Role of transforming growth factor-β1 in experimental chronic cyclosporine nephropathy

FUAD S. SHIHAB, TAKESHI F. ANDOH, AMIE M. TANNER, NANCY A. NOBLE, WAYNE A. BORDER, NORA FRANCESCHINI, and WILLIAM M. BENNETT

Division of Nephrology, University of Utah and Department of Veterans Affairs Medical Center, Salt Lake City, Utah, and Division of Nephrology, Hypertension and Clinical Pharmacology, Oregon Health Sciences University, Portland, Oregon, USA

Role of transforming growth factor (TGF)-β1 in experimental chronic cyclosporine nephropathy. The pathogenesis of fibrosis in chronic cyclosporine (CsA) nephropathy remains unknown. Since TGF-β1 plays a key role in the fibrogenesis of a number of renal diseases, we studied a salt-depleted rat model of chronic CsA nephropathy which shows similarity to the structural and functional lesions described in patients. Pair fed rats were treated with either CsA (15 mg/kg/day s.c.) or an equivalent dose of olive oil and sacrificed at 7 and 28 days. Characteristic histologic changes of proximal tubular injury, tubulointerstitial fibrosis and arteriolopathy developed in CsA-treated rats at day 28. They were accompanied by physiologic changes of increased serum creatinine, decreased creatinine clearance, increased enzymuria and decreased concentrating ability. CsA-treated rats showed a progressive increase in mRNA expression of TGF-β1 and matrix proteins at days 7 and 28. Most of the changes were in the tubulointerstitial and vascular compartments by immunofluorescence with a predominant involvement of the medulla as compared to cortex. The mRNA expression of plasminogen activator inhibitor, a protease inhibitor stimulated by TGF-β1, followed TGF-β1 and matrix proteins, suggesting that the fibrosis of chronic CsA nephropathy likely involves the dual action of TGF-β on matrix deposition and degradation.

The introduction of cyclosporine A (CsA) into clinical practice has resulted in a major improvement in the short term outcomes of solid organ transplantation [1] and the treatment of autoimmune diseases [2]. However, with increased use has come the recognition that CsA has severe deleterious effects on renal structure and function making chronic nephrotoxicity a major limiting side effect [3]. Acute renal dysfunction due to CsA is reversible since it involves renal hemodynamic dysfunction and is not associated with any permanent histologic changes [4]. On the other hand, chronic CsA nephrotoxicity may progress to an irreversible renal lesion characterized by striped interstitial fibrosis, tubular atrophy and hyalinosis of the afferent arterioles [5, 6].

Chronic CsA nephrotoxicity has been described in kidneys of recipients of renal and other organ allografts, as well as in patients treated with CsA for autoimmune diseases [7–9]. In addition, progression to end-stage renal disease with chronic CsA use is well documented [10]. However, the exact mechanism underlying the development of fibrosis in chronic CsA nephrotoxicity remains poorly understood [11]. The observed striped interstitial fibrosis does not improve consistently with discontinuation of the drug and occurs even in patients treated with low doses of CsA [6]. Because the fibrotic lesion of chronic CsA nephrotoxicity has been difficult to reproduce in animals, the lack of an animal model of chronic CsA nephrotoxicity has hampered the study of its pathogenesis. Using the observation that sodium depletion exacerbates CsA nephrotoxicity, a reproducible animal model of chronic CsA nephrotoxicity was recently established [12]. In this model, CsA treatment in rats on low salt diet induced physiologic and histologic features that resemble the human lesion described in patients on long-term CsA therapy [13].

To date, no study has looked at the components of the fibrotic lesion of chronic CsA nephrotoxicity in vivo and none has attempted to correlate the renal fibrosis or the expression of ECM components with any growth factor. Transforming growth factor-β (TGF-β) has been implicated in the fibrosis of a number of chronic diseases of the kidney and other organs [14, 15]. TGF-β directly stimulates the synthesis of individual ECM components [16–18]. It also blocks ECM degradation by decreasing the synthesis of proteases and stimulating protease inhibitors like plasminogen activator inhibitor-1 (PAI-1) [19]. In addition to its effect on matrix accumulation, TGF-β also promotes immune suppression [20, 21].

Since TGF-β is a key fibrogenic cytokine, we hypothesized that TGF-β overexpression might be responsible for the fibrosis of chronic CsA nephrotoxicity. To test this hypothesis, we utilized this animal model with lesions similar to human chronic CsA nephropathy. Our findings indicate that fibrosis is mostly expressed in the medulla, that all major components of the ECM are elevated and that their expression correlates with an increased expression of TGF-β and PAI-1. These results suggest that the ECM accumulation observed with chronic CsA therapy likely involves the dual action of TGF-β on ECM deposition and degradation.

Methods

Experimental design

Adult, male Sprague-Dawley rats (Charles River, Wilmington, MA, USA), 225 to 250 g, were housed in individual cages in a temperature and light controlled environment, and received a low salt diet (0.05% sodium, Teklad, Premier, WI, USA), with water.
ad libitum. After one week on the low salt diet, weight matched pairs of rats were randomly assigned to receive daily subcutaneous injections of CsA 15 mg/kg (Sandimmune®, Sandoz Research Institute, East Hanover, NJ, USA), or an identical volume of the vehicle olive oil as a control. Control rats were fed the exact amount of food consumed the day before by the CsA-treated rats. Animals were divided into two groups of 16 CsA-treated and 16 control rats. Eight rats from each group were studied at days 7 and 28. Body weight was recorded daily. At the end of each treatment period, 24-hour quantitative urine samples were collected in metabolic cages (Nalge Co., Rochester, NY, USA) and systolic blood pressure (BP) was measured by tail plethysmography (Narlo Bio-systems, Houston, TX, USA). The following day animals were anesthetized with ketamine and a blood sample was obtained. After opening the abdomen through a midline incision, the abdominal aorta was cannulated retrogradely below the renal arteries with an 18-gauge needle. With the aorta occluded by ligation above the renal arteries and the renal veins opened by a small incision for outflow, the kidneys were perfused with 20 ml of cold heparinized saline. The kidneys were removed, the cortex was carefully dissected from the medulla, and the tissues were processed for evaluation by light microscopy, RNA analysis and immunohistochemistry.

**Functional studies**

Urinary and plasma osmolality were measured by flame photometer (Instrumentation Laboratories, Lexington, MA, USA). Urinary and plasma sodium were measured by flame photometer depression (Omett A, Precision Systems Inc., Natick, MA, USA). Urinary and serum creatinine, urinary protein (Uprot), urinary alanine aminopeptidase (AAP) and urinary N-acetyl β-D-glucosaminidase (NAG) were measured by a Cobas autoanalyzer (Roche Diagnostics, Div. Hoffman-La Roche Inc., Nutley, NJ, USA). CsA blood levels were determined in whole blood by a specific radioimmunoassay (Instar Corp., Stillwater, MN, USA). The creatinine clearance (Ccr), fractional excretion of sodium (FENa) and free water reabsorption (TcW) were calculated using standard formulae.

### Table 1. Physiologic parameters in CsA and placebo (VH) treated rats at 7 and 28 days

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VH</td>
<td>CsA</td>
</tr>
<tr>
<td>Scr mg/dl</td>
<td>0.37 ± 0.02</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>Ccr ml/min/100 g</td>
<td>0.55 ± 0.02</td>
<td>0.41 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FE&lt;sub&gt;Na&lt;/sub&gt; %</td>
<td>0.06 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Urine AAP IU/gCr</td>
<td>45 ± 10</td>
<td>168 ± 34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urine NAG IU/gCr</td>
<td>25 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>UV ml/24 hr</td>
<td>10.2 ± 1.2</td>
<td>12.9 ± 1.4</td>
</tr>
<tr>
<td>U&lt;sub&gt;Osm&lt;/sub&gt; mOsm/kg</td>
<td>1,848 ± 253</td>
<td>1,037 ± 178&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TcW ml/24 hr</td>
<td>49 ± 6</td>
<td>28 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of eight rats. Abbreviations are: VH, placebo; Scr, serum creatinine; Ccr, creatinine clearance; FE<sub>Na</sub>, fractional excretion of sodium; AAP, alanine aminopeptidase; NAG, N-acetyl β-D-glucosaminidase; UV, urinary volume; U<sub>Osm</sub>, urinary osmolality; TcW, free-water reabsorption.

<sup>a</sup> P < 0.01 vs. VH

<sup>b</sup> P < 0.05 vs. VH

### Morphology

Renal tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Two to four micron thick sections were stained with periodic acid-Schiff's reagent (PAS) and Trichrome. Horizontal and sagittal sections were both performed. The histologic findings were subdivided into three categories: proximal tubular injury, interstitial inflammation and scarring, and arteriolopathy. Findings ascribed to tubular injury included cellular and intercellular vacuolization, tubular collapse (unassociated with interstitial fibrosis or tubular membrane thickening) and tubular distention. Features of interstitial inflammation were mononuclear infiltrates, edema and vacuolization of interstitial cells. The findings of scarring were matrix-rich expansion of the interstitium with distortion of the tubules and thickening of the tubular basement membranes. Renal arteriolopathy in chronic CsA-induced nephrotoxicity was characterized by hyalinization and destruction of the afferent arterioles. The hyalinization consisted of hyalin deposition within the tunica media of afferent arterioles and terminal portion of interlobular arteries. A minimum of 20 fields at 100× magnification were assessed and graded in each biopsy by an observer masked to treatment groups using a color image analyzer (Olympus C1A-102, Olympus Inc., Tokyo, Japan).

The following semiquantitative score was used to assess the extent of changes in each category. For tubular injury, the following score was utilized: 0 = no tubular injury; 0.5 = < 5% of tubules injured; 1 = 5 to 20% of tubules injured; 1.5 = 21 to 35% of tubules injured; 2 = 36 to 50% of tubules injured; 2.5 = 51 to 65% of tubules injured and 3 = > 65% of tubules injured. Tubulointerstitial fibrosis was estimated by counting the percentage of injured areas per field of cortex and medulla, and was scored semiquantitatively using the following: 0 = normal interstitium; 0.5 = < 5% of areas injured; 1 = 5 to 20% of areas injured; 1.5 = 21 to 35% of areas injured; 2 = 36 to 50% of areas injured; 2.5 = 51 to 65% of areas injured and 3 = > 65% of areas injured. Hyalinosis of the afferent arterioles was semiquantitatively determined by counting the percentage of juxtaglomerular afferent arterioles available for examination with a minimum of 100 glomeruli per biopsy assessed: 0 = no arterioles injured; 0.5 = < 15% of arterioles injured; 1 = 15 to 30% of arterioles injured; 1.5 = 31 to 45% of arterioles injured; 2 = 46 to 60% of arterioles injured; 2.5 = 61 to 75% of arterioles injured and 3 = > 75% of arterioles injured.

### RNA analysis

After separating the cortex from the medulla, the tissue was finely minced with a razor blade on ice. Total RNA was prepared by lysis in 4 M guanidine isothiocyanate containing 1% 2-mercaptoethanol and 0.5% lauryl sarcosyl and ultracentrifugation of the lysate on a cesium chloride cushion. After resuspending in Tris-EDTA buffer, RNA concentrations were determined using spectrophotometric readings at Absorbance<sub>260</sub>. Thirty micrograms of RNA were electrophoresed in each lane in 0.9% agarose gels containing 2.2 M formaldehyde and 0.2 M Mops (pH 7.0) and transferred to a nylon membrane (ICN Biomedicals, Inc., Costa Mesa, CA, USA) overnight by capillary blotting. Nucleic acids
Fig. 1. Histologic changes in experimental chronic CsA nephrototoxicity. Micrograph showing the renal cortex of a salt-depleted rat given CsA, 15 mg/kg/day during four weeks. Striped tubulointerstitial fibrosis and tubular atrophy are extensive in this animal (A). There is also focal smooth muscle injury of the glomerular afferent arteriole showing hypertrophied and granular eosinophilic transformation (B) (periodic acid-Schiff, magnification ×200).
were crosslinked by ultraviolet irradiation (Stratagene, La Jolla, CA, USA). The membranes were prehybridized for two hours at 42°C with 50% formamide, 10% Denhardt’s solution, 0.1% SDS, 5× standard saline citrate (SSC), and 200 μg/ml denatured salmon sperm DNA. They were then hybridized at 42°C for 18 hours with cDNA probes labeled with 32P-dCTP by random oligonucleotide priming (Boehringer Mannheim Corp., Indianapolis, IN, USA). The blots were washed in 2× SSC, 0.1% SDS at room temperature for 15 minutes and in 0.1× SSC, 0.1% SDS at 50°C for 15 minutes. Films were exposed at —70°C for different time periods to ensure linearity of densitometric values and exposure time. Autoradiographs were scanned on a laser densitometer (Ultrascan XL; Pharmacia LKB Biotechnology, Inc.). The density of bands for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to control for differences in the total amount of RNA loaded onto each gel line. For quantitative purposes, the values were divided by the density of bands for GAPDH in the same lane. The following probes were used for Northern blot analysis.

A mouse TGF-β1 cDNA probe (plasmid Mu15) was kindly provided by R. Derynck [22]. A rat PAI-1 cDNA probe [plasmid pBluescript SK(−)] was obtained from T.D. Gelehrter [23]. Plasmid P16, which contains a human biglycan insert, was a gift of L.W. Fisher [24]. A rat decorin cDNA probe (plasmid pGEM4) was provided generously by K.L. Dreher [25]. A rat procollagen α1 cDNA (plasmid pα1R1) was obtained from D. Rowe [26]. A rat GAPDH cDNA probe (plasmid pBluescript KS II) was a gift from J.M. Blanchard [27].

**Immunohistochemistry**

Immunofluorescence microscopy was performed on tissues snap frozen in cold isopentane with a cryostat microscope (Miles Scientific, Naperville, IL, USA) as previously described [28]. Four micrometer cryostat sections were fixed in acetone and washed in PBS, pH 7.4. The deposition of the following ECM components, decorin, tenascin and fibronectin-cellular containing extra domain A (fibronectin EDA+) was performed. The primary antibody for decorin was a rabbit anti-human decorin antibody and was provided by E. Ruoslahti (La Jolla, CA, USA) [29]. The other primary antibodies used were rabbit anti-human tenasin (Life Technologies, Gaithersburg, MD, USA) and mouse anti-human fibronectin EDA+ (Sera-lab, Crowley Down, UK). The secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated F(ab')2 donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) and FITC-conjugated F(ab')2 rat antimouse IgG (Jackson Immunoresearch). A minimum of 20 randomly selected areas per sample were observed at ×250 magnification. The severity of staining for the glomerular, tubulointerstitial and vascular compartments was evaluated by an observer blinded to the treatment groups using the following semiquantitative scale: 0 = diffuse, very weak or absent staining, 1 = staining involving less than 25%, 2 = staining involving 25% to 50%, 3 = staining involving 50% to 75% and 4 = staining involving 75% to 100%. Photographs were obtained at identical exposure and development time intervals.

**Statistical analysis**

Results are presented as mean ± SEM. Multiple comparisons were done by analysis of variance. Comparisons between CsA and VH animals were done by two-tailed unpaired Student’s t-test or Mann-Whitney test, as appropriate. The level of statistical significance was chosen as P < 0.05.

**Results**

**Physiologic studies**

Although weight gain was progressive in the two treatment groups, animals treated with CsA failed to gain as much weight as those receiving vehicle (VH) with the difference achieving statistical significance (P < 0.01) at day 28. CsA-induced hypertension was not observed in this model; in fact, in CsA-treated rats, mean systolic BP tended to be numerically lower than in VH-treated rats, although the difference was not statistically significant. There was a progressive increase in CsA whole blood level in CsA-treated animals: 2930 ± 250 ng/ml at day 7 and 5827 ± 301 ng/ml at day 28.

CsA induced a significant increase in serum creatinine (S_{Cr}) only at day 28 (P < 0.01) (Table 1). On the other hand, the C_{Cr} was already lower in the CsA-treated rats at day 7 (P < 0.05) and was progressively lower at day 28 (P < 0.01) (Table 1). There was no significant proteinuria in the CsA-treated rats or the placebo animals.

The fractional excretion of sodium (FE_{Na}) was significantly decreased compared to normal in CsA-treated and placebo animals at all time points, reflecting the low sodium diet (Table 1). CsA caused an increase in the urinary excretion of AAP at days 7 and 28 that was significant at day 7 (P < 0.01; Table 1). Urinary NAG excretion was also higher at both time intervals, but did not reach statistical significance (Table 1). Urinary concentrating ability was clearly impaired as indicated by the progressive and significant increase in urinary volume by day 28 (P < 0.01) and decrease in U_{GFR} by day 7 (P < 0.05) and day 28 (P < 0.01) (Table 1). There was also a decrease in TcW at both time intervals, although it was statistically significant only at day 7 (P < 0.05; Table 1).

**Histologic changes**

CsA-treated rats had characteristic morphologic findings similar to the chronic human lesion, mostly evident at day 28. There was a mononuclear cell infiltrate and an early tubular injury by day 7. However, the typical lesion of chronic CsA nephropathy was observed by day 28 (Fig. 1). Findings ascribed to tubular injury consisted of cellular and intercellular vacuolization, tubular collapse and tubular dilatation. Striped interstitial fibrosis was present with progressive inflammatory cell infiltrates associated with thickening of the tubular basement membranes (TBM) and Bowman’s capsule. There was focal disruption of the TBM in some areas of interstitial infiltrate with loss of tubular definition.

| Table 2. Semiquantitative scoring by light microscopy of the extent of changes observed in the chronic CsA lesion for proximal tubular injury, tubulointerstitial fibrosis and arteriolopathy |
|---|---|---|---|---|
| | Day 7 | Day 28 |
| | VH | CsA | VH | CsA |
| Proximal tubular injury | 0.1 ± 0.1 | 0.6 ± 0.2 | 0.1 ± 0.1 | 1.5 ± 0.2* |
| Tubulointerstitial fibrosis | 0 ± 0 | 0 ± 0 | 0 ± 0 | 1.6 ± 0.3* |
| Arteriolopathy | 0 ± 0 | 0 ± 0 | 0 ± 0 | 1.6 ± 0.2* |

Data are mean ± SEM of eight rats. VH is control. *P < 0.01 vs. VH
The vascular lesion was characterized by arteriolar hyalinosis consisting of hyalin deposition within the tunica media of afferent arterioles and the terminal portions of the interlobular arteries. The extent of changes was graded (Table 2) using a 0 to 3+ semiquantitative scale, with 3+ indicating extensive changes. In this model, while no early histologic changes were observed by day 7, changes of chronic CsA nephrotoxicity manifested as proximal tubular injury, tubulointerstitial fibrosis and arteriolopathy developed by 28 days ($P < 0.01$) when compared to VH-treated rat kidneys.

Expression of TGF-β1 and PAI-1 mRNA

TGF-β1 mRNA was progressively increased by day 7 ($P < 0.001$) and more so at day 28 ($P < 0.001$) in the CsA-treated rats when compared to control VH rats (Figs. 2 and 3). The mRNA expression of PAI-1, a protease inhibitor that blocks ECM degradation by the plasmin protease system and is directly stimulated by TGF-β, is shown in Figures 2 and 4. PAI-1 mRNA, in parallel to TGF-β1 mRNA expression, was progressively up-regulated in the CsA-treated rats at seven days ($P < 0.001$) and

![Fig. 2. Northern blotting of mRNA from cortex and medulla in CsA and VH-treated rat kidneys. Total RNA was isolated from whole cortex and medulla at days 7 and 28 from rats treated with CsA 15 mg/kg/day or VH control and was hybridized with cDNA probes to (A) TGF-β1, (B) PAI-1, (C) biglycan, (D) decorin, (E) type I collagen, and (F) GAPDH. Molecular size markers are shown on the right.](image-url)
Fig. 3. Quantitation of mRNA expression of TGF-β1 in CsA and VH-treated rat kidneys relative to GAPDH. Total RNA was isolated from whole cortex and medulla at days 7 and 28 and was hybridized with a cDNA probe to TGF-β1. Symbols are: (■) CsA 7 days; (□) VH 7 days; (■) CsA 28 days; (□) VH 28 days. N = 8 for each group. **P < 0.001 compared to VH control.

more so at 28 days (P < 0.001) as compared to control VH rats (Fig. 4).

Fig. 4. Quantitation of mRNA expression of PAI-1 in CsA and VH-treated rat kidneys relative to GAPDH. Total RNA was isolated from whole cortex and medulla at days 7 and 28 and was hybridized with a cDNA probe to PAI-1. Symbols are: (■) CsA 7 days; (□) VH 7 days; (■) CsA 28 days; (□) VH 28 days. N = 8 for each group. **P < 0.005 compared to VH control.

Extracellular matrix deposition in the kidney

Northern blot analysis. The proteoglycans biglycan and decorin are components of the ECM. Their mRNA expression was elevated in this model suggesting active matrix synthesis (Figs. 2 and 5). Biglycan mRNA expression closely followed TGF-β1 mRNA expression and was progressively elevated at seven days (P < 0.005 for cortex and < 0.001 for medulla) and at 28 days (P < 0.001) when compared to control VH. The behavior of the other proteoglycan, decorin, was somewhat different. At day 7, it was not statistically elevated when compared to VH control but was definitely up-regulated by day 28 (P < 0.001). Collagen type
of the changes were statistically significant except for an
histology of chronic CsA nephrotoxicity (Fig. 8). In the glomeruli,
compartments of the kidney, in accordance with the characteristic
statistical significance in the tubulointerstitium and vascular com-
pared to VH controls (Fig. 7). Most of the changes reached
did not reach statistical significance until day 28 (P < 0.01) when
fibronectin EDA+ at day 7 (P < 0.001) that
decreased by day 28 to control level.
The deposition of decorin followed its mRNA expression and
that of tenascin and fibronectin EDA+ (Figs. 7 and 8). Most of
the changes were observed at day 7 in the vessels (P < 0.001) and
tubulointerstitium (P < 0.001) with no elevated expression in the
glomeruli when compared to VH controls.

**Differential expression in cortex and medulla**

The differential expression in the cortex versus the medulla for
TGF-β1, PAI-1 and the ECM proteins was examined by Northern
blots. As shown in Figure 9, the changes observed in mRNA
expression for TGF-β1, PAI-1, type I collagen and the proteogly-
cans, biglycan and decorin, although present in the cortex, was
more dramatic and significant (P < 0.001) in the medulla as
compared to the cortex at both days 7 and 28.

**Discussion**

This report describes an animal model of chronic CsA nephro-
toxicity that mimics the functional and histopathological findings
observed in the human lesion of chronic-CsA induced injury. Salt-depletion has been shown to accelerate CsA nephropathy
with the lesion developing by three to four weeks in the rat [12]
instead of three months or longer [30]. In this model, we found
that the histologic changes were accompanied by physiologic changes namely increased creatinine, decreased creatinine clear-
ance, increased tubular enzymuria, and a loss of medullary
concentrating ability. While enzymuria stabilizes, the other phys-
ologio markers of CsA toxicity progress with continued treat-
ment.
The matrix expansion observed in this model was most marked
in the interstitial and vascular compartments of the kidney. This is
in accordance with the characteristic histology of chronic CsA
nephropathy that is interstitial fibrosis and arteriolopathy [5].
Although present in the cortex, the observed changes were mostly
in the medulla and were manifested by a dramatic increase in the
presence of TGF-β1, PAI-1 and matrix proteins. These changes
were associated with a decrease in medullary concentrating
ability. In accordance with our observation, recent data have
shown that the early changes of CsA nephropathy are first seen in
the medullary compartment of the kidney [31, 32].
The common belief is that the chronic form of CsA injury is a
consequence of CsA-induced renal vasoconstriction. According to
this notion, the arteriolar vasospasm with resultant impairment in
renal blood flow leads to tubulointerstitial fibrosis. Data to
support the role of ischemia as a primary stimulus for tubuloin-
terstitial fibrogenesis have been offered [33]. However, more
recent studies have suggested that there can be a dissociation
between the functional and histological injuries induced by CsA
[13]. CsA withdrawal led to improved GFR but the degree of
tubular atrophy and interstitial fibrosis actually progressed [13]. In
our model, while acute tubular enzymuria stabilized, the associ-
ated histologic changes continued to progress during the fol-
low-up period and were dominated by renal fibrosis. Our findings
also indicate that the observed fibrosis was associated with an
increased deposition of the three major components of the ECM:
proteoglycans, glycoproteins, and collagens.

Certain matrix components have been shown to be up-regu-
lated with the administration of CsA. In cell cultures treated with
CsA, collagen type I and IV mRNAs were elevated in murine

![Fig. 6. Quantitation of mRNA expression of type I collagen in CsA and
VH-treated rat kidneys relative to GAPDH. Total RNA was isolated from
whole cortex and medulla at days 7 and 28 and was hybridized with a
cDNA probe to type I collagen, N = 8 for each group. Symbols are: (■)
CsA 7 days; (□) VH 7 days; (■) CsA 28 days, (□) VH 28 days. *P < 0.005
and **P < 0.001 compared to VH control.](image-url)
Fig. 7. Immunofluorescence micrographs of CsA and VH-treated rat kidneys. Kidney sections from CsA treated rats for seven days (A, D, and G), for 28 days (B, E, and H) and VH controls (C, F, and I) were stained with an antibody to tenascin (A, B, and C), fibronectin EDA+ (D, E, and F) and decorin (G, H, and I). The photographs were taken under identical conditions with equal exposures of 60 seconds (magnification ×250).
Shihab et al: TGF-β1 in CsA nephropathy

**Fig. 9.** Quantitation of mRNA expression of TGF-β1, PAI-1 and matrix proteins in cortex versus medulla in CsA-treated rat kidneys. Total RNA was isolated from whole cortex (■) and medulla (□) at days 7 (A) and 28 (B), and was hybridized with cDNA probes to TGF-β1, PAI-1, biglycan, decorin, and type I collagen. Abbreviations are: biglycan (BG), decorin (DEC), and type I collagen (COL I). **P < 0.001 compared to cortex.

**Fig. 8.** Quantitation of deposition of extracellular matrix components in CsA and control treated rat kidneys by immunofluorescence. (A) Glomerular staining, (B) tubulointerstitial staining, and (C) vessels staining scores at days 7 and 28. Symbols are: ■ CsA 7 days; (●) VH 7 days; (□) CsA 28 days, (●) VH 28 days. Abbreviation Fibro-EDA+ is fibronectin EDA+.

*P < 0.005 and **P < 0.001 compared to VH control.
tubular cells [34], while no change was observed in a variety of rat and human renal cells [35]. On the other hand, collagen type III synthesis was increased in human mesangial cells and renal fibroblasts treated with CsA [35]. In addition, procollagen α1(I) mRNA was shown to be elevated in the renal cortex of CsA-treated rats [36]. CsA was also recently shown to enhance TGF-β mRNA expression in normal human T cell culture [37]. Another cytokine, platelet-derived growth factor (PDGF), was shown to be present in the arteriolar walls of CsA-treated rats [38]. However, while most of the experiments done were in vitro, no study has looked at the components of the fibrotic lesion of chronic CsA nephropathy in vivo, and none has attempted to correlate the renal fibrosis with any growth factor.

Transforming growth factor-β (TGF-β) has been implicated in the fibrosis of a number of chronic diseases of the kidney and other organs [14, 15]. In addition, it is known to induce its own production [39] and its persistent expression following repeated injuries can lead to a cycle of continued TGF-β production [14]. TGF-β causes fibrosis by stimulating synthesis of individual ECM components and simultaneously blocking ECM degradation [16–19]. In this model, we have observed an elevated expression of representative members of the proteoglycans, glycoproteins and collagen families. Their expression correlated with a parallel and significant increase in TGF-β1. In addition, the protease inhibitor PAI-1 was up-regulated and its expression followed closely the expression of TGF-β1 and the ECM proteins studied. Since TGF-β1 directly stimulates PAI-1 expression [19], our data suggest that the observed ECM accumulation in this model likely involves the dual action of TGF-β1 on ECM deposition and degradation.

What causes the elevation of TGF-β remains unclear. Since CsA directly stimulates TGF-β in vitro, it has been proposed that the immune suppressive activities and renal fibrosis due to CsA are both mediated by TGF-β [37, 40, 41] and may explain the elevated expression of TGF-β1 in our model. It is also possible that renal ischemia produced by the vasoconstriction of CsA elevates TGF-β [42]. Since the current model is produced by salt-depletion, the renin-angiotensin system (RAS) is activated [32]. Interestingly, there is evidence to suggest that activation of the RAS up-regulates TGF-β. The renal RAS is induced by CsA and plays an important role not only in the regulation of glomerular hemodynamics but also in glomerular growth and sclerosis [43]. Angiotensin (Ang) II and CsA have been separately shown to stimulate fibrogenesis by mechanisms independent of their vasoconstrictive action [33, 44]. Chronic infusion of Ang II in rats produce considerable interstitial fibrosis [45]. Ang converting enzyme inhibitors and Ang II receptor antagonists attenuate interstitial fibrosis and progressive glomerulosclerosis in several disease models and slow disease progression in several human studies [46, 47]. Kagami et al have recently demonstrated a link between Ang II and TGF-β by showing that Ang II stimulation of ECM protein synthesis in rat glomerular mesangial cells is mediated by both an increase in TGF-β synthesis and an increase in the conversion from latent to active TGF-β [48]. In addition, CsA administration in salt-depleted rats caused a significant increase in juxtaglomerular (JG) apparatus renin, and blockage of the RAS with a competitive Ang II receptor antagonist, losartan, reduced the formation of interstitial fibrosis [49]. While the peripheral renin activity is often normal or suppressed in CsA-treated renal transplants, there is abundant evidence of JG cell hyperplasia within the kidney in such individuals similar to other one-clip, one-kidney models of hypertension [50].

In conclusion, by using a model of chronic CsA nephropathy that mimics the human lesion, we have shown that matrix proteins are increased, mostly in the medulla, and more specifically in the interstitial and vascular compartments of the kidney. We have linked the expression of TGF-β1 to matrix expansion and have shown that both TGF-β1 and PAI-1 are increased. We propose that TGF-β1 is a key fibrogenic cytokine involved in the development of chronic CsA nephropathy by enhancing ECM deposition and inhibiting its degradation. However, other factors may also be involved in the fibrosis of chronic CsA nephropathy. Whether long-term immunosuppression with CsA inevitably leads to renal fibrosis is unknown and has not been excluded by any properly controlled clinical study.

Acknowledgments

A preliminary report of this work was presented at the Annual Meeting of the American Society of Transplant Physicians, Chicago, IL in May 1995. This work was supported in part by the Ogden Research Foundation (F.S.S.) and a grant from the Oregon Health Sciences University Foundation and the Clinical Research Group of Oregon (W.M.B.).

Reprint requests to Fuad S. Shihab, M.D., Division of Nephrology, 48312 Medical Center, University of Utah, 50 N. Medical Drive, Salt Lake City, Utah 84132, USA.

References

18. BALZA E, BORSI L, ALLEMANNI G, ZARDI L: Transforming growth factor β regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. FEMS Lett 228:42—44, 1988
24. FISHER LW, TERRY M, YOUNG MF: Deduced protein sequence of small proteoglycan (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species. J Biol Chem 264:4571—4576, 1989
35. GHIGGIERI GM, ALBIERI PI, OLEGGI R, VALENTI F, GINEVA F, PEREFUPO GF, GUSMAN R: Cyclosporine enhances the synthesis of selected extracellular matrix proteins by renal cells "in culture". Transplantation 57:1382—1388, 1994