Enhanced Collagen Synthesis in Cultured Skin Fibroblasts from Insulin-Dependent Diabetic Patients with Nephropathy

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Abstract. Excessive production and deposition of extracellular matrix proteins are characteristic features of diabetic nephropathy. This study tests the hypothesis that cells from diabetic patients who develop nephropathy have a disturbance in collagen metabolism compared with cells from diabetic patients without complications. Kinetics of overall collagen metabolism and total protein synthesis were examined in serially passaged, subconfluent, quiescent skin fibroblasts cultured in either normal (5 mM) or high (25 mM) glucose concentrations from 14 insulin-dependent diabetic (IDDM) patients with nephropathy; 14 IDDM patients without nephropathy matched for age, diabetes duration, and body mass index; and 14 healthy subjects. Fibroblasts were incubated in the presence of 2 μCi/ml [3H]proline, and after labeling the incorporation of [3H]proline into total protein, collagen (collagenase-sensitive material), and noncollagen proteins (collagenase-resistant material) was determined at different time points. Collagen degradation was determined in pulse-chase experiments by following the residual collagen-bound radioactivity after incubation for 8 h with 10 μCi/ml [3H]proline. In high glucose concentrations (25 mM), overall collagen synthesis (measured as [3H]proline incorporation into extracellular and intracellular collagenase-sensitive material) was significantly greater in the patients with nephropathy (mean ± SEM after a 24-h labeling period: 7189 ± 671 dpm/10⁶ cells) than in the patients without (4341 ± 267 dpm/10⁶ cells; P < 0.01) or healthy control subjects (3836 ± 234 dpm/10⁶ cells; P < 0.01). No significant differences were observed in noncollagen protein production or in collagen degradation rates among the three groups of subjects. In the presence of normal glucose concentrations (5 mM), collagen synthesis was lower in all groups studied, but the differences between IDDM patients with nephropathy and those without remained unaltered. These results suggest that long-term cultured fibroblasts derived from diabetic patients with nephropathy exhibit an abnormality in collagen metabolism. Cells from long-standing diabetic patients without nephropathy have normal collagen metabolism. The increased collagen synthesis is likely to be intrinsic to those diabetic patients susceptible to nephropathy and may play an important role in the sclerotic processes that occur in the kidneys, arteries, and heart. (J Am Soc Nephrol 8: 1133–1139, 1997)

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without nephropathy and nondiabetic healthy control subjects who do not develop sclerotic damage.

Cultured skin fibroblasts are a useful tool in the investigation of various genetic errors of metabolism (12), and our earlier results suggest that these cells may help identify intrinsic abnormalities of cell function that may participate in the pathogenesis of diabetic cardiorenal complications.

We report here the kinetics of overall collagen production and total protein synthesis in quiescent skin fibroblasts, cultured in normal- and high-glucose concentrations, from IDDM patients with or without nephropathy and from healthy nondiabetic control subjects.

Materials and Methods

Patients

Fourteen IDDM patients with overt diabetic nephropathy, defined as a urinary albumin excretion (AER) persistently greater than 300 μg/min in sterile urine, duration of diabetes > 10 yr, classic glomerulosclerotic lesions on renal biopsy, and concomitant retinopathy, were recruited from the outpatient clinic at Guy's Hospital. Fourteen healthy subjects and 14 long-term IDDM patients without family history of hypertension with normal albumin excretion rate (AER < 20 μg/min) served as control subjects. The two diabetic groups came from the same clinic population and had a similar age, diabetes duration, body mass index, and sex distribution. Nondiabetic control subjects were younger but otherwise comparable to the diabetic patients (Table 1). All subjects were of European origin and gave informed consent to participate in the study, which was approved by the Committee on Ethical Practice of Guy's Hospital.

On the morning of the skin biopsy, the patients' height and weight without shoes and in light indoor clothing were recorded, and blood was taken for determination of glycosylated hemoglobin (Corning gel electrophoresis, Ciba-Corning, Palo Alto, California) and serum creatinine (Jaffe reaction rate method, Hitachi autoanalyzer, Boehringer Mannheim, Lewes, United Kingdom). Arterial blood pressure was measured with a standard mercury sphygmomanometer to the nearest mmHg in the dominant arm after at least 10 min rest in the supine position. Mean blood pressure was calculated as diastolic blood pressure plus one-third pulse pressure. GFR was determined by plasma clearance of $^{51}$Cr-ethylenediaminetetra-acetic acid within 1 mo of the skin biopsy in the diabetic patients only (13). Three timed overnight urine samples were collected for measurement of urinary AER (14), and the median value was used for classification. The metabolic control of the diabetic patients was estimated by calculating the average glycosylated hemoglobin from all values available over the previous 4 yr. The median (range) number of measurements available per patient was 12 (9–18).

All patients with proteinuria were on antihypertensive medication, which included calcium antagonists, angiotensin-converting enzyme inhibitors, vasodilators, and loop diuretics either alone or in combination. The patients with normalalbuminuria were taking no drug other than insulin. The diabetic patients withheld their morning insulin injection until after the skin biopsy, and the patients taking antihypertensive drugs were asked to stop them at least 36 h before the study.

Cell Culture

A skin biopsy was taken from the anterior surface of the left forearm by excision under local anesthetic (ethyl chloride), and specimens were diced into 0.5-mm fragments. Fragments were seeded for fibroblast culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) containing either a physiologically normal D-glucose concentration of 5 mM or a high D-glucose concentration of 25 mM. D-Mannitol was added to 5 mM glucose medium to achieve equal final osmolality in the medium. After the fourth passage, cells were harvested and stored in liquid nitrogen. For each experiment, fibroblasts were then thawed and grown as described previously (4). All studies were performed between the 6th and 10th passage, and each experiment in sets of up to three patients from each group was carried out always after an identical number of passages.

Estimation of Collagen Synthesis

Cell Incubation. A total of $5 \times 10^5$ cells was seeded in 25-mm$^2$ flasks, and at subconfluence, fibroblasts were made quiescent by incubation in DMEM with 0.4% FCS for 24 h. That the cells were in a quiescent steady state, in which cell proliferation has almost completely ceased, was confirmed by direct cell count in parallel experiments in which fibroblasts were grown in the same conditions. There were no significant changes in cell number between 12 and 36 h.

Quiescent medium was then replaced by 3 ml of DMEM containing 0.4% FCS, 100 mg/L L-ascorbic acid (to stimulate collagen synthesis), $50 \mu g/ml \beta$-aminopropionitrile fumarate (to retard cross-linking), 2 μCi/ml $[^3H]$-proline, and cells incubated for 12, 24, or 36 h. In preliminary experiments, we found that under these conditions collagen in the medium accounts for >95% of total collagen synthesized. All determinations at each experimental time point for each patient were carried out in triplicate. Parallel experiments were conducted to correct data of proline incorporation for cell number. To this end, cells were trypsinized for 4 min at 37°C, followed by neutralization of trypsin with 1 ml/flask of DMEM plus 10% FCS. Triplicate counts were then performed by standard hemocytometry, averaged, and expressed as number of cells per flask.

Processing of the Medium and Cells. For the collagen assay, after incubation with radioactive proline, cells were solubilized with 0.5N NaOH and collected with the medium into 5 ml of 0.5 M Tris-HCl, pH 7.4, containing 0.11 M NaCl, 1 mM unlabeled proline, and 1 mg/ml albumin. Proteins were precipitated for 30 min with 10 ml of ice-cold 20% TCA, and the precipitates were collected by centrifugation for 15 min at 3000 × g. The supernatant was removed, and the precipitate was dissolved and washed twice in 10 ml of ice-cold 10% TCA. The resultant precipitate was dissolved in 0.6 ml of 0.2 M NaOH. Then 0.2 ml was removed for counting to represent the radioactivity incorporated into total protein. Duplicate, 0.2-ml aliquots from the remaining sample were neutralized by 0.16 ml of 0.15 M HCl and 0.1 ml of 1 M Hepes/Tris buffer, pH 7.3, and added with 10 μl of 25 mM CaCl$_2$, 20 μl of 62.5 mM N-ethylmaleimide (to inhibit residual contaminating proteolytic activity), and 20 μl of elution buffer (50 mM Tris/HCl, pH 7.4; 0.005 M CaCl$_2$) or 20 μl of elution buffer containing purified collagenase (3000 U/ml in 1 ml of 50 mM Tris/HCl, pH 7.4; 0.005 M CaCl$_2$).

After a 90-min incubation period at 37°C with and without collagenase, the samples were placed on ice and precipitated with 0.5 ml of ice-cold 10% TCA plus 0.5% tannic acid. Acid-insoluble material (noncollagen protein) was pelleted by centrifugation. The supernatants were transferred to a counting vial for determination of radioactivity. The amount of radioactivity solubilized by collagenase subtracted from the radioactivity of tubes without collagenase (unspecified digest) is a measure of $[^3H]$proline incorporated into collagen. The pellets were then solubilized with 1 ml of 0.2 M NaOH and counted to represent the radioactivity incorporated into non-collagenous material.
Estimation of Collagen Degradation

Collagen degradation was measured in eight subjects only in each group by following the residual, collagen-bound radioactivity in a pulse-chase experiment (18). Fibroblasts (5 x 10^5) were seeded in 25-mm² flasks and at subconfluence made quiescent by incubation in DMEM with 0.4% FCS for 24 h. Cells were then pulse-labeled in 3 ml of DMEM containing 0.4% FCS, 100 mg/L L-ascorbic acid (to stimulate collagen synthesis), 50 μg/ml β-aminopropionitrile fumarate (to retard cross-linking), and 10 μCi/ml [5-3H] proline for 8 h. After labeling, the radioactive incubation medium was removed, cell layers were washed five times with warm PBS buffer, and 3 ml of chase medium (identical to labeling medium except that 1 mM unlabeled L-proline was substituted for [5-3H] proline) was added. The cells were then chased in this medium for 2, 6, and 24 h. Cell layers and medium were processed and then digested with collagenase as described above. After determination of collagenase-sensitive radioactive material, the percentage of collagen degradation was calculated using the following formula: Percentage of collagen degraded = [(Disintegration per minute [dpm] of collagen in the sample at t₀) - (dpm of radioactivity in the sample at tₓ)] x 100/(dpm of collagen in the sample at t₀), where t₀ indicates time 0, and tₓ indicates a period of 2, 6, or 24 h.

Statistical Analyses

For each individual experiment, the mean of triplicate determinations was calculated. Statistical calculation was performed by ANOVA, and comparisons between groups were conducted using the Newman-Keuls test. Spearman’s regression analysis was performed to determine correlations between different parameters. A two-tailed P value < 0.05 was considered significant. Data are given as mean ± SEM unless otherwise stated.

Results

The two diabetic groups had similar glycosylated hemoglobin levels, but mean blood pressure was higher in the diabetic group with nephropathy compared with the other two groups. In the group with nephropathy, serum creatinine was higher and GFR was lower (Table 1), but 8 of 14 of these IDDM patients still had GFR values >90 ml/min per 1.73 m².

Table 1. Clinical features of insulin-dependent diabetic patients (IDDM) with and without nephropathy and healthy control subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>IDDM with Nephropathy</th>
<th>IDDM without Nephropathy</th>
<th>Healthy Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (male/female)</td>
<td>10/4</td>
<td>9/5</td>
<td>11/3</td>
</tr>
<tr>
<td>Age</td>
<td>45 ± 8</td>
<td>44 ± 9</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23 ± 3</td>
<td>23 ± 2</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>Duration of diabetes (yr)</td>
<td>25 ± 7</td>
<td>24 ± 6</td>
<td></td>
</tr>
<tr>
<td>Glycosylated hemoglobin (%)</td>
<td>8.8 ± 0.4</td>
<td>9.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>97 ± 6^b</td>
<td>92 ± 6</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>Albumin excretion rate (μg/min)</td>
<td>860 (302–4047)</td>
<td>7 (1–16)</td>
<td>6 (4–15)</td>
</tr>
<tr>
<td>Serum urea (mmol/L)</td>
<td>4.95 ± 0.53</td>
<td>4.16 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (μmol/L)</td>
<td>115 ± 40^b</td>
<td>90 ± 12</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>GFR (ml/min per 1.73 m²)</td>
<td>74 ± 21^b</td>
<td>121 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

^a Values are mean ± SD except for albumin excretion rate, which is given as median and range.

^b P < 0.01 versus healthy controls and IDDM without nephropathy.
Collagenase-sensitive material) in quiescent fibroblasts from nine IDDM patients with nephropathy (1.75 ± 0.29 \times 10^6 in IDDM patients without nephropathy, and 1.78 ± 0.28 \times 10^6 in IDDM patients without nephropathy, and 1.82 ± 0.24 \times 10^6 in healthy control subjects).

At all time points, overall collagen production was significantly higher in fibroblasts from IDDM patients with nephropathy compared with that of fibroblasts from IDDM patients without nephropathy and healthy control subjects (Figure 2) (P < 0.05 by ANOVA).

Noncollagen protein synthetic rates (measured as [3H]proline incorporation into intracellular and extracellular-collagenase-resistant material) were similar at all time points in all three groups (Table 2), suggesting that the higher total protein synthesis in IDDM patients with nephropathy was selectively ascribable to increased collagen production.

A reduction in collagen degradation rates would also result in the observed increase in collagen production. In pulse-chase experiments, however, the rate of decline of labeled [3H]proline was similar among the three groups, indicating no differences in collagen degradation (Figure 3).

**Total Protein and Collagen Synthesis in Normal Glucose Medium (5 mM)**

To evaluate whether the differences observed in collagen metabolism between diabetic patients with and without nephropathy could be accounted for by a differential effect of high glucose concentration in different patients, we studied 24-h total protein and collagen synthesis in fibroblasts cultured in normal glucose medium (5 mM) from five IDDM patients with nephropathy, five without nephropathy, and five healthy control subjects.

Total protein synthesis in medium containing 5 mM glucose was significantly lower in the three groups of subjects compared with that observed in medium containing 25 mM glucose, but, again, it was higher in IDDM patients with nephropathy compared with the other two groups. The difference reached conventional significance only between IDDM patients with nephropathy and healthy control subjects (Table 3). Although in normal glucose concentration overall collagen synthesis was reduced in all three groups in comparison with culture in 25 mM glucose, it remained significantly higher in fibroblasts from IDDM patients with nephropathy compared with that of fibroblasts from IDDM without nephropathy and healthy control subjects (P < 0.05 for both) (Figure 4). The results of the noncollagen protein synthesis were similar in all groups, confirming that the higher total protein synthesis in IDDM patients with nephropathy was due to increased collagen production. Cell density was similar in the three groups (1.75 ± 0.29 \times 10^6 in IDDM patients with nephropathy, 1.78 ± 0.28 \times 10^6 in IDDM patients without nephropathy, and 1.82 ± 0.24 \times 10^6 in healthy control subjects).

**Discussion**

This study demonstrates an enhanced overall collagen production in quiescent skin fibroblasts of IDDM patients with nephropathy. Because collagen degradation rates were similar in all subjects, these results are consistent with an increased net collagen synthesis in patients with diabetic nephropathy. Moreover, high glucose concentrations increased overall collagen synthesis in cells from all subjects, exaggerating the difference already present in normal glucose concentration between cells from IDDM patients with nephropathy and those from IDDM patients without nephropathy and nondiabetic control subjects. The specific collagen types synthesized were not characterized in this study, and further studies are needed to examine whether a specific type of collagen is responsible for the differences observed among the groups.

In the diabetic kidney, mesangial expansion predominantly due to an increase in extracellular matrix proteins, is the lesion that most closely correlates with severity of clinical nephropathy (19). It is accompanied by interstitial expansion, matrix deposition, and arteriolosclerosis (20). In several animal mod-
Table 2. Time course of noncollagen protein synthesis (measured as \[^{3}\text{H}]\)proline incorporation into extracellular and intracellular collagenase-resistant proteins) in quiescent fibroblasts from nine IDDM with nephropathy, nine IDDM without nephropathy, and nine healthy subjects\(^{a}\)

<table>
<thead>
<tr>
<th>[^{3}\text{H}])proline Incubation</th>
<th>IDDM with Nephropathy</th>
<th>IDDM without Nephropathy</th>
<th>Healthy Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11692 ± 1418</td>
<td>9906 ± 680</td>
<td>9409 ± 1021</td>
</tr>
<tr>
<td>24</td>
<td>13524 ± 1488</td>
<td>12022 ± 1409</td>
<td>10624 ± 1063</td>
</tr>
<tr>
<td>36</td>
<td>16217 ± 1568</td>
<td>15881 ± 900</td>
<td>12451 ± 1072</td>
</tr>
</tbody>
</table>

* Values are expressed as disintegrations per minute per \(10^6\) cells and given as mean ± SEM.

differ between different individuals, but this would suggest an intrinsic difference. This interpretation would be consistent with our experiments, which reveal a heterogeneity of response to hyperglycemia in different individuals and relate these responses to the presence or absence of kidney complications. It is remarkable that cells from long-standing diabetic individuals with normoalbuminuria who are at very low risk of ever developing complications display a collagen synthetic rate not different from nondiabetic healthy control subjects. This was in spite of a slightly older age in the diabetic control group compared with healthy subjects, a condition that would tend to accentuate potential differences in that older cells are more likely to have a higher proportion of terminally differentiated cells that produce extracellular matrix (23). Our experiments were performed in cells cultured in high or normal glucose medium, but the cell culture conditions were exactly the same for all subjects, and therefore the hyperglycemic milieu per se cannot explain the differences observed. Although collagen production was already higher in fibroblasts from IDDM patients with nephropathy in normal glucose concentrations, high glucose concentrations further increased collagen synthesis, thereby providing a biochemical explanation of the interaction between diabetic milieu and intrinsic features of individual patients.

Botstein et al. (24) have also reported that there is a marked heterogeneity in the capacity of human foreskin fibroblasts to incorporate \[^{3}\text{H}]\)proline into collagen. Serially passaged dermal fibroblasts cultured from patients with scleroderma were found to produce more collagen than fibroblasts from healthy subjects (24). These observations are consistent with a selection of populations of fibroblasts in vivo characterized by high rates of collagen synthesis.

The heterogeneity in the patterns of collagen synthesis among the three groups of subjects in our study could be related to differences in growth rates and the effects of in vitro aging on cell phenotype (6). These phenomena cannot be excluded, but are probably not directly relevant to our present data, because cells from all subjects were studied after the same number of passages and experiments were performed in quiescent nonproliferating subconfluent cells. The effects of antihypertensive therapy in the IDDM patients with nephropathy, even if it persisted after several cell passages in vitro, is also unlikely to explain the differences among the groups. The available evidence from animal work would suggest that some
Table 3. Total protein synthesis (measured as \[^{3}H\]proline incorporation into extracellular and intracellular proteins) in quiescent fibroblasts cultured in either 5 or 25 mM glucose medium from five IDDM with nephropathy, five IDDM without nephropathy, and five healthy control subjects\(^{a}\)

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM</td>
</tr>
<tr>
<td>IDDM with nephropathy</td>
<td>18604 ± 2466(^{b})</td>
</tr>
<tr>
<td>IDDM without nephropathy</td>
<td>13335 ± 1150</td>
</tr>
<tr>
<td>Normal control subjects</td>
<td>11947 ± 1214</td>
</tr>
</tbody>
</table>

\(^{a}\) Values are expressed as disintegrations per minute per \(10^6\) cells and given as mean ± SEM.

\(^{b}\) \(p < 0.05\) versus healthy control subjects.

\(^{c}\) \(p < 0.01\) versus 5 mM glucose.

of the antihypertensive drugs used, particularly the angiotensin-converting enzyme inhibitors, would reduce collagen production (25–27). Thus, antihypertensive therapy would tend to reduce, rather than enhance, the difference. It is also unlikely that the increased collagen production in proteinuric patients is secondary to the impairment in renal function, because no relationship was observed between the decline in GFR and collagen production.

Other cell function abnormalities have recently been described in fibroblasts from IDDM patients with nephropathy. A reduced fractional incorporation of \(^{3}H\)glucosamine into heparan sulfate was found in skin fibroblasts from patients with nephropathy compared with nondiabetic controls, suggesting a possible altered regulation in the biosynthesis of glycosaminoglycans (28). However, clear differences between diabetic patients with and without nephropathy were not found. Our group and others (4,5,29) have reported an overactivity of sodium-hydrogen antiport specifically in the group of diabetic patients that had developed nephropathy. Because sodium-hydrogen antiport can be activated by extracellular matrix molecules (30), any interaction between extracellular matrix production and sodium-hydrogen exchange might be of particular importance in view of the relevance of this ion transporter to processes of hypertrophy, hyperplasia, or both. Interestingly, high glucose concentrations significantly increased sodium-hydrogen antiport activity in cells from IDDM patients with nephropathy, exaggerating differences already present between patients with and without nephropathy under culture conditions of normal glucose concentration (31).

All of these observations of abnormalities in cell function, despite serial passaging in identical media \textit{in vitro}, suggest a likely intrinsic component in the cell-altered response to diabetics in the subset of patients at risk of kidney disease. We report here that the increased synthesis of collagen represents a critical abnormality in this process and helps explain the fibrotic lesions that occur in the kidney, arteries, and heart of diabetic patients who develop nephropathy.

![Figure 4](image-url). Effect of culture in normal (5 mM) and high (25 mM) glucose on individual values of \[^{3}H\]proline incorporation into extracellular and intracellular collagen after a 24-h incubation period in quiescent fibroblasts from five IDDM patients with nephropathy (■), five IDDM patients without nephropathy (■), and five normal subjects (△).
Acknowledgments

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