Diabetic retinopathy: quantitative variation in capillary basement membrane thickening in arterial or venous environments

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Abstract

Diabetes mellitus was induced in male beagles by a single injection of an alloxan and streptozotocin cocktail and fasting blood sugar levels maintained between 15 and 20 mmol/l. Five years after induction of diabetes, three diabetic animals were sacrificed, together with sex and age-matched controls, and the retinas fixed for either transmission electron microscopy (TEM) or trypsin digestion. In TEM specimens, capillaries in close proximity to the major vessels were designated as either AE (arterial environment) or VE (venous environment) and the thickness of their basement membranes (BMs) measured using an image analyser based two dimensional morphometric analysis system. Results show that the BMs of retinal capillaries from the diabetic dogs were significantly thicker than those from control dogs. Furthermore, within the diabetic group the AE capillaries had thicker BMs than VE capillaries ($p \leq 0.05$). The controls, however, showed no significant difference in BM thickness between AE and VE capillaries. Although many of the capillaries designated as AE or VE would actually have been derived from the opposite side of the circulation, with respect to BM thickness, they conformed to values of their specific group. The conclusion is that diabetic capillaries are more vulnerable to BM thickening in an arterial environment than in a venous environment.

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It is now established that the diabetic dog develops a retinopathy that is morphologically identical to the preproliferative changes observed in the human retina.^{1,2} The various pathological manifestations of this condition, such as microaneurysms, zones of capillary non-perfusion, pericyte cell death, and thickening of vascular basement membranes (BM) have been consistently reported in this animal model.^{3–5} Of all these changes, BM thickening has received most attention, being the major and earliest morphological characteristic of diabetic microangiopathy, not only in the retina,^{6–8} but in the kidneys,^{9–10} muscle,¹¹ and skin^{12–13} of humans and diabetic animals.

Several studies have sought to measure BM thickening of the retinal capillaries and have quantitatively confirmed BM thickening during diabetes.^{14–17} Despite this, it remains unknown if BM thickening is more marked in any particular portion of the retinal circulation. Ashton¹⁸ suggested that increased mural thickening occurs at the arterial side of the circulation in diabetics,

particularly affecting the precapillary arterioles. Also, workers in our laboratory have previously observed localised capillary BM thickening within the diabetic retina, with vessels showing grossly thickened BMs occurring in the same capillary bed as vessels in which the BM thickness was within normal limits.¹⁹ The subjective impression in this study was that BM thickening was greater in capillaries situated close to arteries than in those close to veins.

In the present study we have investigated, quantitatively, the possibility of a difference in BM thickness between capillaries residing in tissue environments dominated by either major retinal arteries or veins.

Materials and methods

Male beagle dogs were made diabetic with a cocktail of streptozotocin and alloxan according to Anderson *et al.*²⁰ They were maintained as moderate diabetics with fasting blood sugars between 15 and 20 mmol/l. Blood glucose levels were monitored daily and regulated with a single daily injection of Insulatard isophane insulin (20–40 units/day) given subcutaneously before breakfast.

Age and sex-matched control dogs were also maintained under identical conditions to the diabetics. After 5 years' duration of diabetes, three diabetic animals and three controls were sacrificed and the right eye of each processed for trypsin digest examination according to the method described by Kuwabara and Cogan.²¹ The digests were stained with periodic acid Schiff and haematoxylin.

Following removal of the anterior segment and vitreous, the left eye cup was fixed for transmission electron microscopy (TEM) by overnight immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 10 mmol magnesium chloride. Tissue blocks straddling the main blood vessels in the central retina were dissected out, post fixed in osmium tetroxide, dehydrated, and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate before examination in the electron microscope.

The criteria for selecting capillaries were the presence of a single discontinuous layer of pericytes and no more than three endothelial cell junctions per vessel profile. These were deemed more reliable criteria than size alone, since they allowed exclusion of capillary sized arterioles. All the retinal capillaries observed in close proximity (60–80 μ m) to the major vessels were designated as AE – that is, from an arterial environment, or VE, from a venous environment

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Figure 1 Trypsin digest from a normal dog showing that the capillaries (small arrows) lying between adjacent arteries (A) or between an arteriolar bifurcation (large arrows) are derived predominantly from the associated major vessel (magnification $\times 250$).

(see rationale for capillary designation below) and it was also noted if they occurred in the outer plexiform layer or inner plexiform/nerve fibre layer of the retina. Capillaries were then photographed at magnifications ranging from 7000–9000, the images transferred to an image analysis system (Fenestra, Confocal Technologies Ltd, Liverpool), and calibrated according to magnification. The two dimensional thickness of the BM of each capillary was measured by randomly placing a grid over each image and, at sites where the BM was intercepted by a grid line, the shortest distance across the BM was measured. Around 25–30 measurements of BM thickness were made per capillary and from these the mean BM thickness for every capillary sampled was calculated. For each dog, the capillaries measured were taken from TEM blocks from five different regions of central retina. Within the diabetic group of dogs a total of 41 capillaries

Figure 2 Trypsin digest from a normal dog showing retinal veins (large arrows) with a considerable overlap of small arterioles and arterial capillaries (small arrows) (magnification $\times 250$).

were measured; 26 were AE and 15 VE, and for the control group of dogs 77 capillaries were analysed comprising 48 AE and 29 VE. Vessels which had lost their pericyte covering were excluded from the quantitative study. The results were analysed by a two level nested analysis of variance (ANOVA) and also by a two way ANOVA with replication. To ensure that an adequate number of capillaries had been sampled, graphs of the cumulative means for capillary BM thickness were plotted.

Capillary designation

Trypsin digests show that in comparison with the regular arrangement of alternating arteries and veins found in animals such as the rat, the retinal vasculature in the dog is highly asymmetrical. While there is a relatively regular pattern in the equatorial to peripheral retina, with considerable arteriovenous overlap, major portions of the central retina are dominated by arteries and arterioles, with little venous overlap. As a consequence of this arrangement, capillaries lying between adjacent arterioles or between arteriolar bifurcations in the central retina tend to be derived from the associated major vessel (Fig 1). In contrast, the major veins in the central retina are heavily overlapped by arterioles and their capillaries (Fig 2). It was therefore decided to sample capillaries adjacent to either the major arteries or veins in the expectation that a sufficiently large sample would expose any real difference in BM thickness between the two groups.

Results

TRYPsin DIGESTS

Following 5 years of diabetes in dogs, trypsin digest preparations revealed numerous pericyte ghosts in the retinal capillaries, although acellular capillaries were rare and microaneurysms were not observed. In terms of gross architecture and layout, there were no significant differences between normal and diabetic dogs in the time period covered by the present study.

TRANSMISSION ELECTRON MICROSCOPY

AE capillaries in diabetic dogs displayed markedly thickened BMs compared with AE capillaries in control animals (compare Figs 3 and 4). VE capillaries from diabetics also showed increased BM thickening (Fig 5) when compared with control capillaries. The most extreme BM thickening occurred in diabetic AE capillaries with no pericyte covering although such vessels were excluded from the quantitative study.

Mean values of two dimensional BM thickness (μm) of AE and VE capillaries from control and diabetic dogs are presented in Table 1. A two way ANOVA carried out on these data showed that the BMs of retinal capillaries from the diabetic dogs were significantly thicker than those from the control animals ($p \leq 0.005$) (Fig 6).

Within the diabetic group, a two level nested ANOVA showed that AE capillaries had significantly thicker BMs than VE capillaries ($p \leq 0.01$)

Figure 3 Transmission electron micrograph of a retinal capillary from a control dog showing an endothelial cell (E), pericyte (P), and basement membrane (arrows). L = lumen, G = glial cell (magnification $\times 9000$).

(Fig 6). Within the control group there was no significant difference in mean BM thickness between AE and VE capillaries.

In both diabetic and normal dogs, there was no significant difference in the BM thickness of capillaries sampled in the outer or inner retinal layers. Comparable numbers were measured from each layer (Table 1).

A graph plotted of the cumulative means showed that a more than adequate number of capillaries were examined in the study to outweigh any overlap of arterial and venous components of the retinal circulation (Fig 7). Also, a nested ANOVA showed no significant variation in mean values of BM thickness between the three dogs in the control group (Table 2). Similarly, there was no significant difference between individuals in the diabetic group (Table 2).

Discussion

Capillary BM thickening is the most widely

reported lesion of diabetic microangiopathy, being observed consistently in human diabetes and experimental diabetes in several different animal models. That microaneurysms were not encountered in the present study was attributed to the moderate diabetic state, breed, and environmental conditions of the animals. Although it is known that development of thickened BMs is related to prolonged hyperglycaemia,²²⁻²⁴ the pathogenesis of this phenomenon remains to be elucidated adequately. The current investigation has shown that after 5 years of diabetes in dogs, BM thickening is more marked in capillaries, when residing in an environment dominated by a major arterial vessel. Vessels in venous environments were still thicker than non-diabetic equivalents. The phenomenon of vessels with grossly thickened BMs resident in the same capillary bed as others with BMs within the normal range has been previously observed but not quantified.¹⁹

There has been much speculation about the causal factors of BM thickening in diabetic capillaries and it has been proposed that excessive polyol pathway activity leading to damaging accumulations of intracellular polyalcohols in vascular cells may account for increased biosynthesis of BM components.²⁵⁻²⁸ Others have emphasised increased non-enzymatic glycosylation of BM components in diabetes.²⁹⁻³¹ Glycosylated collagens and other BM proteins are known to be less susceptible to protease digestion which may reduce BM modification by vascular cells and lead to a net increase in BM thickness in diabetes.^{32,33} There is also evidence for reduced activity of enzymes involved in BM catabolism³⁴ and increased activity of enzymes involved in BM synthesis.^{35,36} BM proteins may also be altered through oxidative damage by free radicals generated by the autoxidation of glucose or glucose protein adducts³⁷⁻⁴¹ and this, in combination with compromised scavenger systems, may increase the free radical induced modifications of BM components in diabetes.⁴²

Owing to the degree of arteriovenous overlap in the retinal circulation of the dog, not all of the capillaries, designated either AE or VE for the purposes of the present study, were truly arterial or venous in terms of their distance from a precapillary arteriole or a postcapillary venule. It was therefore surprising that the thickest BMs in the VE group were never greater than the thinnest in the AE group. Considering the inevitability of arteriovenous overlap, if the BM thickness was related to the distinctive arterial or venous nature of the vessel, it would be expected that the AE group would include some vessels with a BM thickness approaching the greatest value recorded for the VE group and vice versa. However, the greatest value recorded for BM thickness in the venous group was less than the smallest recorded for the arterial group. Accordingly, it would appear that venous capillaries suffer more BM thickening when resident in an arterial environment while arterial capillaries suffer less in a venous environment.

As BM thickening in diabetes has been related to hyperglycaemia,²² it could be speculated that the differential BM thickening in the present study is associated with the relative concentra-

Figure 4 Transmission electron micrograph of an arterial environment capillary from a 5 year diabetic dog showing the thickened basement membrane (arrows). E = endothelial cell, P = pericyte, L = lumen (magnification $\times 9000$).

Table 1 Mean two dimensional values of capillary basement membrane (BM) thickness for normal and diabetic dogs. The results are presented as mean BM thickness values (µm) for all capillaries measured (value shown in bold with SD) and also, for capillaries appearing in the outer and inner retinal layers. The proportion of inner and outer capillaries for each group appears in brackets

	Diabetic capillaries			Control capillaries		
	Overall mean thickness (µm)	'Outer' capillaries	'Inner' capillaries	Overall mean thickness (µm)	'Outer' capillaries	'Inner' capillaries
Arterial environment capillaries	0.258 (0.148)	0.248 (52.4%)	0.268 (47.6%)	0.142 (0.036)	0.138 (33.3%)	0.146 (66.7%)
Venous environment capillaries	0.173 (0.055)	0.171 (47.6%)	0.175 (52.4%)	0.152 (0.332)	0.141 (42.8%)	0.163 (57.2%)

Figure 5 Transmission electron micrograph of a venous environment capillary from a 5 year diabetic dog. Note that the thickening of the basement membrane (arrows) is not as extreme as in the arterial environment capillary. E=endothelial cell, L=lumen (magnification ×9000).

Figure 6 Graph showing the basement membrane thickness of AE and VE designated capillaries in diabetic and control dogs. Means (SD) are indicated. (AE=capillaries in an arterial environment; VE=capillaries in a venous environment.)

tions of glucose in the arterial or venous environments. However, at present, it is not known if such a gradient exists in the retina during chronic hyperglycaemia. It is also possible that the differential capillary BM thickening in the diabetic animals may be related to the tissue oxygen tension and increased oxidative stress in the arterial regions. Oxygen tension in the retinal neuropile of the pig is known to be higher in the vicinity of retinal arterioles than in the remainder of the extracellular space of inner retina.⁴³ Also, the high tissue oxygen tension in the juxta-arteriolar region is reflected by the capillary-free zone around the arterioles in human retina.

Figure 7 Plots of running means for capillary basement membrane (BM) thickness from (a) diabetic dogs and (b) control dogs. (■) Arterial environment capillaries; (□) venous environment capillaries.

Table 2 Results from a two way ANOVA showing comparison between diabetic and normal and between arterial environment (AE) and venous environment (VE) capillaries

Source of variation	df	SS	MS	F
Subgroups	3	0.545	0.1812	
A (diabetics/controls)	1	0.31	0.31	21.15***
B (AE/VE capillaries)	1	0.1038	0.1038	7.08***
A×B interaction	1	0.1312	0.1312	8.95***
Within groups (error)	248	3.63624	0.01466	
Total	251	1.98175		

F 0.01 (1,248)=6.63**, F 0.005 (1,248)=7.88***.

However, it has recently been suggested that the combination of glycation and oxidation, a phenomenon described as 'glycooxidation', may be more important in protein modifications of the extracellular matrix occurring in diabetes than either process alone.⁴⁴ Therefore, increased BM thickening of retinal capillaries residing in arterial environments, as described in the present study, may result from a complex interplay between hyperglycaemia and enhanced oxidation in such environments.

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