CHAPTER 9

Research summary

DR is a clinically well-defined, sight-threatening, chronic microvascular complication that affects virtually all patients with diabetes mellitus. DR is characterized by gradually progressive alterations in the retinal microvasculature, leading to areas of retinal nonperfusion, increased vasopermeability, and in response to retinal non-perfusion, pathologic intraocular proliferation of retinal vessels. Most diabetes researchers and clinicians are aware of the major advances made in understanding the pathobiology of proliferative diabetic retinopathy. However, mechanisms underlying the progressive alterations in retinal microvessels, which precede and stimulate neovascularization are less well known. Elucidation of the mechanisms by which chronic hyperglycemia causes intraretinal vasoregression will provide new targets for pharmaceutical intervention, before irreversible retinal ischemia and secondary proliferative retinopathy necessitate damaging treatments such as panretinal laser photocoagulation.

As described in **chapter 1**, the primary and predominant pathological changes in the diabetic retinal microvasculature are the loss of pericytes and endothelial cells and the progressive occlusion of capillaries. The mechanisms causing vasoregression in the diabetic retina are complex and not completely elucidated. The specific biochemical changes that occur under diabetic conditions are generally discussed as inducer of apoptosis and destructive signaling pathways in pericytes and endothelial cells. However, growing evidence suggests that alternative mechanisms including activation of ligand-receptor systems that determine the fate of retinal capillaries during vascular development and maturation are also involved in pericyte loss during incipient DR.

Ang-2 is among the relevant growth factors induced by hypoxia and hyperglycemia and acts dependent on the VEGF environment either proangiogenic or vasoregressive. Ang-2 is upregulated at the angiogenic front of a growing vasculature and induces destabilization of endothelial cells via down-regulation of intercellular junction molecules and promotes endothelial cell proliferation in vitro. It is not known, however, what is the precise role of Ang-2 in physiological and pathological angiogenesis. In **chapter 2,** we showed that overexpression of Ang-2 in the retina enhances retinal vascular outgrowth and capillary density during physiological angiogenesis with pericyte coverage being reduced. Similarly, intraretinal vascular regrowth and preretinal neovascularization are increased in the model of oxygen-induced retinopathy, and newly formed vessels were also pericyte-deficient. In line with the notion that capillaries with reduced pericyte coverage are more susceptible to

angiogenic signals, Ang-2 overexpressing mice revealed endothelial cell proliferation and enhanced vascular sprouting. Together, these data from chapter 2 demonstrated that Ang-2 is an important regulator of pericyte recruitment and investment to the growing vasculature under physiological and pathological conditions, and pericyte deficient capillaries show enhanced angiogenesis and endothelial cell proliferation under Ang-2 overexpression in a hypoxic environment. Furthermore, our data indicate that in the mature retinal vasculature pericytes are particular sensitive to changes in Ang-2 expression.

Pericyte loss and vasoregression are the typical hallmarks of incipient DR. It is clear however, that these vascular lesions do not only occur in the diabetic retina. Such lesions can also be found in retinas from humans that are over 50 years of age. Glatt et al. demonstrated reduced numbers of pericytes and increased acellular capillary formation in the aging retinae of Sprague Dawley rats and Hughes and colleagues found reduced contacts between endothelial cells and pericytes in retinas of aging Wistar rats. In a comparative study between non-diabetic and diabetic rats, it has recently been demonstrated that the lesions in aged rat retinas have striking anatomical and histological similarities to those found in diabetic rat retinas. It is therefore conceivable that the mechanisms underlying age-related and diabetic retinal vasoregression bear similarities. It has been suggested that the age-related imbalance of pro- and anti-angiogenic factors may contribute to the vascular changes observed in aging and diabetic retinas. In **chapter 3**, we evaluated age dependent retinal vascular changes and alterations in gene expression of growth factors in healthy and in heterozygous Ang-2 deficient mice. Our data show that Ang-2 deficiency protects from age-related decrease of the number of endothelial cells and pericytes and significantly decelerated the formation of acellular capillaries. Moreover, gene expression analysis revealed that Ang-2 deficiency prevented the decrease of VEGF and Ang-1 protein observed in aged wild-type mice. These data suggest that the vasoprotective effect of Ang-2 gene dose reduction results from consequently higher levels of survival factors, such as VEGF and Ang-1, although the underlying mechanisms of this interaction remain to be examined. Furthermore, the results illustrates that the ratio of these factors is crucial for capillary cell survival in the mature vasculature.

Our previous work and the data generated in chapter 2 and 3 uncovered that Ang-2 is

linked to pericyte loss, thus plays an important role in diabetic retinopathy. Overexpression of human Ang-2 in the mouse retina during development resulted in reduced pericyte coverage and accelerated retinal angiogenesis. However, the further fate of retinal capillaries in this gain-of-function model is unknown, and a final proof for the causal role of Ang-2 in DR was still missing. In **chapter 4** we showed that overexpression of Ang-2 in retinas of healthy mice causes pericyte loss and the formation of acellular capillaries equivalent to the vasoregression found in diabetic mice. If the Ang-2 overexpressing mice are diabetic, a significantly worse vascular pathology is present compared to wild type counterparts. These results provide further evidence that Ang-2 plays a critical role in retinal vascular maintenance and in retinal vascular damage of early diabetic retinopathy by regulating pericyte coverage. Nevertheless, the mechanisms by which Ang-2 causes pericyte drop-out remained unclear.

Data from human and animal studies have suggested that diabetic pericyte loss is the result of apoptosis induced by activation of NFkB. However, the frequency of apoptotic pericytes detected in retinal digest specimens is too low to explain the amount of pericytes lost after several months of diabetes. Thus, other mechanisms than apoptosis must also be involved. The developmental origin and the morphological diversity of pericytes in retinal capillaries indicate that pericytes consist of a heterogeneous population. In **chapter 5** we determined the pattern of pericyte loss in the diabetic retina by performing quantitative retinal morphology in healthy and diabetic mice. Moreover, to investigate the impact of Ang-2 on pericytes, we examined healthy and diabetic retinas from mice with different levels of Ang-2 expression. We categorized pericytes into three classes, one being located at vessel branches („saddle pericytes“), another being located on straight parts of capillaries, and a third one showing different degrees of detachment from adjacent endothelial cells („migrating pericytes“). We show that saddle pericytes remained unaffected by diabetes, while pericytes on straight parts of capillaries were reduced in diabetic retinas. The decrease of pericytes on straight capillaries is paralleled by an increased number of migrating pericytes, exclusively detaching from straight capillary parts. Migrating pericytes from the saddle position were never observed suggesting that these pericytes might represent a distinct population from a different origin. In Ang-2 overexpressing non-diabetic animals, the numbers of migrating pericytes are also increased whereas in Ang-2 deficient non-diabetic mice, the numbers are reduced.

Together, the data from chapter 5 strongly support that migration of pericytes is an alternative mechanism contributing to the loss of pericytes in the diabetic retina, that involves the Ang-Tie ligand-receptor system.

Avoiding early damage in the diabetic retina, such as pericyte loss and vasoregression, is the goal of primary prevention of the disease. In chapter 6 to 8, we studied two promising therapeutic agents, Epo and carnosine, which are characterized by their vaso- and neuroprotective function. Recently, it has been reported that Epo plays an important role in the pathogenesis of proliferative diabetic retinopathy and experimental inhibition of EPO is as effective as inhibition of VEGF in the ROP model. However, the impact of Epo on vasoregression in experimental diabetic retinopathy has not been assessed yet. In **chapter 6** we show that low-dose Epo treatment of diabetic rats over 3 months, reduces oxidative stress, inhibits methylglyoxal-modification of retinal proteins and ameliorates pro-survival signals in the diabetic retinas. Furthermore, Epo treatment prevents Ang-2 upregulation paralleled by reduced pericyte loss and reduced glial activation. As described in the follow-up study in **chapter 7**, the formation of acellular capillaries and the loss of pericytes were significantly reduced in treated diabetic animals after 6 months of hyperglycemia, demonstrating that low-dose Epo treatment could sufficiently prevent the development of vasoregression in diabetic retinopathy. Of note, the increased levels of leukostasis, recently proposed to be involved in the pathogenesis of vasoregression in diabetes, were not affected by Epo treatment, suggesting that vasoregression in the diabetic retina can successfully be prevented without correcting leukostasis.

A number of reports demonstrated that the endogenous dipeptide carnosine suppresses the progression of secondary complications of diabetes, such as diabetic cataract, nephropathy and neuropathy. However, the therapeutic potential of carnosine in the treatment of DR has not been tested. In **chapter 8**, we supplemented diabetic rats with carnosine and examined the effect on biochemical, growth factor, vascular and neuroglial changes. We show that carnosine treatment prevented diabetic pericyte loss and acellular capillary formation. In contrast to the effects observed in Epo treated animals, the protective effect of carnosine is independent of ROS- and AGE-inhibition. However, vascular protection by carnosine was associated with normalized Ang-2 levels and increased expression of Hsp-27 in activated glial cells. The data indicate the importance of the neuroglial compartment of the retina in the pathogenesis of DR and suggest that Ang-2

expression in the diabetic retina might also be modulated independent of methylglyoxal. Furthermore, we demonstrate that carnosine treatment induces loss of photoreceptor cells. Together, the results show that vascular protection in the diabetic retina is achievable by targeting other factors than hyperglycemia-induced biochemical changes. Inhibition of Ang-2 upregulation and induction of protective Hsp might be effective therapeutic approaches in the treatment of diabetic retinopathy. Since retinal glial cells are a major source of Ang-2 and Hsp under diabetic conditions, it is apparent that the neuroglial retina should be considered as a therapeutic target to prevent vascular damage.

In summary, the results achieved in this thesis emphasize the importance of the Ang-2 in diabetic vasoregression. Beside direct hyperglycemic toxicity, the initiation and propagation of diabetic retinopathy strongly depends on firm pericyte coverage and the complex interplay between survival factors, such as VEGF, Hsp and other growth factors like the angiopoietins. Furthermore, recent evidence and data generated in this thesis introduced the neuroglial retina as a major target of hyperglycemic damage and a key component in the pathogenesis of incipient DR. Of note, neuroglial cells contribute substantially to the overall production of the above mentioned key factors of DR. Understanding of the interaction between neuroglial and vascular cells and the importance of factors released by these cells is of utmost importance for the development of novel and specific molecular drug targets with the potential to prevent or arrest the progression of intraretinal vasoregression in DR.