Association of polymorphisms within the transforming growth factor-β1 gene with diabetic nephropathy and serum cholesterol and triglyceride concentrations

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SUMMARY AT A GLANCE

Diabetic nephropathy is characterized by ultrastructural changes in all renal compartments, leading to glomerular basement thickening, glomerular and tubular hypertrophy, mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis. The 869 T > C, 915 G > C polymorphisms of TGF-β1 are associated with diabetic nephropathy (DN).

Diabetic nephropathy (DN) is the leading cause of chronic kidney disease and one of the main mechanisms by which diabetes results in increased mortality.1 The public health impact of DN is expected to grow in years to come due to the increasing prevalence of diabetes in many countries.2 In Mexico, in 2005 the total cost of diabetes treatment was reported as $US 317 631 206 and the direct cost for patients with DN was $US 32 972 722.3 DN is characterized by ultrastructural changes in all renal compartments, leading to glomerular basement thickening, glomerular and tubular hypertrophy, mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis.4 Albuminuria is a clinical marker for DN. High blood pressure, declining renal function, uraemia and end-stage renal disease (ESRD) are the intermediate and long-term outcomes of the disease. These processes are triggered by the interaction of haemodynamic and...
metabolic disturbances, changes in the expression of growth factors and cytokines, and genetic susceptibility. Metabolic derangements are contributing factors to having atherosclerosis and the progression of renal disease. Hypercholesterolaemia and hypertriglyceridaemia aggravate renal injury primarily by podocyte damage. Moreover, Chade et al. showed in animal models that hypercholesterolaemia not only promotes fibrogenic activity and renal inflammation but also downregulates renal metalloproteinases, thereby blunting extracellular matrix degradation and facilitating renal scarring.

There is strong evidence implicating genetic susceptibility factors associated to diabetic nephropathy. Several single nucleotide polymorphisms (SNP) have been linked with an increased likelihood of having DN. It has been proposed that the transforming growth factor-β (TGF-β) gene participates in the development of renal hypertrophy and in the accumulation of extracellular matrix in diabetes. TGF-β is a bifunctional growth regulator: it inhibits cell growth of most cell types by Smad-dependent mechanisms, but also stimulates the growth of mesenchymal cells. TGF-β is also an anti-inflammatory factor that inhibits migration and differentiation of inflammatory cells, by specifically targeting anti-inflammatory factor that inhibits migration and differentiation of inflammatory cells, by specifically targeting differentiation of inflammatory cells, by specifically targeting the self-tissues. Several TGF-β1 gene polymorphisms are associated with DN. The polymorphism 869 T>C, which changes codon 10 (Leucine/Proline) as well as the polymorphism 509 C>T are associated with the circulating concentration of TGF-β1 protein, and both polymorphisms have been associated with DN traits. At the molecular level, there is differential regulation of TGF-β1 expression due to the presence of polymorphisms in the regulatory gene region, which promote defective binding affinity of transcription factors. However, there are discrepancies in the results reported for this gene in previous case–control studies. The aim of the present study was to assess the association of three TGF-β1 gene variants with DN and other metabolic parameters such as cholesterol and triglycerides in a sample of individuals from Mexico City.

**METHODS**

**Study group**

The sample included 439 patients with type 2 diabetes mellitus; 233 with diabetic nephropathy (DN+) and 206 without (DN–). Only 228 DN+ and 192 DN– were genotyped. All participants were inhabitants of Mexico City, and had had diabetes for more than 15 years. Blood samples were drawn after a 12 h fasting period.

Patients with type 1 diabetes or other causes of nephropathy were excluded. The participants were recruited from the Primary Care Medical Units of the Mexican Social Security Institute (IMSS) in Mexico City and signed an informed consent letter. The project was approved by the Ethics and Evaluation Committee of the IMSS, according to the declaration of Helsinki.

**Biochemical profile**

All biochemical tests were performed in sera as well as in urine using the ILab 350 equipment (Instrumentation Laboratory, Barcelona, Spain). Albumin excretion and creatinine clearance were measured in a 24 h urine collection.

**DNA purification**

DNA was extracted from peripheral blood using silica columns (Qiagen, Chatsworth, CA, USA) following the manufacturer’s recommendations. The DNA concentration was measured using a Lambda 25 spectrometer (Perkin Elmer, Pomona, CA, USA). DNA integrity was verified in 0.8% agarose gels, stained with ethidium bromide.

**Identification of TGF-β1 SNP**

The 869 T>C (Leu10Pro) and 915 G>C (Arg25Pro) TGF-β1 gene variants were identified using the restriction fragment length polymorphism technique. The primers were: forward 5′-TTCCCTCG AGGCCCTCC-3′ and reverse 5′-GGCGGAGCTTGGAGGATC-3′. A 294 bp product was obtained, which included two SNP of the signalling peptide, the 869 T>C and 915 G>C. The restriction enzymesMspAI and BglI (New England Biolabs, Beverly, MA USA) were used, respectively, to identify the presence or absence of the polymorphisms as described by Ohtsuka et al., but with some modifications. Briefly, the polymerase chain reaction (PCR) was done by initial denaturation at 94°C for 2 min, 35 cycles at 96°C for 75 s, 62°C for 75 s, 73°C for 75 s and a final extension at 73°C for 5 min. The amplifications were performed in an Applied Biosystems GeneAmp PCR system 2700 thermocycler (Foster City, CA, USA). The PCR product was subjected to enzymatic digestion with the corresponding enzymes and following the manufacturer’s instructions. Then, electrophoresis in agarose gels at 3.2% for 869 T>C and 2.5% for 915 G>C were performed in horizontal chambers (SubCell GT; Bio-Rad, Richmond, CA, USA) using Tris-borate/ethylene diamine tetra acetate (TBE) 1% buffer at 110 V for 2.5 h, to identify the genotypes according to the band patterns. SNP 869 T>C produced the T allele that was identified in 161, 67, 40 and 26 bp bands, and the C allele in 149, 67, 40 and 12 bp bands. For SNP 915 G>C, the G allele were identified in 131 103 and 60 bp and allele C in 163 and 131 bp bands. SNP −800 G>A was performed by the TaqMan assays-by-design probe (Applied Biosystems). The forward primer sequence was 5′-TGGAGTGCTTGGAGGACTC-3′ and the reverse primer sequence was 5′-GCCATCCTCCCCTCATC-3′. The reporter 1 sequence was 5′-CCTCACAATGTCACCAC-3′ and reporter 2 sequence was 5′-CCTCACAATGTCACCAC-3′. At least 20 samples were repeated for each SNP.

**Statistical analysis**

Deviations from Hardy–Weinberg were tested using an exact test (available at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Linkage disequilibrium (LD) patterns and haplotype frequencies were estimated using an expectation maximization method implemented in the program EMLD (University of Texas, Houston, TX, USA). Tests of association were performed using χ²-test. Values of P < 0.05 were considered significant. Finally, a multivariate logistic regression
analysis was carried out using DN as dependent variable and including in the model age, sex, duration of diabetes, hypertension, TGF-β1 polymorphisms, insulin, statins and fibric acid use, cholesterol and triglyceride levels and treatment for lipid control. Data were analyzed with the SPSS Statistic Package ver. 15 (Chicago, IL, USA).

RESULTS

Demographic and biochemical characteristics of study groups

Table 1 shows the demographic and biochemical characteristics of the DN+ and DN− cohorts from Mexico City. There were no differences between both groups, except for age. The average age of the DN− group was 3 years older than the average age of the DN+ group (P = 0.001).

Diabetic nephropathy was defined as the presence of albuminuria and/or decreased glomerular filtration rate (GFR). We included 40 (18%) patients in stage 1 of chronic kidney disease (a ratio of the concentration of albumin to creatinine in spot urine sample of ≥30 mg/g with a normal or increased GFR); 51 patients (23%) in stage 2 (GFR 60–89 mL/min per 1.73 m²); 45 patients (20%) in stage 3 (GFR 30–59 mL/min per 1.73 m²); 17 patients (8%) in stage 4 (GFR 15–29 mL/min per 1.73 m²); and 70 patients (31%) in stage 5 (GFR <15 mL/min per 1.73 m² or dialysis). DN− patients were confirmed by the absence of microalbuminuria (albumin : creatinine ratio in spot urine sample of <30 mg/g) and serum creatinine below 132.18 μmol/L. No glomerular filtration rate was estimated in these subjects. Twenty-seven percent of DN− and 31% DN+ subjects were treated with bezafibrate or pravastatins for lipid control.

Association of TGF-β1 polymorphisms with DN, total cholesterol and triglycerides

None of the SNP showed significant deviations from the Hardy–Weinberg equilibrium in either study group or in the whole sample. The 869 T > C and 915 G > C polymorphisms showed a significant association with DN. Frequency of CC/CT genotypes for 869 T > C was higher in DN+ patients than in the DN− (Table 2). The CC/CT versus TT genotype had an odds ratio (OR) of 1.818 (95% confidence interval (CI) = 1.128–2.930) under a dominant model (P = 0.016). For the 915 G > C polymorphism, there were significant differences in CC + CG versus GG genotype frequencies between DN− and DN+ (P = 0.008, OR = 4.073, 95% CI = 1.355–12.249). There were no significant differences for SNP −800 G > A (Table 3).

We analyzed the LD pattern between the 869 T > C and 915 G > C SNP. The two polymorphisms did not show sig-
DISCUSSION

The TGF-β1 gene plays an important role in the development of renal disease. In this study, we confirm that two polymorphisms of the TGF-β1 gene (869 T > C and 915 G > C, Table 3) are associated with DN in a sample from Mexico City. In addition, the 869 T > C SNP is associated with total cholesterol and triglyceride levels: subjects with the CC + CT genotype showed higher plasma levels than subjects with the TT genotype.

The distribution of the TGF-β1 869 T > C genotypes in our sample from Mexico City was similar to the distribution reported by Wong et al. in a Chinese sample. Mittal et al. found that the 915 G > C and 869 T > C polymorphisms increase the risk of end-stage renal disease. Coll et al. also reported an association between 915 G > C and chronic renal disease in Europeans. In contrast, Khalil et al. reported that in white patients with chronic kidney failure, GG homozygotes have an increased risk for developing advanced kidney failure. On the other hand, Prasad et al. reported that this variant is non-informative because the minor allelic frequency is too low in Asian Indians. These contrasting reports emphasize the need to carry out additional studies in other populations.

We observed that total cholesterol and triglyceride levels were associated with the 869 T > C polymorphism: plasma levels were higher in subjects with the CC + CT genotype compared to the TT subjects (Table 4). This association has not been reported previously. It is known that hypercholesterolaemia and hypertriglyceridaemia participate in renal damage by altering the function and structure of podocytes. Joles et al. reported podocyte damage evidenced by de novo expression of desmin and ultrastructural changes, high levels of glomerular macrophages, interstitial myofibroblast activation and tubulointerstitial injury in rats on a hypercholesterolaemia diet for 13 weeks. Recently, Chade et al. demonstrated that pigs fed for 16 weeks with a cholesterol-rich diet have endothelial dysfunction, increased intrarenal oxidative stress, inflammation and activation of the endothelin and TGF systems, as well as decreased matrix metalloproteinase expression and activity. These changes were reversible when animals returned to their normal diet; thus, early control of lipid disorders may be useful to preserve renal function. One of the proposed pathways between hypercholesterolaemia and TGF-β1 is that Ox-LDL stimulates its receptor. This route leads to glomerular injury by inducing formation of foam cells that are associated with later glomerulosclerosis and interstitial injury. Also, it has been proposed that a relation exists between hypercholesterolaemia, TGF-β1 levels, and anti-Ox-LDL, resulting in activation of the innate immune response, inflammation, and fibrosis.

In summary, we show that the 869 T > C and 915 G > C polymorphisms of the TGF-β1 gene are associated with DN. This finding is important because DN leads to end-stage renal disease, one of the most prominent causes of morbidity and mortality. In addition, the TT genotype of 869 T > C variant is associated with cholesterol and triglyceride concentrations. More studies are required to identify the role of lipids and the TT genotype of 869 T > C TGF-β1 variant in DN.

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