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Human Glomerular Basement Membrane: Chemical Alteration in Diabetes Mellitus

Abstract. *The human glomerular basement membrane belongs to the collagen family of proteins. It contains about 7 percent carbohydrate, half of which occurs as glucosylgalactose disaccharide units linked to hydroxylysine. Glomeruli from diabetics contain increased amounts of basement membrane material. In addition, these membranes show a distinct chemical alteration characterized by a significant decrease in lysine, accompanied by an equivalent increase in hydroxylysine and hydroxylysine-linked disaccharide units.*

Thickening of the capillary basement membrane, as observed with the electron microscope, is a characteristic pathological alteration in many tissues of human diabetics (*1*). Under the light microscope, this may be seen as an increase in material which reacts intensely with the periodic acid–Schiff stain. In the renal glomerulus, alterations are particularly prominent and involve, in addition to the thickening of the basement membranes of the capillary loops, the accumulation of material in the mesangial region similar to that of basement membranes; this thickening eventually results in the characteristic Kimmelstiel-Wilson nodular lesions (*2*).

The glomerular basement membrane is believed to function as the major filtration barrier between the blood and the urine (*3*). In diabetes, where it undergoes marked thickening, it becomes defective in this function, and patients with diabetic nephropathy often lose large quantities of protein in the urine. This suggests that diabetes leads not only to an increased amount of basement membrane material but also to alterations in its structure.

Using methods previously described for the bovine membrane (*4*), we have extensively studied the composition of human glomerular basement membrane. Basement membranes were isolated from normal and diabetic human kidneys obtained at autopsy by a modification of the method of Krakower and Greenspon (*5*). In this procedure steel sieves are used to disrupt the renal cortex and to separate the glomeruli from other tissue elements (*4*). Basement membranes were obtained from the isolated glomeruli after ultrasonic treatment. The purity of the preparations was evaluated under the electron microscope and by chemical analysis. Electron microscopic examination showed that the preparations of basement membrane had an amorphous appearance, similar to that seen in the intact glomeruli; only negligible amounts of fibrillar collagen could be seen. The average content of DNA was 0.36 percent of the weight of the dry membrane, and the average total phosphorus was 0.14 percent. The total lipid content of the basement membranes was less than 1 percent, and no significant amounts of covalently bound fatty acids could be detected. Normal and diabetic basement membranes were not significantly different in these analyses.

Table 1. Composition of normal human glomerular basement membrane. Values represent the average of analyses performed on six pools each of which contained the glomerular basement membranes from 15 to 20 pairs of kidneys. The components were analyzed by the methods previously used (*4*, *9*).

Component	Residue weight (g/100 g dry wt. ± S.D.M.)	Residues per 1000 total amino acid residues
Hydroxyproline	7.51 ± 0.35	84.1
Aspartic acid	5.87 ± 0.37	64.6
Threonine	2.72 ± 0.23	34.1
Serine	3.49 ± 0.23	50.8
Glutamic acid	8.83 ± 0.87	86.7
Proline	6.09 ± 0.69	79.4
Glycine	9.95 ± 0.37	220.9
Alanine	3.87 ± 0.18	68.9
Valine	2.93 ± 0.24	37.4
Methionine	1.27 ± 0.08	12.7
Isoleucine	2.53 ± 0.13	28.3
Leucine	4.88 ± 0.23	54.8
Tyrosine	1.97 ± 0.21	15.4
Phenylalanine	2.90 ± 0.34	26.3
Hydroxylysine	2.39 ± 0.19	21.3
Lysine	2.91 ± 0.21	28.8
Histidine	1.66 ± 0.08	15.4
Arginine	5.79 ± 0.36	47.1
Half-cystine	1.71 ± 0.09	21.0
Tryptophan	0.25	2.0
Amide nitrogen	0.87	(64.9)
Glucose	2.03 ± 0.07	15.8
Galactose	2.34 ± 0.13	18.3
Mannose	0.55 ± 0.02	4.32
Fucose	0.14 ± 0.007	0.98
N-Acetylglucosamine	1.21 ± 0.02	7.40
N-Acetylgalactosamine	0.16 ± 0.004	0.99
N-Acetylneuraminic acid	0.62 ± 0.025	2.67
Glucosylgalactosylhydroxylysine		16.13 ± 0.76

Compositional studies on the basement membrane of normal human kidney indicated that it belongs to the collagen family of proteins (Table 1). More than one-fifth of the total amino acid residues was glycine; substantial amounts of hydroxyproline and hydroxylysine were also present. The total carbohydrate content made up 7.05 percent of the dry weight of the membrane, and the sugars were identified as glucose, galactose, mannose, fucose, glucosamine, galactosamine, and *N*-acetylneuraminic acid. The presence of this large amount of carbohydrate, as well as the occurrence of half-cystine and a high content of hydroxylysine, clearly differentiates the analyses of this material from those of vertebrate fibrillar collagen (*6*).

Digestion of the membrane with collagenase and Pronase, followed by separation of the glycopeptides by gel filtration on Sephadex G-25 and Sephadex G-50, was performed as previously described (*7*). Analyses of the glycopeptides obtained in this manner indicated that the carbohydrate is distributed in two distinct types of units.

One unit is a heteropolysaccharide made up of galactose, mannose, hexosamine, sialic acid, and fucose residues; it accounts for 45 percent of the carbohydrate in the membrane. If we assume that this unit contains three mannose residues, as in the heteropolysaccharide units of the bovine glomerular basement membrane and many other glycoproteins (*8*), then its average composition would consist, in addition to the mannose, of 3.7 galactose, 5.9 hexosamine, 2.7 sialic acid, and 1.2 fucose residues. From the amino acid content of the glycopeptides contained in this unit, it is likely that it is linked to the protein through a glycosylamine type of bond to asparagine.

The other carbohydrate unit was a disaccharide consisting of glucose and galactose linked by a β-glycosidic bond

to the hydroxyl group of hydroxylysine. Structural studies indicated that the disaccharide was 2-*O*-α-D-glucosylgalactose, as has been found for the hydroxylysine-linked carbohydrate of the bovine glomerular basement membrane, the lens capsule, and ichthyocol (*9, 10*). The disaccharide unit accounts for 55 percent of the carbohydrate in the normal basement membrane. The stability of the glycosidic bonds of the hydroxylysine-linked carbohydrate unit to alkaline hydrolysis permitted the determination of the intact unit, glucosylgalactosylhydroxylysine, on the amino acid analyzer (Technicon). The total glucose values of the basement membrane agreed well with values obtained for the glucosylgalactosylhydroxylysine content, an indication that glucose is contained exclusively in this unit (Table 1).

In addition to the studies in Table 1 which were carried out on pools from normal human kidneys, the glomerular basement membranes of eight individual diabetics and eight age-matched normal controls were analyzed. All of the diabetic kidneys obtained at autopsy were from cases in which the disease was well documented, and in all except two the known duration of the disease was 12 years or more. The ages of diabetics and normals studied ranged from 23 to 68 years. The degree of mesangial and basement membrane change was moderate to severe as judged by light microscopic examination of kidney sections stained with hematoxylin and eosin and with periodic acid–Schiff reagent. It could be shown that abnormal glomeruli had been isolated from the diabetic kidneys by the procedure used in this study, because both nodules and diffuse thickening of the basement membrane were unequivocally demonstrated with the periodic acid–Schiff stain.

Increased deposition of basement membrane-like material was also shown by the finding of a significant increase in the hydroxylysine and glucose content of the dry, washed glomeruli of the diabetics compared to the normals (*11*). These two components were considered sensitive indicators of the amount of basement membrane material present.

Comparison of the analyses of the normal and diabetic glomerular basement membranes revealed that only two amino acids, lysine and hydroxylysine, showed differences significant to levels of P less than .01 (Table 2). Diabetic basement membranes showed an increase in the hydroxylysine values, with a corresponding decrease in the lysine content. The sum of these two amino acids, which differ from each other only by a hydroxyl group, is similar in diabetics and normals (Table 2). Glucose, which occurs uniquely in the hydroxylysine-linked disaccharide unit, was significantly elevated in the diabetic membranes. On the other hand, mannose and hexosamines, which occur only in the heteropolysaccharide unit, were similar in diabetic and normal membranes. From the molar ratios of glucose and mannose in their respective units, a ratio of 11.3 disaccharide units to 1 heteropolysaccharide unit can be calculated for the normal basement membranes (Table 2). In the membranes of the diabetics, there is an increase in this ratio to 14.6 to 1. In the diabetic glomerular basement membranes, the amount of glucosylgalactosylhydroxylysine determined after alkaline hydrolysis correlated well with the glucose values, an indication that in the diseased membranes, as in the normals, all of the glucose is present as the disaccharide (Table 2). The percentage of hydroxylysine residues substituted with the disaccharide showed no significant difference between the normal and diabetic (Table 2) because the elevation in the hydroxylysine was accompanied by a proportional increase in the glucose (disaccharide) values.

Comparison of the other amino acids of normal and diabetic glomerular basement membranes showed no differences except for a slight increase in the glycine and hydroxyproline content of the diabetic membranes ($P < .05$, $P > .025$). The increases in these amino acids could result from some further differences between the normal and diabetic membranes, or could indicate the presence of a small amount of fibrillar collagen in the diabetic preparations. However, the occurrence of some fibrillar collagen in the diabetic samples could not be responsible for the highly significant increases observed in the hydroxylysine and glucose contents of the diabetic membranes, because compared with the basement membrane fibrillar collagen is particularly low in these components (*10*). Indeed, any contamination with fibrillar collagen woud tend to decrease rather than to increase the hydroxylysine and glucose content.

Because of the slow turnover of the basement membrane, the membrane isolated from diabetic patients includes material which was synthesized both before and after the onset of the metabolic disturbances and therefore does not give a complete picture of the degree of alteration which occurs in this membrane in the diabetic state. Therefore, any changes which occur in the diabetic membrane would be minimized in these analyses by the presence of normal (prediabetic) basement membrane material.

It is interesting that in diabetes, a disease with abnormal carbohydrate metabolism, there is an increase in the covalently bound carbohydrate in the glomerular basement membrane. This increase is presumably preceded by an increase in the number of attachment points on the peptide chain, which are produced by an increased hydroxylation of lysine to form hydroxylysine.

Studies with the kidney galactosyltransferase enzyme, which is responsible for the glycosylation of hydroxylysine on the peptide chain, have shown that the native basement membrane has no hydroxylysine residues available for carbohydrate attachment (*12*), an indication that the 78 percent substitution of this amino acid by carbohydrate is a maximum value. The newly formed hydroxylysine residues, therefore, would serve as the sites for placement of new

Table 2. Comparison of several components of the normal and diabetic human glomerular basement membrane. Values represent averages of analyses performed on the kidneys of eight normal individuals and eight diabetics; NS, not significant.

Component	Residues per 1000 total amino acid residues ± S.D.M.		P value
	Normal	Diabetic	
Hydroxylysine	24.7 ± 1.06	30.21 ± 0.96	< .01
Lysine	25.4 ± 0.94	19.70 ± 0.34	< .001
Lysine plus hydroxylysine	50.1 ± 1.49	49.9 ± 0.98	NS
(Lysine/hydroxylysine)	(1.03 ± 0.09)	(0.65 ± 0.03)	< .002
Glucose	17.8 ± 1.01	22.8 ± 1.15	< .01
Mannose	4.73 ± 0.25	4.70 ± 0.17	NS
Hexosamine	10.26 ± 0.46	10.47 ± 0.32	NS
(Disaccharide/heteropolysaccharide)*	(11.3)	(14.6)	
(Percentage of glycosylation of hydroxylysine)†	(72.5 ± 4.3)	(75.7 ± 4.2)	NS
Glucosylgalactosylhydroxylysine	15.9 ± 1.08	20.8 ± 1.31	< .025

* Calculated as glucose/(mannose divided by 3). † Calculated as (glucose/hydroxylysine) times 100.

carbohydrate units. It is interesting that the asparagine-linked heteropolysaccharide unit does not increase in amount in the diabetic membrane. This could be due to the fact that asparagine residues are put into the peptide chain in a coded manner during the synthesis of the peptide chain and cannot be increased in number after the protein leaves the ribosome, as is possible for the hydroxylysine residues.

The sequence of events between insulin deficiency and this structural alteration in the basement membrane is not understood. The assembly of the basement membrane involves several steps after the ribosomal ones, including hydroxylation and carbohydrate attachment. These steps are open to regulation by environmental influences. It is conceivable that in diabetes the high concentration of glucose or of some metabolic derivative of this sugar could function in regulating both the hydroxylation and the carbohydration of the basement membrane. In addition, the overall rate of the synthesis of this membrane could be influenced by the availability of sugar nucleotides for attachment to the peptide chain.

The alterations which occur in the diabetic membrane involve lysine, or its derivatives hydroxylysine and glycosylated hydroxylysine. Both lysine and hydroxylysine participate in the formation of cross-links of the peptide chains of collagens and elastin (*13*). It has been postulated that glycosylation may help regulate cross-linking of collagen molecules (*14*). The substitution of carbohydrate on hydroxylysine residues may have the function of making them no longer available for participating in the ε-deamination and the subsequent condensation which are involved in cross-link formation.

If such cross-links also exist in the glomerular basement membrane, the increase in glycosylation of hydroxylysine would reduce the availability of this amino acid to participate in their formation. Such a defect in cross-linking and the effect of the extra and bulky carbohydrate substituents on the packing of the peptide chains could contribute to the increased permeability of the basement membrane seen in diabetes mellitus.

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L-Glutamic Acid Decarboxylase: A New Type in Glial Cells and Human Brain Gliomas

Abstract. *Human glial cells grown in culture and gliomas and white matter contain an* L-*glutamic acid decarboxylase which is stimulated markedly by carbonyl-trapping agents. In contrast,* L-*glutamic acid decarboxylase activity of human cerebral gray matter is strongly inhibited by carbonyl-trapping agents. These results suggest a glial localization of the new type of* L-*glutamic acid decarboxylase.*

The α-decarboxylation of L-glutamic acid is catalyzed in mammalian kidney and other nonneuronal tissues by a second L-glutamic acid decarboxylase (GAD II) (*1, 2*) that differs markedly from the previously described, partially purified and characterized L-glutamic acid decarboxylase (GAD I) (*3*). The GAD I is inhibited by anions and completely inhibited by various carbonyl trapping agents at $10^{-3}M$ (*4*) and has largely a synaptosomal localization (*5*). In contrast, GAD II is activated by high concentrations of anions and carbonyl trapping agents (*1, 2*). The GAD II activity measured in the presence of $10^{-3}M$ aminooxyacetic acid hemihydrochloride (AOAA) is primarily mitochondrial in kidney and in developing chick embryo brain (*2*). The GAD II activity became apparent when purified adult mouse brain mitochondrial fractions were assayed with $10^{-3}M$ AOAA, which suggests that in homogenates GAD II activity may be obscured by the presence of GAD I under the usual assay conditions (*6*).

Table 1. L-Glutamic acid decarboxylase activities in cortical gray and white matters of human cerebrum. The values are averages of closely checking triplicate determinations performed on adult human brain obtained 2 hours after death.

Tissue	Specific activity (μg GABA/g protein/min)	
	Standard assay	+AOAA ($10^{-3}M$)
Cortical gray matter	98.6	29.6
White matter	44.1	80.2

Samples of cortical gray and white matters of human brain (2 hours postmortem, taken at autopsy) were homogenized in ice-cold distilled water and the protein concentrations per assay were adjusted to 0.5 mg/ml. Protein was determined by the method of Lowry *et al.* (*7*). The GAD activity was determined in the absence and presence of $10^{-3}M$ AOAA by the radiometric method of Roberts and Simonsen (*4*). The standard assay was performed in $0.1M$ potassium phosphate buffer (*p*H 6.5) containing $10^{-3}M$ aminoethylisothiouronium bromide and $10^{-4}M$ pyridoxal phosphate. Human glial cells cultured from autopsy material in medium 199 containing 10 percent newborn calf serum and 5 percent fetal calf serum and subcultured in the usual manner were supplied by H. Kihara (Pacific State Hospital, California). Human gliomas (astrocytomas, surgical specimens) were supplied by B. Crue (City of Hope).

Table 2. L-Glutamic acid decarboxylase activities of glial cells grown in culture and gliomas of human brain.

	Specific activity (μg GABA/g protein/min)	
	Standard assay	+ AOAA ($10^{-3}M$)
	Glial cells	
Sample 1	42.4	160.4
Sample 2	36.1	141.0
Sample 3	45.0	144.5
	Gliomas	
Case 1	19.1	81.0
Case 2	24.1	79.1
Case 3	21.0	85.0