Lack of association between TGF-β-1 genotypes and microalbuminuria in essential hypertensive men

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Abstract

Background. Polymorphisms within the gene for transforming growth factor (TGF)-β-1, a pro-fibrogenic cytokine pathophysiologically involved in hypertension and hypertensive target damage, might modulate the biological activity of the encoded protein. Through that mechanism, they might contribute to microalbuminuria, a marker of subclinical renal damage and a correlate of systemic inflammation and endothelial dysfunction in hypertension, a possibility never before tested. For this reason, we assessed the association of four TGF-β-1 polymorphic variants (C->509T, Leu10Pro, Arg25Pro, Thr263Ile) with albuminuria in uncomplicated essential hypertensive men, using (circulating active + acid-activatable latent) TGF-β-1 levels as an indirect index of their in vivo biological activity. Because of the close pathophysiological link of TGF-β-1 with the renin–angiotensin system, we also tested the behaviour of the angiotensin converting enzyme (ACE) deletion/insertion (D/I) polymorphism.

Methods. Albuminuria (three overnight collections), office and 24-h BP, left ventricular mass index (LVMI), BMI, renal function, glucose, lipids, plasma TGF-β-1 (n = 162, ELISA) were measured in 222 genetically unrelated, never-treated, uncomplicated Caucasian hypertensive men. ACE D/I polymorphisms were analysed by the polymerase chain reaction technique or a

Conflict of interest statement. None declared.

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5’ nuclease assay with further restriction analysis when required.

Results. Urine albumin levels or microalbuminuria (albuminuria ≥ 15 µg/min) did not differ by TGF-β-1 genotypes, but both parameters were more frequent in ACE D/D homozygotes. Plasma TGF-β-1 was similar across genetic backgrounds and was unrelated to albuminuria. Cardiovascular, renal, metabolic parameters were homogeneously distributed across genotypes.

Conclusions. In contrast to its link with the ACE D/I genotype, microalbuminuria was independent of TGF-β-1 polymorphism in this group of never-treated, uncomplicated essential hypertensive men.

Keywords: angiotensin converting enzyme deletion/insertion polymorphism; circulating TGF-β-1 levels; hypertensive target organ damage; microalbuminuria; TGF-β-1 polymorphisms

Introduction

Transforming growth factor-beta (TGF-β)-1, a modulator of multiple biological processes including cell growth and proliferation, inflammation, endothelial and vascular smooth muscle cell function and extracellular matrix metabolism [1], may play a role in human hypertension [2] and cardiovascular disease [3]. Experimental and clinical data support its involvement in renal glomerulosclerosis, tubulointerstitial fibrosis and progressive renal failure [4], and studies in essential hypertensive patients found an association between circulating TGF-β-1 levels and microalbuminuria (MA) [5,6], an integrated marker of cardiovascular risk [7], multiple biological processes including cell growth and pro-

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Several single nucleotide polymorphisms (SNPs) exist within the gene encoding TGF-β-1, including a C-509T non-coding variant in the promoter region and coding variants in exons 1 (T-868C and G-913C giving rise to amino acid substitutions at position 263 (Thr263→Pro and Arg25→Pro, respectively) and 5 [788C/T giving rise to amino acid substitutions at position 263 (Thr263→Ile)] [11]. Experimental evidence has shown an influence of the first three genetic variants on either circulating TGF-β-1 levels or biological activity [12–16], while the Thr263→Ile SNP, located within the cleaved part of the TGF-β-1 proprotein, may affect its intracellular activation process thus reducing the release of the active protein [11]. Thus, TGF-β-1 SNPs, through their influence on the levels and/or biological activity of the encoded protein, may influence urinary albumin excretion (UAE) in non-diabetic hypertensive patients. However, that possibility has not been tested so far.

On that background, we analysed the association of the C-509T, Leu10→Pro, Arg25→Pro and Thr263→Ile genetic variants within the TGF-β-1 gene in a large and carefully screened group of non-diabetic hypertensive men. We also measured circulating (active + acid-activatable latent) TGF-β1 as an indirect index of the biological activity of those SNPs. Because of the interrelationship between TGF-

β1 and the renin—angiotensin system (RAS) [17] and the known link between UAE and angiotensin-converting enzyme (ACE) deletion/insertion polymorphism (D/I) (e.g. 18,19), we also analysed the impact of that genetic configuration in our patients.

Material and methods

Subjects

The analysis was carried out on 222 sedentary, genetically unrelated Caucasian hypertensive men screened in the context of a preventive programme offered to never-treated male patients referred to our unit for hypertension screening (see Table 1, left-hand column, for general characteristics of the sample). Main inclusion criteria were casual BP >140/90 mmHg on at least three occasions as outpatients, fasting blood glucose levels ≤126 mg/dl, serum creatinine ≤1.4 mg/dl, no proteinuria at the dipstick test, negative urinalysis, normal sediment, ejection fraction >50% and no history of valvular or cardiovascular diseases.

In all, renal ultrasound scans showed normal-sized kidneys and no evidence of cortical scarring or obstructive uropathy. Angiograms, when indicated, had excluded renal artery stenosis, while routine clinical and biochemical evaluations excluded other secondary forms of hypertension.

Evaluations were completed in a 2-week period. The study was carried out in accordance with the Declaration of Helsinki, and the protocol approved by the University Ethics Committee.

UAE determination

Urinary albumin was measured by nephelometry (Behring Nephelometer Analyzer II, Behring Diagnostics GmbH, Marburg, Germany) using a commercially available kit (Roche, Italy). To minimize the confounding influence of daily physical activity and facilitate the collection procedure, urine was collected from 8:00 p.m. to 8:00 a.m. during three consecutive days.

Genetic determinations

DNA was isolated from frozen whole blood samples containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant by a commercial kit for DNA extraction (Nucleon BACC2, Amersham Biosciences, Uppsala, Sweden) and resuspended in water. Genomic DNA (100–200 ng) was amplified in a Perkin Elmer 9600 thermocycler by the polymerase chain reaction (PCR) in a 20–50 µl reaction mixture containing 10 mmol/l Tris-HCl, PH 8.8, 50 mmol/l KCl, 1.5 mmol/l MgCl2, 0.1% Triton X-100, 200 pmol of each dATP, dCTP, dGTP, dTTP; variable amounts of each primer and 0.5 U of thermostable taq DNA polymerase (Hotmaster TAQ Eppendorf, Hamburg, Germany). All samples were analysed on 3.5% agarose gel staining. Genotyping was performed in a blinded fashion.

C-509T polymorphism. The fragment containing the C-509T SNP was amplified using forward (5’AGGGCATGCCACCGGTCG3’; 0.4 µmol) and reverse (5’GAAGGAGGGTCTGTCAACAT3’; 0.4 µmol) primers; the annealing temperature was 61°C. For the polymorphism of the promoter region, primer-induced restriction analysis with BSU 36 I enzyme was performed as previously reported [20].

Leu10→Pro and Arg25→Pro polymorphisms. For amplification, forward primer (5’TTCCTTCAGGGCCCTCTCA; 0.4 µmol) and reverse primer (5’GAAGGAGGGTCTGTCAACAT3’; 0.4 µmol) were used at an annealing temperature of 60°C. The Leu10→Pro and Arg25→Pro polymorphisms were assayed by restriction analysis with the MSPA11 and BglII, respectively, as described by other authors [21].

Thr263→Ile polymorphism. The genotype was identified by 5’ nuclease assay. The fragment containing the Thr263→Ile SNP was amplified using forward (5’ACGCCCTTTCTGCTCTCAGGC CCT; 0.15 µmol), forward2 (5’AGGGCCACATTGCGCATGAGT CGGCTTTTCCCTGTCTCAGGCA3’; 0.09 µmol) and reverse (5’AGGAGGGACCATGCGTACAGGCTA; 0.10 µmol) primers at an annealing temperature of 58°C [22].

ACE D/I polymorphisms were identified as described elsewhere [23].
Circulating TGF-β1 determination

Plasma TGF-β1 levels were determined in 162 samples collected in 1.5% EDTA and kept at -70°C until assayed by using a specific solid-phase quantitative sandwich enzyme immunoassay technique with a Quantikine human TGF-β1 ELISA kit (R&D Systems). The biologically active TGF-β1 protein concentration was determined after acidification and tested at 1:300 dilution. A standard curve was constructed using recombinant β1 263Ile allele 11% 11% 11%

Table 1. Age, rare allele frequencies and clinical characteristics of the sample as a whole and by microalbuminuria

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (n = 222)</th>
<th>Normoalbuminuria (n = 168)</th>
<th>Microalbuminuria (n = 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51 ± 11</td>
<td>51 ± 11</td>
<td>51 ± 11</td>
</tr>
<tr>
<td>UAE (µg/min)</td>
<td>10 (9)</td>
<td>7 (5)</td>
<td>27 (30)*</td>
</tr>
<tr>
<td>TGF-β1-509T allele</td>
<td>40%</td>
<td>40%</td>
<td>41%</td>
</tr>
<tr>
<td>TGF-β1-1 10 Pro allele</td>
<td>50%</td>
<td>50%</td>
<td>55%</td>
</tr>
<tr>
<td>TGF-β1-1 25 Pro allele</td>
<td>7%</td>
<td>8%</td>
<td>5%</td>
</tr>
<tr>
<td>TGF-β1-263He allele</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
</tr>
<tr>
<td>ACE I allele</td>
<td>33%</td>
<td>35%</td>
<td>21%*</td>
</tr>
<tr>
<td>Plasma TGF-β1 (ng/ml)</td>
<td>5.1 (7.5) (n = 162)</td>
<td>5.1 (7.5) (n = 123)</td>
<td>5.2 (7.6) (n = 39)</td>
</tr>
<tr>
<td>Estimated GFR (ml/min× 1.73 m²)</td>
<td>93 ± 21</td>
<td>92 ± 20</td>
<td>96 ± 23</td>
</tr>
<tr>
<td>Smokers</td>
<td>32%</td>
<td>26%</td>
<td>43%</td>
</tr>
<tr>
<td>ASBP (mmHg)</td>
<td>133 ± 16</td>
<td>131 ± 15</td>
<td>139 ± 18*</td>
</tr>
<tr>
<td>ADBP (mmHg)</td>
<td>86 ± 9</td>
<td>85 ± 9</td>
<td>88 ± 12*</td>
</tr>
<tr>
<td>SBP office (mmHg)</td>
<td>153</td>
<td>151</td>
<td>159*</td>
</tr>
<tr>
<td>DBP office (mmHg)</td>
<td>96</td>
<td>95</td>
<td>98</td>
</tr>
<tr>
<td>LVM (g/m²)</td>
<td>57 ± 13</td>
<td>55 ± 12</td>
<td>61 ± 16*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.9 ± 3.0</td>
<td>26.8 ± 2.8</td>
<td>27.5 ± 3.4'</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>98 ± 12</td>
<td>100 ± 11</td>
<td>100 ± 13</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>46 ± 13</td>
<td>46 ± 12</td>
<td>47 ± 12</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>137 (92)</td>
<td>137 (89)</td>
<td>127 (107)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>214 ± 43</td>
<td>212 ± 42</td>
<td>216 ± 42</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>138 ± 42</td>
<td>137 ± 40</td>
<td>140 ± 36</td>
</tr>
</tbody>
</table>

Arithmetic means ± SD or geometric means (interquartile range) for skewed distributions and percentages for proportions.

For abbreviations and definitions see the text.

*P <0.01, P <0.05.

Statistics

Differences among continuous variables and proportions were tested by ANOVA and chi-square statistics, respectively, and strength of association by Spearman rho statistics. The association of MA (coded as 1 and 0 as present or absent, respectively) with genotypes and other continuous and categorical covariates was analysed by multivariate logistic regression using odds ratios (ORs) and 95% confidence interval (CI)s as estimates of relative risk. Descriptive statistics for continuous parameters were arithmetic means ± SD or geometric means (interquartile range) for skewed data and percentages for proportions. The two-tailed statistical significance was set at P < 0.05. Estimates accounting for allele frequency in our population and assuming a multiplicative effect on disease risk and best-case scenario of typing susceptibility variant itself (or a perfect proxy), allowed us to predict that the size of our sample could discriminate only an allelic OR >2 [26].

Results

MA was present in 54 (24%) patients characterized by higher office and 24-h BP, greater LVM, heavier weight and more frequent smoking habits. TGF-β1 rare allele frequencies and plasma levels of the cytokine did not differ by MA status while the ACE I allele was less prevalent (and therefore the D allele more frequent) among microalbuminuric patients (Table 1).

Genotypic proportions for the C-509T, Arg25→Pro, Leu10→Pro and Thr263→He variants were in Hardy-Weinberg equilibrium and in line with the expected distribution in previous population studies (Table 2) [3]. Age, plasma TGF-β1, 24-h BP, LVMI, BMI, smoking...
status (Table 3), renal and metabolic parameters (not shown) were homogeneously distributed across genetic backgrounds.

MA frequency and UAE levels did not differ by TGF-β-1 genotypes either per se (Figure 1 and Table 3) or after stratification for the different ACE D/I genotype (data not shown). Both parameters were higher in ACE D/D than in D/I subtypes, with 24-h SBP representing an additional independent contributor (OR: 1.26/10 mmHg, 95% CI: 1.05–1.42/10 mmHg, P = 0.012) to MA.

The plasma TGF-β-1 levels showed no statistical association with either UAE or L VMI (rho = 0.1 and 0.2, respectively, P = 0.2, n = 162).
Discussion

In showing no association with MA, our data do not support the hypothesis of a significant contribution of TGF-β-1 polymorphisms to subclinical renal damage in non-diabetic hypertensive subjects. Although, to our knowledge, this is the first study to address this issue in non-diabetic, uncomplicated hypertensive patients, similarly negative results were reported in regard to the association of TGF-β-1 SNPs with diabetic nephropathy [27,28] and coronary artery disease [20,29]. However, further evaluation is worthwhile since the evidence in the diabetic setting is controversial [22,30] and discrepancies also extend to cardiovascular disease (see [3] for a review).

Although the C-509T and Leu<sup>10</sup>→Pro variants of the TGF-β-1 gene were associated by some Authors with increased serum levels of the encoded protein [12,15], we could not confirm those data in our sample, in line, however, with negative conclusions of others [31,32]. The <i>in vitro</i> TGF-β-1 biological activity was increased in patient mutants for the Arg<sup>25</sup>→Pro variant [16], but our data are not pertinent in that regard. On the other hand, no previous study has, to our knowledge, described the behaviour of circulating TGF-β-1 in subjects categorized by the Thr<sup>293</sup>→Ile SNP. In disagreement with previous clinical studies [5,6] and for unclear reasons, we also found no difference in circulating TGF-β-1 levels between normo- and microalbuminuric essential hypertensive patients, nor any association with UAE. Still, it is possible that hypertension as such conditions higher TGF-β-1 levels [5] since our series did not include a normotensive control group. From a broader perspective, the homogeneous distribution of L VMI across TGF-β-1 genotypes and the missing statistical association with circulating TGF-β-1 levels do not support its role in promoting left ventricular adaptation to hypertension [5] and susceptibility to hypertensive cardiac organ damage [33].

A second interesting outcome of this study relates to the confirmation [18,19] of the greater renal risk carried by the ACE D/D versus D/I and I/I genotypes. This positive finding has to be seen in contrast not only with the herein explored neutral role of the TGF-β-1 polymorphisms but also with other polymorphic variants of genes encoding products with a relevant impact on vascular and renal biology such as e-NOS [34] documented in previous analyses in this same group of patients. A similar behaviour was shown by the α-adducin variants although an epistatic interaction emerged with the ACE genotype [23]. Overall, the data are consistent with an overwhelming influence of the RAS over other biological mechanisms controlling glomerular haemodynamics. In fact, the ACE DD genotype associates with higher circulating and tissue ACE levels [35], the rate limiting step in biologically active angiotensin II production [36] and an effective stimulus for increased local angiotensin II production [37]. In turn, angiotensin II increases oxidative stress through NADH/NADPH oxidase stimulation and, in concert with other mediators such as cytokines, NO, endothelin-1, prostaglandins and Rho-protein pathway, promotes vascular inflammation, increases capillary permeability and impairs endothelial function [38].

In concordance with that concept, elevated plasma renin activity in previous reports in hypertensive patients [39].

Our results have to be interpreted in the context of some limitations. First, we may evidently have missed low-entity associations between TGF-β-1 polymorphisms and MA because of insufficient statistical power. As anticipated in the Statistics section, our sample was empowered by its size (<i>n</i> = 222) to discriminate only an allelic OR > 2 [26], i.e. genetic influences associated with a large biological impact of the homozygous susceptibility genotype as it was the case for the ACE DD genotype even in our relatively limited number of subjects. On the other hand, one might question the biological relevance of TGF-β-1 SNPs provided with lower, if any, phenotypic penetrance whose evaluation needs extremely large samples difficult to be collected, particularly for the Arg<sup>25</sup>→Pro and Thr<sup>293</sup>→Ile allelic variants characterized by a low prevalence of heterozygosis. Second, although we measured circulating TGF-β-1 levels as an index of the biological activity of the cytokine, that determination is hampered by the presence of multiple TGF-β-1-containing protein complexes, including various latent forms and many non-covalent complexes with a range of serum components and extracellular matrix proteins. Moreover, local rather than systemic alterations in TGF-β activity may be pathophysiologically relevant [3]. However, our results are supported by those of Cha et al. who reported no differences between normo- and microalbuminuric non-diabetic subjects in urinary TGF-β-inducible gene h3 transcript, a protein whose expression may represent a better index of the biological activity of the cytokine in tissues [40]. Third, we studied an all-male hypertensive group and the influence of the TGF-β-1 polymorphism may differ by gender [12,16]. Restriction to an all-male Southern European cohort may also influence the generalizability of our findings to other genetic groups even within the same ethnicity. Fourth, allelic association studies are subject to confounding and we cannot exclude the obscuring influence of some unmeasured factors in our patients, particularly when dealing with a rather remote phenotype such as MA subject to modulation by a host of other environmental and biological factors [7].

In conclusion, our study does not support the assumption that genetic variations in the TGF-β-1 locus may confer significant susceptibility to the development of MA as a marker of preclinical renal damage in uncomplicated essential hypertensive men.

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Conflict of interest statement. None declared.

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