Mast cell infiltration and chemokine expression in progressive renal disease

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Background. Mast cells are growth factor–rich, bone marrow–derived cells that infiltrate injured tissue where they have been implicated in the pathogenesis of progressive fibrosis.

Methods. Mast cell infiltration and the expression of related chemoattractants was examined following 5/6 nephrectomy, a model of progressive, nonimmune-mediated renal injury. In addition, expression of the profibrotic cytokine, transforming growth factor-β (TGF-β) within mast cells and the effects of renoprotective therapy with angiotensin-converting enzyme (ACE) inhibition were also determined.

Results. Renal injury was accompanied by mast cell infiltration, in close proximity to areas of tubulointerstitial fibrosis. Mast cells displayed toluidine blue metachromasia and were immunopositive for TGF-β1 as well as chymase and tryptase. The expression of several mast cell chemokines, including stem cell factor, interleukin-8 (IL-8), and also TGF-β1, were increased in 5/6 nephrectomized kidneys. ACE inhibition with ramipril led to a reduction in renal injury in association with attenuation of mast cell infiltration and chemokine expression.

Conclusion. Mast cell infiltration and related chemokine expression are prominent and early features following renal mass reduction and may contribute pathogenetically to progressive renal injury.

Activation and phenotypic changes to resident renal cells, such as myofibroblasts, contribute to the pathogenesis of progressive kidney disease [1]. Studies conducted over the past decade have also indicated a role for infiltrating inflammatory cells, principally macrophages in renal disease progression [2, 3]. However, other cellular components of the immune system, including mast cells, have additionally been implicated in the pathogenesis of nonimmune renal disease [4].

While mast cells are traditionally known for their role in allergic immunoglobulin E (IgE)-mediated reactions, there is also a nonimmune-related mast cell phenotype that predominates in connective tissue, rather than at mucosal surfaces [5]. These nonimmune mast cells participate in cell migration, differentiation, and, in particular, the synthesis of extracellular matrix and the formation of fibrotic scar tissue [5]. Indeed, mast cell infiltration has not only been documented in a wide range of human renal diseases [6–11], but a close relationship with the extent of interstitial fibrosis and impaired renal function has also been reported [8].

Mast cells, derived from hematopoietic progenitors, leave the bone marrow and migrate to areas of inflammation. A number of factors responsible for this directional migration and tissue maturation of mast cells have been identified [12]. These include the CXC family of chemokines [13], stem cell factor (also known as kit-ligand, steel factor, and mast cell growth factor) [14, 15], and transforming growth factor-β (TGF-β) [16].

The present study first sought to examine the development of mast cell infiltration and the expression of related chemoattractants following subtotal nephrectomy, a model of progressive, nonimmune mediated renal injury. This study also sought to determine whether the profibrotic cytokine, TGF-β, found in mast cells at other sites, was also present in kidney mast cells. In addition, the study also sought to determine the effects of renoprotective therapy with angiotensin-converting enzyme (ACE) inhibition on mast cell numbers and chemokine expression.

METHODS

To determine the time course of mast cell infiltration and chemoattractant expression, 40 male Sprague-Dawley rats weighing 200 to 250 g were randomized to five groups of 10 animals each. Ten animals were assigned to undergo sham surgery and the remaining 30 underwent
subtotal nephrectomy. Among the latter, rats were assigned for sacrifice at 4 weeks and 12 weeks after surgery. Animals studied for 12 weeks were further assigned to treatment with or without the ACE inhibitor, ramipril (3 mg/L drinking water). Anesthesia was achieved by the intraperitoneal administration of pentobarbital (6 mg/100 g body weight) (Boehringer Ingelheim, Artarmon, NSW, Australia). The control group underwent sham surgery consisting of laparotomy and manipulation of both kidneys before wound closure. The other 30 rats all underwent subtotal nephrectomy performed by right subcapsular nephrectomy and infarction of approximately two thirds of the left kidney by selective ligation of two of three to four extrarenal branches of the left renal artery [17]. Rats were housed in a temperature (22°C)-controlled room with ad libitum access to commercial standard rat chow (Norco Co-Operative Ltd., Lismore, NSW, Australia) and water during the entire study. At sacrifice, the remnant (left) kidney was then sliced sagitally and one half immersion-fixed in 10% neutral buffered formalin and embedded in paraffin for histological and immunohistochemical studies. The remaining half was snap-frozen in liquid nitrogen and stored at –80°C for subsequent gene expression analyses. All experiments adhered to the guidelines of the Animal Welfare and Ethics Committee of St. Vincent’s Hospital and the National Health and Medical Research Foundation of Australia.

Renal function

Body weight was measured weekly. Plasma urea and creatinine were measured by autoanalyzer (Beckman Instrumentals, Palo Alto, CA, USA) at the beginning and end of the study. Glomerular filtration rate (GFR) was measured prior to sacrifice by a single shot of 99m-Tc-dihydrotetracyantranilic acid (Te99m, DTPA) clearance [18]. Systolic blood pressure was measured in conscious rats using an occlusive tail-cuff plethysmograph attached to a pneumatic pulse transducer (Narco Bio-system, Inc., Houston, TX, USA) [19]. Before sacrifice, rats were housed in metabolic cages for 24 hours for subsequent measurement of urinary protein excretion using the Coomassie brilliant blue method.

Histochemistry and immunohistochemistry

Four micron sections were cut and examined after either histochemical or immunohistochemical staining. Histochemical staining comprised the use of either hematoxylin and eosin, Masson’s trichrome, and periodic-acid Schiff (PAS) stains to examine extracellular matrix. Mast cells were identified in serial sections stained with toluidine blue, chymase, tryptase, and TGF-β. Macrophages were identified by immunostaining with a monoclonal antibody specific for the monocyte/macrophage antigen, ED-1 (Serotec, London, UK). Toluidine blue staining was performed by immersion of sections in 0.1% toluidine blue (Sigma Chemical Co., St. Louis, MO, USA) for 1 minute at room temperature with mast cells, identified by their characteristic metachromasia [20]. Mast cells and macrophages were quantified in 25 randomly selected, nonoverlapping fields and expressed as the number of mast cells/mm². Immunostaining for chymase, tryptase, and TGF-β1 was also performed using a monoclonal antichymase (Chemicon, Temecula, CA, USA), monoclonal antitryptase (Chemicon) and polyclonal rabbit antihuman TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Stem cell factor (SCF-1) was localized using a polyclonal rabbit antirabbit antibody (Santa Cruz Biotechnology). Immunohistochemistry was performed, as previously described [21] with visualization using the indirect avidin-biotin complex (ABC) method.

Renal structure

Changes in kidney structure were assessed in a masked protocol in at least 25 randomly selected tissue sections from each group studied. Sections were stained with either Mayer’s hematoxylin and eosin to examine cell structure, PAS, or Masson’s modified trichrome to demonstrate collagen matrix [22].

Glomerulosclerosis. In 4 μm kidney sections stained with PAS, 50 to 80 glomeruli from rats were examined. The degree of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4 as previously described [23]: grade 0, normal; grade 1, sclerotic area up to 25% (minimal); grade 2, sclerotic area 25% to 50% (moderate); grade 3, sclerotic area 50% to 75% (moderate to severe); and grade 4, sclerotic area 75% to 100% (severe). A glomerulosclerotic index (GSI) was then calculated using the formula:

\[
GSI = \sum_{i=0}^{4} Fi (i)
\]

where Fi is the % of glomeruli in the rat with a given score (i) [18].

Tubulointerstitial fibrosis. The accumulation of matrix within the tubulointerstitium was estimated on Masson’s trichrome–stained sections using computer-assisted image analysis, as previously reported [24, 25]. Briefly, five random nonoverlapping fields from each rat were captured and digitized using a BX50 microscope attached to a Fujix HC5000 digital camera (Fuji, Tokyo, Japan). Digital images were then loaded onto a Pentium III IBM computer (Gateway, Los Angeles, CA). An area of blue on a trichrome-stained section was selected for its color range and the proportional area of tissue with this range of color was then quantified. Calculation of the proportional area stained blue (matrix) was then determined using image analysis (AIS, Analytical Imaging Station Version 6.0, Toronto, Ontario, Canada).
**RNA extraction and cDNA synthesis**

Frozen kidney tissue, stored at −80°C, was homogenized (Polytron, Kinematica Gmbh, Littau, Switzerland) and total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY, USA). The purified RNA was dissolved in sterile water and quantified spectrophotometrically (OD260). RNA quality was verified on a 1% denaturing agarose gel. Four micrograms of total RNA was treated with RQ1 DNase (1 U/μL) (Promega, Madison, WI, USA) to remove genomic DNA. The DNase treated RNA was reverse transcribed with avian myelomatosis virus (AMV) reaction buffer, 0.5 μL of 10 mmol/L desoxynucleoside triphosphate (dNTP) (Roche Diagnostics, Mannheim, Germany), 2.5 μL of 10 mmol/L diethyl pyrocarbonate (DEPC) water was added. Tubescriptase (25 U/μL) (Roche Diagnostics) and 4.5 μL of diethyl pyrocarbonate (DEPC) water was added. Tubes were incubated at 37°C for 60 minutes, after which cDNA was stored at −20°C for future use.

**Quantitative real-time polymerase chain reaction**

Using rat specific sequence primers (Table 1), the gene expression of three mast cell chemokines, interleukin-8 (IL-8)/gro-α, SCF-1, and TGF-β1, were quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR), as previously described [26]. A commercial, predeveloped 18S control kit with [TAMRA] on the 3’ end (PE Biosystems, Foster City, CA, USA) was used as the housekeeping gene to control for inequalities of loading. Primers and probes for target genes were obtained from PE Biosystems. Both primers included a fluorescence reporter (6-carboxyfluorescein [FAM]) at the 5’-end and a fluorescent quencher (6-carboxytetra-methylrhodamine [TAMRA]) at the 3’-end. For the relative quantification of the target gene and the endogenous reference 18S ribosomal RNA (18S), real-time quantitative RT-PCR was performed using an GeneAmp 5700 Sequence Detector (PE Biosystems) according to the manufacturer’s instructions. The derived normalized values were the averages of four runs. Results were expressed as the ratio of IL-8/gro-α, TGF-β1, or SCF mRNA to 18S relative to sham kidney, which were arbitrarily assigned a value of 1.

**Statistics**

All data are shown as mean ± SEM unless otherwise specified. Data were analyzed by analysis of variance (ANOVA) using the StatView IV program (Brainpower, Calabasas, CA, USA) on a Macintosh G4. Comparisons between group means were performed by Fisher’s least significant difference method. A P value of less than 0.05 was considered statistically significant.

**RESULTS**

**Clinical parameters**

Subtotal nephrectomy led to the development of progressive renal scarring with associated hypertension, proteinuria and impaired glomerular filtration rate (GFR). All of these changes were attenuated with ramipril treatment (Table 2).

**Mast cells and macrophages**

Only an occasional mast cell was identified in the kidneys of sham-operated rats. In contrast, significant infiltration of toluidine blue–positive mast cells were noted in the tubulointerstitium of rats that had undergone subtotal nephrectomy (Fig. 1). In particular, mast cells were noted in areas of tubular dilatation and interstitial fibrosis, but not noted within the glomerular tuft. The extent of mast cell infiltration was greater at 12 weeks than at 4 weeks and was significantly reduced in kidneys from ramipril-treated rats. In serial sections, toluidine blue–stained mast cells also showed positive immunostaining for chymase, tryptase, and TGF-β1 (Fig. 2). Mast cells were mostly found in areas that also included abundant ED-1–labeled macrophages (Fig. 3). At 12 weeks, the ratio of macrophages to mast cells was 15:1 (macrophages 404 ± 11/mm², mast cells 26 ± 6/mm²; P < 0.01).

**Mast cell chemokines**

Expression of IL-8/gro-α was increased at 4 weeks and to a greater extent at 12 weeks after subtotal nephrectomy (Fig. 4). TGF-β mRNA followed a similar chronology. In contrast, SCF was not elevated at 4 weeks but only at 12 weeks after renal mass reduction. The over-expression of all three mast cell chemokines, IL-8/gro-α, TGF-β, and SCF, was attenuated by ramipril (Fig. 4). SCF expression was mostly undetectable in sham-operated rats but was present in tubules of rats that had undergone 5/6 nephrectomy (Fig. 5).

**Table 1. Primers and probes for rat interleukin-8 (IL-8)/gro-α, stem cell factor (SCF) and transforming growth factor-β (TGF-β1)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8/gro-α</td>
<td>AGAACATCCAGAATTTGGAAGGTGTAG</td>
<td>GTCGGCTAGTACTCGTGTTCG</td>
<td>CCGCAGAACCACACTGCA</td>
</tr>
<tr>
<td>SCF</td>
<td>CAGTTAATAGGAAAGCCGCAAGTCG</td>
<td>GCCGGCAGTGCACATTG</td>
<td>CTGAAAGCCGGCCCTACGACTGAC</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>AGAAGTCACCCGCGTGCCTA</td>
<td>TGTGTATGIGTCTTGGTGGTTCGTA</td>
<td>TGTTGACGCCAACAAGCAAT</td>
</tr>
</tbody>
</table>

**Example of a table showing primers and probes for specific gene expression.**
Table 2. Clinical parameters at end of study

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>4 weeks</th>
<th>12 weeks</th>
<th>12 weeks + angiotensin-converting enzyme inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Body weight g</td>
<td>491 ± 13</td>
<td>324 ± 9</td>
<td>436 ± 19</td>
<td>401 ± 23</td>
</tr>
<tr>
<td>Systolic blood pressure mm Hg</td>
<td>115 ± 3</td>
<td>176 ± 8a</td>
<td>180 ± 15a</td>
<td>121 ± 8b</td>
</tr>
<tr>
<td>Glomerular filtration rate mL/min/L</td>
<td>4.0 ± 0.2</td>
<td>0.6 ± 0.3a</td>
<td>0.7 ± 0.2a</td>
<td>1.7 ± 0.1b</td>
</tr>
<tr>
<td>Urinary protein g/day</td>
<td>13 ± 2</td>
<td>26 ± 16a</td>
<td>31 ± 86a</td>
<td>23 ± 6a</td>
</tr>
<tr>
<td>Glomerulosclerotic index</td>
<td>0.3 ± 0.0</td>
<td>2.0 ± 0.3a</td>
<td>3.1 ± 4a</td>
<td>1.5 ± 0.4a</td>
</tr>
<tr>
<td>Tubulointerstitial fibrosis % area</td>
<td>0.0 ± 0.1</td>
<td>2.0 ± 0.6a</td>
<td>3.6 ± 1.0a</td>
<td>1.4 ± 0.4a</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM.

*P < 0.01 vs. sham; †P < 0.01 vs. 12 weeks

**DISCUSSION**

The pathogenesis of progressive renal injury following an initial insult remains incompletely understood. While the role of macrophages and their chemotactic factors in kidney disease has been widely examined [2, 27], other bone marrow–derived cells have not been extensively studied. The present study identifies a number of novel findings in relation to the pathogenesis of renal injury that follows renal mass reduction, a nonimmune model of progressive renal disease [28]. First, mast cell infiltration was a prominent and early feature following renal injury. Second, mast cells in the injured kidney were found to contain TGF-β, as well as chymase and tryptase. Third, we found that a range of mast cell chemo- kines were elevated in the setting of renal injury, with early induction of TGF-β and IL-8/gro-α, while SCF was expressed later in the course of disease. Finally, ACE inhibitor treatment was associated with attenuation in chemokine expression and mast cell infiltration.

In addition to their well-established role in allergic disease, mast cells have also been repeatedly implicated in the pathogenesis of fibrotic scarring [29]. For instance, mast cells are particularly abundant at the site of active lesions in scleroderma [30], a chronic disorder characterised by progressive skin and extradermal fibrosis affecting multiple tissue sites, including the kidney. Mast cell infiltration has also been documented in a range of kidney diseases where their presence in the tubulointerstitial correlates closely with collagen–synthesizing myofibroblasts [31]. In particular, mast cells have been noted to be most abundant in diabetic nephropathy [11], IgA nephropathy [7], and in rapidly progressive glomerulonephritis, where mast cells numbers correlates closely with both tubulointerstitial pathology and declining GFR [8].

Mast cells secrete a range of factors that may contribute to renal injury, including endothelin [21], growth factors, and the proteolytic enzymes implicated in the pathogenesis of renal disease. For instance, the major mast cell enzyme, tryptase, promotes renal fibroblast proliferation and collagen synthesis, particularly in combination with heparin, another mast cell secretory product [10]. Chymase, in addition to its potential role in angiotensin II generation, also converts the latent, biologically inactive form of TGF-β into an active form recognized by its type II receptor [32]. TGF-β is a potent profibrotic growth factor that is widely implicated in the pathogenesis of progressive renal disease [33]. While TGF-β containing mast cells have been previously reported [32, 34], the present study is the first to identify them within the kidney. Indeed, the presence of both TGF-β and chymase within mast cell granules, as demonstrated in this study, suggests that mast cell degranulation will be followed by the liberation and subsequent activation of TGF-β.

Identification of TGF-β containing mast cell infiltration suggests a role in renal fibrosis. Other TGF-β containing bone marrow–derived cells, such as macrophages, were also identified in the present study as even more abundant. However, the enormous secretory, storage, and synthetic capacity of the mast cell [35] suggests that...
it may contribute disproportionately to the quantity of TGF-β in the local milieu.

The profibrotic contents of the mast cell suggest that it has an active, pathogenetic role in renal fibrosis, rather than that of a bystander [12]. In the present study, mast cells were closely associated with areas of tubulointerstitial fibrosis and tubular atrophy. While not specifically tested in the kidney, the active role of the mast cell in the process of organ injury has recently been definitively shown in a mouse model of heart failure [36]. Using the W/Wv mouse, Hara et al [36] demonstrated a significant reduction in both cardiac fibrosis and ventricular dysfunction when these mast cell–deficient animals were subjected to aortic banding.

Several factors have been identified as mast cell chemotactants. These include chemokines such as IL-8, growth factors like TGF-β, and the ligand for the mast cell c-kit receptor, SCF [14–16]. Chemokines are a family of cytokines responsible for the chemotaxis of inflammatory cells. It consists of four subfamilies that are classified on the basis of their constituent cysteine residues as C, CC, CXC, and C(X3)C chemokines [37]. Recent studies have indicated that of this array, only a subtype of the CXC chemokines, those with a conserved glutamate-
leucine-arginine (ELR) motif are chemotactic for mast cells [13]. In the present study, IL-8/gro-α was dramatically up-regulated early in the course of renal disease and also increased in the later disease stages, commensurate with both progressive injury and mast cell infiltration.

In the present study, early up-regulation of TGF-β was also noted. While TGF-β in the kidney is mostly known for its role in tissue fibrosis, its other actions include it being a potent chemoattractant for mast cells [16]. Thus, the finding in the present study that TGF-β is expressed in renal mast cells raises the potential for a vicious cycle, whereby TGF-β produced by mast cells leads to TGF-β–dependent mast cell chemotaxis, more TGF-β, and an exuberant fibrotic response in neighboring tissues.

In contrast to most other bone marrow–derived cells, mast cells are extruded as immature precursor cells that differentiate and mature at the site of their migration [38]. The cellular and soluble components that lead to mast cell differentiation and maturation are not well understood, although SCF acting through its cognate receptor, c-kit, is thought to play a pivotal role in a range of aspects of mast cell differentiation and function [14]. Consistent with findings in chronic glomerulonephritis [9], the present study also documented increased SCF in association with renal injury and localized its expression to areas of tubulointerstitial injury, as previously reported in human biopsies [9]. In addition to its chemotactic actions, SCF, through its interaction with c-kit, is also a potent mediator of mast cell degranulation [39].
The finding in the present study of the late overexpression of SCF suggests that this factor may have a role in the perpetuation of mast cell–mediated renal injury rather than the initiation of the pathologic process.

In the present study, ACE inhibition not only attenuated the structural and functional injury in subtotal nephrectomized rats, but also reduced mast cell infiltration and chemokine expression. In particular, IL-8/gro-α, dramatically overexpressed following subtotal nephrectomy, was normalized by ACE inhibition. Together, the findings of the present study indicate that mast cell infiltration and related chemokine expression are prominent and early features following renal mass that may contribute pathogenetically to progressive renal injury.

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