Variation in Thickness of the Capillary Basement Membrane in Single Muscles of Diabetic Subjects

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SUMMARY

Thickness of skeletal muscle's capillary basement membrane was measured in gastrocnemius and quadriceps femoris muscles of diabetic subjects. At least two different sites of each muscle studied were biopsied. Comparison of these biopsy samples revealed a marked and significant variation in thickness of the capillary basement membrane at different sites within the same muscle. In one subject so studied, this variation was found regardless of the method of fixation or the method of measurement of capillary basement membrane thickness used. Variation was present in diabetics suffering from long-term complications of the disease as well as in diabetics free of such complications. A reevaluation of the biologic significance of basement membrane thickness measurements, as presently applied, is indicated in the light of the variability of base-line values. DIABETES 28: 548–551, June 1979.

The phenomenon of basement membrane thickening of the skeletal muscle capillaries of diabetics is reported by many investigators,[1–10] yet the cause, prevalence, and significance of the lesion remain controversial. In addition, the interpretation of quantitative measurements of basement membrane, particularly as they relate to the onset, duration, and control of diabetes as well as to the age and sex of the individual, is a matter of current debate.[4,11–20]

A key issue in the controversy is the order in which the two phenomena of basement membrane thickening and glucose intolerance present themselves in the person who will be recognized as having diabetes. Siperstein et al.[1,17] presented data that they feel demonstrate that basement membrane thickening occurs before glucose intolerance and may in fact be its cause; Williamson et al.[2,4] feel that their data suggest the opposite.

In attempting to resolve the controversy, it is important to establish whether or not capillary basement membrane thickness, as it is currently quantified, is constant within a radius of 2–6 cm throughout a particular muscle from a diabetic subject.

Accordingly, individual muscles from diabetic subjects were biopsied at discretely different sites in order to examine the quantitative consistency of the capillary basement membrane lesion.

Different methods of tissue fixation for electron microscopy are currently in use for the analysis of basement membrane thickness. Primary fixation by glutaraldehyde is favored by Williamson et al.[2] while Siperstein et al.[1] favor osmic acid for this purpose. The question of whether or not such a difference in procedure will alter the final results of basement membrane thickness measurement is another topic to be considered in this study.

SUBJECTS AND METHODS

Subjects. Five adult male diabetic subjects were included in the study. In three of the subjects (E.J., G.M., and R.S.), biopsies were performed on the amputated limb immediately (less than 10 min) after removal. In one subject (R.C.), biopsies were performed during surgical repair of a broken leg, and, in a volunteer (G.P.), biopsies were performed under local anesthetic using a Vim-Silverman biopsy needle. Table 1 summarizes the pertinent information regarding these subjects. In all subjects, all biopsy samples were taken on the same day.

Biopsy location. All gastrocnemius samples were taken from the lower half of the muscle. The samples of quadriceps femoris muscle were taken from the region midway between knee and hip.

Fixation. The procedure for fixation of the samples from the four different gastrocnemius muscles followed the technique reported by Williamson.[2] Briefly, the tissue was fixed in

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TABLE 1
Subjects included in study

Subject	Age (yr)	Known duration of diabetes (yr)	Treatment	Muscle studied	Complications
E.J.	55	Diagnosed at time of amputation	—	Gastrocnemius	Gangrene, retinopathy
G.M.	61	17	Diet	Gastrocnemius	Chronic skin ulcer (foot)
R.S.	56	12	Insulin	Gastrocnemius	Gangrene, retinopathy
R.C.	22	4	None at present; insulin for 2 months in 1972*	Gastrocnemius	None
G.P.	23	14	Insulin	Quadriceps femoris	None

* Present diabetic status confirmed, while hospitalized, by a repeated fasting blood glucose above 110 mg/dl and by frequent glycosuria.

glutaraldehyde and postfixed in osmium tetroxide. Ethanol dehydration was followed by substitution with propylene oxide and embedment in Epon-812 resin.

Each biopsy sample from the quadriceps femoris muscle studied was divided in two, and one of the halves was fixed as stated above for the gastrocnemius muscle samples. The other half of each sample was fixed in osmium tetroxide and postfixed in formaldehyde as described by Siperstein et al.[1]

Electron microscopy. Samples from the gastrocnemius muscles were photographed with an RCA EMU-3H electron microscope at a single, calibrated, tap setting. Samples from the quadriceps femoris muscle were photographed with a Zeiss 9S electron microscope at a magnification of 2000. Throughout the study the filament of the microscope was not changed, and weekly calibration procedures indicated a stable magnification at the selected setting. For subject G.P., four blocks of tissue for each biopsy site were cut and two grids from each block were examined. For all other subjects, three blocks of tissue per site were cut and two grids from each block were examined.

Measurement of basement membrane thickness. For all samples, measurement of the basement membrane was performed using the method of Siperstein et al.[1] In addition, the method of Williamson et al.[2] was used to measure the quadriceps femoris samples. For all subjects, 12 capillaries per site were measured.

RESULTS

Effect of fixation on basement membrane thickness. Tables 2 and 3 summarize the information obtained by analysis of four quadriceps femoris muscle biopsy samples that were divided in two and subsequently processed by different fixation procedures. Measurement by the method of Siperstein et al.[1] resulted in a mean basement membrane thickness for the four sites of 1965 Å when primary fixation was by glutaraldehyde followed by osmium and a mean thickness of 2016 Å when primary fixation was by osmium followed by formaldehyde (Table 2A). The difference between these means (1965 vs. 2016) was not significant (Student's t test: $n = 8$, $P > 0.05$), nor was the difference between the two means from any one of the four individual sites significant. Measurement by the method of Williamson et al.[2] resulted in a mean basement membrane thickness for the four sites of 1296 Å when primary fixation was by glutaraldehyde followed by osmium and a mean thickness of 1311 Å when primary fixation was by osmium followed by formaldehyde (Table 2B). These differences (1296 vs. 1311) also were not significant (Student's t test: $n = 8$, $P > 0.05$), nor was the difference between the two means from any one of the four individual sites significant.

Effect of variation of site in the same muscle on thickness of basement membrane. All gastrocnemius muscle biopsy samples were fixed in glutaraldehyde followed by osmium. Of the four muscles studied, all had significantly different basement membrane thicknesses at different sites within the same muscle (Tables 4 and 5). This significant variation in location was also found in the subject in whom the quadriceps femoris muscle was studied, regardless of the method of fixation or the method of measurement used (Tables 2A, 2B, 3A, and 3B).

One subject (E.J.) demonstrated increasing basement

TABLE 2
Capillary basement membrane thickness in tissue fixed and measured by two different methods (All biopsies were obtained from a single subject on the same day. Measurements are Mean ± SD in angstroms.)

	Primary fixation with glutaraldehyde	Primary fixation with osmium
A. Measurement method of Siperstein		
Site I	1931 ± 570	1925 ± 1056
Site II	2597 ± 872	2431 ± 837
Site III	1962 ± 644	2196 ± 795
Site IV	1372 ± 266	1533 ± 321
Mean of 4 sites	1965 / 588	2016 / 723
B. Measurement method of Williamson		
Site I	1203 ± 332	1347 ± 775
Site II	1763 ± 689	1592 ± 187
Site III	1292 ± 668	1315 ± 449
Site IV	818 ± 225	989 ± 291
Mean of 4 sites	1296 / 477	1311 / 425

TABLE 3
Statistical significance of the differences between identical muscle biopsy sites fixed and measured by two different methods

	Primary fixation with osmium	Primary fixation with glutaraldehyde
A. Measurement method of Siperstein		
Site I vs. II	$P < 0.05$	$P < 0.05$
Site I vs. III	$P > 0.05$	$P > 0.05$
Site I vs. IV	$P < 0.01$	$P < 0.01$
Site II vs. III	$P > 0.05$	$P > 0.05$
Site II vs. IV	$P < 0.001$	$P < 0.001$
Site III vs. IV	$P < 0.01$	$P < 0.01$
B. Measurement method of Williamson		
Site I vs. II	$P < 0.05$	$P < 0.05$
Site I vs. III	$P > 0.05$	$P > 0.05$
Site I vs. IV	$P < 0.01$	$P < 0.01$
Site II vs. III	$P < 0.05$	$P < 0.05$
Site II vs. IV	$P < 0.001$	$P < 0.001$
Site III vs. IV	$P < 0.01$	$P < 0.01$

membrane thickening as the site's distance below the knee increased. No such relationship was found in the other subjects. It is important that E.J. had the highest values for basement membrane thickness in the study, although his diabetes was only diagnosed at the time of the biopsy. His excessive basement membrane thickness is explained by his having had diabetes for many years before examination by us; his diabetes was undiagnosed because of renal disease that markedly decreased his urinary glucose.

DISCUSSION

A wide range of studies concerned with the quantification of the thickness of muscle capillary basement membrane was published. The subjects of these studies included adults,[1-10] children,[21,22] and patients with congestive heart failure,[23] as well as dogs,[16,24] the giraffe,[25] numerous small animals,[26-28] and black Celebes apes.[29] Muscles chosen for study include the quadriceps femoris,[1,5,25] deltoid,[1] pectoralis,[25] gastrocnemius,[1,5,25] abdominal rectus,[5,25] and pronator teres.[23]

The majority of these studies compared normal, control populations with diabetic populations with the intention of defining the basement membrane lesion relative to some aspect of diabetes or its treatment. Increased thickness of basement membrane is taken by Siperstein as being a marker of genetic disease in humans.[1]

The variation found when comparing anatomically different muscles is well-documented. Williamson et al.[25] report that, in nondiabetic humans and in the giraffe, the thickness of the basement membrane varies considerably between samples obtained from different skeletal muscles of the same individual. They postulate that this variation is related to the regional differences of venous hydrostatic pressure. Siperstein et al.[1] and Vracko[5] reported that thickness of the basement membrane varies in muscle samples taken from different body sites of the same individual in both diabetic and nondiabetic humans. These studies are in mutual agreement and imply that comparisons of basement membrane thickness are meaningful only if the samples obtained are from the same muscle in each subject.

However, the variation between sites within the *same* muscle has been less thoroughly studied. In the present study, basement membrane thickness was measured at discretely different sites in each muscle studied. In addition, all subjects had diabetes. The results clearly demonstrate that significant variation in basement membrane thickness can occur within a single muscle of a diabetic subject even when comparing sites were separated by as little as 2 cm. Whereas

TABLE 4
Gastrocnemius muscle biopsy measurements

Subject and site number	Site location (distance below knee)	Basement membrane thickness Mean ± SD (angstroms)
E.J. I	20 cm lateral side	8037 ± 3397
E.J. II	22 cm lateral side	9318 ± 3338
E.J. III	22 cm medial side	9318 ± 3729
E.J. IV	24 cm lateral side	10566 ± 2245
E.J. V	26 cm lateral side	14500 ± 2775
E.J. VI	26 cm medial side	15400 ± 6235
G.M. I	20 cm lateral side	3477 ± 955
G.M. II	22 cm lateral side	1117 ± 280
G.M. III	24 cm lateral side	1913 ± 810
R.C. I	24 cm lateral side	1333 ± 318
R.C. II	24 cm medial side	1560 ± 242
R.S. I	24 cm lateral side	3165 ± 1055
R.S. II	26 cm lateral side	2001 ± 626

TABLE 5
Significance of difference when comparing sites within the same muscle

	Level of significance
Subject E.J.	
Site I vs. II	$P > 0.05$
Site I vs. III	$P > 0.05$
Site I vs. IV	$P < 0.05$
Site I vs. V	$P < 0.001$
Site I vs. VI	$P < 0.001$
Site II vs. III	$P > 0.05$
Site II vs. IV	$P > 0.05$
Site II vs. V	$P < 0.001$
Site II vs. VI	$P < 0.001$
Site III vs. IV	$P > 0.05$
Site III vs. V	$P < 0.001$
Site III vs. VI	$P < 0.001$
Site IV vs. V	$P < 0.001$
Site IV vs. VI	$P < 0.001$
Site V vs. VI	$P > 0.05$
Subject G.M.	
Site I vs. II	$P < 0.001$
Site I vs. III	$P < 0.001$
Site II vs. III	$P < 0.01$
Subject R.S.	
Site I vs. II	$P < 0.01$
Subject R.C.	
Site I vs. II	$P < 0.05$

diabetic complications were present in three subjects (Table 1), two (R.C. and G.P.) showed excellent general health and a lack of diabetic complications. Thus, the finding of variation of the basement membrane thickness was not confined to persons in whom basement membrane thickness was grossly increased (as in E.J. who had values easily 10 times normal) or who had other evidence of complications from diabetes (E.J., G.M., and R.S.). Siperstein et al.[1] reported that when the biopsy needle is redirected to obtain an adjacent, duplicate sample the basement membrane thickness of the two samples varies by an average of only 6.9%; the study was performed on nondiabetics. Judging from this, it would seem that the sample size is sufficient and, within a small area (adjacent duplicates), the basement membrane thickness in nondiabetic patients is consistent. These findings, however, do not compare truly different sites and refer only to normal individuals, in whom the basement membrane is thin.

Much work has been done[1,2,30] to elucidate the possible sources of error inherent in the techniques and equipment used in the process of quantification of the basement membrane of muscle capillaries. Discourse on the subject includes arguments concerning the efficacy of different fixatives in helping to discern the differences between diabetic and normal basement membranes: Siperstein et al.[17] feel that the procedure of using osmium tetroxide as a primary fixative followed by formaldehyde actually yields a result that allows greater differentiation. The present study is not in agreement with those findings, however, and in fact supports the opposite findings of Williamson et al.,[31] who report that the use of different fixatives does not significantly alter the results of basement membrane measurements. Other investigators also find no difference when using different fixatives.[27]

Considering the wide variation in basement membrane thickness at different biopsy sites in the same muscle, it is not surprising that the evidence supporting a correlation between the duration of diabetes or control of the disease and the thickness of the basement membrane is not conclusive. Any relationship will be approximate because of the spontaneous variation of the capillary basement membrane thickness found within a single diabetic muscle.

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