Reduced Synthesis of Basement Membrane Heparan Sulfate Proteoglycan in Streptozotocin-induced Diabetic Mice*

(Received for publication, August 6, 1982)

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In diabetes, certain basement membranes become thicker yet more porous than normal. To identify possible changes in the basement membrane, we have grown the Engelbreth-Holm-Swarm tumor, a tissue that produces quantities of basement membrane in normal mice and in streptozotocin-treated, insulin-deficient, diabetic mice. The level of laminin, a basement membrane-specific glycoprotein, and the level of total protein were slightly elevated in the diabetic tissue. In contrast, the level of the basement membrane specific heparan sulfate proteoglycan was only 20% of control. The synthesis of this proteoglycan was also reduced in the diabetic animals, while the synthesis of other proteoglycans by tissues such as cartilage was normal.

The synthesis of the heparan sulfate proteoglycan in diabetic animals was inversely related to plasma glucose levels showing an abrupt decrease above the normal range of plasma glucose. Insulin restored synthesis to normal but this required doses of insulin that maintained plasma glucose at normal levels for several hours. Since the heparan sulfate proteoglycan in the basement membrane restricts passage of proteins, its absence could account for the increased porosity of basement membrane in diabetes. A compensatory synthesis of other components could lead to their increased deposition and the accumulation of basement membrane in diabetes.

Basement membranes are thin, extracellular matrices that underlie various epithelia and endothelia and, among other functions, regulate the passage of macromolecules across these tissues (1-4). Considerable progress has been made in defining their chemistry. Basement membrane-specific components include: type IV collagen, the structural component (5, 6); laminin, a glycoprotein to which epithelial cells attach (7-9); and a heparan sulfate proteoglycan (10, 11) which regulates the permeability of the basement membrane by limiting the penetration of charged macromolecules (10, 12, 13). While heparan sulfate is found in many tissues, the protein portion of the basement membrane heparan sulfate proteoglycan is specific for that tissue (11).

Patients with chronic diabetes often develop degenerative changes leading to nephropathy, neuropathy, and retinopathy. A histological feature common to these pathological situations is that the basement membranes associated with the capillaries become thickened, yet more porous to the passage of macromolecules (14-19).

Previous studies of the basement membrane in animal models of diabetes indicate that there are alterations in the production of its components. These include an increased synthesis of collagen, presumably type IV (20-22), and of laminin (23). In contrast, the incorporation of [35S]sulfate into basement membrane materials, a measure of the synthesis of sulfated proteoglycans, is decreased in diabetes (23-26).

Studies on normal basement membranes are complicated by the fact that they are small, relatively inert structures which are difficult to separate from surrounding tissue elements. For this reason, we have utilized the EHS tumor which produces a homogeneous matrix of basement membrane as a source of basement membrane components and as a model to study their synthesis in normal and in disease states (7, 11, 27, 28). When this tumor was grown in genetically diabetic (db/db) mice, we found decreased amounts of the basement membrane-specific heparan sulfate proteoglycan (23) compared to the levels found in mice with a wild type genetic background. This could cause the diabetic basement membranes to be more permeable to proteins than normal. The thickening of the basement membranes in glomerular capillaries and other sites of high fluid flow could result from an increased synthesis of basement membrane components to compensate for the loss of the proteoglycan.

The genetically diabetic mouse, db/db, is a model for non-insulin-dependent diabetes since it develops high serum levels of glucose in the presence of high levels of insulin due to end organ receptor deficiencies (29, 30). It was of interest to conduct similar studies on basement membrane produced in insulin-deficient mice whose diabetes was induced with streptozotocin. These studies show that the content and the synthesis of the heparan sulfate proteoglycan are reduced in the insulin-deficient diabetic mice and can be restored by the addition of insulin.

EXPERIMENTAL PROCEDURES

The EHS tumor was grown intramuscularly in the hind limbs of C57BL/6J mice. A day after tumor injection, the mice were fasted
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were determined by a glucose oxidase assay (31). In most of the studies reported here, we utilized tumor tissue from animals with blood glucose levels greater than 250 mg/dl and considered these animals as diabetic. However, in studies designed to establish the relationship between blood glucose levels and proteoglycan synthesis, we studied tumors from streptozotocin-injected animals with a broader range of plasma glucose levels.

Matrix macromolecules produced by the tumors were radiolabeled by intraperitoneal injection of 100 μCi of [2,4,5,3H]proline and 100 μCi of [35S]sodium sulfate (Amersham Corp.). Other mice were labeled with 100 μCi of [3H]glucosamine and [35S]sodium sulfate.

The tumor tissue was harvested and then digested with papain (1 mg/ml) in 1.0 M guanidine hydrochloride, 0.1 mM ethylenediamine tetraacetate and 1 mM phenylmethylsulfonyl fluoride (5 ml/g of tumor, wet weight). Insoluble material was removed by centrifugation at 3000 rpm for 20 min, and the extracts were dialyzed exhaustively against 0.05 M Tris-HCl, pH 7.2, containing 0.85% NaCl and 2 mM sodium sulfate. Aliquots were counted in a Beckman LS8100 scintillation counter in Instagel scintillation cocktail (Packard Instrument Co.,). Approximately 70% of the incorporated [35S]sulfate was present in extracts of control as well as diabetic tissue. Chromatography of the labeled macromolecules in the extracts on DEAE-cellulose showed that over 90% of the [35S]sulfate was incorporated into the proteoglycan fraction with a maximum of 9% incorporated into proteins and other materials.

Radiolabel incorporation by other tissues and in plasma was also determined 2½ h after insulin administration. Liver, kidney, lung, heart, and xiphoid process were rinsed briefly in 3.4 M NaCl, 0.05 M Tris-HCl, pH 7.2, and then each organ from each mouse was homogenized individually in 2 ml of 5 M guanidine hydrochloride extraction buffer described above. The tissues were extracted overnight. Extracts were dialyzed and counted as described above. Blood plasma from radiolabeled mice was obtained by centrifugation in heparinized Natelson capillary tubes (Lancer). Plasma was dialyzed and counted. The significance of differences between group means was analyzed by the two-tailed t test.

Laminin and proteoglycan in tumor extracts were measured by the ELISA (32). Briefly, polystyrene or polyevinyl Multi-wall plates (Dyne-tech Laboratories, Inc.) were coated with 500 μg of either laminin or proteoglycan and then antibody specific for each substance was added to the wells. Antibody that bound to each substrate was detected using peroxidase-conjugated second antibody. Laminin and heparan sulfate proteoglycan in extracts of the tumor were assayed based on their ability to compete with the substrate for the specific antibody and were quantitated by comparison with known amounts of these materials over the range of 4 ng/ml to 10 μg/ml. Each sample was analyzed in triplicate, and duplicate analyses of each sample gave values within 10%. The Lowry technique (33) was used to measure total protein content of extracts of the EHS tumor. In the experiment shown in Table I individual analyses were performed on tumor extract from each of 8 diabetic and 10 control animals. Similar results were obtained in other experiments.

Proteoglycans were isolated by chromatography on a DEAE-cellulose column equilibrated with 8 M urea, 0.05 M Tris-HCl, pH 6.8, and eluted with a linear salt gradient of 0-0.75 M NaCl. The size of the biosynthetically labeled proteoglycan was determined by chromatography on a Sepharose CL-4B column, equilibrated in 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. Glicosaminoglycans were prepared from the proteoglycan by digestion of the material 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. The size of the glycosaminoglycans was determined by chromatography on a Sepharose CL-6B column, equilibrated in 4 M guanidine hydrochloride, 0.05 M Tris-HCl, pH 6.8. Proteoglycan synthesis was also measured after insulin was administered to diabetic animals. Various injection (intramuscular), regimens of insulin (Sigma, St. Louis, MO), including 10 units of insulin 18 h before radiolabeling, 5 units of insulin twice daily for 5 days, and 1 unit of insulin twice daily for 5 days. The insulin-treated animals received a final injection of 2 units of insulin and all animals received 100 μCi each of [35S]sulfate and [3H]proline 2½ h before harvesting the tumors. Other animals were given a single injection of 10 units of insulin either 18 h before or with the radiolabeled compounds. To monitor the effects of these levels of insulin on plasma glucose, animals were given single injections of either 1, 2, or 10 units of insulin, and their blood glucose levels were measured as described above.

RESULTS

Quantitation of the components extracted from the tumor matrices showed that less heparan sulfate proteoglycan was present in the tumor tissue from diabetic mice than in similar tissue from control animals (Table I). In different experiments, the level of proteoglycan was always found to be less than half of that present in control tissue. Since the antibody used in this assay recognizes the protein core of the proteoglycan, the tumor from animals treated with streptozotocin must contain fewer proteoglycan molecules in the basement membrane material. In contrast, the laminin and total protein content of the tumor tissue showed no significant differences between diabetic and control animals.

Reduced levels of proteoglycan could be due to reduced synthesis. This was investigated by measuring the levels of [35S]sulfate incorporated into macromolecules at various times after administration of isotope. We also injected the animals with [3H]proline and measured the incorporation of this amino acid into nondialyzable materials to correct for differences in overall synthesis of proteins from one tumor to another. There was no significant difference in protein incorporation between normal and diabetic animals and the level of incorporation remained stable from 2½ to 24 h (not shown). The incorporation of [35S]sulfate by diabetic tissue was reduced 2½ h after isotope injection while the incorporation of [3H]proline was somewhat increased particularly in diabetic animals with the highest glucose levels (Fig. 1A). The ratio of [3S]sulfate to [3H]proline incorporated into tumor macromolecules was determined from 2½ h to 9 days after injection of labeled precursors (Fig. 1B and C). The ratio of 35S/3H in tumor tissue was only 50% of control at 2½ h and this difference was maintained for 3-4 days in vivo. After 4 days, the ratio began to decline in control tissue (Fig. 1C). Total glucosamine incorporation into proteoglycans isolated from a DEAE-cellulose column was also depressed in the tumor from diabetic animals (data not shown).

We also investigated the incorporation of [35S]sulfate and [3H]proline into other mouse tissues (Table II). No significant differences in incorporation were found in the liver, kidney, cartilage, lung, heart, or blood plasma between normal and streptozotocin-treated animals. These data support the idea that the decrease in [35S]sulfate incorporation into the basement membrane type of proteoglycan in diabetes is specific and not the result of a general decrease in the synthesis of all kinds of sulfated macromolecules. Further, the data indicate that the handling of [35S]sulfate is not different in the diabetic

| TABLE I | Quantitation of components of the EHS tumor grown in control and streptozotocin-treated mice |
|---------|-----------------------------------------------|
| Values represent mean ± S.D. of individual analysis of tumors from each of 10 control and 8 streptozotocin-treated diabetic mice. Numbers in parenthesis give the percentage of the control value. Heparan sulfate proteoglycan and laminin were quantitated by the ELISA assay (32). Extractable protein was quantitated by the Lowry procedure (33). |

| Component          | Control  | Diabetic |
|--------------------|----------|----------|
| Heparan sulfate proteoglycan (μg/mg protein) | 9.0 ± 3.6 | 1.8 ± 0.7 (20) |
| Laminin (μg/mg protein) | 190 ± 20 | 230 ± 70 (121) |
| Protein (mg/g tumor) | 54 ± 9 | 65 ± 9 (120) |
| Tumor wet weight (g) | 7.1 ± 2.4 | 6.8 ± 2.7 (96) |
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![Graph 1](image)

**Fig. 1.** Incorporation of [\textsuperscript{3}H]proline and [\textsuperscript{35}S]sulfate into macromolecules from control and streptozotocin-treated mice. Mice were injected intraperitoneally with [\textsuperscript{3}H]proline and [\textsuperscript{35}S]sulfate either 2½ h prior to sacrifice (A) or at the intervals indicated (B and C). The incorporation of [\textsuperscript{3}H]proline was somewhat elevated particularly in the animals with the highest levels of blood glucose. B and C show that the incorporation of [\textsuperscript{35}S]sulfate relative to [\textsuperscript{3}H]proline was considerably lower in diabetic than control tissue 2½ h after isotope administration, and this difference was maintained for several days.

![Graph 2](image)

**Fig. 2.** Chromatography of proteoglycans extracted from diabetic and control tumor tissue. A and B are the Sepharose CL-4B column chromatograms using a column (85 × 1.5 cm) equilibrated and eluted with 4 M guanidine, 0.05 M Tris-HCl, pH 7.2. C and D are the DEAE-cellulose column chromatograms using a column (9 × 1.8 cm) equilibrated with 8 M urea, 0.05 M Tris-HCl, pH 6.8. A linear gradient of 400 ml, 0 to 0.75 M NaCl, was used for elution. Equal amounts of [\textsuperscript{35}S]sulfate counts per min were applied to each column.

animals; otherwise the incorporation of [\textsuperscript{35}S]sulfate would be altered.

The proteoglycan extracted from tumor tissue grown in normal and diabetic mice as well as the glycosaminoglycan chains prepared from them were characterized by molecular sieve and ion exchange chromatography. The data indicate that the intact proteoglycan molecules from diabetic and control mice are of similar size (Fig. 2) and contain glycosaminoglycan chains of similar length (Fig. 3). The ionic strength at which the proteoglycans eluted from DEAE-cellulose was identical (Fig. 2) indicating that the net charge and the degree of sulfation of each proteoglycan must be similar.

Considerable variation was observed in blood glucose levels among animals treated with streptozotocin, presumably reflecting the degree of damage to the pancreatic cells (Fig. 4). Those animals whose blood glucose was greater than 250 mg/dl at harvest synthesized minimal amounts of proteoglycan (Fig. 4). Many animals treated with streptozotocin had blood glucose levels near control values. Some of these incorporated [\textsuperscript{35}S]sulfate into proteoglycans at values approaching controls,

**Table II**

| Tissue    | Ratio of [\textsuperscript{35}S]/[\textsuperscript{3}H]proline | % of Control |
|-----------|---------------------------------------------------------------|--------------|
| Tumor     | 1.28 \pm 0.27                                                 | 60           |
| Liver     | 0.17 \pm 0.03                                                 | 82           |
| Kidney    | 0.24 \pm 0.03                                                 | 83           |
| Cartilage | 0.33 \pm 0.05                                                 | 88           |
| Lung      | 0.21 \pm 0.01                                                 | 114          |
| Heart     | 0.28 \pm 0.04                                                 | 114          |
| Plasma    | 0.68 \pm 0.02                                                 | 88           |

* Mean \pm S.D. of analyses of tissue from 15 control and 14 streptozotocin-treated diabetic mice and tumor from 34 control and 42 diabetic mice.

† Significant at 0.005 level.

‡ Not significant.

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Significant at 0.005 level.

Not significant.
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We tested the ability of various regimens of insulin treatment (intramuscular) to restore proteoglycan synthesis. Twice daily administration of 1 or 2 units of insulin to diabetic animals for the 5 days prior to death plus 2 units of insulin administered with labeled tracers did not restore the incorporation of \[^{35}S\]\textit{S} sulfate into proteoglycan. In contrast, administration of 10 units of insulin 18 h prior to labeling plus 2 units of insulin with isotope 2½ h prior to sacrifice was effective in restoring synthesis. In both cases blood glucose levels were normalized at the time of sacrifice (Fig. 5).

Administration of a single dose of 10 units of insulin 18 h prior to radiolabeling did not restore synthesis (\[^{35}S\]/H, 0.89 ± 0.25 in diabetics versus 1.51 ± 0.43 in controls), and blood glucose levels were elevated at the time of sacrifice (479 versus 133 in the nondiabetic). The data suggest that lowered glucose levels while in others, with similar levels of plasma glucose, lower synthesis was observed. These studies suggest that there is a critical level of glucose, insulin, and/or some other factor at which the synthesis of the heparan sulfate proteoglycan is reduced.

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must be sustained, requiring rather high and repeated insulin doses, in order to restore synthesis. This conclusion was supported by measurements on diabetic animals given 1, 2, or 10 units of insulin. A similar decrease in glucose was achieved in each case, but lowered glucose was maintained much longer with the highest dose of insulin (Fig. 6).

**DISCUSSION**

Our studies indicate that both the level and the synthesis of the basement membrane-specific heparan sulfate proteoglycan are reduced in the EHS tumor when it is grown in mice made diabetic by treatment with streptozotocin. Reduced heparan sulfate proteoglycan levels were demonstrated by ELISA and by observing the relative incorporation of \[^{35}S\]sulfate and \[^{3}H\]proline. Reduced synthesis of proteoglycans has been identified previously in diabetic basement membrane, and the levels of heparan sulfate glycosaminoglycan have recently been reported to be reduced in basement membranes from glomeruli of diabetic individuals (24–26). Other investigators have shown in other tissues with different models of diabetes that the synthesis of basement membrane collagen is increased (5, 20–22). The wet weight, total protein, and laminin content in the tumor tissue from the diabetic mice studied here were not increased. Similar results have been reported recently (34) in studies in the same system. Furthermore, the reduction in sulfate incorporation is specific for the basement membrane and was not observed in other tissues which produce other kinds of proteoglycans.

The lower levels of the heparan sulfate proteoglycan in diabetic tumor tissue could be due either to a reduced rate of synthesis or to an increased turnover. If the turnover of the proteoglycan were increased, one would expect the difference between normal and diabetic tissue would increase with time. Instead, these studies indicate that the synthesis of proteoglycan was significantly reduced in the diabetic tissue at 2½ h but that the proteoglycan formed was relatively stable. Since the proteoglycan extracted from the tissue of diabetic animals was normal in size and charge as well as in the size and charge of attached glycosaminoglycan side chains, these studies indicate that decreased synthesis, not turnover, is responsible for the reduced amount of proteoglycan found in the diabetic tissue. This is in contrast to our previous studies on the genetically diabetic mouse (db/db) in which the turnover of the heparan sulfate proteoglycan was found to be elevated (23). It is not unexpected that quite different results would be noted in such distinct models of diabetes.

The reason for the decreased proteoglycan synthesis in streptozotocin-treated animals is unclear and could be due, for example, to low insulin levels, to elevated glucose levels, or to other factors. The greatest reduction in proteoglycan synthesis was seen at the higher glucose levels with less change in animals having glucose levels closer to normal. This suggests that elevations of blood glucose may be responsible for the degenerative changes in diabetes of long duration but it would also be expected that the levels of insulin would show an inverse relationship to blood glucose. A direct cause/ effect relationship to either material cannot be established by our study.

Administration of 1 or 2 units of insulin for up to 5 days lowered blood glucose transiently but failed to restore the level of \[^{35}S\]sulfate incorporation. Administration of a high dose of insulin (10 units) 18 h before labeling in conjunction with a lower dose just prior to labeling restored \[^{35}S\]sulfate incorporation to normal levels. Thus, restoration of heparan sulfate synthesis may require near normal levels of blood glucose and/or insulin levels for a sustained period. The reason for such high doses of insulin is unclear. Since the mice can tolerate the levels used, it appears that the metabolism of these animals is such that pathways affected by diabetes require elevated insulin levels for normalization. It is also possible that insulin at high doses is acting indirectly, possibly in place of some other factor. For example, it has been demonstrated that insulin can bind to somatomedin receptors on target cells and can produce somatomedin-like responses at high levels (35). In any event, it would appear that serum glucose and insulin must be maintained at normal levels for an extended period for synthesis of the proteoglycan to be restored (36).

A possible model to account for the thickening of certain basement membranes in diabetes is presented in Fig. 7. Since the proteoglycan appears to be responsible for the filtration function of the basement membrane because of its location and negative charge (10, 12, 13), reduced proteoglycan levels would be expected to make the basement membrane more permeable to anionic proteins. This change could induce a compensatory synthesis of basement membrane components to correct for the increased porosity. With time, the unbalanced synthesis of the components could lead to the deposition of a basement membrane that is thicker, albeit more porous, than normal. Presumably, the basement membranes most affected would be those in capillaries and glomeruli in which the highest rates of filtration occur. These data would

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**Fig. 7.** Schematic representation of possible events leading to thickening of the basement membrane in diabetes. The left-hand panel depicts normal synthesis of basement membrane in which all components are produced in relatively appropriate quantities and form a functional basement membrane. In diabetes, decreased synthesis of proteoglycan coupled with normal or even stimulated synthesis of other basement membrane components results in a thicker basement membrane, yet one that is lacking the proper amount of proteoglycan for regulation of filtration.
also suggest that damage is done to the basement membrane primarily at times when glucose levels are elevated, and the long term complications of diabetes could be due to cumulative damage.

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J. Biol. Chem. 1983, 258:11672-11677.