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ORIGINAL ARTICLES – Laboratory science

Diabetic retinopathy: morphometric analysis of basement membrane thickening of capillaries in different retinal layers within arterial and venous environments

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Abstract

***Aims*—To assess quantitatively variations in the extent of capillary basement membrane (BM) thickening between different retinal layers and within arterial and venous environments during diabetes.**

***Methods*—One year after induction of experimental (streptozotocin) diabetes in rats, six diabetic animals together with six age-matched control animals were sacrificed and the retinas fixed for transmission electron microscopy (TEM). Blocks of retina straddling the major arteries and veins in the central retina were dissected out, embedded in resin, and sectioned. Capillaries in close proximity to arteries or veins were designated as residing in either an arterial (AE) or a venous (VE) environment respectively, and the retinal layer in which each capillary was located was also noted. The thickness of the BM was then measured on an image analyser based two dimensional morphometric analysis system.**

***Results*—In both diabetics and controls the AE capillaries had consistently thicker BMs than the VE capillaries. The BMs of both AE and VE capillaries from diabetics were thicker than those of capillaries in the corresponding retinal layer from the normal rats ($p \leq 0{\cdot}005$). Also, in normal AE and VE capillaries and diabetic AE capillaries the BM in the nerve fibre layer (NFL) was thicker than that in either the inner (IPL) or outer (OPL) plexiform layers ($p \leq 0{\cdot}001$). However, in diabetic VE capillaries the BMs of capillaries in the NFL were thicker than those of capillaries in the IPL ($p \leq 0{\cdot}05$) which, in turn, had thicker BMs than capillaries in the OPL ($p \leq 0{\cdot}005$).**

***Conclusions*—The variation in the extent of capillary BM thickening between different retinal layers within AE and VE environments may be related to differences in levels of oxygen tension and oxidative stress in the retina around arteries compared with that around veins.**

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Thickening of capillary basement membranes (BM) during diabetes is well documented both in human diabetics as well as in animal models of experimental diabetes.[1–10] Many studies carried out previously on BM thickening of retinal capillaries have included data from all capillaries within the retina regardless of their location with respect to the different layers within the retina. Only two investigations have included an assessment of variation in capillary BM thickening between different retinal layers.[5 11] These studies both found significant differences in the BM thickness of capillaries from the different retinal layers in normal rats: capillaries in the nerve fibre layer (NFL) having significantly thicker BMs than capillaries located within either the inner plexiform layer (IPL) or the outer plexiform layer (OPL). After 12 months of diabetes Fischer and Gärtner[5] reported that although capillary BM thickness had increased in all three retinal layers, capillaries in the NFL still had significantly thicker BMs than capillaries in either the IPL or OPL.

Furthermore, a recent study carried out in our laboratory, on diabetic dogs, showed that retinal capillaries located in a tissue environment dominated by a major retinal artery had significantly thicker BMs compared with those of capillaries found in a venous environment.[7]

The present study assesses quantitatively the combined effects of the two variables mentioned above – that is, location within different retinal layers and closeness to major arteries or veins, on the extent of capillary BM thickening in the rat after 12 months' duration of diabetes. The information from this investigation may help to establish the factors which are influential in causing BM thickening during diabetes.

Materials and methods

Diabetes was induced in a colony of male Wistar albino rats (weighing approximately 250 g) by a single injection of streptozotocin (40 mg/kg). After 12 months' duration of diabetes (fasting blood glucose ≥ 15 mmol/l), six of the diabetic animals together with six age and sex-matched control rats were sacrificed by a lethal

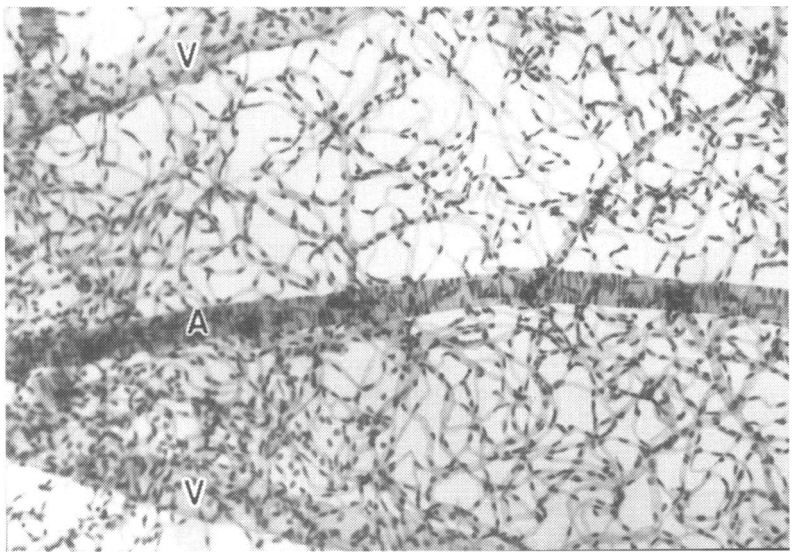

Figure 1 Trypsin digest of the retinal vasculature from a control rat showing the alternating arrangement of the major arteries and veins. V=vein; A=artery. ×250.

injection of sodium pentobarbitone. The eyes were enucleated, the anterior segments removed, and the posterior eye cup immersed overnight in 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer containing 10 mmol magnesium chloride. After fixation, blocks of retina straddling the major arteries and veins in the central to equatorial region of the retina were dissected out, post fixed in 1% osmium tetroxide and embedded in Spurr's resin. Ultrathin sections for transmission electron microscopy (TEM) were cut from blocks taken from the right eye of each animal and then stained with uranyl acetate and lead citrate.

Trypsin digest preparations were also prepared, according to the method described by Kuwabara and Cogan[12] in order to examine the retinal vascular pattern of the rat. These preparations showed that the retinal vasculature of the rat has a very symmetrical arrangement; the major retinal arteries alternate with the major veins and there is little arteriovenous overlap (Fig 1). Therefore, the majority of capillaries found in close proximity to either retinal arteries or veins tend to be derived from the associated major vessel.

The criteria for selecting capillaries have been described in a previous study.[7] However, briefly, a vessel was designated a capillary if a single discontinuous layer of pericytes was present and there were no more than three endothelial cell junctions per vessel profile.

Images of all capillaries in each section examined in the TEM were taken at 9K and transferred directly to an image analyser system (Kinetic Imaging Ltd, Liverpool). Capillaries in close proximity (60–80 μm) to major retinal arteries were designated as residing in an arterial environment (AE) while those capillaries close to retinal veins were considered to be resident in a venous environment (VE). The retinal layer in which each capillary was located—that is, the nerve fibre layer (NFL), the inner plexiform layer (IPL), and the outer plexiform layer (OPL) was also recorded.

The method used to measure the two dimensional (2D) thickness of the retinal capillary BM has been described in detail previously[7]; however, a brief description will also be given below. A 2D grid of lines 15 mm apart was superimposed over each capillary profile on the image analyser and, at the points where a grid line intercepted the BM, the shortest distance across the BM was measured. This method resulted in approximately 25–30 measurements of BM thickness per capillary and these values were then used to determine the mean BM thickness for each individual capillary sampled. For both control and diabetic groups, the BM thickness was measured for 180 AE capillaries and 180 VE capillaries, comprising 60 capillaries from each of the three retinal layers – that is, the NFL, IPL, and OPL. The results were then analysed by a two way analysis of variance (ANOVA) as well as by a two tailed Student's *t* test.

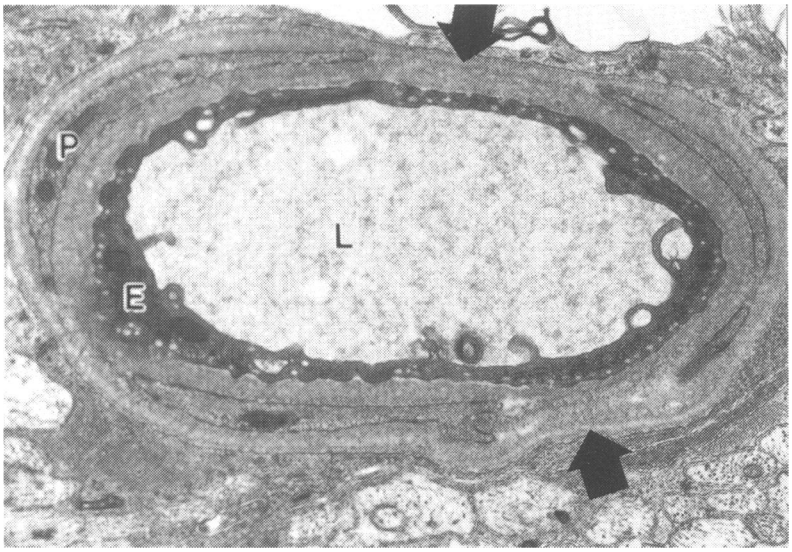

Figure 2 Transmission electron micrograph of a retinal capillary from the nerve fibre layer of a 1 year diabetic rat showing a grossly thickened basement membrane (arrows). E=endothelial cell; P=pericyte; L=lumen. ×12 600.

Results

TEM examination of the retinas from the 12 month diabetic rats showed increased thickening of the capillary BM compared with those of the control rats. The most extreme thickening of the BM was observed in diabetic capillaries located in the NFL close to the inner limiting membrane (Fig 2). Pericyte loss was also observed in a few capillaries from two of the six diabetic animals examined (Fig 3), although these vessels were excluded from the quantitative study. All six diabetic rats had visible cataracts at the time of sacrifice.

The mean values for the 2D BM thickness (μm) of AE and VE capillaries from the NFL, IPL, and OPL of both control and diabetic rats are shown in Table 1. Both methods of statistical analysis used – that is, the *t* test and the ANOVA produced the same results although the probability levels varied between the two tests. In the present results section the probability values given will be those resulting from the ANOVA.

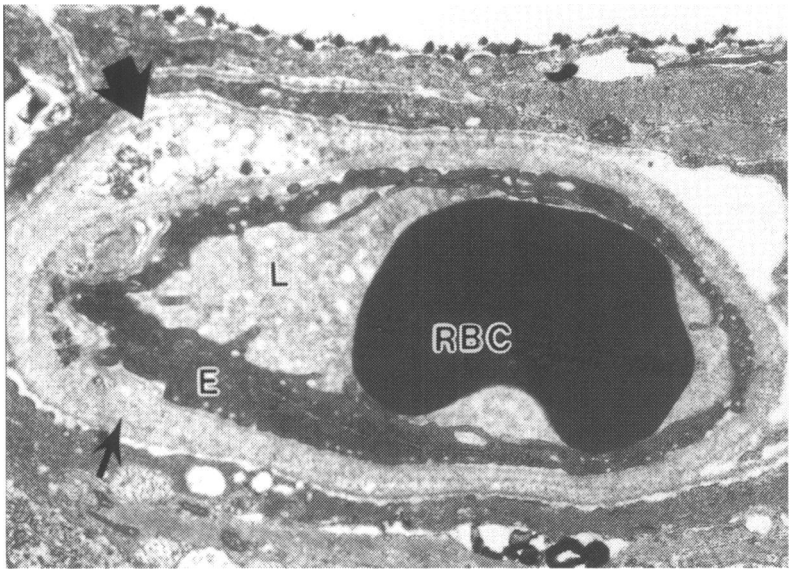

Figure 3 Transmission electron micrograph of a retinal capillary in the nerve fibre layer of a 1 year diabetic rat showing a pericyte ghost (large arrow) and thickened basement membrane (small arrow). E=endothelial cell; L=lumen; RBC=red blood cell. ×10 800.

Results show that the BMs of both AE and VE capillaries from diabetic animals were significantly thicker than those of capillaries in the corresponding retinal layers from the control animals ($p \leq 0.005$). In the control rats, the AE capillaries had significantly thicker BMs compared with the VE capillaries in the same retinal layer ($p \leq 0.005$). However, in the diabetic group the BMs of the AE capillaries were thicker than those of the VE capillaries only in the NFL and OPL ($p \leq 0.005$). In the IPL of diabetics the mean BM thickness of AE capillaries was greater than that of VE capillaries although the difference was not statistically significant.

In both AE and VE capillaries from control rats, and in AE capillaries from the diabetics, the BM in NFL capillaries was significantly thicker than that in those of either the IPL or OPL ($p \leq 0.005$). There was no significant difference in capillary BM thickness between IPL and OPL capillaries in either normal or diabetic animals. However, in the VE capillaries from the diabetic animals, the BMs of capillaries in the NFL were significantly thicker than those in the IPL ($p \leq 0.005$) which, in turn, had thicker BMs than capillaries located in the OPL ($p \leq 0.005$).

Discussion

BM thickening is one of the most widely studied morphological changes occurring to the microvascular system during diabetes. Although the disease is characterised by hyperglycaemia, it is not clear which of the many sequelae of hyperglycaemia contribute to the development of the vascular disease. A number of factors have been implicated in contributing to increased BM thickening during diabetes. These include increased polyol pathway activity within the microvascular cells[13 14] and changes in the activities of enzymes involved in BM synthesis or breakdown.[15–17] Important biological properties of BMs, such as their susceptibility to proteolytic resorption, may also be altered in diabetes through non-enzymatic glycosylation and associated oxidative modification of the BM proteins.[18–22]

The results of the present investigation show that, within the retina of both control and 1 year diabetic rats, capillary BM thickness varies between the different retinal layers, with the capillaries located in the nerve fibre layer consistently having thicker BMs than capillaries found in either the inner or outer plexiform layers. Similar differences in capillary BM thickness with respect to retinal layers have been reported previously in both normal and diabetic rats.[5 11] It is interesting that the values which Sosula *et al* [11] reported for the mean BM thickness of capillaries in each of the three vascularised layers of normal albino rats approximate to the measurements for AE capillaries of control animals in the present study. Also, their values for hooded rats, which were somewhat lower than the albinos, closely parallel those of VE capillaries. The results of Fischer and Gärtner[5] for the 'average basal lamina width' of retinal capillaries in the NFL, IPL, and OPL of control rats were lower than our values for the AE capillaries of normal rats, but were similar to those of VE capillaries. In the present study we found that in normal rats the BMs of AE capillaries were significantly thicker than those of VE capillaries. Therefore, in morphometric studies of BM thickness it is important that equal numbers of AE and VE capillaries should be included in order to ensure an accurate determination of the mean BM thickness.

The values for the thickness of retinal capillary BM in 1 year diabetic rats which Fischer and Gärtner[5] reported were considerably lower than the results from the present investigation. However, the discrepancy between these studies could be due to differences in the diabetic state of the animals. In Fischer and Gärtner's study the rats were classified as diabetic if their fasting blood sugar levels exceeded 170 mg/100 ml (9·4 mmol/l),[23] whereas, in the present study the rats in the diabetic group had blood sugar levels of 15–20 mmol/l.

The present study showed that within each of the three retinal layers studied in both control and diabetic rats, the BMs of capillaries located close to major retinal arteries were significantly thicker than those of capillaries found in close proximity to retinal veins. A previous study on 5 year diabetic dogs also showed that retinal capillaries residing in an arterial environment had significantly thicker BMs than those from a venous environment regardless of whether they were actually arterial or venous capillaries.[7] Thus, the data

Table 1 Mean basement membrane thickness values (μm) for retinal capillaries from the nerve fibre layer (NFL), inner plexiform layer (IPL), and outer plexiform layer (OPL) in arterial (AE) and venous (VE) environments from control and 1 year diabetic rats. (Values=mean (SEM). For each value shown n=60)

	Controls		*Diabetics*	
	AE	*VE*	*AE*	*VE*
NFL	0·164 (0·032)	0·146 (0·020)	0·381 (0·139)	0·307 (0·138)
IPL	0·114 (0·019)	0·101 (0·018)	0·233 (0·100)	0·200 (0·025)
OPL	0·120 (0·019)	0·108 (0·020)	0·186 (0·058)	0·150 (0·014)

from the present investigation together with those from previous studies collectively indicate that in diabetes, retinal capillary BM thickening varies with respect to different retinal layers and relative to arterial or venous environments.

In the present study normoglycaemic control animals showed the same local gradations in capillary BM thickening as the diabetics. As the control animals were age-matched to the diabetic group (15 months), regional differences of BM thickness in the retinal capillaries of the controls may represent an age-related change. Diabetes-like modifications in long half-life components of the extracellular matrix such as BM proteins are known to occur during the aging process owing to the combined effects of glycation and oxidative damage.[24 25] Thus, the increased BM thickening of AE capillaries in normal rats may be related to increased oxidative stress at the arterial side of the circulation.[26] Likewise, the greater thickening of BMs in capillaries within the nerve fibre layer may result from the higher tissue oxygen tension close to the vitreous body.[26] That such gradients of capillary BM thickness relative to the retinal arteries and vitreous body were accentuated in the diabetic animals is probably the result of the massively increased glycation of BM components in diabetes. BM protein modification caused by oxidative damage is also likely to be increased in diabetes through the action of oxidising free radicals generated by products of protein glycation.[19 27 28] Such oxidative damage may be further exacerbated by depletion of antioxidant defences in diabetic animals.[29]

Recently there has been considerable interest in the idea that oxidative stress may represent a common pathway linking several pathophysiological mechanisms of tissue damage to the pathogenesis of diabetic complications.[19] Also, glycoxidation, a process combining glycation and oxidation, has been implicated as being more important in the modification of proteins of the extracellular matrix than either process alone.[25] Therefore, it is possible that the increased hyperglycaemia in diabetes induces glyco-oxidative changes in BM proteins which are exacerbated by increased oxidative stress at sites where the microenvironment is rich in oxygen, such as close to major retinal arteries or the vitreous body.

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