Table 1. Difference of tonsils with (+) and without (−) IgA nephropathy (IgAN)

Characteristic of tonsils	IgAN(+)	IgAN(−)	Reference number
T cell area (T nodules)	Expanded	Not expanded	29
Reticulization of crypt epithelium	Reduced	Not reduced	31
IgA cells:IgG cells	>1	<1	32–34
Polymeric IgA cells	Increased	Not changed	32–34
Polymeric IgA:IgA	Increased	Not changed	35
Follicular dendritic cells	IgA1+	IgA1−	36
J chain mRAN-positive cells	Increased	Not changed	37
Adhesion molecules CD31, CD54	Increased	Not changed	38
CD5+ B cells	Increased	Not changed	39

signals, but also on the expression of various complementary adhesion and costimulatory molecules [19]. In additional, Mitogen-triggered T cells from tonsils produced both of Th1- and Th2-type cytokines, clearly exhibiting their pluripotentiality for support of cell-mediated and antibody responses. The antigen-specific T cells produced interferon-gamma (INF-γ) and lower levels of interleukin-5 (IL-5). These results suggest that tonsils of the nasopharyngeal-associated lymphoreticular tissues represent a distinct component of the mucosal-associated lymphoreticular tissues with features of both systemic and mucosal compartments [20].

DIFFERENCE OF TONSILS WITH AND WITHOUT IgA NEPHROPATHY

There are significant differences in histologic structure, proportional ratios of cells, and cell adhesion molecules in tonsils with and without IgAN (Table 1).

Difference of tonsillar histologic structure

The enlarged primary T nodules in tonsils, which are defined as sum of the small areas of accumulating T lymphocytes and apparent nodule, composed predominantly of small T lymphocytes, were a characteristic feature of tonsils in patients with IgAN. Most T nodules in patients with IgAN were enlarged, especially in younger patients, and a few T nodules contain high endothelial venules and nonlymphoid cells. In contrast, T nodules in patients with habitual tonsillitis do not expand, and nonlymphoid cells and high endothelial venules are distributed peripherally around the nodules [29]. The basic structure and functional unit of reactive lymph nodes is composed of two separate T nodules and B-lymphoid follicles. These composite nodules play a major role in the triggering, helper T-cell–dependent stimulation and subsequent maturation of antigen-responsive B cells into antibody-secreting plasma cells [30]. With regard to his relationship between T-cell and B-cell domains, the enlarged primary T nodules reminds us that extrafollicular maturation of the stimulated B lymphocytes into plasma cells may occur more frequently in the tonsils of patients with IgAN than in patients with habitual tonsillitis. Another study demonstrated abnormal reticulization of tonsillar crypt epithelium in patients with IgAN. Tonsils of controls with recurrent tonsillitis or tonsillar hypertrophy showed well-developed reticular crypt epithelia with lymphoepithelial symbiosis, and the nonreticulated area was less than 7% of the total crypt epithelia per overall section. In IgAN tonsils, however, nonreticulated crypt epithelium was frequently observed and, in the advanced stage of IgAN, exceeded 50% of total crypt epithelia [31].

Difference of tonsillar cells

Primary IgAN is characterized by renal deposits of polymeric IgA (J chain-positive), the origin of which is not confirmed, yet. The study by Bene et al [32] showed that in controls with recurrent tonsillitis, IgG secreting cells were predominant (IgG secreting cells 65% and IgA plasma cells 29%), while in the IgAN patients, the plasma cells percentages was of an inversion (IgG 37% and IgA 56%). This increment in the IgA population was paralleled by an augmentation of the number of dimeric IgA secreting cells (75% of IgA plasma cells), stained both for cytoplasmic IgA and J chain [32]. The study by Nagy and Brandtzaeg [33] and a later multicenter study by Bene et al [34] also demonstrated a similar result. In additional, after 7 days of culture with pokeweed mitogen, the percentage of tonsillar cells producing polymeric IgA is significantly higher in the IgAN patients than in the controls suffering from chronic tonsillitis [35]. The IgA1 subclass was found in follicular dendritic cells (FDC) of the tonsil of IgAN patients, but not in FDC of non-IgAN controls. On the other hand, IgA2, IgG, IgM, and C3 did not show any differences in distribution between the two groups [36]. In situ hybridization (ISH) study for the detection of J chain mRNA within IgA plasma cells revealed J chain mRNA-positive cells were identified in germinal centres, and within the subepithelial and interfollicular zones of tonsils. Combined immunofluorescence and fluorescent ISH showed a greater proportion of J chain mRNA-positive interfollicular IgA cells in the patient tonsils compared with the controls. In additional, the finding of excess numbers of J chain-positive IgA-negative cells was found within germinal centers of tonsils in IgAN patients [37]. These results demonstrate immune abnormalities within the tonsil as a central feature of abnormal polymeric IgA biology in IgAN, which is in keeping with the hypothesis favoring a tonsil origin for the mesangial IgA present in their kidneys.

Abnormalities in the partition of IgA- and IgG-producing cells in the tonsils of patients with IgAN have been suggested to result from a dysregulation of cell

trafficking and homing through high endothelial venules in this lymphoid tissue. Study demonstrated a significant enhancement of cell adhesion molecules, CD31 and CD54, expression on high endothelial venules of tonsils from patients with IgAN compared with controls [38]. In additional, the number of $CD5^+$ B cells isolated from the tonsil germinal centers of IgAN patients is increased. These $CD5^+$ B cells are likely IgA1 antibody-producing cells. Moreover, these $CD5^+$ B cells show a reduced susceptibility to Fas-mediated apoptosis [39].

RELATIONSHIP BETWEEN TONSILLAR IgA AND GLOMERULAR IgA

Both IgA produced by tonsil cells and IgA deposited in glomerular mesangium with IgAN are mainly J chain-positive polymeric IgA [35, 37, 40, 41]. Studies demonstrated they were consistent in some cases. The antibodies eluted from renal tissues of patients with IgAN specifically bound with the nuclear regions of tonsillar cells. The binding of eluted antibodies and tonsillar cells was completely inhibited by the addition of antihuman IgA antisera, but not inhibited by human IgA myeloma proteins. The eluted antibodies bound with tonsillar cells from the same patients, but only 10% of them bound with the tonsillar cells obtained from other patients with IgAN. This result suggests that IgA antibodies deposited in glomeruli specifically bind with tonsillar cells obtained from patients with IgAN [42]. The study by Tokuda et al [43] offered another evidence of binding of IgA produced by tonsillar B lymphocytes to the glomerular mesangium of IgAN. They first made heterohybridoma cells of human tonsillar B lymphocytes from IgAN patient with mouse myeloma cells and cultured them. The culture medium was analyzed by Western blot analysis using antihuman IgA antibody, and both IgA1 and IgA2 were demonstrated to be produced. The specimens of the biopsied kidney tissue of IgAN were washed with 0.02 mol/L citrate buffer (pH 3.2) to remove deposited IgA from glomerulus. The specimens were then incubated with the culture media of hybridoma cells, and immunofluorescence analysis using fluorescein isothiocyanate (FITC)-conjugated antihuman IgA antibody was performed. The result demonstrated that IgA deposit was efficiently removed by washing with citrate buffer and was recovered after incubation with the culture medium of hybridoma cells [43].

TONSIL STIMULATION AND IgAN

Method and judging criteria of tonsil provocation test

The methods of tonsil provocation test include direct or indirect tonsil stimulation using Tonsil Provocator producing an ultrashort wave (each tonsil for 5 minutes), mechanical tonsil stimulation (tonsil massage, each tonsil for 5 minutes) and injecting hyaluronidase (2000 U/mL, each tonsil for 0.5 mL) into tonsils. In general, four criteria are used to judge the results of tonsil provocation test. Any one of four criteria positive is regarded as tonsil provocation test positive: (*1*) an increase of white blood cell count over 1200/mm^3 after 3 hours; (*2*) an increase in body temperature over 0.55°C after 15 minutes; (*3*) enhancement of erythrocyte sedimentation rate over 12 mm after 1 hour; and (*4*) worsened skin eruption or deterioration of urinary findings after 3 hours, which is defined as urinary protein increased by more than 30 mg/dL or erythrocyte count in the sediment increased by more than 10/hpf, as compared with that before the test [44, 45].

Effect of tonsil stimulation on IgAN

Although the pathogenesis of IgAN still remains uncertain, it is well known that IgAN patients often show gross hematuria or deteriorated urinary findings after upper respiratory tract infections such as tonsillitis, it is supposed that tonsil inflammatory stimulation may be related to IgAN. Masuda et al [46] reported that a tendency of decreasing levels of serum complement combined with an increase of CIC was observed within 1 week after tonsil provocation test in several cases of IgAN associated with chronic tonsillitis [46]. Shiraishi et al [44] performed the tonsil provocation test in 11 cases with pustulosis palmaris et plantaris (PPP) and seven cases with IgAN. Analysis of the provocation test proved positive in three of 11 cases (27%) with PPP and in five of seven cases (71%) with IgAN [44]. Yamabe et al [45] studied effect of ultrashort wave stimulation of tonsils on urinary findings in patients with IgAN. In 62 patients with IgAN and 20 patients with other renal diseases, tonsils were directly stimulated by Tonsil Provocator producing an ultrashort wave to 40.68 MHz each tonsil for 5 minutes. Forty (65%) of 62 patients with IgAN showed deterioration of urinary findings after the stimulation compared with 6 (30%) of 20 patients with other renal diseases. The deterioration of urinary findings was significantly more frequent in IgAN than in other renal diseases. In additional, previous episodes of gross hematuria following upper respiratory tract infections and the level of serum secretory IgA were higher in IgAN patients with deterioration of urinary findings after tonsil stimulation than in those without deterioration [45]. Matsuda et al [47] evaluated the effects of the mechanical tonsil stimulation on the serum and urinary concentrations of macrophage-colony-stimulating factor (M-CSF) in patients with IgAN associated with chronic tonsillitis. The serum and urinary levels of M-CSF in the groups with mild and severe IgAN were significantly higher than those in the chronic tonsillitis group without IgAN. Enhanced urinary excretion of M-CSF prolonged for 7 days after tonsil stimulation in the severe IgAN group; in contrast, the urinary M-CSF

level was increased for only 2 days after tonsil stimulation in the mild IgAN group. The urinary M-CSF level was not changed in the chronic tonsillitis group after tonsil stimulation. These results suggest that tonsil stimulation contributes to the progression of IgAN via enhancement of glomerular production of M-CSF [47].

However, the usefulness of the tonsillar provocation test in IgAN is now doubted. Even otolaryngologists [46] who initially claimed that the tonsillar provocation test was of clinical value in patients with IgAN have already changed their opinion. Their late results showed that there was no statistically significant difference between positive and negative patients in the rate of remission of proteinuria based on any parameter of the tonsillar provocation test at any time after surgery [48, 49]. Moreover, the Japan Society of Stomato-Pharyngology officially reported the lack of value of tonsillar provocation test in determining the indications for tonsillectomy in IgAN patients [50]. We think that an increase of white blood cell count, an increase of body temperature and enhancement of erythrocyte sedimentation rate after tonsillar provocation test may not be of any clinical value in patients with IgAN, but deterioration of urinary findings after tonsillar stimulation may be significant and suggest that tonsils are related to kidneys. A questionnaire survey also showed that 51.6% of 154 medical doctors who had reported case of IgAN answered that urine protein was the most important factor in any estimation of the provocation test [51].

TONSILLAR INFECTION AND IgAN

Tonsillar bacterial infection and IgAN

Suzuki et al [52] reported that the antigen and antibodies of outer membranes of *Haemophilus parainfluenzae*, a common bacterium on the tonsils, were present in glomerular mesangium and sera of IgAN, respectively, suggesting that *H. parainfluenza* infection may have a role in the etiology of IgAN [52]. Further studies showed that tonsillar lymphocytes from patients with IgAN revealed a significantly higher stimulation index to *H. parainfluenza* antigens (thymidine incorporation in tonsillar lymphocytes with *H. parainfluenza*/thymidine incorporation in unstimulated tonsillar lymphocytes) than controls. The lymphocytes from patients with IgAN also showed a significantly higher level of IgA antibody and IgA1 antibody against *H. parainfluenza* antigens in culture supernatants than lymphocytes from controls [53]. In vivo study showed that mouse glomerular deposition of *H. parainfluenza* outer membrane antigens and IgA, and increases in the amount of mesangial matrix were observed after administration of *H. parainfluenza* outer membrane antigens orally or intraperitoneally, respectively. Levels of IgA antibodies against *H. parainfluenza* outer membrane antigens were significantly increased in administration groups compared with controls. That is, administration of *H. parainfluenza* outer membrane antigens to mice may induce glomerular deposition of IgA and mesangial proliferation, resembling the changes seen in IgAN, with increases in IgA antibodies against *H. parainfluenza* outer membrane antigens [54]. Furthermore, production of cytokines IL-10 and transforming growth factor-β (TGF-β) was enhanced by stimulation with *H. parainfluenza* outer membranes in tonsillar mononuclear cells from IgAN [55]. These results suggest that *H. parainfluenza* antigens stimulate tonsillar T and B lymphocytes in patients with IgAN to produce cytokines and IgA antibody and that an immune response to *H. parainfluenza* antigens may play a role in the pathogenesis of IgAN in some cases.

In additional, Rekola et al [56] reported that 38 of 187 IgAN patients had possible acute glomerulonephritis at the onset of their disease. Antistreptococcal antibodies increased in forty-three percent of the patients. Thirty-three percent of the patients had different groups of beta-hemolytic streptococci isolated from their throats. This result indicates a possible role of beta-hemolytic streptococci, a most common bacterium in tonsils or throat, in the pathogenesis of some IgAN cases [56].

Tonsillar viral infection and IgAN

Regarding relationship between viral infection in tonsils and IgAN, there is a adult case report in which granular depositions of adeno- and herpes simplex viral antigens were detected in the glomerular mesangial areas in IgAN patients associated with episodes of recurrent tonsillitis and in the tonsillar epithelial cells by *H. parainfluenza* immunofluorescence [57]. The later study showed that the detection ratio of Epstein-Barr virus in the patients with glomerular lesions, such as IgAN and membranous nephropathy, was significantly greater than those without. However, the detection of Epstein-Barr virus was not disease specific [58]. Kunimoto et al [59] investigated viral infections in the tonsils, pharynx, and renal tissues of patients with IgAN using cell culture, polymerase chain reaction, and immunofluorescent techniques, and measured antibody titers against numerous types of viruses. As a result, no evidence was obtained that the viral infections play a significant role in the pathogenesis of IgAN [59].

EFFECT OF TONSILLECTOMY ON IgAN

Effect of tonsillectomy on immune system

As described above, human tonsil tissues are located at the gateway of the respiratory and alimentary tract, belong to the mucosa-associated lymphoid tissue, and play a role in the systemic immune and the local mucosal immune. What effect does tonsillectomy have on

Table 2. Effect of tonsillectomy on urinary findings and renal function

Author	Year	Tonsillectomy +/−	Follow-up *months*	Urinary remission	Urinary improvement	Renal survival	Reference number
Masuda	1988	16/0	36	56.3%			46
Tamura	1993	26/0	24		46%		71
Sugiyama	1993	28/0	61	32%	60.7%		79
Bene	1993	34/0	48	UP 3.5 g/day →	0.9 g/day	Stable	69
Iino	1993	35/15	36	54.8%/53.8%	61%/46%	96.8%/76.9%	78
Tomioka	1996	104/0	12	38%	94%		80
Barta	1996	35/40	144	6m UP 1.4 g/day →	0.92 g/d	88.6%/80.0%	70
Rasche	1999	16/39	41			NS	74
Hotta	2001	250/79	75	Total 48%			81
Xie	2003	48/70	193			89.6%/63.7%	17

Abbreviations are: UP, urine protein; NS, non-statistic difference.

the systemic and local mucosal immune? Studies demonstrated that tonsillectomy decreased the levels of serum IgA and salivary secretory IgA, especially in children, several months or years after operation [60–62]. However, these changes do not cause significant immune deficiency and are clinically insignificant. Moreover, these alterations do not increase incidence of immunomodulated diseases, such as infections of the upper respiratory tract [63]. These may be because the concentrations of serum IgA and salivary secretory IgA are higher before operation in some tonsillectomy cases than that of nontonsillectomy controls. Tonsillectomy decreased significantly the IgA levels compared with preoperation, but there is no significant difference compared with normal nonoperative controls [27]. Recent study demonstrated children with chronic tonsillitis have increased levels of $CD19^+$ B lymphocytes compared to healthy controls in the pre-operative period. The percentage of B lymphocytes bearing CD23 was found to be significantly higher in patients, most likely representing in vivo B lymphocyte activation due to chronic antigenic stimulation. After the tonsillectomy, despite ongoing B lymphocyte activation, $CD8^+$ T lymphocyte levels increased and B cell levels returned to normal [64].

Tonsillectomy may also lead to certain changes in the cellular immune systems in some boys, including slightly increased percentages of $CD21^+$ cells, raised counts of $CD4^+$ cells, absolute and relative increases in DR^+ cells and a raised $CD4^+DR$ count [63]. Peripheral blood $CD8^+$ cells, $CD45RA^+CD4^+$ cells, and $CD8^+CD11b^-$ cells increase significantly after tonsillectomy, compared with their preoperative values in patients with IgAN accompanied by chronic tonsillitis. In some cases, the preoperative serum tumor necrosis factor-alpha (TNF-α), and INF-γ levels were higher than normal before surgery, but decreased after surgery. These results suggest that tonsillectomy suppresses a decrease in suppressor T cells in patients with IgAN and corrects abnormal cell-mediated immune responses in these patients [65].

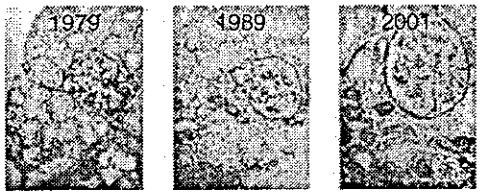
In additional, tonsillectomy has no effect on complement and saliva-derived nonimmunoglobulin host defense factors, such as lysozyme, salivary peroxidases, thiocyanate, hypothiocyanite, and agglutinins, except lactoferrin, which declined significantly [66]. The effects of tonsillectomy on serum and salivary secretory IgG, IgM, and IgE remain still controversial [67, 68].

Effect of tonsillectomy on urinary finding and renal function

Studies demonstrated that tonsillectomy can improve the urinary finding and keep stable renal function in some patients with IgAN (Table 2). Bene et al [69] followed up the evolution of urinary protein and serum creatinine in 34 patients with IgAN, and Barta et al [70] followed up 35 IgAN patients after tonsillectomy. The urinary protein and microhematuria decreased significantly from 6 months after tonsillectomy than that before operation, and no significant variation was observed in the levels of creatininemia [69]. Furthermore, tonsillectomy stopped gross hematuria in more than two thirds of patients [70]. Tamura et al [71] reported that 46% IgAN patients with chronic tonsillitis showed distinct improvement in urinary findings after the tonsillectomy. Akagi et al [48] followed up 24 patients with IgAN for more than 2 years after tonsillectomy. Remission of proteinuria was observed in 41.7% of the patients 6 months after surgery and in 50.0% 2 years after surgery [48]. The clinical remission rate of urinary finding and the stable renal function rate in tonsillectomy patients with IgAN were significantly higher than that in nontonsillectomy patients [72].

Effect of tonsillectomy on renal histologic findings

A repeat renal biopsy study for 35 patients demonstrated that renal histologic finding improved distinctly after the combined therapy of methylprednisolone pulse, prednisolone, antiplatelet, and tonsillectomy in IgAN patients [73]. The interval between the first and second biopsy was 18 to 138 months (mean, 77.1 months) in that study. Mesangial proliferation and interstitial mononuclear cell infiltration were significantly reduced in second biopsy specimens. Acute inflammatory glomerular lesions, such as endocapillary proliferations, glomerular tuft necrosis, and cellular crescents, were present in 32



BP (mm Hg)	132/88	120/80	120/80
UP (g/d)	0.4	0.4	0.5
U-RBC (/hpf)	2.3	2	2
Scr (mg/dL)	1.0	1.1	1.4
Ccr (mL/min)	113	78.9	56

Fig. 3. Effect of tonsillectomy on renal histological findings. This patient was born in 1952, diagnosed with IgA nephropathy (IgAN) by the first renal biopsy in 1979, received tonsillectomy in 1982, discovered hypertension in 1985, and received antihypertensive therapy. The patient underwent the second biopsy in 1989 and the third biopsy in 2001. Renal specimen was performed by periodic acid-Schiff (PAS) staining (original magnification ×50).

patients in first biopsy specimens, whereas these were no longer present in any of the second biopsy specimens. Although there was no significant difference in percentage of globally sclerotic glomeruli between the first and second biopsy specimens, the percentage of segmentally sclerotic glomeruli was significantly lower in second biopsy specimens. The distribution of IgA mesangial deposits had diminished in most patients, and no IgA deposits were seen in second biopsy specimens from eight patients. Impact of isolated tonsillectomy on renal histologic findings was unknown. We followed up a repeated biopsy patient with IgAN and tonsillectomy. He did not receive other drug therapy except for antihypertension. The first renal biopsy showed the marked mesangial proliferation, marked IgA deposit in glomerular mesangium, and almost normal renal tubules and blood vessel. After 10 years, the second biopsy showed the moderate mesangial proliferation, moderate IgA deposit, mild-to-moderate renal tubular atrophy, and mild arteriole sclerosis. The third biopsy after 22 years showed the enlarged glomeruli, mild mesangial proliferation, negative IgA deposit, marked tubular atrophy, and moderate arteriole sclerosis. The results of this patient demonstrated that tonsillectomy can improve IgA deposit and mesangial proliferation and cannot impact renal damage induced by other causes such as hypertension (Fig. 3).

Effect of tonsillectomy on long-term renal survival

Rasche, Schwarz, and Keller [74] reported that there was no significant correlation between tonsillectomy and ESRD by observing 16 IgAN patients with tonsillectomy and 39 patients without tonsillectomy, and introduced that tonsillectomy does not reduce the risk of developing renal failure [74]. The mean observation time after renal biopsy was relatively short (3.4 ± 4 years) in that study. Another study demonstrated that ESRD was detected in four of 35 IgAN patients after 10 years after tonsillectomy, in eight patients of 40 nontonsillectomy controls [70]. We [17] followed up 118 patients with idiopathic IgAN patients, including 48 tonsillectomy patients and 70 nontonsillectomy patients, for 192.9 ± 74.8 months (48~326 months). In that study, we used three different statistical methods, including the chi-squared test, Kaplan-Meier method with log-rank test, and Cox regression proportional hazards model in order to establish the efficacy of tonsillectomy in IgAN patients. Baseline characteristics at the time of renal biopsy, pathologic finding, and therapy during observation were not significant different between tonsillectomy and nontonsillectomy patients. A mean 15 years after diagnostic biopsy, only five (10.4%) of 48 tonsillectomy patients entered dialysis, whereas 18 (25.7%) of 70 nontonsillectomy patients required dialysis, by chi-squared test, $P = 0.0393$. Kaplan-Meier analysis showed the renal survival rates of tonsillectomy patients were statistically higher than those of non-tonsillectomy (log-rank test, $P = 0.0329$). For example, renal survival rates were 89.6% and 63.7% in the patients with and without tonsillectomy, respectively, at 240 months after renal biopsy. Cox regression analysis showed that the relative risk for terminal renal failure in patients with tonsillectomy was lower compared to nontonsillectomy patients (hazard ratio 0.22, 95% CI 0.06 to 0.76, $P = 0.0164$). The results of these three statistical analyses were consistent. All revealed that tonsillectomy had a favorable effect on long-term renal survival in patients with IgAN [17].

Indications and limitations of tonsillectomy

Tonsillectomy and adenoidectomy procedures are among the oldest surgical procedures still performed today. Otolaryngologically, the two main indications for tonsillectomy are upper airway obstruction due to tonsillar hypertrophy and recurrent acute or chronic tonsillitis. Adenoid hypertrophy with upper airway or eustachian tube obstruction and recurrent acute or chronic adenoiditis or otitis media are main indications to perform an adenoidectomy [75]. Nephrologically, indications for tonsillectomy are to date still unclear. In fact, many factors have effect on the efficacy of tonsillectomy in patients with IgAN, such as urinary finding and grades of renal damage. In general, the efficacy of tonsillectomy in patients with hemoturia type IgAN, especially presenting hemoturia after tonsil infection, is good [76]. We have showed that with a mild renal damage condition, in which the amount of urine protein excretion was less than 1.0 g/24 hours and global glomerular sclerosis less than 25%, none of 26 patients with tonsillectomy needed dialysis, whereas five (13.2%) of 38 patients without tonsillectomy required dialysis [17]. The percentage entering

dialysis in the tonsillectomy patients with moderate renal damage, such as urinary protein was more than 1.0 g/24 hours, but global glomerular sclerosis was less than 25% of total, was less than half of that in the nontonsillectomy patients [17]. On the other hand, the patients with a marked renal damage, in whom both the amount of urine protein excretion was more than 1.0 g/24 hours and global glomerular sclerosis was more than 25% of total or crescent formation was more than 25% of total might develop renal failure even if tonsillectomy was performed, that is, tonsillectomy is mainly indicated for patients with mild or moderate IgAN [17, 77, 78]. Rupture of the glomerular basement membrane occurred more frequently in the noneffective tonsillectomy than in the effective tonsillectomy group [79, 80]. Hotta et al [81] conducted a retrospective investigation of the renal outcome in IgAN patients with a median observation period of 75 months after tonsillectomy and steroid pulse therapy. Their results showed that there were no significant differences between the tonsillectomy and nontonsillectomy groups regarding the incidence of progressive renal functional loss defined as a 50% increase in baseline serum creatinine, but a combination of tonsillectomy and steroid pulse therapy had a significant impact on clinical remission by multivariate Cox regression analysis [81]. Sato et al [82] retrospectively investigated 70 patients with advanced IgAN (serum creatinine $\geq$1.5 mg/dL) classified into three groups according to their treatment regimens, that is, steroid pulse with tonsillectomy (30 patients), conventional steroid (25 patients), and supportive therapy (15 patients). During the mean follow-up period of 70.3 (12 to 137) months, 41.4% of patients reached ESRD (13.3% vs. 56.0% vs. 73.3%). The incidence of ESRD in the patients treated by steroid pulse with tonsillectomy was significantly lower than that in the patients treated by conventional steroid and supportive therapy at a baseline creatinine level of 1.5 to 2 mg/dL, but no statistical difference was observed at a level of >2 mg/dL [82]. These results suggest tonsillectomy combined with steroid pulse therapy may be effective in the IgAN patients with a baseline creatinine level of $\leq$2 mg/dL, whereas when serum creatinine >2 mg/dL, tonsillectomy may not change renal outcome even if that combines steroid therapy.

CONCLUSION

First, human tonsils are lymphoid organs and play a role in production of antibodies and local mucosal immune, especially in children. Tonsillectomy decreases the levels of serum IgA and salivary secretory IgA, but these changes do not cause significant immune deficiency and do not increase incidence of the upper respiratory tract infections. Second, tonsils are closely related to IgAN and polymeric IgA1 deposited in glomerular mesangium is at least in part of tonsillar origin. However, it is unclear that why do IgA-producing cells be predominant in tonsils with IgAN and how does IgA produced by tonsils deposit in mesangium. Third, tonsillectomy can improve the urinary findings, keep stable renal function, and have a favorable effect on long-tern renal survival in some IgAN patients. The indications of tonsillectomy in patients with IgAN include mainly the deterioration of urinary findings after tonsillar infection, mild or moderate renal damage. However, tonsillectomy may not be enough and may not change the prognosis in IgAN patients with marked renal damage. Unfortunately, studies regarding tonsillectomy were performed until now in a retrospective style and little information has been available about the side effect or complication of the operation in IgAN patients. In order to further clarify the clinical efficacy and security of tonsillecteomy, randomized prospective controlled trials are necessary because of the high degree of variability of IgAN.

ACKNOWLEDGMENTS

This study was supported by a grant from the Creative Research Group of the National Foundation Committee of Natural Science of P.R. China (30121005), and a grant from the Key Technologies Research and Development Programme of the Tenth Five-year Plan of P.R. China (2001BA701A14a). We are grateful to Dr. Minoru Sakatsume and Dr. Mitsuhiro Ueno for their help.

Reprint requests to Professor Xiangmei Chen, Kidney Center of PLA, Department of Nephrology, Chinese General Hospital of PLA, Fuxing Road 28, Beijing 100853, China.
E-mail: xmchen@public.bta.net.cn

REFERENCES

1. Berger J, Hinglais N: Les depots intercapillaires d'IgA-IgG. *J Urol Nephrol* (Paris) 74:694–695, 1968
2. Berger J. IgA glomerular deposits in renal disease: *Transplant Proc* 1:939–944, 1969
3. Emancipator SN: IgA nephropathy: morphologic expression and pathogenesis. *Am J Kidney Dis* 23:451–462, 1994
4. D'Amico G: The commonest glomerulonephritis in the world: IgA nephropathy. *Q J Med* 245:709–727, 1987
5. Julian BA, Waldo FB, Rifai A, Mestecky J: IgA nephropathy, the most common glomerulonephritis worldwide: A neglected disease in the United States? *Am J Med* 84:129–132, 1988
6. Levy M, Berger J: Worldwide perspective of IgA nephropathy. *Am J Kidney Dis* 12:340–347, 1988
7. D'Amico G, Colasanti G, Barbiano di Belgioioso G, *et al*: Long-term follow-up of IgA mesangial nephropathy: Clinico-histological study in 374 patients. *Semin Nephrol* 7:355–358, 1987
8. Ibels LS, Gyory AZ: IgA nephropathy: Analysis of the natural history, important factors in the progression of renal disease, and a review of the literature. *Medicine* (Baltimore) 73:79–102, 1994
9. Hogg RJ, Silva FG, Wyatt RJ, *et al*: Prognostic indicators in children with IgA nephropathy—Report of the Southwest Pediatric Nephrology Study Group. *Pediatr Nephrol* 8:15–20, 1994
10. Radford MG Jr, Donadio JV Jr, Bergstralh EJ, Grande JP: Predicting renal outcome in IgA nephropathy. *J Am Soc Nephrol* 8:199–207, 1997
11. Nishi S: The prognosis of IgA nephropathy—Favorable or poor? *Intern Med* 40:679–680, 2001
12. Maisonneuve P, Agodoa L, Gellert R, *et al*: Distribution of primary renal diseases leading to end-stage renal failure in the United

States, Europe, and Australia/New Zealand: Results from an international comparative study. *Am J Kidney Dis* 35:157–165, 2000
13. Briganti EM, Dowling J, Finlay M, *et al*: The incidence of biopsy-proven glomerulonephritis in Australia. *Nephrol Dial Transplant* 16:1364–1367, 2001
14. Donadio JV, Grande JP: IgA nephropathy. *N Engl J Med* 347:738–748, 2002
15. Lomax-Smith JD, Zabrowarny LA, Howarth GS, *et al*: The immunochemical characterization of mesangial IgA deposits. *Am J Pathol* 113:359–364, 1983
16. Tomino Y, Sakai H, and Special Study Group (IgA Nephropathy): Clinical guidelines for immunoglobulin A (IgA) nephropathy in Japan, second version. *Clin Exp Nephrol* 7:93–97, 2003
17. Xie Y, Nishi S, Ueno M, *et al*: The efficacy of tonsillectomy on long-term renal survival in patients with IgA nephropathy. *Kidney Int* 63:1861–1867, 2003
18. Hellings P, Jorissen M, Ceuppens JL: The Waldeyer's ring. *Acta Otorhinolaryngol Belg* 54:237–241, 2000
19. van Kempen MJ, Rijkers GT, Van Cauwenberge PB: The immune response in adenoids and tonsils. *Int Arch Allergy Immunol* 122:8–19, 2000
20. Boyaka PN, Wright PF, Marinaro M, *et al*: Human nasopharyngeal-associated lymphoreticular tissues. Functional analysis of subepithelial and intraepithelial B and T cells from adenoids and tonsils. *Am J Pathol* 157:2023–2035, 2000
21. Matthews JB, Basu MK: Oral tonsils: An immunoperoxidase study. *Int Arch Allergy Appl Immunol* 69:21–25, 1982
22. Brandtzaeg P, Surjan L Jr, Berdal P: Immunoglobulin-producing cells in clinically normal, hyperplastic and inflamed human palatine tonsils. *Acta Otolaryngol Suppl* 360:211–215, 1979
23. Goumas P, Troubouкis D, Toska N, *et al*: Immunohistochemical study of the palatine tonsils. *Laryngol Rhinol Otol* (Stuttg) 67:34–37, 1988
24. Moro I, Komiyama K, Iwase T, *et al*: Immunoglobulin subclasses in chronic tonsillitis. *J Oral Pathol* 17:475–477, 1988
25. von Gaudecker B, Muller-Hermelink HK: The development of the human tonsilla palatina. *Cell Tissue Res* 224:579–600, 1982
26. Brandtzaeg P: The B-cell development in tonsillar lymphoid follicles. *Acta Otolaryngol Suppl* 523:55–59, 1996
27. Jung KY, Lim HH, Choi G, Choi JO: Age-related changes of IgA immunocytes and serum and salivary IgA after tonsillectomy. *Acta Otolaryngol Suppl* 523:115–119, 1996
28. Bernstein JM: Mucosal immunology of the upper respiratory tract. *Respiration* 59 (Suppl 3):3–13, 1992
29. Kawaguchi M, Sakai T, Sakamaki A, *et al*: Expanded primary T nodules in the palatine tonsils from patients with IgA nephropathy. *Acta Otolaryngol Suppl* 508:36–42, 1993
30. van den Oord JJ, de Wolf-Peeters C, Desmet VJ: The composite nodule. A structural and functional unit of the reactive human lymph node. *Am J Pathol* 122:83–91, 1986
31. Sato Y, Hotta O, Taguma Y, *et al*: IgA nephropathy with poorly developed lymphoepithelial symbiosis of the palatine tonsils. *Nephron* 74:301–308, 1996
32. Bene MC, Faure G, Hurault de Ligny B, *et al*: Immunoglobulin A nephropathy. Quantitative immunohistomorphometry of the tonsillar plasma cells evidences an inversion of the immunoglobulin A versus immunoglobulin G secreting cell balance. *J Clin Invest* 71:1342–1347, 1983
33. Nagy J, Brandtzaeg P: Tonsillar distribution of IgA and IgG immunocytes and production of IgA subclasses and J chain in tonsillitis vary with the presence or absence of IgA nephropathy. *Scand J Immunol* 27:393–399, 1988
34. Bene MC, Hurault De Ligny B, Kessler M, Faure GC: Confirmation of tonsillar anomalies in IgA nephropathy: A multicenter study. *Nephron* 58:425–428, 1991
35. Egido J, Blasco R, Lozano L, *et al*: Immunological abnormalities in the tonsils of patients with IgA nephropathy: inversion in the ratio of IgA: IgG bearing lymphocytes and increased polymeric IgA synthesis. *Clin Exp Immunol* 57:101–106, 1984
36. Kusakari C, Nose M, Takasaka T, *et al*: Immunopathological features of palatine tonsil characteristic of IgA nephropathy: IgA1 localization in follicular dendritic cells. *Clin Exp Immunol* 95:42–48, 1994
37. Harper SJ, Allen AC, Bene MC, *et al*: Increased dimeric IgA-producing B cells in tonsils in IgA nephropathy determined by in situ hybridization for J chain mRNA. *Clin Exp Immunol* 101:442–448, 1995
38. Kennel-De March A, Bene MC, Hurault de Ligny B, *et al*: Enhanced expression of CD31 and CD54 on tonsillar high endothelial venules in IgA nephropathy. *Clin Immunol Immunopathol* 84:158–165, 1997
39. Kodama S, Suzuki M, Arita M, Mogi G: Increase in tonsillar germinal centre B-1 cell numbers in IgA nephropathy (IgAN) patients and reduced susceptibility to Fas-mediated apoptosis. *Clin Exp Immunol* 123:301–308, 2001
40. Rifai A, Small PA Jr, Teague PO, Ayoub EM: Experimental IgA nephropathy. *J Exp Med* 150:1161–1173, 1979
41. Harper SJ, Feehally J: The pathogenic role of immunoglobulin A polymers in immunoglobulin A nephropathy. *Nephron* 65:337–345, 1993
42. Tomino Y, Sakai H, Endoh M, *et al*: Cross-reactivity of IgA antibodies between renal mesangial areas and nuclei of tonsillar cells in patients with IgA nephropathy. *Clin Exp Immunol* 51:605–610, 1983
43. Tokuda M, Shimizu J, Sugiyama N, *et al*: Direct evidence of the production of IgA by tonsillar lymphocytes and the binding of IgA to the glomerular mesangium of IgA nephropathy patients. *Acta Otolaryngol Suppl* 523:182–184, 1996
44. Shiraishi S, Tomoda K, Matsumoto A, *et al*: Investigation of the local provocation test to PPP and IgA nephritis. *Acta Otolaryngol Suppl* 523:178–181, 1996
45. Yamabe H, Osawa H, Inuma H, *et al*: Deterioration of urinary findings after tonsil stimulation in patients with IgA nephropathy. *Acta Otolaryngol Suppl* 523:169–171, 1996
46. Masuda Y, Terazawa K, Kawakami S, *et al*: Clinical and immunological study of IgA nephropathy before and after tonsillectomy. *Acta Otolaryngol Suppl* 454:248–255, 1988
47. Matsuda M, Shikata K, Wada J, *et al*: Increased urinary excretion of macrophage-colony-stimulating factor (M-CSF) in patients with IgA nephropathy: Tonsil stimulation enhances urinary M-CSF excretion. *Nephron* 81:264–270, 1999
48. Akagi H, Nishizaki K, Hattori K, *et al*: Prognosis of tonsillectomy in patients with IgA nephropathy. *Acta Otolaryngol Suppl* 540:64–66, 1999
49. Akagi H, Hattori K, Doi A, *et al*: Tonsillar provocation test for tonsillectomy in patients with IgA nephropathy. *Stomato-pharyngol* 11:297–305, 1999
50. Kataura A: Evaluation of the provocation test of tonsils: A report from the Committee for the standardization of diagnostic criteria for tonsillar focal infections. *Stomato-pharyngol* 9:213–221, 1997
51. Akagi H, Kosaka M, Doi A, *et al*: Results of a survey of the relationship between palatine tonsil and IgA nephropathy. *Nippon Jibiinkoka Gakkai Kaiho* 102:305–310, 1999
52. Suzuki S, Nakatomi Y, Sato H, *et al*: *Haemophilus parainfluenzae* antigen and antibody in renal biopsy samples and serum of patients with IgA nephropathy. *Lancet* 343:12–16, 1994
53. Suzuki S, Fujieda S, Sunaga H, *et al*: Immune response of tonsillar lymphocytes to *Haemophilus parainfluenzae* in patients with IgA nephropathy. *Clin Exp Immunol* 119:328–332, 2000
54. Yamamoto C, Suzuki S, Kimura H, *et al*: Experimental nephropathy induced by *Haemophilus parainfluenzae* antigens. *Nephron* 90:320–327, 2002
55. Fujieda S, Suzuki S, Sunaga H, *et al*: Bacterial infection and production of immunoglobulin in the tonsil. *Rinsho Byori* 47:724–729, 1999
56. Rekola S, Bergstrand A, Bucht H, Lindberg A: Are beta-haemolytic streptococci involved in the pathogenesis of mesangial IgA-nephropathy? *Proc Eur Dial Transplant Assoc Eur Ren Assoc* 21:698–702, 1985
57. Tomino Y, Yagame M, Omata F, *et al*: A case of IgA nephropathy associated with adeno- and herpes simplex viruses. *Nephron* 47:258–261, 1987
58. Iwama H, Horikoshi S, Shirato I, Tomino Y: Epstein-Barr virus detection in kidney biopsy specimens correlates with glomerular mesangial injury. *Am J Kidney Dis* 32:785–793, 1998

59. Kunimoto M, Hayashi Y, Kuki K, *et al*: Analysis of viral infection in patients with IgA nephropathy. *Acta Otolaryngol Suppl* 508:11–18, 1993
60. Ostergaard PA: IgA levels and carrier rate *of Haemophilus influenzae* and beta-haemolytic streptococci in children undergoing tonsillectomy. *Acta Pathol Microbiol Scand* [C] 84:290–298, 1976
61. D'Amelio R, Palmisano L, Le Moli S, *et al*: Serum and salivary IgA levels in normal subjects: comparison between tonsillectomized and non-tonsillectomized subjects. *Int Arch Allergy Appl Immunol* 68:256–259, 1982
62. Cantani A, Bellioni P, Salvinelli F, Businco L: Serum immunoglobulins and secretory IgA deficiency in tonsillectomized children. *Ann Allergy* 57:413–416, 1986
63. Bock A, Popp W, Herkner KR: Tonsillectomy and the immune system: A long-term follow up comparison between tonsillectomized and non-tonsillectomized children. *Eur Arch Otorhinolaryngol* 251:423–427, 1994
64. Ikincigullari A, Dogu F, Ikincigullari A, *et al*: Is immune system influenced by adenotonsillectomy in children? *Int J Pediatr Otorhinolaryngol* 66:251–257, 2002
65. Kanamoto Y, Shibata R, Ozono Y, Harada T: Effect of tonsillectomy on peripheral blood T cell surface markers and cytokine production in patients with IgA nephropathy accompanied by chronic tonsillitis. *Nippon Jinzo Gakkai Shi* 36:1296–1302, 1994
66. Kirstila V, Tenovuo J, Ruuskanen O, *et al*: Longitudinal analysis of human salivary immunoglobulins, nonimmune antimicrobial agents, and microflora after tonsillectomy. *Clin Immunol Immunopathol* 80:110–115, 1996
67. Ostergaard PA: IgA levels and carrier rate of pathogenic bacteria in 27 children previously tonsillectomized. *Acta Pathol Microbiol Scand* [C] 85:178–186, 1977
68. Sainz M, Gutierrez F, Moreno PM, *et al*: Changes in immunologic response in tonsillectomized children. I. Immunosuppression in recurrent tonsillitis. *Clin Otolaryngol* 17:376–379, 1992
69. Bene MC, Hurault de Ligny B, Kessler M, *et al*: Tonsils in IgA nephropathy. *Contrib Nephrol* 104:153–161, 1993
70. Barta J, Kovacs T, Fazekas A, *et al*: Does tonsillectomy cause any change in long-term course of IgA nephropathy? *Orv Hetil* 137:2903–2906, 1996
71. Tamura S, Masuda Y, Inokuchi I, *et al*: Effect of and indication for tonsillectomy in IgA nephropathy. *Acta Otolaryngol Suppl* 508:23–28, 1993
72. Kosaka M: Long-term prognosis for tonsillectomy patients with IgA nephropathy. *Nippon Jibiinkoka Gakkai Kaiho* 101:916–923, 1998
73. Hotta O, Furuta T, Chiba S, *et al*: Regression of IgA nephropathy: a repeat biopsy study. *Am J Kidney Dis* 39:493–502, 2002
74. Rasche FM, Schwarz A, Keller F: Tonsillectomy does not prevent a progressive course in IgA nephropathy. *Clin Nephrol* 51:147–152, 1999
75. Paulussen C, Claes J, Claes G, Jorissen M: Adenoids and tonsils, indications for surgery and immunological consequences of surgery. *Acta Otorhinolaryngol Belg* 54:403–408, 2000
76. Zhou WG, Wang TF, Xue Y, *et al*: Chronic tonsillitis and IgA nephropathy. *Chin Med J* (Engl) 106:770–772, 1993
77. Hattori K: Therapeutic effects and prognostic factors of tonsillectomy for IgA nephropathy in long-term follow-up. *Nippon Jibiinkoka Gakkai Kaiho* 101:1412–1422, 1998
78. Ino Y, Ambe K, Kato Y, *et al*: Chronic tonsillitis and IgA nephropathy. Clinical study of patients with and without tonsillectomy. *Acta Otolaryngol Suppl* 508:29–35, 1993
79. Sugiyama N, Shimizu J, Nakamura M, *et al*: Clinicopathological study of the effectiveness of tonsillectomy in IgA nephropathy accompanied by chronic tonsillitis. *Acta Otolaryngol Suppl* 508:43–48, 1993
80. Tomioka S, Miyoshi K, Tabata K, *et al*: Clinical study of chronic tonsillitis with IgA nephropathy treated by tonsillectomy. *Acta Otolaryngol Suppl* 523:175–177, 1996
81. Hotta O, Miyazaki M, Furuta T, *et al*: Tonsillectomy and steroid pulse therapy significantly impact on clinical remission in patients with IgA nephropathy. *Am J Kidney Dis* 38:736–743, 2001
82. Sato M, Hotta O, Tomioka S, *et al*: Cohort study of advanced IgA nephropathy: Efficacy and limitations of corticosteroids with tonsillectomy. *Nephron Clin Pract* 93:c137–c145, 2003

Kidney International, Vol. 65 (2004), pp. 2228–2237

Role of mast cells in the development of renal fibrosis: Use of mast cell–deficient rats

SHINOBU MIYAZAWA, OSAMU HOTTA, NAOKO DOI, YUMIKO NATORI, KIYOTAKA NISHIKAWA, and YASUHIRO NATORI

Department of Clinical Pharmacology, Research Institute, International Medical Center of Japan, Tokyo, Japan; Department of Nephrology, Sendai Shakaihoken Hospital, Sendai, Japan

Role of mast cells in the development of renal fibrosis: Use of mast cell–deficient rats.

Background. Recent clinical studies have shown that the number of interstitial mast cells increases in various types of renal disease and correlates well with the magnitude of interstitial fibrosis. The present study was conducted to assess the role of mast cells in renal fibrosis by examining an experimental glomerular disease.

Methods. A rat model of chronic glomerular disease, puromycin aminonucleoside-nephrosis, was induced in mast cell–deficient (*Ws/Ws*) and normal (+/+) rats.

Results. The area of interstitial fibrosis was widely distributed at 6 weeks in both groups of rats; however, unexpectedly, the area of interstitial fibrosis was greater in *Ws/Ws* rats than in +/+ littermates. Biochemical analysis of the hydroxyproline content confirmed the more severe fibrosis in the *Ws/Ws* rats. The number of mast cells increased in both *Ws/Ws* and +/+ rats, concomitant with the development of interstitial fibrosis, but was confirmed to be lower in *Ws/Ws* than in +/+ rats. There were no differences in the numbers of interstitial macrophages and T lymphocytes between the two groups. Reverse transcription-polymerase chain reaction analysis of cytokine expression revealed that the level of mRNA for transforming growth factor-β (TGF-β), a potent profibrotic cytokine, was higher in *Ws/Ws* rats. In addition, heparin, one of the major components of mast cells, inhibited the expression of TGF-β mRNA in rat fibroblasts in culture.

Conclusion. These results suggest that mast cells do not play a major role in the pathogenesis of interstitial fibrosis in puromycin aminonucleoside nephrosis. Rather, they might be protective or ameliorative in this model through the inhibition of TGF-β production by heparin, and possibly in other models and also in humans.

Mast cells are known to be immune-effector cells that augment inflammatory reactions. It is widely accepted that mast cells are involved in a number of allergic inflammatory diseases as well as in host defense against pathogens. In addition, several lines of evidence suggest that mast cells may participate in fibrotic processes. Mast cells are found in fibrogenic lesions in various tissues in human diseases, such as pulmonary fibrosis [1, 2], hepatic cirrhosis [3, 4], scleroderma [5, 6], and keloid [7]. Mast cells serve as a rich source of several mediators [8], including histamines, proteoglycans, and proteolytic enzymes (proteases), as well as a number of cytokines [9], some of which are reported to be mitogenic [10] and chemotactic [11] for fibroblasts, and to stimulate the production of the extracellular matrix (ECM) by fibroblasts [9, 10, 12]. Furthermore, mast cells themselves produce components of the ECM [13]. Therefore, for these reasons they are considered to play a profibrogenic role in the above-mentioned diseases.

In recent years, mast cells were shown to be present in the interstitial area of human renal biopsy tissues from patients with various renal diseases, such as IgA nephropathy [14–16], rapidly progressive glomerulonephritis [17], focal and segmental glomerulosclerosis [15], diabetic nephropathy [15, 18], and kidney graft rejection [16, 19, 20]. Furthermore, the number of interstitial mast cells in these glomerular diseases correlates well with the degree of interstitial fibrosis. Tubulointerstitial lesions including interstitial fibrosis are considered to be prognostic features of various glomerular diseases; regardless of its primary causes, decreased renal function correlates most closely with pathologic changes in the tubulointerstitium, which include interstitial fibrosis, tubular atrophy, and loss of peritubular capillaries [21, 22]. Based on these observations, mast cells are suggested to contribute to the renal deterioration in glomerular diseases by inducing interstitial fibrosis [14, 15, 17]. However, there have been no studies that directly show the involvement of mast cells in the pathogenesis of interstitial fibrosis in the kidney.

To elucidate the role of mast cells in the interstitial fibrosis in progressive glomerular diseases, we

Key words: mast cell, *Ws/Ws rat*, renal fibrosis, animal model, transforming growth factor-β.

Received for publication January 26, 2003
and in revised form September 5, 2003, and December 11, 2003
Accepted for publication January 15, 2004

investigated the accumulation of mast cells in an animal model of glomerular disease that is accompanied by interstitial fibrosis and renal deterioration. Furthermore, we utilized mast cell–deficient *Ws/Ws* rats to test if mast cells contribute to the development of the interstitial fibrosis. *Ws/Ws* rats have very few mast cells in their skin and other tissues [less than 1% of the number in control (+/+) littermates] because of a small deletion in the tyrosine kinase domain of the *c-kit* gene [23–25]. Unexpectedly, in this study we found that the degree of interstitial fibrosis was more severe in *Ws/Ws* rats than in the control littermates, suggesting that mast cells do not contribute to the development of interstitial fibrosis in this model. Rather, they may actually play a beneficial role in the process of fibrosis in the kidney.

METHODS

Animal model

Puromycin aminonucleoside (PAN) nephrosis model was induced in male mast cell–deficient *Ws/Ws* and their normal +/+ littermates (Japan SLC; Hamamatsu, Japan, $N = 12$ each), weighing 140 to 160 g, by the method of Jones et al [26], with slight modifications. In brief, left unilateral nephrectomy through a flank incision was performed on each rat under sodium pentobarbital (45 mg/kg body weight) anesthesia. Five days after the nephrectomy, the rats received an intraperitoneal (i.p.) injection of PAN (Sigma Chemical Co., St. Louis, MO, USA) dissolved in 0.9% saline (15 mg/mL) and given at a dose of 15-mg/100 g body weight. Second, third, and fourth doses of PAN (4.3 mg/100 g body weight, i.p.) were administered at 3, 4, and 5 weeks, respectively, after the initial dose. Control animals did not receive any surgical procedures or injections ($N = 10$ each). All animals were maintained on a standard rat diet and had free access to water throughout the course of the experiment. The animals were housed individually in metabolic cages to obtain 24-hour urine once every week. They were sacrificed sequentially at 2 ($N = 5$ each) and 6 weeks ($N = 5$ each except for $N = 4$ for PAN-injected *Ws/Ws* rats; see below) after the initial dose of PAN, namely, 1 week after the first and the last injections, respectively. Three of the PAN-injected *Ws/Ws* rats and 2 of the PAN-injected +/+ rats died of chronic renal failure by 6 weeks. These rats were found dead in their cages in the morning, and thus their kidneys could not be used for histologic studies. These animals were excluded from the subsequent analysis. At sacrifice, blood samples were collected by heart puncture under anesthesia with diethylether. The kidneys were perfused via the abdominal aorta with ice-cold saline, and pieces of renal tissues were fixed in 10% buffered formalin or periodate-lysine-paraformaldehyde (PLP) solution. Pieces of renal cortex were also used for RNA preparation and hydroxyproline analysis (see below). Urinary protein was quantified by the biuret method. Levels of blood urea nitrogen (BUN) were measured with a kit designed for clinical use (Wako Pure Chemical Industries, Osaka, Japan).

Kidney tissue preparation

The kidney tissues fixed in 10% buffered formalin were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, periodic acid-Schiff (PAS), or Masson trichrome. The degree of interstitial fibrosis was semiquantitatively analyzed by inspection of Masson trichrome–stained sections and graded on a scale of 0 to 3 as follows: (*0*), no apparent damage; (*1*) mild damage, with lesions involving less than 5% of the cortex; (*2*) moderate damage, involving 5% to 20% of the cortex; and (*3*) severe damage, involving more than 20% of the cortex. Pieces for cryostat sectioning were fixed in PLP solution for 4 hours, washed several times in phosphate-buffered saline (PBS) containing 7% sucrose, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA), and snap-frozen.

Immunohistochemical study

To block endogenous peroxidase activity, we treated sections of frozen or paraffin-embedded kidney tissues with methanol containing 0.6% hydrogen peroxide for 15 minutes and then washed them with PBS. They were stained by the standard avidin-biotin peroxidase technique with sheep antirat mast cell protease (RMCP) I (mast cell–specific antibody; Moredun, Scotland, UK), mouse monoclonal antibody ED-1 (specific for rat monocytes/macrophages), W3/25 ($CD4^+$ cells), or OX8 ($CD8^+$ cells) at 4°C overnight. The sections were incubated with the corresponding second antibodies, biotinylated donkey antisheep IgG or horse antimouse IgG, and stained with the reagents of an ABC staining kit (Vector Laboratories, Inc., Burlingame, CA, USA). Sections were also stained with horseradish peroxidase–conjugated mouse antihuman α-smooth muscle actin (α–SMA, a marker for myofibroblasts) monoclonal antibody (Dako Corp., Carpinteria, CA, USA). All sections were then developed with 3, 3′-diaminobenzidine solution as chromogen and counterstained with methylgreen. With the aid of a 10 × 10 eyepiece grid, the numbers of monocytes/macrophages, $CD4^+$, and $CD8^+$ cells in the interstitium were counted manually in 6 random nonoverlapping cortical fields (×400) of sections made from each experimental animal. RMCP I–positive interstitial mast cells were also counted in 20 random cortical fields (×100). The results of cell counting were expressed as the number per square millimeter.

Cell cultures

A cell line of renal fibroblasts (NRK49F) derived from rat kidney was obtained from The European

Table 1. Nucleotide sequences of the polymerase chain reaction (PCR) primers, the expected sizes of the amplified products, annealing temperature, and cycle numbers of PCR reactions

Primers			Product	Annealing temperature	Cycles
RMCP II	5′ primer	5′-ACGAAAGTTACAACTCCGTTC-3′	475 bp	60°C	32
	3′ primer	5′-GTGTCTCAAGCAGGTCAGG-3′			
RMCP V	5′ primer	5′-GTCTATAACCGTCCTCCTAG-3′	381 bp	54°C	38
	3′ primer	5′-TTTTGCATCTTCTTGGGGTTG-3′			
RMCP VIII	5′ primer	5′-GGCCATCCTACCAGTCAACAC-3′	341 bp	60°C	30
	3′ primer	5′-CACCATTGAGTTGAGCTTTGCG-3′			
GAPDH	5′ primer	5′-GGGAGCCAAAAGGGTCATCATCTC-3′	516 bp	64°C	18
	3′ primer	5′-CCATGCCAGTGAGCTTCCCGTTC-3′			

All of the PCR reactions were performed as described in the Methods section.

Collection of Cell Cultures (Salisbury, UK) and maintained in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS), glutamine, and nonessential amino acid equilibrated with 5% CO_2-95% air at 37°C. For experiments, cells grown to 80% confluence in 100-mm dishes were exposed to fresh medium with or without 100 μg/mL heparin (sodium salt; Sigma) for 6 hours and then used for RNA preparation.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA fractions were prepared from pieces of renal cortex or cultured cells by the guanidinium thiocyanate-phenol-chloroform method [27], and 5 μg of RNA was reverse-transcribed by use of oligo(dT) primers in 20 μL of buffer as previously described [28]. For the analysis of levels of mRNA for RMCPs II, V, and VIII, 1 μL of this reaction mixture containing the cDNA was amplified by PCR in 50 μL of 10 mmol/L Tris-HCl (pH 9.0) containing 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 5′ and 3′ primers (Table 1), and Taq DNA polymerase (Promega, Madison, WI, USA). Aliquots were electrophoresed on a 2% agarose gel and visualized with ethidium bromide under ultraviolet illumination. For the quantitative measurement of cytokine mRNA levels, real-time PCR was performed by using an ABI PRISM 7700 Sequence Detector and TaqMan® Pre-Developed Assay Reagents for Gene Expression Quantification System (Applied Biosystems, Foster City, CA, USA). Using multiple reporter dyes, we assayed the mRNA levels of each cytokine and endogenous control (GAPDH) in the same tube and expressed cytokine mRNA levels as the ratio relative to the endogenous control.

Hydroxyproline analysis

The amount of hydroxyproline in the renal cortex was measured according to Kivirikko et al [29] as an index of collagen content. At sacrifice, pieces of renal cortex for the hydroxyproline assay were weighed, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent use. The samples were hydrolyzed in 1 mL of 6N hydrochloric acid at 110°C for 18 hours in tightly capped tubes. After the hydrolysates had been neutralized with sodium hydroxide, their hydroxyproline content was assessed colorimetrically at 560 nm with *p*-dimethylaminobenzaldehyde. Results were expressed as ng per mg wet renal cortex.

Statistical analysis

All values were expressed as the mean ± SD. Data were analyzed by the Mann-Whitney *U* test, and a difference with a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

PAN nephrosis in *Ws/Ws* and +/+ rats

In PAN nephrosis model induced by uninephrectomy and subsequent administration of PAN, increases in urinary protein excretion and BUN level were observed in both mast cell–deficient *Ws/Ws* and their control (+/+) littermates; there was no difference in either parameter between the two groups (Fig. 1). At 6 weeks, the level of urinary protein excretion markedly decreased because of the loss of functioning nephrons (end-stage renal failure).

Renal fibrosis induced in *Ws/Ws* and +/+ rats by PAN

Interstitial fibrosis occurred in both PAN-treated *Ws/Ws* and +/+ rats at 2 weeks at the border of the cortex-medulla. A significant expansion of the fibrotic area was observed at 6 weeks in both strains. Unexpectedly however, the fibrosis was more severe in the *Ws/Ws* rats than in their +/+ littermates (Fig. 2). No obvious fibrosis was observed in the control animals. The magnitude of fibrosis was semiquantitatively determined with light microscopy (Fig. 3). The degree of fibrosis was similar in both groups at 2 weeks, but was greater in *Ws/Ws* rats than in +/+ rats at 6 weeks.

To confirm these histologic results, we measured the hydroxyproline content in the renal cortex (Fig. 4). At 2 weeks, although the content of this collagen marker

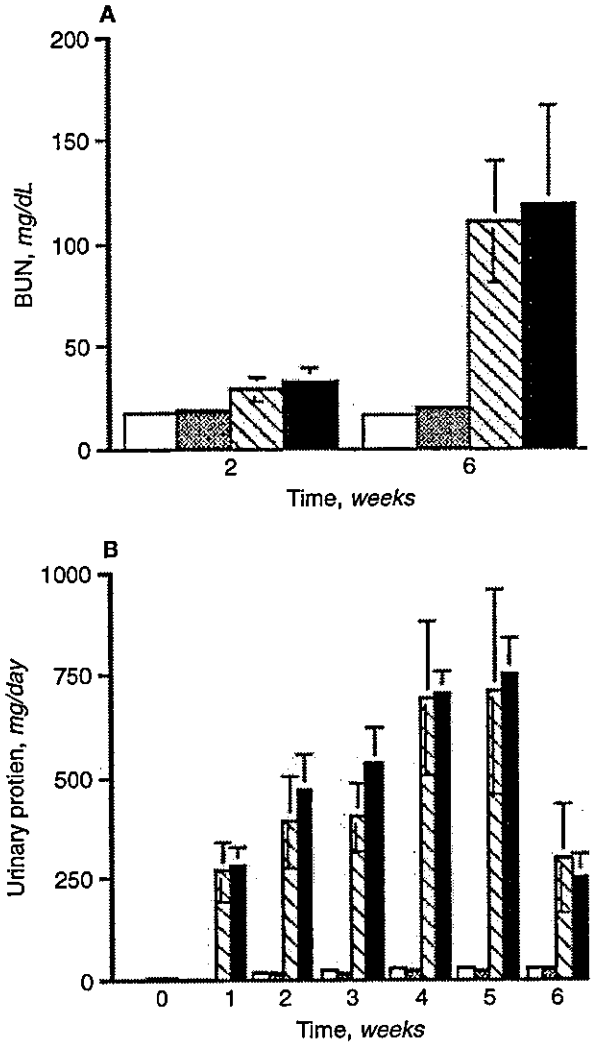


Fig. 1. Clinical course of puromycin aminonucleoside (PAN) nephrosis in *Ws/Ws* and +/+ rats. Blood urea nitrogen (*A*), urinary protein excretion (*B*). Control +/+ (□); Control *Ws/Ws* (■); PAN-treated +/+ (▨); PAN-treated *Ws/Ws* rats (■).

in PAN-treated animals was slightly higher than that in control animals, the difference was not statistically significant; and the level was similar in PAN-treated *Ws/Ws* and +/+ rats. At 6 weeks, the content was significantly greater in PAN-treated animals than in control animals. Moreover, the level in the PAN-treated *Ws/Ws* rats was significantly higher than that in the PAN-treated +/+ rats. Thus, the fibrotic changes in the interstitium assessed by both histologic and biochemical methods were greater in *Ws/Ws* rats than in their +/+ littermates.

Interstitial mast cells in PAN-induced nephrosis

Mast cells were immunohistochemically identified by their immunoreactivity, indicating the presence of RMCP I, a specific marker for mast cells [30, 31]. An almost complete absence of mast cells in the skin of *Ws/Ws* rats was confirmed by immunostaining for RMCP I (data not shown). Practically no mast cells were detectable in the kidney of control *Ws/Ws* and +/+ rats. However, in the kidney tissues of PAN-treated +/+ rats, mast cells were observed in the interstitium, where they showed a scattered distribution (Fig. 5A). Most were localized in the peritubular region (Fig. 5B).

To confirm the increase in the number of mast cells in the kidney, we also examined mRNA levels of other specific markers for mast cells (i.e., RMCPs II, V, and VIII). By RT-PCR analysis, mRNAs for all of these proteases were consistently found in disease-induced rats, but not in control rats, by the experimental procedures used (Fig. 6).

The time-kinetics of the number of interstitial mast cells determined by immunohistochemistry is illustrated in Figure 7. After administration of PAN, the number of mast cells increased significantly in both *Ws/Ws* and +/+ rats, but was lower in the former than in the latter. While the number of mast cells gradually increased in PAN-treated +/+ rats with the expansion of the interstitial fibrosis, the number in PAN-treated *Ws/Ws* rats decreased with the continued expansion. The number in PAN-treated *Ws/Ws* rats was one half at 2 weeks and one fifth at 6 weeks compared with that in the PAN-treated +/+ rats.

Other interstitial cells in PAN-induced nephrosis

Because thymocytes are *c-kit*–positive cells, the defect in *c-kit* carried by *Ws/Ws* rats could potentially affect T-cell development, although there have been no reports showing such T-cell deficits. It is also known that myofibroblasts express *c-kit* [32]. To clarify the influence of the *c-kit* deletion in these types of cell in the kidney of *Ws/Ws* rats, we examined the levels of monocytes/macrophages, CD4–, CD8–, and α–SMA–positive cells in the interstitium (Table 2). Low numbers of these types of cells were detected in the interstitium of the control animals. After the disease induction, their numbers increased in both *Ws/Ws* and +/+ rats, but there were no differences in them between the two strains. Most of the monocyte/macrophages and CD4– and CD8– positive cells were found in the interstitial lesion in clusters, especially in the area around glomeruli and disrupted tubuli, and the localization was different from that of the mast cells.

Cytokine mRNA levels in renal cortex

To investigate the possible mechanism for the increase in fibrosis in these *Ws/Ws* rats, we measured mRNA levels of cytokines that might affect the progression of fibrosis. As shown in Figure 8, the levels of mRNA for two potentially profibrotic cytokines, TGF-β and interleukin (IL)-4,

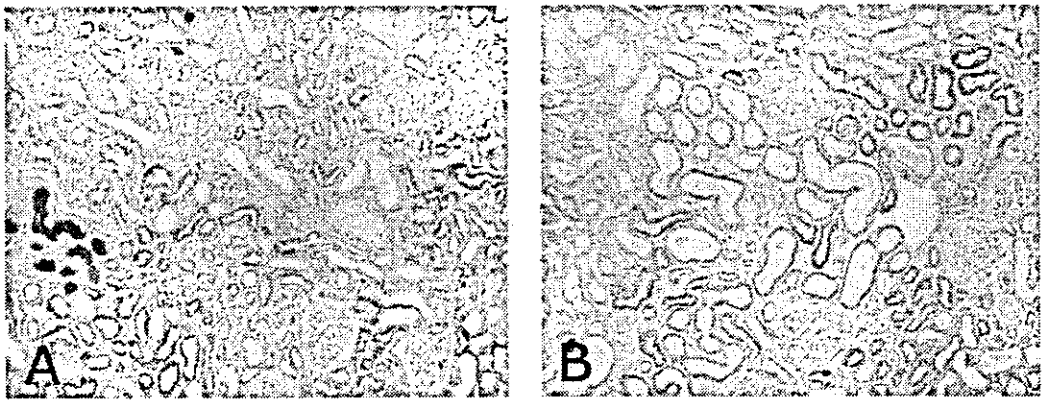


Fig. 2. Representative histologic changes in the kidney in puromycin aminonucleoside (PAN) nephrosis. Kidney tissues were obtained at 6 weeks from PAN-treated +/+ rats (*A*) and PAN-treated *Ws/Ws* rats (*B*). Masson-trichrome staining. Original magnification ×100.

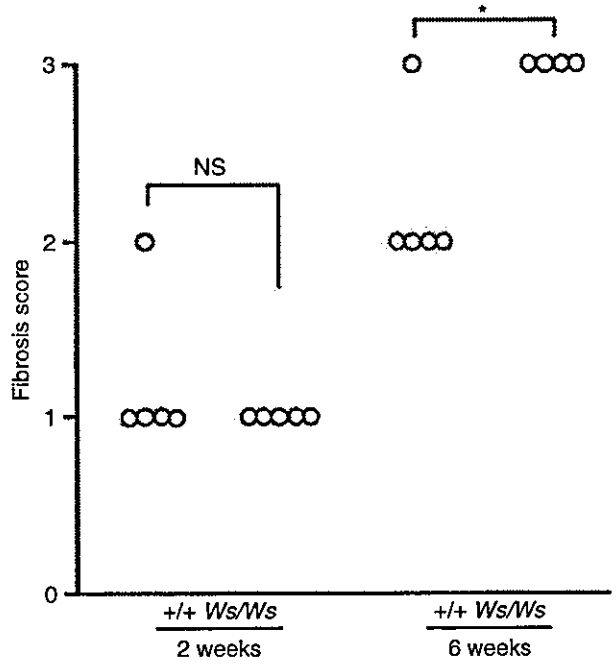


Fig. 3. Semiquantification of the fibrotic interstitium in puromycin aminonucleoside (PAN) nephrosis. Kidney tissues were obtained at 2 and 6 weeks from PAN-treated +/+ and *Ws/Ws* rats. $^{*}P < 0.05$.

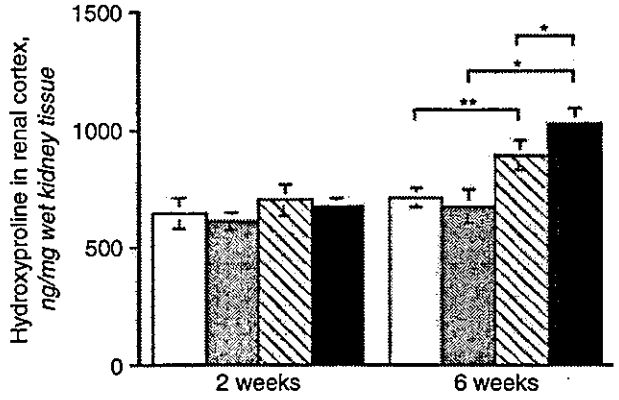


Fig. 4. Hydroxyproline content in renal cortex of rats with puromycin aminonucleoside (PAN) nephrosis. Control +/+ (□); control *Ws/Ws* (■); PAN-treated +/+ (▨); PAN-treated *Ws/Ws* rats (■). $^{*}P < 0.05$; $^{**}P < 0.01$.

were higher in the *Ws/Ws* rats than in the +/+ ones. The differences between the two groups in the level of TGF-β mRNA at 2 weeks, and in that of IL-4 mRNA at 6 weeks, were statistically significant. There were no differences in mRNA levels of IL-2, IL-10, and interferon (IFN)-γ in the renal cortex between the two strains. These results suggest that the enhanced production of TGF-β and/or IL-4 in *Ws/Ws* rats may have caused the enhanced fibrosis in the kidney.

Effect of heparin on the expression of TGF-β1 mRNA in renal fibroblast in culture

Although mast cells have been shown to produce TGF-β and IL-4 in culture [9], they also produce other cytokines and factors that may modulate the production of these cytokines by other types of cells. Heparin is a major component stored in secretory granules of mast cells and is known to influence cytokine production in culture [33]. Therefore, using a cell line of interstitial fibroblasts derived from normal rat kidney, we investigated if heparin could influence the production of TGF-β in culture. As shown in Figure 9, the expression of TGF-β mRNA in renal fibroblast was inhibited by heparin.

DISCUSSION

In progressive human glomerular diseases, regardless of their type, the number of interstitial mast cells

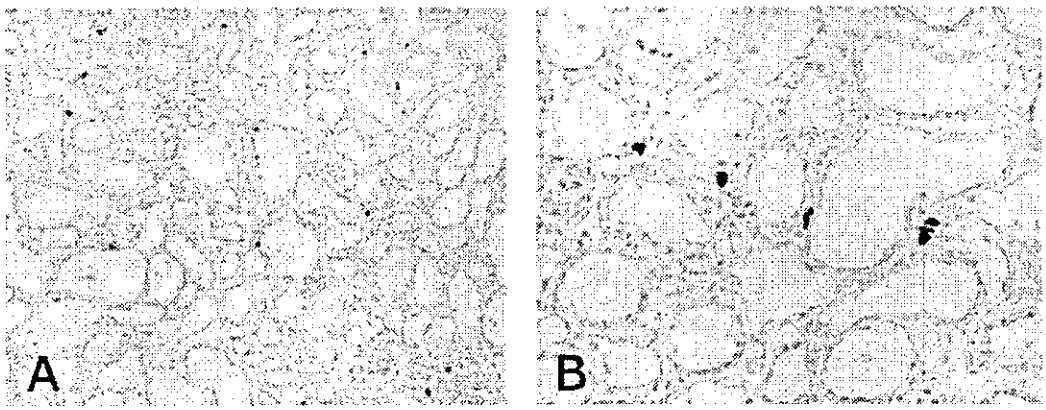


Fig. 5. Immunostaining for rat mast cell protease (RMCP) I, a specific marker for mast cell, in puromycin aminonucleoside (PAN) nephrosis. Representative kidney sections obtained at 6 weeks from PAN-treated +/+ rats are shown. RMCP I-positive cells with a scattered distribution are seen in the interstitium (*A*). These cells are mostly located in the peritubular region (*B*). Original magnification, ×100 (A) and ×200 (B).

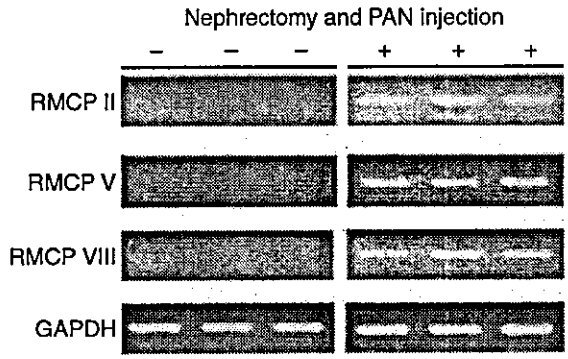


Fig. 6. Expression of mRNA for rat mast cell proteases (RMCPs) II, V, and VIII. RNA extracted from 3 animals of each group [2-week puromycin aminonucleoside (PAN)-treated +/+ rats and control +/+ rats] was used for reverse transcription-polymerase chain reaction (RT-PCR) analysis. Each lane represents the mRNA expression of each animal. The GAPDH band is shown as the internal control.

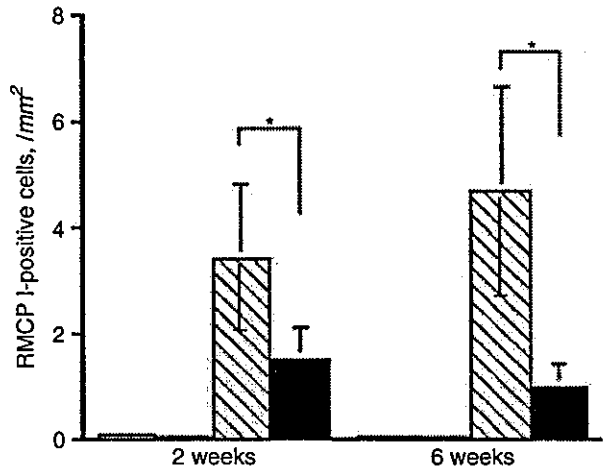


Fig. 7. Numbers of interstitial rat mast cell protease (RMCP) I-positive cells in puromycin aminonucleoside (PAN) nephrosis. Control +/+ (□); control *Ws/Ws* (■); PAN-treated +/+ (▨); PAN-treated *Ws/Ws* rats (■). $^*P < 0.05$.

increases as interstitial fibrosis expands [14, 15, 17]. The present study has demonstrated that in PAN nephrosis, a rat model of progressive glomerular disease, the number of interstitial mast cells also increased concomitant with the expansion of interstitial fibrosis. We also found that the number of interstitial mast cells increased in the crescentic glomerulonephritis model in Wistar Kyoto rats (our unpublished observations). These results indicate that, like in humans, mast cells accumulate in the interstitial area in progressive renal diseases in rats when interstitial fibrosis develops.

Because PAN nephrosis can be induced in any strain of rat, using mast cell–deficient *Ws/Ws* rats, we then investigated the suggested causal link between mast cell accumulation and interstitial fibrosis. Unexpectedly, interstitial fibrosis was markedly worse in these rats. Although mast cells were not completely absent in the diseased kidney of *Ws/Ws* rats, their number at 6 weeks in PAN-treated *Ws/Ws* rats was approximately one fifth of that in the PAN-treated control littermates. Because it was shown that the number of mast cells in the whole body of *Ws/Ws* rats is decreased by aging [23], we considered that the decrease in the number of interstitial mast cells from 2 to 6 weeks in the rats resulted from aging and is irrelevant to the development of interstitial fibrosis. The observed interstitial mast cells in the *Ws/Ws* rats may have resulted from the accumulation of the remaining small number of mast cells from other parts of the body [34]. In contrast, there were no differences between the two strains in the levels of interstitial T lymphocytes and myofibroblasts, two types of cell that could be affected by the *c-kit* gene mutation in *Ws/Ws* rats. Taken together, these results suggest that the reduction in the number of interstitial mast cells in *Ws/Ws* rats resulted in the exacerbation of interstitial fibrosis in this model.

Table 2. Interstitial cells in +/+ and *Ws/Ws* rats at 6 weeks

	Control		PAN nephrosis	
	+/+	*Ws/Ws*	+/+	*Ws/Ws*
ED-1 (monocytes/macrophages)[a]	83.8 ± 13.3	80.0 ± 10.6	570.0 ± 40.1	592.0 ± 46.3
W3/25 ($CD4^+$ cells)[a]	157.4 ± 14.4	165.8 ± 11.8	593.9 ± 42.4	580.0 ± 36.0
OX8 ($CD8^+$ cells)[a]	21.1 ± 10.3	16.6 ± 4.8	116.5 ± 12.7	112.8 ± 12.6
α-SMA (myofibroblast)[b]	1.2 ± 0.3	1.3 ± 0.3	11.6 ± 1.1	10.5 ± 3.3

α-SMA, alpha-smooth muscle actin.
[a]Data are the numbers of interstitial cells per mm^2 expressed as mean ± SD ($N = 5$).
[b]Data are % interstitial positive area expressed as mean ± SD ($N = 5$).

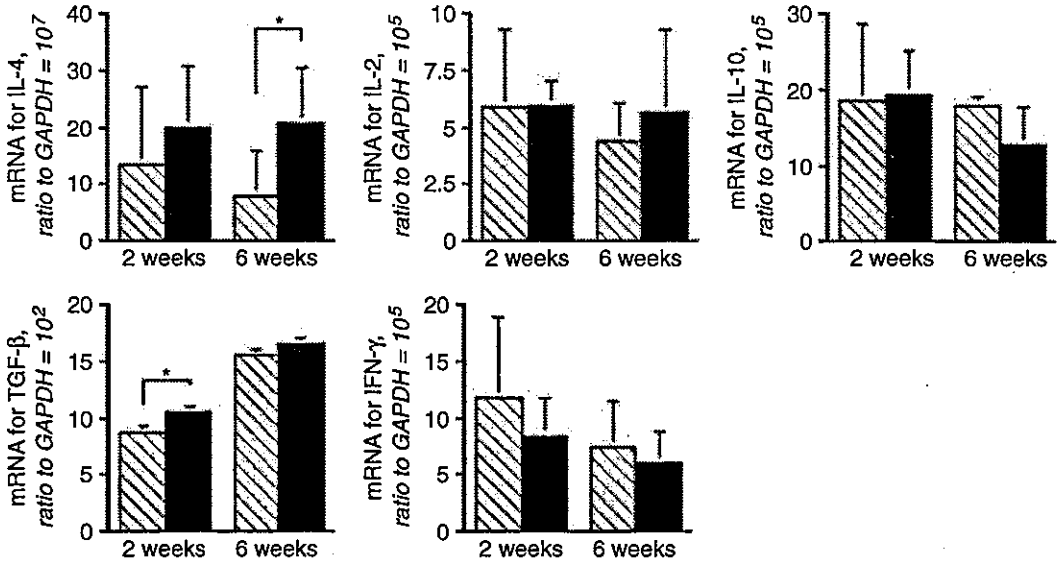


Fig. 8. Expression of cytokine mRNA levels in renal cortex of rats with puromycin aminonucleoside (PAN) nephrosis. Data are expressed as the ratio to the internal control. PAN-treated +/+ (▨); PAN-treated *Ws/Ws* rats (■). *$P < 0.05$.

It was demonstrated earlier that myofibroblast proliferation is paralleled by or precedes the development of interstitial fibrosis in human and animal models [35], but the result of the present study is not consistent with those previous studies. It could be possible that there is some unknown effect that originated from the mutated *c-kit* gene that changed the functions of myofibroblasts in *Ws/Ws* rats. In addition, we cannot exclude the possibility that the mutated *c-kit* gene causes other abnormalities than mast cell deficiency, although there have been no reports so far that show such defects in *Ws/Ws* rats except for anemia in their early life [23].

Our present study using mast cell–deficient *Ws/Ws* is the first one to our knowledge to reveal the role of mast cells in renal fibrosis. There have been some earlier studies in which the involvement of mast cells in fibrosis of lung [36, 37], liver [36, 38], and skin [39, 40] was examined by using mast cell–deficient W/W^v mice and/or *Ws/Ws* rats. Some of these studies showed no difference in the magnitude of fibrosis between the mast cell–deficient animals and their control +/+ littermates, which suggested that mast cells do not appear to be necessary for the induction of fibrosis. In the present study, our histologic and biochemical analysis revealed that the fibrosis was more severe in *Ws/Ws* rats than in +/+ littermates at 6 weeks after the induction of PAN nephrosis. These data are consistent with other studies on lung [36, 37] and liver [36] fibrosis, which showed that the increase in the hydroxyproline content of the tissues of deficient animals was greater than that in control +/+ littermates.

The mechanism by which greater renal interstitial fibrosis occurred in *Ws/Ws* rats with PAN nephrosis is unclear. We investigated the possibility that levels of cytokines that potentially enhance directly or indirectly the progression of fibrosis are changed in *Ws/Ws* rats with PAN nephrosis. The cytokines tested in this study included Th1 and Th2 cytokines, the balance of which is suggested to affect fibrotic changes [41]. Our results showed that mRNA levels of TGF-β and IL-4 were higher in *Ws/Ws* rats than in controls in the PAN nephrosis model,

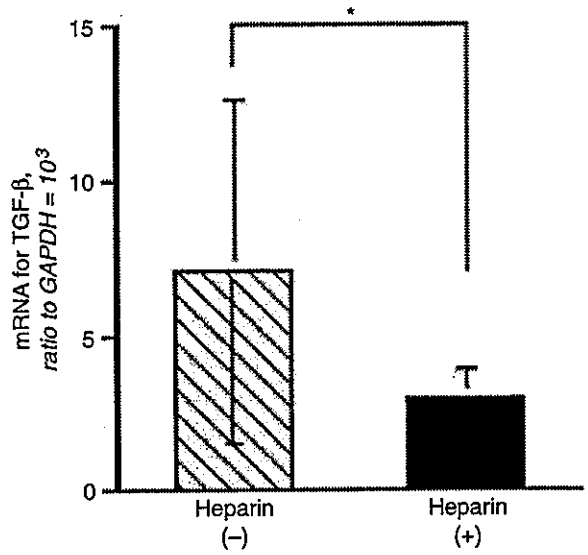


Fig. 9. Inhibition of expression of transforming growth factor (TGF)-β1 mRNA by heparin. Rat renal fibroblasts were cultured in the absence (▨) or presence (■) of heparin for 6 hours. Data are expressed as mean ± SD of 5 samples.

suggesting that the deficiency of mast cells (i.e., in *Ws/Ws* rats) may have caused an increase in their expression, resulting in more fibrosis in the *Ws/Ws* rats.

Mast cells contain a variety of mediators that have been shown to directly or indirectly affect the progression of fibrosis in tissues [42]. Although mast cells have been shown to produce TGF-β and IL-4 in culture [9], they also produce other cytokines and factors that may modulate cytokine production by other types of cells. Heparin, a major component stored in secretory granules of mast cells, was shown to have effects on cell proliferation [43] and cytokine production [33], both of which are involved in the pathogenesis of fibrosis. Heparin was also shown to inhibit DNA synthesis by human renal fibroblasts when added alone, although it enhanced DNA synthesis when added with tryptase, a proteolytic enzyme secreted by mast cells [44]. The present study demonstrated that heparin inhibited the gene expression of TGF-β, the most potent fibrogenic cytokine, in renal fibroblasts in culture. A recent study also demonstrated that heparin inhibited the production of TGF-β by cultured human proximal tubular epithelial cells, another source of the cytokine in diseased kidney [45]. Therefore, it is conceivable that heparin secreted from mast cells may inhibit TGF-β production by these cells in the diseased kidney and that this action may have contributed to the less fibrosis in the control +/+ littermates in the PAN nephrosis model. We also tested the effect of heparin on IL-4 production by these cells, but the production level was very low, and thus the effect of heparin could not be detected (our unpublished observation).

The results of the present study using mast cell–deficient animals seem to be in conflict with a suggested role of mast cells in renal diseases. The profibrogenic role of mast cells has been proposed mostly based on histologic observations of clinical samples and results of in vitro studies using cultured cells [14–20, 44]. However, the histologic studies only showed a correlation between the two phenomena, mast cell accumulation and fibrosis, and do not provide evidence of a causal link between them. In vitro studies, on the other hand, give data on the potential effects of mast cells or their secretory components on fibrosis, but do not provide information about the overall effect of mast cells. Even if whole components stored in mast cells or mast cells themselves are used in a culture system, one cannot provide all cells and extracellular circumstances of the kidney in the in vitro system. Further studies are needed to elucidate the role of mast cells in the mechanism of fibrosis, but we consider that experiments using animal models as well as intervention studies in humans will be necessary to assess the effect of mast cells as a whole on fibrosis.

In the kidney of the animal models of glomerular diseases we tested, mast cells were localized in a scattered distribution in the interstitium, whereas interstitial mononuclear cells (MNC), mostly consisting of monocytes/macrophages and T lymphocytes, were present in clusters. In human glomerular diseases on the other hand, mast cells were restricted to the area of interstitial fibrosis where only a few T lymphocytes and macrophages were concomitantly present, but were rarely found in the interstitial lesion where the influx of MNC was pronounced (our unpublished observations). This interstitial influx of MNC presumably precedes the interstitial fibrosis and is suggested to be involved in the mechanism of fibrotic changes [46]. Therefore, in consideration of these findings, together with the results of the present study, it is conceivable that mast cells are not involved in the development of fibrosis because of their absence in prefibrotic lesions in contrast to T lymphocytes or macrophages, but are attracted in fibrotic lesion and possibly participate in the attenuation or resolution of the fibrotic lesions. Unfortunately, in the rodent models it was rather difficult to discriminate prefibrotic lesions from established areas of fibrosis, probably because of the short duration and/or acute inflammation after the disease onset.

CONCLUSION

In the present study, we investigated the role of mast cells in the development of renal fibrosis by using mast cell–deficient rats. In human studies, mast cells are thought to be one of the cell types contributing to the interstitial fibrosis. However, it could be mentioned from our data that mast cells are not simply fibrogenic. Rather, they might have a potential to be protective or

ameliorative in this model and possibly in other models and also in humans.

ACKNOWLEDGMENTS

We are grateful to Dr. Takashi Oda, National Defense Medical College, for his advice concerning the hydroxyproline analysis. This work was supported in part by a Health Science Research Grant (Research on Specific Diseases) from the Ministry of Health and Welfare. Portions of this work were presented at the 2001 Annual Meeting of the American Society of Nephrology and reported in abstract form (*J Am Soc Nephrol* 12:711A, 2001).

Reprint requests to Dr. Yasuhiro Natori, Department of Clinical Pharmacology, Research Institute, International Medical Center of Japan, Toyama 1-21-1, Shinjuku-ku, Tokyo 162-8655, Japan.
E-mail: natoriya@ri.imcj.go.jp

REFERENCES

1. Pesci A, Bertorelli G, Gabrielli M: Mast cells in fibrotic lung disorders. *Chest* 103:989–996, 1993
2. Pesci A, Majori M, Piccili ML, *et al*: Mast cells in bronchiolitis obliterans organizing pneumonia: Mast cell hyperplasia and evidence for extracellular release of tryptase. *Chest* 110:383–391, 1996
3. Murata K, Okudaira M, Akashio K: Mast cells in human liver tissue. Increased mast cell number in relation to the components of connective tissue in the cirrhotic process. *Acta Derm Venereol* 73:157–165, 1973
4. Armbrust T, Batusic D, Ringe B, *et al*: Mast cell distribution in human liver disease and experimental rat liver fibrosis: Indications for mast cell participation in development of liver fibrosis. *J Hepatol* 26:1042–1054, 1997
5. Hawkins RA, Claman HN, Clark RAF, *et al*: Increased dermal mast cell populations in progressive systemic sclerosis: A link in chronic fibrosis? *Ann Intern Med* 102:182–186, 1985
6. Rothe MJ, Nowak M, Kerdel FA: The mast cell in health and disease. *J Am Acad Dermatol* 23:615–624, 1990
7. Craig SS, DeBlois G, Schwartz LB: Mast cells in human keloid, small intestine, and lung by an immunoperoxidase technique using a murine monoclonal antibody against tryptase. *Am J Pathol* 124:427–435, 1986
8. Schwartz LB: Mast cells: Function and contents. *Curr Opin Immunol* 6:91–97, 1994
9. Gordon JR, Burd PR, Galli SJ: Mast cells as a source of multifunctional cytokines. *Immunol Today* 11:458–464, 1990
10. Ruoss SJ, Hartmann T, Caughey GH: Mast cell tryptase is a mitogen for cultured fibroblasts. *J Clin Invest* 88:493–499, 1991
11. Gruber BL, Kew RR, Jelaska A, *et al*: Human mast cells activate fibroblasts: Tryptase is a fibrogenic factor stimulating collagen messenger ribonucleic acid synthesis and fibroblast chemotaxis. *J Immunol* 158:2310–2317, 1997
12. Gordon JR, Galli SJ: Promotion of mouse fibroblast collagen gene expression by mast cells stimulated via the Fc epsilon RI. Role for mast cell-derived transforming growth factor beta and tumor necrosis factor alpha. *J Exp Med* 180:2027–2037, 1994
13. Thompson HL, Burbelo PD, Gabriel G, *et al*: Murine mast cells synthesize basement membrane components. A potential role in early fibrosis. *J Clin Invest* 87:619–623, 1991
14. Ehara T, Shigematsu H: Contribution of mast cells to the tubulointerstitial lesions in IgA nephritis. *Kidney Int* 54:1675–1683, 1998
15. Hiromura K, Kurosawa M, Yano S, *et al*: Tubulointerstitial mast cell infiltration in glomerulonephritis. *Am J Kidney Dis* 32:593–599, 1998
16. Roberts IS, Brenchley PE: Mast cells: The forgotten cells of renal fibrosis. *J Clin Pathol* 53:858–862, 2000
17. Toth T, Toth-Jakatics R, Jimi S, *et al*: Mast cells in rapidly progressive glomerulonephritis. *J Am Soc Nephrol* 10:1498–1505, 1999
18. Ruger BM, Hasan Q, Greenhill NS, *et al*: Mast cells and type VIII collagen in human diabetic nephropathy. *Diabetologia* 39:1215–1222, 1996
19. Lajoie G, Nadasdy T, Laszik Z, *et al*: Mast cells in acute cellular rejection of human renal allografts. *Mod Pathol* 9:1118–1125, 1996
20. Yamada M, Ueda M, Naruko T, *et al*: Mast cell chymase expression and mast cell phenotypes in human rejected kidneys. *Kidney Int* 59:1374–1381, 2001
21. Risdon RA, Sloper JC, de Wardener HE: Relationship between renal function and histological changes found in renal-biopsy specimens from patients with persistent glomerular nephritis. *Lancet* 2:363–366, 1968
22. Cameron JS: Tubular and interstitial factors in the progression of glomerulonephritis. *Pediatr Nephrol* 6:292–303, 1992
23. Niwa Y, Kasugai T, Ohno K, *et al*: Anemia and mast cell depletion in mutant rats that are homozygous at "white spotting (Ws)" locus. *Blood* 78:1936–1941, 1991
24. Tsujimura T, Hirota S, Nomura S, *et al*: Characterization of *Ws* mutant allele of rats: A 12-base deletion in tyrosine kinase domain of *c-kit* gene. *Blood* 78:1942–1946, 1991
25. Onoue H, Maeyama K, Nomura S, *et al*: Absence of immature mast cells in the skin of Ws/Ws rats with a small deletion at tyrosine kinase domain of the c-kit gene. *Am J Pathol* 142:1001–1007, 1993
26. Jones CL, Buch S, Post M, *et al*: Pathogenesis of interstitial fibrosis in chronic purine aminonucleoside nephrosis. *Kidney Int* 40:1020–1031, 1991
27. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
28. Ou ZL, Natori Y, Doi N, *et al*: Competitive reverse transcription-polymerase chain reaction for determination of rat CC and C chemokine mRNAs. *Anal Biochem* 261:227–229, 1998
29. Kivirikko KI, Laitinen O, Prockop DJ: Modifications of a specific assay for hydroxyproline in urine. *Anal Biochem* 19:249–255, 1967
30. Gibson S, Miller HR: Mast cell subsets in the rat distinguished immunohistochemically by their content of serine proteinases. *Immunology* 58:101–104, 1986
31. Gibson S, Mackeller A, Newlands GF, *et al*: Phenotypic expression of mast cell granule proteinases. Distribution of mast cell proteinases I and II in the rat digestive system. *Immunology* 62:621–627, 1987
32. Powell DW, Mifflin RC, Valentich JD, *et al*: Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 277:C1–9, 1999
33. Weigert C, Brodbeck K, Haring HU, *et al*: Low-molecular-weight heparin prevents high glucose- and phorbol ester-induced TGF-beta 1 gene activation. *Kidney Int* 60:935–943, 2001
34. Tei H, Kasugai T, Tsujimura T, *et al*: Characterization of cultured mast cells derived from *Ws/Ws* mast cell-deficient rats with a small deletion at tyrosine kinase domain of *c-kit*. *Blood* 83:916–925, 1994
35. Remuzzi G, Bertani T: Pathophysiology of progressive nephropathies. *N Engl J Med* 339:1448–1456, 1998
36. Okazaki T, Hirota S, Xu ZD, *et al*: Increase of mast cells in the liver and lung may be associated with but not a cause of fibrosis: Demonstration using mast cell-deficient Ws/Ws rats. *Lab Invest* 78:1431–1438, 1998
37. Mori H, Kawad K, Zhang P, *et al*: Bleomycin-induced pulmonary fibrosis in genetically mast cell-deficient WBB6F1-W/Wv mice and mechanism of the suppressive effect of tranilast, an antiallergic drug inhibiting mediator release from mast cells, on fibrosis. *Int Arch Allergy Appl Immunol* 95:195–201, 1991
38. Sughara A, Tsujimura T, Fujita Y, *et al*: Evaluation of role of mast cells in the development of liver fibrosis using mast cell-deficient rats and mice. *J Hepatol* 30:859–867, 1999
39. Everett ET, Pablos JL, Harley RA, *et al*: The role of mast cells in the development of skin fibrosis in tight-skin mutant mice. *Comp Biochem Physiol A Physiol* 110:159–165, 1995
40. Yamamoto T, Takahashi Y, Takagawa S, *et al*: Animal model of sclerotic skin. II. Bleomycin induced scleroderma in genetically mast cell deficient WBB6F1-W/Wv mice. *J Rheumatol* 26:2628–2634, 1999
41. Lukacs NW, Hogaboam C, Chensue SW, *et al*: Type 1/type 2 cytokine paradigm and the progression of pulmonary fibrosis. *Chest* 120:5S–8S, 2001
42. Metcalfe DD, Baram D, Mekori YA: Mast cells. *Physiol Rev* 77:1033–1079, 1997

43. Del Vecchio PJ, Bizios R, Holleran LA, *et al*: Inhibition of human scleral fibroblast proliferation with heparin. *Invest Ophthalmol Vis Sci* 29:1272–1276, 1988
44. Kondo S, Kagami S, Kido H, *et al*: Role of mast cell tryptase in renal interstitial fibrosis. *J Am Soc Nephrol* 12:1668–1676, 2001
45. Yard BA, Chorianopoulos E, Herr D, *et al*: Regulation of endothelin-1 and transforming growth factor-beta1 production in cultured proximal tubular cells by albumin and heparan sulphate glycosaminoglycans. *Nephrol Dial Transplant* 16:1769–1775, 2001
46. Muller GA, Markovic-Lipkovski J, Frank J, *et al*: The role of interstitial cells in the progression of renal diseases. *J Am Soc Nephrol* 2:S198–S205, 1992

Extracellular Matrix Glycoprotein Biglycan Enhances Vascular Smooth Muscle Cell Proliferation and Migration

Ryoko Shimizu-Hirota, Hiroyuki Sasamura, Mari Kuroda, Emi Kobayashi, Matsuhiko Hayashi, Takao Saruta

Abstract—Proteoglycans are produced and secreted by vascular smooth muscle cells, but the pathophysiological role of these glycoproteins in the vasculature is an enigma. Because the small leucine-rich proteoglycan (SLRP) biglycan is overexpressed in arteriosclerotic lesions, we produced mice constitutively overexpressing biglycan in the vascular smooth muscle, in order to examine the effects on vascular pathology. In the aorta and renal vasculature, increased vascular proliferation was seen both in the basal state and after infusion of angiotensin II (Ang II) in the transgenic mice compared with wild-type controls. In addition, the combination of biglycan overexpression and Ang II infusion resulted in marked increases in vascular smooth muscle cell proliferation and migration in the coronary arteries, as well as increases in fibrosis surrounding the vessels. In vitro, biglycan caused an increase in thymidine incorporation and migration of vascular smooth muscle cells, whereas these parameters were unchanged or reduced in endothelial cells. Moreover, addition of biglycan resulted in an increase in cdk2 expression and decrease in p27 levels in the vascular smooth muscle cells. These results suggest that this extracellular matrix SLRP may be involved in the regulation of vascular smooth muscle growth and migration through cdk2- and p27-dependent pathways. Furthermore, changes in biglycan expression could be a factor influencing the susceptibility of arteries to vascular injury, and may play a direct role in the pathogenesis of vascular lesions. (*Circ Res.* 2004;94:1067-1074.)

Key Words: proteoglycan ■ vascular injury ■ angiotensin

The cells in the vascular wall are held together by a complex network of macromolecules that collectively form the extracellular matrix. The species of glycoproteins known as proteoglycans are an important component of this matrix. These glycoproteins consist of a core protein covalently bound to one or more glycosaminoglycan (GAG) side chains.[1] Based on the composition of the GAG moiety, the proteoglycans may be classified into chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate proteoglycans. In the vascular wall, a major proteoglycan synthesized by endothelial cells and vascular smooth muscle cells is the proteoglycan biglycan, which consists of a core protein bound to two chondroitin sulfate/dermatan sulfate side chains.

Biglycan is a member of the small leucine-rich proteoglycan (SLRP) family of proteoglycans, which are characterized by the presence of repeated sequences containing a high proportion of leucine residues.[2] Recent studies have suggested that these SLRPs are not simply an inert structural component of the extracellular matrix, but may be actively involved in the control of collagen deposition, and the activation and inactivation of cytokines and growth factors.[1,3]

Studies from our and other laboratories have shown that biglycan expression is markedly altered in disease states, and that therapeutic intervention with hormones or antihypertensive agents can alter biglycan expression in vivo and in vitro.[4–6] Of importance, biglycan expression in the blood vessel wall has been shown to be increased in atherosclerotic lesions and restenotic lesions both in animal models[7,8] and in human samples.[9,10]

At present, the functions of biglycan in the blood vessel wall are still unclear. In this study, we used a vascular smooth muscle cell–specific promoter to amplify biglycan expression in the vasculature, and examined the susceptibility of these vessels to vascular injury mediated by angiotensin II infusion. We also examined if biglycan can affect growth and migratory properties in vascular smooth muscle cells in vitro.

Materials and Methods

Production of Transgenic Mice Overexpressing Biglycan in the Vasculature

A transgenic construct was produced by ligating the human smooth muscle α-actin promoter[11] (pBS-HSMA-EA4.7, generously provided by Dr Miwa, Osaka University, Osaka, Japan) upstream of the human biglycan cDNA[2] (P16, generously provided by Dr Fisher, National Institute of Dental and Craniofacial Research, Bethesda, Md). Purified DNA was microinjected into fertilized ova of C56BL/6J mice using standard techniques. Transgenic mice were identified by PCR and Southern blotting, and further experiments

Original received August 20, 2003; resubmission received February 6, 2004; revised resubmission received March 5, 2004; accepted March 9, 2004.
From the Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan.
Correspondence to Hiroyuki Sasamura, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail sasamura@sc.itc.keio.ac.jp

Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000126049.79800.CA

were performed on two independent transgenic lines. All animal experiments were performed in accordance with institutional guidelines.

Analysis of Transgene Expression by RT-PCR-RFLP and Immunohistochemistry

Total RNA was purified from aorta, kidney, heart, lung, testis, and skeletal muscle by the acid guanidine-phenol-chloroform method, and quantified by absorbance at 260 nm in a spectrophotometer. Semiquantitative RT-PCR was performed using protocols described by us previously.[5,6] The biglycan primers used corresponded to an area of complete homology in the nucleotide sequences of human and mouse biglycan cDNA and spanned several introns in the genomic sequence. Moreover, the amplified cDNA products could be distinguished by the digestion with the restriction enzyme *SacI* because the human cDNA contains an internal *SacI* site, resulting in two DNA fragments (228 bp and 177 bp) after digestion of amplified DNA derived from transgenic transcription products, whereas the native biglycan transcript yielded one fragment of size 405 bp. Thus, comparison of the abundance of the DNA of these sizes allows an estimate of the relative levels of mRNA expression derived from the native biglycan gene and transgenic biglycan cDNA, respectively. RT-PCR of transforming growth factor-β_1 (TGF-β_1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as described.[5,12]

Immunohistochemistry of biglycan expression in the aorta was performed using an anti-human biglycan antibody (LF-51)[2] kindly provided by Dr Fisher (National Institute of Dental and Craniofacial Research, Bethesda, Md). Deparaffinized samples were pretreated with chondroitinase ABC for 1 hour, then subjected to immunohistochemistry as described later.

Animal Treatments and Assays

Eight-week-old male heterozygous transgenic mice were infused with Ang II (1 μg/kg/min) or vehicle (saline) for 2 weeks using osmotic minipumps (Alzet). Wild-type littermates were used as controls. Blood pressures were measured by tail-cuff plethysmography. Mice were euthanized by ether anesthesia and tissue samples of aorta, heart, and kidneys were fixed by rapid immersion in 4% paraformaldehyde (PFA), before embedding in paraffin. Preliminary experiments were performed to compare the results seen in mice perfusion-fixed at blood pressure before euthanasia versus mice that had not been perfusion-fixed. These experiments confirmed that similar differences between the groups were obtained with or without perfusion-fixation at blood pressure. Aortic sections were stained with Azan, whereas heart and kidney sections were stained with the Masson trichrome stain. Plasma renin activity was measured by standard techniques.[5]

Measurement of Collagen Content

Cardiac collagen content was assessed by estimation of hydroxyproline content. Cardiac sections were hydrolyzed by treatment with 6 mol/L HCl at 115°C for 24 hours, then hydroxyproline content in the lyophilizes samples was estimated by reaction with chloramine T and p-dimethylaminobenzaldehyde.[13]

Morphometric and Immunohistochemical Analyses

Media thickness and lumen diameters in the vasculature were measured using NIH image computer software. The lumen diameter was calculated from the lumen circumference, assuming that the vessel is circular, and the media/lumen ratio calculated by dividing the media thickness by the lumen diameter. For the assessment of perivascular fibrosis, the area of fibrosis (collagen deposition stained with aniline blue) immediately surrounding the coronary arterial wall was measured, and the fibrotic index was determined as the area of fibrosis divided by total vessel area. To estimate the incidence of coronary stenosis/occlusion, the three main coronary arteries were examined, and the heart was defined to have a coronary stenotic lesion if at least one neointimal lesion occupying >30% of the lumen area was seen. Immunohistochemical analyses were performed on PFA-fixed paraffin-embedded 5-um sections by the streptavidin-biotin-peroxidase method. Briefly, after deparaffination and quenching of endogenous peroxidase with 0.3% hydrogen peroxidase in methanol for 30 minutes, serial sections were incubated with 2% normal goat serum to reduce nonspecific background staining, then incubated with polyclonal antibodies directed against proliferating cell nuclear antigen (PCNA), α-smooth muscle actin, CD68, or CD31 at a dilution of 1:100. Negative control experiments were performed by replacing the primary antibodies with normal rabbit serum. Subsequently, biotinylated secondary antibody and then streptavidin conjugate were applied. Positive staining was visualized with either DAB or tetra-methyl-benzidine using a commercially available kit (Toyobo).

In Vitro Studies

In vitro studies were performed on rat VSMCs[4] and bovine aortic endothelial cells. Assessment of cell proliferation, collagen synthesis, cell migration, and Western blot analysis of cell cycle proteins was performed based on previously described protocols,[14,15] which are presented in the expanded Materials and Methods section of the online data supplement (available at http://circres.ahajournals.org).

Statistical Analyses

Results are expressed as the mean±SEM. Statistical comparisons were made by ANOVA followed by Fisher's post hoc test. Growth curves were compared using repeated measures ANOVA. Values of $P<0.05$ were considered statistically significant.

Results

Expression of Human Biglycan in Biglycan Transgenic Mice

The transgene construct contained the mouse α-smooth muscle actin promoter ligated to the coding sequence of human biglycan cDNA. This promoter effects a pattern of transgene expression similar to that of the endogenous α-smooth muscle actin, which is the dominant actin isoform in vascular tissue. Six founder mice containing the human biglycan transgene were identified by PCR and Southern blot analysis, and two lines were propagated and used for subsequent experiments. Figure 1 shows the results of a representative experiment to examine the expression of biglycan in the aorta of transgenic mice and their nontransgenic littermates. The presence of the biglycan transgene DNA in tail genomic DNA was assessed using PCR primers spanning intron 2, which yielded a 263-bp PCR product in the case of the intronless transgene, and 550-bp PCR product in the case of the native biglycan gene. Expression of biglycan mRNA in the aorta was assessed by RT-PCR. We have shown that RT-PCR performed in the linear phase of the amplification reaction enables a comparison of total biglycan mRNA expression between different animals.[5] Moreover, because the primers correspond to identical sequences of the human and mouse biglycan cDNA sequences, and only human biglycan cDNA sequence contains a *SacI* site, relative levels of transgene and native biglycan mRNA expression in the same animal could also be directly assessed by comparison of the amplified bands after the addition of a *SacI* digestion step after RT-PCR. These methods suggested that biglycan mRNA was increased (≈2- to 4-fold) in the transgenic mice aorta. In contrast, biglycan mRNA from the transgene was below detectable levels in other tissues such as kidney, heart, lung, testis, and skeletal muscle (Figure 1C). To further confirm overexpression of human biglycan in the vasculature,