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CHAPTER

6

Pericyte in the eye

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Abstract

Pericytes in the retina differ from pericytes in many other organs by their high density and their cooperative role in the neurovascular unit. Their diverse ontogeny and the fact that not one pericyte marker identifies the entire population suggest also functional plurality in the retina, including invading cells of mesenchymal origin. Further, to establish factors determining pericyte recruitment, modifiers of pericyte adhesion and homeostasis, such as notch-3 and angpt l-4, have been recently identified, expanding the understanding of pericyte function in the retina. Also, the role of pericytes as part of the neurovascular unit has been appreciated, given that the neuroglia determines pericyte survival and motility under disease conditions. Pericyte dropout is not unique in the diabetic retina, and non-diabetic animal models may prove useful in the search for mechanisms involved in disease-associated dysfunction of the neurovascular unit.

Introduction

The retina is the organ with the most abundant pericyte decoration. This abundance and the combination of accessibility and availability of mouse models have made the mouse retina a popular research tool for vascular biologists. As part of the neurovascular unit and as an important player in providing a tight blood–retinal barrier, the pericyte attracts interest from many disciplines. Pericyte loss in early diabetic retinopathy continues to direct attention to pericyte biology in general, as the mechanisms associated with diabetes and leading to pericyte loss are far from being understood.The pericyte remains a mysterious cell for several reasons: (a) the unclear ontogeny, (b) the lack of a pan-pericyte marker, and (c) the functional diversity of the different populations in the body. For the purpose of this review, it remains unclear to which extent pericytes in the retina represent pericyte biology in general and how much of the in vitro data from pericytes in culture apply to pericyte function in the retina. This review will focus on the pericyte in the retina as opposed to pericytes in other organs and emphasize distinctive features by which the retinal pericyte is characterized. Pericytes may control endothelial cell survival and proliferation, vascular tone, and permeability, and therefore emerge as a key player in microvascular homeostasis and response to injury.

Identification and ontogeny

Pericytes are regular components of capillaries in the retina [*17*]. They share a common basement membrane with endothelial cells, where they communicate with each other via physical contacts and paracrine signaling. In contrast to retinal arteries and arterioles, where the coverage consists of single or multilayers of vascular smooth muscle cells (vSMC), retinal capillaries are covered by individual pericytes. The density of pericyte coverage of capillaries varies from organ to organ, and the retina has been reported to host the highest relative ratio of pericytes to endothelial cells (1:1), even higher than the brain [*65*]. The ratio may be needed for the tightness of the blood–retinal barrier in the presence of a high blood flow [*63*]. During the early 1960s, Kuwabara and Cogan proposed a method to dissect the retinal vasculature from the neuroglia by the differential susceptibility of both compartments towards enzymatic digestions [*39*]. This method yields a definitive specimen for exact quantitative analysis of the ratio of endothelial cells to pericytes in different species, since pericytes are identified by the protuberant position within the capillary basement membrane and the shape [*12*] (Fig. 1).

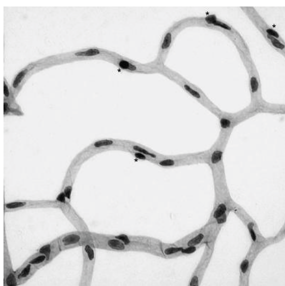

Fig. 1. Retinal digest preparation of a rat retina. *Asterisks* denote pericytes. PAS staining. Original magnification, ×200.

A major limitation in studying pericyte functionality and structure–function relation has been the lack of a pan-pericyte marker [*25*]. Recent reviews have delineated the counts of markers that are validated (PDGF-Rß, NG2, CD 13, ASMA, and desmin) and new markers with interesting but still incompletely known properties (RGS5, SUR2, Kir6.1, CD248) [*4*]. Several lineage-tracing studies have indicated that due to the multiple and mixed origin of mural cells, vascular cells may represent a mosaic [*47*]. In contrast to the mesenchymal origin of mural cells in many parts of the body, which are located at the outside of a vessel, most if not all mural cells in the CNS are derived from the neural crest, as summarized by Armulik et al. in a recent review [*4*]. However, as shown by Heglind et al., only brain surface vessels of mice with a ß-gal reporter under control of Foxs1 promoter depicted labeling of smooth muscle cells (SMCs) and pericytes [*32*]. Although the retina is an ontogenic diencephalic outpouch of the CNS, the cells labeled in the retina by the same marker were located in the outer nuclear layer, suggesting that these are not pericytes, but neurons. Pericytes which invade the retina during the formation of the primary retinal vascular plexus may be derived from two other potential sources. According to detailed studies in the mouse by Fruttiger, retinal vessels form through angiogenesis. In this process, pericytes co-invade the retina on an astrocytic template together with endothelial cells [*22*]. Their potential derivation from the bone marrow is demonstrated by using mice in which the GFP reporter is under control of the bone marrow-specific stem cell antigen-1 promoter (sca-1) [*45*]. Sca-1-positive cells were found to invade the retina on the extraluminal surface of the primitive vascular system, migrating to the angiogenic front and sites of vascular remodeling in physiological postnatal and pathological angiogenesis of the mouse retina suggesting that BM-derived cells contribute to pericyte recruitment to developing retinal capillaries (Fig. 2). Noteworthy, recruitment of NG2- and PDGFRb-positive pericytes from bone marrow has been demonstrated in postnatal angiogenesis in tumors [*57*]. The alternative origin of pericytes is the mesoderm as suggested by studies of Tidhar et al. They demonstrated that pericytes marked by a reporter construct under control of an adipose tissue-specific promoter (aP2) were present in the sprouting front of the developing retinal capillaries and in adult retinal capillaries (Fig. 3) [*69*]. This XlacZ4 mouse is widely used as a model to study the role of pericytes and SMC because it expresses a reporter gene under control of a pericyte/SMC-specific promoter. However, according to our analysis, only 52 % of retinal pericytes express the XlacZ4, and the expression is context dependent [*55*]. Common to all markers mentioned above is that they fail to recognize all pericytes at all stages [*5*]. In the human retinal vasculature which forms before birth, vasculogenesis dominates [*49*].

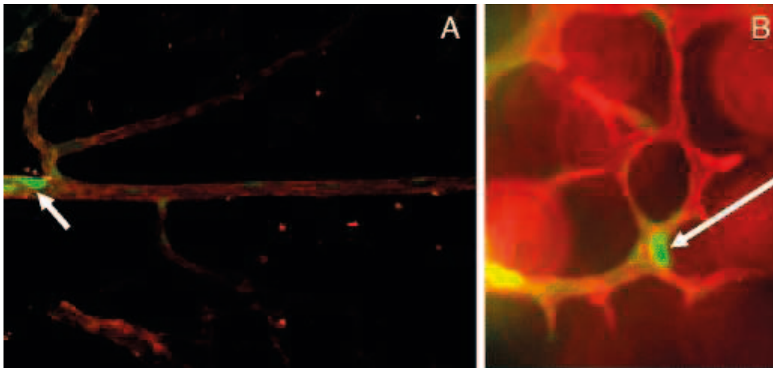

Fig. 2. Retinal whole mount preparations of a mouse with a sca-1-GFP transgene at postnatal day p5, counterstained with isolectin B4 [*red*]. **a** Partial overview depicting a GFP-positive perivascular cell in an arteriole [marked with a *white arrow*]. **b** GFP-positive cell in the vicinity of a sprouting capillary

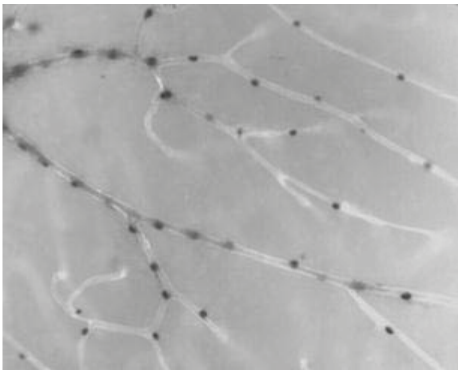

Fig. 3. A retinal whole mount preparation of an adult XlacZ mouse. LacZ-labeled cells are pinching out towards the midcapillary parts of the superficial capillary layers.

In general, pericytes can transdifferentiate into various other cell types. For instance, in the brain, pericytes can assume phagocytotic properties similar to macrophages or transdifferentiate into fibroblasts, and pericytes isolated from multiple other human organs including skeletal muscle, pancreas, adipose tissue, and placenta showed myogenic, osteogenic, chondrogenic, and adipogenic potentials [*6*, *13*, *19*, *71*]. Specific for the retina, pericytes are able to differentiate along the osteogenic pathway in vitro [*19*, *62*]. However, it is presently unknown whether retinal pericytes transdifferentiate into mesenchymal and neuroglial cells in the retina in vivo. The stromal vascular fraction (SVF) of bone marrow and adipose tissue harbors adhesive cells with high plasticity. These cells have been denoted

mesenchymal stromal cells but also as mesenchymal stem cells (MSC), based on their plasticity towards cartilage, bone, and fat cell differentiation. Moreover, these MSC can differentiate into SMC which, in vitro, are difficult to distinguish from pericytes [*14*]. In recent years, the origin of adipose-derived stem cells (ADSC) has been particularly well studied. The SVF-derived ADSC are derived either from the perivascular pericytes or from adventitial cells [*70*]. Adventitial cells lack pericytic markers such as NG-2, RGS-5, and PDGFRβ. The March lab has shown that endothelial cells interact with ADSC in co-cultures. In a study on tissue engineering of vascularized adipose tissue using endothelial cells, ADSC, and ADSC-derived adipocytes, the adipocytes physically interacted with and stabilized newly formed vascular structures [*66*]. The molecular phenotype of these pericyte-like adipocytes was, however, not dissected. On monolayers of ADSC, endothelial cells form sprouting networks that are comparable to sprouting networks on Matrigel [*50*]. Moreover, a number of studies have shown that ADSC and bone marrow MSC integrate into endothelial sprouting networks on Matrigel [*10, 70*]. We have shown that ADSC not only support formation of endothelial tubes, but that multicellular vascular structures are formed that are maintained for several weeks through interaction with ADSC (Przybyt et al., unpublished data). Solid in vivo evidence that ADSC can acquire a pericytic phenotype or even replace pericytes is scarce. However, we have shown in a murine model for retinal pericyte loss that ADSC can normalize pathologic capillaries. These ADSC acquired a typical pericytic position in the vasculature (Przybyt et al., unpublished data). This shows both that there exists a reciprocal plasticity between ADSC and pericytes and that ADSC are a promising tool to alleviate retinal pericyte loss. Yet, also endothelial plasticity might extend as far as a mural phenotype. We have shown that TGFβ can promote endothelial to mesenchymal transition [EndMT] of adult as well as precursor endothelial cells. The phenotype of the cells after EndMT was typically smooth muscle like [*51, 38*]. We have shown that conditioned medium of cells after EndMT promotes sprouting angiogenesis in a Tie-2-dependent fashion [*51*]. Yet, it remains to be confirmed that upon EndMT, endothelial cells acquire a genuine pericyte phenotype and integrate and stabilize newly formed vasculature. Zimmerlin and co-workers recently proposed a challenging hypothesis that pericytes are the major source not only of supra-adventitial arteriolar-derived ADSC, but also of circulating endothelial progenitor cells. This again emphasizes the high plasticity of all microvascular cellular constituents.

Pericyte recruitment signals

Developmental angiogenesis in the retina defines endothelial heterogeneity with an impact on the recruitment of pericytes and is subdivided into two major phases, sprouting angiogenesis where endothelial cells differentiate into tip and stalk cells (environment sensing/migration and proliferation), leading to an immature vascular plexus, and maturation, which involves recruitment of perivascular cells, arrangement of the basement membrane, and formation of junctions and transport systems [*15*].

Pericytes express PDGFRβ, and their attachment to vessels is dependent on PDGF released from endothelial cells [*43*]. During developmental angiogenesis, tip cells express more PDGF-B than stalk cells which is consistent with the observation that pericytes immediately co-migrate with the angiogenic sprout [*25*]. During this phase, the PDGF-ß receptor is most prominently expressed on pericytes, while it is not detectable in pericytes of the adult retina. Perinatal death of mice with a genetic ablation of PDGF-B or receptor PDGFR-ß precludes retinal studies. Studies in the brain of homozygous PDGF-B mice showed impaired pericyte recruitment and microaneurysm formation which is highly

reminiscent of early diabetic retinopathy [*43*]. In subsequent studies using heterozygous PDGF-B mice, it was indicated that recruitment of pericytes was defective and that a secondary effect of pericyte coverage on endothelial survival was observed in adult mice [*30*]. Mice with an endothelium-restricted ablation of PDGF-B revealed a dose-dependent effect on pericyte recruitment and vasoregression. Pericyte loss up to 50 % in brain capillaries was accompanied by capillary dropout in the retina, whereas pericyte deficits exceeding 50 % in the brain induced secondary proliferative retinopathy [*18, 68*].

A second receptor–ligand system exists which determines pericyte recruitment in the retinal microvessels. Current data suggest that angiopoietin-1 (Ang-1) is produced by pericytes in the retina which provides stabilizing signals to endothelial cells, thereby reducing permeability and promoting vascular maturation [*27,23*]. The reciprocal orientation of the Ang-Tie and the PDGFB/PDGF-ßR system with overlapping functional roles underlines the importance of a stable microvascular network in the retina [*23*]. Ang-1 signals via the receptor tyrosine kinase Tie2 whose signaling is differentially regulated through the natural antagonist angiopoietin-2 which can inhibit phosphorylation of Tie2, induced by Ang-1 [*46*]. Injection of recombinant Ang-2 into the vitreous of a non-diabetic rat reproduces pericyte dropout, and constitutive overexpression of Ang-2 in the posterior retina induces pericyte reduction in the adjacent deep capillary layer of the retina [*31*]. The expression of Tie2 may not be endothelial cell specific as evidence by Tie2 reporter mice which demonstrated Tie2 expression in pericytes of angiogenic vessels ([*9*]; HP Hammes and U Deutsch, unpublished). In turn, Ang-1 overexpression or absence of Ang-2 results in increased vascular branching and remodeling into larger capillary diameters on the arteriolar site of the network suggesting that the ratios of growth factors rather than absolute levels determine pericyte function in vivo [*21*].

Recently, another player of the angiopoietin family, angiopoietin-like-4 (ANGPTL4), was identified as having a role in pericyte recruitment, physiological and pathological angiogenesis, and permeability. ANGPTL4 knockout mice presented with a 20 % reduction in NG2-positive pericytes, a pertubation of caveolae, and adhesion molecules such as VE-cadherin and ZO-1, and substantially increased leakage of retinal capillaries. It was noted that ANGPTL4 is not involved in pericyte recruitment per se, but pericytes fail to spread on angptl4-deficient endothelial cells [*54*].

Yet another ligand which determines retinal pericyte investment is Notch3, which regulates arterial–venous differentiation within developing arteries. In the human cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy, Notch3 is mutated leading to degeneration of mural cells. In the retina of Notch3 knockout mice, pericyte apoptosis is increased, and the numbers of ASMA-positive pericytes are reduced [*40, 34, 59*]. Co-validation with the pericyte marker NG2 revealed a 58 % loss of mural cells [vSMCs and pericytes] in retinal vessels. Concomittantly, pericytic Ang2 expression was reduced in Notch3−/− mice indicating a mediator function, in particular since angiogenesis in the ROP model was reduced [*44*].

It is long known that TGF-ß, which is produced both in endothelial cells and in mesenchymal cells, is involved in the final stabilization of microvessels (see [*33*] for an overview). The collaborative expression and functional activation of TGF-ß requiring both cell divisions underscore the complexity of TGF-ß function which is further augmented by the cooperativity with other signaling molecules such as Notch. Since most of the models with a

genetic ablation of TGF-ß, its receptors, and the downstream signaling pathways are embryonically lethal, a distinct role in the retina is difficult to identify. In the brain, some link to reduced mural cell coverage in TGF-ß receptor signaling [SMAD-4] knockout mice has been identified [*41*].

Despite differences between the retina and brain with regard to the pericyte ontogeny and function, like the blood–brain barrier, the blood–retinal barrier integrity depends on tight inter-endothelial and endothelial–pericyte connections [*1*]. Of particular importance for the cohesion of adjacent capillary endothelial cells is VE-cadherin, while N-cadherin promotes the attachment of pericytes to the underlying endothelium and thereby ensures the integrity of BRB [*16*, *25*]. VE- and N-cadherin are regulated by S1P, a sphingolipid metabolite found in high concentrations in platelets and blood and on pericytes. Studies in mice in which the receptor for S1P is specifically deleted from endothelial cells demonstrated that S1P-receptor signaling in the endothelium is critical for the regulation of pericyte recruitment, attachment, and vascular maturation [*3*]. More recently, McGiure demonstrated that retinal pericyte-derived sphinogosine 1-phosphate increases the barrier properties of endothelial cells by induction of VE- and N-cadherin and downregulation of Ang-2 [*48*]. Whether pericyte-derived S1P and subsequent expression of N-cadherin are altered in the diabetic retina is not clear so far.

Pericytes in the neurovascular unit

Pericyte coverage of retinal capillaries and proper function of the blood–retinal barrier are closely linked. Like in the brain, the BRB consists of the endothelial lining of retinal capillaries, pericytes in basement membrane pockets, enwrapped by glial cells (astrocytes in the superficial layer and Müller cells in the deep capillary layer), neurons, and microglia around the superficial vascular layer [*11*]. In general, the tight association between retinal vascular and glial cells implies a close functional relationship between these two cell types and suggests that changes in one have profound consequences for the other. Like the brain, the retina contains glial cells, which are normally quiescent but adopt a reactive state during infection, injury, and also diabetes. In diabetic rats, activation of the principle glia cell, the Müller cell, induces the expression of a variety of angiogenic growth and trophic factors, cytokines, acute-phase response proteins, and other inflammatory genes, which can have an impact on retinal vasculature [*8*].

As mentioned above, tightness of the BRB depends on several factors including proper function of junctional molecules between endothelial cells, undisturbed heterotypic cell contacts between endothelial cells and pericytes, and regular perivascular cell homeostasis. These characteristics preclude passive transport of cells and proteins, but also of therapeutic agents from into retinal tissues. The dominant role in BRB tightness is played by the endothelium, and multiple components have been identified whose disturbance is associated with a breakdown of the blood retinal barrier (reviewed in [*11*]). The most prominent role for pericytes in this context relates to the production of basement membrane components, adhesion, and tight junction molecules, including proteoglycans, N-cadherin, claudins, JAMs, ZO 1 and 2, and occludin. These cells also express several barrier-related transporters like ABCG2, P-gp, MRP 1, and GLUT-1 suggesting an active role in NVU communication [*61*]. The strongest permeability-enhancing cytokine—vascular endothelial growth factor—does not cause changes in pericyte recruitment, indicating that permeability-enhancing mechanisms targeting the endothelium overrule mechanisms affecting pericyte survival

[*67*]. Pericytes can respond in vitro to inflammatory stimuli by production of cytokines such as interleukins, NO, and CCLs, at least in the brain, suggesting that, under disease conditions, pericytes can affect BRB patency [*37*]. Contractility of pericytes has been invoked as an essential property of the cells that determines capillary permeability [*26*, *56*]. Pericytes contain both smooth muscle and nonsmooth muscle isoforms of actin and myosin, however, with an uneven distribution within the pericyte population [*52*]. Several factors have been identified that regulate pericyte contractility. Among the vasoconstrictive cytokines and hormones are alpha 2-adrenergic agonists, cholinergic agonists, histamine, serotonin, angiotensin II, and endothelin-1, whereas beta-2 adrenergic agonists, NO, and atrial natriuretic peptide lead to a dilatation of the pericyte-covered capillaries [*60*]. As demonstrated in in vivo studies, pericytes in brain and retinal capillaries constrict in response to electrical stimulation, superperfusion with ATP and noradrenalin. These data suggest that pericytes control capillary blood flow in response to local modulation by vasoactive mechanisms [*53*]. However, Armulik et al. have expressed concern about pericytes controlling blood flow, which applies in particular to the retina [*4*]. The absence of in vivo data showing blood flow changes in retinal capillaries, the difficulty to unequivocally identify pericytes in vivo, and the physical constitution of midcapillary pericytes in the retina represent some of the concerns which have to be addressed by future research.

Lessons from animal models

Pericytes are lost during aging and in all diabetic models studied so far in our laboratory, using retinal digest preparations and quantitative retinal morphometry [*27*, *20*]. In rodent models, the pericyte dropout starts around 2 months after diabetes induction and thus precedes the development of acellular capillaries (vasoregression) by several months. Vasoregression is considered the most predictive diabetes-induced lesion in the retina apart from increased vascular permeability. However, since rodents do not have a macula, their type of capillary leakage may not directly correspond to the type of leakage in diabetic macular edema in humans.

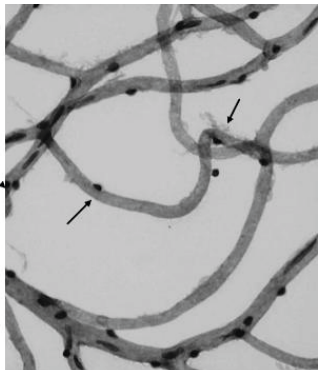

Fig. 4. Postmortem retinal digest preparation of a type 2 diabetic patient. Note the capillary area in which only pericytes have survived [marked by the *arrows*].

Pericyte dropout in diabetes occurs throughout the entire retina at a comparable extent, indicating a causal link to the metabolic changes that appear in diabetes [*35*]. As the perimacular retinal network is particularly sensitive to increased leakage, the question was raised whether pericyte dropout occurs preferentially in this area. However, preliminary studies in retinae of human type 2 patients revealed that pericytes drop out in the perimacular network at the same level as in the rest of the retina (R Schlingemann, I Klaassen, and HP Hammes, unpublished).

The onset and time course of pericyte loss in the human aging and diabetic retina are unknown as appropriate tissue samples are missing. However, in rodent animal models of DR, either spontaneously diabetic or by chemical induction, a significant loss of pericytes occurs already after 2 to 3 months depending on the strain used and severity of hyperglycemic metabolism. After 6 months of experimental diabetes, pericyte numbers are reduced by 15–50 % (Pfister et al., unpublished observations; [*36*, *31*]), and other typical morphological signs of DR, such as acellular capillaries, develop [*27*]. The underlying causes and mechanisms of early pericyte dropout in DR and the aging retina remain still unclear.

On one side, biochemical alterations, such as increased production of ROS, AGEs and the activation of biochemical pathway as the hexosamine pathway and the PKC pathway, directly damage vascular and nonvascular cells of the retina [*28*]. However, there is only little information whether and how retinal microvascular pericytes in situ are specifically damaged by hyperglycemia, and reports about hyperglycemia-induced apoptosis are contradictory. Activation of pro-apoptotic signaling and apoptosis in pericytes, but also in endothelial and neuroglial cells, has been documented in diabetic retinas of rodents and patients [*29*, *42*,*58*]. Biochemical changes, such as intracellular ROS, AGE, and other aforementioned bioactive intermediates of glycolysis, are themselves harmful for cells when present in abundance, and activation of downstream targets of these biochemical changes, such as RAGE, PKC, and NF-κB, might contribute to the activation. In vitro, AGEs can induce dose- and time-dependent apoptotic effects on pericytes, and it has been recently demonstrated that AGEs, like tumor necrosis factor TNF-α, induce pericyte apoptosis through activation of the transcription factor FOXO1, mediated in part by p38 and JNK MAP kinases [*2*]. Repeated injection of high doses of AGE-modified proteins can induce selective pericyte loss in non-diabetic rats after 2 weeks [*73*]. Moreover, endogenous AGEs can accumulate in pericytes [*7*]. It is thus speculated that pericytes take up AGEs from the circulation or from the direct vicinity suggesting a clearing function under specific conditions. Since the tissue load with AGEs changes over time in the aging retina and diabetes, the role of pericytes as AGE removing cell compartment becomes increasingly relevant. However, the time course of AGE accumulation in pericytes is inconsistent with the time course of pericyte loss in diabetes, indicating that AGEs are not solely responsible for pericyte loss. Furthermore, NF-κB activation and increase of extracellular matrix protein βIG-H3 is regarded as key mediator for retinal cell apoptosis, but more recently, it has been demonstrated that hyperglycemia persistently activates PKC-δ and p38 mitogen-activated protein kinase [MAPK] leading to pericyte apoptosis and vasoregression independently of NF-κB [*24*]. Altogether, a number of possible mechanisms have been suggested to explain vascular cell apoptosis in the diabetic retina. It is clear, however, that the frequency of apoptotic pericytes in retinal digest specimens is low and does not explain the number of pericytes lost after several months of diabetes. Alternatively to early pericyte loss in diabetic retinopathy being the result of hyperglycemic injury, a different concept was proposed. It is possible that pericyte loss is an active process involving migration of pericytes away from the

capillaries, driven by the angiopoietin-Tie system. As described above, gain of function experiments in non-diabetic animals revealed the induction of pericyte dropout in the vicinity of the Ang-2 overexpressing site. Superimposition of diabetes aggravated the most important vascular readout, i.e., the formation of acellular capillaries. Loss of function studies in the presence of diabetes yielded the prevention of pericyte dropout and the reduction of acellular capillary formation [*55*]. Ang-2 is expressed in three cell types of the retina, i.e., the endothelial cell, the Müller cells, and the horizontal cells. In situ hybridization of diabetic retinae with Ang-2 yielded the expression particularly in Müller cells. The regulation of Ang-2 in chronic hyperglycemia has been recently addressed, and it was found that increased glucose flux in renal microvascular endothelial cells caused increased modification of the corepressor mSin3A by methylglyoxal resulting in recruitment of the enzyme O-GlcNAc transferase to an mSin3A-Sp3 complex. Subsequently, Sp3 modification by O-linked *N*-acetlyglucosamine decreased its binding to a glucose-responsive GC box in the Ang-2 promoter and the activation of Ang-2 transcription [*72*]. The same mechanism was operative in retinal Müller cells consistent with in vivo data from retinae of diabetic rats and mice. These data are consistent with the novel hypothesis, i.e., that pericyte loss is actively induced by glial cells overexpressing Ang-2 in response to high glucose.

Future questions

From the above, it shines through that the retina and its pericytes may be different from other tissues, given the facts about ontogeny, relative density on the capillaries, and communication within the neurovascular unit. Even more complexity is added in disease, in particular when considering two recent observations. Simonavicius et al. reported that pericytes are able to promote endothelial cell death in an endosialin-dependent fashion, suggesting that pericytes have opposing effects on endothelial cells [*64*]. In line with this study in preclinical models of physiological and pathological angiogenesis, we observed in human retinae of type 2 diabetes capillary areas which were devoid of endothelial cells, but not of pericytes (Fig. 4). This strongly votes for a differentiated view on pericyte pathobiology in the retina.

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