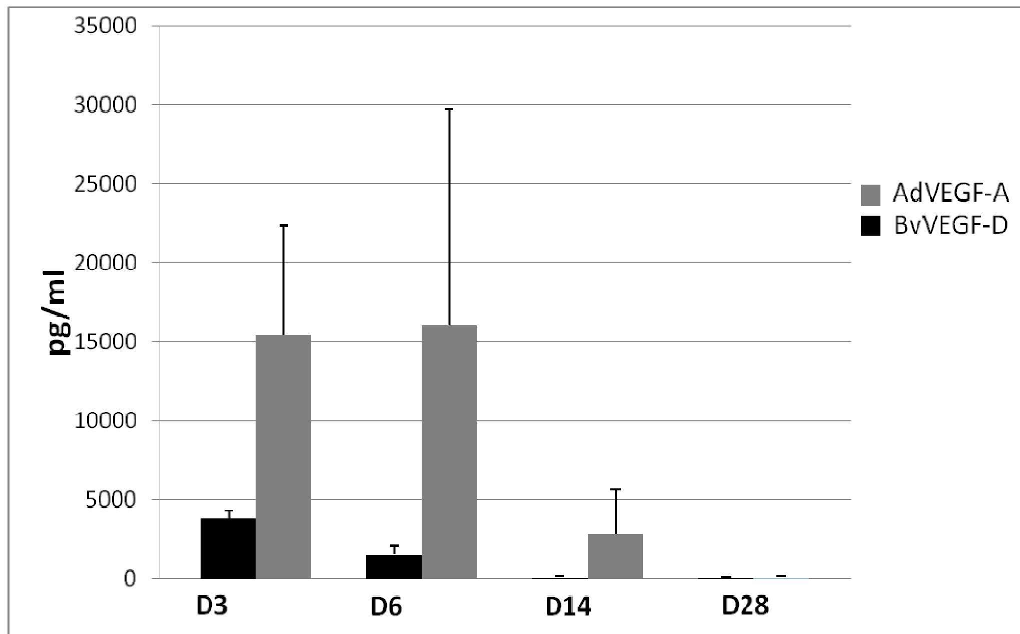

Figure 15. Time curve for adenoviral and baculoviral gene transfer.

5.3 Assessment of Baculoviral and Adenoviral Gene Transfer

The efficiency of baculo- and adenovirus-mediated gene transfer was directly assessed by examination of GFP-positive or LacZ-positive cell layers in the anterior and posterior parts of the eye. Baculoviral transfection was highly efficient for photoreceptor cells and RPE, and to a lesser extent for the inner retina. Adenoviral gene expression was detected mostly in the anterior segments, especially in the iris and the ciliary body, in the NFL and GCL of the retina. Also VEGF-D$^{\Delta N\Delta C}$ expression showed similar pattern in the retina and RPE layer after baculoviral and adenoviral transduction (Figure 16). Adenovirus mediated expression of VEGF-D$^{\Delta N\Delta C}$ in the vitreous humour was significantly higher than that of baculovirus at 10^9 and 10^{10} IU/ml. Both BacVEGF-D$^{\Delta N\Delta C}$ and AdVEGF-D$^{\Delta N\Delta C}$ caused dilatation of capillaries in all parts of the eye already at a dose of 10^8 IU/ml ($p < 0.05$). Both baculoviral and adenoviral gene transfers caused similar inflammatory response: macrophage invasion into the anterior segments, the retina and the optic nerve head of the eye. Inflammation-like alterations in the choriocapillaries were observed in BacVEGF-D$^{\Delta N\Delta C}$ treated eyes, but not in AdVEGF-D$^{\Delta N\Delta C}$ treated ones at 6 days after the gene transfer.

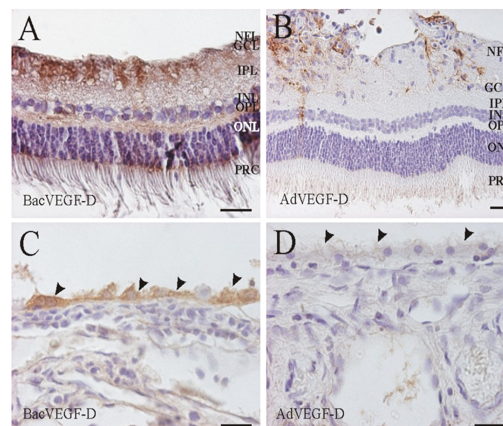

Figure 16. VEGF-D$^{\Delta N\Delta C}$ expression in the retina and RPE layer after baculoviral (A, C) and adenoviral (B, D) transduction. Scale bar = 20 µm.

5.4 IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mouse

Fasting glucose levels of 15-month-old diabetic IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice were significantly elevated on Western diet compared to LDLR$^{-/-}$ApoB$^{100/100}$ controls (8.2±2.1 versus 4.6±1.1 mmol/L, $P<0.05$), but with normal diet, there was no significant difference in glucose values between diabetic and control animals. *In vivo* examination of the mice eyes in biomicroscopy revealed no significant changes in neither diabetic animals fed with normal or Western diet nor in controls. The pupils dilated normally with mydriatics and retinal photographs of the mice revealed no clinical diabetic changes in the retinas neither in the diabetic nor in the control animals. The number of capillaries in IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice was not significantly increased compared to control LDLR$^{-/-}$ApoB$^{100/100}$ mice. The number of pericytes did not differ between the diabetic and control animals. Capillaries were similarly situated in the nerve fiber layer and in the outer plexiform layer and there were no haemorrhages or other signs of hyperpermeability of the vessels in the retinal layers. In paraffin embedded samples stained with haematoxylin-eosin, the altered morphology of the entire retina was found in all IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice fed with normal diet. There were displaced amacrine cells in the inner plexiform layer which stained positively with calbindin, a specific marker for amacrine cells. The morphology of the inner nuclear, outer plexiform and outer nuclear layers was abundantly altered in the diabetic IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$mice. Photoreceptor atrophy and thinning of the outer nuclear layer with large acellular areas were also present (Figure 17). There were no differences between the control and the diabetic mice in the retinal expression of heat shock proteins. However, caspase-3 was abundantly positive in the inner segment of photoreceptor cells indicating mitochondrial oxidative stress in the IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice. No such staining was observed in their littermate LDLR$^{-/-}$ApoB$^{100/100}$ controls. To evaluate the retinal structure of diabetic IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice, rhodopsin, a transmembrane protein that initiates the visual transduction cascade was studied with antibody against rhodopsin. Rhodopsin staining was reduced in the retinas of diabetic animals, consistent with the decreased number of photoreceptor cells.

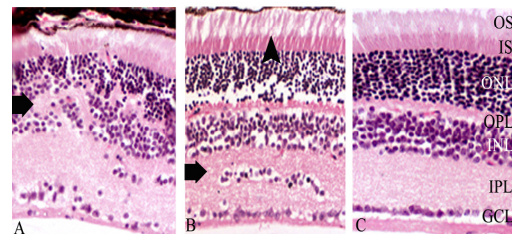

Figure 17. Haematoxylin-eosin stained samples show displaced cells and altered morphology throughout the retina of IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice fed with normal diet (large arrows; A, B). Photoreceptor atrophy is also present (arrowhead; B). Control animals have normal retinal morphology (C).

5.5 Summary of findings in this study

Study	Model	Conclusions
I	Growth factor expression in diabetic retinopathy	Different growth factor patterns between type 1 and type 2 diabetic proliferative retinopathy
II	Adenoviral VEGF-A_{165} gene transfer in the vitreous humour	Adenovirally delivered VEGF-A_{165} causes neovascularization in the retina and in the anterior segments
III	Baculoviral VEGF-$D^{\Delta N \Delta C}$ gene transfer in the vitreous humour	Baculoviral gene transfer is efficient in transducing photoreceptor layer and RPE
		VEGF-$D^{\Delta N \Delta C}$ causes blood retina barrier breakdown but no neovascularization in the eye
IV	Ocular findings of diabetic IGF-II/$LDLR^{-/-}$ $ApoB^{100/100}$ mice	Diabetic IGF-II/$LDLR^{-/-}$ $ApoB^{100/100}$ mice reveal photoreceptor atrophy and altered retinal morphology

6 DISCUSSION

6.1 Angiogenic factors in diabetic retinopathy

Several angiogenic factors and their receptors are found in the neovascular tissue and in vitreous humour in diabetic retinopathy. VEGF-A and ANG-2 were equally abundant in the vitreous humour of studied diabetics. The amount of VEGF-A in the vitreous humour was at the same level as in previous studies, and also increased ANG-2 levels have been found in the vitreous humour of diabetic patients (Aiello et al., 1994; Watanabe et al., 2005). ANG-2 expression has been demonstrated at the outer border of the inner nuclear layer, which is in close proximity to the deep retinal capillaries. The localized production of ANG-2 in the region of the deep capillaries may be the reason for their increased sensitivity to VEGF (Hackett et al., 2000). ANG-2 deficient mice develop abnormal retinal vascular bed consisting of some formation of the superficial capillaries, but display an almost complete lack of vessel penetration into the retina resulting in the absence of the intermediate and deep capillary plexus. In addition, ANG-2 deficient mice fail to develop ischemia-induced retinal neovascularization (Hackett et al., 2002). Furthermore, inhibition of Tie-2, when combined with inhibition of VEGF, suppresses retinal angiogenesis more efficiently than inhibition of VEGF alone, suggesting that signaling of both Tie-2 and VEGF plays a potential role in ischemia-induced retinal angiogenesis (Takagi et al., 2003). ANG-2 and its signaling pathways provide new molecular targets for the development of new treatments for ocular neovascularization.

In our study, type 1 diabetics had much more VEGF-A in the samples than other measured growth factors. In type 2 diabetics, VEGF-D was equally or even more abundantly present than VEGF-A in the neovascular tissues in more than 50% of the patients. VEGF-C was more abundantly present than VEGF-B in the neovascular tissues in type 1 diabetics. With type 2 diabetics, the situation was opposite. VEGF-A, VEGF-C and VEGF-D angiogenic effects are mediated by VEGFR-2. VEGF-A and VEGF-B signal through VEGFR-1. In addition, VEGFR-3 mediates the actions of VEGF-C and VEGF-D. VEGFR-2 is usually seen as the most important mediator of blood vessel growth. Signalling of VEGF-A by VEGFR-2 is fast and transient whereas signalling of VEGF-D is slower and more long-lasting (Jia et al., 2004). VEGFR-3 is mainly expressed on lymphatic endothelium and VEGF-C and -D are thought to play a role in the maintenance of lymphatic endothelium and in lymphangiogenesis (Iljin et al., 2001; Stacker et al., 2001). However, they have also been found in vascular structures (Rutanen et al., 2003; Witmer et al., 2001; Zhao et al., 2007). Expression of VEGFR-3 has been found particularly in areas with leaky retinal microvessels (Witmer et al., 2002). In the present study we found more immunoreactive VEGFR-1 and VEGFR-3 than VEGFR-2 in the neovascular tufts in both types of diabetics suggesting the important role of these receptors in the pathogenesis of diabetic retinopathy and in the formation and maintenance of microvessels such as retinal capillaries. VEGF-B mRNA is expressed in the developing retina (Simpson et al., 1999; Yonekura et al., 1999). However, VEGF-B expression appears not to be up-regulated in hypoxia, and the physiological role of VEGF-B remains essentially unknown (Gollmer et al., 2000). In addition, VEGF-B is unlikely to be necessary for the development of the retinal vasculature in an animal model under normal conditions (Reichelt et al., 2003). Recent reports have shown that VEGFR-1, mediator for action of VEGF-B, is also found in non-vascular retinal tissues in the mouse and primate eye (Robinson et al., 2001; Witmer et al., 2002). The fact that neural retina is also damaged in diabetic retinopathy may explain the existence of VEGF-B in diabetic neovascular tissues.

Our finding showing VEGF-C expression

in proliferative diabetic retinopathy is in line with a recent study of Zhao and coworkers (Zhao et al., 2007). In addition, VEGF-C is shown to be regulated by both VEGF-A and high glucose and promote angiogenesis *in vitro* enhanced by the presence of VEGF-A (Zhao et al., 2006). VEGF-D was not found in the vitreous humour in any patient in this study indicating that VEGF-D binds to retinal tissues more efficiently than the other growth factors in diabetic patients. VEGF-D has also recently been found in the subretinal vascular membranes of patients with AMD suggesting that it also plays some role in the pathogenesis of choroideal angiogenesis (Ikeda et al., 2006). It has been shown that cell–cell adhesion and cell attachment independently regulate the VEGF-D mRNA expression in human RPE *in vitro* suggesting that the breakdown of these interactions causes the overexpression of VEGF-D (Ikeda et al., 2006).

The difference in growth factor and growth factor receptor expression in the retinas between type 1 and type 2 diabetics might contribute to the differences in their pathogenesis and clinical course. Type 1 diabetics have a much higher risk of developing proliferative retinopathy than type 2 diabetics and in type 2 diabetics, diabetic maculopathy is the most prevalent sight-threatening manifestation of retinopathy (Henricsson et al., 1996; Klein et al., 1984). In the pathogenesis of vascular diseases in type 2 diabetes, the damage and dysfunction of endothelial cells have shown to play a crucial role (Tooke, 2000). Similarly to studies in RPE cells, VEGF-D may contribute to the breakdown of the inner blood retina barrier in diabetic patients leading to maculopathy and oedema in type 2 diabetics. Macrophages secrete many angiogenic and lymphangiogenic factors, including VEGF-D (Cursiefen et al., 2004). Inflammation has shown to be involved in many ocular neovacular diseases (Killingsworth et al., 1990; Meleth et al., 2005). Although the precise role of inflammation in the development of diabetic retinopathy is still unclear, it is possible that inflammatory mechanisms accelerate proliferative retinopathy in type 2 diabetics by VEGF-D dependent pathways. On the other hand, the fact that type 1 diabetics had more VEGF-A in the neovascular tissues than VEGF-D, suggests that with type 1 diabetics, hypoxia is more important in the development of proliferative retinopathy than inflammation. In order to stop the proliferative process, it might also be beneficial to prevent inflammation in the eye especially in type 2 diabetics.

HIF-1α is activated in hypoxic conditions preceding the development of various retinopathies and stimulates the expression of proangiogenic growth factors (Semenza and Wang, 1992; Wiesener et al., 1998). HIF-1α has been found in the epiretinal membranes of diabetic patients without previous laser photocoagulation (Abu El-Asrar et al., 2007). It was also demonstrated that HIF-1 mediates the hypoxic upregulation of VEGF and ANG-2 in vascular ECs (Yamakawa et al., 2003). Hyperglycemia can also regulate HIF-1α protein stability by interfering with its proteosomal degradation (Catrina et al., 2004). Also in this study, HIF-1α was detected both in type 1 and type 2 diabetic eyes despite of the previous panretinal laser photocoagulation. Normally, the retinal vessels are autoregulated in hypoxic conditions. However, the pathologic changes in retinal vasculature in diabetic retinopathy cause the distraction of the normal autoregulatory mechanism leading to active angiogenic process and proliferation. Manipulation of the HIF-1α pathway in the treatment of diabetic retina might be an attractive choice in addition to targeting VEGF and other growth factors.

Nuclear factor κ B (NFκB) is an ubiquitous transcription factor that, by regulating the expression of multiple inflammatory and immune genes, plays a critical role in host defense and in chronic inflammatory diseases (Barnes, 1997). NFκB is activated under hypoxic conditions, in retinal ECs or pericytes exposed to elevated glucose

concentration and in retinas of diabetic rats (Cummins and Taylor, 2005; Kowluru et al., 2003; Zheng et al., 2004; Zheng et al., 2004). Specific NFκB inhibitors were able to inhibit cell death *in vitro* and suppress retinal neovascularization in an OIR mouse model (Yoshida et al., 1999; Zheng et al., 2004). It has also been demonstrated that VEGF is able to activate NFκB in ECs (Grosjean et al., 2006). In previous studies, elevated levels of NFκB, chemokines and cell adhesion molecules have been identified in the eyes with proliferative diabetic retinopathy (Harada et al., 2006; Meleth et al., 2005). In this study, macrophages and NFκB were abundantly present in the neovascular samples in both types of diabetes. In diabetic rats, non-steroidal salicylate-based anti-inflammatory drugs have been shown to significantly inhibit the degeneration of retinal capillaries and prevent ganglion cell loss. The salicylate-mediated inhibition of early stages of diabetic retinopathy is due at least in part to inhibition of the diabetes-induced activation of NFκB and other transcription factors in the retina (Zheng et al., 2007).

All patients in our study had had previous panretinal laserphotocoagulation because of the proliferative retinopathy and in this sense, groups of type 1 and type 2 diabetic patients are comparable. The quantity of retinal photocoagulation was on average 1125 applications in type 1 and 1660 applications in type 2 diabetics. The mean time scale of laserphotocoagulation before vitrectomy was on average 8 months in type 1 diabetics and 16 months in type 2 diabetics. Laser photocoagulation has been shown to upregulate the expression of VEGF and transcription factors in photocoagulated human RPE cells *in vitro* 6 hours after photocoagulation. 72 hours after photocoagulation the expression of VEGF was decreased to the level before photocoagulation (Ogata et al., 2001b). In an *in vivo* study, changes in growth factor expression following laser photocoagulation were observed only in burn areas and mainly confined to the RPE and outer nuclear layer. The immunoreactivity was increased within the outer nuclear layer of burn areas during the healing process but returned to normal by 42 days (Xiao et al., 1999). Based on these reports it is presumable that the laser photocoagulation causes only temporal increase on growth factor expression.

6.2 Antiangiogenic strategies

With the current anti-VEGF treatment the ocular neovascular diseases are not cured, the progression of the disease is just slowed down. Current therapies to inhibit pathological angiogenesis in the eye use mostly inhibitors of one or two growth factors. This strategy has had only limited success (Afzal et al., 2007). This might be explained by other angiogenic factors involved that are not affected by the anti-VEGF agents. Potential future approaches to pathological angiogenesis could be based on angiogenesis inhibitors that simultaneously target several angiogenic factors or on blocking some master modulators such as HIF-1α. However, it must be remembered that angiogenic growth factors also have important roles and protective properties in the normal eye and blocking the action of VEGF completely can be harmful in the long run.

Most of the existing angiogenic inhibitors are large proteins or peptides. There is currently no effective drug delivery route for the administration of these agents into the most susceptible sites for ocular NV, the retina and the choroid. With systemic administration the drug is not able to efficiently reach the retina and choroid because of the blood retina barrier. In addition, many diseases involving ocular neovascularization are chronic disorders which require a long-term administration of drugs. Recent angiogenic inhibitors have a relatively short half-live, which means these agents need to be injected repeatedly (Cao, 2001). Furthermore, most of the angiogenic inhibitors are effective only in preventing the development of ocular neovascularization

(Das and McGuire, 2003; Zhang et al., 2001). Clinically, most ocular neovessels are formed in an indefinite period. Therefore, the angiogenic inhibitors regressing existing ocular neovascularization would be more useful in the treatment of ocular neovascular diseases.

6.3 VEGF-A and VEGF-D in a rabbit model

Type 1 diabetic patients had abundant VEGF-A in the samples. In type 2 diabetic patients, VEGF-D was equally or even more abundantly present than VEGF-A. Based on these results, following studies with rabbits were focused on VEGF-A and VEGF-D effects in ocular tissues. In addition, we wanted to explore adenovirus and baculovirus as gene transfer vectors for more sustained therapy for ocular angiogenesis compared to current treatment. Adenovirally delivered intravitreous VEGF-A_{165} caused increased dilation, tortuosity and leakage of the retinal vessels, breakdown of the blood-retina barrier and dilation of the capillaries in the optic nerve head and the anterior segments resembling the changes of diabetic retinopathy. Long lasting hyperglycaemia causes microvascular damage leading to pericyte loss, basement membrane thickening, microaneurysm formation and capillary hypoperfusion resulting in hypoxia and ischaemia (Garner, 1994). In healthy monkey eyes, intravitreal injection of VEGF caused retinal capillary nonperfusion documented by FAG. This might be explained by EC hyperplasia and subdivision or complete obstruction of vessel lumen by dividing ECs (Tolentino et al., 2002). Ischaemia leads to overexpression of VEGF-A which promotes neovascularization (Ozaki et al., 1997; Yla-Herttuala and Alitalo, 2003). In our present study, high doses and/or long follow-up VEGF-A_{165} caused neovascularization in the anterior segments, the retina and the optic nerve head, whereas the choroidal vessels remained unchanged. The changes were blocked by soluble KDR, which confirms the significant role of VEGF-A_{165} and VEGFR-2 in causing these effects. Overexpression of VEGF-A in the RPE is shown to induce vascular leakage, new choroidal blood vessel growth, the development of CNV and neural retina degeneration (Spilsbury et al., 2000). The development of CNV requires VEGF to be secreted from the RPE toward the inner choroid where high levels of VEGF receptors have been found (Blaauwgeers et al., 1999). Transgenic mice expressing VEGF in photoreceptor cells develop subretinal neovascularization of retinal vasculature origin and not from the choroid (Okamoto et al., 1997; Tobe et al., 1998a). VEGF expressed by the photoreceptor cells may be unable to pass through the outer blood-retina barrier to exert any effect on the choroid (Tobe et al., 1998a).

VEGF-$D^{\Delta N\Delta C}$ caused a dose-dependent enlargement of capillaries and break down of the blood-retina barrier. However, intravitreal injection of VEGF-$D^{\Delta N\Delta C}$ was not capable of inducing neovessel formation in the eye although other effects were similar to VEGF-A. VEGF-$D^{\Delta N\Delta C}$ *in vitro* leads to an approximately 2-fold greater increase in the number of bovine aortic endothelial cells after 3 days compared to the control. However, this effect is approximately 5-fold less potent than $VEGF_{165}$ (Achen et al., 1998).

6.4 Diabetic mouse model

The progression of diabetic retinopathy has not undeniably shown to correlate with dyslipidemia (Kohner et al., 1998; Stratton et al., 2001). The plasma lipid levels did not differ between diabetic IGF-II/$LDLR^{-/-}$ $ApoB^{100/100}$ and control $LDLR^{-/-}ApoB^{100/100}$ mice on neither of the diets. Therefore, the alterations in the retinal morphology are not caused by dyslipidemia. Hyperglycemia is a well known risk factor for diabetic retinopathy. However, there is no glycemic threshold for the development of long term complications in diabetic patients (Diabetes Control and Complications Trial, 1996). In diabetic IGF-II/$LDLR^{-/-}ApoB^{100/100}$ mice, the

plasma glucose levels were only moderately increased simulating early diabetes treated with diet therapy in humans. However, the morphology of the retina was abundantly altered especially in the outer part already with the normal diet. Despite of this, there were no signs of microvascular damage. Hyperglycaemia causes the onset of diabetic retinopathy and the cascade of metabolic and biochemical changes long before disease pathology is detectable (Brownlee, 2001). In addition, hyperglycaemia is associated with biochemical alterations and apoptosis of neuronal and vascular cells in the retina (Gardner et al., 2002; Mizutani et al., 1996). Demonstrated by psychophysical and electrophysiological experiments, anomalies are developing especially in rod vision during the preclinical period of diabetic retinopathy although the fundus is normal. This is caused by changes in the circulation and slight reduction in oxygen supply in the retina (Arden, 2001). These findings are confirmed with diabetic cats, whose retinal oxygen tension is reduced when compared to control animals, even with no fluorescein angiographic evidence of actual capillary dropout (Linsenmeier et al., 1998).

In the retina, caspase-3 is activated in disorders that result in photoreceptor degeneration. Activation of caspase-3 has been noted in the rd-1 mouse, in the rhodopsin mutant rat, in chemically induced models of retinal degeneration, and in photoreceptor degeneration due to exposure to blue light (Jomary et al., 2001; Kim et al., 2002; Liu et al., 1999; Wu et al., 2002; Yoshizawa et al., 2000). There is increasing evidence that diabetic patients with retinitis pigmentosa have a reduced risk of the development of preproliferative diabetic retinopathy (Arden, 2001; Arden et al., 1998). It has been proposed that the loss of rods during retinitis pigmentosa leads to a net reduction in oxygen usage by the retina, a phenomenon that is intimately related to the high oxygen demands of these cells in combination with the dark adaptation response (Arden et al., 2005; Penn et al., 2000). There is enhanced oxygen usage by rods under scotopic conditions, and, in some pathologies, this physiological phenomenon could serve to exacerbate pathologic hypoxia (Braun et al., 1995; Yu et al., 2000). Diabetic rho$^{-/-}$ mice have been shown to suffer less retinal hypoxia and reduced pathologic symptoms when compared with their diabetic controls with no retinal degeneration (de Gooyer et al., 2006). It has been suggested that photoreceptor metabolism could play a contributory role in the initiation and progression of diabetic retinopathy even before there is overt microvascular damage (Arden, 2001; Lahdenranta et al., 2001). It is also possible in our model, that the lack of microvascular diabetic retinopathy is due to excessive photoreceptor atrophy.

6.5 Viral vectors

The *in vivo* gene therapy approach in rodent models of ocular neovascularization has been shown to be efficient in multiple reports. Also the safety data of different viral vectors in larger animals is accumulating. Adenoviruses are relatively easy to produce, have a good capacity of 30 kb, and with an appropriate promoter can mediate good expression levels in many types of cells (Mori et al., 2002c). Baculoviruses cannot replicate in vertebrate cells and are also capable of transducing differentiated, nondividing cells (Hu, 2006; Mähönen et al., 2007). In addition, they have a low cytotoxicity in mammalian cells even at a very high virus load and they can be easily produced in high titers (Airenne et al., 2003; Laitinen et al., 2005). Furthermore, the large size of the baculovirus makes it possible to simultaneously transfer more than one gene. AAVs are substantially more difficult to produce than Ad vectors and have limited capacity of less than 5 kb. Lentivirus vectors have a similar transgene capacity (8–10 kb) but they can easily be generated in high titres (Lever et al., 2004). The major disadvantage with adenoviral vectors is that they induce an immune response that leads to inflammation, and mediates the destruction of transduced

cells reducing transgene expression (Loewen et al., 2004). Baculovirus causes a similar inflammatory response in transduced eyes. Fortunately the anterior chamber, the subretinal space and to a lesser extent the vitreous cavity are sheltered from a full immunological response that limits the immune response against the vector. However, AAV and lentivirus appear to invoke minor immune response and therefore mediate prolonged transgene expression (Bennett, 2003).

Intravitreally administered adenoviruses transduce genes in the nerve fibre and the ganglion cell layers in the retina and also in the anterior segment of the eye. On the other hand, baculovirus-mediated intravitreal gene transfer had the highest efficiency in the photoreceptor layer and the RPE followed by the inner retina. Intravitreally administrated AAVs transduce ganglion cells, trabecular meshwork cells and various cells of the inner nuclear layer (Ali et al., 1998b; Borrás et al., 2006; Grant et al., 1997; Martin et al., 2002; Surace and Auricchio, 2008). Lentiviral intravitreal gene transfer does not cause transgene expression in the retina (Greenberg et al., 2007). In order to transduce deeper layers of the retina, adenoviruses, AAVs and lentiviruses should be administered subretinally, whereas baculoviruses are able to transduce genes to deeper layers of the retina even after intravitreal injection.

Although intravitreal injection is somewhat less efficient than subretinal injection particularly when gene delivery to the outer retina is concerned, there are clinical aspects which favour this technique. The subretinal space is small, hence the subretinal injection is technically more difficult and only a small volume can be injected. Retinal neovascularization for example in proliferative diabetic retinopathy arises from the retina extending into the vitreous gel, and is therefore easily accessed with intravitreal techniques.

Viral vectors are in general more efficient than non-viral vectors in delivering genetic material into cells *in vivo*, and about 70% of the clinical trials are performed using viral vectors (Gene Therapy Clinical Trials). However, their safety is a concern. To achieve extended duration of transgene expression for gene therapeutic applications, the transgene should be either integrated into the genome or administered repeatedly. Chromosomal integration poses the risk of insertional mutagenesis and repeated administration of viruses might provoke an immune response with serious or even fatal consequences (Thomas et al., 2003). Non-viral vectors are safer than virus-mediated gene delivery, but far less efficient and shorter in their duration (Abdallah et al., 1995; Herweijer and Wolff, 2003; Niidome and Huang, 2002). A limitation of this approach is that long-term therapeutic transgene expression has been difficult to achieve due to the inefficiency of delivery.

6.6 Ocular gene therapy

The eye is one of the most suitable targets for gene therapy. It is easily accessible and allows restricted application of therapeutic agents with limited risk of systemic effects with a single injection. In addition, the effects of the treatment may be monitored by a variety of non-invasive examinations such as ophthalmoscopy, ERG and OCT. The amount of virus injected into the retina is about 1/1000 of the amount used for systemic diseases. A virus delivered to the eye is unlikely to cause any systemic disease (Bennett and Maguire, 2000). Gene transfer experiments have demonstrated that it is possible to deliver transgenes to the retina *in vivo* in stable and efficient fashion with minimal toxicity (Hauswirth and Beaufrere, 2000). However, the optimal gene transfer vector is not developed yet. Permanent models of retinal and choroidal neovascular disorders in larger animals would simulate clinical conditions better than recently used transient models. Attachment of regulation elements or tissue-specific promoters into the vector construct would increase their efficiency and safety in gene therapy.

Major steps have been taken forward in the treatment of inherited diseases in the eye. A large number of inherited ocular diseases have been defined at the molecular level; there are approximately 150 retinal disease loci with 90 disease-causing genes identified to date (Bainbridge et al., 2006). However, there are still many unsolved problems. Several diseases have complexed pathways which complicate the treatments. Phenotypes of retinal diseases vary from early and severe to late and progressive. Slowly progressive diseases have a much wider therapeutic window than more acute, aggressively progressing and those with an earlier onset. AMD and diabetic retinopathy are chronic diseases and because of that permanent expression of therapeutic genes is required. Also the therapy should occur early enough before the retina is permanently damaged.

Encouriging results in animal models have led to phase I clinical trials in patients with neovascular AMD. The improvement of gene therapy vectors has prolonged the expression period of the introduced gene after a single injection (Cao, 2001). Thus, gene therapy with angiogenesis inhibitors may be an option to repeated administrations that are costly and increase the risks of complications. Inhibition of CNV by gene therapy is also reached without delivery of vector intraocularly (Gehlbach et al., 2003a; Saishin et al., 2005).

In diabetic retinopathy, the complexicity of the disease offers several alternative targets for therapeutic intervention. In addition to targeting angiogenic factors, gene transfer of proteins targeted to aldose reductase, AGEs or PKC offers different options to influence the disease. RNA interference offers a potential approach for therapeutic gene silencing for downregulation of pathogenic proteins in acquired disorders (Campochiaro, 2004; Reich et al., 2003). Also inhibition of inflammation involved in many ocular neovascular diseases offers an alternative treatment pathway. Recent success in clinical trials of gene therapy has demonstrated that gene-based therapies can be effective in patients (Bainbridge et al., 2008; Maguire et al., 2008). Although ocular neovascular disorders are not life threatening, we should not underestimate the impact of visual impairment on the quality of life.

7 CONCLUSIONS

Significant progress has been made in the understanding of molecular pathogenesis of several retinal neovascular disorders providing multiple new targets for therapeutic interventions. In this study, we evaluated which factors are involved in the pathogenesis of proliferative diabetic retinopathy and retinal neovascularization. Multiple factors, suitable as targets of therapeutic interventions are present in the neovascular tissues of proliferative diabetic retinopathy. There are also differences in the expression pattern of angiogenic factors between type 1 and type 2 diabetic retinopathy. These may partly explain the differences in their clinical course. In type 1 diabetics, VEGF-A was much more abundant than the other growth factors but in type 2 diabetics, VEGF-D was equally or more abundantly present than VEGF-A in the neovascular tufts in over 50% of cases. ANG-2 seems to be an important factor in neovessel growth both in type 1 and type 2 diabetics. These differencies should be considered when developing new treatment options for patients with proliferative diabetic retinopathy. In type 1 diabetics, the first targets should be VEGF-A, VEGFR-2 and ANG-2 while in type 2 diabetics, preventing the action of VEGF-D and other VEGFRs should also be considered.

Intravitreal overexpression of VEGF-A_{165} led to the break down of the blood retina barrier, increased permeability and ultimately a dose-dependent neovessel formation in the rabbit eye. Blocking the action of VEGF-A_{165} prevented the progression of the angiogenic process. Intravitreally administred VEGF-$D^{\Delta N \Delta C}$ leads to similar breakdown of the blood retina barrier and dilatation of

capillaries as VEGF-A_{165} but it was not capable of neovessel formation alone.

Adenovirus and baculovirus vectors turned out to be efficient vectors for ocular gene transfer. However, both vectors initiated an immune response in the target tissue which limited the expression of the transducted gene to a few weeks. In this study, intravitreally injected adenovirus vector was particularly efficient in delivering agents to the ganglion cell layer and inner retina. Baculoviruses showed transduction in the RPE and photoreceptor layer even after the intravitreal injection and could therefore offer a novel choice for the development of treatments for certain diseases, such as AMD.

Diabetic IGF-II/LDLR$^{-/-}$ApoB$^{100/100}$ mice showed abundant changes in retinal morphology with no signs of microvascular damage normally seen in diabetic retinopathy possibly due to excessive photoreceptor atrophy. This model might useful in studying early diabetic changes and changes related to oxidative stress including retinal degenerations.

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