Relationships Between Microvascular Function and Capillary Structure in Diabetic and Nondiabetic Human Skin

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Despite the commonly held view that abnormalities in capillary morphology, in particular thickening of the capillary basement membrane, are partly responsible for diabetic ischemia, few studies have correlated anatomic and hemodynamic variables in the same diabetic subjects. In a previous study of 24 type II (non-insulin-dependent) diabetic subjects and 24 age-matched control subjects, we showed that a standard finger exercise vasodilated cutaneous forearm vessels nearly equally (51%), but the postarteriolar flow responded differently between groups. Nondiabetic subjects increased flow by recruitment of capillaries, whereas diabetic subjects did so by capillary flow augmentation. Moreover, resting permeability–surface area product (PS) to pentetic acid was 85% higher in diabetic than nondiabetic subjects. In this study, these same subjects had their forearm skin biopsied and examined morphometrically by electron microscopy for capillary radius, basement membrane thickness, endothelial cell density, and a folding index of luminal membrane reduplication. All morphological variables were correlated stepwise in a saturated, analysis of covariance model with the physiological results. The correlations were sparse and specifically excluded basement membrane thickness. The highest r^2 value was .432 between resting PS and a ratio of capillary density to endothelial cell number per capillary. These studies show little evidence that diabetic microvascular physiological variables are tightly connected to morphometric changes except for minor permeability changes, which rise with capillary density and decrease with endothelial cell number. Because PS to pentetic acid is increased in diabetic subjects at any level of capillary density, it seems reasonable that permeability may be increased above that of nondiabetic subjects. However, such a conclusion is tentative because anatomic measurement of capillary density gives only the maximum estimate of capillaries in the tissue, which may be greater than the number of capillaries with flow at the time of physiological measurement. *Diabetes* 38:1245–50, 1989

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Received for publication 6 March 1989 and accepted in revised form 14 June 1989.

Abnormalities in the structure of capillaries have been noted in naturally occurring and experimental diabetes mellitus for >20 yr (1–9). In addition, microcirculatory functional disturbances, particularly in vasoactivity and permeability, have also been well described in the diabetic state (5,10–24). Potential linkages between structure and function have rarely been explored, but some studies have noted a relationship between autoregulatory malfunctions and *p*-aminosalicylic acid–positive material in terminal arterioles (22). Systematic studies of the relationships between vasoactive behavior and microvascular morphology are essential for understanding such interactions and to explore whether knowledge of microvascular structure is refined enough to allow for such interrelationships. In this study of type II (non-insulin-dependent) diabetic subjects and age-matched control subjects, we examined possible relationships between microvascular structure and resting and vasodilated flow and permeability characteristics of the cutaneous circulation (25).

RESEARCH DESIGN AND METHODS

Complete anatomic and physiological methods for measurements in the cutaneous microcirculation have been published elsewhere (25,26). Twenty-four nondiabetic subjects 59 ± 1.5 (mean ± SE) yr of age were matched by age (range 44–72 yr), sex, and weight to 24 type II diabetic subjects who were free of hypertension, proliferative retinopathy, macrovascular disease, proteinuria, and other kidney disease. General subject characteristics, methods for measurement

of forearm circulatory characteristics by tissue-clearance techniques, and results are thoroughly presented in a previous paper (25).

After informed consent, patients were studied for cutaneous forearm blood flow and permeability–surface area product (PS) to In-111-diethylenetriamine pentaacetic acid salt of sodium (pentetic acid) injected intradermally over the midanterior forearm. Methods have been described for measuring blood flow in detail by Sejrsen (27), Chimoskey (28), and Daly and Henry (29), and for PS by Gosselin and Stibitz (30), Gosselin and Audino (31), and Katz and McNeill (25). Measurements were made at rest and during 90 s of repetitive finger exercise at 0.16 W.

With Δ to represent the change from rest to repetitive exercise, we computed two possible fates for postarteriolar blood flow (Q) as it rises from Q to $Q + \Delta Q$. Changes in capillary density in the tissue bed are given by the recruitment response $\Delta n/n = \Delta PS/PS$, in which $\Delta n/n$ is the fractional change in capillary density (30). Changes in capillary blood flow are given by the augmentation response

$$\Delta b/b = \frac{\Delta Q/Q - \Delta PS/PS}{1 + \Delta PS/PS}$$

which results from

$$\Delta Q/Q = \Delta n/n + \Delta b/b + (\Delta n/n)(\Delta b/b)$$

in which $\Delta b/b$ is the fractional change in capillary blood flow. The results from this portion of the study have been reported previously (25).

Morphological analyses of skin capillaries have been presented in part previously (26). In brief, the methods were as follows: after the tissue-washout studies were completed, skin biopsies were taken from the midventral forearm skin after 1% lidocaine injection of the epidermis with a 3-mm disposable skin-punch biopsy with paper tape closure of the wound. Skin biopsies were fixed for 24 h in cold Karnovsky's solution (32). After fixation, the biopsies were divided into 1-mm-thick slices by cutting on a transverse plane through the dermal-epidermal interface. The slices were osmicated for 2 h in 2% OsO_4 (buffered to pH 7.4 with phosphate), dehydrated in a series of graded alcohols and propylene oxide, and embedded in epoxy resin (33). One-micrometer-thick sections for light microscopy were cut with glass knives and stained with paragon. Before thin sectioning, blocks were trimmed to ensure that capillary sampling would be restricted to <1 mm below the dermal-epidermal junction. Thin sections were cut with diamond knives, collected on copper grids, and doubly stained with uranyl acetate and lead citrate. Sections were examined in a Philips 300 electron microscope (Mahwah, NJ) operated at 60 kV. The calibration of the microscope was stable throughout the study. Ten capillaries from each biopsy were photographed and enlarged to ×16,000 on 8 × 10-in paper. A test grid was printed during each enlarging session to determine the exact enlargement factor. The prints were morphometrically analyzed with a Zeiss Videoplan (Thornwood, NY). Morphometric analysis was done by J.L.B., who did not have knowledge of or access to clinical patient information.

Measurement was carried out by a collapsed-ellipse technique described previously (26). In these techniques, the elliptical section was mathematically collapsed on the right circular cylinder of the capillary by methods measuring thickness or by annular areas. This was done to avoid overestimating mean capillary width (w) measured on elliptical sections. The formula used was

$$w = \sqrt{A_E^2/(\pi^2 a^2) + (r/a) \times A_{AnE}/\pi} - A_E/(\pi a)$$

where A_E is the internal or luminal area of the capillary outside the abluminal surface of the endothelial cells, r and a are the minor and major capillary hemiaxes, and A_{AnE} is the annular elliptical area of the basement membrane.

The collapsed-ellipse technique also allows for the measurement of a folding index (FI) of the internal lamina of the capillary basement membrane given by

$$FI = P_E \pi r/(4A_E E)$$

where P_E is the measured internal perimeter of the basement membrane and E is the elliptical integral given by

$$E = \int_0^{\pi/2} \sqrt{1 - [1 - (r/a)^2]\sin^2 \phi}\, d\phi$$

The value of FI gives the fraction of extra membrane in the capillary that exists above that required for an ellipse of hemiaxes r and a.

The capillary density (no. capillaries/g tissue) was derived by counting all the capillaries in 50–100 1-μm-thick sections and measuring the area density of capillaries (n_a) in rectangular grids of 13,774 μm² inserted into the ocular of the light microscope. The volume number density is derived in the APPENDIX to be

$$n = \frac{n_a}{2(1 \times 10^{-4} + 0.67l + 1.4827r - w)}$$

where 1×10^{-4} cm is section thickness, l is mean capillary length (0.04 cm), and r is capillary radius or minor hemiaxis.

The final measurement was a count of the number of endothelial cells per capillary (Endo). This count was simply made from the five capillaries used for basement membrane widths. The endothelial cells were identified by J.L.B. and P.C.J. with care taken to count only those cells with nuclei and clear plasmalemmal boundaries between counted cells. The measurement may underestimate cell number by not taking into account cytoplasmic processes of other cells with nuclei that were not in the plane of the section, but such processes were not seen. Moreover, such an error would be a small correction, probably random, among all capillaries of both groups. The results of five capillaries in each subject were averaged and used for comparisons.

Because of the uncertainty of whether distributions of results were normal, comparisons were carried out as follows. Differences between control and diabetic subjects on these measures have been reported before (25): although $\Delta Q/Q$ in both groups was 51–53%, this change was brought about by recruitment (i.e., more open capillaries) in nondiabetic subjects ($\Delta n/n$ = 125%) but by augmentation (i.e., increased flow per capillary) in diabetic subjects ($\Delta b/b$ =

139%). PS at rest in diabetic subjects was significantly greater than in nondiabetic subjects (85% greater). The research questions herein concern whether these physiological characteristics are predictable from anatomic traits and whether that predictability differs in extent or form between nondiabetic and diabetic subjects.

The distributions of test variables in these subjects are quite positively skewed. To reduce this skewness and the influence it grants to extreme data values in analysis, the logarithms of those variables for which logarithms could be taken were analyzed rather than their raw values. This approach has the side benefit that to fit linear-regression models to logged data is implicitly to fit power functions, and prediction equations constructed of products and ratios of powers of variables are more useful for stimulating model development than are simple linear equations. All analyses were run with the Statistical Package for the Social Sciences (SPSS^x, Chicago, IL).

Our objective was to determine if there were relationships between physiological and anatomic variables in the nondiabetic and diabetic groups and to ascertain whether these relationships were different between groups. An illustration of how this is conveniently accomplished is the following linear case. Suppose x and y are, respectively, anatomic and physiological variables that are linearly related but with intercepts and slopes for both groups that may be different. If we introduce the dummy variable G, which equals 0 for the diabetic subjects and unity for the nondiabetic group, the complete linear relationship for both groups is

$$y = p + (q \times G) + r \times x + s\,(G \times x)$$

where p, q, r, and s are constants determined by regression analysis. For the diabetic group, $y = p + rx$, which is examined by comparing the slopes r and $r + s$ to 0. The differences between groups would be measured by comparing q to 0 for the intercept and s to 0 for the slope. The steps would be the same for nonlinear relationships. This technique allows exploration of a saturated analysis of covariance model of each of the physiological traits on the anatomic variables. Completely separate regressions can thereby be estimated for nondiabetic and diabetic subjects in a single analysis.

Group comparisons of means were also carried out. For morphological nondirectional differences between groups, the unpaired t test (probability given by $2P$) and the Mann-Whitney U test ($2P_{MW}$) were used. Differences were considered not significant if $2P$ or $2P_{MW} > .05$. Coefficients of determination (r^2) were significant if $P < .05$.

RESULTS

Morphometric data. Differences between nondiabetic and diabetic subjects were unimpressively marginal. The measure w was 1025.3 ± 86.0 (SE) nm in nondiabetic and 1167.4 ± 82.8 in diabetic ($2P$ NS, $2P_{MW} = .091$) subjects, and FI was slightly greater or not significantly greater in nondiabetic than diabetic subjects (1.190 ± 0.014 vs. 1.157 ± 0.012; $2P = .088$, $2P_{MW} = .049$). No differences were seen in Endo (3.0 ± 0.1 cells/capillary for each group), radius (2746.4 ± 102.7 for nondiabetic and 2685.4 ± 90.0 for diabetic subjects), or number of capillaries per gram ($2.65 \times 10^5 \pm 1.10 \times 10^4$ for nondiabetic and $2.62 \times 10^5 \pm 1.18 \times 10^4$ for diabetic subjects). Thus, in general, little evidence supports important anatomic differences in means between the groups, although differences in population distributions were apparent, with width predominating in diabetic subjects and membrane wrinkling predominating in nondiabetic subjects.

We previously reported that the most striking physiological differences between nondiabetic and diabetic subjects was in the resting PS to pentetic acid and its exercise-induced response to exercise. Nondiabetic PS was 1.77 ± 0.20 ml · min^{-1} · (100 g)$^{-1}$, whereas diabetic PS was 3.29 ± 0.35 ml · min^{-1} · (100 g)$^{-1}$ ($2P = .0003$). During exercise, nondiabetic subjects increased capillary density ($\Delta n/n = \Delta PS/PS$) by 125 ± 27% ($2P = .0001$), whereas diabetic subjects vasodilated by increasing individual capillary blood flow by 139 ± 38% ($2P = .001$).

The saturated model of partial regression showed results that were as noteworthy for those morphological variables that were not correlated to physiological findings as for those showing relationships. The only morphological variable with variance that did not contribute significantly to that of any physiological variables was capillary basement membrane width.

Moreover, even when a physiological variable had a significant regression on one or more morphological variables, there was only one instance in which both diabetic and nondiabetic physiological variables depended on the same morphological variables. This occurred when the logarithm of PS was the dependent variable. With an overall r^2 of .432 ($F = 10.923$, $P = .0000$), it was determined that a best fit occurred with

$$\text{nondiabetic PS} = 3.197 \times 10^{-5} \times \frac{\text{density}^{0.9590}}{\text{Endo}^{1.0662}}$$

and

$$\text{diabetic PS} = 3.197 \times 10^{-5} \times \frac{\text{density}^{1.0092}}{\text{Endo}^{1.0662}}$$

The difference between the exponents of density in the two equations was 0.0502 ± 0.0112 (mean ± SE) with $P \leq .0001$.

The fairly unimpressive 43.2% predictive value of these model equations is shown by Fig. 1, in which the curves of prediction are different but not closely predictive of the data points. Thus, the saturated covariance computations of logarithmic transformation of the data show only a single relationship in which independent variables are shared between patient groups, and this relationship predicts only 43.2% of the variance of the PS values.

DISCUSSION

Although it has tacitly been assumed that disturbances in capillary morphology, and basement membrane thickening in particular, are at least partly responsible for a decline in vascular function leading to tissue ischemia, there have been relatively few studies that specifically support this thesis.

We have shown a clear-cut physiological separation between diabetic and nondiabetic subjects in that the former have high resting PS and an inability to recruit new capillaries

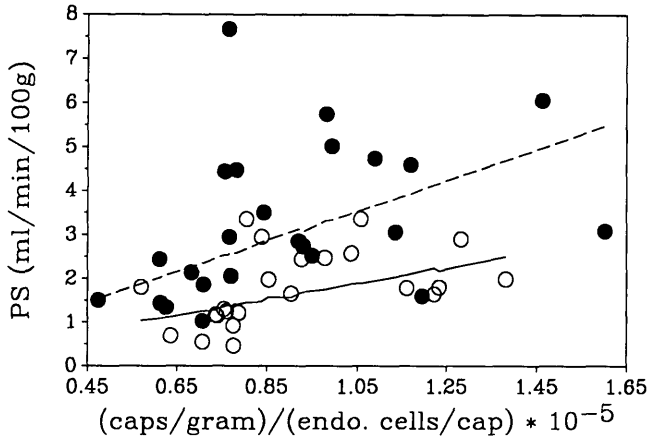

FIG. 1. Plot of permeability–surface area product (PS) vs. density/number of endothelial (Endo) cells per capillary (cap) ratio for data from nondiabetic (○) and diabetic (●) subjects and for fitted curves where density is raised to *a* power and Endo is raised to *b* power (*solid line*, nondiabetic subjects; *dashed line*, diabetic subjects). Predictability of these 2 *lines* is only 43.2%. Irregularities in *lines* are reflection of fact that PS is plotted against density/Endo ratio, but curve is function of this ratio with numerator and denominator raised to different powers. a = 0.959 for nondiabetic and 1.009 for diabetic subjects. b = 1.062 for nondiabetic and diabetic subjects. PS = 3.197×10^{-5} (density^a/Endo^b). r^2 = .432, P = .0000.

in the skin with standard exercise (25). Despite this physiological difference, which may be a forerunner of ischemia, there are very few morphological or demographic correlates of these findings. The high PS values in diabetic subjects are probably caused by a rise in permeability, because capillary density and surface area (proportional to nr) are the same in both groups, and basement membrane width is only marginally different between groups. Note that of all the morphological variables studied, the only one not showing a relationship at any stage of correlation to PS, $\Delta n/n$, or $\Delta b/b$ was basement membrane width.

The physiological differences between groups are easily incorporated into a construct that is still speculative, albeit compatible with many other studies of diabetic ischemia that might follow this path: through an unknown diabetic stimulus (such as ischemia from hemorheologic disturbance, for example), diabetic capillaries at rest are already recruited maximally. When a need for more oxygen delivery is manifest, the diabetic subjects must increase capillary blood flow by upstream dilation with subsequent capillary hypertension rather than by recruitment through vasodilation of precapillary sphincters. The absence of recruitment necessarily leads to capillary hypertension because an increased flow through a fixed vascular bed requires an elevated driving force. Although flow is increased, cell-to-blood diffusion distances are not reduced, whereas capillary hypertension may eventually damage the capillaries, leading to a slow dropout and exacerbation of elongated cell-to-blood diffusion distances. Direct visual support for such a thesis has been obtained, indicating a long-term decrease in capillarity in the cremaster muscle of the *db/db* mouse (34), the same muscle of the adult streptozocin-administered mouse (35), the human diabetic conjunctiva (36), and the diabetic rat mesentary exposed to vasodilating hypoxic superfusion (37). Other evidence of abnormal diabetic microvascular vasoactive phenomena has been reported in various cutaneous beds (16,20,22,24). Much evidence further supports the thesis that capillary hypertension may be the final common path for capillary destruction in many tissues in diabetes, including the kidney, skin, adipose tissue, eye, and brain (38–41).

It could be that our diabetic subjects, who were carefully chosen for lack of end-organ disease, were in the early phase of this process with high resting PS and high $\Delta b/b$ in compensation for minor tissue hypoxia. This compensation response may be inhibited by high blood glucose, and it could be important to determine if normalization of blood glucose raises both PS and $\Delta b/b$. It is conceivable that this might cause further capillary hypertension, unless normalization of blood glucose had salutory effects on a more proximal step of the process, such as obliterating the hemorheological minor-tissue hypoxia leading to increased PS. This question might be assessed with studies of subjects on insulin pumps.

The few morphological-physiological correlations found in this study suggest potentially important relationships, but they emphasize that a critical role for membrane thickness is lacking. In this absence of correlation between basement membrane thickness and physiologically important variables, this study is supported by negative searches for such correlations in type II diabetic muscle capillary basement membranes by Ellis et al. (42).

The only clear-cut relationships common to both diabetic and nondiabetic groups arose through partial regression analysis of logarithmic transformations of physiological and anatomic variables. The solitary relationship arising in both groups was a direct one between PS and the ratio of capillary density to Endo, with minor deviations of exponents of numerator and denominator from unity. Although the relationship between PS and a function of capillary density to Endo accounts for 43% of the variance of PS in both groups, it is apparent that if this is the strongest relationship seen, then little can be offered linking anatomic with physiological variables with any degree of predictability. Nonetheless, it is conceivable to develop a hypothesis consistent with the results, i.e., that PS increases with density because surface area increases, and PS decreases with Endo because larger numbers of endothelial cells may leave more tortuous passageways between cells. However, because capillary density for each group is the same, and the exponent relating PS to density is higher for diabetic than nondiabetic subjects, it seems reasonable to conclude that permeability might be increased across the diabetic capillary for molecules the size of sodium–pentetic acid molecules. Such a conclusion is tentative because anatomic measurement of capillary density may overestimate the capillaries with flow in living skin.

Although this study shows only minimal relationships between physiological and anatomic variables, there are various reasons why a type II error in such studies could obscure a significant relationship. First, the appropriate variables may not have been measured, but it is not readily apparent what these might have been. Second, the tissue-preparation methods could obscure the important differences seen elsewhere, such as between diabetic and nondiabetic striated muscle capillaries (3). Third, the degree of correlation of physiological variables to anatomic variables is related to the range of the anatomic variables. For any slope, it is generally true that the correlation is higher as the range of the independent variables rises. This effect is be-

yond our control, but at least it does not appear to be systematic in these studies, because the range of anatomic variables between groups is similar.

The absence of an easy explanation of high PS by morphological markers in diabetic subjects at rest suggests that a focus for future studies might be those cellular features that may increase permeability in diabetic capillaries. These might include larger or shorter passageways and other related electrostatic or geometric structures.

ACKNOWLEDGMENTS

We are grateful to Berniece Williams for secretarial assistance.

This study was performed in part with support of a merit review project grant from the Veterans Administration Central Office, a biomedical research support grant from the University of Arizona College of Medicine, and National Institutes of Health Grant 1-RO-1-DK-38163-02.

Portions of this work were presented to the Western Association of Physicians, Carmel, California, 16–19 February 1987.

APPENDIX

Our object was to determine the density of capillaries per gram of tissue (n) from capillaries per unit area (n_a) determined by light microscopy. Exhaustive studies by Wiebel (43,44) and others allowed us to state an explicit relationship for the number of particles per unit volume of tissue as

$$n = \frac{n_a}{t + \text{mean } H - 2h} \times \frac{1}{d} \tag{A1}$$

where n is particles per gram, n_a is particles per slide area, t is thickness of the section, H is the mean distance between two horizontal planes that are tangent to the extreme borders of the particle, h is the thickness of the rim or cap of the particle, and d is the gram density of the tissue. Although this formula is adequate for spheres where H is simply twice the radius, more complicated figures, such as hairpin-shaped capillaries fairly well aligned with one another, require some modification of this formula. The straightforward variables are easily dispensed with as follows: t is on average 1×10^{-4} cm, and d can be considered 1 g/cm^3. For looped capillaries largely aligned with each other, they are enclosed on only one end rather than being particles enclosed on two ends. Thus, $2h$ becomes w where w is the average basement membrane width determined for each patient.

To determine H for a cylinder, which is the mean tangent or caliper diameter of a right circular cylindrical capillary in the observer's field, it can be intuitively seen that if all capillaries are viewed along the long axis, H would be $2r$, whereas if viewed horizontally to a field of vertically oriented tubes, H would be l; the actual value for H falls between $2r$ and l.

Weibel cautioned that the precise value of H is most often not feasible to determine and that "one will have to be satisfied with an approximation." His approximation for right circular cylinders is a reasonable starting point (ref. 43, Fig. 2.35). A brief synopsis of his treatment, with modifications for our skin biopsy material, is as follows. Tissues are considered to be sectioned parallel to the x- and z-plane or perpendicular to the y-axis. A typical cylinder is positioned with its long axis running through the origin and subtending an angle θ to the z-axis. Simple trigonometry shows H, the viewer's diameter of the object, to be

$$H(\theta) = l \cos\theta + 2r \sin\theta \tag{A2}$$

Weibel then shows that if the cylinder has a totally random orientation to the z-axis, then the average H is expressed as

$$H = \frac{\int_0^{\pi/2} H(\theta) \sin\theta \, d\theta}{\int_0^{\pi/2} \sin\theta \, d\theta} = \int_0^{\pi/2} H(\theta) \sin\theta \, d\theta \tag{A3}$$

or

$$H = \frac{l}{2} + \frac{\pi r}{2} \tag{A4}$$

In the case of our hairpin capillaries, which are oriented in a semiparallel array, Eq. A4 would not give the proper weight to l and r as contributors to H. In this study, the mean ± SE angle of inclination of the section plane to the long axis of the capillary was 41.9 ± 1.47° for nondiabetic subjects and 42.2 ± 1.79° for diabetic subjects (26). This angle of inclination is the complement of θ. From these data and the fact that they were derived from 24 subjects in each group, the 99% confidence limits for the angle of inclination for nondiabetic subjects is 37.8–46.1°, whereas that for diabetic subjects is 37.2–47.3°. Thus, for all patients taken together, 99% of the angles with the z-axis (θ) are between 42.7 and 58.8° and not between 0 and 90° as demanded by Eq. A3. Thus, for our data, the mean tangent diameter of the cylinder resulted from substituting Eq. A2 into Eq. A3 with the new limits

$$H = \frac{\int_{42.7}^{52.8} (l \cos\theta \sin\theta + 2r \sin^2\theta) \, d\theta}{\int_{42.7}^{52.8} \sin\theta \, d\theta} =$$

$$\frac{\frac{l}{2} \sin^2\theta \Big|_{42.7}^{52.8} + 2r \left[\tfrac{1}{2}\theta - \tfrac{1}{4}\sin 2\theta\right] \Big|_{42.7 \text{ in rads}}^{52.8 \text{ in rads}}}{-\cos\theta \Big|_{42.7}^{52.8}} =$$

$$\frac{l/2 \, [0.6345 - 0.4599] + 2r \, [0.4608 - 0.2408 - 0.3726 + 0.2492]}{-0.6046 - (-0.7349)}$$

or

$$H = 0.67l + 1.4827r \tag{A5}$$

One consideration remained to solve Eq. A1 for our case. Because the capillaries are in a hairpin configuration, roughly half of the capillaries viewed will be limbs carrying blood to the turn, and half will be carrying blood away. Because they are limbs of the same capillary, the number per volume n is related to half of the apparent number per area ($1/2n_a$). This gives the final solution for Eq. A1 as

$$n = \frac{n_a}{2(1 \times 10^{-4} + 0.67l + 1.4827r - w)} \tag{A6}$$

in units of capillaries per gram. For l, we used an average value of 0.04 cm (45).

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