Molecular Comparison of Calcineurin Inhibitor–Induced Fibrogenic Responses in Protocol Renal Transplant Biopsies

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The calcineurin inhibitor cyclosporine (CsA) induces a fibrogenic response that may lead to scarring of the renal allograft. This study investigated whether tacrolimus, a novel calcineurin inhibitor, exerts fibrogenic effects to a similar extent. Sixty patients were enrolled in a randomized study: 29 received CsA, and 31 received tacrolimus. Patients were subjected to tailored exposure-controlled calcineurin inhibitor regimens. Protocol biopsies were obtained at the time of transplantation and 6 and 12 mo after transplantation. Cortical TGF-β and collagens α1(I) and α1(III) mRNA steady-state levels were determined with real-time PCR. The extent of protein deposition of TGF-β, α-smooth muscle actin, and interstitial collagens in the renal cortex was quantified with computer-assisted image analysis. The extent of interstitial collagen deposition measured with Sirius red and the accumulation of α-smooth muscle actin and TGF-β protein after 6 and 12 mo were similar for both immunosuppressive regimens. mRNA levels of TGF-β and collagens α1(I) and α1(III) were not significantly different in the treatment groups either. It is concluded that the fibrogenic response in renal allografts is similar in patients who receive CsA-based regimens and patients who receive tacrolimus-based regimens.


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It was the objective of this study to compare the fibrogenic effect of CsA and tacrolimus at the mRNA and protein levels. Randomized patient groups were used in a prospective manner. Protocol biopsies were taken before transplantation and 6 and 12 mo after transplantation. Controlled target area-under-the-concentration-over-time-curve guided dosing of both calcineurin inhibitors was used to maintain efficacy and minimize toxicity. This was accomplished using a population-based pharmacokinetic model together with limited sampling combined with Bayesian estimation.

Materials and Methods

Patient Groups, Drug Monitoring, and Study Design

It has been estimated that a controlled trial regarding the effects of two different immunosuppressive regimens powered to demonstrate a 10% difference in renal allograft fibrosis at 6 mo posttransplantation would require 23 patients to be randomly assigned to each treatment arm (25). Therefore, 65 patients were enrolled in an open, prospective, randomized, clinical trial at the Leiden University Medical Center in The Netherlands. Before transplantation, patients were randomly assigned 1:1 to receive either a CsA microemulsion–based (Neoral; n = 33) or tacrolimus-based (Prograf; n = 32) immunosuppressive regimen. CsA and tacrolimus were started orally 3 h before surgery (initial dose 4 mg/kg twice per day for CsA and 0.1 mg/kg for tacrolimus). In the first week, target 12-h trough levels were aimed at 225 ng/ml (range 200 to 250 ng/ml) and 12.5 ng/ml (range 10 to 15 ng/ml) for CsA and tacrolimus, respectively. CsA and tacrolimus AUC0 to 12h, were estimated at weeks 2, 4, 6, 8, 12, 17, 21, 26, 39, and 52 with a population-based two-compartmental pharmacokinetic model that was combined with Bayesian fitting and limited sampling. After each AUC assessment, doses were adjusted to comply with predefined target AUC: CsA AUC0 to 12h was 5400 ng·h/ml within the first 6 wk (corresponding with a mean trough level of 225 ng/ml) and 3250 ng·h/ml after the initial 6 wk (corresponding with a mean trough level of 125 ng/ml). Tacrolimus AUC0 to 12h was 210 ng·h/ml within the first 6 wk (corresponding with a mean trough level of 12.5 ng/ml) and 125 ng·h/ml after 6 wk (corresponding with a mean trough level of 7.5 ng/ml). Immunosuppressive co-medication consisted of prednisolone (in both groups 100 mg at days 1 to 3, 50 mg at day 4, 20 mg at days 5 to 14, 15 mg at days 15 to 21, and 10 mg after day 22), mycophenolate mofetil (1000 mg twice per day in the CsA group and 500 mg twice per day in the tacrolimus group), and basiliximab prophylaxis (20 mg intravenously at day 0 and day 4). Drugs that are known to alter concentrations of the calcineurin inhibitors were prohibited.

Protocol biopsies were taken before transplantation (t0) and 6 and 12 mo after transplantation. Biopsies were scored according to the Banff criteria. These were excluded from further study. Four patients from the CsA cohort and one patient from the tacrolimus cohort showed morphologic signs of acute rejection in the protocol biopsies and were excluded. Thus, in our study, 29 patients who received CsA and 31 patients who received tacrolimus were included.

Control Groups

mRNA transcripts from 16 kidneys with normal histology (12 samples from the unaffected part of a tumor nephrectomy kidney and four cadaver donor kidneys that initially were intended for transplantation purposes) and 28 acute rejection biopsies (20 Banff type Ia and 8 Banff type Ib) were studied.

RNA Extraction

Frozen biopsy tissue was available for 34 t0 biopsies, 53 6-mo biopsies, and 48 12-mo biopsies. A 2-μm section from each biopsy was analyzed with light microscopy to demonstrate the presence of the renal cortex according to a procedure that was described in a previous study (27). Ten to fifteen 10-μm sections of renal cortex were cut in a Leica CM3050 S cryostat, collected in an Eppendorf tube, and stored at −70°C until usage. RNA extraction was performed with RNeasy spin columns (Qiagen, Westburg, The Netherlands). RNA concentration was determined with photospectometry. The mean A260 to A280 ratio of the samples was between 1.8 and 2.0. A maximum amount of 1 μg of RNA was used as input for cDNA synthesis (mean input 0.75 ± 0.3 μg of RNA). The cDNA reactions (24 μl total volume) consisted of the following reagents: 0.5 mM dNTP, 100 ng oligo dT (Roche, Mannheim, Germany), 500 ng random hexamer primers (Invitrogen, Breda, The Netherlands), 5 U of avian myeloblastosis virus–reverse transcriptase (RT-AMV) (Roche), 20 U of rRNasin (Promega Benelux BV, Leiden, The Netherlands), and 1× reverse transcriptase buffer (Roche).

Real-Time PCR

A real-time PCR with the ABI Prism 7700 Sequence Detector System (Perkin Elmer Biosystems, Foster City, CA) was performed. This procedure was described in detail previously (28). cDNA samples were diluted at a ratio of 1:50, and 5 μl of the dilution was used for PCR. mRNA levels of TGF-β and collagens α1(I) and α1(III) were quantified and normalized for the mean of mRNA levels of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase and hypoxanthine guanine phosphoribosyltransferase-1. A previous study showed that there is a significant correlation between these housekeeping genes (29). In our study, levels of glyceraldehyde-3-phosphate dehydrogenase significantly correlated with those of hypoxanthine guanine phosphoribosyltransferase-1 (r = 0.61, P < 0.001). Primer and probe sequences and PCR conditions were described previously (29). To allow comparison of samples from different PCR plates, we included in each PCR run a 1:5 dilution range of a reference sample.

Immunohistochemistry and Quantification of Stainings

Paraffin-embedded tissue was available for 54 6-mo biopsies and 54 12-mo biopsies. Four-micrometer sections were cut and used for immunohistochemistry. For detection of deposition of TGF-β, slides were heated in a 0.01-M citrate buffer solution for 10 min and subsequently cooled for 20 min. Slides then were incubated for 1 h at room temperature with polyclonal antibodies against TGF-β before 30 min of incubation with anti-rabbit EnVision (Dako, Glostrup, Denmark) as the secondary antibody. The rabbit polyclonal anti-human TGF-β antibody that was raised against a synthetic peptide that corresponded to the C-terminal amino acids 371 to 390 of human TGF-β1, had been synthesized in our laboratory (30,31). Antibody specificity was confirmed further by Western blotting and immunoabsorption assays. To detect α-smooth muscle actin (α-SMA)–positive cells, slides were stained for 1 h with mAb against α-SMA (Promega Benelux BV) and thereafter incubated for 30 min with anti-mouse EnVision (Dako). NovaRed (Vector, Burlingame, CA) was used to visualize the immunohistochemical signal in both stainings. Further details on staining protocols were described in a previous report (32). For each immunohistochemical assessment, all sections were stained simultaneously in one session. The extent of interstitial collagen accumulation was visualized in both paraffin sections of 6-mo and 12-mo biopsies and frozen sections of t0
biopsies with Sirius red staining, a process that was described previously (33). Slides from frozen biopsies first were fixed in formalin for 10 min before Sirius red staining.

Digital analysis was performed on each biopsy with a Zeiss microscope that was equipped with a full-color 3CCD camera (Sony DYC 950p, Sony Corp., Tokyo, Japan) and KS-400 image analysis software version 3.0 (Zeiss-Kontron, Eching, Germany) to quantify the extent of staining. An average of 10 microscopic sections were examined from each slide, commencing at the capsular side and following in a linear manner. Using the ×20 objective, an average of 63.2 ± 19.5% of the total cortex was analyzed in each biopsy. For both Sirius red and the α-SMA staining, blood vessels larger than adjacent tubules were excluded. The procedure was described previously (28).

Statistical Analyses

Differences between groups were assessed with independent-samples t test. Correlations between expression levels were analyzed with Pearson correlation tests. Analyses were performed with SPSS software (version 10.0; SPSS, Inc., Chicago, IL). P < 0.05 was considered statistically significant.

Results

Patient Population

Twenty-nine participants received CsA, and 31 received tacrolimus. Demographic characteristics in the treatment groups were similar, as depicted in Table 1. There were no significant differences between treatment groups regarding the donor’s and the recipient’s age and gender, cold ischemia time, occurrence of delayed graft function, donor source, BP, antihypertensive medication use, total HLA mismatches, incidence of cytomegalovirus infection, and GFR 6 and 12 mo after transplantation. There was no significant difference in the number of patients who showed borderline subclinical rejection after 6 and 12 mo between treatment groups. In these groups, GFR was not different for the patients with borderline subclinical rejection and for those without. During the first 12 mo after transplantation, 10 biopsies were taken on clinical indication in addition to the protocolized biopsies (five in each treatment group). These were taken within the first 2.5 mo after transplantation. In the CsA group, four biopsies showed signs of acute rejection and one was taken because of delayed graft function (no morphologic abnormalities). In the tacrolimus group, three biopsies were taken because of delayed graft function and one because of a slight increase in creatinine (all demonstrating no morphologic abnormalities). The fifth biopsy taken showed signs of acute transplant glomerulopathy.

Calcineurin-inhibitor nephrotoxicity (CIN) was regarded as the presence of nodular arteriolar hyalinosis and/or striped

<table>
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<tr>
<th>Table 1. Clinical parameters of CsA- and tacrolimus-based regimensa</th>
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<td>Cold ischemia time (h)</td>
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<td>12 mo</td>
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*Patients with acute rejection (AR), as defined by the Banff 97 classification, in the 6- or 12-mo protocol biopsies were excluded from the study. Data are means ± SD. CsA, cyclosporine; CMV, cytomegalovirus.*
fibrosis and tubular microcalcification. We found CIN to be present in 13% of the biopsies that were obtained at 6 mo and in 18% of the biopsies that were obtained at 1 yr. No biopsies showed signs of intimal arteritis. During the course of the study, an increase in vascular fibrous intimal thickening was found in 12 patients (nine in the tacrolimus group and three in the CsA group).

mRNA Assessments
The mRNA levels of TGF-β and collagens α1(I) and α1(III) at t0, after 6 mo, and after 12 mo for the study groups are summarized in Table 2. In both treatment groups, the mean TGF-β mRNA levels 6 and 12 mo after transplantation were significantly higher than those at the time of transplantation (P < 0.005). No significant difference was found in TGF-β mRNA levels between treatment regimens at t0 (CsA 0.14 ± 0.09 versus tacrolimus 0.11 ± 0.08), at 6 mo (CsA 0.54 ± 0.30 versus tacrolimus 0.53 ± 0.37), or at 12 mo (CsA 1.03 ± 0.60 versus tacrolimus 0.91 ± 0.62; Figure 1A). There were no significant differences in collagens α1(I) and α1(III) mRNA expression levels at t0, after 6 mo, or after 12 mo in the treatment groups either (Figure 1, C and D).

The tacrolimus recipients showed significant correlations at each time point between mRNA expression levels of TGF-β with collagens α1(I) and α1(III) (6 mo r = 0.80 and 0.77; 12 mo r = 0.71 and 0.55). There was a significant correlation between collagens α1(I) and α1(III) at 6 mo (r = 0.90, P < 0.01) and at 12 mo (r = 0.87, P < 0.01) in the patients who were treated with tacrolimus. Significant correlations were found at 6 mo of TGF-β with both collagens α1(I) and α1(III) (r = 0.46 and r = 0.68, P < 0.05) for the patients who were treated with CsA. This significance was also found at 12 mo (r = 0.45 and r = 0.72, P < 0.05). Collagens α1(I) and α1(III) correlated significantly in the CsA treatment group after both 6 and 12 mo (r = 0.86 and r = 0.80, respectively).

Interstitial Protein Deposition
The extent of protein deposition of TGF-β and α-SMA and the extent of Sirius red staining for both treatment groups are summarized in Table 2. Consistent with the findings for TGF-β mRNA expression, no significant difference in TGF-β protein expression levels was seen between treatment groups after 6 mo (CsA 5.61 ± 6.12% versus tacrolimus 5.48 ± 4.04%) and after 12 mo (CsA 4.48 ± 3.08% versus tacrolimus 4.64 ± 4.46%; Figure 1B).

The Sirius red staining was performed in duplicate on all sections. A significant correlation was found between duplicate measurements (6 mo r = 0.75, 12 mo r = 0.80; P < 0.001). For each patient, the mean of the duplicate measurements was calculated. There was no significant difference in the extent of Sirius red staining between CsA and tacrolimus treatment regimens (t0: CsA 14.2 ± 5.8% versus tacrolimus 14.8 ± 6.4%; 6 mo: CsA 24.3 ± 5.0% versus tacrolimus 23.4 ± 4.0%; 12 mo: CsA 23.4 ± 5.1% versus tacrolimus 23.5 ± 4.5%; Figure 2A). In accordance with the results found for Sirius red, the extent of α-SMA staining after 6 mo (CsA 7.08 ± 2.60% versus tacrolimus 6.88 ± 2.39%) and after 12 mo (CsA 7.75 ± 2.78% versus tacrolimus 7.01 ± 2.49%) was not significantly different between the treatment groups (Figure 2B).

Correlations between Demographics and Fibrogenic Markers
Two correlations were found between demographics, as depicted in Table 1, and the results found in mRNA and protein evaluation. At 12 mo, donor age and mRNA levels for both collagens α1(I) and α1(III) at 12 mo correlated with each other (collagen α1(I) r = −0.351, P < 0.05; α1(III) r = −0.447, P < 0.005).

Discussion
Chronic graft damage can be attributed partly to the toxic effect of immunosuppressive agents (7). CsA has been shown to cause fibrosis (8), but it is controversial whether tacrolimus induces a similar effect on renal allografts (9,10). The objective of our study was to compare the fibrogenic effect of CsA and tacrolimus at the mRNA and the protein level in whole cortical tissue of protocol transplant biopsies from rejection-free patients who were exposed to tailored regimens of these drugs. With both CsA and tacrolimus, a progressive increase in expression of fibrogenic molecules was observed over time. We found no significant difference in expression of fibrosis-related components between treatment groups.

Table 2. Fibrogenic molecules in CsA- and tacrolimus-based regimens

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<th>CsA</th>
<th>Tacrolimus</th>
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<tr>
<td></td>
<td>t = 0</td>
<td>6 mo</td>
<td>12 mo</td>
<td>t = 0</td>
</tr>
<tr>
<td>TGF-β1 mRNA</td>
<td>0.14 ± 0.09</td>
<td>0.54 ± 0.30b</td>
<td>1.03 ± 0.60b</td>
<td>0.11 ± 0.08</td>
</tr>
<tr>
<td>Collagen α1(I) mRNA</td>
<td>0.16 ± 0.21</td>
<td>2.84 ± 4.48c</td>
<td>1.92 ± 2.79</td>
<td>0.17 ± 0.31</td>
</tr>
<tr>
<td>Collagen α1(III) mRNA</td>
<td>0.18 ± 0.30</td>
<td>1.38 ± 1.82c</td>
<td>1.70 ± 2.96</td>
<td>0.17 ± 0.34</td>
</tr>
<tr>
<td>TGF-β staining</td>
<td>—</td>
<td>5.61 ± 6.12</td>
<td>4.48 ± 3.08</td>
<td>—</td>
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<tr>
<td>α-SMA deposition</td>
<td>—</td>
<td>7.08 ± 2.60</td>
<td>7.75 ± 2.78</td>
<td>—</td>
</tr>
<tr>
<td>Sirius red (%)</td>
<td>14.2 ± 5.8</td>
<td>24.3 ± 5.0b</td>
<td>23.4 ± 5.1b</td>
<td>14.8 ± 6.4</td>
</tr>
</tbody>
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aData are means ± SD. α-SMA, α-smooth muscle actin.

bP < 0.005 versus t0.

cP < 0.05 versus t0.
Opposing results have been found in recently published studies of renal protocol transplant biopsies with respect to the influence of CsA and tacrolimus on fibrogenesis and intragraft expression levels of TGF-β and ECM molecules (22–24). Most of these studies included biopsies that were taken within 1 yr after transplantation and demonstrated lower TGF-β expression levels in tacrolimus-treated recipients (18,19,22–24). This may suggest that fibrosis develops slower in tacrolimus-treated patients than in patients who were treated with CsA. However, several of these studies included renal biopsies that were taken for diagnostic purposes. Thus, acquired results may be distorted through the presence of infiltrating cells as a result of acute rejection or through the presence of chronic damage that resulted from long-term medication use. Furthermore, previous studies in protocol biopsies did not examine mRNA and protein levels simultaneously and did not answer conclusively the question of whether increments in mRNA levels of fibrogenic molecules result in increased deposition of the corresponding proteins.

Previous studies showed that the extent of fibrosis, demonstrated by Sirius red staining, in 6-mo protocol biopsies predicts renal function deterioration (34,35). We found that the extent of

Figure 1. Fibrogenic mRNA molecules and TGF-β protein expression in protocol biopsies of cyclosporine (CsA-) and tacrolimus-treated patients. mRNA expression and protein deposition were determined using real-time PCR and immunohistochemistry, respectively. (A) TGF-β mRNA levels in protocol biopsies at t0, after 6 mo, and after 12 mo did not significantly differ between treatment regimens. As a comparison, expression was measured in 28 biopsy samples with acute rejection (AR); TGF-β mRNA levels were significantly higher in clinical rejection biopsies than in protocol biopsies. (B) TGF-β protein staining in protocol biopsies after both 6 and 12 mo was not significantly different in the immunosuppressive medication groups either. (C and D) No significant difference was found between medication regimens for both collagen α1(I) and collagen α1(III) mRNA levels at any time.
fibrosis, measured with digital analysis of Sirius red staining, did not significantly differ between CsA and tacrolimus in our cohort at any moment. The development of interstitial fibrosis is for a large part mediated by the action of interstitial myofibroblasts, which are positive for \( \alpha \)-SMA (36). We performed immunohistochemical staining for \( \alpha \)-SMA in our patient cohorts and did not find a significant difference between medication groups.

We found higher mRNA expression levels of TGF-\( \beta \) and collagen \( \alpha \)(I) in acute rejection biopsies than in the protocol biopsies without rejection (Figure 1). This is another indication that the expression of fibrogenic molecules can be affected by the presence of such rejection episodes. Recipients who showed clinical or morphologic signs of acute rejection in their protocol biopsies therefore were excluded from this study. No significant differences were found for TGF-\( \beta \) mRNA expression levels and TGF-\( \beta \) protein deposition between patients who were treated with either CsA or tacrolimus. This finding is in accordance with previous findings for TGF-\( \beta \) mRNA expression in glomeruli (22). No significant correlation between TGF-\( \beta \) mRNA levels and TGF-\( \beta \) protein levels was found, which might be because the antibodies against TGF-\( \beta \) visualize both its active and its latent and inactivated forms. Because TGF-\( \beta \) mRNA levels significantly correlated with collagens \( \alpha \)(I) and \( \alpha \)(III) mRNA levels in this study, we suppose that mRNA assessment gives a good impression of the extent of TGF-\( \beta \) activity in this case.

In both tacrolimus and CsA treatment groups, TGF-\( \beta \) mRNA levels progressively rose from their time of transplantation until 12 mo later. Because the patients who had been treated with tacrolimus and CsA showed no differences with respect to TGF-\( \beta \) mRNA expression levels at any moment, these findings suggest that the two drugs have a similar effect on TGF-\( \beta \) mRNA synthesis. Similarly, mRNA levels of interstitial collagens \( \alpha \)(I) and \( \alpha \)(III) rose over time in both treatment groups. CsA has been known to target the promoter fragment of collagen III (37) and in the process specifically affects the rate of synthesis of the mRNA transcript. A similar mechanism may be involved in the case of collagen \( \alpha \)(I). It is not clear whether tacrolimus also has the capacity to interact with response elements in the interstitial collagen genes.

Our study puts forward results that seem to be at variance with the results in previous studies in the literature, because no significant difference in fibrogenic response of the kidney graft between tacrolimus and CsA was observed in this study. This difference may be because, in this study, patients received tailored calcineurin-inhibitor regimens. The calcineurin inhibitor regimens follow a population-based two-compartmental pharmacokinetic model, which requires only limited sampling and is combined with Bayesian estimation. As previous studies have shown, this method gives an accurate and precise estimation of systemic exposure while being very flexible in allowing nonrigid sampling times as long as dosing and sampling times are recorded accurately (38,39).

It seems that this study is the first to have compared predefined area-under-the-concentration-over-time-curve guided dosing regimens of tacrolimus and CsA through an assessment of the expression of TGF-\( \beta \) and ECM molecules in protocol renal allograft biopsies that are free of rejection. CsA and tacrolimus have a similar inducing effect on intragraft TGF-\( \beta \) and collagens \( \alpha \)(I) and \( \alpha \)(III) mRNA levels within the first 12 mo after transplantation. The extent of deposition of TGF-\( \beta \) protein and interstitial collagens did not significantly differ between recipients of either CsA or tacrolimus, which shows that the fibrogenic response of renal allografts is similar for either calcineurin inhibitor.

**Figure 2.** The extent of interstitial collagen deposition and the accumulation of \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) protein. (A) Sirius red staining was performed to assess the extent of interstitial collagen accumulation. No difference in interstitial staining was found between CsA and tacrolimus treatment regimens. (B) \( \alpha \)-SMA staining was performed as a marker for interstitial myofibroblasts. There was no significant difference in the extent of \( \alpha \)-SMA staining between treatment groups after 6 and 12 mo.
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