Alterations in the Basement Membrane (Heparan Sulfate) Proteoglycan in Diabetic Mice

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SUMMARY

We have grown the EHS (Engelbreth-Holm, Swarm) tumor in normal and genetically diabetic mice (db/db) and measured some components of basement membrane produced in the tumor. These studies showed similar amounts of total protein in control and diabetic tissue and similar patterns of proteins on SDS gel electrophoresis of extracts of the tissue. Laminin, a basement membrane specific glycoprotein utilized as an attachment factor by epithelial cells, was present in increased amounts in diabetic tissue. In contrast, the amount of BM-1 (heparan sulfate) proteoglycan was reduced. Less ^{35}S-sulfate was incorporated into this proteoglycan, and the proteoglycan, but not its component glycosaminoglycans, was heterogeneous in size. The data indicate that either the synthesis of proteoglycan was decreased or its degradation was increased in diabetic tissue. Since the heparan sulfate proteoglycan serves to block the passage of anionic macromolecules through the basement membrane, decreased levels could account for the increased porosity of diabetic basement membrane. Compensatory synthesis of the basement membrane components to restore normal permeability could account for the thickened basement membranes observed in diabetes. DIABETES 31:185–188, February 1982.

Basement membranes are thin extracellular matrices[1] that separate epithelial and endothelial cells from underlying stroma and prevent the passage of certain macromolecules.[2–5] In diabetics, marked thickening of basement membranes is seen in capillaries and the kidney glomeruli and this change is believed to underlie premature degeneration of the kidneys, blood vessels, and eyes.[6,7] Although the basement membranes are thicker, they are more porous.[8–11]

As a model system we have used the EHS tumor, which produces basement membrane components that are immunologically and chemically similar to those obtained from normal tissue.[12–15] Here we analyzed the basement membrane components extracted from the EHS tumor grown in normal and genetically diabetic (db/db) mice.

MATERIALS AND METHODS

Tissue from the EHS tumor was grown intramuscularly in the hind limbs of littermate 7-wk-old C57BL/KsJ (db/db) mice, heterozygous mice (db/+), and wild type mice (+/+).[16,17] Three weeks later, serum glucose levels were assayed and found to be higher than 250 mg/dl for the db/db mice, while the levels in heterozygote and wild type mice were less than 100 mg/dl. Tumor tissue was harvested and the weights of the tumors from diabetic and normal mice were similar.

Tumor from 9 db/db mice, 3 db/+ mice, and 5 +/+ mice were pooled separately and dispersed by passage through a syringe. About 5 g of tissue from each group was incubated for 18 h in 25 ml of Minimal Essential Eagle's Medium containing 50 mg/ml lincocin (Gibco, Grand Island, New York) and 100 U/ml mycostatin plus 200 μCi of ^{3}H-glucosamine and 250 μCi of ^{35}S-sulfate. Subsequently the tissue was extracted at 4°C for 4 h with 5 ml/g wet weight of tissue with 0.5 M NaCl containing 0.05 M TRIS-HCl, ph 7.2, 4 mM ethylenediaminetetraacetate (EDTA), and 1 mM phenylmethylsulfanyl-fluoride (PMSF). The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant fluid was removed and the residue was then extracted overnight at 4°C with 4 M guanidine hydrochloride containing EDTA and PMSF (1.3 ml/g tissue). The extracts were dialyzed exhaustively against 0.05 M TRIS-HCl, pH 7.2, containing 0.85% NaCl and 2 mM sodium sulfate.

Laminin and proteoglycan in tumor extracts were measured by the enzyme-linked immunosorbent assay (ELISA).[18] Briefly, polystyrene Titertek plates (Dynatech Lab. Inc., Alexandria, Virginia) were coated with 500 ng of either laminin or proteoglycan and assayed as described[18] by the

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TABLE 1
Laminin and protein content in normal and diabetic tissue*

	Laminin (mg/mg protein)	Laminin (mg/g tissue)	Protein (mg/g tissue)
db/db (9)	0.28	4.4	15
db/+ (3)	0.18	2.5	14
+/+ (5)	0.16	2.6	17

The extracts of db/db, db/+, +/+ mice were pooled separately and aliquots were used for the analyses. Numbers of mice in each group are indicated within parentheses.
* Total laminin and total protein content extractable from EHS tumor grown in genetically diabetic (db/db), heterozygous (db/+), and control (+/+) mice. An analysis of duplicate samples of the pooled tissue differed by less than 10%.

inhibition test with the standards ranging from 4 ng/ml to 10 μg/ml. Each sample was tested over a range of dilutions and duplicate analyses on the samples gave values within 10%. Total protein was measured by the Lowry technique.[19] The proteins in the extracts was also analyzed by electrophoresis.[20]

Samples containing labeled proteoglycan were chromatographed on a Sepharose CL-4B column (1.8 × 180 cm) equilibrated with 4 M guanidine hydrochloride, 0.05 M TRIS-HCl, pH 6.8. Fractions of 5.5 ml were collected and a portion of each fraction was assayed for radioactivity in Instagel (Packard) with a Beckman LS 8100 liquid scintillation counter. Extracts were also digested with papain (1 mg/ml) in 1.0 M sodium acetate (pH 6.5) at 60°C overnight. The papain was inactivated by addition of 2-iodoacetamide. Samples were then chromatographed on a Sepharose CL-6B column (1.5 × 90 cm), equilibrated with 4 M guanidine hydrochloride. Fractions of 5.5 ml were collected and assayed for radioactivity. Samples were also digested using either heparanase[14] or chondroitinase ABC[21] and the digests were analyzed on paper chromatography.[21]

RESULTS

The proteins in NaCl and guanidine extracts of the tumor were examined and no significant differences were observed in either the amount of protein (Table 1) or in type as assayed by one-dimensional slab gel electrophoresis (data not shown). Assays for laminin were performed on the extracts using the ELISA assay.[18] These studies (Table 1) showed that the extracts of tumor grown in diabetic (db/db) mice had almost twice the laminin content, relative to either wet weight of tumor tissue or to protein content, as extracts from tumor grown in nondiabetic mice. This indicates that there is an increase in laminin in the diabetic state which is consistent with previous observations of an increase in basement membrane (type IV) collagen.[22–24]

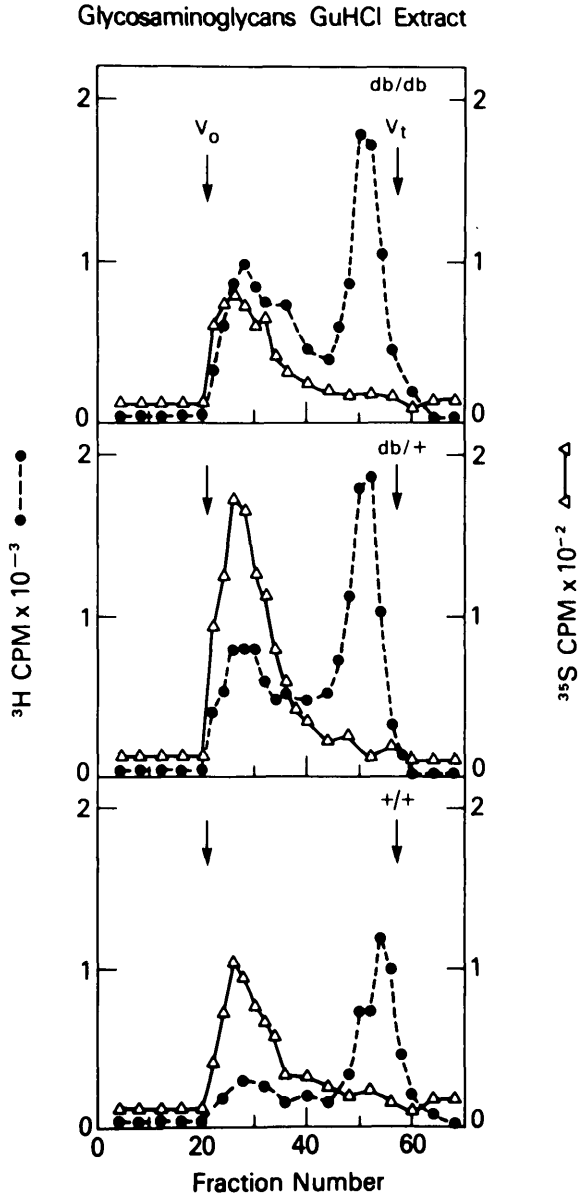

FIGURE 1. Sepharose CL-6B column chromatography of guanidine extracts of EHS tumor tissue after papain digestion.

TABLE 2
Incorporation of ^{3}H-glucosamine and ^{35}S-sulfate into normal and diabetic tissue*

	^{3}H-glucosamine (cpm × 10^{-4}/g tumor)	^{35}S-sulfate (cpm × 10^{-2}/g tumor)	^{35}S/^{3}H
db/db (9)	1.8	3.3	0.02
db/+ (3)	2.3	10.5	0.05
+/+ (5)	2.0	7.1	0.04

The extracts of db/db, db/+, and +/+ mice were pooled separately and aliquots were used for the analyses. Numbers of mice in each group are indicated within parentheses.
* In vitro synthesis of basement membrane macromolecules by EHS tumor tissue grown in diabetic, heterozygous, and control animals radiolabeled with ^{3}H-glucosamine and ^{35}S-sodium sulfate. Two aliquots of each sample were counted and did not differ by more than 10%.

The synthesis of proteoglycans by normal and diabetic tumor tissue was studied by following the incorporation of ^{3}H-glucosamine and ^{35}S-sulfate. There was a slight decrease in the level of ^{3}H-glucosamine incorporation and a marked reduction in the level of ^{35}S-sulfate incorporation in the tissue from diabetic (db/db) mice (Table 2). A similar decrease in ^{35}S-sulfate incorporation has been seen in the glomerular basement membrane of diabetic rats.[25] A decrease in heparan sulfate in aortas of diabetic rats has also been reported.[26]

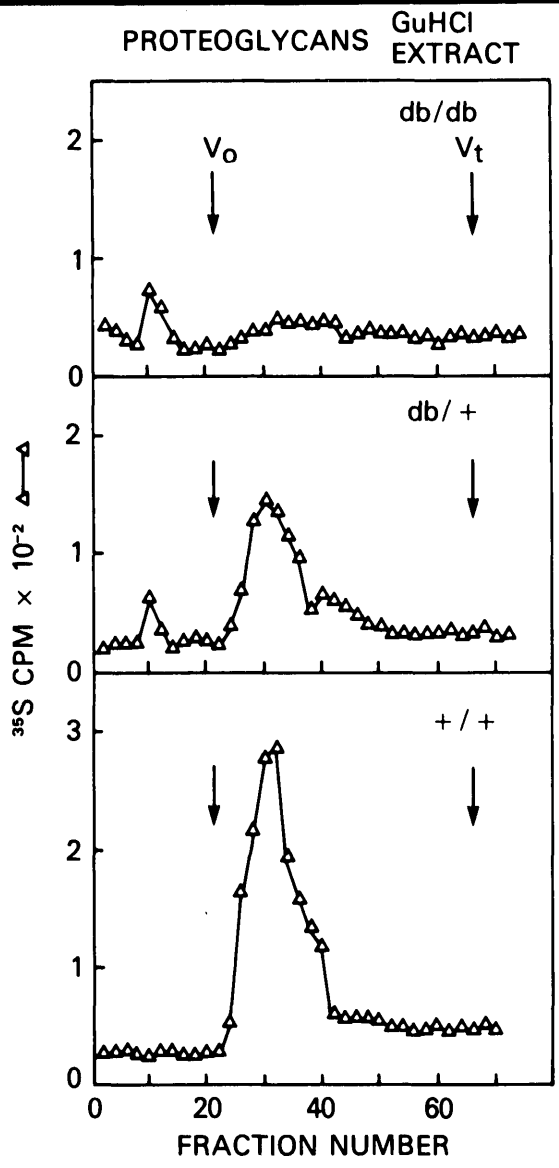

FIGURE 2. Sepharose CL-4B column chromatography of guanidine extracts of EHS tumor tissue.

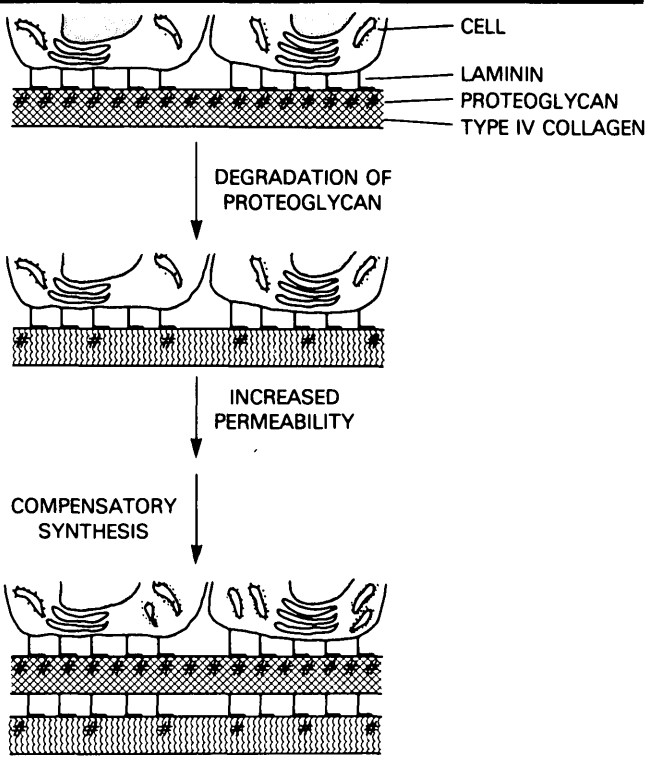

FIGURE 3. Schematic representation of possible events leading to the thickening of basement membrane in diabetes. At the top of the figure, synthesis of the common constituents of basement membrane (laminin, type IV collagen, and the heparan sulfate proteoglycan) has been completed and the functional basement membrane is intact. In the diabetic state, the level of the heparan sulfate proteoglycan decreases due either to increased degradation (middle) or inadequate synthesis (not depicted). As a result of the loss of proteoglycan, the basement membrane becomes more porous. We propose that a high porosity of the basement membrane triggers the compensatory synthesis of more basement membrane components which leads to an increase in type IV collagen and laminin as shown at the bottom.

Since ^{35}S-sulfate is incorporated in large part into proteoglycans, enzymes known to degrade specific glycosaminoglycans were used to characterize the ^{35}S-labeled material. Heparinase degraded the ^{35}S-sulfate labeled macromolecules from the tissue grown in normal, heterozygous, and diabetic mice, while chondroitinase ABC had little or no effect (data not shown). These results indicate that the ^{35}S-sulfate was incorporated into a heparan sulfate proteoglycan.

Quantitative ELISA assays[18] for BM-1 proteoglycan core protein were carried out using antibody specific for the core protein. These studies confirmed that the extracts of diabetic tumor tissue (db/db) contained less BM-1 proteoglycan (10.9 μg/mg protein) than tumor tissue from wild type (+/+) animals (25.6 μg/mg protein).

The heparan sulfate containing macromolecules were studied further by molecular sieve chromatography. When the glycosaminoglycans prepared by papain digestion were chromatographed on a molecular sieve column of Sepharose CL-6B, no difference in size was noted between the GAG from the tumors grown in control and diabetic animals (Figure 1). Using molecular sieve chromatography, the intact proteoglycan material from the control tissue consisted of high molecular weight molecules, while the material from diabetic tissue was more heterogeneous (Figure 2) in size and contained lower molecular weight components. These studies confirm that there is less proteoglycan and it is reduced in size in the diabetic state.

DISCUSSION

We demonstrate that the level of laminin is increased and the amount of the proteoglycan is decreased in tumor grown in diabetic mice. Both the amount of ^{35}S-sulfate incorporated into proteoglycans and the level of proteoglycan core protein are reduced, indicating that fewer intact proteoglycan molecules are present. Decreased amounts of proteoglycan are not limited to tumor tissue grown in db/db mice, but also occur in tumor grown in mice treated with streptozotocin (manuscript in preparation).

The reason for the decreased levels of BM-1 proteoglycan is unclear. Reduced synthesis or increased degradation could result in reduced levels of proteoglycan. Additionally, an altered structure of the molecule could result in increased susceptibility to degradation. Further studies are

required to define the exact nature of the mechanism responsible for the reduced levels of BM-1 proteoglycan.

It is possible that the decrease in BM-1 proteoglycan observed here could also occur as an early consequence of human diabetes. We propose (Figure 3) that in the different forms of diabetes there is a net reduction in the level of proteoglycan in the basement membrane. As a result, the basement membranes would have less negative charge from the heparan sulfate side chains and, therefore, become more permeable to anionic proteins. This could induce a compensatory synthesis of basement membrane components to correct the increased porosity. Either decreased synthesis or increased turnover could reduce the amount of proteoglycan in the diabetic state relative to other components but initiate the new synthesis of all components. Over time, repeated cycles of this process could lead to a grossly thickened basement membrane. Presumably, the basement membranes most affected would be those in capillaries and glomeruli in which the highest rates of filtration occur.

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