TGF-β1 Gene Mutations in Insulin-Dependent Diabetes Mellitus and Diabetic Nephropathy

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Abstract. PCR assays were established for easy and fast analysis of two transforming growth factor-β1 (TGF-β1) gene mutations, a C to T transition at position 76 in exon 5 resulting in a change from threonine to isoleucine in position 263 (Thr263Ile) of the propeptide and a deletion of a C in the intron sequence eight bases prior to exon 5 (713–8delC). These mutations were evaluated in insulin-dependent diabetes mellitus (IDDM) patients (n = 137) and control subjects (n = 105) and in IDDM patients with (n = 170) and without (n = 99) nephropathy. After evaluating intra- and interindividual variation in TGF-β1 expression levels, the TGF-β1 mRNA level in phorbol 12-myristate-13-acetate-stimulated (1 ng/ml) lymphocytes from individuals with different TGF-β1 genotypes was also studied. No association of the two TGF-β1 sequence variations with IDDM in general was found. However, a weak but significant association of the Thr263Ile mutation with diabetic nephropathy was found (P = 0.03). No correlation between TGF-β1 transcription level and genotype of any of the two studied polymorphisms was found. However, significant interindividual differences in TGF-β1 mRNA levels were observed between the tested individuals (P < 0.0001) compatible with a genetic control mechanism of TGF-β1 synthesis at the mRNA level.

Transforming growth factor-β is a pleiotropic cytokine, and much evidence has accumulated to suggest that dysfunction of TGF-β regulation can lead to pathologic conditions (1–3). Transgenic mice that overproduce transforming growth factor-β1 (TGF-β1) die just before or within hours after birth (4), and TGF-β1 knockout mice are born apparently normal but die within 3 wk from cachexia due to massive inflammation (5,6). Also, TGF-β1 has been implicated in several inflammatory (5) and autoimmune (7–9) diseases, partly due to its regulatory effects on macrophage functions. TGF-β1 regulates production of almost every known molecule of the extracellular matrix (e.g., collagens, fibronectin, tenasin, and proteoglycans) (10). A large number of pathologies involve modifications of extracellular matrix, either in the initial triggering of the disease or in its aggravation. Hence, the effect of TGF-β1 on matrix formation is indicative of its potential role in such disorders (10–12). The central feature of diabetic nephropathy is an altered composition of the extracellular matrix, including thickening of the glomerular basement membrane and expansion of the mesangial matrix (13). It has been hypothesized—based on studies in human and animal models—that hypersecretion of TGF-β1 triggered by diabetes might be a major cause of these processes, thereby contributing to diabetic nephropathy (14–17). Furthermore, in patients with diabetes, the level of intraglomerular TGF-β1 mRNA was positively correlated with the staining intensity of collagen type IV in the mesangium, glomerular basement membrane, and Bowman’s capsule (18). Recently, it was demonstrated that diabetic patients had increased renal production of TGF-β1 protein (19). Thus, the case for TGF-β1 as a mediator of diabetic nephropathy is well established (20,21), and TGF-β1 is an obvious candidate gene for conferring susceptibility to diabetic nephropathy.

Recently, we (22) identified two polymorphisms of the TGF-β1 gene by single-strand conformational polymorphism scanning: a C to T transition at position 76 in exon 5 (C788-T), corresponding to position 788 in the TGF-β1 mRNA, resulting in a change from threonine to isoleucine in position 263 (Thr263Ile) of the propeptide; and a deletion of a C in the intron sequence eight bases prior to exon 5 (713–8delC). Thus, both of these mutations may have functional significance. Preliminary data suggested that the 713–8delC polymorphism had a higher prevalence in osteoporotic women and correlated to low bone mass in this group (22). In the present study, we evaluated these two polymorphisms in an insulin-dependent diabetes mellitus (IDDM) case-control study, since the pleiotropic effects of TGF-β1 on the immune system may modulate susceptibility to IDDM in general (e.g., TGF-β1 has a potentiating effect on interleukin-1-mediated impairment of pancreatic islet cell function) (23). Then, we analyzed the polymorphisms in relation to diabetic nephropathy, in which a more specific role for TGB-β1 has been suggested (20,21).

The gene for TGF-β1 is on chromosome 19q13 and comprises seven exons, of which part of exon 5, exon 6, and part of exon 7 encode the active TGF-β1 (24).
Materials and Methods

Study Population

The study population comprised the following subgroups. Group 1: 137 unrelated IDDM patients of Danish Caucasian origin. They were all identified through the outpatient clinic at Steno Diabetes Center, fulfilled the World Health Organization diagnostic criteria for IDDM, and were all diagnosed before the age of 30. Group 2: 105 ethnically matched, unrelated control individuals, none of whom had a family history of IDDM or other endocrinopathies. Group 3: 170 IDDM patients with nephropathy, i.e., albumin excretion rate (AER) >300 mg/24 h in more than two consecutive 24-h urine collections. They had no clinical or biochemical evidence of ongoing nondiabetic kidney or renal tract disease and AER was <30 mg/24 h within the first 5 yr from diabetes onset. Group 4: 99 IDDM patients without nephropathy, i.e., AER <30 mg/24 h, after disease duration of more than 20 yr. The majority of these patients received no medication except for insulin. If treated with antihypertensive drugs, only patients that were normoalbuninuric prior to treatment and after more than 5 yr of treatment were included in this group. Group 5: For functional studies, individuals from group 1 were selected according to their TGF-β1 genotype. The mean ± SD disease duration was 10.0 ± 5.22 yr in these patients. None of them had symptoms or clinical evidence of nephropathy. The mean ± SD HbA1c in this group at the time of blood sampling was 7.9 ± 0.63% (range, 6.9 to 8.9). Group 6: To evaluate intra- and interindividual variations of TGF-β1 expression levels, we initially analyzed six individuals. They were all healthy and free of any allergic manifestations for 12 wk before blood sampling and had no infectious diseases including colds for 4 wk before blood sampling. They received no medication and no dietary supplementation with vitamins or minerals for 4 wk before blood sampling. All participants gave informed consent, and the study was approved by the appropriate ethics committees.

DNA Preparation and Mutation Screening

Whole blood was collected in ethylenediamine tetra-acetic acid-containing Vacutainer tubes, and DNA was extracted from leukocytes according to standard procedure (25).

For screening of the two polymorphisms, we developed two PCR-based assays. For the 713–8delC polymorphism, the following primers were used: 5'-ATG TCC CCT ATC CCC TCA CTC-3' and 5'-TCC TGC iTC TCA TOG CCC T-3' (P3), 5'-TGG CCA CCA TTC 7-1-1 ACC ACC AGA TOC CAG-3'. The PCR product was incubated with the restriction endonuclease Bssl (New England Bio-labs, Hitchin, United Kingdom) and analyzed on a 1.5% agarose gel. For the Thr263Ile polymorphism, we developed a mutagenically separated PCR (MS-PCR) assay (26). In the MS-PCR setup, three primers were used: two allele-specific “MS-PCR” primers with both sequences derived from the same DNA strand and one primer with a sequence derived from the complementary strand. The two MS-PCR primers differed in length, and at five nucleotide positions: three at the 3' end and two in positions corresponding to the 5' end of the primer. Deviations from wild-type sequence in the MS-PCR-primer sequences are indicated in **italics**. MS-PCR primers: 5'-ACC GGC CCT TCC TGC TCC TCA TGG CCC 7-3' (P3), 5'-TGG CCA CCA TTC ATG GCA TGA GTG GCC TCT TCC TGC TCA TGG ACA C'3- (P2). Complementary strand primer (P1): 5'-AAG GCC TCC ATC CAG GCC TCT GTC ACA AAG CTC AC-3'. These three primers were used in a single reaction mix containing 100 ng of target DNA, 20 μM dNTP, 1.5 mM MgCl₂, 0.1 μM P1, 0.09 μM P2, 0.15 μM P3, and 0.5 U Duralze™ II DNA polymerase (Finnzymes OY, Espoo, Finland). The products were analyzed on a 1.5% agarose gel. The MS-PCR assay was validated by sequencing six individuals with the wild-type sequence and six individuals heterozygous for the mutation. Complete accordance between sequencing and MS-PCR data was found (data not shown).

Lymphocyte Stimulation

To analyze intra- and interindividual variation in TGF-β1 expression levels and to test for the possible effect of the 713–8delC mutation on either exon skipping or cryptic splicing, or test for any effect of the studied mutations on quantitative mRNA expression, cDNA was prepared from phorbol 12-myristate 13-acetate (PMA)-stimulated lymphocytes from selected individuals. Peripheral blood mononuclear cells (8 × 10⁶ cells/ml) from individuals positive and negative for the two mutations were stimulated with 1 ng/ml PMA (Life Technologies, Paisley, United Kingdom) for 6 h. Then the cells were harvested, washed in phosphate-buffered saline, and pelleted by centrifugation.

cDNA Synthesis

Poly(A)+ mRNA was isolated directly from the cell pellets using the Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway), according to the manufacturer’s protocol. cDNA was prepared from mRNA using the cDNA Cycle™ kit from Invtrogen (San Diego, CA).

cDNA Analysis

Primers were designed to amplify the sequence, including both mutations, around the splicing site of intron 4/exon 5 to evaluate possible exon skipping. These primers were used on genomic DNA and cDNA. Primers were designed so they resulted in fragments of different lengths on genomic DNA and cDNA, respectively.

Reverse Transcription-PCR

Quantitative PCR for TGF-β1 was performed using the same primers as above. As an internal control, primers for the cyclophilin (CP) gene were included in the same PCR mixture. CP primers: 5'-CAA GAT CGA GGT GGA AAG GC-3' and 5'-GTC GCC TCC ACC AGA TG CAG CAG-3'. The mixture contained 300 ng of target cDNA, 50 μM dNTP, 0.25 MBq of α33P-CTP, 2.0 mM MgCl₂, 0.6 μM of each primer, and 1 U TaqGold™ DNA polymerase (Perkin Elmer, Foster City, CA). Twenty-five cycles were performed which ensured that both products were in the log-linear amplification phase (data not shown). After separation of the PCR products on a 6% acrylamide gel, the gel was dried and exposed to PhosphorImager (Molecular Dynamics, Kent, United Kingdom) screens. After PhosphorImager scanning of the screens, quantification of the 33P-labeled PCR bands was performed using the ImageQuant 3.3 (Molecular Dynamics) software, and TGF-β1 mRNA expression was calculated relative to the expression of the internal CP housekeeping gene included in each PCR (TGF-β1/CP × 100).

Statistical Analyses

Genotype frequencies were compared using the Fisher exact test. For analyzing lymphocyte mRNA quantitative data, one-way ANOVA was used for comparison of the individuals and the Mann-Whitney test for comparison of two groups. P values (two-sided) of <0.05 were accepted as significant unless otherwise stated.

Results

The two PCR-based screening assays showed the following patterns: In the wild-type sequence, Bssl revealed three fragments of 33, 44, and 127 bp, respectively, whereas one Bssl
Typing data were not available for one person for the Thr263Ile mutation.

Figure 1. Mutagenically separated PCR (MS-PCR) assay for the Thr263Ile polymorphism of transforming growth factor-β1 (TGF-β1). Lanes demonstrate examples of three homozygous and three heterozygous individuals. The size of the amplification product is shown to the left and the allelic variant to the right. For details, see DNA Preparation and Mutation Screening.

recognition site was eliminated in the 713-8delC variant resulting in fragments of 44 and 160 bp. In the presence of Thr263, a PCR product of 150 bp was amplified in the MS-PCR assay, whereas a 129-bp fragment was amplified in the presence of the Ile263 variant (Figure 1).

No linkage of specific mutant alleles of the two polymorphisms was observed despite the fact that they are separated by only 40 nucleotides. No significant differences in genotype frequencies were observed for any of the two polymorphisms between randomly selected IDDM diabetic patients and control subjects (P = 0.6 for the 713-8delC variant and P = 0.4 for the Thr263Ile mutation) (Table 1). Between IDDM patients with nephropathy and normoalbuminuric IDDM patients, no difference was found for the intron four 713-8delC mutation (P = 0.7) (Table 2). However, a significant difference in the frequency of the Thr263Ile mutation was observed. Of 98 normoalbuminuric patients for whom typing was available, none had the Ile263 variant, whereas this variant was found in nine (5.3%) patients with nephropathy (P = 0.03) (Table 2).

Interestingly, we have identified no individuals homozygous for the rare alleles of the two TGF-β1 gene variants, although this was expected with the numbers included in the present study and other studies (reference 22 and unpublished data).

Table 1. Frequencies of genotypes of the 713-8delC and the Thr263Ile mutations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>713-8delC (CC/CC)</th>
<th>713-8delC (CC/CC)</th>
<th>P Value</th>
<th>Thr263/Ile263</th>
<th>Thr263/Thr263</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDDM patients</td>
<td>n = 137</td>
<td>10</td>
<td>127</td>
<td>14</td>
<td>120b</td>
<td>0.4</td>
</tr>
<tr>
<td>Control subjects</td>
<td>n = 105</td>
<td>5</td>
<td>100</td>
<td>0.6</td>
<td>7</td>
<td>98</td>
</tr>
</tbody>
</table>

*Thr, threonine; Ile, isoleucine; IDDM, insulin-dependent diabetes mellitus.
bTyping data were not available for three patients for the Thr263Ile mutation.

Table 2. Frequencies of genotypes of the 713-8delC and the Thr263Ile mutations in IDDM patients with and without nephropathy

<table>
<thead>
<tr>
<th>Genotype</th>
<th>713-8delC (CC/CC)</th>
<th>713-8delC (CC/CC)</th>
<th>P Value</th>
<th>Thr263/Ile263</th>
<th>Thr263/Thr263</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephropathy</td>
<td>n = 170</td>
<td>10</td>
<td>160</td>
<td>9</td>
<td>161</td>
<td>0.03</td>
</tr>
<tr>
<td>No nephropathy</td>
<td>n = 99</td>
<td>4</td>
<td>95</td>
<td>0</td>
<td>98a</td>
<td>0.03</td>
</tr>
</tbody>
</table>

aTyping data were not available for one person for the Thr263Ile mutation.
polymorphisms ($P = 0.26$ for the $713-8$delC mutation and $P = 0.17$ for the Thr263Ile mutation) was demonstrated. However, significant interindividual differences in TGF-β1 mRNA levels were observed between the tested individuals ($F = 18.386; P < 0.0001$, ANOVA) when TGF-β1 mRNA level was expressed as TGF-β1/CP × 100 (Figure 2).

**Discussion**

We have established PCR assays for easy and fast analysis of two TGF-β1 gene mutations. No association of the two TGF-β1 sequence variations with IDDM in general was found. However, we found a weak but significant association of the Thr263Ile mutation with diabetic nephropathy. Given the low frequency of the Ile263 variant, the observed association may also be due to a type 1 error. However, the nephropathic and non-nephropathic cohorts were sufficiently large to yield 80% power to detect a 6% deviation in carriage rate of the Ile263 variant with $P < 0.025$. Case-control studies are likely to be inflated by several pitfalls; however, the impact of the present results should be emphasized due to the strict categorization of IDDM patients as either having diabetic nephropathy or being non-nephropathic with a diabetes duration exceeding 20 yr, thereby having low risk of later developing diabetic nephropathy (27,28).

We observed no correlation between TGF-β1 transcription levels and genotypes of any of the two studied polymorphisms; however, it should be noted that only groups of three individuals with the mutations were tested. However, significant interindividual differences in TGF-β1 mRNA levels were observed between the tested individuals compatible with a genetic control mechanism of TGF-β1 synthesis at the mRNA level. If this is the case, then genetic variations other than the ones tested in the present study are most likely responsible. Very preliminary data, based on twin analysis and regression procedures, have suggested that the concentration of TGF-β1 in serum is partly under genetic control (29). Whether polymorphisms of the promoter region account for this, however, is not clear at present (29). Also, at the posttranscriptional and translational levels, the regulation of TGF-β1 is rather complex. The substitution of threonine with isoleucine in position 263 is 16 amino acids from the activation cleavage site in the amino terminal direction. The Thr263 codon is conserved in genomes of human, mouse, rat, pig, dog, and green monkey and therefore is supposed to be of functional importance. Threonine has one hydroxyl group, whereas the side chains of isoleucine are nonpolar. This substitution with its potential effect on the structure of the propeptide therefore could affect the stability of latent TGF-β1 and/or the activation of TGF-β1. Such mechanisms could lead to reduced concentrations of latent TGF-β1 and subsequently active TGF-β1 at the tissue level. The Thr263Ile substitution may also affect the cleavage site, leading to an altered NH$_2$-terminal part of the TGF-β1.

**Figure 2.** Mean ± SD TGF-β1 mRNA levels from phorbol 12-myristate 13-acetate (1 ng/ml)-stimulated peripheral blood mononuclear cells from nine different individuals (U, A, B, . . . , I). U represents an unstimulated sample from one of the individuals. TGF-β1 mRNA values are expressed relative to the expression of the internal cyclophilin (CP) housekeeping gene included in each PCR (TGF-β1/CP × 100). Values represent mean of three independent experiments, i.e., RNA and cDNA preparation, reverse transcription-PCR, and quantification of mRNA levels by phospho-imaging from the same pool of stimulated cells. Genotypes of the analyzed individuals: A, C, and F: 713-8delC [CC/CC] - Thr263/Thr263; D, H, and I: 713-8delC [CC/CC] - Thr263/Thr263; B, E, and G: : 713-8delC [CC/CC] - Thr263/Ile263. No correlation between TGF-β1 transcription levels and genotypes was demonstrated. However, significant interindividual differences in TGF-β1 mRNA levels were observed between the tested individuals ($F = 18.386; P < 0.0001$, ANOVA). (Inset) PhosphorImager scan of five samples from one experiment.
molecule. We have not been able to address these possibilities specifically in the present study. Hence, the disease-mechanistic significance of the observed association is not clear.

The 713-8delC mutation is located in the intron sequence eight nucleotides upstream from exon 5. Fifteen percent of human genetic diseases are caused by point-sequence variations, causing either exon skipping or cryptic splicing (30). Both exon skipping and cryptic splicing would result in a truncated propeptide, and absence of active TGF-β1. We have PCR-amplified the sequence including exons 4 to 6 from cDNA prepared from heterozygous and homozygous individuals. This revealed exactly the expected fragments and showed no differences between heterozygous and homozygous individuals, thus not supporting the idea that the 713-8delC mutation may affect the splicing site.

In conclusion, the present study suggests that the Thr263Ile mutation is associated with nephropathy in IDDM patients. By reverse transcription-PCR, we demonstrated interindividual variation in PMA-induced TGF-β1 mRNA levels compatible with a genetic control of TGF-β1 synthesis or, alternatively, different priming of the leukocytes due to, e.g., current inflammatory status. However, no clinical evidence for ongoing infectious or inflammatory disease or information about medical treatment at the time of blood sampling was present. Disease duration and metabolic control were comparable within this group. The mean HbA1c in the group was 7.9% (range).

Whether the Thr263Ile mutation can serve as a risk marker for later development of nephropathy in diabetic patients remains to be demonstrated. However, other study populations should be investigated first to confirm or refute the present data, and a further search for polymorphisms controlling TGF-β1 production is warranted.

References