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Angiopoietin-2 Deficiency Decelerates Age-Dependent Vascular Changes in the Mouse Retina

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Key Words
Angiopoietin • Retina • Age • VEGF • Vascular regression

Abstract
Retinae of aged humans show signs of vascular regression. Vascular regression involves a mismatch between Angiopoietin-2 (Ang-2) and vascular endothelial growth factor (VEGF) expression. We used heterozygous Ang-2 deficient (Ang2LacZ) mice to evaluate murine retinal vascular changes and gene expression of growth factors. Vascular changes were assessed by quantitative retinal morphometry and gene expression levels of growth factors were measured by quantitative PCR. The numbers of endothelial cells and pericytes did not change in the Ang2LacZ retinae with age, whereas they decreased throughout the age spectrum studied in the wild type retinae. Moreover, vascular regression significantly decelerated in the heterozygous Ang2LacZ retinae (200% to 1 month), while the formation of acellular capillaries was significantly increased at 13 months in the wild type retinae (340% to 1 month). Gene expression analysis revealed that VEGF, Ang-1, PDGF-B and Ang2 mRNA levels were decreased in the wild type retinae at 9 month of age. However, the decrease of Ang-2 was smaller compared with other genes. While VEGF levels dropped in wild type mice up to 60% compared to 1 month, VEGF increased in heterozygous Ang-2 deficient retinae at an age of 9 months (141% to 1 month). Similarly, Ang-1 levels decreased in wild type mice (45% to 1 month), but remained stable in Ang2LacZ mice. These data suggest that Ang-2 gene dose reduction decelerates vasoregression in the retina with age. This effect links to higher levels of survival factors such as VEGF and Ang-1, suggesting that the ratio of these factors is critical for capillary cell survival.

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Introduction

Vascular changes have been first described in aging human retina by Kuwabara et al. [1]. Acellular capillaries (AC) were frequently observed in human retinae over the age of 50, and were neither associated with visual impairment nor with additional age-associated systemic disease. Glatt et al. performed quantitative analysis in aging retinae of Sprague Dawley rats [2]. They demonstrated reduced numbers of pericytes in rat retinae during aging, with the most decrement between 8 and 12 months. Furthermore, increased acellular capillary formation in the aging retinae was documented in this study [2]. Recently, Hughes and colleagues focussed on pericyte changes in the aging retina of Wistar rats. While the numbers remained unaffected, a reduced contact between endothelial cells and pericytes was noted [3].

Pericytes sitting on retinal capillaries are fundamental for vessel stabilization [4]. Angiopoietins (Ang) are involved in the interaction between endothelial cells and pericytes [5]. Ang1 induces pericyte recruitment and the subsequent stabilization of newly formed vessels during development [6]. In cooperation with VEGF Ang-1 enhances endothelial cell stabilization and reduces VEGF-induced vascular permeability [7-9]. Both Ang-1 and its natural antagonist Ang-2, bind to the endothelial specific receptor Tie2. While Ang-1 activates Tie2 phosphorylation, Ang-2 was identified as an antagonist, which blocks Ang-1-induced Tie2 phosphorylation by competitively binding to Tie2. Ang-2 is a context-dependent molecule in angiogenesis. Ang-2 together with abundant VEGF levels promotes angiogenesis, and with low or absence of VEGF induces vasoregression [6, 10]. Ang-2 has been characterized as a destabilization factor for vessels. Our previous studies have shown that injection of recombinant Ang-2 into the adult retina results in pericyte loss [11]. Ang-2 is upregulated prior to pericyte loss in diabetic retinopathy. Ang-2 deficiency prevents hyperglycemia-induced pericyte loss and reduces the formation of acellular capillaries (vasoregression) [11]. Genetically overproduced Ang-2 in the retina enhances angiogenesis [12]. Ang-2 can loosen the interaction of endothelial cells and pericytes, and hence promotes vascular leakage [13]. Another pericyte recruiting factor is PDGF-B, which plays an important role in pericyte coverage of newly formed vessels [14, 15]. PDGF-B deficient mice exhibit retinal vascular abnormalities, such as increased acellular capillary formation and increased pericyte loss, resembling diabetic retinopathy [16].

The mechanisms of age-related retinal vaso-regression are unknown. It has been suggested that the age-related imbalance of angiogenic and antiangiogenic factors may contribute to the vascular changes [17, 18]. According to the properties of Ang-2, we hypothesized that Ang-2 plays a central role in vasoregression of the aging retina. Therefore, we used heterozygous Ang2 deficient mice to examine the age-dependent vascular changes by quantitative retinal morphometry and the expression of angiogenesis-related genes by real time PCR.

Materials and Methods

Animals

Experiments were performed according to the guidelines of the statement of animal experimentation issued by the association for Research in Vision and Ophthalmology and were approved by the Institutional Animal Care and Use Committee.

Heterozygous Ang2LacZ mice, originally kindly provided by Regeneron Pharmaceutics, Inc. in New York, and their wild type littermates were used in this study. Mice were kept under a 12 hr light and 12 hr dark rhythm and were fed with a standard chow. Identification of positive transgenic mice was performed by genotyping as previously described [19]. Mice of different age were sacrificed for quantitative analysis of the retinal vasculature and of mRNA expression of angiogenesis-related genes. The eyes were enucleated and frozen immediately at -80°C until further analysis.

Retinal digest preparation

Retinal digest preparation was performed with retinae of 1, 5, 9, and 13 months old animals as previously described [11, 20]. In brief, eyes were fixed in 4% paraformadehyde for 48 hrs. Then, the retinae were dissected under an operating microscope. After incubation in aqua bidest for 30 min, the retinae were subjected to the digestion solution (3% trypsin dissolved in 0.2M Tris buffer) at 37°C for 3 hrs. The retinae were cut four times in the periphery and were transferred onto object slides to isolate retinal vasculature by dropping water. Finally, the isolated retinal vessels were air dried and stained with Periodic Acid Schiff (PAS) and hematoxylin.

Quantitative retinal morphometry

Quantitative retinal morphometry has been described previously [11, 21]. Briefly, analysis was carried out on the retinal digest preparations in a circular area in the middle third of the retina. Endothelial cells and pericytes were identified by shape of their nuclei and localization of nuclei in relation to the capillaries. Determination of numbers of endothelial cells and pericytes was undertaken in 10 randomly selected fields using an image analyzing system (CUE-2; Olympus Opticals, Hamburg, Germany), and numbers were standardized to the relative retinal area (numbers of cells per mm^2 of retinal area). Segments of acellular capillaries were counted in ten randomly

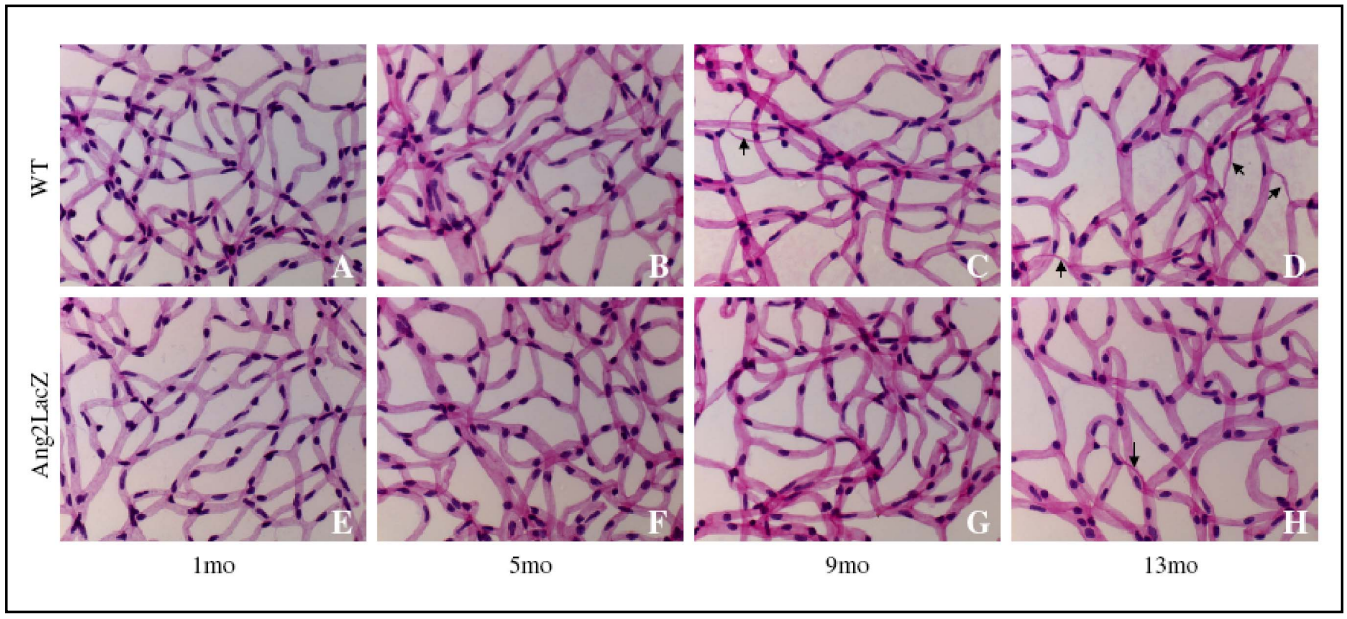

Fig. 1. Representative example of retinal vascular morphometry. Formation of acellular capillaries increases in the retina after 9 months in wild type retina, while decelerated formation occurs in heterozygous Ang-2LacZ retina. Arrows: acellular capillaries. A. B. C. D: wild type. E. F. G. H: heterozygous Ang-2LacZ. A and E: 1 month. B and F: 5 months. C and G: 9 months. D and H: 13 months. n=4-7. Original magnification: 400x.

selected fields with a integration ocular and numbers were normalized to each mm^2 retinal area.

Electron microscopy

For electron microscopy, retinae of 13-month-old mice were fixed for 4 hours in 2.5% glutaraldehyde (Paesel and Lorei, Hanau, Germany) in 0.1 M cacodylate buffer (pH 7.4). Specimens were washed in pure cacodylate buffer, postfixed overnight in 1% OsO4 in cacodylate buffer for 1h, dehydrated in ascending series of ethanol and propyleneoxide, bloc-stained in uranyl-acetate for 4 h and flat-embedded in Araldite (Serva, Germany). Using an ultramicrotome (Ultracut, Leica, Bensheim, Germany), semi- (1µm) and ultrathin sections (50 nm) were cut. Ultrathin sections were stained with lead citrate, mounted on copper grids and finally analysed with a Zeiss EM 10 (Oberkochen, Germany) electron microscope.

RNA isolation and quantitative PCR

According to the properties of Ang-2 and our previous studies, which demonstrated an altered regulation of Ang-2 much earlier than the morphological changes in the retina, analysis of RNA expression was undertaken in the retinae at 1, 3 and 9 months of age. Retinal RNA was isolated from individual retinae and homogenized in 1ml Trizol reagent (Invitrogen Karlsruhe, Germany) according to the manufacturer's instructions. Then, total RNA was purified utilizing the RNeasy Kit (Qiagen, Germany) with DNase-treatment to completely remove the genomic DNA according to the instructions of the manufacturers. Subsequently, RNA was reverse transcribed using Superscript II RNaseH-Reverse Transcriptase (Invitrogen Karlsruhe, Germany) and subjected to Taqman analysis using the Taqman 2xPCR master Mix (Applied Biosystems, Weiterstadt, Germany). The Mix contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive reference Rox and optimized buffer components. Quantitative PCR was performed in the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). The expression of genes was analysed by the Ct method using 18s ribosomal RNA as endogenous control. All primers were designed according to the specific sequences in the Genbank and purchased from IBA (Göttingen, Germany). Primers and FAM-labeled Tagman TAMRA probes were mouse VEGF forwards 5'-CGA GAT AGA GTA CAT CTT CAA GCC G-3' reverse 5'-TCA TCG TTA CAG CAG CCT GC-3' probe 5'-CTG TGT GCC GCT GAT GCG CTG-3'; mouse Ang-1 forwards 5'-CAA CAA CAG CAT CCT GCA GAA-3' reverse 5'-CTT TAG TGC AAA GGC TGA TAA GGT T-3' probe 5'-CAA CAA CTG GAG CTC ATG GAC ACA GTT CA-3'; mouse PDGF-B forwards 5'-GGT CAG CGC CGA GGG-3' reverse 5'-GCG GAT GGA GTG GTC GC-3' probe 5'-ATC CCA TTC CTG AGG AAC TGT ATG AAA TGC TG-3'; mouse Ang-2 forwards 5'-TGC GGA AAT CTT CAA GTC AGG -3' reverse 5'-CCT TGA TCT CCT CTG TGG AGT TG-3' probe 5'-CAC CAG TGG CAT CTA CAC ACT GAC CTT CC-3' mouse 18S forwards 5'-ACC ACA TCC AAG GAA GGC AG-3' reverse 5'-TTT TCG TCA CTA CCT CCC C-3' probe 5'-AGG CGC GCA AAT TAC CCA CTC CC-3'.

Statistical analysis

Data are given as mean ± SD unless otherwise stated. Data were evaluated by analysis of variance (ANOVA) with Bonferroni posttests to show differences between groups. A value of $p < 0.05$ was considered as statistically significant.

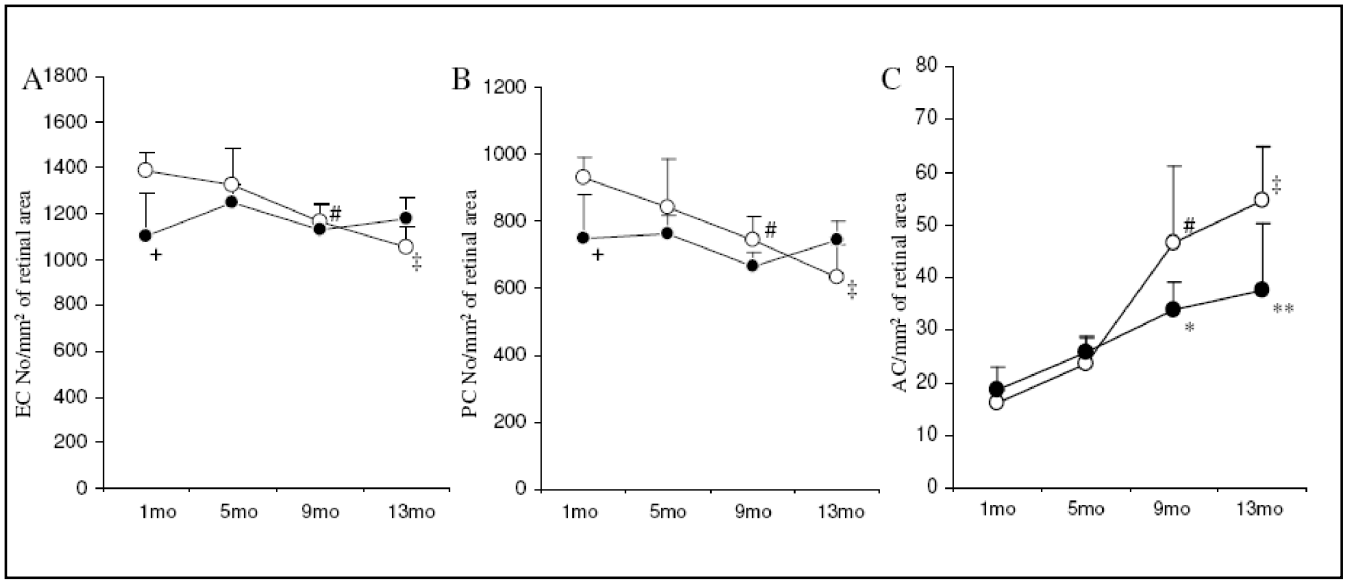

Fig. 2. Quantitative retinal morphometry of wild type (○) and Ang2LacZ (●) retinae at 1, 5, 9 and 13 months of age. Numbers of endothelial cells (A), pericytes (B) and acellular capillaries (C) are shown. n=4-7. + $p<0.05$, # $p<0.01$, ‡ $p<0.001$ vs. 1-month wild type retinae; * $p<0.05$ vs. 1-month Ang2LacZ retinae; ** $p<0.05$ vs. 1-month Ang2LacZ retinae and age-matched wild type retinae.

Results

Ang2 deficiency abrogates age-dependent vascular changes in the mouse retina

To investigate the effect of Ang-2 deficiency on vascular changes in the retina with age, we first assessed the vascular morphology in a time course between 1 and 13 months of age using digest preparations (Fig. 1). Ang2LacZ retinae showed decreased numbers of endothelial cells and pericytes at 1 month (EC: 1100±193/mm² of retinal area in Ang2LacZ vs. 1387±79/mm² of retinal area in wild type; PC: 747±130/mm² of retinal area in Ang2LacZ vs. 930±59/mm² of retinal area in wild type). As shown in Fig. 2A, capillary endothelial cell numbers decreased in the wild type retinae at 9 and 13 month of age compared to genotype-matched 1-month old retinae (9 months: 1165±83/mm² of retinal area; 13 months: 1050±91/mm² of retinal area), whereas Ang2LacZ retinae did not change in the numbers of endothelial cells throughout all time points studied (9 months: 1129±112/mm² of retinal area; 13 months: 1178±92/mm² of retinal area). Similary, pericytes reduced in the wild type retinae at 9 and 13 months compared to their genotype-matched 1-month old retinae (9 months: 745±68/mm² of retinal area; 13 months: 632±99/mm² of retinal area). Pericyte coverage at 9 and 13 months in Ang2LacZ was similar to its 1-month retinae as depicted in Fig. 2B (9 months: 663±43/mm² of retinal area; 13 months: 742±56/mm² of retinal area).

Second, acellular capillary segments were quantified to assess age-dependent vasoregression in this model (Fig. 2C). In wild type retinae, formation of acellular capillaries was increased significantly at 9 (46.7±14.5 AC/mm² of retinal area, $p<0.01$) and 13 months up to 340% (54.4±10.3 AC/mm² of retinal area, $p<0.001$) compared to the numbers of 1-month wild type (16.0±2.6 AC/mm² of retinal area). Ang-2 heterozygous retinae demonstrated similar numbers of acellular capillaries at 1 month and 5 months as the wild type retinae (1 month: 18.8±4.3 AC/mm² of retinal area; 5 month: 25.8±3.0 AC/mm² of retinal area). There was also a significant increase of acellular capillaries at 9 and 13 months compared to 1 month (9 months: 33.8±5.1 AC/mm² of retinal area; 13 months: 37.6±12.7 AC/mm² of retinal area, Fig. 1A-H, Fig. 2B), but the increase (up to 200% at 13 months) was significantly lower than in the wild type mice ($p<0.05$). Finally, we studied the ultrastructural morphology of endothelial cells and pericytes at 13 months by means of electron microscopy. No changes were observed regarding the physical contact between endothelial cells and pericytes, the viability or cytomorphological features such as organelle content or nucleus size, and thickness of the perivascular basal lamina (data not shown).

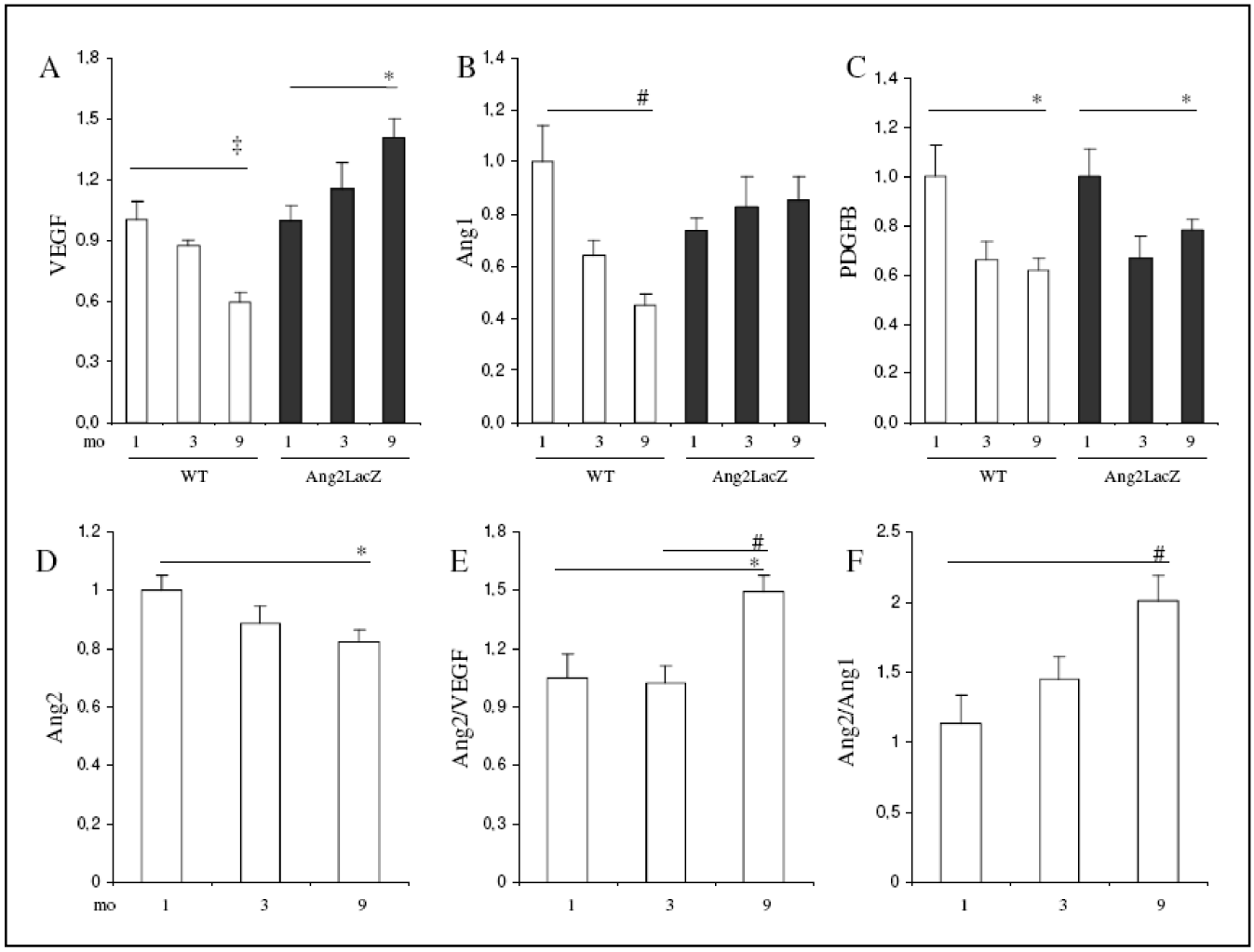

Fig. 3. Expression levels of VEGF, Ang-1, PDGF-B and Ang-2 in 1-, 3- and 9-month retinae. mRNA levels of VEGF (3A), Ang-1 (3B) and PDGFB (3C) were detected in wild type (□) and heterozygous Ang2LacZ (■) retinae. mRNA levels of Ang-2 (3D), ratio of Ang-2 to VEGF (3E) and Ang-1 (3F) were assessed in wild type retinae. Quantitative analysis was performed by real time PCR. Data represent the mean ± SEM for n = 6 individual retinae. Data are expressed in fold to 1-month wild type. * $p<0.05$, # $p<0.01$, ‡ $p<0.001$.

Association of survival factors in the retina with age

Given the role of growth factors in vessel survival, we further assessed the expression of survival factors in the retinae in relation to age. RNA expression of VEGF, Ang-1 and PDGFB was assessed from 1 to 9 months of age before severe vasoregression (at 13 months) occurs. In the wild type mice, survival factors were expressed at 1 month of age, i.e. at the end of retinal vascular development (Fig. 3A-C). VEGF, Ang-1 and PDGF-B RNA expressions were significantly reduced in wild type retinae at 9 months up to 60%, 45% and 62% compared to 1 month, respectively. Heterozygous Ang-2-LacZ mice expressed the same RNA level of VEGF as wild types at 1 month. However, RNA expression of VEGF increased with age in the heterozygous Ang-2LacZ mice and was increased to 141 % at 9 months of age compared with the expression level at 1 month (Fig. 3A). Ang-1 remained constant during the observation period in the heterozygous Ang-2LacZ mice (Fig. 3B). PDGF-B expression in heterozygous Ang-2LacZ mice remained similar to wild type mice at 1, 3 and 9 months, respectively (Fig. 3C). Moreover, we examined the expression of Ang-2 in wild type retinae. Compared with the survival factors VEGF and Ang-1, Ang-2 mRNA level was also significantly decreased at 9 months compared with the level at 1 month.

However, the reduction (18%) was smaller than that of other survival genes. The ratio of Ang-2 to VEGF and Ang-1 showed a significant increase at 9 months in the wild type retinae compared with the ratio at 1 month.

Discussion

In this study, we demonstrate a role for Ang-2 in age-dependent vasoregression. We show that Ang-2 deficiency decelerates the formation of acellular capillaries in the retinae with age as a sign of vascular involution and thus age-dependent regression. The improved vascular outcome is associated with augmented transcription of the retinal vascular survival factors VEGF and Ang-1.

Vascular regression involves a mismatch between Ang-2 and other angiogenic growth factors [6, 22, 23]. Ang-2, as a natural antagonist of Ang-1, plays a central role in destabilizing capillaries [24]. Consistent with this concept, our quantifications show that vascular cells are maintained and age-induced retinal vascular regression is decelerated in Ang-2 deficient mice. Our data demonstrate a simultaneous decrease of endothelial cell and pericyte numbers after 9 months of age in wild type retinae. The finding is in line with the results of Glatt et al. [2], suggesting that aging has an effect on endothelial cells survival and pericyte coverage in the retina. Association with a significant decrease in endothelial cell and pericyte numbers suggests impaired renewal of age-dependent accumulated cellular damage. We also observed a significant increase in vascular regression expressed by enhanced formation of acellular capillaries after 9 months in the wild type retinae, indicating a similar pattern of age-dependent vascular change in mouse and rat retina. These data show that vascular regression during age occurs by the combined loss of endothelial cells and pericytes. The finding of the protective effect of Ang-2 deficiency on vascular changes in aging is consistent with our previous observations showing that hyperglycemic vascular damage is partially prevented by heterozygous Ang-2 deficiency in experimental diabetic retinopathy. These data indicate the importance of Ang-2 as a risk factors-mediating molecule in aging as in diabetic retinopathy. An alteration of the contact between endothelial cells and pericytes was not noted in our study, probably because of species difference and/or a shorter observation period from 1 to 13 months compared to the study of Hughes et al. [3].

Expression data of survival factors in the wild type mice reveal that the regression of capillaries in the aging wild type retina is a result of relative decreasing expression of the survival factors VEGF, Ang-1 and PDGFB. Aging seems to be controlled by a balance between regression and survival factors. The regression of capillaries in the aging retina is associated with a shift in the balance of survival factors toward regression factors. VEGF is an essential growth factor for survival of endothelial cells, and Ang-1 plays a crucial role in the recruitment of pericyte to the capillaries and in endothelial maturation. Ang-1 can protect from vascular damage induced by VEGF in several animal models. Decreased expression of VEGF in wild type retinae with age is consistent with the presence of acellular capillaries in the retinae after 9 months of age. In agreement with the finding by Baffert et al. [25], who demonstrated age-related VEGF dependency of tracheal capillaries, a decreased expression of VEGF can be observed with age in our study, indicating age related sensitivity of vessels to VEGF. Increased age-dependent vascular regression seems to be predominantly related to the reduction of VEGF-mediated signaling in the retinae with age. Elevated expression of VEGF in Ang-2 deficient retinae with age suggests that VEGF contributes to improved retinal capillaries under Ang-2 deficiency. The expression of Ang-1 in Ang2LacZ retinae is maintained throughout the time points observed in the study. The level of Ang1 under Ang-2 deficiency may in combination with VEGF support survival of vascular cells. PDGFB expression levels do not change between groups, although it is changing over time. Since both, wild type and Ang2LacZ mice, have similar pericyte numbers after 13 months, it appears that PDGF-B and Ang-2 may function through two independent pathways in the retinae with advancing age. These data indicate that the decelerated vasoregression in the retinae of heterozygous Ang-2 mice links to higher levels of survival factors such as VEGF and Ang-1.

Pericytes can also act as survival promoters. Pericytes protect the endothelium and have a survival effect on endothelial cells [5]. Under certain conditions, pericytes can be lost or migrate away from capillaries, leaving vessels only with endothelial coverage facilitating damage of endothelial cells. Modified pericyte coverage in PDGF-B knockout mouse demonstrated that fewer pericytes sensitize retinal vessels to augmented hyperglycemic damage in experimental diabetic retinopathy [26]. Aging results in loss of 32% pericytes at 13 months compared to 1-month retinae. Loss of

pericytes in wild type aging retina indicates aging as an injury factor to pericytes. In Ang-2 deficiency, pericyte loss is protected during aging, therefore, exhibiting more stabilized vasculature with less vascular changes in aging retinae.

Together, our study suggests an important role for the Angiopoietin-Tie system in vascular aging.

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