Immunohistochemical Analysis of Renin Activity in Chronic Cyclosporine Nephropathy in Childhood Nephrotic Syndrome

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Abstract. Although the pivotal role of activation of the intrarenal renin-angiotensin system (RAS) has been demonstrated in the rat model of chronic cyclosporine (CyA) nephropathy, it is still unclear whether intrarenal RAS activation is responsible for chronic CyA nephropathy in humans. Therefore, the distribution of renin in formalin-fixed, paraffin-embedded renal biopsy specimens obtained from 26 children who had idiopathic nephrotic syndrome (NS) and who were treated with long-term moderate-dose CyA was examined with the use of immunohistochemistry with rabbit polyclonal anti-human renin antibody. Nineteen patients had steroid-dependent NS, and 7 had steroid-resistant NS. However, CyA treatment led all of the latter patients into complete remission. All of the patients underwent renal biopsies at the start and the end of CyA treatment. In the pre-CyA specimens, immunoreactivity to renin was detected mainly in those parts of arterioles within the anatomically well-defined juxtaglomerular apparatus. In the post-CyA specimens, it was also detected in those parts of the vessels upstream from the juxtaglomerular apparatus. Moreover, the ratio of the number of renin-positive cells to the number of glomeruli (histologic renin index) increased significantly with long-term CyA treatment (from 1.26 ± 0.24 to 4.30 ± 0.40, $P < 0.0001$). Eleven of the post-CyA specimens showed mild or moderate CyA-associated arteriolopathy (CAA), whereas 15 showed no CAA. The histologic renin index was significantly higher in specimens with CAA than in those without CAA (5.16 ± 0.59 *versus* 3.67 ± 0.48, $P = 0.031$). Seven CAA-positive patients underwent repeat biopsies 6 to 12 mo after discontinuing the CyA. Their specimens showed an improvement in the CAA and significantly lower histologic renin index compared with the post-CyA (from 4.18 ± 0.69 to 2.10 ± 0.25, $P = 0.018$). Eleven of the post-CyA specimens showed mild to moderate interstitial fibrosis, and 15 showed no fibrosis. There was no significant difference in immunoreactivity to renin between the specimens with interstitial fibrosis and those without. However, patients with interstitial fibrosis had significantly longer periods of heavy proteinuria during CyA therapy, because of either steroid-resistant NS or frequent relapses of NS (83 ± 18 *versus* 35 ± 12 d, $P = 0.030$). These findings indicate that long-term CyA treatment for idiopathic NS in children may stimulate renin production in arterioles. They also suggest that CyA-stimulated intrarenal RAS activation is responsible for the development of CAA and that CyA-induced interstitial fibrosis is potentiated by long-term heavy proteinuria and is at least partly independent of CyA-stimulated intrarenal RAS activation.

Cyclosporine (CyA) is a relatively new agent that is useful in the management of steroid-dependent or steroid-resistant nephrotic syndrome (NS), in place of corticosteroids and alkylating agents (1–6). The beneficial effects of CyA, however, often are accompanied by side effects. Of greatest concern is chronic CyA nephropathy, characterized by arteriolar lesions (CyA-associated arteriolopathy [CAA]), tubulointerstitial lesions, and focal glomerulosclerosis (7,8).

Rats that are maintained on a low-salt diet and given CyA develop arteriolar hyalinosis and striped interstitial fibrosis, similar to the tissue changes seen in chronic CyA nephropathy in humans (9–11). The rat model shows enhanced renin expression in the juxtaglomerular apparatus (JGA); the nephropathy can be improved by angiotensin-converting enzyme inhibitors and also angiotensin II type I receptor antagonists (12,13), suggesting that activation of the tissue renin-angiotensin system (RAS) is, at least in part, responsible for the development of chronic CyA nephropathy in rats.

However, it is still unclear whether activation of the intrarenal RAS is responsible for chronic CyA nephropathy in humans. To elucidate the role of RAS activation in human chronic CyA nephropathy, we studied the distribution of renin in formalin-fixed, paraffin-embedded renal biopsy specimens obtained from 26 children who had idiopathic NS and who were treated with long-term moderate-dose CyA. The children underwent sequential biopsies at the start and the end of CyA treatment.

Materials and Methods

This study was performed at Kobe University Hospital from 1991 to 1999. All patients and/or their parents/guardians gave their informed consent.

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Patients

We studied 26 children (20 male and 6 female) who had idiopathic NS and who were treated with long-term moderate-dose CyA. Nineteen patients had steroid-dependent NS, and 7 had steroid-resistant NS. However, CyA treatment led all of the latter patients into complete remission within 3 mo after the start of CyA treatment. The definitions and criteria for NS, remission, relapse, steroid dependence, and steroid resistance were those used by the International Study of Kidney Disease in Children (14,15). *NS* was defined as heavy proteinuria (urinary protein excretion $\geq$ 40 mg/m^2 per hour) with hypoalbuminemia ($\leq$ 25 g/L). *Remission* was defined as a reduction in urinary protein excretion to less than 4 mg/m^2 per hour (Albustix 0 to trace) for 3 consecutive days. *Relapse* was defined as a reappearance of proteinuria of $\geq$ 40 mg/m^2 per hour (Albustix 2+ or greater) for 3 consecutive days. *Steroid dependence* was defined as a remission within 4 wk of beginning prednisolone therapy, with relapse occurring when the dose of prednisolone was reduced to below a critical level or within 2 wk of discontinuation of therapy. *Steroid resistance* was defined as a failure to achieve response despite 4 wk of prednisolone therapy.

Patients were followed up at least once a month during the study. At each follow-up visit, the patients were asked about their symptoms, and tests and measurements, including a blood count, serum creatinine, creatinine clearance, blood urea nitrogen, urinary protein excretion, BP, body weight, and body height, were performed. *Hypertension* was defined as being present if the mean BP exceeded the upper normal limit of that of healthy Japanese children (mean + 2SD).

Steroid Therapy

Only prednisolone was used for steroid therapy. The initial attack was treated with 2 mg/kg per day prednisolone, given in three divided doses (maximum dose, 80 mg/d) for the first 4 wk, followed by alternate-day prednisolone, with 1.3 mg/kg given as a single dose on the morning of every other day for 4 wk (total 8 wk). Patients who had not entered complete remission despite 4 wk of prednisolone therapy (steroid-resistant NS) were changed to alternate-day prednisolone, with 2 mg/kg given as a single dose on the morning of every other day. This dose of prednisolone was continued in conjunction with CyA until complete remission was achieved. The dose of prednisolone was then decreased by 0.5 mg/kg every 2 wk. Relapses were treated with 2 mg/kg per day prednisolone, given in three divided doses (maximum dose, 80 mg/d) for the first 4 wk, followed by alternate-day prednisolone, with 2 mg/kg given as a single dose on the morning of every other day for 2 wk, after which the dose was decreased by 0.5 mg/kg every 2 wk (total 12 wk).

CyA Treatment

In patients with steroid-dependent NS, treatment with CyA (Sandimmune® oral solution, Novartis Pharmaceutical Corporation, Osaka, Japan) was started at a dose of 100 to 150 mg/m^2 per day in two divided doses after the patients had attained remission with prednisolone therapy. In patients with steroid-resistant NS, CyA treatment was started to induce remission, so these patients had heavy proteinuria at the start of CyA treatment. The dose of CyA for all patients was adjusted to a target trough level of 60 to 100 ng/ml, as measured by monoclonal antibody fluorescence polarization immunoassay. The protocol for prednisolone therapy for relapses during CyA treatment was the same as that described above. No drugs that might have contributed to CyA nephrotoxicity were given during CyA treatment.

Immunoperoxidase Staining of Renin

Rabbit polyclonal antibody to renin was provided by Professor A. Fukamizu (Tsukuba, Ibaragi, Japan). The antibody was made by immunization of rabbits with human active renin purified from recombinant human prorenin. Previous studies demonstrated that the antibody recognized human active and inactive renin (16,17). Formalin-fixed, paraffin-embedded renal biopsy specimens were sectioned, deparaffinized, and incubated with the antibody (1:2000) for 60 min at room temperature, then stained by standard immunoperoxidase procedures, according to the manufacturer's instructions (VECTASTAIN® ABC system, Vector Laboratories, Inc., Burlingame, CA).

Histologic Evaluation

From each case, we prepared two to four serial periodic acid-Schiff (PAS) stains, a Masson's trichrome stain, and a renin stain. We also prepared a PAS-renin double stain in some cases to examine the relationship between renin immunoreactivity and CAA. An investigator who was blinded to the treatment status reviewed the pre- and post-CyA treatment renal biopsies.

The number of arterioles and JGA seen in each cross section sometimes varied markedly, even in serial sections obtained from the same specimen. In post-CyA specimens, immunoreactivity to renin was often seen in those parts of arterioles upstream from the JGA, so the number of renin-positive cells per arteriole or per JGA was also variable, even in the same specimen. However, the number of glomeruli seen in each cross section tended to be stable in the same specimen. Therefore, we determined the ratio of the number of renin-positive cells to the number of glomeruli (histologic renin index) to evaluate the renin immunoreactivity in the renal cortices.

CAA was defined as arteriolar lesions showing hyalinosis. *Hyalinosis* was identified as lumpy PAS-positive protein deposits within or on the outer aspects of the arteriolar walls. The degree of CAA was determined as follows: grade 0, no CAA; grade 1 (mild), the ratio of the number of CAA-positive arterioles to the number of glomeruli seen in each cross section is less than 10%; grade 2 (moderate), the ratio is between 10 and 20%; and grade 3 (severe), the ratio is more than 20%.

CyA-induced interstitial fibrosis in cortex was graded semiquantitatively on PAS-stained and Masson's trichrome–stained sections with $\times$40 objective. A minimum of 20 fields were assessed in each biopsy. The findings of interstitial fibrosis consisted of matrix-rich expansion of the interstitium with distortion and collapse of the tubules and thickening of the tubular basement membranes. The following semiquantitative grade was used: grade 0, no interstitial fibrosis; grade 1, less than 25% of the interstitial space showing fibrosis; grade 2, 25 to 50% of the interstitial space showing fibrosis; and grade 3, more than 50% of the interstitial space showing fibrosis.

Statistical Analyses

The values are expressed as mean $\pm$ SEM. We used the Wilcoxon rank sum test and Mann-Whitney U test to compare paired and unpaired two-group means, respectively. We used Fisher's exact test to evaluate the association between categorical variables. The differences were evaluated with a Stat View® software package (Abacus Concepts Inc., Berkeley, CA); those at $P < 0.05$ were considered significant.

Results

The mean age at the start of CyA was 7.5 $\pm$ 0.9 yr (range, 1 to 16 yr). The mean period of CyA treatment was 30 $\pm$ 2 mo

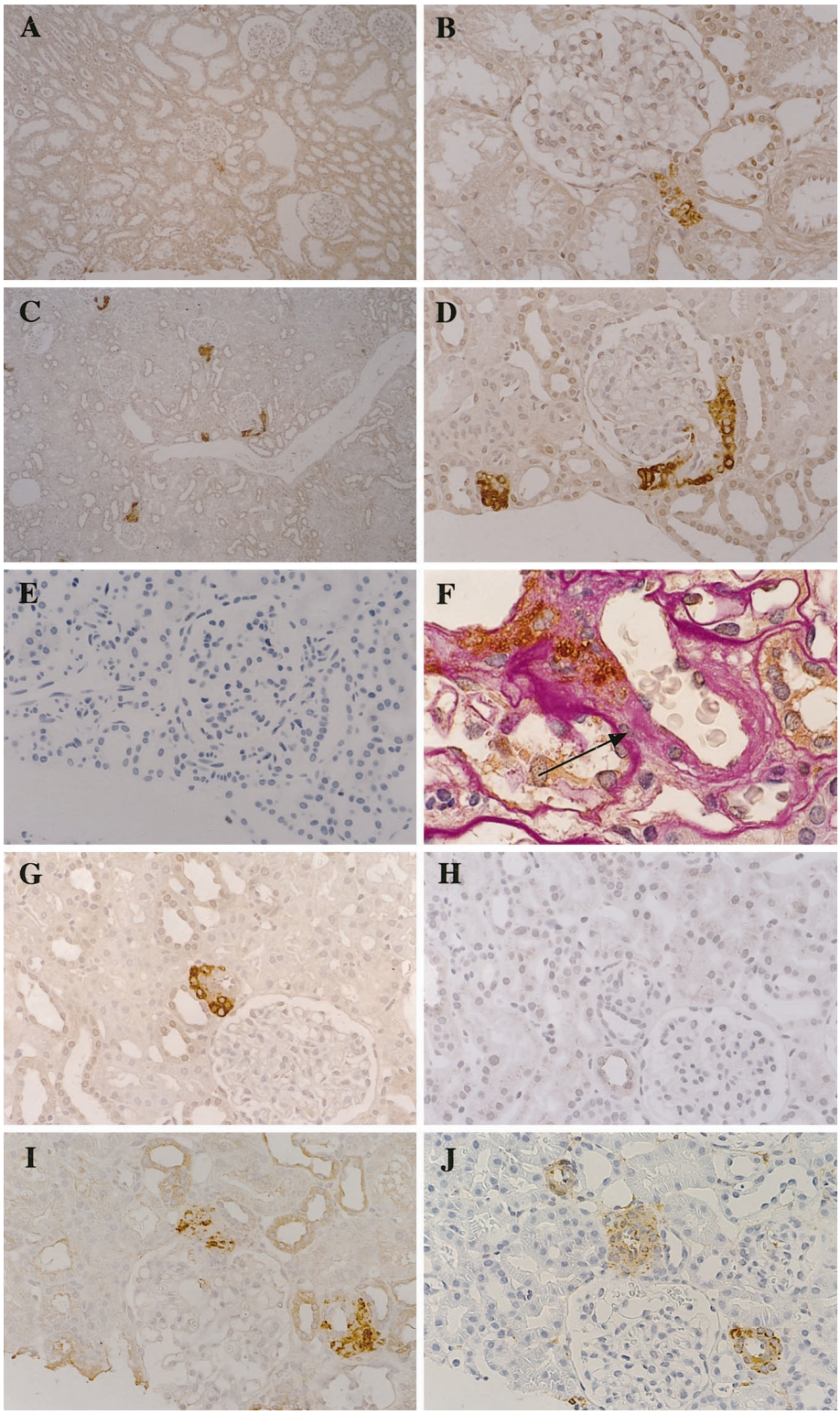

Figure 1. Immunoreactivity to renin, and lesions of cyclosporine (CyA)-associated arteriolopathy (CAA). Immunoreactivity to renin was detected mainly in those parts of the arterioles within anatomically well-defined juxtaglomerular apparatus (JGA) in a pre-CyA specimen (A and B). In a post-CyA specimen obtained from the same patient, immunoreactivity to renin was also detected in parts of the vessels upstream from the JGA and was greater than that found in the pre-CyA specimen (C and D). The immunoreactivity observed was absent when an irrelevant antibody was used as the primary antibody (E). CAA lesions (hyalinosis, arrow) were often found near areas of enhanced immunoreactivity to renin (F). The immunoreactivity observed was not detectable when a rabbit polyclonal antibody to renin absorbed by preincubation with excess human renin was used as the primary antibody (G, anti-renin antibody; H, anti-renin antibody absorbed by excess renin). Immunostaining of serial sections using the antibody to renin (I) or an antibody to α-smooth muscle actin (J) as the primary antibody suggested that the immunoreactivity to renin was localized in arteriolar smooth muscle cells. Magnifications: $\times$45 in A, C; $\times$180 in B, D, E, G, H, I, J; $\times$450 in F.

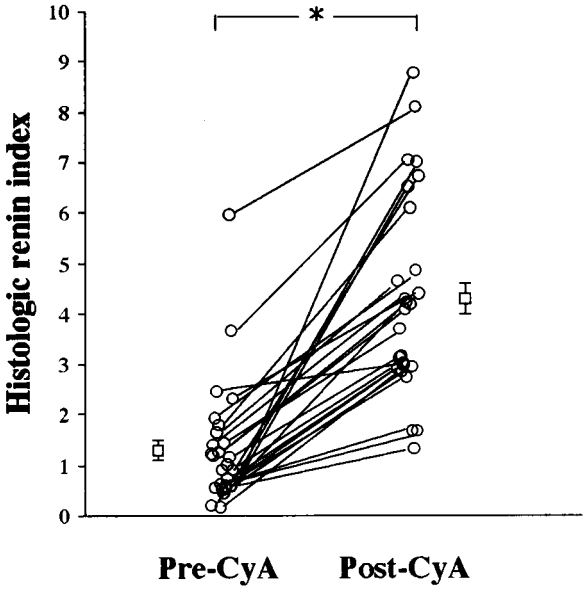

Figure 2. Long-term CyA treatment and immunoreactivity to renin. The histologic renin index was significantly increased by long-term CyA treatment in children with idiopathic nephrotic syndrome ($n = 26$). Open squares and error bars denote mean and SE, respectively. *, $P < 0.0001$.

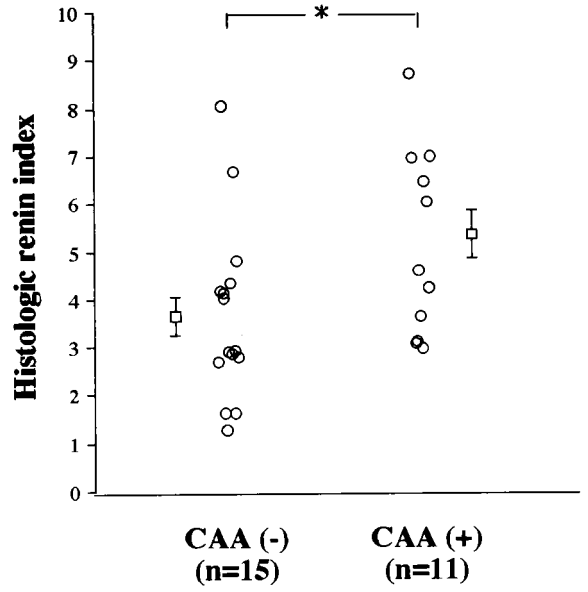

Figure 3. Development of CAA and immunoreactivity to renin. Long-term CyA treatment resulted in mild to moderate CAA in 11 patients. The histologic renin index in specimens with CAA was significantly greater than that without CAA. Open squares and error bars denote mean and SE, respectively. *, $P = 0.031$.

(range, 22 to 57 mo). All of the patients ($n = 26$) underwent renal biopsies at the start and the end of CyA treatment, and at least 10 glomeruli were present in each biopsy. The pre-CyA biopsies showed minimal-change NS in 20 patients, diffuse mesangial proliferation in 4, and focal segmental glomerulosclerosis in 2. However, all of the post-CyA biopsies showed minimal-change NS. All of the pre-CyA biopsies showed no or negligible tubulointerstitial lesions. No patients developed hypertension or impaired creatinine clearance. The mean BP and creatinine clearance at the end of CyA treatment were similar to those at the start of it (mean BP, from 82 ± 2 to 80 ± 1 mmHg, $P > 0.05$; creatinine clearance, from 172 ± 7 to 163 ± 6 ml/h per 1.73 m^2, $P > 0.05$).

In pre-CyA specimens, immunoreactivity to renin was detected mainly in those parts of the arterioles within anatomically well-defined JGA (Figure 1, A and B). In post-CyA specimens, it was also detected in those parts of vessels upstream from the JGA (Figure 1, C and D). We did not detect this immunoreactivity when we used an irrelevant antibody (rabbit IgG against mouse Ig; Zymed Laboratories, San Francisco, CA; Figure 1E) or a rabbit polyclonal antibody to renin absorbed by preincubation with excess human renin (Calbiochem-Novabiochem Corporation, Darmstadt, Germany; Figure 1, G and H) as the primary antibody. This indicated that the immunoreactivity observed was specific to renin. Immunostaining of serial sections using either the antibody to renin or an mouse monoclonal antibody to α-smooth muscle actin (IBL Research Products Co., Cambridge, MA) as the primary antibody suggested that the immunoreactivity to renin was localized in arteriolar smooth muscle cells (Figure 1, I and J).

The histologic renin index was significantly increased by long-term CyA treatment (from 1.26 ± 0.24 to 4.30 ± 0.40, $P < 0.0001$; Figure 2). Eleven post-CyA specimens showed mild (grade 1, $n = 8$) or moderate (grade 2, $n = 3$) CAA, and 15 showed no CAA. The doses of CyA given were similar in both groups (4.27 ± 0.43 *versus* 4.17 ± 0.36 mg/kg per d, $P > 0.05$). The mean BP at the end of CyA treatment was similar in both groups (79 ± 3 *versus* 80 ± 1 mmHg, $P > 0.05$). CyA treatment significantly increased the histologic renin index in both groups (with CAA, from 1.29 ± 0.27 to 5.16 ± 0.59, $P = 0.0033$; without CAA, from 1.24 ± 0.38 to 3.67 ± 0.48, $P = 0.0007$). The histologic renin index was significantly higher in specimens with CAA than in those without CAA (5.16 ± 0.59 *versus* 3.67 ± 0.48, $P = 0.031$; Figure 3), although the histologic renin index in both groups was similar in the pre-CyA specimens (1.29 ± 0.27 *versus* 1.24 ± 0.38, $P > 0.05$). CAA lesions were often found next to areas that showed enhanced immunoreactivity to renin (Figure 1F). Seven CAA-positive patients developed frequent relapses after the discontinuation of CyA. These patients underwent repeat biopsies an average of 10 ± 0.8 mo (range, 6 to 12 mo) after CyA discontinuation. The specimens obtained after CyA discontinuation showed a significantly lower histologic renin index compared with the post-CyA specimens (from 4.18 ± 0.69 to 2.10 ± 0.25, $P = 0.018$; Figures 4, A and B, and 5A). The CAA improved significantly (mean CAA grade, from 1.14 ± 0.14 to 0.29 ± 0.18, $P = 0.034$) and disappeared in five of these patients after CyA discontinuation (Figures 4, C and D, and 5B). The interstitial fibrosis in these patients improved slightly but not statistically significantly after CyA discontinuation (mean fibrosis grade, from 0.71 ± 0.18 to 0.29 ± 0.18, $P = 0.083$).

Eleven post-CyA specimens showed mild to moderate in-

terstitial fibrosis, and 15 showed no fibrosis. The doses of CyA were similar in both groups (4.50 ± 0.46 *versus* 4.01 ± 0.33 mg/kg per d, $P > 0.05$). There was no significant difference in immunoreactivity to renin between the specimens with interstitial fibrosis and those without (4.64 ± 0.66 *versus* 4.05 ± 0.50, $P > 0.05$). However, patients with interstitial fibrosis had significantly longer periods of heavy proteinuria during CyA therapy, because of steroid-resistant NS or frequent relapses of NS (83 ± 18 *versus* 35 ± 12 d, $P = 0.030$), although the duration of CyA therapy was similar in both groups (33.1 ± 3.8 *versus* 27.0 ± 1.9 mo, $P > 0.05$). Indeed, patients who had heavy proteinuria for more than 30 d during the CyA treatment exhibited significantly higher rates of positive interstitial fibrosis (64 *versus* 17%, $P = 0.021$) and developed significantly

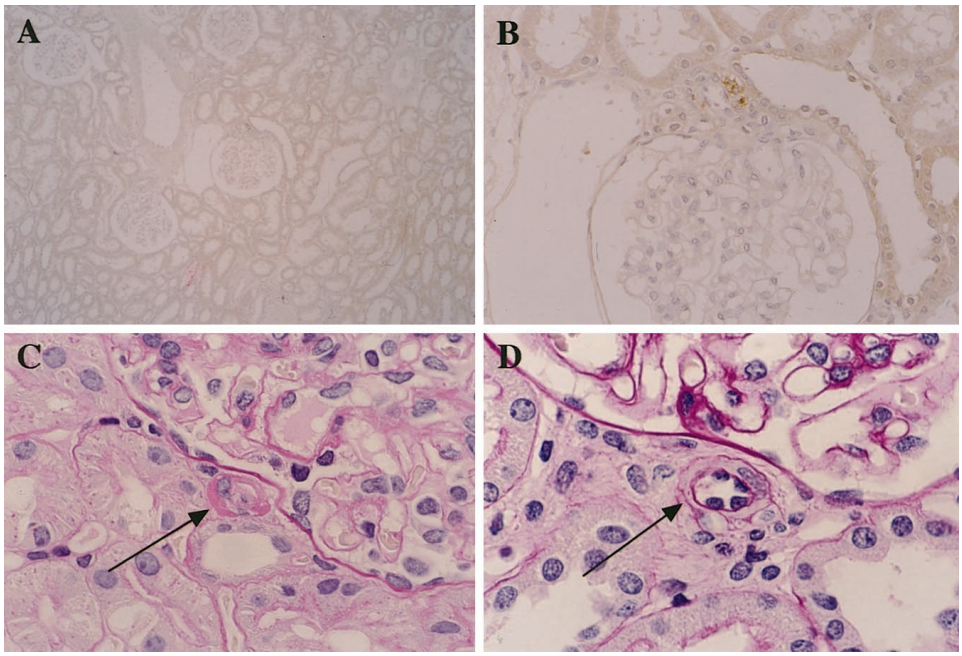

Figure 4. Immunoreactivity to renin and CAA after CyA discontinuation. After CyA was discontinued, the immunoreactivity to renin in a specimen obtained from the same patient shown in Figure 1 decreased to a similar level to that of the patient's pre-CyA specimen (A and B), and the CAA disappeared (C, post-CyA, arrow indicates a lesion of arteriolar hyalinosis; D, after CyA discontinuation, arrow indicates an intact arteriole). Magnifications: ×45 in A; ×180 in B; ×450 in C and D.

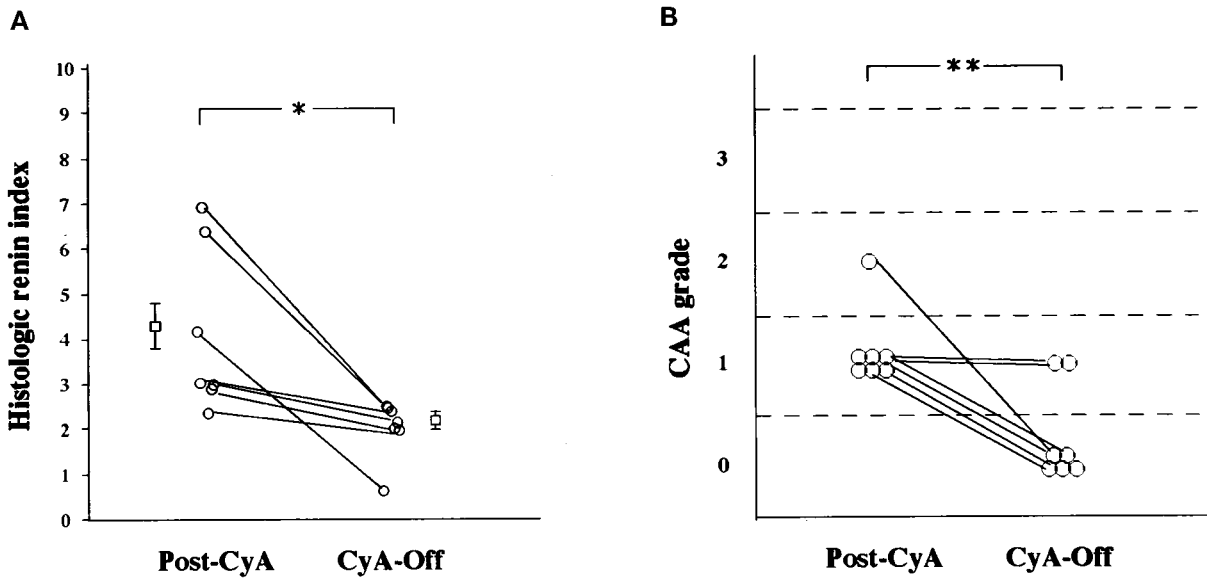

Figure 5. Reduction of immunoreactivity to renin and improvement of CAA after CyA was discontinued. CyA discontinuation for 6 to 12 mo significantly decreased the histologic renin index ($n = 7$). Open squares and error bars denote mean and SE, respectively (A). The degree of CAA was significantly improved after CyA was discontinued (B). *, $P = 0.018$; **, $P = 0.034$.

more severe interstitial fibrosis (mean fibrosis grade, 0.71 ± 0.16 *versus* 0.17 ± 0.11, $P = 0.015$).

Discussion

This study has provided the following pieces of evidence that the development of CAA in childhood idiopathic NS is closely related to renin expression in the arterioles: (*1*) long-term CyA therapy induced change in the distribution pattern of immunoreactivity to renin and an increase in the histologic renin index, (*2*) the histologic renin index was significantly higher in specimens with CAA than in those without CAA, (*3*) CAA lesions were often found near areas of enhanced immunoreactivity to renin, and (*4*) the histologic renin index was significantly reduced and CAA was improved after CyA discontinuation. The pathogenic role of enhanced renin expression in CAA can be explained in several ways. Enhanced arteriolar renin expression may activate the systemic RAS, which, in turn, results in systemic hypertension, inducing renal arteriolar hypertension and arteriolar damage. However, this theory is unlikely, as chronic CyA treatment did not induce systemic hypertension in our study. Moreover, it has been reported that chronic CyA treatment does not activate systemic RAS in humans (18–21). Alternatively, renin or prorenin *per se* may be toxic to vascular smooth muscle cells in the renal arterioles. However, this is also unlikely, because treatment with angiotensin-converting enzyme inhibitor and/or angiotensin II type I receptor antagonists improve CAA, although they enhance immunoreactivity to renin in the rat model of chronic CyA nephropathy (13). Therefore, intrarenal—*i.e.*, tissue RAS—activation is the most likely mechanism responsible for CAA, although the effects of tissue RAS inhibition on chronic CyA nephropathy in humans need to be investigated.

Strøm *et al.* (22) reported that in biopsy specimens of CyA-treated transplanted kidneys, there was a slight but not significant increase in the proportion of renin-positive arterioles in CyA-treated groups without CAA compared with the control groups but that with increasing CAA there was a decrease in the proportion of renin-positive arterioles. These findings seem to be inconsistent with our results, which indicated that CyA treatment significantly increased renin expression even in specimens without CAA and that renin expression was significantly enhanced in specimens with CAA, compared with those without CAA. However, our study, unlike that of Strøm *et al.*, used serial biopsy specimens obtained from the same patients, and this may have affected the effects that we observed after CyA treatment. Also, the degree of CAA was much milder in our study than in their study. Indeed, in their study, the number of renin-positive cells in specimens with moderate CAA was significantly greater than that in specimens without CAA, but the number of renin-positive cells in specimens with severe CAA dropped toward the baseline. Differences in patients' distribution and CAA severity may explain the discrepancy between the results of their and our studies.

Gardiner *et al.* (23) demonstrated a reduction in the numbers of renin-containing cells in renal allograft biopsies after patients were switched from CyA to azathioprine treatment. However, they did not demonstrate a relationship between renin expression and chronic CyA nephropathy, particularly CAA. It is interesting to note that in our study, patients were able to recover from mild to moderate CAA after CyA discontinuation. CAA disappeared in five of seven patients, and their biopsy specimens showed a significant reduction in the number of renin-containing cells after CyA discontinuation. Thus, our study demonstrated for the first time that there was an association between the reduction in the number of renin-producing cells and improvement of CAA after CyA discontinuation. Ponticelli and Passerini (24) reported that in patients with steroid-dependent NS, CyA should be discontinued after 2 yr. If the patient relapses, they treat him or her with steroids for 6 to 12 mo, then again with CyA for 1 to 2 yr, to minimize the toxicity of prolonged administration of CyA. The data in the present study strongly support their treatment strategy for patients with steroid-dependent NS, as CyA discontinuation can induce improvements in chronic CyA nephropathy, especially CAA.

In our study, immunoreactivity to renin was not related to the existence and the severity of CyA-induced interstitial fibrosis, suggesting that the development of CyA-induced interstitial fibrosis is due to mechanisms that are at least partly independent of CyA-stimulated intrarenal RAS activation. Pichler *et al.* (13) reported that interstitial fibrosis mediated by CyA in rats can be prevented partly by an angiotensin II type I receptor antagonist or by hydralazine and furosemide. They speculated that lowering BP or inducing vasodilation *per se* could prevent the development of CyA-induced interstitial fibrosis, supporting our conclusion that the fibrosis is, at least in part, independent of intrarenal RAS activation. The mechanisms of development of CyA-induced interstitial fibrosis are still unclear. However, the results of our study suggest that long-term proteinuria during CyA treatment has a pivotal role in CyA-induced interstitial fibrosis. It is interesting that the interstitial fibrosis did not deteriorate but slightly (not statistically) improved after CyA discontinuation, although long-term proteinuria as a result of frequent relapses had been present during the period between CyA discontinuation and the repeat renal biopsies. Moreover, it has been reported in rats that long-term (6 mo) massive proteinuria induced by multiple injections of anti-rat slit diaphragm monoclonal antibody results in tubular epithelial cell injury and cell infiltration but not interstitial fibrosis (25). Collectively, these findings suggest that CyA and long-term proteinuria can synergistically induce interstitial fibrosis by unknown mechanisms. Additional studies are required to shed light on these mechanisms.

In summary, the results of our study indicate that long-term CyA treatment for idiopathic NS in children may stimulate renin production in arterioles. They also suggest that CyA-stimulated intrarenal RAS activation may be responsible for the development of CAA and that CyA-induced interstitial fibrosis may be potentiated by long-term proteinuria and is, at least partly, independent of CyA-stimulated intrarenal RAS activation.

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