Immunohistochemical Quantification of Heparan Sulfate Proteoglycan and Collagen IV in Skeletal Muscle Capillary Basement Membranes of Patients With Diabetic Nephropathy

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In IDDM patients, an increased permeability of the glomerular capillaries has been associated with a general loss of negatively charged heparan sulfate proteoglycans (HSPGs) within basement membranes (BMs). In the present study, we used immunohistochemical staining to quantify heparan sulfate (HS), HSPG core protein, and collagen IV in capillary basement membranes of skeletal muscle biopsies taken from 9 healthy control subjects (C) and 20 IDDM patients: 7 with normal albumin excretion rate (<30 mg/24 h) (D0), 5 with incipient nephropathy (albumin excretion rate 30-300 mg/24 h) (Dx), and 8 with clinical nephropathy (albumin excretion rate >300 mg/24 h) (D2). In the capillaries, staining was measured by a scanning and integrating microspectrophotometer. A significant difference in the absorbance of HS was found among the four subgroups (means ± SD): 0.477 ± 0.082 (C), 0.627 ± 0.081 (D0), 0.542 ± 0.098 (Dx), and 0.371 ± 0.118 (D2) (P = 0.006). Similarly, an overall significant difference in the absorbance of collagen IV was demonstrated (means ± SD): 0.836 ± 0.111 (C), 0.838 ± 0.300 (D0), 0.970 ± 0.173 (Dx), and 0.512 ± 0.248 (D2) (P = 0.02). No statistical difference in the absorbance of core protein was demonstrated among the groups. Within the diabetic groups, HS was inversely correlated to albuminuria (r = -0.76, P = 0.003) and albuminuria corrected for creatinine clearance (r = -0.69, P = 0.008). Because, in IDDM patients with albuminuria, alterations of the content of HS and collagen IV within the capillary BM have been demonstrated immunohistochemically, not only in the glomerular filtration barrier, but also in the skeletal muscle capillary BM, we suggest that these changes reflect universal quantitative or qualitative alterations within the capillary filtration barrier.


A

ccording to the Steno hypothesis (1), in IDDM patients, albuminuria reflects not only severe renal disease, but also a generalized vascular dysfunction, demonstrated by increased cardiovascular morbidity and mortality (2,3). The nature of such universal alterations is unknown, and might be due to both increased capillary pressure (4) and structural alterations of the macromolecular pathway between the capillary lumen and the lymphatic capillaries (i.e., the extracellular matrix [ECM]) (5). Within the ECM, heparan sulfate proteoglycans (HSPGs) constitute a major class of proteoglycans that are formed especially in basement membranes (BMs). HSPGs are formed by a central core protein to which the anionic glycosaminoglycan heparan sulfate (HS) is linked (6).

In diabetic nephropathy, renal pathobiocchemical alterations are characterized by accumulation of BM-like material within the glomeruli (7) and a decreased number of anionic sites (i.e., HS or HSPG) within the glomerular basement membrane (GBM) (7-12). In rats, loss of GBM-HS induces albuminuria (13), and in humans a negative correlation between urinary albumin excretion rate (AER) and the content of HS within the GBM has been demonstrated (12). However, patients with diabetic nephropathy suffer from not only increased macromolecular permeability within the glomeruli, but also an increased extrarenal capillary permeability, as indicated by a 50% increase of the transcapillary escape rate of albumin and other plasma proteins (14,15). Therefore we hypothesized that deficiency of HS, both in glomeruli and in the vessel walls of extrarenal tissue, is the common link between albuminuria and macrovascular disease in diabetic patients with nephropathy. The aim of the present study was to evaluate possible alterations of the amount of HS in extrarenal tissue among healthy subjects and IDDM patients with and without albuminuria. We performed a quantification of HS, HSPG core protein, and collagen IV by immunohistochemistry on the BMs of skeletal muscle capillaries.

RESEARCH DESIGN AND METHODS

Subjects. Nine healthy control subjects (C) and twenty IDDM patients participated in the study. The diabetic patients were recruited from the outpatient clinic of the Steno Diabetes Center, and the control subjects were selected from the same geographic area. All IDDM patients had diabetes diagnosed before age 31 years. Diabetes duration was >10 years. All patients, except one, were on insulin treat-
TABLE 1

<table>
<thead>
<tr>
<th>Control subjects</th>
<th>D2</th>
<th>D1</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>4/5</td>
<td>4/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>25±3</td>
<td>25±3</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>9 (6/4.5)</td>
<td>12 (8/11)</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>73±7</td>
<td>72±12</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23±2.6</td>
<td>24±2.0</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>123±60</td>
<td>125±56</td>
</tr>
<tr>
<td>Albuminuria/creatinine clearance (mg/g)</td>
<td>0.077 (0.024–0.146)</td>
<td>0.064 (0.037–0.113)</td>
</tr>
<tr>
<td>Values missing from one subject.</td>
<td></td>
<td></td>
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</tbody>
</table>

Data are means ± SD or median (range). *p < 0.05 between diabetic groups.}
The sections were incubated overnight at 4°C. After being washed in three changes (5 min each) of TBS, secondary antibodies diluted 1:250 (biotinylated anti-mouse IgM antibody) and 1:200 (biotinylated rabbit anti-mouse IgG antibody) were added; the sections were then incubated for 30 min. Another three changes (5 min each) in TBS were performed, after which the sections were incubated for 30 min with alkaline phosphatase-conjugated avidin according to the manufacturer's prescription (K0670; Dako). This was followed by three changes (5 min each) in TBS. Finally, alkaline phosphatase activity was revealed by development for 20 min in 5-bromo-4-chloro-indolyl phosphate/nitroblue tetrazolium medium containing 1 mM N4-vanadate (Sigma, St. Louis, MO). The medium was prepared by mixing the following solutions: 10 ml precipitation buffer (100 mM Tris buffer [pH 8.8], 5 mM MgCl2, and 100 mM NaCl), 20 ml 5-bromo-4-chloro-indolyl phosphate solution (BCIP; 10 mg BCIP [Sigma, St. Louis, MO] in 200 μM N,N-dimethylformamide) and 140 ml nitroblue tetrazolium solution (NBT; 15 mg NBT [Sigma, St. Louis, MO] in 200 μM N,N-dimethylformamide).

All procedures for collagen IV, HSPG, and HSPI core protein for all subjects were performed in one setting.

**Quantitative procedure.** A minimum of 40 muscle capillaries cut in cross section and located between at least three adjacent muscle fibers (e.g., Fig. 1A) were randomly selected from at least two sections on slides with the same code. If it was impossible to identify 40 capillaries for quantification, the specimen was excluded. A randomly selected area of the stained BM in each of the capillaries was scanned with a Vickers M88A scanning and integrating microspectrophotometer (Vickers, York, U.K.) with the following machine settings: ×100 objective (scanning area 3.1 μm²), light at the isobestic wavelength (585 μm) of NBT dyes, and a scanning spot diameter of 0.2 μm. Within each area, the individual spot readings were automatically integrated by the instrument. The mean integrated absorbance over all areas was then calculated by reference to a standard calibration graph constructed by measuring a series of neutral density filters with known absorbance values. Because this procedure was performed on each experimental day, any day-to-day variation in the sensitivity of the microspectrophotometer was eliminated. Moreover, measurements of the neutral density filters showed that the instrument was linear within the range of absorbance values recorded in the capillaries. Finally, the mean formazan deposition measured in the absence of the primary antibody was subtracted from that measured in the presence of antibody to give a measure (± SD) of the specific antibody binding alone.

**Metabolic control and renal function.** HbA1c was measured by high-performance liquid chromatography (Bio Rad Diamat, Richmond, CA; normal range 4.2-6.2%). Blood glucose was measured by means of Hypocount 1B (Hypoguard LD, Woodbridge, Suffolk, U.K.). Serum and urinary creatinine was measured by a kinetic Jaffe's method (interassay coefficient of variation <0.5%). Urinary albumin concentration was determined using an enzyme-linked immunosorbent assay method (inter- and intrassay coefficient of variation <9%) (17). In diabetic nephropathy, the glomerular filtration rate and creatinine clearance show a strong inverse correlation with the percentage of occluded and globally sclerosed glomeruli (18). We therefore corrected the albuminuria (mg/24 h) for the creatinine clearance (ml/min), yielding a ratio expressed in μg/ml (Table 1). This ratio of albuminuria reflects the albumin filtration in still-functioning glomeruli (12).

**Statistical analysis.** The slides were decoded at the end of the experiment. Data were processed using the StatGraphics statistical software package (STSC, Rockville, MD) and Unistat for Windows (Unistat, London, U.K.). Results are expressed as means ± SD, except for results from AER. AER was not normally distributed, and the results are therefore given as median and range. Differences between groups were assessed by unpaired Kruskal-Wallis tests. Correlations were expressed by Spearman's correlation coefficients (r). P values <0.05 were accepted as significant.

**RESULTS**

Clinical characteristics of the study groups are given in Table 1. The diabetic groups were comparable regarding age, diabetic duration, daily insulin dosage, and BMI. Among diabetic patients, systolic and diastolic blood pressure was significantly higher in patients with albuminuria. A similar increase was observed for HbA1c, although it was not significant.

Because of the exclusion criteria related to the quantitative analysis, HS, HSPG core protein, and collagen IV were quantified in an unequal number of subjects. HS was quantified in 24 subjects. An overall significant difference was found among the four subgroups (P < 0.006) and among the diabetic groups (P = 0.01; Fig. 2A). This was mainly due to an increase in HS absorbance within the normoalbuminuric group of...
IDDM patients and a reduction of HS absorbance in the clinical nephropathy patients: 0.477 ± 0.082 (C), 0.627 ± 0.031 (D0), 0.542 ± 0.098 (D1), and 0.371 ± 0.118 (D2). Within the diabetic patients, a significant inverse correlation between HS absorbance and albuminuria (r = -0.76, P = 0.003) or albuminuria corrected for creatinine clearance (r = -0.69, P = 0.008; Fig. 3) was observed.

Collagen IV was quantified in 28 subjects. An overall significant difference in absorbance was found among the four subgroups (P = 0.02) and among the three diabetic groups (P = 0.03; Fig. 2B). This was mainly due to a decrease of collagen IV within the group of patients having clinical nephropathy: 0.836 ± 0.111 (C), 0.888 ± 0.300 (D0), 0.970 ± 0.173 (D1), and 0.512 ± 0.248 (D2). Within the diabetic group, collagen IV absorbance revealed a significant inverse correlation with albuminuria corrected for creatinine clearance (r = -0.57, P = 0.02). The coefficient of variation was 3.5, 2.2, and 2.8%, respectively, when three sections stained for collagen IV were measured on each of 10 days.

HSPG core protein was quantified in 28 subjects. Among the groups no significant difference in absorbance was revealed: 0.985 ± 0.150 (C), 0.868 ± 0.146 (D0), 1.126 ± 0.24 (D1), and 0.894 ± 0.097 (D2) (P = 0.12; Fig. 2C).

No correlation between HS absorbance and HbA1c, postprandial blood glucose, or diabetes duration was demonstrated in the entire population or within the diabetic groups. Also, the absorbance of collagen IV and HSPG core protein was unrelated to HbA1c. Furthermore, if the diabetic subjects enrolled for the measurements of HS, collagen IV, or HSPG core protein absorbance were stratified into two groups defined by a HbA1c level above or below the median value, no significant difference in absorbance was demonstrated between groups.

**DISCUSSION**

Previously, a comparable decrease of GBM-HS or HSPG core protein has been demonstrated in diabetic kidney disease, although the results have been restricted by the use of semiquantitative analysis (7,9,10,12). In the present study, a reproducible quantitative immunohistochemical analysis was used to estimate the concentration of HS, HSPG core protein, and collagen IV within capillary BMs of skeletal muscle biopsies. The present data document for the first time an extrarenal reduction of capillary BM-HS in patients with diabetic nephropathy.

Such an alteration of BM-HS absorbance in IDDM patients may be explained by altered antibody-epitope interaction. Recently, we showed that the JM403 binding to HS is dependent on the presence of an N-unsubstituted glucosamine unit in HS, and that the JM403 epitope expression in HS preparations from various sources is inversely correlated with HS sulfation (19). The HS chains are modified posttranscriptionally by N-substitution of the glucosamine units, facilitated by the key enzyme glucosaminyl N-deacetylase/N-sulphotransferase. This enzyme has been found to be decreased in both diabetic animals (20,21) and diabetic patients (22). Thus, in the present study, the increase of HS absorbance in the D0 group might be related to an undersulfation of HS. Consequently, our finding of decreased HS absorbance in patients with albuminuria might be explained by oversulfation of HS; that is, a decrease in the number of N-unsubstituted glucosamine units of HS or a decreased content of HS in the BM of muscle capillaries. Along the same line, our observation of decreased absorbance of HS in the D2 group might be due to an absolute decrease of HS within muscle capillary BM, as was previously demonstrated in the GBM of diabetic patients with albuminuria (11). We therefore suggest that the first change in HSPG in diabetes is probably an undersulfation of HS (the D0 group), followed by an absolute decrease of HS (the D1 and D2 groups), as has been discussed previously (23). An absolute reduction of BM-HS could be genetically determined. Thus an altered gene transcription or posttranscriptional modification of the first domain within the HSPG2-gene coding for the amino acid sequence at the protein HS-attachment sites (24) could be responsible for the observed "loss" of HS. Such a qualitative alteration within the HSPG core protein might not have been detected in the present quantification.

Theoretically, the reduced quantity of HS could be due to masking of epitopes, either by nonenzymatic glycation of the
primary amino group on HIS recognized by JM403, or by increasing amounts of other BM proteins, such as collagen, laminin, or fibronectin (7, 9), perhaps induced by transforming growth factor-[3 overexpression (25). Thus it has been interpreted that structural changes in the GBM prevent the normal organization of LSPG, making it inaccessible to specific antibodies (26), and thereby blurring the use of immunohistochemistry to draw conclusions about quantitative changes in macromolecular compositions. In the present study, the influence of nonenzymatic glycation seems to be negligible, since HIS absorbance did not correlate with metabolic regulation. Also, a relationship between collagen IV and a reduction of HIS seems unlikely, given that a concomitant decrease of both molecules was found in diabetic patients with nephropathy. This is in agreement with the findings of Tamsma et al. (12), who studied the GBM of IDDM patients with advanced nephropathy, but disagrees with findings from other studies in which increasing amounts of GBM-collagen IV in diabetic patients with albuminuria were reported (7, 9, 27). Conflicting results might be due to the inclusion of patients with different degrees of albuminuria, since it has been demonstrated that collagen IV expression is increased in the early stage of diabetic nephropathy and decreased in more advanced stages (28, 29). Further, different results might be obtained due to the use of anti-collagen IV antibodies with different specificities (directed against either α1(1) or α3(1)). We used a commercial monoclonal anti-collagen IV antibody in which the specific binding sites have not been identified.

In accordance with two former studies, we demonstrated that the content of BM-HIS in muscle capillaries is inversely correlated to the degree of albuminuria (9, 12). Because an increased glomerular leakiness of albumin in diabetic patients has been related to an increased systemic transcapillary albumin escape rate (14), we interpret our data as supporting the hypothesis of a functional relationship between the latter could not be measured. In addition, our data increased transcapillary escape rate of albumin (1), although in the present study the latter could not be measured. In addition, our data demonstrated an inverse correlation between collagen IV absorbance and albuminuria corrected for creatinine clearance, thereby indicating that, besides HIS, changes in collagen IV might also contribute to an enhanced diabetes-related ECM permeability.

Acknowledging that capillary wall transport involves mechanisms other than charge-electrostatic interactions in BMs (30), the major impact of the present study is that, using the same primary antibodies, a similar reduction in BM-HIS of muscle capillaries and GBM-HIS (12) is observed in IDDM patients with nephropathy. Accordingly, we suggest that albuminuria in insulin-dependent diabetic patients reflects universal quantitative or qualitative alterations of the capillary filtration barrier.

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