Interstitial Mast Cell Accumulation in AA-type Renal Amyloidosis

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Abstract: Renal interstitial fibrosis is the final common pathway leading to end-stage renal disease in various nephropathies including renal amyloidosis. However, the role of mast cells (MC) in the fibrotic process of renal amyloidosis is still not fully understood. We compared the distribution of MC in renal biopsies from 20 patients with AA type renal amyloidosis and 20 control cases. The immunoreactivity of renal MC to anti–tryptase and anti–chymase was studied. Positively stained cells were counted, and the relative interstitial and fractional areas of anti–α-smooth muscle cell actin (SMA) stained cells were measured. Anti–CD29 mAb was used to detect β1 integrin and anti–basic fibroblast growth factor (bFGF) mAb for the growth factor on MC. Samples showing amyloid deposition contained numerous interstitial tryptase–positive (MC_T) compared to control samples [43.99±6.8 vs. 7.14±1.3/mm²]. A significant relationship was seen between interstitial MCT and creatinine clearance [r=0.759] and the interstitial amyloid area [r=0.853]. A significant relationship was also observed between the MC number and the fractional area of α–SMA positive interstitium [r=0.822] and interstitial fibrotic area [r=0.81]. Double immunostaining demonstrated the intracytoplasmic presence of β1 integrin in 87% of MC_T and correlated significantly with the interstitial amyloid area (r=0.863). Basic FGF was also detected in 85.5% of MC_TC which thus closely correlated with the interstitial α–SMA–area (r=0.781). Our results indicate that MC may play a crucial role in interstitial fibrosis in renal amyloidosis.

Key words: mast cell–renal amyloid–fibrosis–integrin–basic fibroblast growth factor

Introduction

Tubulointerstitial (TI) lesions including interstitial fibrosis are considered to be poor prognostic markers of various renal diseases including renal amyloidosis.1) Changes in the area of interstitial fibrosis correlate better than the amount of amyloid deposition with renal function.1) Despite numerous studies, the exact pathogenic factors involved in TI damage remain unclear. In particular, the role of mast cells (MC) in renal TI lesions has not yet been fully analyzed. MC are present in various renal lesions including diabetes nephropathy2) renal vasculitis,3) IgA nephritis,4) amyloidosis,5) rapidly progressive glomerulonephritis (RPGN)6) and acute cellular rejection of renal allografts.7) There is general agreement that MC increase in number in chronic diseases and different renal lesions.2(5–7)
Immunohistochemical studies have demonstrated the presence of two MC phenotypes. The MC_T phenotype (containing only tryptase) seems to play a role in host defense, whereas the MC_TC phenotype (containing both tryptase and chymase) appears to be a "non-immune system-related" cell involved in fibrosis, wound healing, and sclerosis. The pathophysiological changes induced by MC are in part due to such MC proteases as tryptase or chymase because these serine proteases are involved in the extracellular matrix metabolism. On the other hand, activated MC synthesize, store and release several bioactive mediators mitogenic for fibroblasts and are also known to induce collagen synthesis, and are involved in the extracellular matrix metabolism.

MC hyperplasia and/or accumulation is a common phenomenon in various diseases associated with either high level of serum amyloid A (SAA) and C-reactive protein or SAA-fragments (AA) deposition in tissue. It has been demonstrated that soluble recombinant SAA (rSAA) regulates inflammatory cell migration and SAA-like proteins bind to extracellular matrix (ECM) components and induce T-lymphocyte adhesion. In vitro studies by Herskoviz et al. demonstrated MC adhere to the immobilized ECM–SAA complex through an integrin recognition.

The aim of the present study was to demonstrate (1) the presence of MC in the interstitial renal tissue with amyloidosis A, (2) β1 integrin-expression on MC located around amyloid deposition, and (3) determine the relationship between MC and the interstitial fibrotic process.

Materials and methods

**Tissue samples.** Kidney tissue samples were obtained from 20 randomly selected adult (>16 years old) patients (8 males and 12 females, mean age: 52.2±3.1 years) with amyloid A nephropathy at the time of renal biopsy. Only samples containing at least 10 glomeruli on paraffin blocks were included in this study. All kidney samples showed amyloid deposition in various amounts and different locations by light, immunofluorescence and electron microscopy. The presence of amyloid was also confirmed in all kidney samples by a positive apple–green birefringence under polarized light in Congo red–stained preparations. Control samples consisted of 20 randomly selected renal biopsy samples from adult patients (9 males and 11 females, mean age: 56.2±3.7 years) with thin membranes disease but a normal extraglomerular renal structure, and free of immune deposition, glomerulosclerosis, and inflammation. Clinical and laboratory data were also documented at the time of renal biopsy. The study protocol was approved by the Human Ethics Review Committee of Fukuoka University and a signed consent form was obtained.

**Tissue preparation.** Tissue sections were stained with hematoxylin and eosin, Masson trichrome, periodic acid–Schiff and periodic acid–methenamine–silver stain. To identify mast cells, the sections were stained by toluidin blue. Amyloid was demonstrated by Congo red staining including pretreatment with permanganate and polarizing light as well as by immunohistochemistry.

**Electron Microscopy (EM).** A proportion of the biopsy sample was fixed in 1.4% phosphate buffered glutaraldehyde, postfixed in OsO4 and embedded in Epon resin. Ultra-thin sections were cut and stained by uranyl acetate and lead citrate. All samples were examined by electron microscopy.

**Immunohistochemistry.** The staining method used in the present study for MC has been described previously. Anti–human tryptase and chymase monoclonal antibodies were purchased from Chemicon (Temecula, CA). Anti–CD68, anti–CD20, anti–CD45 and anti–α–smooth muscle actin (α–SMA) mAbs were purchased from Dako (Glostrup, Denmark). Deparaffinized serial sections (3-μm–thick) were washed in 0.05 M Tris–HCl buffer containing 0.145 M NaCl, pH 7.5 (TBS), followed by pretreatment with protease for sec-
tions stained later for chymase or CD68, or autoclave pretreatment (121°C for 10 min) for sections stained for α-smooth muscle actin (α-SMA). Sections were initially treated with 1% skim milk (Difco, USA), and incubated for 1 hr at 20°C with the primary antibody (anti-chymase antibody, 50 μg/ml, anti-tryptase antibody, 30 μg/ml), each dissolved in 3% bovine serum albumin. As a negative control, we used vehicle alone or non-immunized animal immunoglobulin. After incubation, APAAP method was adopted, and the sections were finally stained with 0.01% new Fuchsin (Merk, Germany), 0.01% NaNO₃, 10 μg naphthol AS–BI phosphate (Sigma, St. Louis, MO), 0.1 ml of N, N-dimethyl formamide (Wako, Osaka, Japan) in 40 ml of 0.2 M Tris–HCl buffer (pH 8.2). Tissue for positive control consisted of biopsy samples from the small intestine for chymase and tryptase and renal vasculature for α–SMA.

**Double immunostaining**

**Double immunofluorescence.** After treating deparaffinized sections with 0.005% protease (DAKO, Osaka, Japan) for 10 min, they were incubated with the first mouse antibody against human chymase (Chemicon) for 60 min at 20°C. After washing with TBS, sections were incubated with TRITC (rhodamine)–conjugated second rabbit–antibody against mouse immunoglobulin (Dako) for 60 min at 20°C. Next, the sections were treated with 1% skim milk for 30 min to minimize the background level. The sections were again incubated with the first rabbit antibody against human tryptase (BioPur, Switzerland) for 60 min at 20°C. After washing with TBS, sections were incubated with FITC–conjugated swine–antibody against rabbit immunoglobulin (Dako) for 60 min at 20°C. After incubation, the nuclei were stained with 4’6-diamidino-2-phenylindole (Sigma). Sections were then mounted and examined under a fluorescence microscope (Nikon, Japan). This method was used for the following double staining: anti–tryptase and/or chymase was combined with (1) anti–amyloid–A component (Dako), or (2) basic fibroblast growth factor (Santa Cruz Biotech, Santa Cruz, CA), or anti–CD29 (Serotec, UK) antibodies.

**Double immunohistochemistry.** This method was used to detect the following molecules on the same section: tryptase or chymase with α–SMA, and tryptase or chymase with amyloid A protein. The method was similar to that described above, with the exception of staining MC–proteases by DAB while α–SMA and amyloid A were stained by new–Fuchsin.

**Morphometric analysis.** Several indexes were used to describe the severity of the pathological process based on previously published reports.⁶ (1) Relative interstitial area: A standard point counting method was used to quantitate the relative interstitial area (Aint) of the renal cortex. In this method, sections stained by the PAM method were examined under a high magnification (×400) using a 121–point (100 squares) eyepiece micrometer of 1 mm². A total of 10 consecutive nonoverlapping cortical fields (area, 0.625 mm²) were analyzed in each section of the biopsy. The points overlying the tubular basement membrane and interstitial space were counted while those falling on either Bowman’s capsule or peritubular capillaries were ignored. The points falling on glomerular structures or on larger vessels were excluded from the total count. The results were expressed using the following formula: Aint= [(number of grid intersections on the cortical interstitium/total number of grid intersections)×100]. (2) Interstitial immunoperoxidase staining for α–SMA was quantitated by the same point counting method described above. The fractional area was also calculated using the above formula. (3) The fractional area stained by Masson–trichrome was quantitated by the same point counting method described above and the results were expressed as a fractional area using the above formula. (4) The number of interstitial lymphocytes (CD20, CD45), macrophages (CD68, Ki–1), CD29 or bFGF positive chymase and tryptase positive MC present in the cortical interstitial area were counted under a high magnification (×
400) in 10 adjacent nonoverlapping cortical fields (total area, 0.625 mm² per biopsy specimen). Only cells with clearly identifiable nuclei were counted. Finally, the number of counted cells was expressed as cells per unit area (mm²).

Statistical analysis. All data were expressed as the mean ± SEM. Differences between groups were examined for statistical significance using Student’s t-test and the one way analysis of variance (ANOVA). Associations of categorical variables were examined by the χ² test. The correlation between variables was assessed by a linear regression analysis. A p value of < 5% denoted the presence of a statistically significant difference.

Results

In all renal samples the immunohistologic analysis showed rounded, elongated or polygonal cells and each contained a central nucleus and abundant cytoplasm containing numerous tryptase- or chymase-positive granules or both, thus identifying these cells to be MC. A representative tissue sample containing MC was shown in Fig. 1 and 2. Chymase was not observed in any cells lacking tryptase. MC were localized in the peritubular, perivascular region in close proximity to amyloid-A deposits. MC degranulation was often observed at these sites. These MC were usually not only degranulated but depleted of granules. An electronmicroscopic analysis showed typical MC embedded into the interstitial extracellular matrix, and these cells were found usually in tight relation to the accumulation of the amyloid fibrils (Fig. 3a, b). Furthermore, intraglomerular MC were never found in our tissue samples, although a few intratubular MC were identified. MC were mostly located on the periphery rather than within areas of lymphocytic accumulation. Furthermore, MC were observed in α-SMA positive interstitial area, independent of the presence of amyloid deposits.

The density of both phenotypes of MC was significantly higher in renal amyloid group than in the control (MC_T: 43.99±6.8 vs. 7.14±1.3/mm², MC_TC: 23.2±3.65 vs. 1.9 ±0.86/mm²). Furthermore, MC constituted the third most abundant interstitial inflammatory cell population in renal amyloidosis.

Relationship between MC phenotype and pathological findings. We also analyzed the relationship between the MC phenotype and pathological findings. There was a significant relationship between the glomerular amyloidosis index (GAI) and the number of MC_T (r=0.763, p<0.001). Furthermore, there was also a significant relationship between the number of interstitial T lymphocytes and MC_T (r=0.742, p<0.001) as well as MC_TC (r=0.712, p<0.001). However, no relationship was seen between the density of B cells and MC_T (r=0.21, p>0.05). There was a significant relationship between the number of interstitial CD68+ macrophages and MC_T (r=0.401, p<0.001) and between the relative volume of the interstitium and number of MC_T (r=0.836, p<0.001). Furthermore, a significant relationship was seen between the interstitial amyloid area and number of MC_T (r=0.853, p<0.001) as well as T-cell number (r=0.563, p<0.01). We also found a close relationship between the fractional area of α-SMA (FA-SMA) and number of MC (MC_T: r=0.822 [Fig. 5] and MC_TC: r=0.824, p<0.001). In addition, there was a significant relationship between the fractional area of fibrosis (FAfib) and MC_T (r=0.81, p<0.001) as well as MC_TC (r=0.69, p<0.002).

Double immunostaining showed immunostaining of β1 integrin by anti–CD29–mAb both on the surface and cytoplasm of MC. On average, 87% (38.3±6.4 mm²) of renal MC_T showed β1 staining (Fig. 6). This staining was not dependent on the presence of amyloid deposition. Other cell constituents of the renal tissue, including tubular epithelial cells, vascular endothelial cells and the majority of glomerular cells were also variable reactive for anti–CD29 mAb. A good correlation was demonstrated between the number of CD29+MC_T and T-cell number (r=0.682, p<0.002), FAα–SMA (r=
**Figure 1** A photomicrograph showing the interstitial accumulation of mast cells (arrow) stained with anti-tryptase (DAB) around amyloid deposits (star) stained with anti amyloid-A (new–Fuchsin). (×200).

**Figure 2** A photomicrograph showing intraglomerular and interstitial amyloid deposition [star] (anti amyloid-A, new–Fuchs). Note the accumulation of mast cells [arrow] stained with anti-tryptase (DAB) exclusively around the interstitial amyloid deposits. (×200).
Using the double immunostaining method, bFGF was identified in some interstitial MC [Fig. 8], and was located in the cytoplasm of 50.7% (22.3±1.2 mm²) of interstitial tryp-tase-positive cells and 78.8% (18.2±2.0 mm²) of MCTC cells, independent of the presence or absence of amyloid deposition.

There was a significant relationship between the number of bFGF+MCTC and interstitial fibrotic area (r=0.704, p<0.001) as well as α-SMA+interstitial area (r=0.781, p<0.001, [Fig. 9]) and between bFGF+MCTC and interstitial amyloid positive area (r=0.74, p<0.001).

Clinicopathological correlates. At the time
of biopsy, 18 patients had nephrotic syndrome and 14 patients had elevated serum creatinine concentration (>2.0 mg/dl). The number of MC was not influenced by age or gender, but correlated significantly with creatinine clearance (r=0.76, p<0.001), and the serum creatinine level (r=0.826, p<0.001 [Fig. 10]). Furthermore, there was a significant correlation between the degree of 24-hr proteinuria and number of MC_T (r=0.477, p <0.001). A significantly higher number of MC_T were detected in patients with hypertension (>165/95 mmHg, n=6, 68±9.3 vs. 14 normotensive patients: 34±3.5 cells/mm²).
Figure 2 Renal interstitial mast cells stained with anti-chymase (rhodamine-labeled, a) containing intracytoplasmic bFGF protein (FITC-labeled, b). Note the control bFGF staining (b) on the other interstitial cellular structures (x). (Double immunostaining, ×400)

Figure 7 Correlation between the number of both chymase and CD29 positive interstitial mast cells (MC\textsubscript{TC}) and interstitial amyloid area.
Human MC play a pivotal role in the pathogenesis of acute allergic reactions, but they have also been observed to accumulate at sites of chronic inflammation and/or fibrosis in various disorders thus suggesting that MC are also involved in fibrosis. Very little is known about MC accumulation in amyloidosis, particularly in renal amyloidosis. MC accumulation has been demonstrated in the islets of Langerhans in amyloidosis of the pancreas. Pavone–Macaluso counted numerous MC in the renal parenchyma of three biopsies from patients with amyloidosis associated with pyelonephritis. In contrast, Bohle et al. found only a few MC in 11 biopsies from patients with AA type renal amyloidosis but did not discuss their importance or role in renal lesions. To our knowledge, our study is the first to provide a systematic analysis of the presence of MC in AA–type renal amyloidosis.

We found a significantly high MC density in the renal interstitium of 30 patients with AA–type renal amyloidosis. Both subtypes of MC were found in random distribution, mostly localized close to the interstitial and vascular amyloid deposits. Our analysis showed a significant correlation between the area of interstitial amyloid and the number of MC. MC were also found in close proximity to the glomeruli, which contained amyloid deposits and amyloid filled the Bowman's capsule, but these cells never appeared inside the glomerular capsule. In addition, MC were also found in the non–amyloid interstitial area, although in fewer numbers.

The exact pathomechanism of MC involvement in renal amyloidosis is unclear. However, it is well known that in certain inflammatory diseases including AA amyloidosis, MC hyperplasia has also been observed with increased local and systemic levels of acute phase reactants such as SAA. In this regard, previous studies have shown that SAA and other SAA–like proteins adhere to ECM components, such as laminin (LN) and vitronectin (VN), but not fibronectin (FN) and collagen II. Fig. 2 shows a co–localization of amyloid fibrils and renal interstitial matrix containing collagen fibers. The binding site is located within the amino acid sequence of the AA molecule, the latter also contains cell adhesive epitopes within close proximity (both YIGST and RGD–like).

The affinity of MC to bind to ECM and amyloid proteins has been documented by Herskoviz et al. who demonstrated that human rSAA specifically binds to MC, suggesting the possible presence of SAA receptor on MC. The ECM bound immobilized

**Discussion**

**Figure 9** The relationship between the number of both chymase and bFGF positive interstitial mast cells (MCTC) and the fractional α–SMA positive interstitial area.

**Figure 10** The correlation between the number of typtase positive interstitial mast cells (MCTC) and the serum creatinine–concentration.
SAA also promotes MC accumulation at inflammatory sites, which may in turn modulate the pathological process. The cell–matrix interaction appears to be crucial in the traffic, localization and function of migratory cells within the tissue. Various cell–cell, cell–matrix adhesion molecules have been identified to play important roles in various biological processes. Integrins seem to be the most essential mediator of matrix–cell interaction. Among these, the expression of \( \beta_1 \) integrin (VLA–3, 4, 5) has been demonstrated in human MC, whose adhesion molecule mediates MC adhesion to FN and LN. We believe that ECM-associated SAA or tissue bound–AA also induce MC adhesion through the activation of \( \beta_1 \) integrins. In in vitro studies, Herskoviz et al. demonstrated MC adhesion to the immobilized ECM–SAA complex through specific recognition sites in integrin. Our present work using a double immunostaining method documented for the first time that renal interstitial tryptase and/or chymase–positive MC were also positive for \( \beta_1 \) integrin. These cells were diffusely distributed in the interstitium, mostly close to the amyloid deposits, but some also appeared in areas free of amyloid deposits.

What is the pathologic role of MC in amyloid renal tissues? First, it seems that MC form an integral part of the inflammatory process or reaction of the deposited amyloid in the renal tissue. MC are found at sites of inflammation and wound healing surrounded by other inflammatory cells. In this study, we found a prominent T–cell inflammation that correlated significantly with the volume of interstitial amyloid deposits. In this regard, Bohle et al. also demonstrated a marked increase in number of T cell in amyloidosis kidney compared to normal. Our results also showed that the number MC correlated with amyloid deposition, as well as with the number of T–cells \((r=0.824, p<0.001)\). On the other hand, MC were located in close proximity to other inflammatory cells. This observation is similar to that described by our laboratory in biopsy samples of rapidly progressive glomerulonephritis (RPGN). SAA in its immobilized form induces the adhesion of T–cells and resting MC to subendothelial ECM. We recently demonstrated a significant relationship between the number of MC and T lymphocytes in RPGN. Previous studies have also shown that MC maturation and activation is regulated by various products (interleukin–3, and 4) of T lymphocytes while MC derivatives such as histamine, tryptase, chymase, TNF–\( \alpha \), and interleukins enhance inflammatory cell transmigration, maturation and cytokine-release. A further analysis is necessary to clarify the relationship between these two cell types in inflammatory processes.

Second, there is sufficient evidence to indicate that the presence of MC enhances tissue fibrosis. A high MC density has been demonstrated at wound sites and fibrotic areas in various organs. Furthermore, previous studies have shown a significant relationship between the number of MC and interstitial fibrosis in renal allografts, IgA nephritis, and RPGN. We also herein showed a good correlation between the number of interstitial MC and both the relative interstitial area as well as areas positive for \( \alpha \)–SMA. The latter parameter represents the area of activated interstitial fibroblasts (myofibroblasts), which are important cellular components responsible for interstitial ECM production and accumulation.

In our cases, all MC present in the interstitium were positive for intracytoplasmic bFGF, which is not only a potent angiogenic but also a strong mitogenic polypeptide. The close relationship between interstitial myofibroblasts and MC is well documented. MC products exhibit proliferating activity for fibroblasts, inducing phenotype modulation, which is manifested in \( \alpha \)–SMA expression and regulation of collagen synthesis. In addition, MC proteases (tryptase and chymase) are known to exert a direct metabolic action on ECM, which may modify the extent of fibrotic changes in the amyloid kidney. Interstitial fibrosis in the amyloidosis kidney is well documented and is considered to be a more significant poor prognostic factor than the amount of amyloid deposition. In this regard,
Bohle et al.\(^1\) reported that long–term prognosis of renal amyloidosis is worse when interstitial fibrosis is present at the time of biopsy.

The exact role of MC in the pathomechanism of interstitial fibrosis is not fully understood. It is possible that the intratubular proteinous, amyloid containing casts\(^4\) rupture the tubular basement membrane\(^6\) or absorption of different protein fragments (i.e., amyloid) by proximal tubular epithelial cells from the glomerular primary urine, to become antigen presenting cells.\(^6\) This may trigger an interstitial cellular immune response, T cell and/or MC trafficking, proliferation and activation and finally directly or indirectly induce interstitial fibroblast activation, as discussed earlier.\(^1\)\(^4\)\(^7\) MC–derived chymase is a highly specific and potent angiotensin–II forming enzyme.\(^4\)\(^8\) It is possible that such chymase may locally accumulate in the kidney and thus participate in the generation of renal fibrosis and dysfunction. Ang–II is a profibrotic molecule which, like other MC–products, activates interstitial fibroblasts and stimulates ECM production.\(^4\)\(^9\)\(^50\) In addition to these mechanisms, it is also possible that the SAA molecule serves as a “connector”, in which the RGD motif serves as the ligand for MC integrins, and that another site, distinct from the RGD site, binds to the ECM, thereby providing MC and other inflammatory and resident renal cell cross-talk.

In summary, we have demonstrated in this study that in renal amyloidosis, MC accumulation is an integral part of interstitial damage and may be one of the factors responsible for the inflammatory and fibrotic processes. Our study is the first to demonstrate the interstitial presence of β1 integrins on MC as a mediator of cell–cell and cell–ECM adhesion. The presence of bFGF suggests that these cells may play a crucial role in the activation of interstitial fibroblast, myofibroblast phenotype modulation and finally fibrosis.

References


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