Original Article

Evidence for Abundant Presence of Chymase-Positive Mast Cells in the Kidneys of Patients with Immunoglobulin A Nephropathy: Effect of Combination Therapy with Prednisolone and Angiotensin II Receptor Blocker Valsartan

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Several investigators have reported chymase-positive mast cells in tubulointerstitial damage. However, the significance of the presence of chymase in the pathophysiology of renal diseases is unclear. We investigated relationships among chymase, renal damage, and intra-renal circulation. The participant pool consisted of 52 patients with immunoglobulin A (IgA) nephropathy who underwent renal biopsy. Of these, 18 were examined before and 2 months after the initiation of treatment with prednisolone alone (n=9) or combined with the angiotensin II receptor blocker valsartan (n=9). Biopsied renal specimens were evaluated, and the degree of renal circulation (resistive index; RI) was calculated by measuring flow velocity using Doppler sonography. The number of chymase-positive mast cells as visualized by immunohistochemical staining correlated significantly with both tubulointerstitial damage (ρ=0.69, p<0.001) and RI (r=0.52, p<0.001). Treatment with prednisolone combined with valsartan effectively decreased both chymase-positive mast cells and RI, displaying a significant correlation between these biomarkers (ρ=0.85, p=0.016). However, no such effect was observed with prednisolone alone. The severity of tubulointerstitial damage and the degree of proteinuria were similar in both treatment groups throughout the study term. We concluded that the presence of chymase-positive mast cells and the associated decrease in renal circulation corresponded to disease progression in IgA nephropathy. Combination therapy using prednisolone and valsartan may lead to improvements in intra-renal circulation and to interference in the recruitment of chymase-positive mast cells. (*Hypertens Res* 2008; 31: 1517–1524)

***Key Words*: chymase, immunoglobulin A nephropathy, renin-angiotensin system, ischemia**

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Introduction

Although human chymase is known to produce angiotensin II (AII) from angiotensin I (AI), the role of chymase in clinical pathophysiology remains controversial. Given that several types of mast cells are rich in chymase and that large numbers of mast cells are recruited during inflammation, it is possible that chymase may be released into inflamed tissue. Several reports have considered mast cells a factor associated with worsening nephritis, diabetic nephropathy, and allograft kidney rejection (*1*–*5*).

Recent reports have provided evidence that chymase-positive mast cells or chymase activity are increased in rejected transplanted kidneys (*5*), in stenotic kidneys of renovascular hypertensive model dogs (*6*), and in human renovascular hypertension with severe stenosis of the renal artery (*7*), all of which generally occur under conditions of chronic ischemia. Clarification of the presence of chymase-positive mast cells in pathological cases involving ischemia is worthy of closer scrutiny to investigate the putative role of chymase originating from mast cells.

In glomerular diseases, initial glomerular damage causes injury in downstream peritubular capillaries and results in local chronic ischemia (hypoxia) of the tubulointerstitium (*8*, *9*). Chronic ischemia stimulates the renin-angiotensin system and induces the expression of genes that encode cytokines such as transforming growth factor β1. These proteins are in turn involved in tubulointerstitial damage, causing further deterioration in renal disease (*10*, *11*).

Immunoglobulin A (IgA) nephropathy is present in a large population of patients with glomerulonephritis. In view of the possible relationship between pathological observation of the kidney and the presence of intra-renal chymase, we studied 52 patients with IgA nephropathy from whom renal biopsy samples were available. We report herein that the number of chymase-positive mast cells increased in parallel with the progression of interstitial damage in this disease.

Methods

Subjects and Protocol

Of the patients diagnosed with renal disease at Osaka City General Hospital between 2002 and 2006, a total of 545 Japanese patients provided written informed consent to receive a renal biopsy. From this population, we selected 52 patients (14 men, 38 women) with IgA nephropathy who reported no history of drug treatment prior to biopsy. All patients were hospitalized for diagnosis and treatment of renal disease, both of which were decided on the basis of renal biopsy results. Before histological diagnosis, informed consent to undergo treatment was obtained from all patients. All study protocols were approved by the institutional ethics committee.

The mean age of the studied population was 29 years (range 16–45 years). Of these 52 patients, 18 patients received either prednisolone (0.8 mg/kg/d for 4 weeks followed by gradual tapering with 5 mg/d every 2 weeks, $n=9$) or this same prednisolone regimen combined with the AII receptor blocker valsartan (40 mg/d, $n=9$). Combination treatment with valsartan was conducted in patients with systolic blood pressure ≥120 mmHg or diastolic blood pressure ≥80 mmHg. Biopsy specimens and resistive index (RI) as determined by Doppler sonography were obtained within 1 week of hospitalization. At 2 months following the initiation of treatment, second biopsy specimens and RI were obtained just prior to discharge to examine the effectiveness of the treatments. Additionally, 24-h urine samples were collected for 3 d prior to treatment and posterior to 2 months following the initiation of treatment to be assayed for protein and creatinine. The mean of the three 24-h samples is reported here. During urine collection, blood was sampled for measurement of urea nitrogen and creatinine. Creatinine clearance was calculated by 24-h urinary and serum creatinine levels. Systemic blood pressure was measured three times per day in a supine position, and the mean values for 3 d prior to and 2 months following initiation of treatment are discussed in the Results section.

Histological Study

Tissue specimens obtained by renal biopsy were fixed in phosphate-buffered 4% formaldehyde and embedded in paraffin. Part of each specimen was snap-frozen, stored at −80°C, and used for immunohistochemical staining. The remaining part of each biopsy specimen was stained with periodic acid-Schiff or periodic acid–Schiff–methenamine silver. Biopsy specimens from all subjects contained ≥10 glomeruli. All specimens were evaluated independently by two investigators unaware of the RI and the number of chymase-positive mast cells of that patient. Severity of tubulointerstitial damage was evaluated as the percentage of tubulointerstitial fibrosis, tubular atrophy, and interstitial infiltrates in the cortex (*12*).

Immunohistochemistry

The primary antibody used against mast cell chymase was anti-mast cell chymase (MAB-1254; Chemicon, Temecula, USA). Snap-frozen samples were serially sectioned at 5 μm thickness and fixed in acetone. Sections were incubated with primary antibody overnight at 4°C. The labeled streptavidin-biotin complex system with 3-amino-9-ethylcarbazole color development was used. Sections were faintly counterstained with hematoxylin. Specificity and results obtained with anti-mast cell chymase antibody were checked by omitting the primary antibody and by using a non-immune mouse immunoglobulin G antibody (DAKO, Glostrup, Denmark) as the negative control. The number of chymase-positive mast cells within the surface area of the cortex in biopsy specimens was quantified using the Mac-SCOPE version 2.2 computerized

Table 1. Patient Characteristics

Clinical characteristics	All	ARB (+)	ARB (–)
Gender (male/female)	14/38	3/6	1/8
Age (years)	29±12	25±8	27±9
Blood urea nitrogen (mmol/L)	5.3±1.5	5.7±3.0	4.8±0.8
(mg/dL)	14.8±4.1	16.1±8.5	13.5±2.3
Serum creatinine (μmol/L)	70±27	92±60	63±15
(mg/dL)	0.79±0.31	1.04±0.68	0.71±0.13
Urinary excretion of protein (mg/d)	324 (140, 857)	see Table 4	
Creatinine clearance (mL/min/1.73 m^2)	115±31	see Table 3	
Systolic blood pressure (mmHg)	116±13	see Table 3	
Diastolic blood pressure (mmHg)	69±8	see Table 3	
Mean blood pressure (mmHg)	85±9	see Table 3	

Values for these normally or near-normally distributed data are given as mean±SD, except for urinary excretion of protein, which is given as median followed in parentheses by 25th and 75th percentiles. ARB (+), patients treated with prednisolone and angiotensin II receptor blocker (ARB) (n=9); ARB (–), patients treated with prednisolone alone (n=9).

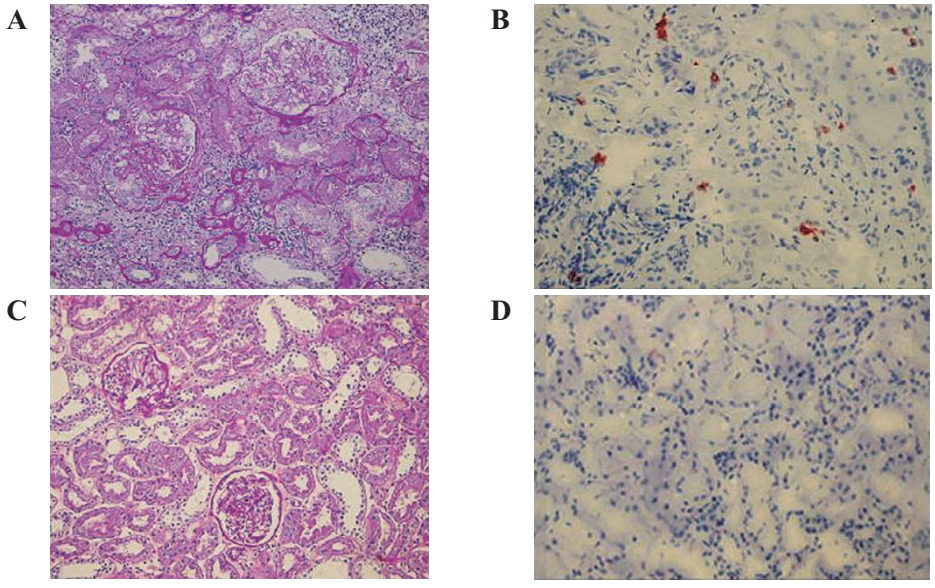

Fig. 1. *Photomicrographs of sections of biopsy specimens from a patient with severe histological damage (A, B) and a patient with mild histological damage (C, D) are shown. A: Periodic acid–Schiff stain (×200). B: Staining of chymase (×400). C: Periodic acid–Schiff stain (×200). D: Staining of chymase (×400).*

morphometry system (Mitani, Fukui, Japan) and expressed as the absolute number of chymase-positive mast cells per mm^2 surface area.

Measurement of RI

RI was measured to assess disturbances in renal circulation (*13*, *14*). Patients first lay at rest in a supine position for at least 10 min, after which RI was measured using a LOGIC 700 ultrasonic unit (GE Medical System, Milwaukee, USA) equipped with a 3–8 MHz convex transducer. Doppler signals were obtained from interlobular arteries by placing the sample volume in medullary pyramids, and the Doppler angle was then corrected in accordance with course of the artery. RI was calculated as (peak systolic velocity – end diastolic velocity)/peak systolic velocity. RI in each patient was expressed as the mean of values for six interlobular arteries. To ensure day-to-day reproducibility of the method for measuring RI, a total of 15 subjects (8 healthy male volunteers, and 2 male patients and 5 female patients with untreated glomerulonephritis) underwent measurement on two different days (interval ≤1 week). The coefficient of day-to-day variance for RI was

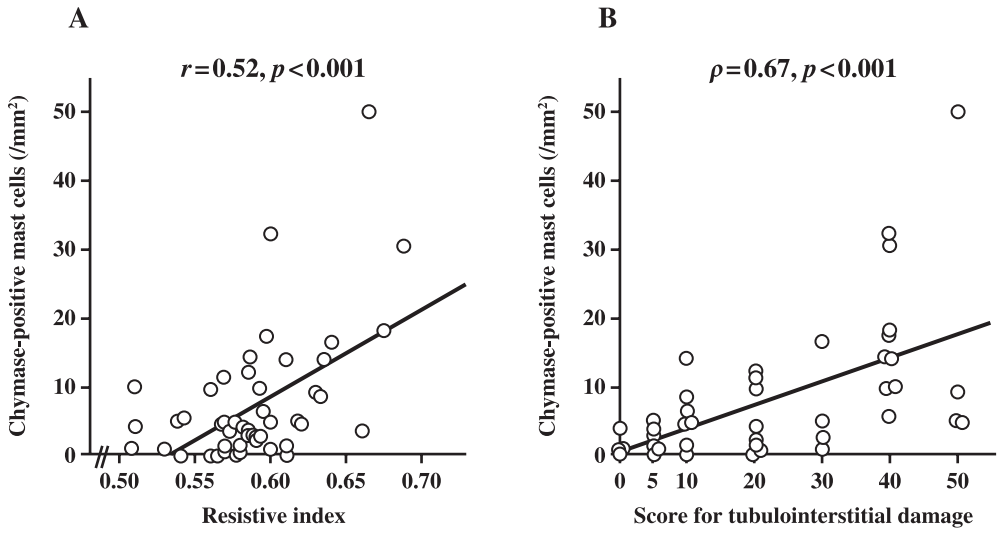

Fig. 2. *Relationships between number of chymase-positive mast cells, resistive index (RI), and score for tubulointerstitial damage in 52 patients with IgA nephropathy. A: Differences in number of chymase-positive mast cells and RI. B: Differences in number of chymase-positive mast cells and score for tubulointerstitial damage.*

2.3%. No specific tendency for day-to-day differences in RI was observed between sexes or health conditions.

Statistical Analysis

Data on patient characteristics are expressed as means and standard deviations. Age, urinary excretion of protein, number of chymase-positive mast cells, and score for tubulointerstitial damage are expressed as medians with 25th to 75th percentiles, as these values did not display normal distributions. The significance of differences between before and after treatments in creatinine clearance, systemic blood pressure, and RI were evaluated using Student's *t*-test for paired samples. The significance of differences between before and after treatments in urinary excretion of protein, number of chymase-positive mast cells, and score for tubulointerstitial damage was evaluated with the Wilcoxon signed-rank test. The correlation between RI and number of chymase-positive mast cells was evaluated by Pearson's correlation. The correlation between score for tubulointerstitial damage and number of chymase-positive mast cells was evaluated using Spearman's correlation. Multivariate analysis of score for tubulointerstitial damage, RI, and number of chymase-positive mast cells was performed by multiple regression analysis. Correlations among changes in urinary excretion of protein, changes in score for tubulointerstitial damage, changes in RI, and changes in chymase-positive mast cells were evaluated with Spearman's correlation. Multivariate analysis of changes in urinary excretion of protein, score for tubulointerstitial damage, RI, and number of chymase-positive mast cells was performed by multiple regression analysis. Statistical analyses were performed using Statistica version 4.1J software (StatSoft, Tulsa, USA). Values of $p<0.05$ were considered as statistically significant.

Table 2. Multiple Linear Regression Analysis of Resistive Index (RI), Score for Tubulointerstitial Damage and Number of Chymase-Positive Mast Cells

	Standardized regression coefficient
	Number of chymase-positive mast cells (*p*)
RI	0.37 (0.002)
Score for tubulointerstitial damage	0.48 (<0.001)

Values are presented as score (*p*).

Results

Table 1 summarizes the baseline characteristics of 52 patients, 18 of whom were treated with prednisolone only or prednisolone combined with valsartan. Figure 1 shows a photomicrograph of sections of the biopsy specimens from two patients with IgA nephropathy. The patient with severe damage had more chymase-positive mast cells in the tubulointerstitial area than the patient with mild damage (Fig. 1B, D, respectively). Chymase-positive mast cells were evident in the tubulointerstitial area of patients with moderate to severe histological damage, but were not evident in glomeruli of all patients. The number of chymase-positive mast cells was significantly correlated with RI (Fig. 2A; $r=0.52$, $p<0.001$, $n=52$) and scored for tubulointerstitial damage (Fig. 2B; $\rho=0.69$, $p<0.001$, $n=52$). In the multiple linear regression

Table 3. Creatinine Clearance, Systemic Blood Pressure and Resistive Index (RI) in 18 Patients before and 2 Months after Start of Treatment

	Before	Treatment	*p*
Creatinine clearance (mL/min/1.73 m^2)			
Total	94±23	94±22	0.88
ARB (+)	93±32	93±31	0.99
ARB (−)	95±8	95±9	0.75
Systolic blood pressure (mmHg)			
Total	121±10	115±7	0.059
ARB (+)	127±10	115±7	0.013
ARB (−)	114±5	115±8	0.71
Diastolic blood pressure (mmHg)			
Total	73±8	66±5	0.032
ARB (+)	76±10	63±4	0.015
ARB (−)	69±3	69±5	0.97
Mean blood pressure (mmHg)			
Total	89±9	82±5	0.034
ARB (+)	93±10	80±5	0.013
ARB (−)	84±4	84±5	0.86
RI			
Total	0.59±0.05	0.57±0.05	0.038
ARB (+)	0.58±0.06	0.56±0.06	0.021
ARB (−)	0.60±0.04	0.58±0.03	0.089

Values for these normally or near-normally distributed data are given as mean±SD. ARB (+), patients treated with prednisolone and angiotensin II receptor blocker (ARB) (n=9); ARB (−), patients treated with prednisolone alone (n=9).

Table 4. Urinary Excretion of Protein, Number of Chymase-Positive Mast Cells and Score for Tubulointerstitial Damage in 18 Patients before and 2 Months after Start of Treatment

	Before	Treatment	*p*
Urinary excretion of protein (mg/d)			
Total	687 (287, 1,091)	182 (104, 270)	<0.001
ARB (+)	944 (261, 2,237)	217 (122, 276)	0.008
ARB (−)	572 (886, 307)	146 (91, 278)	0.008
Chymase-positive mast cells (/mm^2)			
Total	4.3 (1.3, 9.6)	1.8 (0.7, 5.2)	0.015
ARB (+)	5.5 (1.1, 11.0)	1.4 (0.7, 4.6)	0.038
ARB (−)	3.6 (1.4, 6.1)	2.0 (0.7, 5.8)	0.17
Score for tubulo-interstitial damage			
Total	20 (20, 40)	20 (10, 40)	0.20
ARB (+)	30 (20, 40)	30 (10, 40)	0.35
ARB (−)	20 (10, 30)	10 (5, 20)	0.35

Values for urinary excretion of protein, number of chymase-positive mast cells and score for tubulointerstitial damage are given as median followed in parentheses by 25th and 75th percentiles. ARB (+), patients treated with prednisolone and angiotensin II receptor blocker (ARB) (n=9); ARB (−), patients treated with prednisolone alone (n=9).

analysis of these three parameters, both the RI and score for tubulointerstitial damage correlated with chymase-positive mast cells (Table 2).

Table 3 shows creatinine clearance, systemic blood pressure, and RI in the 18 patients measured before and after initiation of treatments with prednisolone alone or with prednisolone and valsartan. Creatinine clearance did not change in either treatment group. However, systolic and diastolic blood pressures decreased significantly in the group with prednisolone and valsartan, but did not decrease in the group with prednisolone only. Additionally, RI significantly decreased following treatment in the group with prednisolone and valsartan, but did not significantly decrease in the group with prednisolone only. Table 4 shows data for urinary excretion of protein, number of chymase-positive mast cells, and score for tubulointerstitial damage in the 18 treated patients: Urinary excretion of protein decreased significantly in both treatment groups. The number of chymase-positive mast cells decreased significantly in patients treated with prednisolone and valsartan, but did not decrease significantly in patients treated with prednisolone only. In both groups, the score for tubulointerstitial damage did not change significantly from the beginning to the end of the study. Analysis of the correlations across the 18 treated patients revealed that changes in the number of chymase-positive mast cells correlated significantly with changes in RI (Fig. 3A; ρ=0.64, p=0.009), but did not correlate with either urinary excretion of protein (ρ=−0.22, p=0.36) or changes in score for tubulointerstitial damage (ρ=−0.058, p=0.75). Decreases in the number of chymase-positive mast cells significantly correlated with decreases in RI in the nine patients treated with prednisolone and valsartan (Fig. 3B; ρ=0.85, p=0.016), but these variables did not significantly correlate in the nine patients not treated with valsartan (Fig. 3C; ρ=0.54, p=0.13). Further supporting the results from the 18 patients as described above, correlation between changes in number of chymase-positive mast cells and changes in urinary excretion of protein was not significant in the group treated with prednisolone and valsartan (ρ=0.20, p=0.57) or in the group treated with prednisolone only (ρ=0.27, p=0.45). The correlation between changes in number of chymase-positive mast cells and changes in score for tubulointerstitial damage was not significant in the group treated with prednisolone and valsartan (ρ=0.27, p=0.44) or in the group treated with prednisolone only (ρ=−0.36, p=0.31). In the multiple linear regression analysis of these four parameters, only changes in RI correlated with changes in chymase-positive mast cells (Table 5).

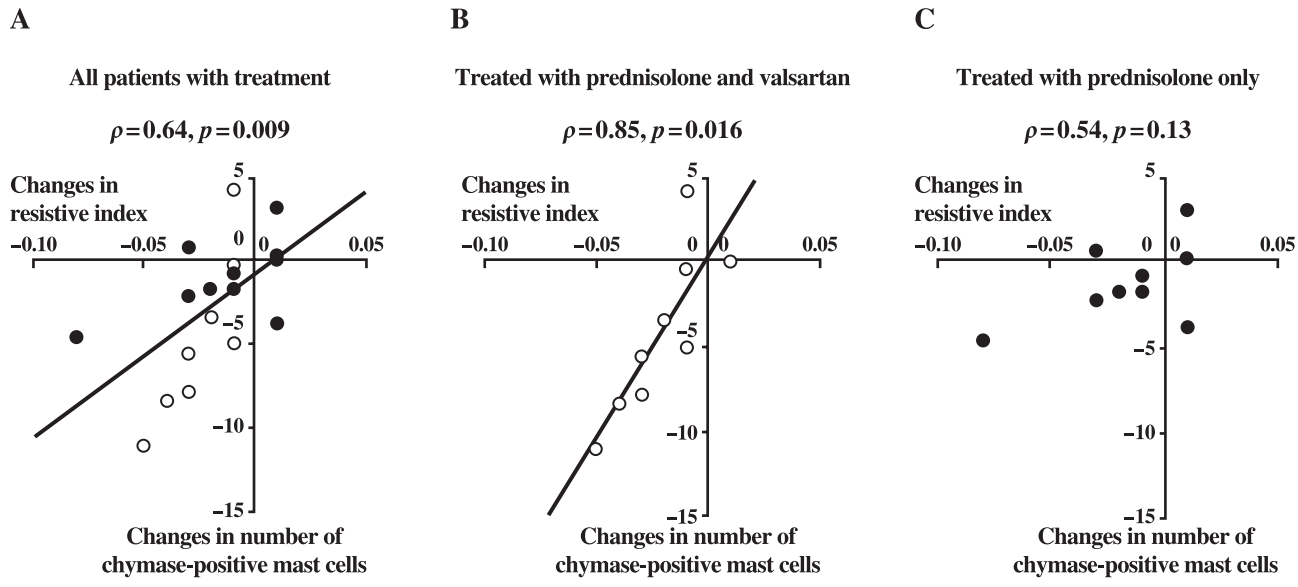

Fig. 3. *Relationships between changes in number of chymase-positive mast cells and changes in resistive index (RI) in 18 patients with IgA nephropathy. A: All patients receiving treatment (n = 18). B: Patients treated with prednisolone and valsartan (n = 9). C: Patients treated with prednisolone alone (n = 9).*

Table 5. Multiple Linear Regression Analysis of Changes in Resistive Index (RI), Urinary Excretion of Protein, Score for Tubulointerstitial Damage and Number of Chymase-Positive Mast Cells

	Standardized regression coefficient
	Changes in number of chymase-positive mast cells (*p*)
Changes in RI	0.52 (0.048)
Changes in urinary excretion of protein	0.28 (0.23)
Changes in score for tubulointerstitial damage	0.15 (0.53)

Values are presented as score (*p*).

Discussion

Mast cells can contain many different types of inflammatory mediators. Among these, human chymase produces a potent vasoconstricting influence. However, the role of chymase in the pathophysiology of glomerulonephritis has remained undefined, since little is known about the extent to which chymase-related AII is present in tissues and the extent to which it affects biological activity. Notwithstanding the many investigators who have tried to identify the specific presence of chymase or chymase-positive mast cells in many types of organs in various diseases, the present study provides preliminary, but substantiated, evidence for the presence of chymase-positive mast cells in the kidney during IgA nephropathy. Indeed, the number of chymase-positive mast cells was increased in tubulointerstitial areas and was related to elevated RI (an index of intra-renal vascular resistance) and severity of tubulointerstitial damage, indicating that tissue lesions, vascular resistance, and chymase-positive mast cells appear to be interrelated. Of note are that following treatment with prednisolone and valsartan, the number of chymase-positive mast cells was decreased and this decrease correlated well with improved renal circulation, as indicated by decreased RI, while no such effect was evident for treatment with prednisolone alone.

Not all mast cells produce chymase, and behavioral and physiological roles are thus likely to differ substantially among types of mast cells. It has been suggested that chymase-positive mast cells cause tubulointerstitial damage in rapidly progressive glomerulonephritis, acute rejection of renal allografts, and diabetic nephropathy (*4*, *5*, *15*). The present report offers an important addition to these suggestions confirming positive and significant correlations between the severity of pathophysiological parameters and chymase-positive mast cells during IgA nephropathy. Nonetheless, there is still no direct evidence that chymase deteriorates tubulointerstitial damage. Therefore, to establish a concrete pathophysiology of chymase in kidney diseases, direct evidence by specific inhibition of this enzyme needs to be shown in a human study.

Proteinuria decreased in both treatment groups, but this decrease was not correlated with the decrease in number of chymase-positive mast cells. Proteinuria depends on various factors, including glomerular injury and glomerular hyperten-

sion. In our present study the decrease in proteinuria due to treatment with prednisolone and valsartan was comparable to that by prednisolone alone. We, thus, do not argue any significance for the specific inhibition of chymase in the treatment of proteinuria at this time, despite a decrease in number of chymase-positive mast cells in the treatment with valsartan. Given that chymase plays a role in proteinuria, the relation between extent of and time-course of development of proteinuria should be carefully studied in the future with a large number of patients.

Fine *et al*. (*8*) and Johnson *et al*. (*9*) proposed a hypothesis of chronic hypoxia in renal diseases, whereby chronic hypoxia (or ischemia) impairs the interstitium and causes tubulointerstitial damage, including fibrosis and peritubular capillary loss. Their argument states that for glomerulonephritis the inflammatory process with chronic ischemia caused by interference of post-glomerular circulation leads to glomerular and tubulointerstitial damage. If chymase-positive mast cells release chymase and this enzyme produces AII locally, such ischemia may be at least partially attributable to the action of locally produced AII mediated chymase.

Renal circulation cannot be directly assessed in humans, but renal flow in interlobular arteries can be evaluated using RI and Doppler sonography (*13*, *16*). This method provides an indirect indication of blood flow and therefore may be somewhat variable, although we obtained good reproducibility for day-to-day measurements (see Methods). RI has been shown to correlate with severity of glomerulosclerosis, tubulointerstitial damage, and arteriosclerosis in renal parenchymal diseases (*14*). RI is also useful for indirect measurement of renal parenchymal flow (conductance of renal vascular bed) in glomerular and post-glomerular circulation. Thus, an elevated RI reflects altered circulation in glomeruli and tubulointerstitium due to improvement of interstitial damage and due to dilatation of arterioles (*16*). Systemic blood pressure is also an important parameter of renal circulation. However, this study was not designed as a cohort study, but rather was conducted as a conventional clinical study. Since valsartan and high blood pressure covaried in this study, no statistical analysis was performed on blood pressure due to the treatment bias. Thus, to what extent blood pressure change affected the chymase results in this present study remains unclear. A cohort study of valsartan treatment in both normotensive and hypertensive patients is needed. In the present study, treatment with prednisolone and valsartan resulted in a decrease in chymase-positive mast cells, which then resulted in subsequent improvements in renal circulation as expressed by RI. Changes in RI caused by treatment with valsartan may thus reflect changes in circulation *per se*, as vascular structures with arteriosclerosis do not change during a time frame as short as 2 months of treatment.

For the present study, the two treatment groups were not divided prospectively or randomly. Nor did we examine patients treated with valsartan only due to ethical regulations. We were thus unable to completely clarify whether the mechanism causing the decrease in chymase-positive mast cells with prednisolone and valsartan treatment was attributable to the suppression of cell infiltration by prednisolone or attributable to an indirect effect involving improvement of circulation through steroid-suppressed inflammation and arteriole dilation by valsartan. Any treatment for immunosuppression can decrease the recruitment of mast cells, but it should be noted that in this study, a significant decrease in number of chymase-positive mast cells was observed in treatment with prednisolone and valsartan, but not in treatment with prednisolone alone, although the degree of tubulointerstitial damage was comparable. Thus, direct immuno-reaction and inflammation may not be major factors modulating the recruitment of chymase-positive mast cells in this disease. Rather, a wide variety of actions of prednisolone and of cardiovascular actions of valsartan are considered to have beneficial effects on the reduction of this type of cell. One possible mechanism may lie in the relation between ischemia and mast cells: We recently reported an increase in the number of chymase-positive mast cells in tubulointerstitial fibrotic lesions of ischemic kidneys in human renovascular hypertension (*7*). In another examination of renal biopsies, we detected increased chymase-positive mast cells in interstitial fibrotic lesions of nephrosclerosis, as well as severe inflammatory lesions, indicating, similar to the present results, that the number of chymase-positive mast cells and RI (renal circulation) change in parallel fashion (data not shown). In addition, several studies have suggested that the fibrosis that occurs through non-immune pathways in various diseases is related to chymase-positive mast cells (*17*). Given that valsartan directly dilates vascular beds and improves circulation by inhibiting AII, valsartan would be effective in the reduction of local ischemia. Improvement of local circulation may thus decrease the number of chymase-positive mast cells. If chymase plays any role in the deterioration of renal tissues, ischemia in renal diseases leads to the recruitment of chymase-positive mast cells and chymase further exacerbates the disease through production of AII. Since this chain of events remains hypothetical, direct evidence is needed to prove the pathophysiological role of chymase by specific inhibition of the enzyme. Angiotensin-converting enzyme inhibitors and calcium channel blockers as vasodilators should also be examined for comparison with the effects of valsartan, in view of their effects on chymase-positive mast cells. Although definitive evidence is still needed, the findings that valsartan effectively reduced the number of chymase-positive mast cells and that valsartan ameliorated renal ischemia lead to our postulation that the therapeutic actions of this type of drug include improved renal circulation and possibility suppression of chymase-positive mast cell recruitment.

Conclusions

Chymase-positive mast cells increased in number in patients with IgA nephropathy and this increase was closely related to

alterations in renal circulation and tubulointerstitial damage (fibrosis). Treatment with prednisolone and valsartan for 2 months reduced the number of chymase-positive mast cells and caused significant recovery of renal circulation. We consider that, in this type of nephropathy, valsartan acts to improve intra-renal circulation, in addition to interfering with the recruitment of chymase-positive mast cells.

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