Brief Genetics Report

Cellular Basis of Diabetic Nephropathy

II. The Transforming Growth Factor-β System and Diabetic Nephropathy Lesions in Type 1 Diabetes

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Transforming growth factor-β (TGF-β) may be critical in the development of diabetic nephropathy (DN), and genetic predisposition is an important determinant of DN risk. We evaluated mRNA expression levels of TGF-β system components in cultured skin fibroblasts (SFs) from type 1 diabetic patients with fast versus slow development of DN. A total of 125 long-standing type 1 diabetic patients were ranked by renal mesangial expansion score (MES) based on renal biopsy findings and diabetes duration. Patients in the highest quintile of MES who were also microalbuminuric or proteinuric and diabetes duration. Patients in the highest quintile who were also normoalbuminuric (n = 16) were classified as “fast-track” for DN, while those in the lowest quintile who were also normoalbuminuric (n = 23) were classified as “slow-track” for DN. Twenty-five normal subjects served as control subjects. SFs were cultured in medium with 25 mmol/l glucose for 36 h. SF mRNA expression levels for TGF-β1, TGF-β type II receptor (TGF-β RI), thrombospondin-1, and latent TGF-β binding protein-1 (LTBP-1) were measured by real-time RT-PCR. LTBP-1 mRNA expression was reduced in slow-track (0.99 ± 0.38) versus fast-track patients (1.65 ± 0.52, P = 0.001) and control subjects (1.41 ± 0.7, P = 0.025). mRNA levels for TGF-β1, TGF-β RI, and thrombospondin-1 were similar in the three groups. Reduced LTBP-1 mRNA expression in SFs from slow-track patients may reflect genetically determined DN protection and suggests that LTBP-1 may be involved in the pathogenesis of DN through the regulation of TGF-β activity.

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Accumulating evidence suggests that the transforming growth factor-β (TGF-β) system plays important roles in the pathogenesis of diabetic nephropathy (DN) (1,2). This intricate system is composed of multifunctional cell-cell signaling proteins that regulate cell proliferation and metabolism of extracellular matrix proteins (3,4). TGF-β is secreted, in most cells, in a large latent complex that has no biological activity (4,5). This complex consists of three components: a homodimer of mature TGF-β, a TGF-β latency-associated peptide (LAP), and a latent TGF-β binding protein (LTBP) (5). Cleavage of both LTBP and LAP is necessary for TGF-β activation (4). Thrombospondin is important in the activation of latent TGF-β (3). The binding of activated TGF-β to its corresponding type II receptor, in turn recruiting the type I receptor, initiates the signaling pathway to its downstream effectors, the Smad proteins (4).

Genetic predisposition or protection may be the most important DN risk determinants in type 1 diabetes. Only about one-half of patients with poor glycemic control develop DN, while some patients do so despite relatively good control (6). Moreover, familial clustering of DN has been demonstrated by several investigators (7,8). Cultured skin fibroblasts (SFs), after several passages under identical in vitro conditions, probably largely reflect the genetically determined behavior of these cells. Thus, cellular studies in diabetic patients could help to identify genetic pathways associated with risk of, or protection from, DN (9,10). The demonstration of cell behavior variability in the TGF-β system in relation to DN risk would support the concept that the TGF-β pathway is involved in this genetic susceptibility. Therefore, we evaluated the mRNA expression levels of several critical genes in TGF-β system, including TGF-β1, TGF-β1 type II receptor (TGF-β RI), LTBP-1, and thrombospondin-1 in cultured SFs from type 1 diabetic patients with rapid or slow development of DN lesions. SF mRNA expression levels for the TGF-β type I and type III receptors, however, were too low to permit accurate measurement.

The patients for this study were selected from 125 patients with ≥8 years of type 1 diabetes with glomerular filtration rate (GFR) ≥30 ml · min⁻¹ · 1.73 m⁻² and ade-
TABLE 1
Demographic, clinical, and glomerular structural characteristics of fast-track and slow-track type 1 diabetic patients and control subjects

|                         | Fast-track | Slow-track | Control subjects | P       |
|-------------------------|------------|------------|------------------|---------|
| n                       | 16         | 23         | 25               |         |
| Male/Female             | 10/6       | 11/12      | 11/14            | NS      |
| Age (years)             | 35.3 ± 7.4 | 38.3 ± 10.1| 41 ± 8           | NS      |
| Diabetes duration (years)| 24.3 ± 7.6 | 18.9 ± 10.2|                  | NS      |
| HbA1c (%)               | 9.5 ± 1.9  | 7.7 ± 1.2  |                  | 0.001   |
| SBP (mmHg)              | 131 ± 14   | 122 ± 11   |                  | 0.036   |
| DBP (mmHg)              | 77 ± 8     | 71 ± 7     |                  | 0.019   |
| Hypertension (yes/no)   | 15/1       | 5/18       |                  | <0.001  |
| Antihypertensive (yes/no)| 13/3      | 1/22       |                  | <0.001  |
| AER (µg/min)            |            |            |                  | NA      |
| Serum creatinine (mg/dl)| 1.2 ± 0.3  | 0.9 ± 0.1  |                  | 0.003   |
| GFR (ml·min⁻¹·1.73 m²)  | 82 ± 27    | 111 ± 14   |                  | 0.001   |
| Retinopathy (none/background/proliferative) | 1/3/12 | 11/1/1 | <0.001 |
| GBM width (nm)          | 745 ± 148  | 436 ± 79   |                  | <0.001  |
| Vv (Mes/glom)           | 0.53 ± 0.1 | 0.21 ± 0.03|                  | <0.001  |
| Vv (MM/glom)            | 0.32 ± 0.1 | 0.11 ± 0.02|                  | <0.001  |
| Vv (MC/glom)            | 0.15 ± 0.06| 0.07 ± 0.01|                  | <0.001  |
| Sv (PGBM/glom)          | 0.05 ± 0.02| 0.12 ± 0.02|                  | <0.001  |
| MES                     | 1.42 ± 0.48| 0.009 ± 0.17|               | NA      |

Data are means ± SD, n, and median (range). DBP, diastolic blood pressure; GBM, glomerular basement membrane; NA, not applicable, different by study design; NS, not statistically significant; SBP, systolic blood pressure; Sv (PGBM/glom), surface density of peripheral GBM; Vv (Mes/glom), mesangial cell fractional volume; Vv (MM/glom), mesangial fractional volume; Vv (MC/glom), mesangial matrix fractional volume.

TABLE 2
mRNA expression levels of TGF-β1, TGF-β RII, thrombospondin-1, and LTBP-1 in SFs from fast-track, slow-track, and control groups

| Genes                | Fast-track  | Slow-track | Control subjects | ANOVA P |
|----------------------|-------------|------------|------------------|---------|
| n                    | 16          | 23         | 25               |         |
| LTBP-1               | 1.65 ± 0.52 | 0.99 ± 0.38| 1.41 ± 0.70      | 0.003   |
| TGF-β1               | 1.26 ± 0.30 | 1.34 ± 0.4 | 1.40 ± 0.43      | 0.54    |
| TGF-β RII            | 1.17 ± 0.23 | 1.12 ± 0.23| 1.10 ± 0.28      | 0.72    |
| Thrombospondin-1     | 1.67 ± 0.43 | 1.74 ± 0.53| 1.56 ± 0.51      | 0.43    |

Data are means ± SD. Relative values of target mRNA in 0.1 µg of total RNA samples are expressed as fold changes to the target mRNA expression level in 0.1 µg of reference standard total RNA.

Seventy-five percent of fast-track and only 4% of slow-track patients had proliferative diabetic retinopathy. Fast-track patients had more advanced glomerular lesions than slow-track patients based on electron microscopy morphometry measurements (Table 1).

SFs from the above-mentioned patients were cultured under identical conditions. mRNA expression levels for LTBP-1 in SFs from slow-track patients were significantly lower than those from fast-track patients (P = 0.001) and control subjects (P = 0.025; Table 2 and Fig. 1). There was no significant difference between fast-track patients and normal control subjects. No significant group differences were found in the mRNA expression levels for TGF-β1, TGF-β RII, or thrombospondin-1 (Table 2). In additional analyses, we determined that the reduced LTBP-1 mRNA expression in SFs from slow-track patients compared with fast-track patients was independent of age, sex, and diabetes duration.

This study describes an SF phenotype of decreased LTBP-1 mRNA expression in slow-track type 1 diabetic patients at low risk for DN. Although fast-track patients had worse glycemia than slow-track patients, the impact of this difference was likely minimal after the cells had undergone several passages under identical culture conditions.

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tions. Moreover, fast-track patients’ LTBP-1 expression levels were similar to those of control subjects. This implies that genetic factors determine the differences in SF behavior between diabetic patients with rapid and slow development of DN. Since control subjects probably represent the range of genetic susceptibility to DN in the general population, the fact that SF LTBP-1 gene expression levels from slow-track patients were lower than those from normal control subjects is consistent with a DN protection factor in these patients.

Although we failed to find significant differences among the three groups in SF gene expression for TGF-β1, its type II receptor and thrombospondin-1, this does not negate the potential importance of these molecules in the pathogenesis of DN. Differences in these molecules might be detectable in other cells (e.g., mesangial cells) or could be elicited by stimuli other than high glucose (e.g., glycation products). There may also be group differences in the protein or activity levels of these molecules that are not reflected in their gene expression levels, and studies along these lines could be of considerable interest. However, currently available data from human studies suggest that there is an increase in renal TGF-β1 mRNA expression (14) and production (15), which is associated with the diabetic state, but that this increase is unrelated to nephropathy risk.

This study may, nonetheless, suggest molecular mechanisms by which the TGF-β system could be a determinant of the known variability in DN risk. LTBP plays an important role in the regulation of TGF-β secretion, local concentration, and activity (3–5). Without LTBP, the secretion and folding of TGF-β are significantly slowed and TGF-β is primarily retained in an immature form in the Golgi apparatus (16). LTBP also participates in targeting and binding the TGF-β complex to extracellular matrix (ECM), resulting in local storage of TGF-β (17). The relevance of LTBP in TGF-β activation is supported by the findings that in vitro TGF-β activity was inhibited by anti-LTBP antibodies or antisense oligonucleotides directed against LTBP-1, and that this effect can be reversed by administration of mature TGF-β proteins (17,18). In addition, the free form of LTBP acts as a matrix component independent of the TGF-β complex, suggesting a role in the structural remodeling of ECM (3). Adult wound-healing studies found increased LTBP-1 expression levels in association with scar formation (19). Also, the intensity of glomerular LTBP-1 staining in IgA nephropathy patients was closely related to the grade of mesangial ECM accumulation (20). These observations suggest a profibrotic role of LTBP-1 through the regulation of TGF-β activity, which, if reflected in renal ECM dynamics, could influence DN risk.

This and other studies have demonstrated associations between DN risk and SF behaviors (9,10). We do not assume that SFs represent a specific renal cell type (e.g., glomerular, interstitial, or vascular cells), but we do argue that SF behaviors reflect one or more intrinsic renal cellular processes related to DN susceptibility.

There is controversy as to the value and choice of housekeeping genes as internal control in PCR studies (21–23). There may be no perfect housekeeping gene, the expression level of which is not influenced by culture conditions or intrinsic cellular variables. The present study used several other strategies for quality control, including assuring RNA integrity by gel electrophoresis, eliminating DNA contamination by primer design and “nonreverse transcription” control, and the requirement for a high degree of assay reproducibility. We determined the minor potential influence of plate position on mRNA measurement in our system. Finally, the results of the measurement of GAPDH in relation to LTBP mRNA levels supports the validity of the strategies for quality control used in these studies.

In summary, these studies support the hypothesis that genetic variability in the TGF-β system is associated with DN risk. The levels of LTBP-1 mRNA expression in SFs could help to create an SF behavior profile useful in identifying the population at high or low DN risk.

**RESEARCH DESIGN AND METHODS**

**Patients.** The overall design of these studies, the clinical and morphometric methods, clinical characteristics, and renal structure-function relationships in these 125 long-standing type 1 diabetic patients have been previously detailed (12). The patients previously designated as fast-track and slow-track for DN based on MES alone (12) were further stratified based on AER in this study, as outlined above.

**Cell culture.** All stored SFs (24) were thawed and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-Life Technologies, Grand Island, NY) supplemented with 25 mmol/l glucose, 10% FCS (Hyclone Lab, Logan, UT), 1.5 mol/l HEPES (Sigma, St. Louis, MO), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (250 ng/ml) for four passages.
**TABLE 3**

| Target genes | Primers and probes (5′–3′) | Product size |
|--------------|-----------------------------|--------------|
| LTBP-1       |                             |              |
| Forward primer | GGGAAACCCACCATCTCTATTAG       | 80 bp        |
| Reverse primer | CAAATTACACTCGGAACTGGT        |              |
| Probe         | AGAATGTCTGACCCTGCGCAACC     |              |
| TGF-β1        |                             |              |
| Forward primer | CTCCTCCGACCTGCCACAGA         | 72 bp        |
| Reverse primer | AACCTAGATGGGCAGATCT          |              |
| Probe         | CCTATACGACCCCACTTCTCG        |              |
| TGF-βRII      |                             |              |
| Forward primer | GCAAGTGGAACTGCAAGAT          | 75 bp        |
| Reverse primer | GAGGAACACATTCTGCAAATTC       |              |
| Probe         | CATGCTCCAGAAGTGCTAGAATCCAGGA |              |
| Thrombospondin-1 |                             |              |
| Forward primer | GCCTGCGACCATTTGT            | 89 bp        |
| Reverse primer | AGGCCGCCTAGCTATT             |              |
| Probe         | AGGACAGATCCGGCAAGTGACTGAAGAG |              |

SFs were then seeded on 75-cm² flasks (Cerning, Cambridge, MA) at a density of 10⁴/cm² and grown in the above medium for 24 h. After synchronization by culture in FCS-deprived DMEM and 25 mmol/l glucose for 48 h, SFs were grown for an additional 36 h in the same culture medium, supplemented with 10% FCS.

**RNA isolation.** Total RNA was isolated using a TRI-REAGENT kit (Molecular Research Center, Cincinnati, OH). Total RNA concentration was quantitated by ultraviolet absorbency at 260 nm. For this assay, each sample was measured in duplicate or triplicate and mean value was used. The correlation coefficient (r) for these repeated measures was 0.98. Total RNA integrity was evaluated by gel electrophoresis using an Agilent 2100 bioanalyzer and RNA 6000 LabChip Kit (Hewlitt Packard, Palo Alto, CA). Samples with 28S/18S band density <2 were discarded. Pooled total RNA from SFs of six control subjects was used as the arbitrary reference standard. A set of dilutions of the reference total RNA (0.4, 0.2, 0.1, 0.04, 0.02, and 0.01 μg/μl) was prepared and stored in aliquots at −70 °C until used.

**Quantitative RT-PCR.** Expression levels of target genes were measured by a fluorescence-based, TaqMan real-time RT-PCR using ABI PRISM 7700 Sequence Detection System (Perkin-Elmer; Applied Biosystems, Foster City, CA). Primers and probes were designed by Primer Express version 1.5 (Applied Biosystems), and were used to produce an amplicon spanning an intron. All the probes contained 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′ end (Table 3).

One-step TaqMan RT-PCR was performed using the TaqMan Gold RT-PCR Kit (Applied Biosystems). The 50-μl reaction mixture contained 1× TaqMan Buffer A, 5.5 mmol/l MgCl₂, 300 μmol/l dNTP, 0.025 units/μl AmpliTaq Gold DNA Polymerase, 0.25 units/μl MultiScribe Reverse Transcriptase, 200–900 mmol/l forward and reverse primers, 0.2 units/μl RNase inhibitor, and 0.1 μg total RNA. After 30 min at 48°C and 10 min at 95°C, the amplification reaction was carried out through 40 cycles at 95°C for 15 s and 55–60°C for 60 s.

The set of six dilutions of the reference standard total RNA was run in triplicate, simultaneously with the unknown samples in a 96-well plate for each batch of assays. Logarithm of relative concentration of target mRNA in the reference standard for each dilution was plotted against the threshold cycle (Ct). Regression analyses were used to generate a best-fit line as the standard curve, and the correlation coefficient (r) of the standard curve was >0.98 for all of the assays. The Ct for unknown samples was interpolated into the regression equation for the standard curve to determine the relative concentration of target mRNA in each unknown sample. The relative value of target mRNA in 0.1 μg of unknown total RNA sample was expressed as fold changes based on the concentration of target mRNA in 0.1 μg of reference total RNA. The mRNA expression assays for all unknown samples were performed in a single run, and each run was repeated two or three times on separate days. All repeat data obtained from two or three repeat runs in the present study had a correlation coefficient (r) of 0.86. The average value for each sample was used for statistical analyses. “No-mRNA template control” and “nonreverse transcription” control were run for each gene. No amplicons were detected in these controls.

In separate experiments, RT-PCR was performed in triplicate in 23 samples randomly allocated to well positions. Agreement between these randomly allocated samples was assessed using an intraclass correlation. This analysis indicated that 89.6% of the total variability was due to the variability between samples. Thus, only ~10% of the variability was due to all other factors, indicating that plate position could have no more than a minor influence on the mRNA level of a given sample. Finally, RT-PCR experiments performed with GAPDH as a housekeeping gene and LTBP-1 indicated that expression of the results as a ratio of these two mRNA levels would not change the outcome of these studies.

**Statistical analysis.** Student’s t tests were used to compare the demographic, clinical, and structural continuous variables between groups, while χ² tests were used for categorical measures. All of the investigators except for the statisticians were blinded to the grouping of patients for the gene expression studies. SF mRNA expression levels among the three groups were compared using ANOVA methods. Comparisons between fast-track, slow-track, and control subjects were made using Fisher’s least significant difference procedure only if the overall F test from the ANOVA was significant at P < 0.05 (26). Values of P < 0.05 for these comparisons were considered statistically significant. Validity of the assumption regarding homogeneity of variance was investigated in all analyses. When required, power transformations of the dependent variable (e.g., square root, logarithmic, etc.) (27) were used to obtain homogeneity of variance among groups before performing hypothesis tests.

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