Pathogenesis of Cyclosporine Nephropathy: Roles of Angiotensin II and Osteopontin[1]

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(J. Am. Soc. Nephrol. 1995; 6:1186–1196)

ABSTRACT

Low-salt-diet, cyclosporine (CsA; 15 mg/kg per day)-treated rats develop striped interstitial fibrosis, arteriolar hyalinosis, and azotemia similar to the chronic nephropathy observed in humans. To examine the role of angiotensin II in this model, rats on a low-salt diet were given CsA, CsA and the angiotensin II receptor Type I antagonist Losartan (10 mg/kg per day), CsA and hydralazine/furosemide, or vehicle. At Day 35, CsA-treated rats had tubular injury, arteriolopathy of the afferent arteriole, increased expression of the monocyte-macrophage adhesive protein osteopontin, interstitial macrophage infiltration, increased interstitial transforming growth factor-β expression, and interstitial fibrosis. This study provides new insight in both pathogenic and therapeutic aspects of CsA nephropathy. The pathogenesis of CsA nephropathy involves the expression of osteopontin by tubular epithelial cells, the level of which closely correlates with the degree of macrophage infiltration and interstitial fibrosis in all groups ($r = 0.79$ and 0.74, respectively; $P < 0.001$). Therapeutic conclusions can be drawn from the observation that both losartan and hydralazine/furosemide reduced osteopontin expression, macrophage infiltration, transforming growth factor-β expression, and interstitial fibrosis, but did not prevent the decrease in GFR. Treatment with losartan, but not with hydralazine and furosemide, markedly reduced arteriolopathy. It was concluded that angiotensin II contributes to the vasculopathy (hyalinosis) induced by CsA. In contrast, the interstitial fibrosis mediated by CsA can be partially prevented by both an angiotensin II Type I receptor antagonist or by hydralazine and furosemide. This suggests that the interstitial fibrosis can be dissociated from the vascular effects of CsA. The beneficial effects of lowering blood pressure or vasodilation *per se* may be difficult to distinguish from the specific effects of angiotensin II receptor blockade.

Key Words: *Cyclosporin A, angiotensin II, osteopontin, macrophages, interstitial fibrosis*

Cyclosporin A (CsA) remains one of the most important immunosuppressive drugs in the management of organ transplantation and autoimmune diseases. Despite its great benefit, CsA may be associated with nephrotoxicity that is either acute and reversible (hemodynamic) or chronic, progressive, and irreversible (structural) (1,2). The chronic changes consist of striped tubulointerstitial fibrosis, tubular injury with tubular dilation and atrophy, nephrocalcinosis, mononuclear infiltration, and hyalinosis of the afferent arteriole. These pathologic findings have been observed in both renal and cardiac allograft recipients, as well as in patients treated for autoimmune diseases (3–5).

Many investigators have attempted to elucidate the pathogenesis of the chronic fibrotic injury and vascular changes induced by CsA. The recent development of an animal model of CsA toxicity, which closely resembles chronic toxicity in humans, has provided new opportunities to investigate the pathogenesis of this complication (6). Specifically, rats maintained on a low-salt diet that are administered CsA develop most of the features of chronic CsA nephrotoxicity, including hyalinization of the afferent arteriole (7,8,9).

Recently, we and others (10–12) have been interested in the role of the renin-angiotensin II (AII) system in mediating CsA nephrotoxicity. First, there is a striking similarity in some of the renal histologic lesions of rats chronically administered AII with those observed in CsA nephropathy (13). Second, most studies (14–17) show that renal tissue and plasma renin levels are elevated in both experimental and human CsA nephrotoxicity. Finally, recent studies suggest that angiotensin-converting enzyme (ACE) inhibitors (11,12) and also AII receptor antagonists (Losartan; Du Pont Merck Pharmaceutical Co., Wilmington, DE) (18) can reduce interstitial fibrosis in experimental models of CsA nephropathy.

In this study, we explore the pathogenesis of the interstitial fibrosis in experimental CsA nephropathy

[1] Received April 14, 1995. Accepted May 19, 1995.

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1046-6673/0604-1186$03.00/0
Journal of the American Society of Nephrology

in rats and the effect of AII receptor blockade. Our studies provide several new insights into this process. First, we provide strong evidence that the interstitial fibrosis is linked to the expression of the macrophage chemoattractant osteopontin, which may mediate the local macrophage accumulation, and transforming growth factor (TGF)-β expression. Second, the fibrosis is partially inhibited both by AII receptor blockade and by other antihypertensives that do not block AII. Finally, the arteriolopathy associated with CsA is reduced by AII blockade but not by hydralazine/furosemide, suggesting a role for AII in its pathogenesis.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 225 to 250 g were housed in individual cages in a temperature- and light-controlled environment. All rats received a low-salt diet (0.05% sodium; Teklad, Madison, Wisconsin).

Drugs

CsA, provided by Sandoz Research Institute (East Hanover, NJ), was diluted in olive oil to a final concentration of 15 mg/mL. **Losartan** (L), provided by Cardiovascular Diseases Research (Du Pont Merck Pharmaceutical Company, Wilmington, DE), was dissolved in sterile water to a final concentration of 10 mg/mL. **Hydralazine** (H) (Sigma Chemical Co., St. Louis, MO) was dissolved in the animals' drinking water to a concentration of 240 mg/L. **Furosemide** (F) (Lympho Med Inc., Rosemont, IL) was dissolved in sterile water to a final concentration of 1 mg/mL.

Experimental Groups

1) **CsA.** Rats received a daily sc injection of CsA, 15 mg/kg for 35 days (N = 8).

2) **CsA+L.** Rats daily received CsA, 15 mg/kg by sc injection, and Losartan, 10 mg/kg by gavage, for 35 days (N = 7).

3) **CsA+H/F.** Rats daily received CsA, 15 mg/kg by sc injection, an sc injection of furosemide at a dose of 1 to 2 mg, and hydralazine, 240 mg/L in the drinking water, for 35 days (N = 9).

4) **Vehicle (VH).** Rats received a daily sc injection of olive oil, 1 mL/kg, for 35 days (N = 8).

5) **VH+L.** Rats received a daily sc injection of olive oil, 1 mL/kg, and Losartan, 10 mg/kg by gavage for 35 days (N = 9).

6) **VH+H/F.** Rats received a daily sc injection of olive oil, 1 mg/kg, an sc injection of furosemide at a dose of 1 to 2 mg, and hydralazine, 240 mg/L in the drinking water, for 35 days (N = 9).

Experimental Protocol

After 1 wk on the low-salt diet, weight-matched rats were randomly assigned to the different treatment groups. Daily body weight and food intake were recorded. After 35 days, systolic blood pressures (SBP) were measured (means of three measurements) in unanesthetized rats by plethysmography with a rat tail manometer-tachometer system (Natsume Seisakusho Co. Ltd., Tokyo, Japan). Rats were placed in metabolic cages (Nalge Company, Rochester, NY) for 24-h urine collection. The following day, animals were anesthetized with ketamine, urine and serum were collected for creatinine clearance and serum creatinine level, and both kidneys were obtained for histologic evaluation. Biopsies were fixed in 10% formalin and methyl Carnoy's and paraffin embedded or frozen in O.C.T (Miles, Elkhart, IN).

Functional Data

Serum and urine creatinine were measured by a Cobas autoanalyzer (Roche Diagnostics, Div. Hoffman-La Roche, Nutley, NJ). Creatinine clearance was calculated by a standard formula.

Morphology

Renal biopsies were fixed in methyl Carnoy's solution and embedded in paraffin. Four-micrometer sections were stained with periodic acid–Schiff's reagent (PAS) and counterstained with hematoxylin. Biopsies were evaluated for interstitial fibrosis, arteriolar hyalinosis, tubular injury, and calcification.

Interstitial fibrosis was scored semiquantitatively by a blinded observer who examined cortical tubulointerstitial fields on PAS-stained renal biopsies using the ×20 objective. A minimum of 30 fields were assessed in each biopsy. The following semiquantitative score was used: **Score 0**, normal interstitium and tubules; **Score 1**, mild fibrosis with minimal interstitial thickening between the tubules; **Score 2**, modest fibrosis with moderate interstitial thickening between the tubules; **Score 3**, severe fibrosis with severe interstitial thickening between the tubules. Pictures representing each individual score are shown in Figure 1.

Arteriolopathy of the afferent arteriole was semiquantitatively estimated by counting the percentage of juxtaglomerular afferent arterioles with arteriolopathy per total number of juxtaglomerular afferent arterioles available for examination with the ×20 objective, with a minimum of 60 glomeruli per biopsy assessed.

Tubular injury was scored semiquantitatively by a blinded observer who examined at least 40 cortical fields (×100 magnification) of PAS-stained biopsies. Tubular injury was defined as tubular dilation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane. Only cortical tubules were included in the following scoring system:

Score 0, no tubular injury; **Score 1**, < 10% of tubules injured; **Score 2**, 10 to 25% of tubules injured; **Score 3**, 26 to 50% of tubules injured; **Score 4**, 51 to 75% of tubules injured; **Score 5**, > 75% tubules injured.

Nephrocalcinosis was defined as the presence of calcium crystals within the tubular lumen and was primarily found at the corticomedullary junction (see Figure 2). Nephrocalcinosis was semiquantitatively assessed by use of the following scoring system: **Score 0**, no nephrocalcinosis present; **Score 1**, mild nephrocalcinosis (< 10% of tubules in corticomedullary junction); **Score 2**, moderate nephrocalcinosis (10 to 25% of tubules in corticomedullary junction); **Score 3**, severe nephrocalcinosis (> 25% of tubules in corticomedullary junction).

Immunohistochemistry

Tissue was sectioned (4 μm) and stained by the use of standard immunoperoxidase or immunofluorescence procedures, as described elsewhere (19–22). The following monoclonal (or polyclonal) antibodies were used as described previously (19,20,23): ED-1 (Bioproducts for Science, Indianapolis, IN), a monoclonal marker of macrophages/monocytes and dendritic cells; an immunoglobulin G (IgG) guinea

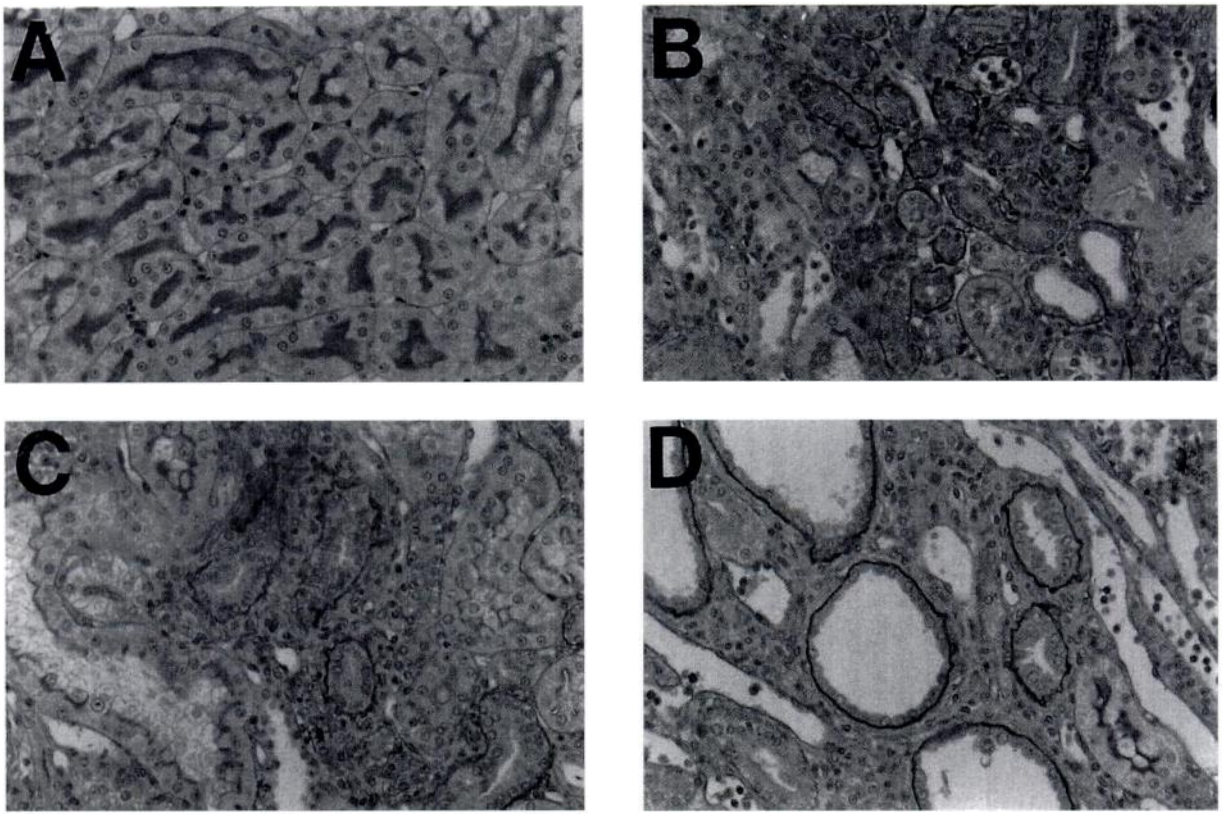

Figure 1. Shown are representative photographs with different scores for interstitial fibrosis (PAS, ×400). The scoring system is detailed in the Methods section. Tubulointerstitial fields are shown with normal interstitium and tubules (Score 0 (A)), mild fibrosis with minimal interstitial thickening between the tubules (Score 1 (B)), modest fibrosis with moderate interstitial thickening between the tubules (Score 2 (C)), and severe fibrosis with severe interstitial thickening between the tubules (Score 3 (D)).

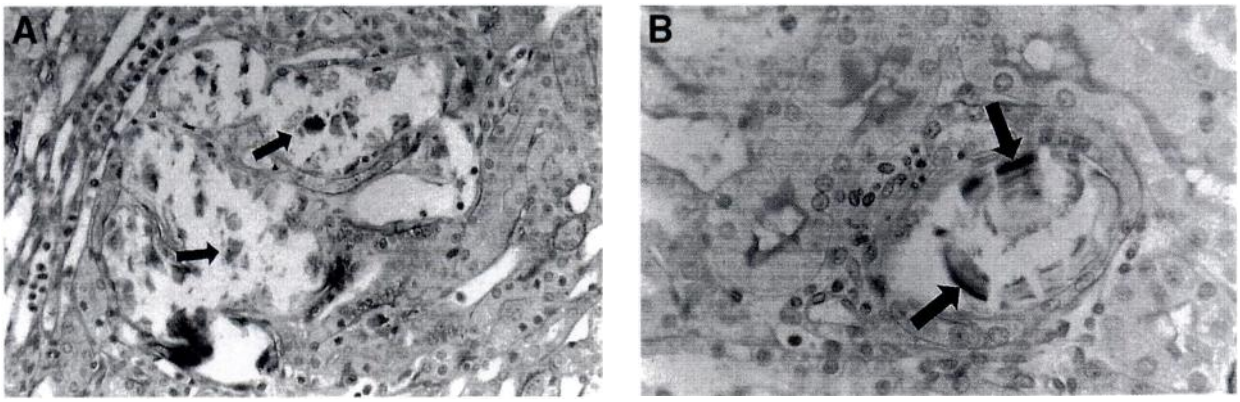

Figure 2. Day 35 CsA-treated animals with typical nephrocalcinosis in the juxtamedullary region (arrows). PAS, ×200 and ×630.

pig anti-rat Type I collagen (gift of L. Iruela-Arispe and E.H. Sage, Seattle WA); polyclonal goat anti-mouse IgG fraction against Type IV collagen (Southern Biotech, Birmingham AL); F37.2D12, a murine monoclonal antibody against human renin (gift of M. Laprade, Sanofi Recherche, Montpellier, France); MPIIIB10, a murine monoclonal antibody against rat osteopontin (obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA); a polyclonal rabbit anti-TGF-β antibody that recognizes all three isoforms (R&D Systems, Minneapolis, MN).

All biopsies were scored in a blinded fashion. Quantitation was performed at ×100 magnification with a 1-mm × 1-mm grid within the microscope eyepiece to demarcate at least 60 tubulointerstitial fields per biopsy exclusive of glomeruli. Renin was semiquantitatively estimated by counting glomeruli with positive juxtaglomerular apparatus staining, with a minimum of 60 glomeruli assessed per biopsy, as described

previously (13) Data are displayed as the percentage of glomeruli with adjacent renin staining. **Osteopontin** (OPN) expression by tubular epithelial cells was semiquantitatively estimated by counting the number of tubules expressing OPN per square millimeter of renal cortex (at least 60 fields per biopsy counted). Interstitial **macrophage** infiltration was quantitated by counting the number of ED-1-positive macrophages/monocytes per square millimeter of renal cortex (at least 60 fields counted per biopsy). Interstitial **Type IV collagen** deposition was scored semiquantitatively in cortical tubulointerstitial fields on renal biopsies that had been immunohistochemically stained for Type IV collagen. A minimum of 30 fields were assessed in each biopsy. The following semiquantitative score was used: **Score 0**, normal interstitial Type IV collagen expression; **Score 1**, minimal increase of interstitial Type IV collagen expression; **Score 2**, modest increase of interstitial Type IV collagen expression; **Score 3**, severe increase of interstitial Type IV collagen expression.

In order to identify the source of TGF-β, double staining for TGF-β and macrophages was performed on pepsin-digested, acetone-fixed, frozen sections(22) by the use of a standard immunofluorescence protocol. The polyclonal rabbit anti-TGF-β antibody was incubated together with a monoclonal antibody directed against macrophages (ED-1) overnight at 4°C followed by an incubation with a biotinylated goat anti-rabbit IgG and fluorescein isothiocyanate–labeled streptavidin D. The monoclonal antibody (ED-1) was detected by a Texas Red–labeled anti-mouse IgG antibody. Slides were cover slipped in Vectashield (Vector, Burlingame, CA) and examined on a fluorescent microscope with appropriate filters.

Statistical Analysis

Results are presented as mean ± SE. Comparisons between the different groups were performed by the use of analysis of variance followed by Fisher's protected least significant difference procedure (Fisher's PLSD test). Linear regression analysis of the relationship of OPN expression with the number of infiltrating macrophages and with the degree of interstitial fibrosis was performed. Statistical significance was defined as $P < 0.05$.

RESULTS

Histologic and Immunohistochemical Changes

CsA is associated with stimulation of the renal **renin-angiotensin system.** A significant increase in renin was observed in CsA- *versus* vehicle-treated animals by immunostaining (Table 1). Treatment with losartan or hydralazine and furosemide alone also increased juxtaglomerular renin staining. However, CsA + H/F treatment was not different from CsA alone, whereas CsA + L increased tissue renin expression when compared with CsA animals (Table 1).

Arteriolopathy of the afferent arteriole was present adjacent to 60.5% of the glomeruli in CsA-treated rats, as evidenced by smooth muscle cell hyperplasia and the accumulation of eosinophilic material in the vascular wall (Figure 3). Losartan dramatically inhibited the development of this arteriolar lesion ($P < 0.0001$), whereas hydralazine/furosemide was without effect (Figure 3; Table 1).

Tubular injury was present in rats on CsA and consisted of tubular dilation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane. There was no protective effect of any form of treatment on tubular injury in CsA-treated animals (Table 1).

OPN, a macrophage chemoattractant, is expressed by tubular epithelial cells in various models of renal injury.(24,25) Treatment with CsA also induced a dramatic up-regulation of OPN expression by tubular epithelial cells as compared with vehicle-treated rats (Figure 4, Table 1). CsA + H/F reduced OPN expression significantly compared with CsA alone. In contrast, CsA + L reduced OPN expression by almost 20%, but this reduction did not reach statistical significance ($P = 0.077$) (Figure 4; Table 1).

CsA-treated rats had a prominent **macrophage** infiltration in the cortical interstitium, particularly in

TABLE 1. Interstitial fibrosis, tubular injury, nephrocalcinosis, arteriolopathy of the afferent arteriole, number of OPN-expressing tubules per square millimeter, number of interstitial macrophages per square millimeter, and juxtaglomerular renin expression in the different groups at Day 35[a]

Groups[b]	Interstitial Fibrosis (0–3)	Tubular Injury (0–3)	Nephrocalcinosis (0–3)	Arteriolopathy (%)	OPN (tubules/mm^2 cortex)	Macrophages (cells/mm^2 cortex)	Tissue Renin (% of glomeruli with positive juxtaglomerular)
CsA	0.8 ± 0.18	2.8 ± 0.45	0.75 ± 0.25	60.5 ± 4.6	81.48 ± 8.61	428.19 ± 32.0	59.0 ± 2.0
CsA+L	0.36 ± 0.12[c]	2.67 ± 0.33	1.5 ± 0.56[d]	18.4 ± 3.3[e]	66.10 ± 8.89	232.77 ± 41.44[e]	69.8 ± 3.0[c]
CsA+H/F	0.26 ± 0.05[e]	2.56 ± 0.18	0.22 ± 0.15	60.5 ± 7.1	39.23 ± 7.65[e]	129.60 ± 11.20[e]	62.7 ± 2.6
VH	0	0.38 ± 0.18[e]	0	0	0.22 ± 0.18	41.05 ± 2.53	37.6 ± 3.3[c]
VH+L	0.09 ± 0.05	1.56 ± 0.38	0.33 ± 0.24	0	7.19 ± 2.5	86.33 ± 10.08	56.4 ± 3.4
VH+H/F	0.09 ± 0.02	1.0 ± 0.29	0	0.13 ± 0.13	10.93 ± 3.34	67.68 ± 11.02	52.4 ± 3.1

[a] Values are expressed as mean ± SE.
[b] CsA, treatment with CsA (15 mg/kg per day); CsA+L, CsA + 10 mg of losartan/kg; CsA+H/F, CsA + hydralazine (240 mg/L) and furosemide (1 to 2 mg); VH, vehicle treatment (1 ml of olive oil/kg); VH+L, vehicle treatment + 10 mg of losartan/kg; VH+H/F, vehicle treatment + hydralazine (240 mg/L) and furosemide (1 to 2 mg).
[c] $P < 0.05$ *versus* CsA.
[d] $P < 0.005$ *versus* CsA + H/F.
[e] $P < 0.0001$ *versus* CsA.

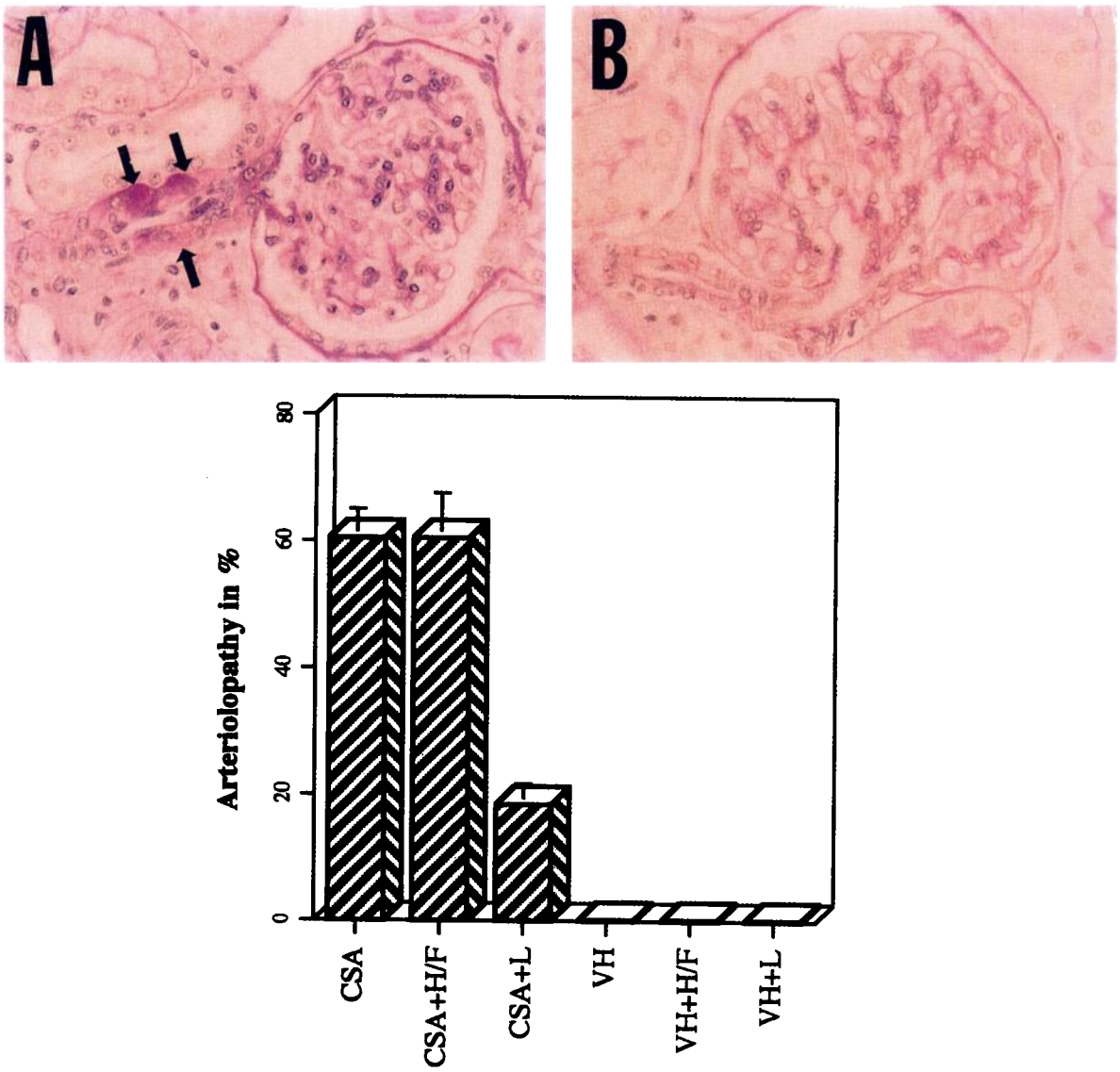

Figure 3. Effect of losartan on CsA-induced arteriolopathy. Whereas the afferent arteriole in CsA-treated animals often displayed a degenerative lesion with accumulation of eosinophilic material (arrows, Panel A), the majority of afferent arterioles in CsA-treated rats on losartan were normal (B). Panel C shows quantification of afferent arteriole arteriolopathy in the different groups displayed as the percentage of glomeruli showing arteriolopathy of the afferent arteriole. Data are mean ± SE.

areas of interstitial fibrosis (Table 2). Both hydralazine/furosemide and losartan reduced the number of infiltrating macrophages significantly in CsA-treated animals (Figure 4; Table 1). VH + L- and VH + H/F-treated animals had a mean of 86 and 68 infiltrating macrophages per square millimeter, respectively, as compared with VH-treated animals, with only 41 ED-1-positive cells/mm^2.

When individual animals in all groups were compared by linear regression, a significant correlation could be demonstrated between the number of infiltrating macrophages and the number of tubules overexpressing OPN (Figure 5a). When individual animals were compared by linear regression, the number of tubules overexpressing OPN also correlated with the extent of interstitial fibrosis (Figure 5b).

Treatment with CsA also induced the expression of TGF-β in the tubulointerstitium (Figure 6). TGF-β expression was primarily noted in interstitial areas between tubules in areas of extracellular matrix expansion. TGF-β expression was highest in CsA-treated animals, whereas it was notably reduced in rats treated with CsA + L and CsA + H/F. In order to address the source of TGF-β, double immunolabeling for TGF-β and macrophages (ED-1) was performed. Only a minor proportion (<10%) of interstitial cells

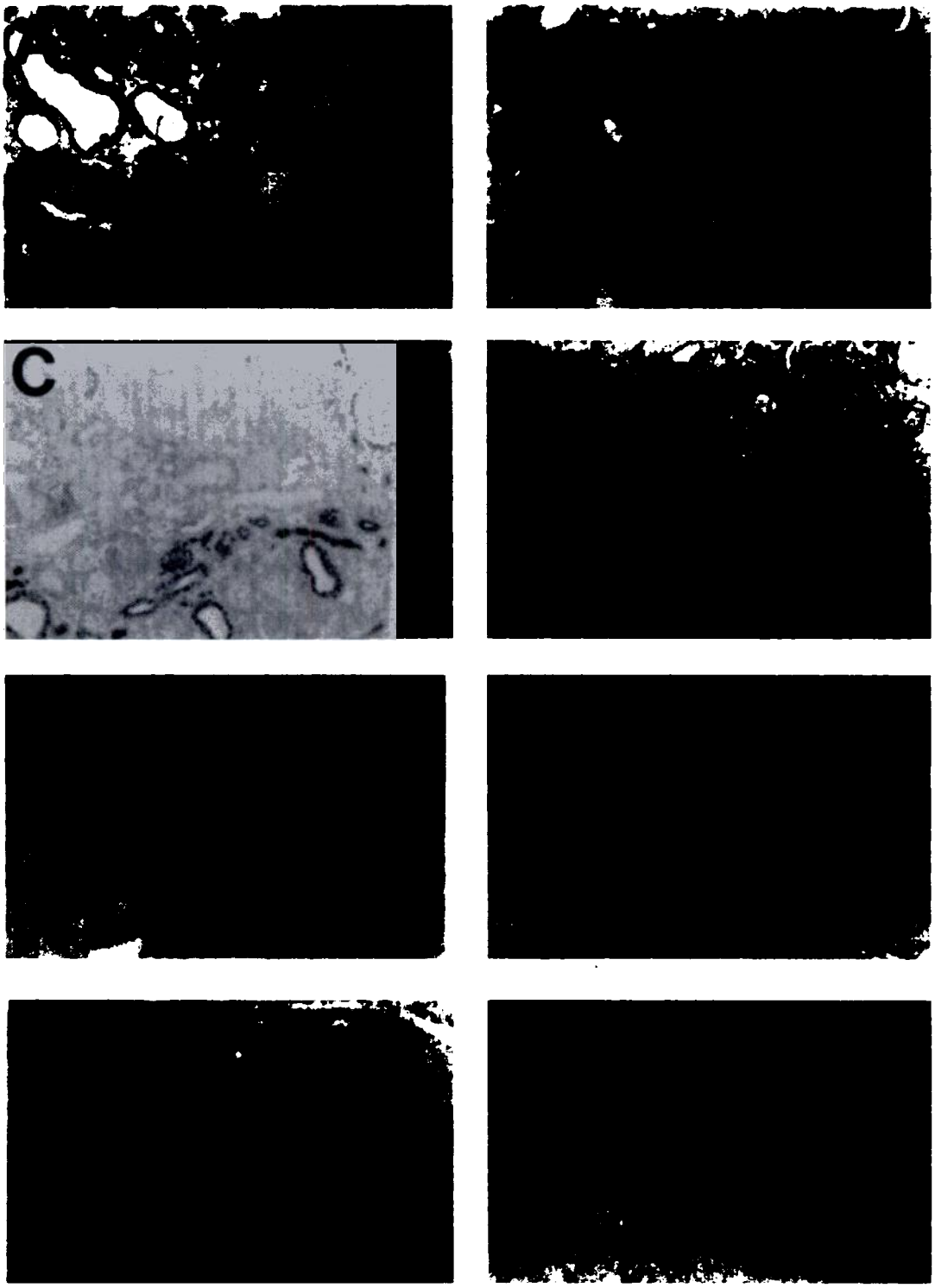

Figure 4. OPN and Macrophage accumulation in CsA nephropathy. CsA-treated rats had a dramatic increase in OPN expression in both proximal and distal tubules (A) that was reduced by losartan (B) or hydralazine/furosemide (C). For comparison, a vehicle-treated animal is shown in Panel D (Day 35, ×200). Similarly, CsA-treated rats also had a severe macrophage influx in the renal cortex (E) that was also reduced by losartan (F) or hydralazine/furosemide (G). Again, for comparison, a vehicle-treated animal is shown in Panel H.

TABLE 2. Change in body weight (Δ body weight), creatinine clearance (GFR), serum creatinine, and SBP in the different groups at Day 35[a]

Groups[b]	Δ Body Wt (g)	GFR (mL/min per 100 g)	Serum Creatinine (Day 35) (mg/dL)	SBP (mm Hg)
CsA	82.1 ± 10.2	0.21 ± 0.04	0.87 ± 0.08	120 ± 6
CsA+L	−8.0 ± 9.7[c]	0.17 ± 0.04[d]	1.63 ± 0.27*	80 ± 4*
CsA+H/F	−26.6 ± 10.6[c]	0.31 ± 0.05	0.85 ± 0.23	95 ± 3*
VH	132.8 ± 6.4[c]	0.52 ± 0.03*	0.39 ± 0.01[c]	133 ± 6
VH+L	101.4 ± 15.6	0.47 ± 0.04	0.48 ± 0.08	92 ± 6
VH+H/F	14.1 ± 12.6	0.53 ± 0.03	0.42 ± 0.02	115 ± 5

[a] Values are expressed as mean ± SE.
[b] CsA, treatment with CsA (15 mg/kg per day); CsA+L, CsA + 10 mg of losartan/kg; CsA+H/F, CsA + hydralazine (240 mg/L) and furosemide (1 to 2 mg); VH, vehicle treatment (1 m of olive oil/kg); VH+L, vehicle treatment + 10 mg of losartan/kg; VH+H/F, vehicle treatment + hydralazine (240 mg/L) and furosemide (1 to 2 mg).
[c] $P < 0.05$ *versus* CsA.
[d] $P < 0.007$ *versus* CsA+H/F.
* $P < 0.005$ *versus* CsA.

containing TGF-β were ED-1-positive macrophages. The identity of the major population of TGF-β-expressing cells was not determined but is likely to be interstitial fibroblasts (Figure 6).

Interstitial fibrosis. CsA-treated animals had significant interstitial fibrosis. Both forms of treatment, CsA + H/F and CsA + L, reduced interstitial fibrosis significantly (Table 1).

Interstitial deposition of collagens. CsA treatment increased the expression of **Type IV collagen** in tubular epithelial cells, tubular basement membranes, and the adjacent interstitium between the tubules. Treatment with hydralazine/furosemide, but not with losartan, significantly reduced the interstitial deposition of Type IV collagen. In vehicle-treated animals, **Type I collagen** was primarily found in the adventitia of blood vessels and in Bowman's capsule. In animals treated with CsA, there was an increase of Type I collagen in areas of severe interstitial fibrosis. However, neither Type I nor Type IV collagen appeared to be a major component of the fibrotic material.

Nephrocalcinosis was present in CsA-treated rats. CsA + H/F-treated animals had less nephrocalcinosis, but this was not statistically significant. Interestingly CsA + L had significantly increased nephrocalcinosis when compared with CsA + H/F. Control animals treated with losartan only (VH + L) developed mild nephrocalcinosis, which was not observed in vehicle-treated animals (Table 1).

Physiologic Studies

The final **body weight** of all CsA-treated rats (regardless of their treatment with L or H/F) was significantly lower compared with that of vehicle controls. Whereas vehicle-treated animals gained 134 g during the 35 days of our study, CsA + L rats lost 8 g and CsA + H/F-treated animals lost a mean of 27 g. These data are shown in Table 2.

Renal Function. Renal function (GFR) in the different groups is summarized in Table 2. All CsA-treated animals had a significant reduction in GFR as measured by creatinine clearance. Animals treated with CsA + H/F had a significantly higher GFR compared with those treated with CsA + L. There was no significant difference between CsA and CsA + H/F.

Systolic blood Pressures. Table 2 summarizes the SBP data of the various groups. CsA treatment alone resulted in a slight but not significant reduction in SBP compared with vehicle-treated animals. Both treatments (CsA + H/F, CsA + L) significantly reduced SBP compared with animals treated with CsA alone.

DISCUSSION

The purpose of this study was to elucidate the pathogenic mechanisms of CsA nephropathy as well as to identify the mechanisms by which the inhibition of AII provides protection. In a previous study, we noted that OPN(26,27) is expressed in CsA nephropathy and that its localization correlated with the accumulation of macrophages.(8) In this study, we have not only demonstrated that the sites of OPN expression are associated with the accumulation of macrophages but we have also provided the first documentation that the number of tubules (*i.e.*, degree) expressing OPN correlates closely ($r = 0.79$) with the number of macrophages in the tubulointerstitium. Furthermore, we were also able to show that the degree of OPN expression also correlates with the severity of interstitial fibrosis ($r = 0.74$). OPN is an Arg-Gly-Asp (RGD)-containing, adhesive glycoprotein that is a potent macrophage chemoattractant (28–30). Recently, we reported that OPN is expressed by proximal and distal tubular epithelial cells in other renal diseases in which tubulointerstitial injury develops, including AII-induced hypertension and several experimental models of glomerulonephritis (24,25). Thus, these studies are consistent with the hypothesis

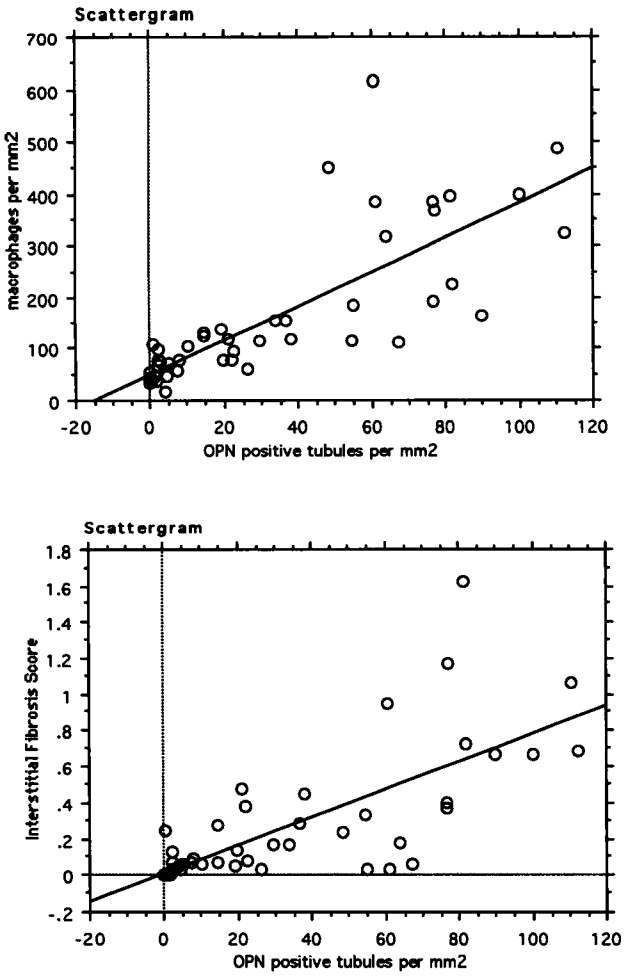

Figure 5. (A) Relationship of macrophages (ED-1-positive cells) with OPN. A significant correlation could be demonstrated between OPN and ED-1-positive cells in individual rats ($r = 0.79$, $P < 0.0001$). (B) Relationship of interstitial fibrosis with OPN. A significant correlation could be demonstrated between OPN and interstitial fibrosis in individual rats ($r = 0.74$, $P < 0.0001$).

that OPN may be one of the chemoattractants that mediates the recruitment of the macrophages.

Another important finding in this study is the demonstration that interstitial TGF-β expression was increased in CsA-treated animals. A number of studies have suggested that TGF-β plays a central role in the pathogenesis of interstitial fibrosis in various diseases (31–33). There are at least three potential sources of the TGF-β found in the tubulointerstitium: interstitial macrophages (34), interstitial fibroblasts (35), and tubular epithelial cells (36). Our double immunolabeling studies suggest that macrophages are not the major source of TGF-β in the interstitium and that other interstitial cells, such as the fibroblast, may be the most likely source (35).

The second part of the study was devoted to exploring the mechanisms by which AII receptor blockade affects CsA nephrotoxicity. AII is of particular interest in CsA nephropathy because AII infusion in rats induces a histologic lesion in the tubulointerstitium similar to that produced by CsA (13). Moreover, AII Type I receptor antagonists and ACE inhibitors reduce fibrosis in this model (12,18). To examine the role of AII, rats were administered losartan at a dose of 10 mg/kg. The dose of 10 mg/kg was chosen because it has previously been shown to significantly block AII Type I receptors (18). A hydralazine/furosemide group was included to control for changes in blood pressure independent of AII. Hydralazine also serves as a non-AII-dependent vasodilator control. The effects of both forms of treatment alone (losartan and hydralazine+furosemide) in vehicle-treated animals were also observed.

A new finding was the observation that the AII receptor antagonist losartan almost completely blocked the arteriolopathy induced by CsA, an effect that was not seen with H/F despite equivalent reduction in SBP (Table 2). This vascular change is thought to be a degenerative lesion of the afferent arteriole and to be pathognomonic for CsA-induced injury. The fact that the arteriolopathy occurs at sites where renin is markedly stimulated suggests that the marked stimulation of renin may also lead to a local generation of AII via the presence of ACE and local angiotensinogen with subsequent AII-mediated injury at these sites. It therefore suggests that the renin-angiotensin system plays a central role in the pathogenesis of this lesion (37–39).

Of interest was the observation that OPN expression was significantly reduced by H/F, and to a lesser extent by losartan (not significant). This was initially puzzling because our original hypothesis was that OPN expression in the CsA model would be mediated by AII. AII induces OPN expression in a variety of cell types *in vitro* (40), and AII induces OPN expression in tubular epithelial cells *in vivo* (24). It is possible that OPN expression was induced not by direct effects of AII, but rather by CsA-induced ischemia, which was partially ameliorated either by AII blockade or by the vasodilatory effect of hydralazine. OPN expression in tubular epithelial cells can be induced by ischemia (41), and ischemia can also lead to tubulointerstitial fibrosis (42). However, an undesirable consequence of the various treatments was that, by lowering blood pressure, perfusion pressure could be excessively reduced, resulting in enhanced ischemia to the cortex, which could account for the low-grade OPN expression and macrophage accumulation noted in VH-treated controls given losartan or hydralazine/furosemide. This could explain why OPN expression and macrophage influx were not completely inhibited.

Another new finding is the observation that both losartan and hydralazine/furosemide were equally effective in reducing interstitial fibrosis. The reduction in fibrosis was also accompanied by decreases in OPN expression, macrophage infiltration, and TGF-β ex-

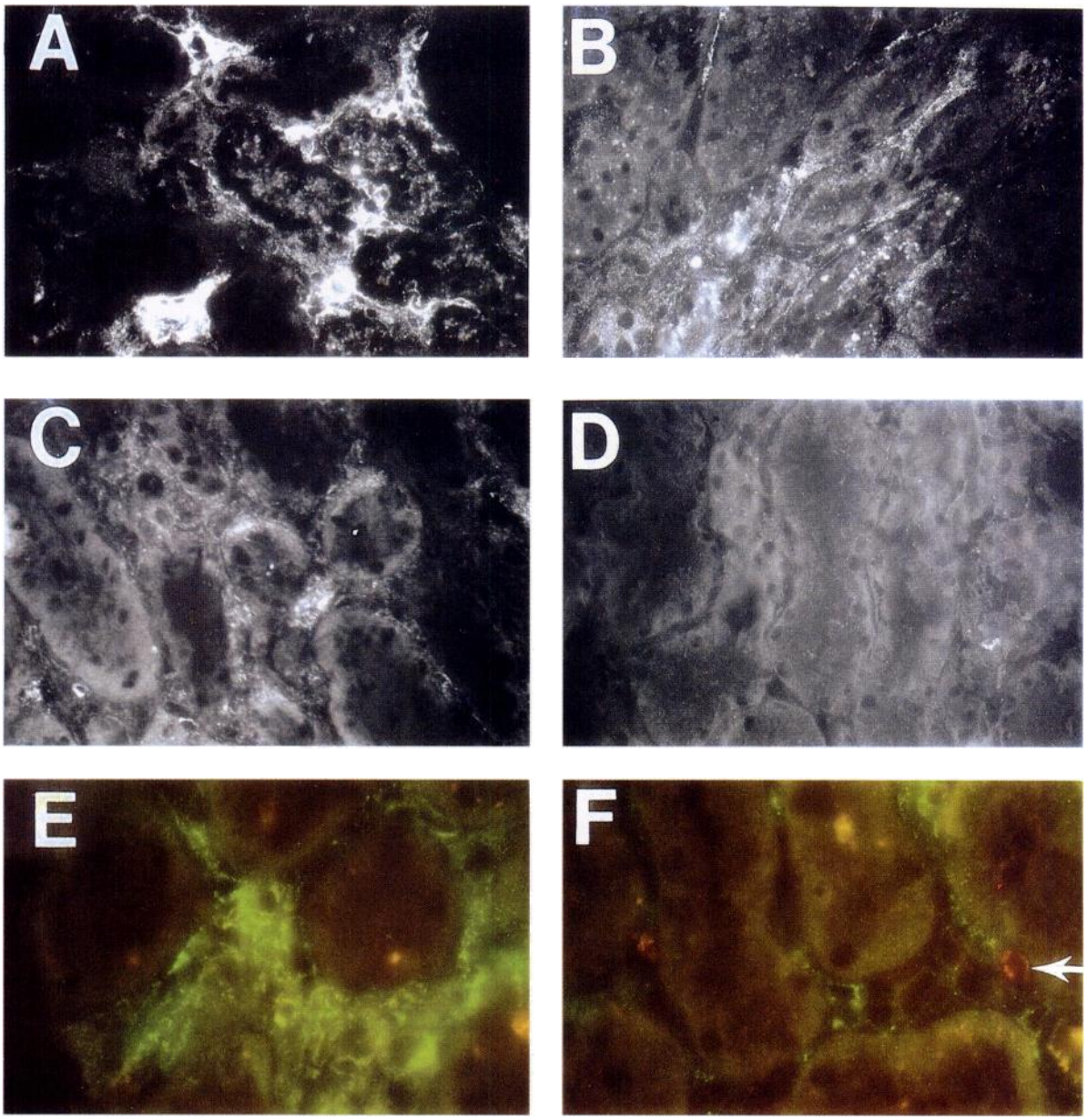

Figure 6. Immunostaining for TGF-β. In CsA-treated rats, a dramatic increase in the expression of TGF-β was noted in interstitial areas (A and E (in color)). CsA+L (B) and CsA+H/F (C)-treated animals had reduced TGF-β expression. A vehicle-treated control is shown in Panel D for comparison. Double immunolabeling showed that most of the TGF-β expression (fluorescent green) was found in interstitial cells that excluded the macrophage marker ED-1 (arrow, shown in red) (original magnification, ×1,000).

pression. The mechanism by which AII blockade and/or hydralazine/furosemide reduce CsA-mediated interstitial fibrosis could relate to the inhibition of the vasoconstrictive effects of CsA, which likely involve endothelin-1, AII, and direct CsA effects and which in turn may result in chronic ischemia to the tubulointerstitium. It has been shown by others (42) that experimental chronic ischemia alone can cause chronic interstitial nephritis. It is conceivable that AII blockade may be acting by reducing vasoconstrictive effects. Similarly, hydralazine/furosemide may protect the kidney from ischemia/fibrosis by the vasodilatory action of hydralazine or by the inhibition of metabolic work by furosemide (43).

The fact that hydralazine/furosemide reduced interstitial fibrosis in this experiment is in contrast to a previous study (18) that showed a protective effect of AII blockade but not of hydralazine/furosemide. This discrepancy may relate to the fact that in the previous study a more severe reduction of SBP was observed

with H/F treatment (mean SBP of 85 *versus* 95 mm Hg in our experiment), thereby possibly compromising the protective effect of hydralazine because of a greater reduction in perfusion pressure in the tubulointerstitium.

Previous studies have documented a reduction in GFR (creatinine clearance) with the chronic administration of CsA (44,45) as well as the deleterious effects of ACE inhibitors or AII blockers on the latter (18,46–48). Because AII has an important role in the regulation of the glomerulular filtration pressure by acting as a potent vasoconstrictor on primarily the efferent arteriole, it is not surprising that blocking AII results in a reduction of the GFR. However, the administration of hydralazine/furosemide had a partially beneficial effect on creatinine clearance, probably as the result of a reduction in the afferent arteriolar resistance, which would thereby increase glomerular filtration.

A result of the antihypertensive drug administration to salt-depleted animals was a decrease in SBP in both losartan- and hydralazine/furosemide-treated rats. Because afferent arterioles may be less able to autoregulate after CsA, excessive hypotension may have compromised perfusion pressure. Furthermore, vehicle-treated rats on a low-salt diet administered hydralazine/furosemide or losartan developed a minor degree of interstitial fibrosis and tubular injury (Table 2). This was only observed in rats with an SBP of less than 80 mm Hg, suggesting a threshold effect (data not shown). This could potentially explain why neither therapy blocked tubular injury.

Another interesting feature of this model was the development of nephrocalcinosis in CsA-treated animals. In this case, the therapeutic interventions had contrary effects. Whereas losartan tended to increase the precipitation of calcium crystals, hydralazine/furosemide reduced the incidence of nephrocalcinosis. A possible explanation for this phenomenon could relate to the fact that hydralazine/furosemide had a partially protective effect on the GFR in CsA-treated rats, thereby resulting in more tubular flow, whereas losartan tended to reduce GFR, which probably facilitated calcium crystal precipitation ($P < 0.02$ for CsA+H/F *versus* CsA+L). This hypothesis is also supported by the fact that animals vehicle treated with losartan developed nephrocalcinosis to a minor degree, which was not observed in vehicle-treated animals given hydralazine/furosemide, despite similar reductions in SBP.

In conclusion, our study demonstrates that the interstitial fibrosis in CsA nephropathy is associated with OPN expression, macrophage accumulation, and TGF-β expression. Treatment with the AII receptor antagonist losartan or hydralazine/furosemide reduces OPN expression, macrophages, TGF-β expression, and interstitial fibrosis. Losartan also reduces the arteriolopathy. Thus, CsA nephrotoxicity results from both AII-dependent and AII-independent mechanisms. Further studies are necessary to elucidate the pathogenic mechanisms involved in this disease.

ACKNOWLEDGMENTS

Supported in part by research grants from the United States Public Health Service (DK 34198, 07467, 07659, 47659, 43422, and 02142). R. Pichler was supported by an Erwin-Schrödinger-Scholarship from the Austrian Science Foundation. N. Franceschini was a recipient of the International Society of Nephrology fellowship grant. We thank Dr. Cecilia M. Giachelli, Jessie Lindsley, Lisa Gunion, Jennifer Withrow, Kathy Gordon, Jeff Pippin, Maureen Reilly, Julie Gray, Kristie Guay, and Gary McNair for their excellent technical advice and assistance.

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