Pericytes and the Pathogenesis of Diabetic Retinopathy

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Pericytes provide vascular stability and control endothelial proliferation. Pericyte loss, microaneurysms, and acellular capillaries are characteristic for the diabetic retina. Platelet-derived growth factor (PDGF)-B is involved in pericyte recruitment, and brain capillaries of mice with a genetic ablation of PDGF-B show pericyte loss and microaneurysms. We investigated the role of capillary coverage with pericytes in early diabetic retinopathy and the contribution to proliferative retinopathy using mice with a single functional allele of PDGF-B (PDGF-B$^{+/-}$ mice). As assessed by quantitative morphometry of retinal digest preparations, pericyte numbers in nondiabetic PDGF-B$^{+/-}$ mice were reduced by 30% compared with wild-type mice, together with a small but significant increase in acellular capillaries. Pericyte numbers were reduced by 40% in diabetic wild-type mice compared with nondiabetic wild-type controls. Pericyte numbers were decreased by 50% in diabetic PDGF-B$^{+/-}$ mice compared with nondiabetic wild-type littermates, and the incidence of acellular capillaries was increased 3.5-fold when compared with nondiabetic PDGF-B$^{+/-}$ mice. To investigate the effect of pericyte loss in the context of ongoing angiogenesis, we subjected mice to hypoxia-induced proliferative retinopathy. As a result, PDGF-B$^{+/-}$ mice developed twice as many new blood vessels as their wild-type littermates. We conclude that retinal capillary coverage with pericytes is crucial for the survival of endothelial cells, particularly under stress conditions such as diabetes. At high vascular endothelial growth factor levels, such as those in the retinopathy of prematurity model, pericyte deficiency leads to reduced inhibition of endothelial proliferation in vivo. *Diabetes* 51:3107–3112, 2002

Microvascular mural cells are referred to as pericytes (1). Several factors are thought to be involved in pericyte recruitment during vascular development and maintenance, including angiopoietin-1 and its receptor tyrosine kinase Tie-2, vascular endothelial growth factor (VEGF)-A and its receptor flk-1, tissue factor, and the platelet-derived growth factor PDGF-B/PDGF-receptor β system (2–6). PDGF-B is critically involved in the recruitment of pericytes to a variety of vascular beds such as brain, kidney, heart, lung, and adipose tissue (7). Studies using PDGF-B– and PDGF-receptor β–deficient mice lead to the concept that mesenchymal progenitor cells are initially recruited around vessels through PDGF-independent mechanisms, while subsequent sprouting involves PDGF-B–dependent comigration/proliferation events (8). Specifically, these mice lack microvascular pericytes in brain vessels and form capillary microaneurysms, which cause cerebral hemorrhages during late gestation (9,10).

Pericyte loss and microaneurysm formation are also hallmarks of early changes in the retinae of diabetic patients (11). After induction of diabetes in rodents, reduction of pericyte numbers in retinal capillaries is the earliest morphological change, followed by the formation of increased numbers of acellular-occluded capillaries, occasional microaneurysms, and thickening of the vascular basement membrane (12). With progressive vascular occlusions in the human diabetic eye, the retina responds with either a progressive increase of vascular permeability, leading to macula edema, or the formation of new vessels that finally proliferate into the vitreous (13). Diabetes accounts for most cases of legal blindness during working age (14).

The cause of pericyte loss during early diabetic retinopathy is unclear. One hypothesis relates to the pericytic accumulation of toxic products such as sorbitol or advanced glycation end products (AGEs) (15,16). Pericyte loss is considered a prerequisite of microaneurysm formation, possibly by local weakening and subsequent outpouching of the capillary wall. In vitro, pericytes control endothelial cell proliferation (6,17), suggesting that pericyte loss may be involved in the pathogeneis of proliferative diabetic retinopathy. However, pericyte loss also occurs in diabetic rodent models, in which proliferative retinopathy is absent. This suggests that pericytes may also control endothelial function during the initial period of nonproliferative diabetic retinopathy.

Several lines of evidence indicate that chronic hyperglycemia is the major cause of vascular endothelial cell injury and that survival/repair mechanisms for vascular cell

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AGE, advanced glycation end product; PDGF, platelet-derived growth factor; ROP, retinopathy of prematurity; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

injury are activated in the early stages of the disease. Consistent with this concept, we have demonstrated upregulation of VEGF and its receptors flk-1 and flt-1 in diabetic rats in the absence of retinal neovascularization (18). The role of early pericyte loss in this setting remains obscure. Because PDGF-B null mice exhibited characteristic lesions in brain capillaries but were not viable, we speculated that a heterzygous deletion of PDGF-B may lead to a gradual pericyte deficit in retinal capillaries, allowing the key question to be assessed: whether pericyte reduction is sufficient to cause capillary pathology similar to that observed in a diabetic retina. Furthermore, two models of different periods of retinal damage in the course of diabetic retinopathy were subsequently used: *1*) the streptozotocin-diabetic model of early retinal damage by hyperglycemia, and *2*) the model of oxygen-induced proliferative retinopathy (resembling human retinopathy of prematurity [ROP]), undergoing the same critical steps of retinal neovascularization as proliferative diabetic retinopathy.

Morphometric parameters of early diabetic retinopathy (pericyte loss and formation of acellular occluded capillaries) were comparatively assessed in streptozotocin-diabetic and nondiabetic PDGF-B$^{+/-}$ mice and their nondiabetic and diabetic wild-type counterparts. The angiogenic responsiveness of retinal capillaries to ischemia was investigated during hypoxia-induced retinal neovascularization in the mouse, which is a model of ROP.

RESEARCH DESIGN AND METHODS

Experiments performed in this study adhered to the Association for Research in Vision and Opthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Animals. Mice with a heterozygous deletion of the PGDF-B gene were used in the study together with wild-type mice of the identical genetic background (C57Bl/6-sv129 hybrids). The gene-targeting strategy and the phenotypes of the PDGF-B null mice have been described earlier (5,9). Accordingly, identification of the PDGF-B status was performed by PCR, as described (5).

Six-week-old mice (weighing 22–26 g, postprandial blood glucose 7.2 ± 0.4 mmol/l) were rendered diabetic by intravenous injection of 100 mg/kg body wt streptozotocin (Roche, Mannheim, Germany). Glucose levels and body weight were monitored consecutively and HbA_{1c} was determined by affinity chromatography (Glyc Affin; Isolab, Akron, OH). Diabetic mice were kept for 6 months, and NPH insulin (Aventis, Bad Soden, Germany) was occasionally given to individual mice to prevent critical weight loss. Age-matched nondiabetic mice served as controls.

Quantitative retinal morphometry. To determine the existence of pericyte loss in PDGF-B$^{+/-}$ mice and the degree of pericyte loss in diabetic mice, retinal digest preparations were used in nondiabetic and diabetic wild-type mice and nondiabetic and diabetic PDGF-B$^{+/-}$ mice (n = 5 for each group). Retinae were obtained after enucleation of the eyes from the animals under deep anesthesia and immediately fixed in 4% buffered formalin. Retinal vascular preparations were performed using a pepsin-trypsin digestion technique as previously described (19). Briefly, a combined pepsin (5% pepsin in 0.2% hydrochloric acid for 1.5 h)-trypsin (2.5% in 0.2 mol/l Tris for 15–30 min) digestion was used to isolate the retinal vasculature. Subsequently, the samples were stained with periodic acid Schiff. The total number of pericytes was counted in 10 randomly selected fields of the retina using an image analyzing system (Systat; Olympus Opticals, Hamburg, Germany), and the numbers were normalized to the relative capillary density (numbers of cells per millimeter squared of capillary area).

The numbers of acellular-occluded segments were counted according to an established method in 10 randomly selected fields to assess the degree of endothelial cell damage under normal and pathological conditions. Samples were evaluated in a masked fashion.

Immunohistochemistry. To determine and confirm the quantity of pericyte loss in nondiabetic PDGF-B$^{+/-}$ mice, sections from paraffin-embedded eyes were stained with a polyclonal anti–PDGF-receptor β antibody (1 μg/ml) (Santa Cruz Biotechnology, Heidelberg, Germany), and immunohistochemistry was performed using the ABC-kit (Vectastain; Linaris, Wertheim, Germany).

Retinal neovascularization in PDGF-B$^{+/-}$ mice. First, we determined the magnitude of pericyte recruitment deficiency in mice at postnatal day 7, i.e., the day at which the exposure to high oxygen starts (ROP model, see below). For proper assessment of pericyte numbers, we used a new mouse model in which retinal pericytes express the LacZ reporter gene (X-LacZ4 mice [20]). The density of Lac-Z–positive cells was assessed in whole-mount retinal preparations. Eyes were obtained from wild-type (n = 5) and PDGF$^{+/-}$ mice (n = 4), which had been crossed into X-LacZ4 mice under deep anesthesia, and fixed in a solution containing 0.2% glutaraldehyde and 1.5% formaldehyde. After 24 h, eyes were opened by equatorial incision, the lens removed, and the retina excised, flattened by peripheral incision, and placed on a glass slide. LacZ staining was performed as described (20). The density of X-lacZ4–positive cells was assessed by counting the numbers of stained cells per microscopic field (an average of 12 fields was counted per retina) randomly selected from the retinal periphery (inside the photo frame) at a 40× magnification. Areas that included larger vessels were excluded from analysis. Analysis was done while blinded to the identity of the samples.

To study the propensity of the retinal vasculature to respond to a hypoxic stimulus, newborn mice were subjected to the model of hypoxia-induced retinal neovascularization resembling human ROP (21,22). Briefly, 7-day-old mice were exposed to 75% oxygen for 5 days with their nursing mothers in an incubator (Stuart Scientific, Redhill, U.K.). At day 12, the mice were returned to room air. To exclude that wild-type and PDGF-B$^{+/-}$ mice differ in hyperoxia-induced occlusions of central retinal vessels, we performed fluorescein angiograms at day 12 as described (22).

At day 17, eyes were enucleated under deep anesthesia, fixed in formalin, and the numbers of new vessel nuclei at the vitreous side of the inner limiting membrane were counted in 10 sections of each mouse eye (n = 10 eyes/group). Samples were quantitated without knowledge of their identity.

Data are given as means ± SD (diabetic model) and means ± SE (ROP model). Differences between groups were tested using the alternate Welsh t test.

RESULTS

To assess the effect of a monoallelic deficiency of PDGF-B on pericyte coverage and retinal pathology, we used retinal digest preparations from PDGF-B$^{+/-}$ and age-matched wild-type mice with the identical genetic background.

Pericyte numbers in PDGF-B$^{+/-}$ mice were reduced by ~30% (normal wild-type 2,350 ± 75 cells/mm^2 of capillary area vs. normal PDGF-B$^{+/-}$ 1,680 ± 140, $P < 0.001$) (Fig. 1). We examined whether the loss of pericytes by quantitative retinal morphometry correlated with the immunohistochemical deficiency of PDGF-receptor β–positive vascular profiles in nondiabetic PDGF-B mice versus wild-type controls. We found approximately threefold fewer labeled capillary profiles in PDGF-B$^{+/-}$ mice than in controls (PDGF-B$^{+/-}$ 2.6 ± 0.5 vs. wild type 8.9 ± 2.3, $P < 0.001$). Concomitant with the reduction in pericyte numbers, PDGF-B$^{+/-}$ mice also showed a slight but significant increase in the numbers of acellular capillaries compared with their nondiabetic wild-type littermates (PDGF-B$^{+/-}$ 29.25 ± 6.95 acellular capillaries/mm^2 of retinal area vs. 19.33 ± 1.53, $P < 0.05$) (Fig. 2). Despite the reduction of pericyte numbers, no further abnormalities in the capillary network were observed; most notably, there were no signs of microaneurysms or vasodilatations in the capillaries. Additionally, arterioles and venules appeared normal.

Next, we combined hyperglycemia with heterozygous PDGF-B deletion and studied the retinal phenotype in these mice.

Nondiabetic PDGF-B$^{+/-}$ mice had blood glucose levels identical to wild-type mice, but had a 13% lower body weight (Table 1). The average glucose values were 7.61 ± 1.03 mmol/l in nondiabetic wild-type mice and 7.34 ± 1.73

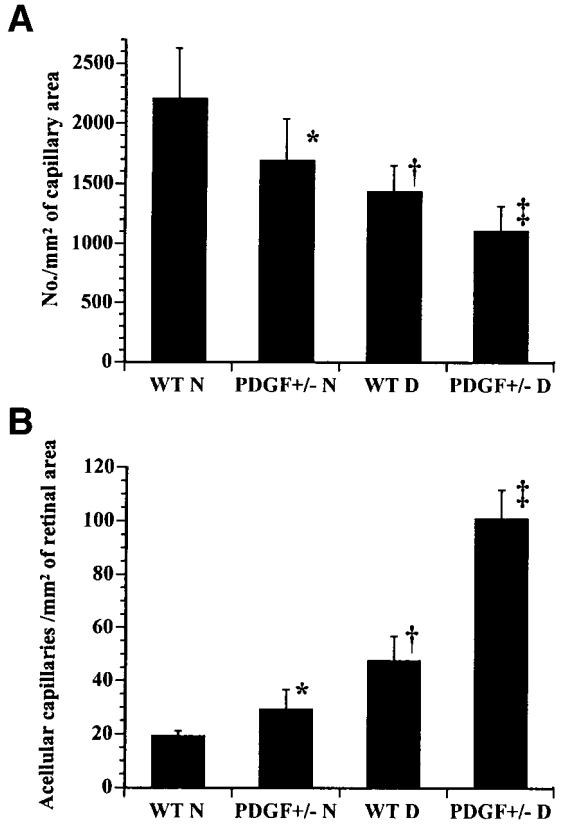

FIG. 1. Quantitative retinal morphometry of normal wild-type (WT N), diabetic wild-type (WT D), normal PDGF-B$^{+/-}$ (PDGF+/− N), and diabetic PDGF-B$^{+/-}$ mice (PDGF+/− D). Shown are numbers of pericytes (*A*) and acellular capillaries (*B*). *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ vs. nondiabetic wild-type mice.

mmol/l in PDGF-B$^{+/-}$ mice during the studies. Diabetes induction led to equivalent degrees of glycemia (mean glucose levels throughout the study: diabetic wild-type mice 27.07 ± 5.86 mmol/l; diabetic PDGF-B mice 26.66 ± 8.62 mmol/l), as indicated by blood glucose and HbA_{1c}, but in contrast to wild-type mice, PDGF-B$^{+/-}$ mice did not gain weight despite equivalent insulin substitution in both groups (data not shown). Wild-type control mice had slightly but significantly higher random blood glucose levels than PDGF-B$^{+/-}$ mice but equal concentrations of HbA_{1c}. Given these data, it was justified to assume similar degrees of glycemia in the experimental settings (normal wild-type versus normal PDGF-B$^{+/-}$ mice and diabetic wild-type versus diabetic PDGF-B$^{+/-}$ mice). Histological examinations of various organs did not reveal any obvious abnormalities in the PDGF-B$^{+/-}$ mice that would explain the difference in body weight compared with wild-type mice (not shown).

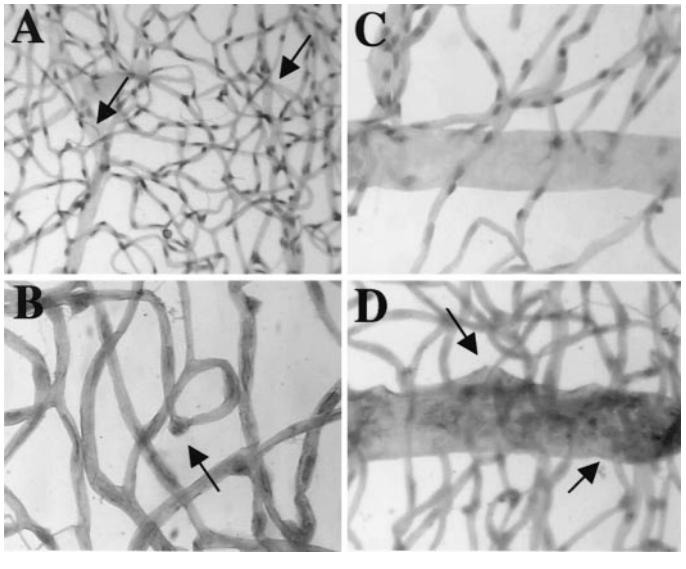

FIG. 2. Acellular capillaries (*A*), microaneuryms (*B*), and irregularities of venoles (*D*) in diabetic PDGF-B$^{+/-}$ mice. *C*: A venolar region of a diabetic wild-type mouse. Original magnification: *A*: 100×; *B*– *D*: 250×.

Consistent with previous data in other diabetic rodent models, a quantitative loss of pericytes was observed in wild-type diabetic mice (1,428 ± 216 cell/mm^2 of capillary area, $P < 0.001$ vs. nondiabetic wild-type mice). The combination of diabetes and heterozygous PDGF-B deletion led to a reduction of pericyte numbers by >50% compared with nondiabetic wild-type mice (1,108 ± 203 cell/mm^2 of capillary area, $P < 0.01$ vs. nondiabetic PDGF-B$^{+/-}$) (Fig. 1*A*).

Diabetes also led to a 2.5-fold increase in the amount (or numbers) of acellular capillary segments in wild-type mice. These occluded segments were uniformly distributed throughout the entire retina. No further abnormalities were found in the capillaries; in particular, formation of microaneurysms was not observed.

Diabetic PDGF-B$^{+/-}$ mice had the most significant lesions in the capillary network of all animal groups studied. Compared with their nondiabetic counterparts, they had a 3.5-fold increase in the numbers of acellular capillaries (101 ± 10.44 acellular capillary segments/mm^2 of retinal area) (Fig. 1*B* and 2*A*), and the formation of small capillary microaneurysms was occasionally recorded (Fig. 2*B*).

Besides these capillary abnormalities, we observed a considerable variation in the width of the retinal venoles. In comparison with diabetic wild-type mice, the venolar index, defined as the ratio between the smallest and the largest vessel diameter at corresponding distances from the retinal center, was 0.92 ± 0.03 in diabetic wild-type mice and 0.77 ± 0.05 in diabetic PDGF-B$^{+/-}$ mice ($P < 0.001$) (Fig. 2*C* and *D*).

In the early postnatal growing mouse, the retina is susceptible to variations in oxygen tension, which is used experimentally to induce retinal neovascularization in the mouse ROP model. We applied this model to examine whether the potential of neovascularization was modified in PDGF-B$^{+/-}$ mice. Beforehand, it was studied whether PDGF-B$^{+/-}$ mice differed from wild-type mice in pericyte numbers at day 7, i.e., the day at which exposure to high oxygen started. Pericytes were identified in these mice by introducing a pericyte-specific LacZ reporter gene through crossbreeding with a newly established mouse line (20). Pericyte numbers were 28.06 ± 3.30 cells/field in wild-type mice and 20.27 ± 3.33 in PDGF-B$^{+/-}$ mice ($P < 0.01$).

Qualitatively, the central avascular areas induced by hyperoxia were comparable between PDGF-B$^{+/-}$ and wild-type mice as assessed by fluorescein angiography at day 12 (not shown). In the ROP model at day 17, wild-type mice exposed to high oxygen for 5 days and then exposed to room air for another 5 days had 28.4 ± 3.7 neovascular nuclei/section. In PDGF-B$^{+/-}$ mice, the neovascular re-

TABLE 1
Physical and metabolic parameters of the animals studied

Parameter	At Start		At Conclusion			
	Wild type	PDGF-B$^{+/-}$	Normal wild type	Diabetic wild type	Normal PDGF-B$^{+/-}$	Diabetic PDGF-B$^{+/-}$
Body weight (g)	25.6 ± 2	22.5 ± 1.76	38.3 ± 6.3	28.5 ± 2.65	37.2 ± 6.5	22.5 ± 4.3
Blood glucose (mmol/l)	7.3 ± 0.38	7.25 ± 0.44	8.9 ± 1.80	26.1 ± 5.02	7.3 ± 0.22	24.4 ± 10.38
HbA_{1c}	—	—	5.1 ± 0.55	14.5 ± 1.88	5.0 ± 0.33	13.2 ± 1.46

Data are means ± SE.

sponse to hypoxia had nearly doubled (53.2 ± 5.4, $P <$ 0,01) (Fig. 3).

DISCUSSION

Pericyte recruitment is essential for the maturation of the developing vasculature. We observed that a 50% reduction in PDGF-B gene dosage is sufficient for a significant reduction in pericyte coverage of retinal capillaries and a moderate but significant increase in the numbers of acellular capillaries in the retina. Thus, the histological features of PDGF-B$^{+/-}$ mice resemble, in part, the very early lesions in a diabetic retina. In the presence of chronic hyperglycemia, PDGF-B$^{+/-}$ mice not only develop an aggravated retinopathy, as reflected by the high numbers of acellular capillaries and the formation of microaneurysms, which is unusual for this short duration of diabetes in rodents, but also venolar abnormalities, which is unprecedented in diabetic rodent models. Finally, the reduced pericyte density in PDGF-B$^{+/-}$ mice alters the angiogenic propensity of the retina in response to hypoxia with a doubling of new preretinal vessels.

Studies in PDGF-B– and PDGF-receptor β–deficient mice have shown that during development, PDGF-B–independent mechanisms, such as the aggregation of smooth muscle cell/pericyte progenitors at sites around distinct types of vessels, and PDGF-B–dependent mechanisms, such as migration and proliferation of progenitor cells, exist in parallel (8). For the mature vascular system, the role of PDGF-B is less clear. Our present data suggest that in the retina PDGF-B dosage is important in determining the pericyte density, i.e., in the absence of one PDGF-B allele, less pericytes are recruited to their final destination. A reduction of pericyte coverage in adult capillaries appears to interfere with the maintenance of an intact mature capillary network; we observed a moderate increase of acellular capillary segments, representing areas of endothelial cell loss. Complementary observations have been reported for the developing retina, in which the expression of α smooth muscle actin by the recruited mural cells correlated with the lack of regression of capillaries in response to hyperoxia (23).

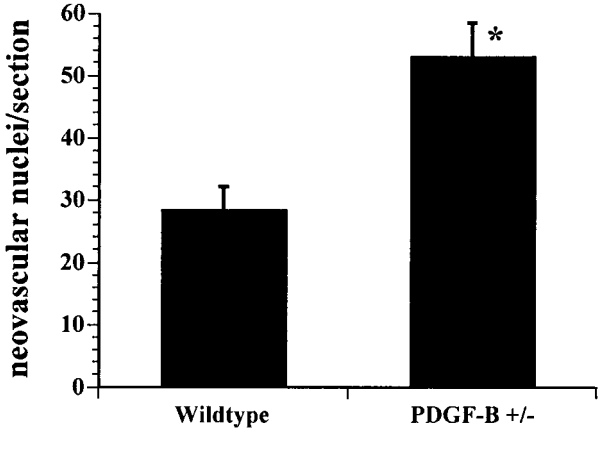

FIG. 3. Increased retinal neovascular response of PDGF-B$^{+/-}$ mice in the ischemic mouse retina. On day 7, both groups of mice, together with the nursing mothers, were exposed to 75 ± 3% % oxygen for 5 days and then returned to room air, which causes retinal ischemia due to VEGF deprivation and vascular obstruction. The neovascular response is assessed at day 17 in consecutive sections, counting new vascular nuclei at the internal site of the inner limiting membrane. *$P < 0.05$ vs. wild-type mice.

The major loss of pericytes in the homozygous PDGF-B–deficient mice leads to capillary dilatation and bleeding (9). In contrast, the phenotype in the heterozygous diabetic PDGF-B$^{+/-}$ mice, however, is characterized by focal endothelial degeneration, as reflected by vascular narrowing and obstructive occlusion. This suggests a survival-promoting role of pericytes for the diabetic endothelium.

Because pericytes but not endothelial cells express PDGF-receptor β in vivo (9,24), it is likely that the effect of PDGF-B loss on endothelial cells is indirect. This is consistent with the suggestion of a close communication between endothelial cells and pericytes/vascular smooth muscle cells (1). Conversely, mice genetically modified to lack the endothelial receptor Tie-2 or its ligand angiopoietin-1 not only develop defects in angiogenesis and vascular remodeling but also exhibit defects in the proper recruitment of pericytes/smooth muscle cells (25,26). This has been explained by the lack of a Tie-2 receptor–transduced signal required for the interaction between endothelial cells and pericytes. Thus, pericyte recruitment defects can be secondary to primary endothelial cell defects.

VEGF has been established as a survival factor in retinal capillary endothelium (27) and tumor angiogenesis (3). Specifically, in a model of conditional deactivation of VEGF production in tumor cells, vessels lacking pericytes are selectively eliminated, whereas vessels invested with pericytes remain intact, proposing that VEGF and pericytes have complementary roles in promoting endothelial cell survival. This possibly applies to the early diabetic retina, in which VEGF levels are comparatively low (although higher than in the unstimulated nondiabetic retina) (18). In contrast, when VEGF levels are high, such as in the ROP mouse model (28), the relative deficiency of capillary pericyte coverage causes increased endothelial cell proliferation, suggesting that the effect of pericytes on endothelial cell survival and proliferation varies with the level of angiogenic factors, such as VEGF (29). Since neovascularizations do not develop in diabetic rodent models, we

used the ROP model for proliferative retinopathy induced by high VEGF. Thus, our conclusions about the role of pericyte loss in the pathogenesis of human proliferative diabetic retinopathy remain speculative at this point.

New findings indicate that the common pathophysiologic mechanism linking chronic hyperglycemia to vascular pathology in diabetes is the mitochondrial overproduction of reactive oxygen species (ROS) leading to the increased formation of AGEs, activation of protein kinase C, activation of the aldose reductase, and deliberation of active nuclear factor kB—mechanisms that have been correlated with the pathogenesis of diabetic microangiopathy (30). PDGF-B can be induced by both ROS and AGE, and PDGF-B is upregulated in glomeruli of diabetic rats (31–33). These findings and our data suggest that hyperglycemia-induced ROS overproduction affects endothelial cell survival in the diabetic vasculature and that retinal capillary coverage with pericytes, partly determined by PDGF-B, may act as a survival signal.

Apoptosis preceding the formation of acellular capillaries in retinae from diabetic rats and humans is one mechanism by which endothelial cells are possibly eliminated from the diabetic capillary (34). The almost 2.5-fold increase in acellular capillary segments of diabetic PDGF-$B^{+/-}$ mice compared with their diabetic wild-type littermates suggests that PDGF-B acts to protect capillaries from hyperglycemic injury.

Endothelial expression of PDGF-B was reported to be restricted to capillary and arteriolar endothelial cells during development because embryonic veins only have rudimentary smooth muscle cell coating at this age (8). Our data suggest that PDGF-B also plays a role in maintaining a coordinated venular structure, providing partial protection against progressive damage on the venular side of a diabetic retina. Venules of diabetic PDGF-$B^{+/-}$ mice showed irregular diameters reminiscent of venous beading in the human diabetic retina.

In summary, our study highlights the importance of the cross-talk between capillary cells as well as the role of capillary pericyte coverage in survival and repair of endothelial cells in the diabetic retina. This also opens new insights into treatment options involving the support of pericyte survival in the diabetic retina.

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