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Basement Membrane Thickness, Insulin Antibodies and HLA-Antigens in Long Standing Insulin Dependent Diabetics with and without Severe Retinopathy

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Summary. The study was designed to show whether there was any relation between muscle capillary basement membrane thickness, HLA-antigens, anti-insulin antibodies and proliferative retinopathy. Electron microscopic measurements of muscle capillary basement membrane thickness were performed on muscle biopsies from 15 insulin-dependent diabetics with severe proliferative retinopathy, 24 insulin-dependent diabetics with minimal retinopathy and 18 age- and sex matched non-diabetics. All the patients had had diabetes for 20 years or more. None had biochemical or clinical evidence of diabetic nephropathy. Basement membrane thickness was measured according to the methods of Siperstein and Williamson. Muscle capillary basement membrane thickening occurred in 32 of 39 diabetics, using the Siperstein method, but patients with proliferative retinopathy did not exhibit thicker basement membranes than patients with no or minimal changes in the retina. There were apparrent differences in HLA-antigens between diabetics with and without proliferative retinopathy, but they did not reach statistical significance. There was no correlation between muscle capillary basement membrane thickness and the quantity of insulin antibodies. The results indicate that factors other than basement membrane thickening and genetic factors in the HLA-region, are responsible for the development of proliferative retinopathy.

Key words: HLA-antigen, muscle capillary basal membrane thickness, insulin-antibodies, insulin dependent diabetes mellitus, retinopathy, proliferative retinopathy.

In general capillary basement membranes are thicker in diabetics than in non-diabetics of the same age [1]. In the kidney the glomerular capillary basement membranes are normal at the onset of diabetes mellitus, but show thickening already after 2–3 years [2]. As the number of vascular diabetic complications increases with the duration of the disease it has been postulated that the capillary basement membrane thickening could be "a useful index, perhaps the best available for monitoring deleterious effects of the diabetic state on the vascular system" [1]. Histological examinations of kidney biopsies however, have failed to prove any correlation of basement membrane thickness with clinical findings [3], and prognostic significance has been negligible [4].

In order to elucidate further, whether thickness of capillary basement membranes bears any relation to grosser organ changes, we have investigated the thicknesses of muscle capillary basement membranes in two groups of insulin-dependent diabetics with a duration of the disease of more than 20 years. In one group the patients suffered from severe retinopathy while the patients in the other group had no or minimal retinal changes. In addition an attempt was also made to determine whether the amount of insulin antibodies and/or genetic factors expressed in the HLA-antigens could be of pathogenetic significance for the development of retinopathy.

Methods

Subjects

Eighteen non-diabetics (11 males and 7 females) with no known disease and with no family history of diabetes (random blood glucose concentration < 5.4 mmol/l) and 39 insulin-dependent diabetics (22 males and 17 females) were studied. Among the diabetics 24 (15 males and 9 females) had no significant retinopathy (2

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Table 1. Clinical details and basement membrane thickness in controls. Individual data including muscle capillary basement membrane thickness of 18 non-diabetics measured with Siperstein's (a) and Williamson's (b) procedure

No.	Sex	Age (years)	% ideal body weight	Blood pressure mmHg systolic	diastolic	Basement membrane thickness mean ± SD (nm) (a)	(b)
1	♂	34	92	125	75	197±15	109± 6
2	♂	58	120	150	95	195±14	126±11
3	♂	39	96	135	80	195±14	118± 7
4	♂	43	103	115	65	119± 5	71± 3
5	♂	46	102	125	80	116±11	73± 8
6	♂	59	110	160	90	138± 6	85± 4
7	♂	54	113	150	95	173± 8	104± 4
8	♂	40	96	135	90	243±13	130± 8
9	♂	46	110	145	95	163± 8	101± 5
10	♂	45	115	145	90	143±10	85± 6
11	♂	42	94	120	70	147± 7	90± 4
12	♀	30	90	105	60	144±27	100±23
13	♀	46	100	145	90	189±21	115±10
14	♀	54	98	140	80	150±12	89± 6
15	♀	47	94	135	90	183±20	115±11
16	♀	42	105	145	90	152± 9	92± 7
17	♀	51	103	150	90	121± 5	79± 4
18	♀	51	85	105	65	174±16	103± 5
Mean		46	101	135	83	163 12	99 7
±SEM		1.8	2.2	3.8	2.7	7.8 1.4	4.1 1.1

microaneurysms or less per retina), 15 (7 males and 8 females) had severe proliferative retinopathy. Eight of this group were blind in both eyes and 4 blind in one eye. All diabetics were seen regularly at the out-patient clinic.

To participate in the investigation both non-diabetics and diabetics had to fulfill the following criteria: body weight within 80–120% of the ideal body weight according to Natvig's table [5]; systolic blood pressure ≤ 160 mmHg and diastolic blood pressure ≤ 95 mmHg; tobacco consumption not exeeding 20 cigarettes daily; alcohol consumption below 25 g daily; oral contraceptives not used. In addition the following criteria were specified for the diabetics: onset of the disease before the age of 35; duration of diabetes of 20 years or more; regular visits to the diabetic centre; no diabetic nephropathy determined by several negative Albustix® tests and serum creatinine within the normal range (66–133 μmol/l in males and 55–124 μmol/l in females). In both groups of diabetics the erythrocyte sedimentation rate (ESR) was below 25 mm/h except in one case where it was 29 mm/h. Ten of the 40 diabetics smoked 10–20 cigarettes/day. Of the ten heavy smokers three had proliferative retinopathy, seven had no or only minimal changes. Of the 25 non-cigarette smokers, ten had proliferative retinopathy, 15 had no or only minimal changes.

Before 1968 patients were treated with mixtures of ox and pig insulin. From 1968 only pig insulin was used, and during the last six months before investigation all patients received highly purified pig insulin (Insulin Leo Retard RI®). The goodness of diabetic control during the diabetic years could not be quantitated accurately, but the records did not reveal any systemic differencies in the two groups of diabetics. None of the controls but 4 of the patients were taking drugs in usual therapeutic doses: hydroflumethiazide (1), oxazepam (1), diazepam (1), and phenemalphenytoin (1). Informed consent was obtained from all patients and controls after careful explanation of the nature and purpose of the investigations.

Analytical Methods

Haemoglobin (Hb), ESR, ECG, blood pressure, serum creatinine, serum cholesterol and serum albumin were determined by standard laboratory procedures. Insulin antibodies were determined according to Andersen et al. [6]. With this technique the antibodies are measured as the insulin-binding capacity of the plasma. In normal non-diabetic individuals the insulin-binding capacity of plasma was below 15μU/ml. Insulin antibodies were determined in the plasma from 39 of the diabetics.

Tissue typing was performed in 37 of the diabetics according to Kissmeyer & Kjerbye [7], and the distribution of the tissue types was compared with a control group of 1967 individuals collected among the Danish population [8].

Muscle biopsies were carried out in all 57 individuals. In one diabetic patient measurement by the method of Williamson could not be done for technical reasons. Biopsies were taken under local subcutaneous anaesthesia (lidocain, 1%) with a punch biopsy needle, from the middle part of the lateral vastus muscle at a depth of 3 cm. The pieces of muscle were immediately fixed in 1% ice-cold osmium tetroxide solution in veronal acetate buffer 450 nosm/kg pH 7.3, dehydrated in a graded series of alcohol and embedded in epon 812. Ultrathin sections were cut with a LKB Ultrotome III, stained with uranyl acetate and lead citrate and examined in a Phillips EM 300 electron microscope at 60 kV. The magnification calibration of the instrument was checked daily by means of a grating replica with 28.800 lines per inch (Ernest F. Fullham cat. no. 1000). Cross sectioned capillaries were photographed at an initial magnification of approximately 2000 X on 35 mm film and enlarged 10 times for subsequent measurements. The muscular capillary basement membrane thickness was measured with an accuracy of 0.1 mm. In each individual 20 vessels were measured and the mean basement membrane thickness was estimated according to two different procedures. In one procedure

Table 2. Clinical details and basement membrane thickness in diabetics with proliferative retinopathy. Individual data including muscle capillary basement membrane thickness measured with Siperstein's (a) and Williamson's (b) procedure, insulin-binding capacity of insulin antibodies (IBC) and HLA antigens on A, B and C locuses in 15 insulin-dependent diabetics with severe proliferative retinopathy

No.	Sex	Age (years)	Duration of diabetes (years)	% ideal body weight	Blood pressure mmHg		Ins. req. (U/kg)	Basement membrane thickness mean ± SD (nm)		IBC (μU/ml)	HLA – type		
					systolic	diastolic		(a)	(b)		A	B	C
1	♂	29	20	85	140	70	1.15	258±20	133±12	3	1, 11	5, 27	w3
2	♂	58	24	120	150	95	0.27	150± 9	91± 5	9	2, 28	12, w15	w3
3	♂	40	26	93	145	95	0.65	205±17	115±10	72	1, 11	13, 8	–
4	♂	48	38	117	140	?	0.65	261±20	136±11	23	2, w19, (w32)	12, w15	w3
5	♂	58	40	97	165	70	0.60	170±26	101±23	23	9, 10, (w25)	w15, 18	w3
6	♂	54	42	115	140	80	0.59	236±20	128± 9	5	1, 3	w15, 8	w3
7	♂	43	42	90	140	85	0.37	384±37	217±16	21	1, 9	w16	–
8	♀	31	24	93	120	80	0.76	298±33	164±23	20	2, 10, (w25)	w15, 18	w3
9	♀	45	21	100	120	80	0.33	270±32	143±15	27	2, 9	w15, 12	w3
10	♀	53	32	99	130	80	0.56	370±23	219±15	38	2, w32	w40	w3
11	♀	48	45	92	160	80	0.54	279±32	176±21	20	3	w15, 7	w3
12	♀	41	37	90	150	90	0.71	303±84	170±32	167	2, 3	12, w15	w3
13	♀	59	30	86	140	90	0.46	458±29	293±23	66	2	w15	w3
14	♀	62	41	93	155	90	0.53	314±26	181±18	10	1, 2	w15, w35	w3, w4
15	♀	35	30	95	120	85	0.46	214±25	110±13	35	9, w19	8, 14, (w16)	–
Mean		47	33	98	141	84	0.58	278 29	159 16	36			
±SEM		2.7	2.2	2.8	3.6	2.1	0.054	21.2 4.4	13.9 1.8	10.7			

(developed and employed by Siperstein et al. [9]) the basement membrane was measured in twenty equidistant places around the periphery of the vessel. In the other method (recommended by Williamson et al. [10]) the muscle capillary basement membrane was measured only at the two points where it was thinnest. These points were at least 0.5 μm apart. All measurements were made without knowledge of the group the patients belonged to.

The following tests were used for statistical evaluation: correlation between age and basement membrane thickness, diabetes duration and basement membrane thickness by the rank correlation test of Kendall; differences between two means by the Mann-Whitney Wilcoxon rank sum test. For comparison of frequencies in two groups, Fisher's exact test was used.

Results

Individual data and results are listed on Table 1–3. Data of controls in Table 1. Table 2 contains the data of diabetics with proliferative retinopathy and Table 3 the data of patients with no or minimal retinal changes. Age, % ideal body weight and blood pressure were not significantly different between groups. Neither were there significant differencies in diabetes duration or insulin requirement between the two diabetic groups (Table 2 and 3). Haemoglobin was 8.7 ± 0.53 mmol/l (mean ± SD) in controls, 8.8 ± 0.93 in diabetics with proliferative retinopathy and 8.8 ± 0.74 in diabetics without proliferative retinopathy. Serum creatinine was 83 ± 11.7 μmol/l, 83 ± 12.7 and 86 ± 13.1 μmol/l, respectively. Serum cholesterol in the patients with proliferative retinopathy was 5.9 ± 0.97 mmol/l and 5.7 ± 0.92 mmol/l in the group without proliferative retinopathy (normal range 4.0–8.0 mmol/l). Serum albumin in the two groups was 37 ± 3.8 and 36 ± 2.9 g/l respectively (normal range 31–42 g/l). Electrocardiography did not reveal signs of heart disease in any of the diabetics involved in the investigation.

Muscle Capillary Basement Membrane Thickness

Fig. 1 shows the thickness of muscle capillary basement membranes in diabetics and non-diabetics. The method of Siperstein et al. yielded an average basement membrane thickness which was about 1.7 times higher than Williamson's method. This ratio between the two techniques was similar in diabetics and in non-diabetics regardless of the thickness.

The diabetics had significantly ($p<0.001$) thicker muscle capillary basement membrane than the non-diabetics (272 ± 80 vs 163 ± 33 nm using the Siperstein method and 155 ± 55 vs 99 ± 18 nm by Williamson's method). Only one non-diabetic had basement membranes thicker than 200 nm using the former method while 32 out of the 39 diabetics had basement membranes thicker than 200 nm. No correlation was found between basement membrane

Table 3. Clinical details and basement membrane thickness in diabetics with minimal changes. Individual data including muscle capillary basement membrane thickness measured with Siperstein's (a) and Williamson's (b) procedure, insulin-binding capacity of insulin antibodies (IBC) and HLA antigens on A, B and C locus in 24 insulin-dependent diabetics with no or minimal changes on the retina by ophthalmoscopy

No.	Sex	Age (years)	Duration of diabetes (years)	% ideal body weight	Blood pressure mmHg		Ins. req. (U/kg)	Basement membrane thickness mean ± SD (nm)		IBC (μU/ml)	HLA – antigens		
					systolic	diastolic		(a)	(b)		A	B	C
1	♂	36	24	108	145	80	0.68	246±27	154±15	13	1, 2	8, 12	–
2	♂	53	24	91	135	90	0.43	233±17	147±14	20	2, 3	8, w15	w3
3	♂	39	26	113	155	95	0.52	249±18	137± 8	25	1, 2	8, w15	w3
4	♂	43	38	101	130	90	0.41	195±12	109± 9	28	3, w24	7, w15	w3
5	♂	51	37	105	110	70	0.57	289±20	151±12	15	〃	〃	〃
6	♂	61	39	107	120	75	0.29	325±48	176±29	4	11, w25	18	〃
7	♂	54	34	91	140	85	0.31	208±15	116±13	19	1, w26	w22, w39	w3
8	♂	35	33	95	150	80	0.45	293±21	168±14	48	1, 2	8, w15	w3
9	♂	52	32	89	135	75	0.40	193±28	108±14	20	2, w30	18, w15	w3
10	♂	46	26	102	135	90	0.71	263±16	156±11	1	〃	〃	〃
11	♂	52	25	101	135	80	0.62	257±17	154±11	54	2	27, w40	w2, w3
12	♂	46	23	92	140	80	0.47	221±22	121±13	40	–	–	–
13	♂	59	35	98	140	85	0.80	216±18	135±10	297	1, 11	8, w15	w3
14	♂	21	20	92	130	85	0.65	401±29	361±18	10	2	w40, w15	w3
15	♂	54	52	117	140	85	0.43	493±46	–	44	3	7, 18	–
16	♀	30	27	114	135	80	0.65	192±11	105± 5		1011, w30	12, 18	–
17	♀	50	22	85	130	80	0.75	127± 6	84± 9	37	2, 3	14, w17	–
18	♀	51	35	110	150	85	0.62	338±20	207±20	147	1, w25	8, 18	–
19	♀	49	38	80	150	80	0.35	421±42	230±48	300	2, w19	12, w15	w3
20	♀	55	36	86	150	90	0.22	194±25	109±13	12	2	w15, w21	w3
21	♀	63	36	88	160	90	0.68	264±17	135±13	18	w33, w32	18, w40	w2
22	♀	42	33	109	140	80	0.73	239±14	132± 6	39	2, 28	8, 12	–
23	♀	58	28	92	150	70	0.45	399±27	212±18	17	1, w24	8	–
24	♀	54	41	84	160	80	0.48	259±22	120± 9	278	1, w26	8, w15	w3
Mean		48	32	98	140	83	0.53	269 21	153 14	62			
±SEM		2.1	1.5	2.2	2.4	1.3	0.032	16.7 2.0	12.1 1.9	18.3			

thickness and age, either among the diabetics or non-diabetis ($r = 0.02$). The duration of diabetes (20–52 years) did not correlate with basement membrane thickness ($r = 0.19$). In patients with proliferative retinopathy the muscle capillary basement membranes was not thicker than in patients without retinopathy ($p > 0.05$).

Insulin antibodies were found in 30 of the diabetics (75%). No correlation was found between the insulin-binding capacity and basement membrane thickness ($r = 0.20$) and no difference in insulin-binding capacity was detected between patients with and without proliferative retinopathy. The thickness of basement membrane in patients with high insulin-binding capacity (≥ 50 μU/ml) was not significantly increased compared to patients with low insulin-binding capacity (< 50 μU/ml). The results of the *HLA-determinations* are shown in Table 4. The well-known accumulation of HLA B8, B18 and Bw15 in insulin-dependent diabetics was confirmed as well as the decrease of B7 [13]. In patients with proliferative retinopathy only the HLA antigen Bw15 deviated significantly ($p < 0.001$) from the controls, but in the larger group without retinopathy B7, B18 as well as Bw15 deviated significantly from non-diabetics. The difference of HLA – A9, B8, B18 and Bw40 antigens between patients with severe retinopathy and patients without retinopathy appeared considerable, but did not reach statistical significance on the 5 per cent level. No difference in basement membrane thickness was found between diabetics with or without tissue types B8 and/or Bw15 ($p > 0.05$). Seven of eight patients with high insulin-binding capacity (≥ 50 μU/ml) had HLA B8 and/or Bw15, but high insulin-binding capacity (≥ 50 μU/ml) was not statistically more frequent in diabetics with the tissue types HLA B8 and/or Bw15 ($p = 0.3$).

Discussion

The two morphometric methods for the estimation of basement membrane thickness have been compared among others by Beauchemin et al. [11], who by

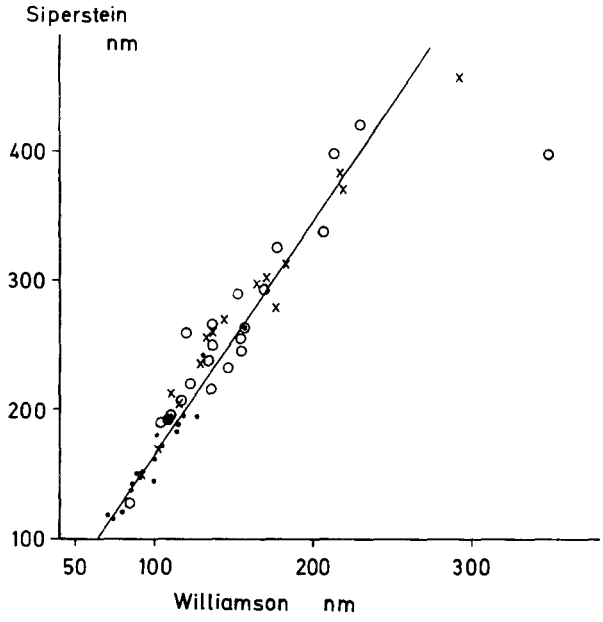

Fig. 1. Correlation of the thickness of the basement membrane of muscle capillaries measured according to Siperstein and Williamson. The biopsies were from 18 nondiabetics and 38 diabetics. • = non-diabetics – o = diabetics with no or minimal changes by ophthalmoscopy – × = diabetics with proliferative retinopathy

means of a nested variance analysis found Williamson's method to be the most precise. The comparison presented in this communication has shown the procedure of Siperstein to be more discriminating, since 82% of diabetics and only one non-diabetic had muscle capillary basement membrane thickness of > 200 nm, whereas by Williamson's method only 74% of the diabetics and 2 non-diabetics had capillary basement membrane thickness of ≥ 120 nm. It therefore seems reasonable to prefer Siperstein's method even if the method of Williamson is easier to perform and less time-consuming [9]. We have not however, used the fixation procedure of Williamson, but the method of Siperstein for both measurements.

It is assumed that the diabetic microangiopathy morphologically expressed as a thickening of the capillary basement membrane is caused by metabolic and hormonal disturbances due to the lack of endogenous insulin. The microangiopathy is assumed to lead to severe organ damage such as nephropathy and possibly plays a role in the development of retinopathy [14]. The present investigation shows, however, that the basement membranes of muscle capillaries may be of normal calibre even in patients who have had diabetes for more than 20 years and who have become blind from retinopathy. Furthermore the investigation shows, that the muscle capillary basement membranes in patients with proliferative retinopathy are not thicker than in diabetics with

Table 4. HLA typing in controls, diabetics with proliferative retinopathy and diabetics with minimal changes. No significant differencies were found between patients with and without proliferative retinopathy

HLA-antigen	Tissue types frequency (per cent)			
	Controls (n=1967)	Non-proliferative (n=21)	Proliferative (n=15)	Total patients (n=36)
HLA-A 1	31.1	38.1	33.3	36.1
HLA-A 2	53.6	52.4	53.3	52.8
HLA-A 3	26.9	19.0	20.0	19.4
HLA-A 9	17.3	9.5	26.7	16.7
HLA-A10	9.6	19.0	13.3	16.7
HLA-A11	10.1	14.3	13.3	13.9
HLA-A28	10.0	4.8	6.7	5.6
HLA-Aw19	17.8	19.0	20.0	19.4
HLA-B 5	10.6	0.0	6.7	2.8
HLA-B 7	26.8	9.5	6.7	8.3
HLA-B 8	23.7	42.9	20.0	33.3
HLA-B12	25.2	19.0	26.7	22.2
HLA-B13	4.3	0.0	6.7	2.8
HLA-B14	4.5	4.8	6.7	5.6
HLA-B18	7.1	28.6	13.3	22.2
HLA-B27	8.6	4.8	6.7	5.6
HLA-Bw15	17.9	47.6	66.7	55.6
HLA-Bw16	5.4	4.8	6.7	5.6
HLA-Bw17	7.7	4.8	0.0	2.8
HLA-Bw21	3.5	4.8	0.0	2.8
HLA-Bw22	3.8	4.8	0.0	2.8
HLA-Bw35	13.1	0.0	6.7	2.8
HLA-Bw40	17.9	14.3	6.7	11.1

One, two and three asterisks indicate significance at the 5, 1 and 0.1% level, respectively. Comparisons are made with the control group by means of Fisher's exact test

long duration of the disease but with no or minimal complications. If one assumes that muscle capillary basement membrane thickness reflects basement membrane thickness in the retina, these observations could be taken to indicate that muscle basement membrane thickness is not a useful index for monitoring deleterious effects of the diabetic state on the vascular system and that other factors should be considered responsible for the development of retinopathy.

The well-known increased frequencies of HLA B8, B15 and B18, and the decreased frequency of B7 in insulin-dependent diabetes [13, 22] was also seen in the total group of patients in this study although the increase of B8 was not significant in this small sample. In fact B8 and A1 had a normal frequency in the group of patients with severe retinopathy which is in contrast to other independent studies [15, 16, 17] which showed a higher frequency of B8 in patients with retinopathy compared to patients without retinopathy. In maturity-onset diabetes, Becker et al. [17] found an increased frequency of B8 in patients

without retinopathy. Considering all available data, however, there is at present no evidence that HLA is involved in the susceptibility to retinopathy once insulin-dependent diabetes has developed. Accordingly, the genetic background for the development of this complication [18] probably involves non-HLA genes.

Seven of the eight patients with high insulin antibody titres (measured as insulin-binding capacity $\geqq 50\ \mu U/ml$) had HLA-antigens B8 and/or Bw15. This is in accordance with the findings of others [19]. However, the findings of Ortved Andersen [20], who found significantly increased basement membrane thickness in muscle capillaries of insulin-dependent diabetics with high insulin antibody titres, could not be confirmed in the present study.

Proliferative retinopathy seems not to be more frequent in heavy cigarette smokers (3 of 10) than in non-cigarette smokers (10 of 25) as it was the case in Paetkau's material [21], but our numbers were very small.

Further studies are needed to isolate factors of importance for the development of late diabetic complications.

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