Differentiating Microaneurysm Pathophysiology in Diabetic Retinopathy Through Objective Analysis of Capillary Nonperfusion, Inflammation, and Pericytes

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Microaneurysms are biomarkers of microvascular injury in diabetic retinopathy (DR). Impaired retinal capillary perfusion is a critical pathogenic mechanism in the development of microvascular abnormalities. Targeting fundamental molecular disturbances resulting from capillary nonperfusion, such as increased vascular endothelial growth factor expression, does not always reverse the anatomic complications of DR, suggesting that other pathogenic mechanisms independent of perfusion also play a role. We stratify the effects of capillary nonperfusion, inflammation, and pericyte loss on microaneurysm size and leakage in DR through three-dimensional analysis of 636 microaneurysms using high-resolution confocal scanning laser microscopy. Capillary nonperfusion, pericyte loss, and inflammatory cells were found to be independent predictors of microaneurysm size. Nonperfusion alone without pericyte loss or inflammation was not a significant predictor of microaneurysm leakage. Microaneurysms found in regions without nonperfusion were significantly smaller than those found in regions with nonperfusion, and their size was not associated with pericyte loss or inflammation. In addition, microaneurysm size was a significant predictor of leakage in regions with nonperfusion only. This report refines our understanding of the disparate pathophysiologic mechanisms in DR and provides a histologic rationale for understanding treatment failure for microvascular complications in DR.

The retinal capillary microaneurysm is a hallmark feature of microvascular disease in diabetic retinopathy (DR) (1–5). It represents a focal insult to the retinal microcirculation and can demonstrate morphologic progression with time (6,7). Despite detailed ultrastructural and histologic studies (4,8), the disease pathways that underlie the heterogeneous structural and functional properties of microaneurysms are yet to be precisely defined. Reconciling the microaneurysm disease paradigm may facilitate the development of nuanced and targeted therapy that addresses the sight-threatening complications of microaneurysms, such as tissue edema. Such studies are also expected to improve our understanding of the pathophysiologic mechanisms that underlie vascular disease progression in DR.

Capillary nonperfusion (CNP), ischemia, and subsequent upregulation of vascular endothelial growth factor (VEGF) play a putative role in the pathogenesis of DR (9,10). However, there are several lines of evidence to implicate non–VEGF-mediated pathways in the development and progression of DR. Clinical evidence to support the latter hypothesis includes the failure of long-term VEGF antagonism to resolve tissue edema (11) or halt the progression of CNP in all patients with DR (12). Data from experimental studies have also suggested that inflammation is another putative factor in the earliest stages of DR (13,14). Focal leukocyte aggregation, increased leukocyte stasis, and interaction of leukocyte-mediated adhesion molecules such as intracellular adhesion molecule-1 with endothelia have been shown to regulate retinal capillary degeneration in DR (15). Other than disease-induced pathogenic factors, the physiologic variation in oxygen

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consumption (16) and the density of glial and neural elements between retinal eccentricities (x and y planes) and between various retinal layers (z plane) (17) are also expected to modulate the natural course of microaneurysms. Refining our understanding of microaneurysm pathobiology therefore requires an in-depth assessment of the interaction between physiologic and disease-induced pathogenic factors in the retinal capillary system.

Over the last 18 years, we have used intravascular antibody labeling of human donor eyes to perform precise quantitative studies of the retinal circulation in health and disease (17–21). A major advantage of this technique is that it obviates the need for tissue digestion, thereby allowing spatial colocalization of vasculature with neural-glial elements. It also allows precise visualization of the vascular elements by minimizing staining of the surrounding tissue. In this study, we leveraged our established perfusion labeling techniques with high-resolution, three-dimensional (3D) confocal scanning laser microscopy to refine our understanding of the pathophysiology of microaneurysms. We performed quantitative analyses of 636 microaneurysms from donor tissue of patients with DR to investigate the role of CNP, inflammation, and pericyte loss in microaneurysm size and leakage. We also investigated whether physiologic variables, such as topographic position of the microaneurysm with respect to retinal eccentricity and retinal layer, modulate microaneurysm size or leakage. The aim of this study was to better define the disease pathways in DR and by doing so investigate new ways to manage the microvascular complications of DR using the microaneurysm as an exemplar.

**RESEARCH DESIGN AND METHODS**

The study was approved by the human research ethics committee at the University of Western Australia (Perth, Western Australia, Australia). All human tissue was handled according to the tenets of the Declaration of Helsinki. The database utilized in this research is available for sharing with qualified researchers interested in this field. Written request is required. Further information regarding this study can be obtained by contacting corresponding author.

**Donor Eyes**

All eyes were obtained from DonateLife WA, the organ and tissue retrieval authority in Mount Hawthorn, Western Australia, Australia, or the Lions Eye Bank, Lions Eye Institute, Nedlands, Western Australia, Australia. Specimens obtained directly by the authors were whole eyes. Specimens obtained via the Lions Eye Bank were posterior segments following removal of the cornea for transplantation purposes. Twenty-seven eyes from 19 donors with a history of diabetes were prospectively collected over a 3-year period. All donors with diabetes had been treated with an oral antihyperglycemic agent and/or insulin. The DR group consisted of 20 eyes from 12 donors that demonstrated histologic vascular alterations that characterize DR, such as microaneurysm, CNP, retinal hemorrhage, and intraretinal microvascular abnormality seen on flat-mount fluorescent microscopy (1,2). The remaining seven eyes from seven donors did not demonstrate evidence of DR on microscopic examination and were excluded from the study. Demographic details and causes of death for all donors included in the analysis are presented in Supplementary Table 1. In addition, 15 age-matched healthy donor eyes from donors without a history of diabetes were used as controls to undertake qualitative comparisons of retinal vascular changes. These control eyes were used in our previous study (19).

**Retina Preparation**

All donor eyes were obtained within 12 h of death. Enucleated eyes were transported in Ringer lactate solution. At the laboratory, the central retinal arteries of eyes were identified and cannulated using 100-µm micropipettes and subsequently perfused with 1% BSA in Ringer lactate solution to wash out residual blood clots at a rate of 60 µL/min. Tissue fixation was achieved with a solution of 4% paraformaldehyde for 20 min followed by 0.1 mol/L phosphate buffer (PB) for 15 min. Eyes from all donors with diabetes were perfused with 0.02 mg FITC (cat. no. L4895; Sigma-Aldrich, Darmstadt, Germany) and 1 µg Hoechst (cat. no. H6024; Sigma-Aldrich) in 0.4 mL PB over 30 s to label vascular endothelium and nuclei. After 12 min, eyes were perfused again with PB for 15 min to wash out excess labels.

Postperfusion, eyes were decannulated and dissected along the equator. The vitreous was carefully peeled from the retina. The posterior segments were then immersed in 4% paraformaldehyde for 12 h. Next, the neuroretina was detached from the retinal pigment epithelium, and the optic nerve head was sectioned to be continuous with the retina. The retina was flat mounted using glycerol as mounting medium.

**Confocal Scanning Laser Microscopy**

A montage of the entire retinal microvasculature of each donor retina was first constructed by acquiring multiple low-magnification retinal scans of lectin-FITC with a Nikon Plan Fluor 4× dry objective lens (numeric aperture [NA] 0.13; field of view 3.74 × 3.74 mm) using a z interval of 20 µm. Images were automatically collated and stitched using Nikon NIS-Elements (Nikon, Tokyo, Japan) to generate a complete retinal vascular map (Fig. 1A). Twenty random 1 × 1 mm regions with microaneurysms were then selected from the map of each donor retina by two observers (D.A. and C.B.). A third observer (B.T.) was instructed to acquire high-magnification, high-resolution images of each of these selected regions using a combination of a Nikon Plan Apo VC 20× dry objective lens (NA 0.75; field of view 0.63 × 0.63 mm) and a Nikon Plan Fluor 40× oil objective lens (NA 0.75; field of view 0.31 × 0.31 mm) or Nikon Plan AP VC.
Figure 1—Perfusion labeling of the retinal circulation. A: Whole-mount retina image labeled using lectin FITC immunofluorescence. The optic nerve head and fovea are marked by blue and red asterisks, respectively. The posterior pole is demarcated by a red circle (dashed line). Microaneurysms can be seen in the central and peripheral retinas. Scale bar 1 mm. B–D: Higher-magnification images of a healthy retina without DR (B), region without CNP (C), and region with CNP (D) are shown. Microaneurysms are indicated by green asterisks, and the area of nonperfusion is indicated by an orange asterisk. Scale bars, 40 μm.
60× oil objective lens (NA 1.4; field of view 0.21 × 0.21 mm) with a z interval of 1 μm. Immunofluorescence labeling using Hoechst (405 nm) and lectin-FITC (488 nm) was visualized via argon laser excitation, with emissions detected through 450- and 561-nm band pass filters, respectively.

**Image Analysis**

Confocal microscopic image files were processed with the image analysis software IMARIS (Bitplane, Zurich, Switzerland). Microaneurysms were measured and analyzed in 3D, and the following parameters were recorded for each microaneurysm:

1) Central or peripheral retina location. For this study, the central retina was considered within 30 degrees of the fovea, which incorporates the entire macula, the temporal arcades, the optic disc, and the nasal retina just beyond the optic disc. Regions located outside this were considered the peripheral retina (Fig. 1A).

2) Regions with or without CNP. Capillary perfusion status was assessed for the location of each microaneurysm using 0.625 × 0.625 mm fields (Fig. 1C and D). Analysis of identical retina eccentricities of age-matched donor eyes from the control group was used as a comparison to determine the presence or absence of CNP in eyes with DR (Fig. 1B).

3) Greatest diameter. Microaneurysms were visualized in 3D, and their geometric dimensions were studied at different angles of rotation. The size of the microaneurysm was denoted as the greatest margin-to-margin diameter size (Supplementary Fig. 1).

4) Layer of the retina. A microaneurysm was assigned to a layer of retina based on its vessel of origin instead of its physical location within the retina, because many large microaneurysms were observed spanning multiple retinal layers (Fig. 2). Colocalization of vascular and nuclear labeling was used to define the location of vascular segments relative to retinal layers. Methods of retinal vascular plexus stratification were described in detail in our previous work, and the same definitions applied to this study (19,21,22). In brief, the central retina contains three plexuses: the superficial vascular plexus (SVP), the intermediate capillary plexus, and the deep capillary plexus (DCP), with an additional retinal peripapillary capillary plexus along the vascular arcades (19,22). The mid-peripheral retina typically contains two plexuses: the SVP and DCP (21). The retinal vasculature anterior to the equator of the globe typically contains a single plexus (21).

For the purposes of this study, the retinal peripapillary capillary plexus was grouped with the SVP. A microaneurysm that originated from a capillary segment adjoining two plexuses was assigned to the more superficial plexus.

5) Microaneurysm leakage. Lectin acts as an intravascular agent within healthy retinal vasculature, with minimal extravasation (19,20) because of its large molecular weight (23), rendering it impermeable to the blood-retina barrier. We have previously shown that lectin extravasation is a marker of microaneurysm leakage in DR (21). Individual microaneurysm leakage was binarily categorized by two masked graders (D.A. and C.B.) as being present or absent. Disagreements were resolved by a third grader (B.T.). If the region immediately surrounding the microaneurysm displayed hyperfluorescence relative to the background, the microaneurysm was classified as leaking (Fig. 3).

6) Presence of inflammatory cells. This was determined by examining the lumen of each microaneurysm for the presence of spherical nucleated cells (24) that were distinguishable from capillary endothelial cells, which have flat and elongated nuclei (Fig. 4A) (19,25,26). No further differentiation of inflammatory cells was performed in this study.

7) Presence of pericyte. Pericytes are dome-shaped cells located on the abluminal aspect of the basement membrane, which is shared with endothelial cells (19,27–29). The presence of pericyte nuclei, cell membrane, and basement membrane can be identified on histologic sections (Fig. 4B). For each microaneurysm, pericytes were graded as being present or absent.

**Statistical Analysis**

Data were analyzed using R software (R Foundation for Statistical Computing, Vienna, Austria). Comparisons between categorical variables were performed using the χ² test. Data involving microaneurysm leakage as the response variable with binary outcomes were analyzed using univariate and multivariate logistic regressions. Data involving microaneurysm diameter were analyzed using univariate and multivariate linear regressions. Normality of data was assessed using the Shapiro-Wilk test. Log transformation was applied for nonnormally distributed data. Regression models included mixed effects to account for multiple sampling regions within the same donor eye. Results of P < 0.050 were considered statistically significant.

**Data and Resource Availability**

The database used in this research is available for sharing with qualified researchers interested in this field. Written request is required. Further information regarding this study can be obtained by contacting the corresponding author.

**RESULTS**

A total of 636 microaneurysms were quantitatively studied using high-resolution confocal microscopic imaging. The DR group consisted of 14 male donor retinas and six female donor retinas. The average age of donor patients in the group was 65.5 ± 12.6 years (range 45–86 years). The average age of donors in the control group was 67.6 ± 20.5 years (range 42–89 years), which was not different to that of patients in the DR group (p = 0.368).
Significant Determinants of Microaneurysm Diameter

Overall mean microaneurysm diameter was 42.7 ± 23.7 μm (median 36.0 μm). The frequency of microaneurysms per 10-μm diameter bracket is summarized in Supplementary Fig. 2. Mean microaneurysm diameters with respect to capillary plexus of origin and retinal eccentricity are summarized in Table 1. When data from all retina eccentricities were pooled, the frequency of microaneurysms in the deep plexus (52.4%) was not different to that of those in the superficial or intermediate plexus (P = 0.88). Microaneurysms of the SVP were significantly larger than those of the DCP in both central (three plexuses) and peripheral (two plexuses) locations (P < 0.001). Microaneurysm diameter was not different between central and peripheral locations (P = 0.899).

Results of univariate and multivariate analyses of microaneurysm diameter are summarized in Table 2. CNP, pericytes, and inflammatory cells were all significant predictors of microaneurysm size in both univariate (all P < 0.001) and multivariate (all P ≤ 0.005) analyses. Age and sex were not significant predictors of microaneurysm size (all P > 0.050).

A total of 66.4% of microaneurysms were seen in regions with CNP. Microaneurysms located in regions with CNP (45.3 ± 25.0 μm) were significantly larger than those in regions without CNP (37.4 ± 19.7 μm; P < 0.001 (Table 3). Pericytes were seen in 39.2% of all microaneurysms (Figs. 4 and 5 and Videos 1 and 2). In regions without CNP, 57.0% of microaneurysms found contained pericytes, but only 30.0% contained pericytes in regions with CNP (P = 0.002). Microaneurysms with pericytes (38.4 ± 19.5 μm) were significantly smaller than those without pericytes (51.0 ± 26.5 μm; P < 0.001). Of all microaneurysms, 31.3% demonstrated inflammatory cells within the lumen (Figs. 4 and 5). Mean diameters of microaneurysms with inflammatory cells (54.8 ± 29.9 μm) were significantly larger than those of microaneurysms without inflammatory cells (37.2 ± 17.7 μm; P < 0.001). Inflammatory cells were found more frequently in microaneurysms located within regions with CNP (34.4%). In regions without CNP (15.5%), significantly fewer microaneurysms contained inflammatory cells (P < 0.001).

The effects of interaction between presence/absence of pericytes, presence/absence of inflammatory cells, and presence/absence of CNP on microaneurysm diameter were explored. In regions with CNP (mean 45.3 ± 25.0 μm), absence of pericytes alone (56.2 ± 26.9 μm) and presence of inflammatory cells alone (56.0 ± 29.6 μm) were significant determinants of increased microaneurysm diameter (both P < 0.001). Microaneurysms with inflammatory cells and without pericytes had a mean diameter of 72.2 ± 28.4 μm and were significantly larger than microaneurysms from all other categories (all P <
Presence of both pericytes and inflammatory cells (mean diameter 67.5 ± 30.5 μm) was observed in only 1.4% (n = 9) of microaneurysms (Supplementary Fig. 3).

In regions without CNP (mean diameter 37.4 ± 19.7 μm), absence of pericytes alone and presence of inflammatory cells alone were not significant determinants of larger microaneurysm diameter (both P > 0.050). However, microaneurysms with both absence of pericytes and presence of inflammatory cells had a significantly larger diameter (63.2 ± 35.6 μm; P = 0.002). Microaneurysms that contained both pericytes and inflammatory cells were not identified in regions without CNP. Microaneurysms with pericytes and without inflammatory cells in regions without CNP had a mean diameter of 32.1 ± 17.6 μm. The mean diameter of these microaneurysms was significantly smaller than that of microaneurysms from all other categories (all P < 0.050).

**Significant Determinants of Microaneurysm Leakage**

Leakage was observed in 58.3% of all microaneurysms. Location of microaneurysm with respect to capillary plexus and eccentricity (peripheral or central location) was not associated with leakage (both P > 0.050). Leaking microaneurysms were significantly smaller than nonleaking microaneurysms (P < 0.001). This association can be visualized in Fig. 6A, where microaneurysms were subdivided into 10-μm diameter brackets. Subanalysis revealed that leaking microaneurysms (38.4 ± 19.7 μm) were...

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**Figure 3**—Determining microaneurysm leakage using perfusion labeling with lectin. Midperipheral retinal section showing two nonleaking microaneurysms (yellow asterisks) and a leaking microaneurysm (blue asterisk) within the same region of interest. The extent of hyperfluorescence that denotes leakage is highlighted (blue dotted circle). A point of focal capillary occlusion is also denoted (red arrow).
significantly smaller than nonleaking microaneurysms in regions with CNP (54.0 ± 28.1 μm; P < 0.001) (Fig. 6B), while diameter was not significantly associated with leakage in regions without CNP (P = 0.302) (Fig. 6C).

Multivariate analysis involving age, sex, diameter, presence of pericytes, presence of inflammatory cells, and CNP showed that only diameter was a significant predictor of leakage (P < 0.001). Presence of pericytes (P = 0.003) and absence of inflammatory cells (P = 0.014) were significantly associated with leakage only in univariate analysis. Age, sex, and CNP were not associated with leakage in either univariate or multivariate analysis (all P > 0.050) (Table 2).

Figure 4—Defining cellular compositions of microaneurysms. A–D: 3D projections of two different microaneurysms (A and B) and 2D cross-sectional images of the same aneurysms (C and D, respectively) are presented. The top row denotes a 30-μm microaneurysm with pericytes that are located on the external aspect of the microaneurysm wall (red arrows). In the same microaneurysm, two endothelial cells with elongated nuclei can be seen within the inner aspect of the wall. Note the difference in morphology of endothelia to pericytes. Significant leakage surrounding the microaneurysm in the top panel is also noted (magenta asterisk). The microaneurysm in the bottom row has a diameter of 65 μm and contains a large aggregation of cells within the lumen. Many of these cells do not contain nuclei and represent red blood cells (red asterisk). Within the lumen of this microaneurysm, there is also evidence of a lymphocyte-like cell with a large, round nucleus (pink asterisk) and a neutrophil-like cell with a polymorphic nucleus (green asterisk). Endothelial cells with nuclei (yellow arrows) are also seen in the microaneurysm. Cell nuclei were false-colored blue. Scale bars, 10 μm.
The frequency of microaneurysm leakage was not different between regions with CNP (55.8%) and regions without CNP (63.6%; P = 0.172) (Table 3). Frequency of leakage was significantly lower in microaneurysms with inflammatory cells (50.7%) than in those without inflammatory cells (62.0%; P = 0.017). Frequency of leakage was also higher in microaneurysms with pericytes (69.0%) compared with those without (46.4%; P = 0.004).

In regions with CNP, microaneurysms with pericytes had a frequency of leakage of 60.3%, similar to those without pericytes (51.2%; P = 0.497). Microaneurysms with inflammatory cells had a lower frequency of leakage (46.6%) than those without inflammatory cells (61.3%; P = 0.004). Microaneurysms with both absence of pericytes and presence of inflammatory cells had the lowest frequency of leakage (36.6%) compared with microaneurysms from all other categories (all P < 0.050).

In regions without CNP, presence of pericytes was associated with a higher frequency of leakage (77.3%; P = 0.012) (Supplementary Fig. 4), while microaneurysms with absence of inflammatory cells (62.0%) had a similar frequency of leakage to those with inflammatory cells (70%; P = 0.349). Microaneurysms without inflammatory cells and with pericytes (74.6%) had a higher frequency of leakage compared with those without inflammatory cells and without pericytes (57.4%; P = 0.048).

**DISCUSSION**

The retina is a nonhomogeneous structure with significant topographic variation in physiologic properties and energy requirements (30,31). Although a range of studies have described the disease-induced pathogenic factors in DR, fewer have focused on the influence of regional variations in the physiologic milieu on microaneurysm formation. In this study, perfusion techniques (17–19,21) were used to label the retinal circulation, and by doing so, we were able to preserve the spatial relationships between capillary beds and retinal layers as well as make comparisons between retinal eccentricities. We demonstrate that the size of microaneurysms is significantly greater in the SVP, suggesting that physiologic factors play a key role in modulating the morphologic properties of microaneurysms. One explanation for this finding is the inherent variations in biomechanical properties between retinal layers (32). Qu et al. (33) investigated the deformability of retinal layers using synchronized acoustic radiation force optical coherence elastography and found that Young’s modulus of the retina comprising the nerve fiber

Table 1—Frequency and diameter of microaneurysms across retinal regions and plexuses

| Location              | Plexus     | Microaneurysm frequency (%) | Mean diameter ± SD, μm |
|-----------------------|------------|-----------------------------|------------------------|
| Central: three retinal plexuses | Superficial | 50 (33.3) | 52.3 ± 36.5 |
|                       | Intermediate | 44 (29.3) | 44.4 ± 28.3 |
|                       | Deep       | 56 (37.3) | 32.5 ± 16.6 |
| Periphery: two retinal plexuses | Superficial | 142 (41.0) | 44.0 ± 23.1 |
|                       | Deep       | 204 (59.0) | 36.8 ± 17.5 |
| Periphery: single retinal plexus | Single plexus | 137 | 51.6 ± 22.9 |

Table 2—Univariate and multivariate analyses for determinants of microaneurysm diameter and leakage

| Microaneurysm analysis | Coefficient, μm | SE, μm | P   | Coefficient, μm | SE, μm | P   |
|------------------------|-----------------|--------|-----|-----------------|--------|-----|
| Diameter               |                 |        |     |                 |        |     |
| CNP                    | 7.89            | 1.95   | <0.001 | 4.91            | 1.59   | 0.002 |
| Pericytes present      | -12.64          | 2.57   | <0.001 | -4.99           | -1.98  | 0.005 |
| Inflammatory cells present | 17.62    | 1.89   | <0.001 | 16.22           | 21.56  | <0.001 |
| Leakage present        | -12.01          | 1.83   | <0.001 | -6.48           | 0.92   | <0.001 |
| Age                    | 0.08            | 0.06   | 0.175 | 0.57            | 0.55   | 0.300 |
| Sex                    | -1.31           | 1.84   | 0.229 | -10.85          | 12.43  | 0.383 |
| Leakege                |                 |        |     |                 |        |     |
| CNP                    | -0.33           | 0.17   | 0.059 | -0.10           | 0.07   | 0.121 |
| Pericytes present      | 0.65            | 0.22   | 0.003 | 0.30            | 0.29   | 0.088 |
| Inflammatory cells present | -0.42     | 0.17   | 0.014 | 0.08            | 0.07   | 0.269 |
| Diameter               | -0.02           | -0.01  | <0.001 | -0.01           | 0.00   | <0.001 |
| Age                    | -0.01           | 0.01   | 0.337 | 0.00            | 0.01   | 0.389 |
| Male sex               | -0.85           | 0.55   | 0.123 | -0.23           | 0.53   | 0.667 |

Bold font indicates significance.
Figure 5—Cellular associations with microaneurysm size and leakage. Microaneurysms in order of increasing diameter are presented. Higher-magnification images of each microaneurysm at different angles of rotation and cross section are presented in insets I and II. A:
layer, ganglion cell layer, and inner plexiform layer was significantly lower than that of other retinal layers. It is therefore plausible that a comparable amount of intraluminal hydraulic force in the SVP results in greater deformation and microaneurysm size than in other plexuses. Our hypothesis requires validation with additional investigative studies.

Progressive capillary occlusion and ischemia are key features in DR (1,34). Retinal ischemia increases VEGF production in retinal ganglion cells and Müller cells (9,35). There are several lines of evidence to suggest that VEGF upregulation is intrinsically linked to the clinical manifestations of DR: 1) microaneurysms are commonly observed at the boundaries of perfused and nonperfused retinas (1,2,5); 2) intravitreal administration of VEGF can induce formation of microaneurysms and simulate human DR in experimental models (36,37); and 3) VEGF immunoreactivity is increased in ischemic retinopathies (10). Our study uses objective histologic assessment of capillary perfusion status as a marker of retinal ischemia. It reaffirms that CNP, ischemia, and, by extension, VEGF upregulation are putative factors in the formation of microaneurysms. Univariate and multivariate analyses revealed that CNP was a significant predictor of microaneurysm size. Taken together, our results suggest that VEGF plays a significant role in the formation of microaneurysms as well as the modulation of their morphologic properties with respect to size.

Spatial and temporal changes in the ultrastructural characteristics of the retinal circulation are associated with leukocyte stasis, implicating inflammation as a central pathogenic factor in DR (13). A key mechanism by which inflammation induces retinal capillary degeneration is via the interaction between leukocyte-released leukotrienes and the capillary endothelium (15). Despite evidence that inflammation plays a critical role in capillary occlusion in early DR (14,38,39), the manner in which it modulates the lifecycle of microaneurysms is unclear. Stitt et al. (4) investigated the ultrastructural stages of microaneurysms using light and electron microscopy and characterized four subtypes of microaneurysms on the basis of their cellular compositions. Type I was characterized by an accumulation of monocytes and neutrophils. Types III and IV contained macrophages. Type II microaneurysms were devoid of inflammatory cells. In our analysis of 636 microaneurysms, we identified inflammatory cells within the lumens of only 31% of microaneurysms. Furthermore, we found that the strongest predictor of microaneurysm size was the presence of inflammatory cells, implying that intraluminal aggregation of inflammatory cells may be a late feature of the microaneurysm lifecycle as it increases in size. Similar to cerebral aneurysms, the intraluminal aggregation of inflammatory cells may result in aneurysm enlargement through signaling of matrix metalloproteinases (40). Furthermore, our data suggest that inflammation is unlikely to be a significant factor in the pathogenesis of microaneurysms but rather an epiphenomenon that is associated with the later stages of the lifecycle. Upregulation of VEGF may play an important role in inflammatory cell recruitment and may precede the accumulation of inflammatory cells within microaneurysms. Evidence to support this speculation is provided in the work by Wang et al. (41), who showed that disrupting Müller cell–derived VEGF in diabetic mice inhibited leukocyte stasis and the overexpression of leukotrienes intracellular adhesion molecule-1 and tumor necrosis factor-α.

In a histopathologic assessment of donor retina, Yanoff (42) quantified the ratio of pericytes and endothelia and showed that a reduction in the pericyte-to-endothelium ratio was strongly correlated with an increase in microaneurysms. In a subsequent paper, Yanoff (3) stated that pericytes were strikingly absent from sites of microaneurysms. Our report supports the principal hypothesis that pericyte loss in DR favors the sequestration and extravasation into the interstitial space (43). Given that pericytes mediate extravasation of leukocytes, it is plausible that pericyte loss in DR favors the sequestration of leukocytes within the microaneurysm lumen. Taken together, we infer and propose the following sequence of alterations in the microaneurysm lifecycle within areas of CNP: 1) ischemia-induced upregulation of VEGF (35), 2) VEGF-mediated pericyte apoptosis (44), and 3)
intralumenal leukocyte sequestration and leukotriene-mediated capillary endothelial damage (43).

Tissue edema resulting from leakage is an important sequela of microaneurysms that can result in irreversible vision loss (45). The pathogenesis of microaneurysm leakage is complex, with many putative factors proposed. In contrast to microaneurysm size, we did not identify any significant physiologic variables that were associated with leakage, such as location of the capillary plexus or retinal eccentricity. The most important predictor of microaneurysm leakage was microaneurysm size, with the rate of leakage being greatest in smaller microaneurysms. Presence of CNP was not a significant predictor of leakage in regression analyses, with rates of leakage similar in regions with CNP (55.8%) and regions without CNP (63.6%). VEGF has been shown to increase retinal vascular permeability in diabetes by reducing interendothelial tight junction proteins, such as occludin (46). Although disruption of the integrity of the blood-retina barrier alone may be sufficient to result in leakage of a retinal capillary, our findings suggest that leakage of microaneurysms requires the interaction of capillary perfusion status and other variables. The most important determinant of microaneurysm leakage in regions with CNP was the presence of inflammatory cells. The presence or absence of pericytes did not alter the rate of leakage in regions with CNP. One explanation for these findings is that leukocytes play a vital role in regulating thrombosis by controlling platelet activation and adhesion and activating the intrinsic and extrinsic coagulation pathways (47). In vivo techniques that facilitate recognition of leukocytes within microaneurysms may therefore aid in the clinical management of microaneurysms. Because of the lower rate of leakage in microaneurysms with inflammatory cells, it may be appropriate to defer treatment of these lesions.

Although VEGF is central to the pathogenesis of DR (9,35,37), this study provides evidence that non–VEGF-dependent pathways also underlie microaneurysm size and leakage. Morphologic characteristics were different in the 35% of microaneurysms found in regions without CNP compared with those found in regions with CNP. This suggests that the biologic mechanisms underlying the lifecycle of these microaneurysms may also be different.

Our study provides several findings to support this claim: 1) microaneurysms in regions without CNP were significantly smaller than those in regions with CNP; 2) unlike that of microaneurysms in regions with CNP, microaneurysm size in regions without CNP was not a predictor of leakage; and 3) unlike with microaneurysms in regions with CNP, we did not find a gradual or significant increase in the size of microaneurysms with pericyte loss or presence of inflammatory cells in regions without CNP. Therefore, in regions without CNP, the major pathogenic factors underlying the lifecycle of microaneurysms may be related to the function of local glia or hydrostatic factors instead of ischemia or VEGF-driven pathways.

Table 3—Microaneurysm frequency, diameter, and leakage with relation to the presence/absence of inflammatory cells and pericytes

| Category | CNP | No CNP |
|----------|-----|--------|
| **Frequency (%)** | 636 (100) | 422 (66.4) |
| **Mean diameter ± SD, μm** | 45.0 ± 25.0 | 37.4 ± 19.7 |
| **Leakage, %** | 55.8 | 35.7 |
| **Frequency (%)** | 249 (39.2) | 145 (23.8) |
| **Mean diameter ± SD, μm** | 41.2 ± 23.5 | 27.7 ± 17.7 |
| **Leakage, %** | 61.3 | 47.9 |
| **Pericytes** | Present | Absent |
| **Inflammatory cells** | Present | Absent |
| **Both present** | 9 (1.4) | 9 (1.4) |
| **Absent and present** | 240 (37.7) | 118 (18.6) |
| **Both absent** | 197 (31.0) | 159 (25.0) |

Microaneurysm frequency percentages were calculated using a denominator of 636 microaneurysms.
Figure 6—Relationship between microaneurysm diameter and leakage. A–C: Comparisons between leaking and nonleaking microaneurysms, per diameter grouping, for all microaneurysms (A), microaneurysms in regions with CNP (B), and microaneurysms in regions without CNP (C) are provided. In contrast to the linear relationship found in combined data and in regions with CNP, there was no association found between microaneurysm diameter and leakage in regions without CNP.
More work is required to reconcile the disease mechanisms of these microaneurysms.

This is one of the largest histopathologic studies of microaneurysms resulting from DR and provides a summary of the quantitative findings of 636 lesions from 20 donor eyes. A major strength of this study is our methodologic technique of perfusion labeling combined with 3D confocal scanning laser microscopy. Our findings suggest that the disease pathways underlying the microaneurysm lifecycle are heterogeneous, and this may have implications for the clinical management of DR. A histopathologic study is an assessment of a single time point in the natural course of any disease, and we emphasize that the proposed disease pathways in this report are inferences based on a large set of microaneurysms at different stages of their lifecycle. Temporal studies using high-resolution imaging, such as adaptive optics, that can assess in vivo cellular details will be useful for validating our histologic results. We did not investigate the role of endothelial cells in the pathophysiology of microaneurysms in the current study. Important factors such as endothelial cell junctions and cytoskeletons and stress fibers could not be assessed using lectin labeling. Additionally, clinical parameters of donors, including diabetes duration, hemoglobin A1c percentage, systemic blood pressure, and prior ophthalmic imaging, were not available, and this is a limitation of the study. Finally, we emphasize that our speculations assume that VEGF is significantly upregulated in regions of CNP only and that regions without CNP do not manifest significant increases in VEGF. We believe this to be a valid assumption because previous work has shown that there is great interindividual variability in the degree of VEGF upregulation as a result of hypoxia in the absence of ischemia (48,49).

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