TGF-\(\beta_1\) DNA Polymorphisms, Protein Levels, and Blood Pressure

Baogui Li, Ashwani Khanna, Vijay Sharma, Tejinder Singh, Manikkam Suthanthiran, Phyllis August

Abstract—Transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)), a multifunctional cytokine with fibrogenic properties, has been implicated in the pathogenesis of the vascular and target organ complications of hypertension. TGF-\(\beta_1\) may also regulate blood pressure via stimulation of endothelin-1 and/or renin secretion. Herein we explored the hypothesis that circulating levels of TGF-\(\beta_1\) protein (quantified using a TGF-\(\beta_1\)-specific sandwich ELISA) are correlates of blood pressure levels. This hypothesis was tested in 98 stable end-stage renal disease (ESRD) patients. (The use of ESRD patients as the study cohort eliminates renal function–dependent alterations in circulating levels of TGF-\(\beta_1\) protein.) In addition, in view of the previously reported correlation among TGF-\(\beta_1\) DNA polymorphisms and systolic blood pressure, TGF-\(\beta_1\) codon 25 genotype and alleles were identified in 71 hypertensive subjects and 57 normotensives using amplification refractory mutation system polymerase chain reaction. Our studies demonstrate for the first time that TGF-\(\beta_1\) levels (209±13 \(\text{ng/mL, mean±SEM}\)) are positive correlates (Pearson correlation analysis) of mean arterial pressure (\(P=0.008\)), systolic pressure (\(P=0.02\)), and diastolic pressure (\(P=0.01\)). We also report that a higher percentage of hypertensives (92%) compared with normotensives (86%) are homozygous for the arginine allele at codon 25. Our observations support the idea that genetically determined TGF-\(\beta_1\) protein concentrations may play a role in blood pressure regulation in humans.

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Key Words: transforming growth factor-\(\beta_1\), renal disease, gene polymorphism

Transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)) is a multifunctional cytokine that regulates cell growth, differentiation and matrix production.\(^1\) It is secreted by several cell types, including peripheral blood mononuclear cells, endothelial cells, and vascular smooth muscle cells, and induces fibrosis in a variety of tissues, including kidney, heart, and blood vessels.\(^2\) Overproduction of TGF-\(\beta_1\), perhaps in part mediated by angiotensin II, has been linked to the sequela of chronic hypertension, including left ventricular hypertrophy,\(^3\) vascular remodeling,\(^4\) and progressive renal disease.\(^5\) Although many of the biological actions of TGF-\(\beta_1\) are mediated in an autocrine or paracrine fashion, data from transgenic mice have demonstrated that high circulating levels can mediate renal fibrosis and loss of function.\(^6\)

In addition to a potential role in contributing to the target organ damage in hypertension, it is possible that TGF-\(\beta_1\) may also determine blood pressure levels, by a number of mechanisms. For example, Kurihara et al\(^7\) have demonstrated that TGF-\(\beta_1\) stimulates the expression of mRNA encoding endothelin in vascular endothelial cells, and recent evidence suggests that TGF-\(\beta_1\) increases renin release from juxtaglomerular cells in the kidney. Data also exist that TGF-\(\beta_1\) and angiotensin II regulate the expression of each other.\(^8\)

At present, the basis for increased TGF-\(\beta_1\) production in humans is unknown; however, recent evidence suggests that genetic factors may play a role. Seven polymorphisms in the TGF-\(\beta_1\) gene have been reported in a large study of patients with myocardial infarction and normal controls.\(^9\) One of these polymorphisms, the presence of the Arg\(^{25}\) allele, was associated with higher blood pressure and a family history of hypertension in the normotensive controls compared with individuals with the Pro\(^{25}\) allele. The Arg\(^{25}\) allele has also been associated with increased TGF-\(\beta_1\) production and fibrosis.\(^10\)

In the current investigation, we explored two hypotheses: (1) that circulating levels of TGF-\(\beta_1\) protein are correlates of blood pressure levels in humans, and (2) that the Arg\(^{25}\) allele is more frequent in hypertensives than normotensives. We measured TGF-\(\beta_1\) protein levels in patients with end-stage renal disease (ESRD) treated with hemodialysis, to control for renal function–dependent alterations in TGF-\(\beta_1\). TGF-\(\beta_1\) genotyping was performed in individuals with hypertension and compared with normotensive controls. In this investigation, TGF-\(\beta_1\) codon 25 polymorphisms were identified for the first time using a novel technique, amplification refractory mutation–polymerase chain reaction (ARMS-PCR).

Methods

Subjects
Blood pressure and protein levels of TGF-\(\beta_1\) were measured in 98 patients with ESRD (study group 1). All patients were treated with...
The patients were clinically stable and free of active infection. Blood pressure measurements were obtained in dialysis patients before hemodialysis. Three measurements were made in the seated position, and the average of these measurements was recorded. TGF-β1 genotyping was performed on an additional study group (study group 2) consisting of 71 treated hypertension patients and 57 normotensive controls. All hypertensive patients had blood pressure $\geq 140/90$ mm Hg on antihypertensive therapy. Normotensive patients had blood pressure $<140/90$ mm Hg without antihypertensive therapy, and had no history of hypertension. The clinical protocols were approved by the Committee for Human Rights in Research at Weill Medical College of Cornell University. Oral consent was obtained for collection of venous blood.

**Measurement of TGF-β1 Protein Levels**

Peripheral venous blood was obtained from the patients and the sera were isolated and stored at $-70^\circ$C until assayed for TGF-β1. Blood samples were drawn before the dialysis procedure. The biologically active TGF-β1 protein concentration was determined using a solid-phase TGF-β1–specific sandwich ELISA (Promega) as described. The sera were activated by acidification and tested at 1:300 dilution. A TGF-β1 standard curve was constructed using 1000, 500, 250, 62.5, 31.25, and 15.6 pg/mL of recombinant human TGF-β1 protein, and a curve-fitting software program was used to quantify TGF-β1 protein concentration in the sera. The minimum level of detection of TGF-β1 with the sandwich ELISA was 25 pg/mL.

**TGF Codon 25 Genotyping by ARMS-PCR**

DNA for ARMS-PCR was extracted from peripheral blood cells as described. ARMS-PCR, an allele-specific PCR, is a powerful new tool for the ready detection of single-nucleotide polymorphisms. Homozygotes can be readily distinguished from heterozygotes, and the presence or absence of a given allele is discerned by inspection.

On the basis of the principle that oligonucleotides that are complementary to a target DNA sequence except for a mismatched 3' terminus will not function as PCR primers, we have designed and synthesized the primers for the identification of TGF-β1 codon 25 genotype and alleles (Arg25/Pro25) (Figure 1). Two complementary reactions were established for each allele, consisting of target DNA, allele-specific ARMS primer, and common primer. PCR products were resolved by agarose gel electrophoresis. A thermal cycler was used, and each experiment had negative and positive controls for the target allele.

**Statistics**

The correlation analysis procedure of SAS (Statistical Analysis Software Program) was used to calculate Pearson’s correlation coefficients between TGF-β1 protein levels and blood pressure in the ESRD population. The general linear models procedure was used to derive the linear regression equation relating TGF-β1 levels to blood pressure.

**Results**

**Patient Characteristics**

The demographics of the ESRD patients (study group 1) are shown in Table 1. The mean age of the patients was 58±2 (mean±SEM) years. There were 51 men and 47 women and 52 blacks and 46 whites. The demographics of study group 2 are also shown in Table 1. There were similar percentages of whites and blacks in the hypertensive and normotensive groups. The hypertensive subjects included 26 blacks and 45 whites and 21 men and 50 women. The normotensive controls included 21 blacks and 36 whites and 19 men and 38 women.
TABLE 1. Study Populations

<table>
<thead>
<tr>
<th>Study group 1, ESRD patients (n=98)</th>
<th>Race (Black/White), n</th>
<th>Gender (Men/Women), n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertensives (n=71)</td>
<td>57±14</td>
<td>26/45</td>
</tr>
<tr>
<td>Normotensives (n=57)</td>
<td>47±11</td>
<td>21/36</td>
</tr>
</tbody>
</table>

TGF-β1 and Blood Pressure

The blood pressure of the ESRD patients was 147/75±24/12 (mean±SD). The TGF-β1 level was 209±133 ng/mL, mean±SD. TGF-β1 levels were significantly associated with systolic (P=0.02), diastolic (P=0.01), and mean arterial blood pressure (P=0.008). (Table 2). The linear regression equation relating TGF-β1 levels to blood pressure and the individual levels of TGF-β1 protein as they relate to MAP, systolic blood pressure, and diastolic blood pressure are shown in Figure 2. We previously reported that TGF-β1 levels are significantly higher in black ESRD patients than in whites (P=0.0001). Gender, independent of race, did not affect TGF-β1 levels.

TABLE 2. TGF-β1 Protein Levels and Blood Pressure: Correlations in 98 ESRD Patients

<table>
<thead>
<tr>
<th>TGF-β1 Protein Levels and BP</th>
<th>Systolic BP</th>
<th>Diastolic BP</th>
<th>MAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>R*</td>
<td>0.22</td>
<td>0.24</td>
<td>0.26</td>
</tr>
<tr>
<td>P*</td>
<td>0.02</td>
<td>0.01</td>
<td>0.008</td>
</tr>
</tbody>
</table>

*R and P values derived by Pearson correlation coefficient.

Discussion

Our investigation demonstrates for the first time that a positive correlation exists between circulating levels of TGF-β1 and blood pressure in humans. Our analysis of TGF-β1, codon 25 DNA polymorphisms using the ARMS-PCR technique reveals that homozygosity for Arg at codon 25, and 8 were heterozygous Arg/Pro. Sixty-five of the 71 hypertensives were homozygous for Arg at codon 25, and 6 were heterozygotes. The frequency of Pro25 allele was 14% in normotensives and 8% in hypertensives.

vocular smooth muscle cell hypertrophy in spontaneously hypertensive rats, which is not observed in normotensive WKY rats. These observations, as well as the finding that direct transfer of TGF-β1 gene into arteries results in fibrocellular hyperplasia suggest that TGF-β1 plays a significant role in vascular remodeling in hypertensive conditions.

Evidence also exists for a role of TGF-β1 in cardiac and renal complications of hypertension. A recent study of 22 hypertensive patients with myocardial hypertrophy has identified TGF-β1 hyperexpression in monocytes from these individuals, when compared to monocytes from normotensive control subjects. TGF-β1 production has also been linked to progressive renal disease in experimental models of hypertensive renal injury, particularly salt-sensitive hypertension.

Increased production of TGF-β1 has been implicated in the progression of human kidney diseases as well, although to our knowledge hypertensive nephrosclerosis has not been studied.

Our data indicating that TGF-β1 correlates with blood pressure levels suggest that TGF-β1 may play a role in blood pressure regulation. TGF-β1 hyperexpression could contribute to hypertension through a number of mechanisms. In addition to stimulation of the expression of endothelin-1, TGF-β1 also stimulates the release of renin from juxtaglomerular cells. Furthermore, increased TGF-β1 resulting in progressive glomerular sclerosis could potentially exacerbate hypertension. Our data do not permit a determination of whether TGF-β1 hyperexpression is the cause of hypertension or the consequence. Our utilization of ESRD patients as a study group eliminated the consideration of renal function–dependent alterations in TGF-β1 protein levels. However, it is also possible that the basis for the correlation between blood pressure and TGF-β1 in patients with ESRD is that those individuals with increased renal fibrosis (and higher TGF-β1 levels) have higher blood pressure because of other mechanisms (eg, increased renal ischemia or decreased sodium excretion).

Several factors may contribute to TGF-β1 overproduction in patients with ESRD or hypertension, including increased angiotensin II, increased systemic blood pressure per se, and increased fluid shear stress. A genetic basis is also possible. Indeed, differential levels of expression of other cytokines, including TNF-α and IL-10, have been reported and linked to DNA polymorphisms in their promoters. Seven DNA sequence polymorphisms have been identified in the human TGF-β1 gene. In the recent ECTIM study of European subjects, systolic blood pressure of Arg25 homozygotes was higher and a history of hypertension more frequent compared with homozygotes or heterozygotes for the Pro25 allele. Moreover, Hutchinson et al demonstrated that patients with pulmonary allograft fibrosis had a higher frequency of the Arg25 allele compared with patients without fibrosis, and Arg25 homozygotes secreted higher amounts of TGF-β1 protein in vitro, compared with Arg25 heterozygotes.

In our investigation, we performed genotype analysis in patients with hypertension rather than ESRD, since sufficient blood was not available from the latter subjects. Furthermore, patients with ESRD may have a variety of underlying diseases that cause renal failure, including diabetes, glomer-
**Figure 2.** Correlation between blood pressure and TGF-β1 protein concentration. The individual values of TGF-β1 are related to mean arterial pressure (A), systolic pressure (B), and diastolic pressure (C). The linear regression equations derived by general linear models' procedure of SAS are also provided as inserts.
ulonephritis, and hypertension, some of which might be associated with polymorphisms in the TGF-$\beta_1$ gene that are independent of elevated blood pressure. TGF-$\beta_1$ protein levels are not yet available in these hypertensive subjects; thus it is not possible to determine whether the Arg$^{25}$ polymorphism is associated with increased protein levels in this population. Nevertheless, our data so far support the findings of the ECTIM study that the Arg$^{25}$ polymorphism is associated with higher blood pressure, although larger numbers than what we have studied appear to be needed to achieve statistical significance. Our preliminary results, demonstrating that the ARMS-PCR technique can be used to study TGF-$\beta_1$ gene polymorphisms, provide a basis for future studies of TGF-$\beta_1$ as a candidate gene in hypertension and for studies of the role of TGF-$\beta_1$ in determination of blood pressure.

### References
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