Expression of TGF-β and fibrogenic genes in transplant recipients with tacrolimus and cyclosporine nephrotoxicity

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Background. Long-term treatment with cyclosporine (CsA) or tacrolimus (Tac) results in chronic nephrotoxicity. Transforming growth factor-β (TGF-β) and other pro-fibrogenic molecules have been known to contribute to this side effect. A comparison of intrarenal expression of TGF-β and other fibrogenic genes in biopsies from patients with either CsA or Tac nephrotoxicity have not been documented. This study compared the expression of TGF-β, collagen, fibronectin, metalloproteinases (MMP-2, -9), tissue inhibitors of metalloproteinases (TIMP-2) and osteopontin in renal biopsies obtained from renal transplant recipients treated with either CsA or Tac as primary immunosuppressive agents.

Methods. Using RT-PCR, intrarenal expression of TGF-β, collagen, fibronectin, MMP-2, MMP-9 and TIMP-2 were studied in renal biopsies from patients with histological diagnosis of CsA or Tac nephrotoxicity and acute rejection. TGF-β protein expression was studied by staining section of biopsies with anti-TGF-β antibody.

Results. Intrarenal expression of TGF-β, collagen, fibronectin, MMP-2, TIMP-2, and osteopontin were significantly increased in patients treated with Tac nephrotoxicity compared with CsA nephrotoxicity. The intrarenal mRNA expression of these genes was higher in patients diagnosed with Tac/CsA nephrotoxicity compared to acute rejection.

Conclusions. This study compares the intrarenal expression of TGF-β and profibrogenic genes in renal transplant recipients treated with Tac and CsA. The results show that patients diagnosed with Tac nephrotoxicity exhibit increased expression of profibrogenic genes compared to CsA nephrotoxicity.

Cyclosporine (CsA) and tacrolimus (Tac) are two primary immunosuppressive agents used for the prevention of acute rejection. However, nephrotoxicity associated with these drugs remains a significant problem. The exact mechanism of the nephrotoxicity in renal allograft recipients remains elusive. Based on clinical and experimental studies, transforming growth factor-β (TGF-β) seems to have emerged as a leading candidate responsible for this effect. A number of in vitro and in vivo studies, including our own, have demonstrated hyperexpression of TGF-β with Tac and CsA treatment [1–13]. Our experimental studies also demonstrated TGF-β to be a potent immunosuppressive cytokine with fibrogenic properties as it induces the production of extracellular matrix proteins, collagen and fibronectin [2]. Therefore, it is conceivable that CsA and Tac exert their nephrotoxic effects via the induction of TGF-β and other pro-fibrogenic molecules.

This study was conducted to compare the intrarenal expression of TGF-β and other fibrogenic genes such as collagen, fibronectin, metalloproteinases (MMP-2, 9), tissue inhibitors of metalloproteinases (TIMP-2), and osteopontin in renal tissues from renal transplant recipients who experienced renal dysfunction when receiving CsA or Tac as primary immunosuppressive drugs.

METHODS

Patients

Renal biopsies from 47 transplant recipients who received either CsA or Tac for maintenance immunosuppression were obtained after an informed consent and an approval from Institutional Review Board. These biopsies were performed for elevated plasma creatinine (>20% baseline) using an 18-gauge Trucut needle with ultrasound guidance. An extra core was obtained and snap frozen in liquid nitrogen for this study. From 1997 to 1998, a total of 47 biopsies were performed. A total of 33 patients were diagnosed with nephrotoxicity: CsA toxicity (N = 16) and Tac toxicity (N = 17). Fourteen patients diagnosed with acute rejection also were included in this study. The mean age at the time of biopsy for these patients was calculated for the three groups: CsA toxicity (45 ± 4 years old), Tac toxicity (49 ± 3 years old) and acute rejection (50 ± 4 years old). Among

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the CsA toxicity, Tac toxicity, and acute rejection patients there were 10, 7 and 8 males, respectively. Similarly, there were 11, 12 and 6 Caucasians in these groups, respectively. There were no significantly differences for these parameters in these groups. At the time of biopsy the degree of renal function was measured by plasma creatinine; for the CsA toxicity, Tac toxicity and acute rejection groups, the creatinine levels were 2.6 ± 0.5, 2.4 ± 0.2 and 2.5 ± 0.4 mg/dL, respectively (NS). The renal dysfunction requiring biopsy occurred after a mean period of 22 months (4 to 78 months) for the CsA toxicity group, 28 months (3 to 84 months) for the Tac toxicity group, and 14.5 months (4 to 48 months) for the acute rejection group. The mean CsA dose and trough level values at the time of biopsy were 325 mg/day and 189 ng/mL (158 to 393 ng/mL), respectively. The corresponding values for Tac were 6 mg/day and 7.2 (4 to 14 ng/mL).

One tissue core was immediately snap-frozen in liquid nitrogen and stored at −80°C. The pathological diagnosis was confirmed by a nephropathologist. The histological findings of acute rejection and CsA/Tac nephrotoxicity were correlated to TGF-β and other fibrogenic molecules in a blinded fashion. The intrarenal expression of TGF-β, collagen, fibronectin, MMPs, TIMP-2, and osteopontin were compared in patients diagnosed with CsA/Tac nephrotoxicity and rejection treated with CsA or Tac.

Detection of mRNA by RT-PCR in kidney biopsies

To study the intrarenal expression of TGF-β and other fibrogenic molecules, total RNA was isolated from a core of biopsy from transplant patients using the SV Total RNA isolation System (Promega, Madison, WI, USA) and the quality of RNA was verified by 260/280 nm ratio. One microgram of RNA was reverse transcribed to cDNA using Superscript First Strand Synthesis system for reverse transcription-polymerase chain reaction (RT-PCR), Life Technologies, Rockville, MD, USA). The amplification by PCR was carried out using 1 μL of cDNA, 2 μL each of 2.5 mmol/L coding and non-coding oligonucleotide primers, and Platinum PCR Supermix (Life Technologies). The primer sequences for TGF-β1 were: coding 5'-ACA GGA AGC GTC ACT TCT CT-3' and non-coding 5'-TCC AAC TGG CAA AC-3' [14]; for β-actin, coding 5'-TCA GGA AGC GTC ACT TCT CT-3' and non-coding 5'-TCC AAC TGG CAA AC-3'; for TGF-β, coding 5'-ACA GGA AGC GTC ACT TCT CT-3' and non-coding 5'-TCC AAC TGG CAA AC-3'; for fibronectin, coding 5'-TCC AAC TGG CAA AC-3'.

For mRNA expression, the CsA toxicity, Tac toxicity, and acute rejection groups, the creatinine values were 325 mg/day and 189 ng/mL (158 to 393 ng/mL), respectively. The corresponding values for Tac were 6 mg/day and 7.2 (4 to 14 ng/mL).

Data analysis

The expression of TGF-β and other fibrogenic molecules were correlated with histologically confirmed acute rejection and CsA/Tac toxicity. The mRNA expression of TGF-β and other fibrogenic molecules also were compared between patients with CsA and Tac nephrotoxicity. Statistical analyses were performed using InStat, a Windows-based statistical program from GraphPad Software (San Diego, CA, USA). The data are expressed as mean ± SEM. Significance was assessed by the Student unpaired t test and a value of P < 0.05 was considered to be significant.
RESULTS

A total of 47 biopsies were performed in 47 transplant recipients. Of these 23 were on CsA as an immunosuppressive agent and the remaining 24 were on Tac. Acute rejection and CsA/Tac nephrotoxicity was diagnosed in 14 and 33 patients and recipients, respectively. CsA and Tac nephrotoxicity was diagnosed in 16 and 17 recipients, respectively.

Renal function

The levels of creatinine of patients diagnosed with Tac or CsA nephrotoxicity were not statistically different (2.4 ± 0.3 vs. 2.1 ± 0.2 mg/dL, P = 0.4).

TGF-β mRNA expression

The results of TGF-β expression are shown in Figure 1. Patients with CsA and Tac nephrotoxicity exhibited significantly increased expression of TGF-β compared to those diagnosed with acute rejection (0.4 ± 0.03, 0.2 ± 0.09 vs. 0.007 ± 0.003, P < 0.001 and <0.05, respectively). The expression of TGF-β mRNA was higher in patients with Tac nephrotoxicity compared to CsA nephrotoxicity (N = 17, 0.4 ± 0.03 vs. N = 16, 0.2 ± 0.09, P < 0.05).

Fibronectin mRNA expression

The results of fibronectin mRNA expression are shown in Figure 2. The expression of fibronectin mRNA was significantly (P < 0.01) increased in patients with Tac nephrotoxicity than with CsA nephrotoxicity (0.4 ± 0.1 vs. 0.1 ± 0.04, P < 0.0001). Similar to TGF-β expression, the expression of fibronectin mRNA was significantly (P < 0.03) higher in patients treated with Tac nephrotoxicity compared those with acute rejection (0.4 ± 0.1 vs. 0.1 ± 0.07). However, the expression in patients with CsA nephrotoxicity was identical to patients with acute rejection (0.1 ± 0.04 vs. 0.1 ± 0.04).

Collagen mRNA expression

The results of collagen mRNA expression are shown in Figure 3. The expression of collagen mRNA was not significantly different between patients with acute rejection
and CsA nephrotoxicity (0.1 ± 0.08 vs. 0.16 ± 0.5). However, the mRNA expression increased threefold in patients with Tac compared to CsA nephrotoxicity (0.3 ± 0.16 vs. 0.1 ± 0.08, P = 0.2).

**TIMP-2 and MMP-9 mRNA expression**

The results of intrarenal expression of metalloproteinase MMP-9 mRNA are shown in Figure 4. The expression of MMP-9 was increased in patients with Tac/CsA nephrotoxicity compared to acute rejection (0.05 ± 0.01). The mRNA expression of MMP-9 in CsA treated patients was significantly higher than with Tac (0.6 ± 0.09 vs. 0.9 ± 0.09, P < 0.05). The results of tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA expression are demonstrated also in Figure 4. The expression of TIMP-2 mRNA was not different between patients with Tac and CsA nephrotoxicity (0.9 ± 0.25 vs. 0.9 ± 0.26; however, levels were higher than patients with acute rejection (0.1 ± 0.01). We also calculated the ratio of MMP-9/TIMP-2 and it was lower in patients diagnosed with CsA nephrotoxicity than Tac nephrotoxicity.

**Osteopontin mRNA expression**

Osteopontin (OPN) is a TGF-β inducible glycoprotein, the synthesis of which in the distal tubules is increased in connection with tubulointerstitial nephritis. The results from our study demonstrate that in patients with nephrotoxicity and those treated with Tac, the expression of OPN mRNA was significantly increased compared to acute rejection (2.1 ± 0.15 vs. 1.6 ± 0.2, P < 0.05) and CsA nephrotoxicity (2.1 ± 0.15 vs. 1.5 ± 0.02, P < 0.02; Fig. 5). No difference was observed among patients with acute rejection and CsA nephrotoxicity.

**Intrarenal expression of TGF-β protein**

The intrarenal expression of TGF-β protein by was examined using an immuno-reaction with anti-TGF-β antibody. The results of TGF-β protein expression in biopsies from patients with CsA or Tac nephrotoxicity are shown from a representative slide of immunohistochemical staining for TGF-β in renal tissues. A and C nephrotoxicity compared to acute rejection (0.05 ± 0.01). The mRNA expression of TIMP-2 and it was lower in patients diagnosed with CsA nephrotoxicity than Tac nephrotoxicity.

**DISCUSSION**

Long-term treatment with CsA is associated with nephrotoxicity [22]. Initially it was believed that Tac treatment would result in decreased nephrotoxicity. However, studies of Randhawa et al [23] and Laskow et al [24] noted that the major toxicities associated with tacrolimus were nephrotoxicity, neurotoxicity and diabetes. Shapiro et al also studied the efficacy and safety of tacrolimus- and cyclosporine-based immunosuppressive regimens in kidney transplant recipients and observed that the side effects of tacrolimus included nephrotoxicity, neurotoxicity,
and diabetogenicity, and they were comparable to those seen with CsA [25]. Platz et al also compared the effects of Tac and CsA in renal transplant patients and showed that the late renal insufficiency appeared in 23.3% of CsA and in 29.4% of Tac treated patients [26]. A number of other studies revealed similar degree of nephrotoxicity in Tac treated patients [27–30]. Besides these clinical findings, similar results have been documented in several animal models [31–32] including those of Shihab et al [8], who also showed the induction of TGF-β following treatment with Tac in salt depleted rats. From our study in combination with these clinical and experimental studies, it is apparent that—like CsA—Tac induces hyperexpression of TGF-β and other fibrogenic molecules in renal transplant patients.

There is a paucity of data demonstrating the differences in CsA and Tac induced nephrotoxicity and their mechanism in renal transplant recipients. To the best of our knowledge, a comparison of intrarenal expression of fibrogenic genes in transplant patients treated with either CsA or Tac has not been explored. Our results demonstrate that tacrolimus treatment significantly increased the intrarenal expression of TGF-β compared to the patients treated with CsA (P < 0.05). In addition, the expression of TGF-β mRNA was significantly (P < 0.05) higher in patients diagnosed with drug nephrotoxicity than with acute rejection. Similarly, significantly more intense staining for TGF-β protein was observed in biopsies from patients with Tac nephrotoxicity compared to CsA nephrotoxicity (Fig. 6).

To further understand the events associated with an increased expression of TGF-β and those leading to nephrotoxicity in renal transplant recipients, we also studied the intrarenal expression of collagen and fibronectin in these patients. Our study demonstrates that in patients diagnosed with Tac nephrotoxicity, mRNA expression of fibronectin was significantly increased compared to acute rejection (P < 0.01) and CsA nephrotoxicity (P < 0.03). Similarly, the expression of collagen was increased 300% in the patients with tacrolimus nephrotoxicity compared to patients diagnosed with CsA nephrotoxicity and those receiving CsA. These results demonstrate that...
Besides up-regulation of TGF-β expression, these drugs cause the increased expression of extracellular matrix proteins, thus facilitating the process of drug-associated renal damage.

During tissue damage, a balance in the synthesis and degradation of extracellular matrix proteins has to be maintained. Metalloproteinases, also termed gelatinases, have been described as extracellular matrix degrading enzymes and are known to play a key role to maintaining this balance [33]. There are number of metalloproteinases numbered from MMP-1 to -17 with a variety of functional roles. Changes in the expression of metalloproteinases translate into altered extracellular matrix protein turnover and may play a role in histopathological changes [34]. Increased levels of metalloproteinases are often associated with disease status and infiltration of inflammatory cells [35]. A selective increase in these proteins may play a role in the ultimate fibrosis and other histological changes irrespective of the insulting injury.

The expression of MMP-9 was statistically increased in patients with CsA nephrotoxicity compared to patients with Tac nephrotoxicity. However, the difference among nephrotoxicity patients was statistically significant compared to patients with acute rejection. It can be interpreted that the increased expression of MMP-9 continues into the chronic phase of renal damage and may contribute to the extensive structural remodeling process that accompanies the nephrotoxic effects. We did not observe a significant difference with regards to MMP-2 mRNA expression among these patients.

Cyclosporine A nephrotoxicity is associated with an increase in glomerular extracellular matrices (ECMs). As discussed above, besides the regulations by MMPs, tissue inhibitors of metalloproteinases (TIMPs) are also considered to contribute to maintain homeostasis in the production and degradation of ECMs in the glomeruli [35]. TIMP-2 is an MMP inhibitor [36]. Paradoxically, it functions in vivo as a cofactor for MMP activation [37] and, therefore, it is pertinent to study the intrarenal expression of TIMP-2 also in these patients. Our study results demonstrate that the expression of TIMP-2, unlike that of MMP-9, was not different among patients with Tac or CsA nephrotoxicity. However, it was significantly higher when compared to acute rejection (P < 0.01). These results might demonstrate that mRNA expression of TIMP-2 and MMP-9 are increased in patients with nephrotoxicity.

We also studied the intrarenal expression of OPN. OPN is a TGF-β inducible glycoprotein that was first characterized in bone tissue and subsequently has been detected in many other tissues, including the kidney [38]. It has been demonstrated that synthesis of OPN in the distal tubules is increased in tubulointerstitial nephritis. More interestingly, it is a chemotactic factor for macrophages and T-cells, and causes the influx of macrophages in acute rejection; its role in various forms of renal injury has been highlighted [39]. Our study demonstrates that the expression of OPN mRNA was significantly increased with Tac nephrotoxicity compared to acute rejection (P < 0.05) and CsA nephrotoxicity (P < 0.03). To the best of our knowledge, mRNA expression of OPN has not been explored in detail, nor has it been compared in patients with Tac or CsA nephrotoxicity.

In summary, this report highlights the expression of TGF-β and pro-fibrogenic molecules in the renal tissues of renal transplant recipients treated with either Tac or CsA. The results demonstrate, to our knowledge for the first time, that the expression of these molecules increases considerably in recipients with Tac nephrotoxicity when compared with CsA nephrotoxicity and with acute rejection irrespective of the drug treatment. Our studies may explain in part the mechanism of nephrotoxicity observed in renal transplant recipients treated with Tac and CsA. We studied only the TGF-β protein expression in these biopsies; however, studies on the expression of other proteins and circulating levels of the protein relevant to fibrogenesis will further delineate the role of these molecules in nephrotoxicity. Also, future studies with specific inhibitors of TGF-β and/or metalloproteinases will be an avenue to alleviate the side effects of these agents.

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