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Morphometric evaluation of capillary basement membrane thickness in the quadriceps muscle of diabetic and nondiabetic Chinese hamsters

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Surnmary. Quadriceps muscle capillaries from 19-23 month old genetically diabetic (XA and AC) and nondiabetic (M) subline Chinese.. hamsters were morphometrically evaluated to determine if capillary basement membrane thickening (CBMT) is a quantifiable complication of diabetes. Significant CBMT was present in the diabetic XA Chinese hamsters (49.37 nm ± 17.81, $p < 0.007$) in comparison with the nondiabetic M. hamsters (34.08 nm ± 9.98). Although there was a trend towards expansion of the muscle capillary basement membranes in the diabetic AC Chinese hamsters, the value was not statistically significant. A nested analysis of variance showed that the greatest source of variation in basement membrane thickness occurred among capillaries within each animal. In addition, a positive correlation ($r = 0.62$; $p < 0.002$) existed between blood glucose levels and CBMT in the XA subline. These data should serve as guidelines for evaluation of antimicrovascular disease compounds which will be tested to determine if they prevent or retard microangiopathy in the diabetic Chinese hamster.

Key Words: Muscle capillaries-Basement membranes-Diabetic

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Introduction

Capillary basement membrane thickening (CBMT) has been recognized as a primary manifestation of diabetic microangiopathy which affects the capillary beds of many tissues and organs, including the eyes, kidneys and skeletal muscle. In severe cases, diabetic microangiopathy appears to be the major etiologic agent underlying blindness, kidney failure or limb loss. Few systematic studies have been conducted in laboratory animals to correlate metabolic perturbations, age, length or severity of diabetes with degree of basement membrane thickening in various tissues. Systematic characterization of diabetic microangiopathy could help to elucidate some of the critica1 factors underlying basement membrane thickening. Since muscle biopsies are easily obtained, CBMT of these capillaries .has received the bulk of attention in human studies. The etiology and pathogenesis of this microvascular complication, however, are still under investigation.

In an effort to understand the progression of muscle CBMT and the potential contribution of metabolic derangement to this disorder, diabetic animal models have been the focal point of intense research. Few animal models (Bloodworth et al.,1969; Creutzfeldt el al., 1970; Yesus et al., 1976; Itabashi et al., 1981), however, exhibit manifestations of the diabetic syndrome which closely resemble those of humans, in particular, CBMT of skeletal muscle capillaries. Therefore, the purpose of this study was to determine if morphometric analysis could demonstrate significant thickening of basement membranes in the quadriceps muscle capillaries of aged (19 - 23 month old) spontaneously diabetic Chinese hamsters of the XA and AC genetic sublines in comparison with age-matched nondiabetics of the M genetic subline.

Table 1. Group rneans* and statistical analysis of rnetabolic data of nondiabetic (M subline) and diabetic (XA and AC sublines) Chinese harnsters

Subline(s)	N = Total Number of Anirnals	Fasting Terminal Blood Glucose (mg/dl)	Fasting Terminal Plasma Insulin (μU/ml)	Fasting Terminal O-hydroxybutyrate (mmol/l)	Pancreatic Insulin (U/g)
M	6	109 ± 25	11 ±11	1.82 k0.37	1.09 ±0.52
XA	12	240 ±103**	5 ± 4**	3.07 ±2.94**	0.29 ±0.09
AC	6	147 ±121	19 k17	1.37 ±0.55***	0.21 k0.14
XA vs M		p < 0.009	NS	NS	p < 0.001
AC vs M		NS	NS	NS	p < 0.002
XA vs AC		NS	p < 0.02	NS	NS

*All values are represented as rnean ±SD
**Mean' ±SD based upon 11 animals
***Mean ±SD based upon 4 anirnals

Table 2. Quadriceps rnuscle capillary minimal basement rnernbrane thickness (M subline) and diabetic (XA and AC subline) Chinese harnsters

M Nondiabetics			XA Diabetics			AC Diabetics		
Sex	Age (mo)	Mean ±SD of 10 Capillaries (nm)	Sex	Age (mo)	Mean ±SD of 10 Capillaries (nm)	Sex	Age (mo)	Mean ±SD of 10 Capillaries (nm)
S	22	31.50± 9.12	♂	22	42.50± 9.18	S	20	42.11 ± 9.05
♀	21	34.98+ 8.38	S	22	47.79± 8.14	S	20	28.53± 4.68
Y	22	30.77± 4.74	♀	20	55.80±1.32	S	21	30.91 ± 8.65
S	20	28.97± 3.92	♀	20	48.07 f12.82	♀	19	72.36 ±40.52
S	20	46.05 ±12.08	0	20	50.08± 9.48	Y	19	53.42 ±10.66
♀	21	32.22± 5.36	♀	22	44.79 ±26.35	S	23	40.93.±10.07
			♀	22	58.65 f18.01			
			S	21	37.95 112.34			
			S	20	57.36 ±14.41			
			Y	21	42.56 ± 9.90			
			♀	20	43.14 ± 8.51			
			♀	20	63.85 119.59			
Grouprneans		34.08 ± 9.98			49.37 117.81			44.71 ±23.40

p values M vs XA (p < 0.007)
M vs AC (NS)
XA vs AC (NS)

Materials and methods

Animals

Twelve genetically diabetic XA subline, six diabetic AC subline, and six nondiabetic M subline animals were selected from the Upjohn Chinese hamster colony. The diabetic XA and AC subline Chinese hamsters exhibit many characteristics of diabetes mellitus including abnormal glucose tolerance, hyperglycemia and glycosuria. The XA hamsters tend to be more glycosuric and less ketonuric than the AC animals (Gerritsen, 1982). Nondiabetic M subline Chinese hamsters, on the other hand, are generally normoglycemic and consistently nonglycosuric (Gerritsen, 1982). Animals of both sexes were age matched (range of 19-23 months). Duration of diabetes in the XA and AC subline animals

Table 3. Analysis of sources of variability for quadriceps muscle capillary basement membrane thickness

Source of Variation	Percent of Variation
location	53.64
hamsters	20.46
sublines	12.30
error	13.60
TOTAL	100.00

was 17-22 months. Food and water were supplied *ad libitum* throughout the study period and animals were housed separately under controlled photoperiod (12 hrs/day; lights on at 0600 hrs) and environmental conditions.

Metabolic studies

At termination, fasting orbital blood samples were collected from both diabetic and nondiabetic animals for analysis of blood glucose (Lloyd et al., 1978), plasma insulin (Zaharto and Beck, 1968) and plasma beta-hydroxybutyrate levels (Lloyd et al., 1978). Group mean values of these parameters were calculated for each subline. Animals were terminated by cervical dislocation and the pancreas was resected for radioimmunoassay of insulin (Chang et al., 1977).

Tissue preparation and rnorphornetric analysis of capillary basernent rnernbranes

Immediately after termination, samples of quadriceps muscle were excised from each animal and immersed in cold Karnovsky's fixative (half strength) for 2-4 hours at 4° C. Following primary fixation, tissues were rinsed in 0.2 M sucrose in 0.1 M cacodylate buffer, post fixed in 1% osmium tetroxide, *en bloc* stained in 2% aqueous uranyl acetate, dehydrated in graded ethanols and propylene oxide and embedded in Polybed 812. All blocks were coded to prevent bias.

Several thick sections were taken from a minimum of 2 blocks per animal, stained with 1% toluidine blue, and examined via light microscopy. Areas containing capillaries were thin sectioned (80-90 nm on a LKB NOVA ultramicrotome, collected on formvar-coated 50 mesh copper grids, stained with uranyl acetate and lead citrate in an LKB Ultrostainer and examined under a Philips 301 transmission electron microscope. The microscope magnification was calibrated with a Pelco 606 carbon grating replica each time the high voltage was activated. At least ten cross sectioned capillaries were selected for photography on the basis of cross sectional appearance and clearly delineated basement membranes. All negatives were printed to a calibrated final magnification of x 22,000 to x 24,000.

For the purpose of this study, the established Williamson two point method for quantitation of minimal basement membrane thickening (MBMT) was used (Williamson et al., 1969). Accordingly, five of the thinnest and most clearly defined basement membrane widths from the short axes of ten capillaries per animal were measured with a Zeiss Videoplan Image Analysis System. Areas where capillary basement membranes were tangentially sectioned or fused with pericyte basement membranes were excluded. All measurements were separated by a linear distance of at least one centimeter. The two minimum measurements for each capillary (ten capillaries per animal) were averaged to quantitate the minimal basement membrane thickness.

Statistics

Metabolic data were analyzed by a one-way ANOVA. The MBMT data were analyzed with an analysis of variance on a 3-factor nested design. Spearman correlation coefficients were calculated to determine if metabolic parameters influenced CBMT. Statistical tests were made to determine if the correlation coefficients were significantly different from zero. Differences were considered significant if $p < 0.05$. An analysis of the sources of variation was performed on the data to evaluate the percentage of variation contributed by measurements among capillaries, animals and sublines.

Results

Metabolic data

Table 1 shows the metabolic data for the nondiabetic and diabetic sublines. The terminal fasting blood glucose of the XA subline was significantly higher in comparison with that of the M subline. The terminal blood glucose of the AC animals did not differ from that of either the XA or M sublines. The fasting terminal plasma insulin of the XA subline was significantly lower than that of the AC subline, while plasma insulin of the M subline did not differ from either diabetic subline. Beta-hydroxybutyrate levels of all three sublines were similar. Pancreatic insulin content in both diabetic sublines was significantly lower than that of the nondiabetic M subline.

Quadriceps rnuscle capillary basernent rnernbrane thickness

Table 2 presents the mean minimal quadriceps muscle capillary basement membrane measurements for each animal and subline. Capillary basement membrane thickness in the XA subline was significantly greater than that of the M subline (Figs. 1, 2). Although the minimal basement membrane thickness of the AC subline displayed a trend towards expansion in comparison with that of the M subline, the difference was not statistically significant.

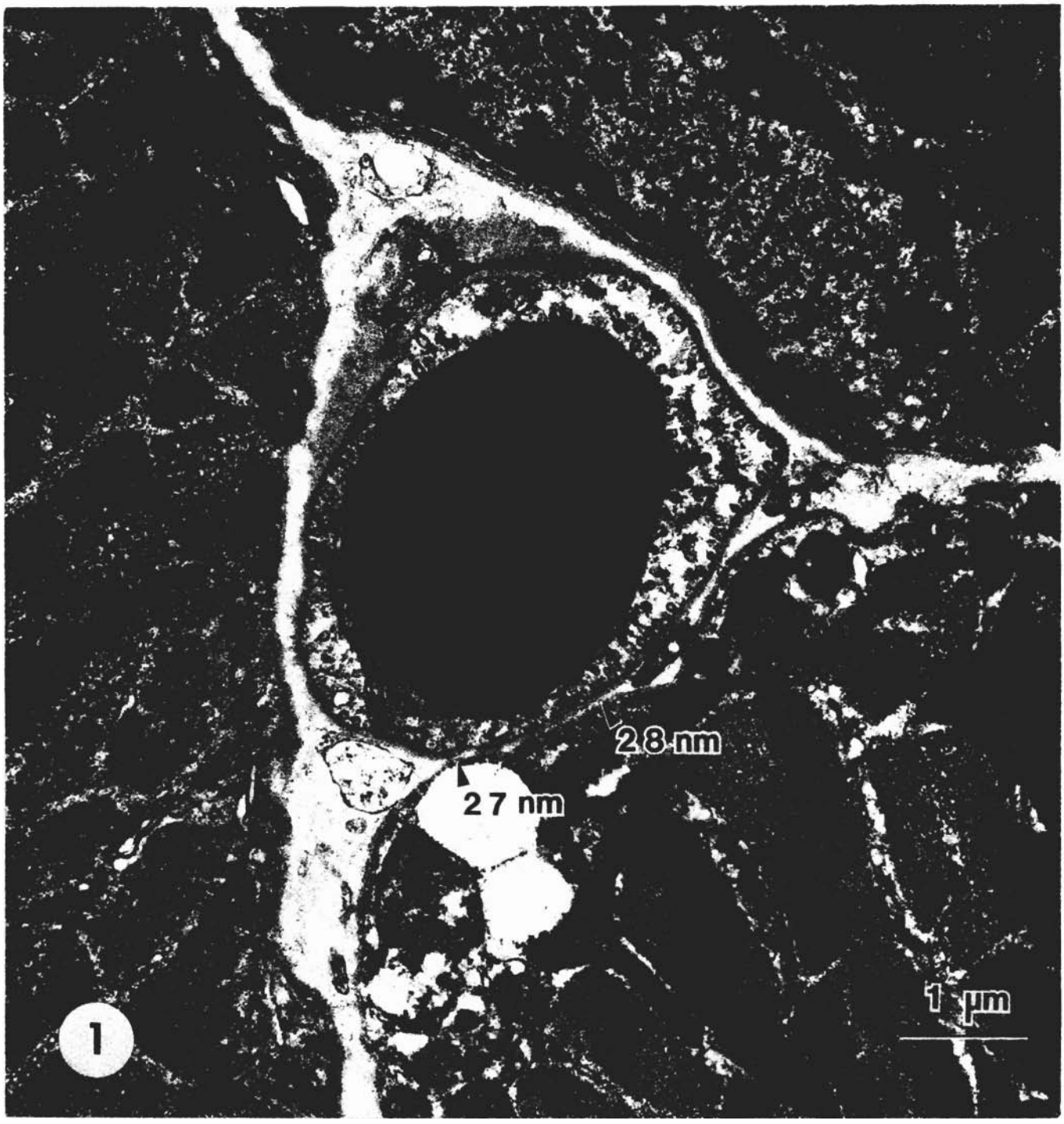

Fig. 1. Quadriceps capillary from a nondiabetic (Msubline) Chinese hamster. The indicated measurements represent the two thinnest areas of clearly defined basement membrane. Note that the minimal basement membrane rneasurements on this figure are less than those in Figure 2. x 25,596

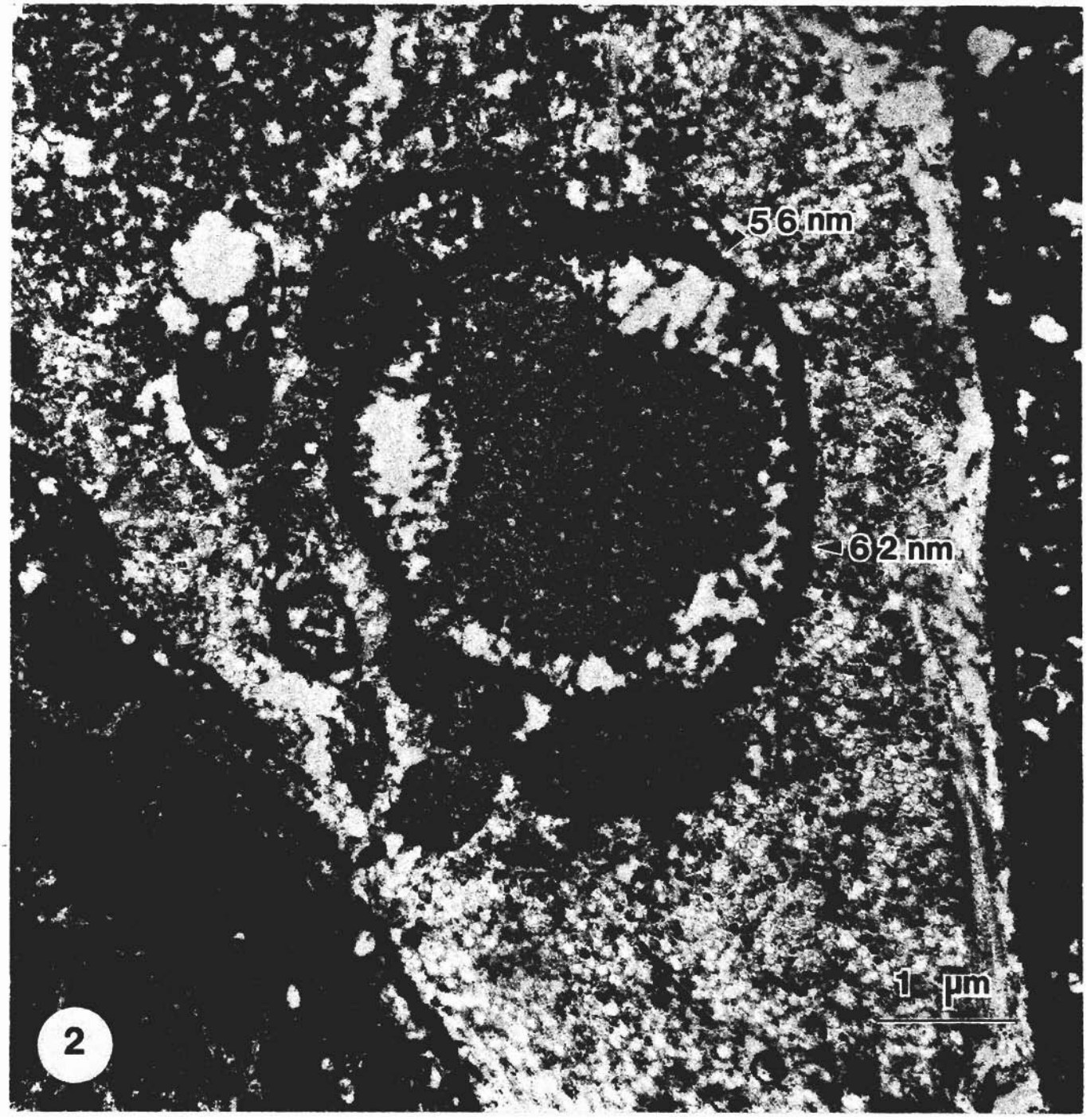

Fig. 2. Quadriceps muscle capillary from a diabetic (XA subline) Chinese hamster. Note that the two minimal basement membrane measurements on this figure are considerably larger than those in Figure 1. x 25,272

Sources of variance

The standard deviations associated with the mean basement membrane measurements for both diabetic sublines were considerably greater than those ofthe M subline. An analysis of variance indicated that the greatest source of variation (53.6%) occurred among the measurements of individual capillaries within each animal (Table 3).

Correlations

The Spearman Correlation Test indicated that a positive relationship existed between CBMT and blood glucose levels ($r = 0.62$; $p < 0.002$) in the XA subline.

Discussion

The current study suggests that quadriceps muscle capillary basement membrane thickening (CBMT) is a quantifiable complication in 19-23 month old diabetic XA subline Chinese hamsters. Similar microvascular complications in skeletal muscle have been thoroughly documented in human diabetics (Kilo et al., 1972; Williamson and Kilo, 1976, 1977; Tilton et al., 1981; Jackson et al., 1982; Ganda et al., 1983). A limited number of diabetic animal models, such as spontaneously diabetic *Mystromys albicaudatus* (Yesus et al., 1976), the KK mouse (Itabashi et al., 1981) and the alloxan-diabetic dog (Bloodworth et al., 1969) have also shown diabetes-related basement membrane thickening in skeletal muscle capillaries. Although the degree of thickening in the Chinese hamster is slightly less than that of other animal models, this discrepancy rnay be due to several factors. First, CBMT in the KK mouse, alloxan-diabetic dog, and the spiny mouse (Creutzfeldt et al., 1970) was quantified by the Siperstein method (Siperstein et al., 1973), which includes measurements of thickened basement membranes due to tangential sections and results in an artificially high value for average basement membrane thickness. Data generated by this type of morphometric analysis are often irreproducible (Gundersen el al., 1978; Camerini-Davalos et al., 1979; Williamson and Kilo, 1979). The Williamson two-point method employed in the current study, however, is a conservative and reproducible estimation of capillary basement membrane thickness. This procedure excludes tangential or undefined areas of basement membrane from measurement and rnay account for the smaller CBMT values in the diabetic Chinese hamster. Second, capillary basement membrane thickness measurements in the KK and spiny mice were made in the gastrocnemius muscle. It has been demonstrated that CBMT increases with an elevation in venous pressure (Williamson et al., 1971). Since venous pressure is highest in the distal lower extremities in comparison with the rest of the body, CBMT in the gastrocnemius muscle tends to be greater than that in the quadriceps muscle in borth diabetic and non-diabetic humans (Williamson and Kilo, 1977, 1983; Tilton et al., 1981; Ganda et al., 1983) and in animal models (Williamson et al., 1971). Third, it is possible that diabetes-related capillary basement membrane thickening in the quadriceps muscle of the diabetic Chinese hamster rnay progress at a slower rate in comparison with other diabetic animal models. Despite these absolute differences,. the relative increase of MBMT in the diabetic XA hamsters in comparison with the nondiabetic M hamsters is similar to that observed between diabetic and nondiabetic animals in other studies (Yesus et al., 1976; Itabashi et al., 1981).

Although considerable variability was present among capillary MBMT measurements in all three sublines, it was more pronounced in the diabetic XA and AC sublines. Statistical analysis determined that the major source of variation was among capillaries within an animal. This variation rnay explain why CBMT in the AC animals was not significantly altered from that of the nondiabetic M animals. Considerable intramuscular variation has also been observed in skeletal muscle capillaries of other diabetic animal models (Yesus et al., 1976; Itabashi et. al., 1981), as well as in human diabetics (Vracko and Strandness, 1967; Peterson and Forsham, 1979; Williamson and Kilo, 1983). It has been hypothesized that this variation rnay be due to a diffuse segmental form of CBMT in which the basement membrane is uniformly thickened around the circumference of the capillary, but not along its length (Williamson and Kilo, 1977). Although the cause of thickening is not understood, it has been suggested that segmental thickening rnay be due to the leakage of glycoproteins at epithelial cell junctions. This rnay induce subsequent localized thickening of basement membrane at these points (Williamson and Kilo, 1976). Theoretically, segmental thickening should be accompanied by a relative increase in standard deviation of the measurements. This association between MBMT and elevated standard deviation has been observed in previous studies of diabetic animal models (Williamson et al., 1971; Yesus et al., 1976) and humans '(Williamson and Kilo, 1976, 1977), as well as in the current study, and provides support for this theory.

The weak positive relationship between CBMT and blood glucose in the current study is supported by clinical findings which índicate that CBMT in diabetic patients increases with hyperglycemia (Sosenko et .al., 1984) and can be prevented by tight metabolic control (Itabashi et al., 1981; Jackson et al., 1982; Camerini-Davalos et al., 1983; Raskin et al., 1983; Siperstein, 1983). However, numerous studies implicate genetic make-up (Itabashi et al., 1981; Williamson and Kilo, 1983), vascular injury (Williamson and Kilo, 1977), increased hydrostatic pressure (Williamson et al., 1971; Tilton et al., 1981; Ganda et al., 1983), or the diabetic milieu (Bloodworth et al., 1969; Fischer et al., 1982; Williamson and Kilo, 1983) as major factors underlying capillary basement membrane thickening in diabetes. In addition, it has recently been shown that similar degrees of renal glomerular CBMT exist in geriatric

diabetic XA and AC subline Chinese hamsters despite differences in genetic background and metabolic data (Diani et al., 1985). These data, coupled with the large degree of variation within animals and sublines in the present study, suggest that CBMT is probably influenced by complex interactions involving metabolic abnormalities, genotype and microenvironmental perturbances.

In summary, quadriceps muscle capillary basement membrane thickness in the diabetic XA subline Chinese hamster is significantly greater than that of the nondiabetic M subline Chinese hamster. These data provide baseline information on CBMT in the Chinese hamster which will be utilized in future studies designed to evaluate the efficacy of antimicrovascular disease drugs in this animal model.

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