

From DEPARTMENT OF CLINICAL NEUROSCIENCE,  
SECTION OF OPHTHALMOLOGY AND VISION,  
ST ERIK'S EYE HOSPITAL  
Karolinska Institutet, Stockholm, Sweden

# EXPERIMENTAL INHIBITION OF PROLIFERATIVE RETINOPATHY

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**Karolinska  
Institutet**

Stockholm 2012

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ISBN 978-91-7457-717-4

To my family



## ABSTRACT

Retinal ischemic diseases like retinopathy of prematurity (ROP), diabetic retinopathy (DRP) and retinal vein occlusion, with preretinal neovascularization (angiogenesis) that may lead to vitreous hemorrhages and retinal detachment with subsequent vision loss and blindness is a global health problem.

By analyzing the potential role of molecules acting at different levels of the angiogenic cascade the presented studies aimed to widen our understanding of retinal ischemic diseases on a molecular, cellular and morphologic level. All studies were based on an experimental model of oxygen-induced retinopathy (OIR) where neonatal mice exposed to a hyperoxic environment develop pathologic retinal neovascularization. Genetically modified mice lacking functional genes (so called knock-out mice (KO)) for matrix metalloproteinase-2 (MMP-2), the adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) or interleukin-10 (IL-10) were studied. In addition, purinergic P2 receptors were investigated in normal mice. Retinal neovascularization was quantified by counting neovascular nuclei in retinal cross-sections. Retinal vessel development and capillary free areas were investigated by fluorescein staining of retinal flatmounts. P2 receptor expression and studies of the inflammatory reaction were performed with immunohistochemistry. *In situ* hybridization and real time-Polymerase Chain Reaction (rt-PCR) were used for identification of mRNA of relevant target molecules.

In the first study MMP-2, a member of an enzyme family essential for degradation of extracellular matrix (ECM) and the vascular basement membrane was not found to interfere with normal retinal vessel development. In OIR there was increased retinal neovascularization in the MMP-2 KO mice pointing at MMP-2 having an anti-angiogenic effect in this model.

In the second study, the expression of P2X<sub>2</sub> and P2Y<sub>2</sub> receptors were found to be up-regulated by oxygen exposure. Retinal neovascularization was strongly inhibited by the P2 antagonists suramin and PPADS and this was accompanied with a down-regulation of P2X<sub>2</sub> receptor expression suggesting a role for P2 receptors in OIR.

In the third study, hyperoxia was seen to reduce A<sub>2A</sub>R mRNA expression but the decline in receptor expression between postnatal day 12 (P12) and P17 seen in control animals was absent in retinas exposed to hyperoxia. The area of vascular regression was smaller in A<sub>2A</sub>R KO mice and there was also reduced retinal neovascularization. Gene microarray expression analyses of retinas of hyperoxia-treated mice showed that the P12 wild-type (WT) mice differed considerably from the P12 A<sub>2A</sub>R KO mice and at all other time-points. The results thus show that the A<sub>2A</sub>R exacerbates the initial vasoobliterative phase in OIR.

In the fourth study, the anti-inflammatory cytokine IL-10 was found to act as a pro-angiogenic factor in the OIR model and occasional macrophages/microglial cells were seen in WT mice.

In conclusion, the presented work has, in different ways, tried to enlighten the complex process of retinal angiogenesis and has identified some potential strategies for treatment of neovascular retinopathies including inhibition of P2 receptor and A<sub>2A</sub>R signaling.

## LIST OF PUBLICATIONS

- I. **Sarman S**, van der Ploeg I, Seregard S, Kvanta A. Retinal vascular development and pathologic retinal angiogenesis are not impaired in matrix metalloproteinase-2 deficient mice. *Curr Eye Res.* 2005 Apr;30(4):259-67.
- II. **Sarman S**, Mancini J, van der Ploeg I, Croxatto JO, Kvanta A, Gallo JE. Involvement of purinergic P2 receptors in experimental retinal neovascularization. *Curr Eye Res.* 2008 Mar;33(3):285-91.
- III. **Sarman S**, Armulik A, Lönnerberg P, Aronsson M, Kvanta A, Betsholtz C, Fredholm B. Mouse retina lacking adenosine A2A receptors show altered responses to both phases of oxygen-induced retinopathy. Submitted
- IV. **Sarman S**, Mia S, Harris B, Kvanta A, Aronsson M, van der Ploeg I. Reduced neovascularisation in interleukin-10-deficient mice in a model of ischemic retinopathy. Manuscript

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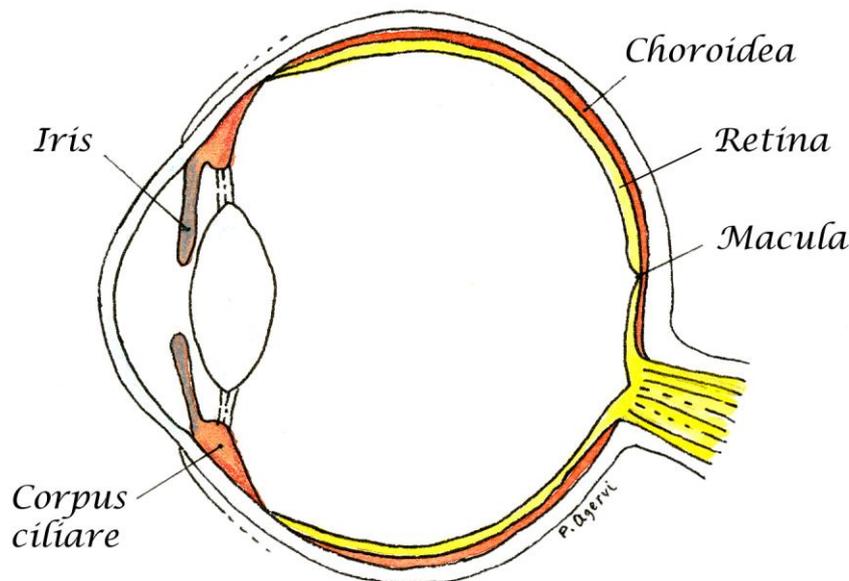
## LIST OF ABBREVIATIONS

A <sub>2A</sub> R	Adenosine A <sub>2A</sub> receptor
Ang	Angiopoietin
ATP	Adenosintriphosphat
C57BL/6J	Laboratory mouse; the most widely used inbred strain, with sequenced genome
cDNA	Complementary or copy DNA (deoxyribonucleic acid)
cRNA	Complementary RNA (ribonucleic acid)
DRP	Diabetic retinopathy
ECM	Extracellular matrix
G proteins	Guanine nucleotide-binding protein
GCL	Ganglion cell layer
IL-10	Interleukin-10, cytokine
ILM	Internal limiting membrane
INL	Inner nuclear layer
IPL	Inner plexiform layer
KO	Knock-out mouse, genetically engineerd mouse
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NFL	Nerv fiber layer
OIR	Oxygen-induced retinopathy
ONL	Outer nuclear layer
OPL	Outer plexiform layer
P	Postnatal day
P receptors	Purinergic receptors
PDGF-B	Platelet derived growth factor-B
PPADS	Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt
Pttg1	Pituitary tumor transforming gene 1
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
Rt-PCR	Real time-polymerase chain reaction
Tie	Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains, receptor for angiopoietins
TIMPs	Tissue inhibitors of metalloproteinases
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
WT	Wild type mouse, normal genom mouse

# 1 INTRODUCTION

## 1.1 THE RETINA

The retina is the innermost coat of the eyewall extending from the optic nerve to the ora serrata in the retinal periphery. It is composed of the outer retinal pigment epithelium (RPE) and the inner neural retina facing the choroid and the corpus vitreum respectively. The inner 2/3 of the retina receives its blood supply through the retinal circulation. The outer 1/3 of the retina instead is supplied through the choroidal circulation. The retina has several layers. From the inner to outer retina the layers they are: 1) the internal limiting membrane (ILM), 2) the nerve fiber layer (NFL), 3) the ganglion cell layer (GCL), 4) the inner plexiform layer (IPL), 5) the inner nuclear layer (INL), 6) the outer plexiform layer (OPL), 7) the outer nuclear layer (the nuclei of the photoreceptors, ONL), 8) the external limiting membrane, 9) the rod and cone inner and outer segments, and 10) the retinal pigment epithelium (RPE).



## 1.2 VASCULAR DEVELOPMENT

At the third week during embryonal development mesoderm cells differentiate into blood cells and blood vessels. These cells, known as angioblasts, form isolated clusters and cords, which gradually become canalized. By endothelial cell sprouting and after fusion they give rise to small blood vessels and a capillary network. (Langman 1980). This de novo formation of blood vessels from precursor cells is called vasculogenesis (Penn 2008). Vasculogenesis can also occur in the adult organism from circulating endothelial progenitor cells (derivatives of stem cells) able to contribute, to varying degrees, to neovascularization. Examples of vasculogenesis in adults are during tumor growth (Gao 2008), revascularization following trauma, e.g., after cardiac ischemia

(Asahara 1999) and in endometriosis (Laschke 2011). The formation of new capillaries and blood vessels from pre-existing ones is called angiogenesis (Carmeliet 2011). It is mainly driven by hypoxia. It occurs physiologically during endometrial growth as part of the menstrual cycle, in wound healing and in ischemic lesions in the heart and brain. It also occurs pathologically in tumour growth and in ischemic retinopathies in the eye.

### **1.3 ANGIOGENESIS**

The field of angiogenesis has evolved rapidly since its discovery some forty years ago. In 1971, Judah Folkman hypothesized that angiogenesis was a process vital for malignant tumor growth in cancer (Folkman 1971) and in 1989 Napoleone Ferrara and coworkers made ground-breaking discoveries identifying and isolating vascular endothelial growth factor (VEGF) as the main growth factor in vessel development (Leung 1989). Further research has revealed that angiogenesis is a complex, multistep, process involving several growth factors, matrix metalloproteinases (MMP) and cytokines.

#### **1.3.1 The angiogenic cascade**

When an angiogenic signal, such as VEGF expression from hypoxic tissue, is received by an existing, quiescent, vessel the basement membrane surrounding the vessel has to be disrupted and the pericytes, become detached from the vessel wall. Also the adhesive function of the junction molecule vascular endothelial cadherin (VE-cadherin) between adjacent endothelial cells is internalised in response to VEGF (Carmeliet 2011). The surrounding extracellular matrix (ECM) needs to be partially degraded to facilitate the cellular infiltration. This proteolytic degradation is mediated by MMPs. As VEGF increases the permeability of the endothelial cell layer in the vessel wall, plasma proteins extravasate and form a new ECM scaffold. Endothelial cells migrate onto this ECM surface. From the original vessel, upon stimulation by VEGF, a leading endothelial cell, “tip cell”, is chosen to lead the growth of the new vessel supported by neighboring endothelial, “stalk cells”. Tip cells have filopodia to sense environmental guidance and grow along a gradient of VEGF (Gerhardt 2003). The tip cell also expresses MMP activity for digesting the surrounding ECM. Later during angiogenesis, when the stalk cells get into contact with pericytes, the endothelial cells down-regulate MMP again. After fusion of neighboring branches the endothelial cell cords are transformed into tubes by the formation of vacuoles within the endothelial cells. To properly seal the new vessels, VE-cadherin-mediated junctions between endothelial cells are reformed (Carmeliet 2011).. Structural support is provided by the deposition of a basement membrane around the new vessel assisted by the release of MMP inhibitors (tissue inhibitors of metalloproteinases (TIMPs)). Thereafter, recruitment of pericytes is initiated by platelet derived growth factor-B (PDGF-B), an association essential for maturation of the endothelial cell tubes into blood vessels. This process is also supported by the binding of the angiopoietin-1 (Ang-1) to its receptor tyrosine kinase with immunoglobulin (Ig)-like and epidermal growth factor (EGF) homology domains 2 (Tie2) and enables a tight endothelial cell-pericyte interaction and further vessel stabilization. Vascular maturation promotes vessel integrity and inhibition of vascular leakage. The onset of blood flow and improved oxygen delivery lowers local VEGF production. The immature capillary plexus is early on excessively branched and have

an abundance of tubes. At this stage of angiogenesis, changes in the local balance of pro- and anti-angiogenic factors may lead to the elimination (pruning) of the new capillaries; for example by endothelial cell apoptosis. The pruning process is most extensive around the oxygen-rich arteries in the developing retina (Eble 2009, Adams 2007, Carmeliet 2011).

### **1.3.2 Pro- and anti-angiogenic factors**

VEGF binds to two related receptor tyrosinase kinases, VEGFR-1(Flt-1) and VEGFR-2 (KDR). These receptors are expressed on the cell surface of most vascular endothelial cells, monocytes, macrophages and hematopoietic stem cells. VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability enhancing effects of VEGF. VEGFR-1 is not directly involved in mitogenesis and angiogenesis. It may, under some circumstances, function as a “decoy” receptor that sequesters VEGF and prevents its interaction with VEGFR-2 (Ferrara 2004).

Platelet-derived growth factor-B (PDGF-B) is expressed by endothelial cells and by pericytes (Potente 2011). PDGF-B and its receptor (PDGFR- $\beta$ ) are essential for the recruitment of mural cells and for the stabilization of nascent blood vessels via coverage with pericytes in the later stages of the angiogenic cascade (Carmeliet 2004).

The Ang-Tie receptor system is an important receptor regulatory signaling system along the VEGF-VEGFR receptor system. The Ang family are protein growth factors that promote angiogenesis. There are four identified Ang: Ang1, Ang2, Ang3 and Ang4. Ang-1 and Ang-2 are antagonistic ligands that bind to the extracellular domain of the Tie2 receptor. Binding of Ang-1 to Tie2 causes tight endothelial cell-pericyte interaction and vessel stabilization promoting vessel integrity that inhibits vascular leakage. Ang-1 and VEGF control vascular maturation by recruiting mural cells to the endothelial plexus. As a result of pericyte-endothelial interaction, mature vessels lose their dependence on VEGF, and thus the plasticity window is closed (Augustin 2009, Singh 2011).

### **1.3.3 Proteolysis of the basement membrane**

MMPs are a family of over 20 zinc-containing enzymes capable of degrading different components of the ECM. They are produced as latent, pro-enzymes, which must be proteolytically processed to be activated. Endothelial cells produce many of the MMPs. MMPs are divided into five subgroups based on their structure and/or substrate specificities. These are the matrilysins (MMP-7 and MMP-26), the collagenases (MMP-1, MMP-8, MMP-13), the stromelysins (MMP-3, MMP-10, MMP-12), the gelatinases (MMP-2, MMP-9) and the membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-24). The gelatinases have a preference for denatured collagens (gelatin) and also degrade types IV, V, VII and X native fibronectin and laminin. During angiogenesis MMPs are essential for the degradation of the vascular basement membrane and remodeling of the ECM in order to allow endothelial cells to migrate and invade the surrounding tissue. In addition, the MMPs assist in detaching pericytes from the vessels undergoing angiogenesis, release ECM-bound angiogenic growth factors (Hashimoto 2002), expose cryptic epitopes in ECM proteins and cleave endothelial cell-cell adhesions. MMP activity is regulated by TIMPs in tissues whereas

in plasma, the main protease inhibitor,  $\alpha$ 2-makroglobulin, is also the predominant MMP inhibitor. The MMPs also have an anti-angiogenic role by generating endogenous angiogenesis inhibitors (eg. endostatin and tumstatin) through cleavage of collagen chains. MMP-2, MMP-7, MMP-9 and MMP-12 also have the capacity to hydrolyze plasminogen into the potent angiogenesis inhibitor angiostatin (O'Reilly 1999) (Rundhaug 2005). Retinal neovascularization was reduced by an inhibitor of MMPs in the murine oxygen-induced retinopathy model (OIR) (Das 1999). Although preclinical studies testing the efficacy of MMP suppression in tumor models were encouraging, the results of clinical trials in cancer patients have been disappointing (Hua 2011).

#### **1.4 INTERLEUKIN-10**

Cytokines are a large and diverse family of small intercellular communication proteins secreted by numerous cells. Cytokines are grouped into lymphokines, interleukins, and chemokines, based on their presumed function, cell of secretion, or target of action. The term *interleukin* was initially used for those cytokines whose presumed targets are principally leukocytes. It is now used largely for designation of newer cytokines and bears little relation to their presumed function. The vast majority of interleukins are produced by T-helper cells. The term *chemokine* refers to a specific class of cytokines that mediates chemoattraction (chemotaxis) between cells. Cytokines, including chemokines and interleukins, also affect angiogenesis. Chemokines recruit leukocytes to ischemic tissues such as hypoxic regions in tumors, dermal wounds, atherosclerotic plaques, arthritic joints and in proliferative retinopathy, where the leukocytes release angiogenic factors in an attempt to re-establish tissue oxygenation (Lewis 1999). But, leukocytes can also produce angiogenesis inhibitors, making their role in initiating or terminating angiogenesis dependent on the temporospatial balance of these modulators (Carmeliet 2004). Interleukin-10 (IL-10) is an anti-inflammatory cytokine that also may be involved in angiogenesis. In a tumor model for melanoma and in hind-limb ischemia, IL-10 has anti-angiogenic properties through down-regulation of VEGF (Huang 1999, Silvestre 2000). But IL-10 also has pro-angiogenic properties by polarizing the macrophage genetic profile towards a pro-angiogenic phenotype in the mouse OIR model (Dace 2008). Likewise, in a model of corneal neovascularization, IL-10 appeared to have a pro-angiogenic effect (Samolov 2010).

#### **1.5 PURINERGIC SIGNALING**

The finding of adenosinetriphosphat (ATP) as a purinergic neurotransmitter in nonadrenergic, noncholinergic nerves in the gut and bladder was a landmark discovery (Burnstock 1972). Since then purinergic signaling has become an extensive field of research. Purines and pyrimidines have major roles in the activities of non-neuronal cells as well as neurons. This includes acting as co-transmitters and neuromodulators in most, if not all, nerve types in the peripheral and central nervous systems, platelet aggregation and vascular endothelial cell-mediated vasodilation (Burnstock 2002). More recently, purinergic signaling has been implicated in cell proliferation, migration, differentiation and death in embryological development, wound healing, restenosis, atherosclerosis, ischaemia, inflammation, neuroprotection and cancer (Burnstock

2006). Purinergic receptors are of two kinds, P1 (adenosine) and P2 (nucleotide). Adenosine receptors are subdivided into four subtypes: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>, all of which couple to guanine nucleotide-binding proteins (G proteins). P2 receptors comprise two types of receptors, P2X and P2Y. The P2X family encompasses seven ligand-gated ion channel receptors and the P2Y family comprises eight G protein-coupled receptors. In the retina, P1 and P2 receptors are expressed by photoreceptors, most neurons, glial cells, the microvasculature and by the RPE (Housley 2009). Adenosine induces retinal hyperemia after ischemia by increasing retinal blood flow through relaxation of pericytes (Ostwald 1997, Li 2001). Adenosine also serves to maintain tissue oxygenation in response to chronic ischemic stress by stimulating angiogenesis (Adair 2005). Adenosine released from hypoxic tissues activates A<sub>2</sub> receptors, which stimulate the release of VEGF from parenchymal cells. VEGF thereafter binds to VEGFR-2 on endothelial cells, stimulating their proliferation and migration (Adair 2005). P2 receptors have been proposed to have a role in retinal development (Sugioka 1999) and in retinal disease, including retinal detachment (Iandiev 2006), diabetic retinopathy, (Sugiyama 2004) and retinal degeneration (Francke 2005).

## 1.6 RETINAL VASCULAR DEVELOPMENT

In mammals, retinal vascularization begins at the optic nerve head and radiates outwards to the ora serrata in the retinal periphery,. In humans the retinal circulation begins to develop at the fourth month of gestation. At that time small branches from the hyaloid artery form the central retinal artery. By the eight-month of gestation, the retinal vessels extend to the ora serrata nasally, but only to the equator temporally. It is not until birth that the temporal vessels reach the ora serrata (Paysse 2011). In mice there are no, or only very small vessels around the optic nerve, at birth (Gyllenstein 1954). The mouse retina is therefore an ideal structure to study the vascular development using retinal flat-mount preparations. The primary vessel plexus at the inner retinal surface subsequently remodels into three parallel but inter-connected networks, located in the nerve fiber layer and the plexiform layers. The superficial vascular plexus forms during the first week after birth by radial outgrowth of vessels from the optic nerve into the periphery, reaching the retinal edges at approximately P8 (postnatal day 8). From P7 onward the superficial capillaries start sprouting vertically to form first the deep and then the intermediate vascular plexus. The deep plexus, located in the outer plexiform layer, forms rapidly and reaches the retinal periphery at approximately P12, followed by the intermediate plexus in the inner plexiform layer between P12 and P15. By the end of the third postnatal week, all three vascular layers are fully mature with multiple interconnecting vessels between the layers (Connolly 1988, Stahl 2010). Development of the retinal vasculature is preceded by an invasion of migrating astrocytes into the retina (Stone 1987, Watanabe 1988). The astrocytes form a cellular network that provides a template for the growing blood vessels (Ling 1988, Fruttiger 1996). Before the astrocytes are covered with blood vessels they experience hypoxia that induces VEGF expression that in turn stimulates angiogenesis (Pierce EA 1996, West 2005). As the expanding vessel network grows over the retinal astrocytes, they differentiate into a more mature phenotype. Proliferation of astrocytes ceases and VEGF is down-regulated (West 2005). The first vascular plexus is dense and uniform. Over time this primitive plexus is re-modeled into a mature, hierarchical, vascular tree. Pruning is particularly evident in the vicinity of arteries where capillary free zones

emerge. This can occur via migration and re-localization of endothelial cells (Hughes 2000) or via selective endothelial cell apoptosis (Ishida 2003).

## **1.7 ROP AS A MODEL FOR RETINAL NEOVASCULARIZATION**

Retinopathy of prematurity (ROP) is a major cause of blindness in children in the developing and developed world. The condition was earlier known as retrolental fibroplasia, and was originally described in the 1940s by Terry who first connected the condition with premature birth (Terry 1944). Excessive oxygen use was early identified as a risk factor for ROP (Ashton 1954). Besides oxygen, gestational age and birth weight are the major risk factors for ROP (Lutty 2006). In a Swedish study of preterm infants born before 27 weeks' gestation, the incidence of ROP was 73% among those children surviving until their first eye examination. The risk of ROP was reduced by 50% for each week of increase in gestational age at birth (Austeng 2009). ROP is a biphasic disease consisting of an initial phase of vessel loss followed by a second phase of vessel proliferation. In humans retinal blood vessel development is initiated during the fourth month of gestation and reaches the retinal periphery just before birth (Roth 1977). Infants born prematurely have incompletely vascularized retinas with a peripheral avascular zone. The gestational age at birth of the premature infant determines the area of the avascular zone (Chen 2007). In premature infants vascular growth that would normally occur in utero slows or ceases and is accompanied by regression of developed retinal vessels. The relative hyperoxia of the extra-uterine environment as well as supplemental oxygen given to premature infants are thought to be responsible for this process. As the infant matures, the non-vascularized retina becomes increasingly metabolically active and, in the absence of an adequate vascular system, leads to tissue hypoxia (Chen 2007). The second phase of ROP is characterized by hypoxia-induced retinal neovascularization. In animal models it is shown that during the first, hyperoxic, phase of ROP retinal VEGF expression is significantly suppressed, contributing to vessel loss (Alon 1995, Pierce 1996, Gao 2002). During the second, hypoxic, phase however, VEGF becomes up regulated in the inner retina (Ozaki 1999) contributing to pathologic neovascularization (Alon 1995, Pierce 1995, Stone 1996, Geisen 2008). Animal studies have also shown beneficial effects of anti-VEGF treatment on hypoxia-driven retinal neovascularization (Aiello 1995, Robinson 1996, Ozaki 2000, Bainbridge 2002). In a patient with ROP, VEGF was found in the retina in a pattern consistent with the results in mice (Young 1997). Established treatments for ROP are cryotherapy and laser photocoagulation of the avascular retina and surgical treatment in the late stage of the disease. Recently intravitreal injection of an anti-VEGF antibody (bevacizumab (Avastin®)) has been used with excellent result in severe ROP (Mintz-Hittner 2011). Uncertainties exist however with respect to dosing, frequency, timing and adjunct therapies. Careful attention must also be given to the potential for systemic complications and long-term effects in infants. The second phase of ROP has common features of neovascularization in other proliferative retinopathies such as advanced diabetic retinopathy (DRP). Despite cryotherapy or laser photocoagulation of the avascular retina and surgical treatment in the late stage of the disease visual acuity in advanced ROP often remain poor.

## **1.8 OTHER DISEASES WITH PATHOLOGIC RETINAL NEOVASCULARIZATION**

DRP is a major complication in diabetes and remains a leading cause of blindness in the working population of developed countries. Nearly all patients with type 1 diabetes will develop some manifestation whereas in type 2 diabetic patients 80% of insulin-dependent patients and 50% of patients not requiring insulin therapy will have DRP within 20 to 25 years following disease onset (Curtis 2009). The incidence of diabetes throughout the world is projected to be approximately 300 million by the year 2025 (Curtis 2009). Proliferative DRP occurs in approximately 50% of patients with type I diabetes and in about 15% of patients with type II diabetes with disease for 25 years (Klein 1984a, Klein 1984b). Thickening of the capillary basement membrane, pericyte and smooth muscle cell dropout, microaneurysms and capillary occlusion all lead to tissue ischemia and eventually retinal neovascularization. Retinal vein occlusions, sickle-cell anemia and other vascular obliterating diseases also lead to tissue hypoxia and pathologic retinal neovascularization. Intraocular VEGF levels are significantly higher in patients with active retinal neovascularization including proliferative DRP and ischemic central retinal vein occlusion than in patients with no proliferative disorder (Aiello 1994). VEGF has also been demonstrated in ocular membranes from patients with proliferative DRP (Malecaze 1994).

## 2 AIMS OF THE PROJECT

To analyze the potential role of molecules acting at different levels of the angiogenic cascade in experimental retinal neovascularization and in normal retinal development

Specifically I wanted to:

Study the expression and role of MMP-2 in experimental retinal neovascularization and in normal retinal development

Study the expression and role of P2 receptors in experimental retinal neovascularization

Study the expression and role of A<sub>2A</sub>R in experimental retinal neovascularization

Study the role of the anti-inflammatory cytokine IL-10 in experimental retinal neovascularization

### **3 MATERIAL AND METHODS**

#### **3.1 LABORATORY MICE, KNOCK-OUT MICE**

Study I: MMP-2 wild-type (WT) and knockout (KO) mice, 0-19 days old, were used throughout the study. The generation of MMP-2 KO mice has previously been described (Itoh 1997)

Study II: Normal C57BL/6J mice, 7-17 days old, were used throughout the study.

Study III: Normal C57BL/6J and A<sub>2A</sub>R WT and KO mice, 7-17 days old were used throughout the study. The generation of A<sub>2A</sub>R KO mice has previously been described (Chen 1999).

Study IV: C57BL/6J and IL-10 KO mice, 7-17 days old, were used throughout the study. The generation of IL-10 KO mice has previously been described (Kühn 1993).

All the animals were treated in accordance with the Association for Research in Vision and Ophthalmology's (ARVO's) Statement for the Use of Animals in Ophthalmic and Vision Research.

#### **3.2 EXPERIMENTAL MODEL OF RETINAL ANGIOGENESIS**

OIR in the mouse is the experimental model of retinal angiogenesis used in all the present studies. It was first described in 1994 and has since become the most popular model to study abnormal angiogenesis in the retina (Smith 1994). It is referred to in the literature under several names including OIR, ischemia-induced retinopathy and pathologic retinal neovascularization model. In this thesis I have chosen to use the term OIR throughout. Many animals such as mice, rats, kittens, and beagle pups have incompletely vascularized retinas at birth and resemble the immature retinal vascular development of premature infants. However the murine model used herein has become the model of choice because it is reproducible and since it is easily quantifiable (Connor 2009, Aguilar 2008). Furthermore, the study of molecular mechanisms involved is greatly facilitated by the genetic tools available in mice. Mice were chosen at P7 since the vascular development of P7 retinas closely resembles that of premature infants including maximal hyaloid vascular regression and minimal retinal vascular development. Neonatal mice are thus exposed to 75% oxygen from P7 until P12. During this period of hyperoxia, vessel regression and the cessation of normal radial vessel growth occurs mimicking the first phase of ROP. The extent of vaso-obliteration can be determined by measuring the non-perfused area in retinal whole mounts. Upon return to room air, the non-perfused portions of the retina become hypoxic, thereby inducing the expression of angiogenic factors leading to retinal neovascularization. The neovascular phase is similar to the second phase of ROP in humans and, in addition, mimics certain aspects of proliferative DRP (Chen 2007). The formation of vascular tufts are measured by quantifying neovascular nuclei extending into the vitreous in cross sections of retina, or by quantifying the area of vascular tufts in retinal whole mounts.

### **3.3 RETINAL FLATMOUNTS**

Study I: Mice were infused with fluorescein isothiocyanate-dextran in the left cardiac ventricle. Eyes were enucleated at P0, P3, P6, P9, and P15 and fixed in paraformaldehyde. Dissected retinas were flatmounted and examined by fluorescence microscopy. The distance from the center of the optic nerve to the leading front of developing vascular plexus in each quadrant was measured. The mean for each individual was used as a single experimental value.

### **3.4 *IN-SITU* HYBRIDIZATION**

Study I: In situ hybridization was performed on P1 to P19 formaldehyde-fixed, serial sections using radiolabelled T3 and T7 ribonucleic acid (RNA) polymerase-derived sense and antisense complementary RNA probes to MMP-2 (Kvanta 2000). After development, the sections were studied by light and dark-field microscopy.

### **3.5 REAL TIME-POLYMERASE CHAIN REACTION (RT-PCR)**

Study I: rt-PCR was performed P12 to P19 in MMP-2 WT mice exposed or not exposed to hyperoxia. Total RNA was extracted from retinas and reverse transcribed to cDNA. Primers were used for GADPH and MMP-2 (Kuzuya 2003).

Study III: rt-PCR was performed P12 to P17 in C57BL/6J mice exposed or not exposed to hyperoxia. Total RNA was extracted from retinas and reverse transcribed to cDNA. Primers were used for GADPH and A<sub>2A</sub>R and A<sub>2B</sub>R (Chunn 2001). Rt-PCR was also performed P7 to P17 in A<sub>2A</sub>R WT and A<sub>2A</sub>R KO mice exposed to hyperoxia. Primers were used for GADPH and Pttg1.

Study IV: rt-PCR was performed P7 to P17 in IL-10 WT mice exposed or not exposed to hyperoxia. Total RNA was extracted from retinas and reverse transcribed to cDNA. Primers were used for GADPH and IL-10. Rt-PCR was also performed in IL-10 WT and KO mice exposed to hyperoxia. Primers were used for GADPH, MMP-2, MMP-9, VEGF, Ang-1 and Ang-2 (Pomye 2001, Brafman 2004, Luo 2004).

### **3.6 IMMUNOHISTOCHEMISTRY**

Study III: Immunohistochemistry was performed at P17 in retinal sections from C57BL/6J mice exposed or not exposed for hyperoxia, with antibodies for P2 receptors. Visualization was done with a fluorescence microscope.

Study IV: Immunohistochemistry was performed at P17 on cryosections from IL-10 WT and KO mice exposed to hyperoxia, using antibodies for macrophages/microglia. Visualization was done with a fluorescence microscope.

### 3.7 MICROARRAY

Study III: To study changes in gene expression over time in retinas in normoxia (P12 and P17) and induced by hyperoxia (P7, P12 and P17) in A<sub>2A</sub>R WT and KO mice DNA microarray was used. RNA samples for each genotype and time point, were analysed for quality and concentration by using the Agilent 2100 Bioanalyzer. 100 ng of total RNA of each sample was reverse transcribed and amplified into cDNA. The cDNA was labeled with a fluorescent dye and hybridized to Gene Chip Mouse Gene 1.0 ST arrays (Affymetrix). Nonspecific binding was washed off and the microarray was dried. A laser excited the fluorescent dyes producing light detected by a scanner. The scanner generated a digital image from the excited microarray. The digital image was further processed by specialized software to transform the image of each spot to a numerical reading.

### 3.8 GENE ONTOLOGY

Study III: Gene Ontology (GO) is a way to use bioinformatics, that is, the creation and maintenance of databases to store biological information, to unify the representation of genes and gene products across species. The GO project provides a study of defined terms representing gene product properties. Three ontologies have been constructed: A. *Biological processes* which refers to a biological objective to which the gene or gene product contributes. Example of a biological process is: “cell growth and maintenance” or “signal transduction”. B. *Molecular function* which is defined as the biochemical activity of a gene product. Example of a molecular function is: “enzyme” or “ligand”. C. *Cellular components* refers to the place in the cell where a gene product is active. Examples of cellular components is: nuclear membrane” or “Golgi apparatus”. Each GO term has a specific term name and a definition with cited sources (Ashburner 2000).

#### 3.8.1 Gene set enrichment analysis (GSEA)

Gene expression analysis with DNA microarrays that simultaneously measures the expression levels of thousands of genes generates huge amounts of data. The challenge no longer lies in obtaining gene expression profiles, but rather in interpreting the results to gain insights into biological mechanisms. The genes from the microarray experiment can be ordered in a ranked list according to their differential expression. A common approach involves focusing on a handful of genes at the top and bottom of the list (i.e., those genes showing the largest difference) to get proposals of biological information. This approach however, has a few major limitations. First no individual gene may meet the threshold for statistical significance, because the relevant biological differences are modest relative to the noise inherent to the microarray technology. Secondly there can be a long list of statistically significant genes without any unifying biological theme. To overcome these analytical challenges, the Gene Set Enrichment Analysis (GSEA) is developed to evaluate microarray data at the level of gene sets. The gene sets are based on prior biological knowledge, e.g., published information about biochemical pathways or coexpression in previous experiments. Given an *a priori* defined set of genes (e.g., genes encoding products in a metabolic pathway, located in the same cytogenetic band, or sharing the same GO category), the goal of GSEA is to determine whether the

members of the gene set are randomly distributed throughout list or primarily found at the top or bottom (Subramanian 2005). In Study III we used GSEA for analysis of different gene sets in the microarray analysis including: “angiogenesis”, “endothelial cells”, apoptosis” and “astrocytes”, however we did not find any significant differences.

### **3.9 STATISTICAL ANALYSIS**

Study I: Student’s two-tailed  $t$  test for unpaired data was used to determine whether there were significant ( $p < 0.05$ ) differences in pathologic retinal angiogenesis between the MMP-2 KO and WT mice.

Study II: Statistical analysis was performed using one-way ANOVA test after logarithmic transformation of the mean nuclear value. A post hoc least significance difference (LSD) test was run to establish which means differed. The level of significance was set at 0.05 to determine whether there were significant differences in pathologic retinal angiogenesis between control, suramin injected and PPADS injected mice.

Study III. Student’s two-tailed  $t$ -test for unpaired data was used to determine whether there were significant differences ( $P < 0.05$ ) concerning  $A_{2A}R$  and  $A_{2B}R$  mRNA expression, area of avascularity and pathologic retinal angiogenesis. In gene-expression profile analysis the R statistics software and the Limma package were used for microarray data and differential gene expression analysis. The Broad Institute Gene Set Enrichment Analysis (GSEA) software was used for analysis of enriched gene ontology terms and cell type specific markers.

Study IV: Student’s two-tailed  $t$ -test for unpaired data was used to determine whether there were significant ( $P < 0.05$ ) differences in pathologic retinal angiogenesis between the IL-10 WT and KO mice. Differences in mRNA expression in the retina at P17 between the IL-10 WT and KO mice were determined by Mann-Whitney U test.  $P < 0.05$  was considered to be significant.

## 4 RESULTS

### 4.1 STUDY I

We investigated the involvement of MMP-2 in both normal retinal vessel development and in OIR. In normal retinal vessel development we found that there was no difference concerning the growth of the retinal vessels between WT, and MMP-2 KO mice. In both groups the retinal vessels were barely seen around the optic disc at P0. The formation of the primary vascular plexus with small branching vessels had a similar appearance and reached the ora serrata by P10 in both WT and MMP-2 KO mice. At P15, the retinal vasculature had a mature appearance in both groups.

In OIR we found neovascular nuclei inside the ILM in both WT and MMP-2 KO mice. However, the amount of neovascular nuclei was significantly higher in the MMP-2 KO mice group. Since the extent of retinal neovascularization is correlated to the degree of inner retinal ischemia, retinas from P12 animals were analyzed for differences in hyperoxia-induced microvascular closure. P12 is the time-point when mice are transferred from the high oxygen chamber to normal environment. However, no apparent difference in the central capillary non-perfusion was detected between WT and MMP-2 KO mice.

The expression of MMP-2 mRNA was analyzed in the retina at P1 to P19 using *in-situ* hybridization. Expression of MMP-2 mRNA coincided with the formation of the INL that appeared around P8. MMP-2 was uniformly distributed. The expression increased gradually until P10 and remained unchanged thereafter. There was no apparent spatial or temporal correlation between MMP-2 expression and the development of the vascular plexus. The overall distribution of MMP-2 mRNA was similar in retinas from OIR and normal mice. This expression was distinct from the expression of VEGF that was strongly up-regulated in the INL during OIR.

MMP-2 mRNA expression was also investigated by rt-PCR. The rt-PCR data showed, in agreement with the *in-situ* hybridization results, that exposure to hyperoxia was not related to an up-regulation of MMP-2 mRNA expression at any time point. During normal retinal vessel development there was a tendency to declining MMP-2 mRNA expression.

### 4.2 STUDY II

The involvement of P2 receptors in OIR was investigated. First the expression of P2X and P2Y receptors was analyzed by immunohistochemistry in mice, exposed to normoxia, and thereafter in the OIR model. P2X2 receptor expression was mainly found in the outer plexiform layer of normoxic mice. In oxygen-treated mice, the expression was similarly found in the OPL. In addition, a strong signal was also found in the inner layers, particularly the IPL. Immunoreactivity to P2X1 and P2X3 was not seen in either normoxic or oxygen-treated animals. In normoxic mice, weak P2Y2 receptor expression was also seen in the GCL and in the NFL. In mice treated with

oxygen, P2Y2 expression showed a similar overall distribution as normoxic mice. The signal was, however, strongly up-regulated.

The role of P2 receptors in OIR was further investigated using the P2 receptor antagonists, suramin and PPADS. In untreated OIR mice, the mean number of nuclei above the ILM was  $25.2 \pm 4.8$ . Systemic treatment with a single dose of either suramin or PPADS resulted in a dramatic decrease in neovascularization ( $5.7 \pm 4.2$  and  $2.3 \pm 0.5$  nuclei, respectively).

The effect of suramin on P2X2 and P2Y2 receptor expression in oxygen-treated mice was further investigated by immunofluorescence. Intraperitoneal injection of suramin at P12 was found to down-regulate the P2X2 receptor expression in the IPL, whereas the expression in the OPL remained unchanged compared to non-suramin-injected mice. By contrast, P2Y2 receptor expression did not show any difference between suramin-treated or non-treated mice.

### 4.3 STUDY III

The importance of the A<sub>2</sub>R in OIR was investigated. First we examined the expression of A<sub>2A</sub>R and A<sub>2B</sub>R mRNA by rt-PCR during normal vascular development and in OIR. At P12 the expression of A<sub>2A</sub>R mRNA was significantly higher in the control group than in the hyperoxia-treated group. In control retinas, A<sub>2A</sub>R expression thereafter declined between P12 and P17. By contrast, in the hyperoxia-treated group, the expression of A<sub>2A</sub>R remained unchanged. At P17, this translated into unaltered A<sub>2A</sub>R expression in the hyperoxia-treated group as compared to the decrease seen in the controls. A<sub>2B</sub>R mRNA expression showed increased expression between P12 and P17 in retinas from the hyperoxia-treated group whereas the mRNA levels were unaltered in control mice. Attempts to confirm and extend the rt-PCR data using immunofluorescence failed as the level of specific fluorescence was low and there was no discernable difference between retinas from WT and A<sub>2A</sub>R KO mice.

Thereafter we wanted to investigate the hyperoxia-induced capillary obliteration at P12 and P17 in WT and A<sub>2A</sub>R KO mice. The avascular area was assessed on whole-mounted retinas. The avascular area at P12 was smaller, though not significantly different in A<sub>2A</sub>R KO mice compared to controls. At P17 the avascular area was however significantly reduced in the A<sub>2A</sub>R KO retinas.

We also used the OIR model to estimate the oxygen-induced formation of neovascular nuclei in WT and in A<sub>2A</sub>R KO mice. Although retinal neovascularization was evident in both WT and A<sub>2A</sub>R KO mice the number of neovascular nuclei was significantly lower in the A<sub>2A</sub>R KO mice.

To characterize in detail the molecular changes potentially responsible for the observed phenotype associated with A<sub>2A</sub>R deficiency in the retina during the OIR phases (i.e. vasoobliteration at P12 and retinal neovascularization at P17) we performed gene microarray expression analyses. P12 and P17 normoxic retinas of WT and A<sub>2A</sub>R KO mice and P7, P12 and P17 retinas subjected to hyperoxia were studied. For mice that

had been in hyperoxia differences were found in gene expression changes from P7 to P12 between WT and A<sub>2A</sub>R KO mice. When analyzing the most significant differences, corresponding to 80 genes, we found that the P12 WT mice differed significantly from the P12 A<sub>2A</sub>R KO mice and also from P7/P17 WT and A<sub>2A</sub>R KO mice. This difference at P12 between WT and A<sub>2A</sub>R KO mice were not seen in retinas from mice subjected to normoxia. There were no connections associated with signal paths or cellular structures among the genes.

No difference was found in gene expression between hyperoxic P7 WT and A<sub>2A</sub>R KO mice. Only small differences were seen between WT and A<sub>2A</sub>R KO mice in normoxia and hyperoxia at P17.

In hyperoxic mice at P12 we identified *Pttg1* to be expressed more than 10 times higher in the A<sub>2A</sub>R KO mice as compared to WT mice. Rt-PCR confirmed this difference in *Pttg1* mRNA expression.

#### **4.4 STUDY IV**

The involvement of IL-10 in OIR was investigated in WT and in IL-10 KO mice. Although pathologic retinal angiogenesis was evident in both WT and IL-10 KO mice the number of neovascular nuclei was three times lower in the IL-10 KO mice group.

To examine whether IL-10 mRNA expression was related to OIR we assessed relative IL-10 mRNA expression levels by rt-PCR. IL-10 mRNA levels were detectable in 1 out of 8 P7 WT mice, in 1 out of 8 P12 mice and in 1 out of 10 P17 mice. In WT mice exposed to hyperoxia, IL-10 mRNA levels were detectable in 2 out of 9 mice on P12 and in 7 out of 10 mice on P17.

By analyzing potential compensatory differences we sought to explain the observed effects of IL-10-deficiency in OIR. With rt-PCR mRNA expression of well-known angiogenic factors was examined. The relative ratios between MMP-2-, MMP-9-, VEGF, Ang-1, and Ang-2 mRNA in relation to GAPDH mRNA levels in IL-10 KO mice P17 compared to P17 WT mice were determined. However, no significant difference between IL-10 KO and WT mice was found for any of the genes examined.

We also studied the potential differences in the presence of retinal macrophages or microglial cells close to neovascular tufts in WT and IL-10 KO mice. However, retinal cryosections, P17, from WT mice stained with anti-F4/80 antibodies showed only occasional macrophages/microglial cells. No macrophages/microglial cells were observed in cryosections from IL-10 KO mice at P17.

## 5 DISCUSSION

In the eye, formation of new vessels in different diseases is a major cause of vision loss and blindness. Several ocular structures can be affected by pathologic vessel growth including the cornea, iris, retina and choroid. In the retina, ischemic retinopathies with associated pathologic retinal neovascularization, such as ROP, DRP and retinal vein occlusions affect millions of people worldwide.

Anti-angiogenic treatment has revolutionized eye-care starting with the management of neovascular age-related macular degeneration (using VEGF inhibitors such as bevacizumab (Avastin®) and ranibizumab (Lucentis®)(Rosenfeld 2006)). VEGF inhibitors have since also been successfully used to manage vascular leakage and macular edema in diabetes (Massin 2010) and retinal vein occlusions (Heier 2012) as well as pathologic vessel growth in ROP (Mintz-Hittner). In treating neovascular conditions with VEGF inhibitors it is however important to be cautious with doses and timing to avoid complications like tractional retinal detachment. The OIR model, used in the present thesis, is suitable as an experimental model for retinal neovascularization in general and for ROP in particular.

In Study I we investigated the role of MMP-2 in normal retinal development and in OIR. We found no difference in normal retinal vessel development between MMP-2 KO and WT mice. At P0 no retinal vessels were seen. By P9 the retinal vessels had grown out, radially from the center of the retina around the optic nerve, to the reach the ora serrata and at P15 both groups had developed a mature appearance. A similar finding has been reported in rats given different MMP antagonists where no effect was observed on the normal retinal vascular development as determined by measuring vascular areas (Barnett 2007).

In the OIR-model we compared WT and MMP-2 KO mice. We found that the extent of neovessels at P17 was significantly higher in the MMP-2 KO mice group as compared to WT mice. Surprisingly, MMP-2 acted as an anti-angiogenic factor in our model. This is in contrast to previous findings showing regression of pathologic neovascularization in MMP-2 KO mice (Ohno-Matsui 2003). The reason for this discrepancy is not clear. One explanation could be the difference in the methods for quantifying retinal neovascularization. Whereas we have used a protocol counting the extraretinal neovascular nuclei on serial sections stained with haematoxylin and eosin (Smith 1994), Ohno-Matsui instead stained the retinal endothelial cells with the lectin *Griffonia simplicifolia* and then measured the total stained area thereby including both intra- and extraretinal vessels. A later work though has also shown reduction in preretinal neovascularization in MMP-2 KO mice (Barnett 2007). Maybe, differences in the susceptibility to OIR are related to genetic variations resulting from differences in genome manipulation. The MMP-2 KO mice in our study contained the commonly used 129 strain known to have increased susceptibility to neovascularization (Rohan 2000).

In Study II we found expression of P2 receptors both in normal retinal development and in OIR. In normoxia, P2X2 receptor expression was detected in the OPL. Mice

exposed to hyperoxia showed P2X2 receptor expression in the OPL but there was also strong P2X2 expression in the inner retinal layers, especially in the IPL. Earlier work on P2X2 expression in adult mice and rats noted P2X2 receptor expression in the inner retinal layers, including the GCL, the IPL, and the INL (Greenwood 1997, Kaneda 2004, Puthussery 2006). The adult adult pattern of P2X2 expression in mice and rats is thus consistent with the P2X2 receptor expression in young mice after exposure to hyperoxia. This difference in P2X2 receptor expression in our young mice and earlier studies in adult mice and rats is not obvious but probably reflects a developmental change.

In normoxia we found P2Y2 receptor expression in the GCL and NFL. This pattern was unchanged in mice exposed to hyperoxia. This finding is consistent with earlier work where P2Y2 immunostaining was mainly found in the innermost retinal layers in adult rat and pig retina (Fries 2004, Iandiev 2006).

Furthermore we found that oxygen treatment induced P2X2 receptor expression in the IPL whereas P2Y2 receptor expression was augmented in the GCL and NFL. This is also consistent with earlier findings showing up-regulation of P2X2 receptors by hypoxia in cultures from the hippocampus (Cavaliere 2003). Mitotic activation induced by hypoxia in retinal microglia cells has been shown to coincide with P2Y2 receptor expression (Morigiwa 2002). Moreover, the mitotic activity was suppressed by the P2 antagonist suramin.

We found that the P2 antagonists suramin and PPADS both reduced retinal neovascularization. Suramin and PPADS both block P2X2 signalling but are only weak P2Y2 antagonists (Lambrecht 2002). In addition to its P2 antagonistic effects, suramin also inhibits angiogenesis by interfering with VEGF and fibroblast growth factor-2 (Waltenberger 1996, Kathir 2006). Unlike suramin, PPADS seems to be a much more specific P2 antagonist with minimal effects on unrelated proteins (Lambrecht 2002). We cannot exclude that the effects of the two P2 antagonists, suramin and PPADS, on retinal neovascularization in the OIR model are, at least in part, due to inhibition of non-P2 proteins. However, the fact that we observed similar inhibition with two unrelated antagonists argues against this. This conclusion is further supported by our observation that suramin down-regulates P2X2 receptor expression in the IPL in OIR. Moreover, the fact that expression of P2X2 receptors in the IPL was not found in normal mice but only in OIR is interesting and suggests that P2X2 receptors in the IPL may be directly involved in the development of neovascularization during OIR.

In Study III we showed that A<sub>2A</sub>R KO mice have less pathologic neovascularization at P17 compared to WT indicating pro-angiogenic properties of the A<sub>2A</sub>R in this model supporting earlier studies (Adair 2005). The most striking result however was the finding that the avascular zone is smaller at P12 in A<sub>2A</sub>R KO mice compared to WT mice. Differences at P12 were also seen in the gene expression profiling experiment where P12 WT mice differed considerably from P12 A<sub>2A</sub>R KO mice and from all other time-points. The smaller avascular zone at P12 in the A<sub>2A</sub>R KO mice could be the result of enhanced vascularization or the result of regressed apoptosis during the hyperoxic phase (ie. P7 to P12). However, gene ontology analysis of gene expression profile data

from P7 to P12 showed no evidence of augmented angiogenesis or regressed apoptosis. Another explanation for the smaller avascular zone at P12 in the A2A KO mice could be that the astrocytes, normally functioning as template for the growing retinal vessels, survived to a greater extent during the hyperoxic phase in our model. Retinal astrocytes play a critical role during normal retinal vascular development by expressing VEGF and thereby initiating endothelial cell proliferation (Stone 1995, Provis 1997, Stalmans, 2002). It is likely that astrocytes also play an important role in promoting normal revascularization during ischemic injury (Chan-Ling 1992). It has also been shown that maintaining retinal astrocytes normalizes revascularization and prevents vascular pathology associated with oxygen-induced retinopathy (Dorrell 2010). Our gene expression profile analysis, however, could not confirm that the astrocytes (or any other nerve cells) were up-regulated.

In Study IV the role of IL-10 in the OIR model was studied. We found that IL-10 KO mice have significantly less neovascular nuclei compared to WT mice. Retinal IL-10 mRNA was also up-regulated in OIR in P17 mice. This indicates that IL-10 has pro-angiogenic properties in OIR. Using rt-PCR we further analyzed whether any other known angiogenic factors, including MMP-2, MMP-9, VEGF or Ang-1 or Ang-2 could compensate for the loss of IL-10. However, we found no difference between the WT mice and the IL-10 KO mice. Overall, our finding that IL-10 having pro-angiogenic properties is consistent with a previous study showing that IL-10 has pro-angiogenic properties in OIR. This study further found that macrophages from WT mice express higher levels of VEGF mRNA compared to macrophages from IL-10 deficient mice (Dace 2008). During pathologic retinal neovascularization in OIR, macrophages have been seen in proximity to the neovascular tufts (Yoshida 2003, Shen 2007). However, in another study it was shown that vitreal macrophages appear to promote aberrant angiogenesis through migration and association with neovascular tufts. This lead the authors to suggest that the contributions of vitreal macrophages may be marginal because only very few macrophages are present in the vitreous. Instead it was speculated that resident microglia could be the main source of the neovascular-associated macrophages (Kataoka 2011). In the present study we looked for retinal macrophages/microglia in cryosections from both WT and IL-10 KO mice at P17. However, we only found occasional F4/80 positive cells in cryosections from WT mice and failed to detect any macrophages /microglia in the IL-10 KO mice. The explanation for this lack of macrophages/microglia in our model is unclear. Further studies are needed to define both the importance of IL-10 and of microglia/macrophages in OIR.

## 6 CONCLUSIONS

Retinal neovascularization is a global health problem in retinal ischemic diseases such as ROP, DRP and retinal vein occlusion. If the retinal complications are not properly managed they frequently lead to severe vision loss and ultimately blindness.

The OIR model is currently the only experimental model for retinal neovascularization. Although there are ischemic models in other species including rat, cat, dog and zebrafish (Kremer 1987, Ricci 1990, McLeod 1996, Zhang 2000, Cao 2008) the murine OIR (Smith 1994) is the most commonly used model allowing reproducibility, low-cost breeding and the use of transgenic techniques including KO mice.

Common to all ischemic retinopathies is the development of new, irregularly growing, blood vessels. It should be pointed out though that the mice used in the OIR model are young and healthy and that the origin of the ischemic retinopathy is secondary to hyperoxic exposure. In contrast, clinical DRP or retinal vein occlusion is instead a consequence of pathologic vascular function.

The work in this thesis has focused on different aspects of retinal angiogenesis and retinal vascular development. The presented studies have in several ways expanded our understanding of the molecular cascade that leads to ocular neovascularization and has revealed some potential treatment strategies for neovascular retinopathies. In the first study, MMP-2 surprisingly showed anti-angiogenic properties. Previous and later studies have instead suggested that MMP-2 has pro-angiogenic properties during pathologic retinal neovascularization (Ohno-Matsui 2003, Barnett 2007). Further studies are thus necessary to establish which, or what group of MMPs, are responsible for the pathologic growth of retinal vessels and to identify the possible presence and extent of redundancy. In the second study, we found P2 receptors to be involved in the pathologic retinal vessel growth, which has not been described before. This opens a new field for research in exploring possible treatment strategies for neovascular retinopathies. In the third study the  $A_{2A}R$  was found to act in a pro-angiogenic manner, exacerbating the initial vasoobliterative phase of ROP suggesting a therapeutic potential for  $A_{2A}R$  inhibition. Finally, in the fourth study we show that IL-10 also has pro-angiogenic properties. However we did not find any evidence of an inflammatory reaction, making further studies necessary to establish the role of IL-10 in retinal angiogenesis.

With the increasing number of key molecules revealed in pathologic vessel growth multiple corresponding drug targets are identified. Suggested by the present thesis, the best treatment strategy is probably to target different steps in the angiogenic cascade such the MMPs, purinergic signaling or inflammation. Continued research in sorting out these complex connections is important.

## 7 SVENSK SAMMANFATTNING

Nybildning av blodkärl sker normalt under fosterutvecklingen men även i vuxen ålder vid sårhäkning, under menstruationscykeln och vid skador orsakade av syrebrist, som efter hjärtinfarkt. Det förekommer även sjuklig, patologisk, nybildning av blodkärl vid tumörtillväxt och vid de sjukdomar i ögat som är orsakade av syrebrist i ögats näthinna, retina.

Syrebrist i näthinnan kan ha olika orsak. Prematuritetsretinopati (ROP) förekommer hos barn som föds för tidigt. I den omogna, ofullständigt kärlförsedda, näthinnan uppstår syrebrist vilket medverkar till frisättning av kärlstimulerande faktorer bland annat vascular endothelial growth factor (VEGF). Detta medför att näthinnans blodkärl börjar växa kraftigt och okontrollerat vilket kan orsaka blödningar, ärrhäkning och näthinneavlossning. Trots laserbehandling och eventuell operation vid avancerade stadier av sjukdomen kvarstår ofta kraftig synnedsättning. Hos vuxna är syrebrist i näthinnan vanligen orsakad av ögonförändringar vid diabetes eller efter kärlocklusion. Näthinnans blodkärl är då ställvis tilltäppta och kan inte leverera tillräckligt med syrerikt blod varför syrebrist uppstår. Detta leder, på liknande sätt som vid ROP, till frisättning av tillväxtfaktorer varpå okontrollerad, patologisk, kärlnybildning sker.

De aktuella studierna har syftat till att vidga vår förståelse för uppkomst av patologisk kärlnybildning i näthinnan på en molekylär och cellulär nivå. Den musmodell för patologisk kärlnybildning, oxygen-induced retinopathy (OIR), som använts innebär att sju dagar gamla möss (vars ögon mycket liknar ögat hos ett för tidigt fött barn) placeras i kuvös med hög syrgashalt (75 % O<sub>2</sub>) under fem dagar. När musen på 12:e levnadsdagen flyttas från kuvösen till vanlig rumsluft (20 % O<sub>2</sub>) uppstår syrebrist i näthinnan och därmed frisättning av kärlstimulerande faktorer vilket leder till okontrollerad kärltillväxt.

I de olika delarbetena har vi studerat molekyler som på olika sätt griper in i den kaskad av biokemiska reaktioner som leder fram till kärlnybildning. Vi har använt genetiskt modifierade möss som saknar funktionella gener (knock-out möss (KO)) för enzymet matrix metalloproteinase-2 (MMP-2), den syrekänsliga adenosine A<sub>2A</sub> receptorn (A<sub>2A</sub>R) eller den inflammations reglerande interleukin-10 (IL-10). Med farmakologiska metoder har syrekänsliga purinerga receptorer studerats. För studierna har olika molekylära, immunohistokemiska samt histologiska metoder använts.

I den första studien noterades att MMP-2, tillhörande en familj av enzymer viktiga för nedbrytning av vävnadens extracellulärmatrix och blodkärlens basalmembran, inte var involverad i näthinnans normala kärlutveckling. Vid OIR sågs däremot en ökad retinal kärlnybildning hos MMP-2 KO mössen, talande för att MMP-2 här hade en kärnhämmande effekt.

I den andra studien fann vi att uttrycket i näthinnan av P2X<sub>2</sub> och P2Y<sub>2</sub> ökade vid exponering för hög syrgashalt. Syrgasorsakad kärlnybildning i OIR modellen hämmades kraftigt av två farmakologiska P<sub>2</sub> receptor antagonister; suramin och PPADS, vilket även åtföljdes av en minskning av mängden P2X<sub>2</sub> receptor.

Sammantaget talar resultaten för att P2 receptorer har en viktig betydelse för uppkomsten av retinal kärlnybildning samt att läkemedel som blockerar P2 receptorer kan minska skadlig kärlnybildning i näthinnan.

I den tredje studien fann vi att en hög syrgashalt minskade  $A_{2A}R$ , medan den minskning av  $A_{2A}R$  mellan levnadsdag 12 och dag 17 som sågs hos kontrollerna saknades hos de möss som vistats i hög syrgashalt. I OIR modellen sågs hos  $A_{2A}R$  KO möss en minskning i både inledande kärlbortfall och senare retinal kärlnybildning. Gen expressions analys med microarray av näthinna från möss som exponerats för hög syrgashalt visade att 12 dagars kontroll möss skiljde sig avsevärt från 12 dagars  $A_{2A}R$  KO möss samt från alla andra tidpunkter. Tillsammans talar fynden för att  $A_{2A}R$  huvudsakligen verkar genom att öka den inledande fasen av kärlbortfall vid OIR. Som en konsekvens av detta skulle hämning av  $A_{2A}R$  kunna vara en möjlig behandling vid ROP.

I den fjärde studien fann vi att IL-10 stimulerar retinal kärlnybildning i OIR modellen. Någon direkt koppling mellan inflammation och IL-10 effekten kunde inte påvisas.

Sammanfattningsvis har vi med de aktuella studierna på olika sätt studerat och klargjort olika mekanismer vid kärlnybildning i näthinnan. Två möjliga behandlingsstrategier vid patologisk kärlnybildning har identifierats, farmakologisk hämning av dels P2 receptor och dels adenosin  $A_{2A}$  receptor signalering.



## 8 ACKNOWLEDGEMENTS

A number of people have contributed, inspired and helped me to accomplish this thesis. I wish to express my sincere gratitude to all of you. I especially want to thank:

Professor *Anders Kvanta*, my supervisor, for introducing me to the field of angiogenesis, for sharing your inspiring enthusiasm for science and for all the encouraging support throughout my scientific education.

Docent *Ingeborg van der Ploeg*, my co-supervisor, for your guidance, support and enthusiasm and for your encouragement that has enabled me to complete this thesis.

Professor *Stefan Seregard*, my co-supervisor and head of the Department of Vitreoretinal Diseases at St Erik's Eye Hospital. For sharing your interest in science and for your generosity with time and equipment.

Dr *Urban Eriksson*, head of the Department of Medical Retina at St. Erik's Eye Hospital, for your support and encouragement and for your generosity with time to write my thesis.

Professor *Bertil Fredholm*, Professor *Christer Betsholtz*, *Annika Armulik* and *Peter Lönnerberg*, co-authors and advisors in molecular biology. Your knowledge, assistance and spiritual conversations have been crucial to these studies.

*Monica Aronsson*, head of the animal department, for your competence and enthusiasm for research, for your skills with all the animals, for working early mornings, late evenings and week-ends and for becoming a very dear friend.

*Berit Spångberg*, *Margareta Oskarsson*, *Eva Vinter Wernersson* and *Emma Lardner* of the Ophthalmic Pathology and Oncology Service, St Erik's Eye Hospital for skillfully cutting and staining the numerous retinal sections required for this study.

Dr *Jiangmei Wu* for helping me with my first pictures in Photoshop.

The nurses, administrative staff and my colleagues at the Posterior Segment Department, St Erik's Eye Hospital: *Gunvor von Wendt*, *Anne-Catherine Söderberg*, *Helle Kalm*, *Gunilla Bjärnhall*, *David Epstein*, *Håkan Morén*, *Jürg Hengstler*, *Lena Ivert*, *Lars Hjelmkvist*, *Matthias Håkansson*, *Dimitrios Kournetas*, *Göran Olivestedt*, *Louise Bergman*, *Johann Gudmundsson*, *Lennart Berglin*, *Charlotta All Eriksson*, *Manoj Kakar* and *Emma Friling* for inspiring lunches and good laughs and for always encouraging me to carry on with my research despite it meant more work for you at the clinic.

All my dear friends that I haven't had time to meet for a long time.

My brothers, *Sten* and *Björn*, who are always giving a helping hand.

My father, *Arthur Sarman* and his wife *Viveca*, for all support and encouragement.

My mother, *Ingegerd Westlander*, for all support and encouragement and for help with taking care of my children at all times.

My children, *Martina*, *Johanne*, *Erik* and *Karin* for being the joy of my life.

My husband, *Johan*, for your never-failing support and love and for always being there for me.

The work published in this thesis was financially supported by grants from the Crown Princess Margaretas Foundation (KMA), the Edwin Jordans Foundation, the Karolinska Institute research grants, the Swedish Diabetic Society, Åke Wibergs Foundation, Sigvard and Marianne Bernadottes Research Foundation for Children's Eye Care, Stiftelsen Synfrämjandets Forskningsfond, Stockholm, Sweden, St. Erik's Eye Hospital Research Foundation, grants from the Swedish Medical Research Council, 2553 and 2039-04-3 and a grant from Austral University. Financial support was also provided through the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet.

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