p38 Mitogen-Activated Protein Kinase Activation and Cell Localization in Human Glomerulonephritis: Correlation with Renal Injury

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Abstract. Activation of the p38 mitogen-activated protein kinase (MAPK) signal transduction pathway plays an important role in the inflammatory response. It was postulated that p38 MAPK is important in the pathogenesis of human glomerulonephritis and contributes to the development of renal injury. p38 MAPK activation was examined by immunodetection for dual phosphorylated p38 (p-p38) in normal human kidney and 77 renal biopsy specimens encompassing a wide spectrum of glomerulonephritides. In normal kidney, p-p38 immunostaining was restricted to the nuclei of a small number of podocytes, parietal epithelial cells, and tubular cells. There was a dramatic increase in the number of p-p38–positive cells in glomeruli and tubules in nonproliferative and proliferative glomerulonephritis and a substantial increase in the number of interstitial p-p38–positive cells in proliferative glomerulonephritis. Double immunostaining identified p38 activation in intrinsic renal cells (podocytes and endothelial and tubular cells), infiltrating macrophage and neutrophils, and myofibroblasts. Renal failure correlated with the number of p-p38–positive glomerular, tubular, and interstitial cells. Proteinuria correlated with the number of p-p38–positive tubular and interstitial cells and the number of p-p38–positive podocytes in nonproliferative glomerulonephritis. Furthermore, glomerular p38 activation correlated with segmental proliferative and necrotic lesions, and interstitial p38 activation correlated with the degree of interstitial inflammation. In conclusion, activation of p38 MAPK in intrinsic renal cells and infiltrating leukocytes correlated with renal dysfunction and histopathology, suggesting an important pathogenic role for p38 MAPK activation in human glomerulonephritis.

Infiltration of inflammatory cells into the kidney has been identified in various forms of human glomerulonephritis. Macrophage and T-cell accumulation within the diseased kidney correlates with renal dysfunction and pathologic damage, suggesting that renal inflammation plays an important role in disease pathogenesis (1, 2). In addition, cytokines and chemokines produced by both leukocytes and intrinsic renal cells mediate leukocyte accumulation in glomerulonephritis (3–5).

Activation of p38 mitogen-activated protein kinase (MAPK), an intracellular transduction pathway, results in the production of proinflammatory mediators. Multiple stimuli, such as ischemic, osmotic, and hypoxic stress, and cytokines such as IL-1 and TNF-α result in a sequential phosphorylation of upstream kinases, such as MAPK kinase 3 and 6 within the cell cytoplasm, leading to phosphorylation and activation of p38 kinase. Activation of p38 results in its translocation into the nucleus and activation of a variety of transcription factors, including those essential for the production of proinflammatory mediators, such as chemokines and cytokines (6–13).

In vitro studies have shown that p38 activation is important for the proinflammatory functions of different leukocyte populations (7, 14–16), and stress stimuli result in p38 MAPK activation in cultured intrinsic renal cells (17, 18). Furthermore, p38 activation has been demonstrated in animal models of nonrenal inflammation and injury (19–22). We have recently demonstrated the importance of p38 MAPK activation in a rat model of acute glomerulonephritis. With the use of a specific inhibitor of p38, renal injury was attenuated by abrogation of glomerular P-selectin expression and a reduction in neutrophil and platelet accumulation (23). Using a different p38 inhibitor, Wada et al. (24) demonstrated a reduction in the number of glomerular crescents in rat crescentic glomerulonephritis, with a concomitant reduction in glomerular macrophage accumulation and a reduction in renal injury in a mouse model of lupus nephritis (25). Despite these in vitro and animal studies, the significance of p38 MAPK activation in human glomerulonephritis remains to be determined.

The aim of this study, therefore, was to investigate the potential role of p38 MAPK activation in the pathogenesis of human glomerulonephritis. We examined p38 activation by immunohistochemistry staining of renal biopsies from a broad...
cross-section of glomerulonephritides. We localized p38 MAPK pathway activation to cell types in human glomerulonephritis and correlated p38 MAPK activation with clinical parameters of renal function and histologic injury.

Materials and Methods

Patients

The Human Ethics Committee of Monash Medical Centre granted permission for the use of human renal biopsy tissue for experimental purposes. Renal biopsies were performed for diagnostic purposes in accordance with best clinical practice, and informed consent for the use of renal biopsy tissue, in excess of that required for diagnostic purposes, was obtained from the patients. Renal biopsies from 85 patients were analyzed. The classification of human renal diseases and clinical parameters are given in Table 1. In addition, biopsies performed for minimal proteinuria (<0.25 g/d) or hematuria (<25 × 10⁷/L urinary glomerular red cells) and in which no renal abnormality was detected were reported as normal and classified as normal human kidney for the purposes of this study.

Antibodies

The following mouse mAb were used in this study: antiphosphorylated p38 (p-p38; Sigma-Aldrich, St. Louis, MO; #M1877), raised against the p38 phosphorylated peptide and recognizing all of the phosphorylated p38 isoforms; anti-p38α (anti-SAPK2α; Upstate, New York, NY; #05-454), recognizing the nonphosphorylated and phosphorylated p38α isoform; KP1, recognizing human macrophages (Serotec, Oxford, UK); UCHL1, recognizing human T cells (Serotec); anti-human neutrophil cathepsin G, recognizing human neutrophils (Becton Dickinson; #554248); anti-CD31, recognizing human endothelium (Dako, Glostrup, Denmark); IA4 and anti-α-smooth muscle actin (α-SMA; Sigma-Aldrich), recognizing human myofibroblasts; and PHM5, recognizing human podocytes (26). Horseradish peroxidase and alkaline phosphatase-conjugated goat anti-mouse IgG and peroxidase-conjugated mouse anti-peroxidase complexes (PAP) were purchased from Dako. The phosphorylated p38 peptide was provided by Cell Signaling Technology (Beverly, MA). Transplant nephrectomy tissue was received from a patient with severe vascular rejection by Cell Signaling Technology (Beverly, MA). Transplant nephrectomy was performed for minimal proteinuria (0.25 g/d) or hematuria (<25 × 10⁷/L urinary glomerular red cells) and in which no renal abnormality was detected. As a control, membranes were stripped using 1 mg/ml mouse anti-p38 antibody in 5% BSA in wash buffer overnight at 4°C. Blots were washed three times and incubated with horseradish peroxidase–conjugated goat anti-mouse IgG (1:10,000 in 10% normal rat serum, 1% normal sheep serum, and 1% FCS) for 2 h at room temperature. Membranes were washed three times, and the membrane-bound antibody detected was incubated with Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and captured on x-ray film. As a control, membranes were stripped using x1 stripping buffer (Chemicon International, Temecula, CA), blocked with 20 ml of blocking buffer for 4 h, then probed with mouse anti-p38α as above.

Immunohistochemistry

Renal biopsies were fixed in 4% formalin for 4 h and washed in 70% ethanol and embedded in paraffin. Three-microgram sections were cut and analyzed for routine diagnostic purposes. Two-color immunohistochemical staining on 3-μm sections was performed as described previously (27). Paraffin sections of formalin-fixed tissues were dewaxed in histosol, rehydrated, and microwave oven heated in 0.1 M sodium citrate for 10 min. The sections were then washed in PBS and blocked with 10% sheep serum and 10% FCS for 30 min and incubated with anti–p-p38 (5 μg/ml) in 10% normal human serum overnight at 4°C. Washed endogenous peroxidase inactivated in 1% H₂O₂ in methanol for 20 min, incubated with horseradish peroxidase–conjugated goat anti-mouse IgG followed by mouse PAP, and developed with 3,3-diaminobenzidine to produce a brown color. Omission

Table 1. Classification and clinical parameters of patients with glomerulonephritis

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of Patients</th>
<th>Age (Years)</th>
<th>Gender (% Male)</th>
<th>Serum Creatinine (μmol/L)</th>
<th>Creatinine Clearance (ml/min)</th>
<th>Proteinuria (g/d)</th>
<th>Hematuria (×10⁴/ml)</th>
<th>ESR</th>
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<tr>
<td>Normal</td>
<td>8</td>
<td>47 ± 7.8</td>
<td>15</td>
<td>77 ± 24.6</td>
<td>114 ± 21</td>
<td>0.27 ± 0.3</td>
<td>53 ± 76</td>
<td>15</td>
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<tr>
<td>TMD</td>
<td>8</td>
<td>36 ± 15.2</td>
<td>66</td>
<td>74 ± 29.8</td>
<td>96 ± 27</td>
<td>0.25 ± 0.1</td>
<td>150 ± 295</td>
<td>11</td>
</tr>
<tr>
<td>MCD</td>
<td>8</td>
<td>39 ± 32.8</td>
<td>86</td>
<td>109 ± 154.4</td>
<td>97 ± 67</td>
<td>9.5 ± 9.1c</td>
<td>30 ± 36</td>
<td>51</td>
</tr>
<tr>
<td>MGN</td>
<td>10</td>
<td>55 ± 18.4</td>
<td>45</td>
<td>90 ± 13.2</td>
<td>97 ± 24</td>
<td>3.7 ± 1.4b</td>
<td>180 ± 312</td>
<td>34</td>
</tr>
<tr>
<td>1°FSGS</td>
<td>9</td>
<td>44 ± 22</td>
<td>25</td>
<td>362 ± 591</td>
<td>65 ± 14a</td>
<td>6.2 ± 3.6b</td>
<td>59 ± 81</td>
<td>32</td>
</tr>
<tr>
<td>IgA</td>
<td>14</td>
<td>44 ± 14.9</td>
<td>51</td>
<td>102 ± 31.1</td>
<td>106 ± 31</td>
<td>1.1 ± 0.8</td>
<td>197 ± 257</td>
<td>54</td>
</tr>
<tr>
<td>Pi GN</td>
<td>10</td>
<td>41 ± 18.6</td>
<td>71</td>
<td>212 ± 107.3ᵃ</td>
<td>61 ± 15ᵃ</td>
<td>1.2 ± 1.3</td>
<td>106 ± 135</td>
<td>75</td>
</tr>
<tr>
<td>SLE III/IV</td>
<td>10</td>
<td>37 ± 13.1</td>
<td>50</td>
<td>129 ± 77.1ᵃ</td>
<td>80 ± 29</td>
<td>3.8 ± 2.8ᵃ</td>
<td>657 ± 450ᵇ</td>
<td>46</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>8</td>
<td>60 ± 15.5</td>
<td>39</td>
<td>548 ± 386.4ᵃ</td>
<td>12 ± 17ᵇ</td>
<td>2.0 ± 1.4</td>
<td>169 ± 152ᵇ</td>
<td>82</td>
</tr>
</tbody>
</table>

ᵃ ESR, erythrocyte sedimentation rate; TMD, thin-membrane disease; MCD, minimal-change disease; MGN, membranous glomerulonephritis; ³° FSGS, primary focal and segmental glomerulonephritis; IgA, IgA nephropathy; Pi GN, postinfectious glomerulonephritis; SLE III/IV, systemic lupus erythematosus WHO class III/IV; Vasculitis pauci-immune segmental necrotizing crescentic glomerulonephritis. Data are presented as the mean ± SD. bP < 0.05, cP < 0.01, dP < 0.001 compared with normal.
of microwave oven treatment of the tissue results in only a weak signal for p-p38 staining. When double labeling, sections were given a second treatment of microwave oven heating, blocked with 10% normal sheep serum and 10% FCS and incubated with anti-podocalyxin (PHM5, 1:600), anti-human macrophage (KP-1, 1 μg/ml), anti-T cell (5 μg/ml), anti-human neutrophil cathepsin G (20 μg/ml), anti-human endothelial cell (CD31, 5 μg/ml), or anti-α-SMA (1A4, 1:2000) overnight at 4°C in 10% normal human serum and washed, peroxidase inactivated in 1% H2O2 in methanol for 20 min, and incubated with horseradish peroxidase–conjugated goat anti-mouse IgG followed by mouse PAP and developed with Vector SG (Vector Laboratories, Burlingame, CA) to produce a gray color. All 77 biopsies were immunostained for p-p38. Double staining for p-p38 and podocalyxin was performed in all cases of normal, thin-membrane disease (TMD), minimal-change disease (MCD), membranous glomerulonephritis, and primary focal and segmental glomerulosclerosis (FSGS). Double staining for p-p38/α-SMA, p-p38/macrophages, p-p38/T cells was performed on three or four cases in each disease group. Double staining for p-p38/neutrophils was performed on four cases of postinfectious glomerulonephritis, World Health Organization (WHO) class III/IV systemic lupus erythematosus (SLE), and ANCA-positive pauci-immune glomerulonephritis.

Specificity of p-p38 immunostaining was demonstrated by abrogation of the staining pattern after preincubation of the anti–p-p38 antibody with a 10-fold molar excess of the p-p38 peptide for 30 min at room temperature before incubation of the sections. Preincubation of the anti–p-p38 antibody with a 10-fold molar excess of a closely related peptide, phosphorylated c-Jun N-terminal kinase, failed to abrogate the staining pattern (data not shown). In addition, an irrelevant isotype-matched primary antibody was substituted for the anti–p-p38 antibody during the staining protocol.

Quantification of p-p38 Staining of Human Renal Biopsies

Nonglobally sclerosed glomeruli (mean, 16 ± 5.2; range, 10 to 23) were counted. The number of p-p38 immunostained cells (one-color staining), including within glomerular crescents, and the number of p-p38–positive podocytes (two-color stained sections) were counted and given as the mean ± SD per glomerular cross-section. Tubular and interstitial p-p38 cells were counted in 20 high-power fields (×400), and data were expressed as the mean number of positive p-p38 cells ± SD per mm2. All counting was performed on blinded slides.

Quantification of Histologic Injury in Human Renal Biopsies

The human renal biopsy specimens were reviewed and analyzed by an independent anatomic pathologist who was blinded to the quantification of p38 immunostaining. The total number of glomeruli in the renal biopsy specimen and the number of globally sclerosed glomeruli were counted. The nonglobally sclerosed glomeruli were analyzed for the presence of crescents, proliferative segmental (or global) and/or proliferative necrotic lesions, and segmental sclerotic lesions. The degree of interstitial inflammation and the degree of interstitial fibrosis/tubular atrophy was scored between 0 and 3 according to the following: 0, 0 to 5%; 1, 6 to 20%; 2, 21 to 40%; and 3, >40% of the biopsy area demonstrating inflammation or fibrosis, respectively (Table 2).

Statistical Analyses

Data are presented as mean ± 1 SD. Comparisons were made between groups by one-way ANOVA, using the Bonferroni correction for multiple comparisons (GraphPad 3.0, San Diego, CA). The Pearson single correlation analysis was used to compare quantification of glomerular, tubular, and interstitial p-p38 staining with renal function, proteinuria, hematuria, and erythrocyte sedimentation rate. The Spearman correlation analysis was used to compare the number of glomerular p-p38–positive cells with the percentage of glomeruli exhibiting segmental lesions (patients with biopsies demonstrating normal kidney or TMD were excluded from the analysis). Significance between the number of tubular and interstitial p-p38–positive cells was compared with the degree of interstitial inflammation and interstitial fibrosis using the Kruskal-Wallis test (STATA 8.0, Stata Corp, College Station, TX; all patients were included in the analysis).

Results

Specificity of p38 MAPK Activation in Human Kidney

Stimulation of a fibroblast cell line, NRK49F, with IL-1 resulted in an upregulation in the signal strength of a band at 38 kD by Western blot, consistent with that expected with increased p-p38 phosphorylation (Figure 1). A band at the same molecular weight was also present in nephrectomy tissue from a patient with severe transplant rejection. Reprobing of the blot with anti-p38α demonstrated a similar signal in the human transplant rejection specimen to that seen with anti–p-p38.

Table 2. Histopathologic features of renal biopsies in patients with glomerulonephritis

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of Patients</th>
<th>% Global Sclerosis</th>
<th>% Crescents</th>
<th>% Gloms Segmental Lesions</th>
<th>% Gloms Segmental Sclerosis</th>
<th>Interstitial Inflammation (0–3)</th>
<th>Interstitial Fibrosis (0–3)</th>
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<tr>
<td>Normal</td>
<td>8</td>
<td>2.4 ± 3.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TMD</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.14 ± 0.38</td>
<td>0.29 ± 0.49</td>
</tr>
<tr>
<td>MCD</td>
<td>8</td>
<td>4.1 ± 7.0</td>
<td>0</td>
<td>0</td>
<td>0.43 ± 0.53</td>
<td>0.89 ± 0.93</td>
<td></td>
</tr>
<tr>
<td>MGN</td>
<td>10</td>
<td>18.7 ± 27.6</td>
<td>4.9 ± 7.8</td>
<td>9.3 ± 18.8</td>
<td>0.74</td>
<td>0.53</td>
<td>0.43 ± 0.53</td>
</tr>
<tr>
<td>1° FSGS</td>
<td>9</td>
<td>10.4 ± 17</td>
<td>0</td>
<td>40.8 ± 29.7</td>
<td>1.5 ± 0.76</td>
<td>0.75 ± 0.89</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>14</td>
<td>14.2 ± 19.1</td>
<td>8.6 ± 26.5</td>
<td>21.3 ± 25.7</td>
<td>1.43 ± 0.51</td>
<td>0.71 ± 0.47</td>
<td></td>
</tr>
<tr>
<td>PI GN</td>
<td>10</td>
<td>5.2 ± 5.3</td>
<td>18.7 ± 9.6</td>
<td>0</td>
<td>0.57 ± 0.53</td>
<td>0.43 ± 0.53</td>
<td></td>
</tr>
<tr>
<td>SLE III/IV</td>
<td>10</td>
<td>7.5 ± 10.3</td>
<td>12.7 ± 14.7</td>
<td>33.6 ± 21.3</td>
<td>0.6 ± 0.7</td>
<td>0.9 ± 0.74</td>
<td></td>
</tr>
<tr>
<td>Vasculitis</td>
<td>8</td>
<td>21.9 ± 12.4</td>
<td>48.2 ± 28.3</td>
<td>52.3 ± 24.7</td>
<td>1.29 ± 0.5</td>
<td>1.57 ± 0.53</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD. Glomeruli with either segmental or global proliferative lesions and with segmental necrotic lesions are included together under % gloms with segmental lesions.
p38 MAPK Activation in Normal Human Kidney

Within normal human kidney, a small number of cells within the glomerulus were p-p38 positive (Figure 2a). The staining pattern was nuclear as expected from in vitro studies in which phosphorylation of p38 leads to a translocation from the cytoplasm to the nucleus (28, 29). These p-p38–positive cells include both parietal epithelial cells of Bowman’s capsule and podocytes. The latter was demonstrated by double immunostaining for a podocyte cytoplasmic marker (Figure 2b). Small numbers of tubular epithelial cells and occasional interstitial cells were stained for p-p38 (Figures 2 and 3). p38 activation was seen in all tubular segments and collecting ducts, with no clear restriction to any one particular region.

p38 MAPK Activation in Human Glomerulonephritis

There was a dramatic increase in the number of p-p38–positive glomerular cells in both nonproliferative and proliferative forms of human glomerulonephritis, although the magnitude of the increase was greater in the proliferative glomerulonephritides (Figures 3a and 4). Many of the cells within crescents of SLE WHO class III/IV glomerulonephritis and ANCA-positive pauci-immune glomerulonephritis were p-p38 positive (Figure 4e).

Of the nonproliferative glomerulonephritides, the number of tubular cells stained positive for p-p38 was increased in MCD (Figures 3b and 5b) but not in membranous glomerulonephritis or primary FSGS (Figure 3b). The greatest increase in the number of p-p38–positive tubular cells, however, occurred in the proliferative glomerulonephritides, with IgA nephropathy having more than a 20-fold increase in the number of p-p38–positive tubular cells compared with normal kidney (Figures 3b and 5c). The increase in p-p38 staining within tubules involved all tubular segments, including the proximal and distal convoluted tubules, and collecting ducts.

The number of positive p-p38 interstitial cells was significantly increased in SLE WHO class III/IV glomerulonephritis and ANCA-positive pauci-immune necrotizing glomerulonephritis (Figures 3c and 5d), compared with normal human kidney or TMD. Specificity of p-p38 immunostaining was demonstrated by abrogation of both the glomerular and tubulointerstitial nuclear staining pattern, after incubation of the p-p38 antibody with a 10-fold molar excess of the p-p38 but not the phosphorylated c-Jun N-terminal kinase peptide (data not shown).

p38 MAPK Activation in Individual Cell Types in Human Glomerulonephritis

Intrinsic glomerular cells and infiltrating cells were examined for the presence of p-p38 by double immunohistochemistry. The increase in the number of p-p38–positive glomerular cells in nonproliferative glomerulonephritides, such as MCD, is largely due to an increase in podocyte p-p38 staining, as demonstrated by double staining for podocalyxin, a cytoplasmic podocyte marker (Figures 3, 6a, and 7). The podocalyxin antigen is lost in proliferative glomerulonephritis; therefore, no assessment was made with regard to podocyte p-p38 immunostaining in this group of diseases. Furthermore, p-p38 immunostaining of endothelial cells is prominent in proliferative glomerulonephritis but not within nonproliferative glomerulonephritis or normal human kidney (Figure 6b).

Infiltrating leukocytes are a feature of both proliferative and nonproliferative glomerulonephritides (although greater in pro-
liferative glomerulonephritides), and myofibroblast accumulation is a feature of sclerosis in chronic or subacute glomerulonephritides. p-p38 expression was examined in macrophages, T cells, neutrophils, and myofibroblasts. Infiltrating glomerular and interstitial macrophages were prominent in SLE WHO class III/IV and ANCA-positive pauci-immune vasculitis and are present in small numbers in membranous and primary FSGS. Most if not all macrophages were p-p38 positive (Figure 6c). Infiltrating neutrophils in postinfectious glomerulonephritis, SLE WHO class III/IV, and ANCA-positive pauci-immune crescentic glomerulonephritis (c). Many p-p38-positive cells (brown) were seen in a fibrocellular crescent (e, arrowheads). Incubation of the p-p38 antibody with the p-p38 peptide before the staining protocol abrogated the nuclear staining pattern in glomeruli and tubules of IgA nephropathy (f). Magnification, ×250.

Figure 3. Quantification of p-p38 immunostaining in glomeruli (a), tubules (b), and the interstitium (c) of normal and diseased human kidney. Renal biopsies of normal kidney, thin membrane disease (TMD), nonproliferative glomerulonephritis (NP GN), and proliferative glomerulonephritis (P GN) were counted for the number of p-p38-positive cells in the glomeruli (a), tubules (b), and interstitium (c). For each disease classification, the data are given as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 versus normal. For abbreviations, see legend to Table 1.

Figure 4. Glomerular p38 MAPK activation in human glomerulonephritis. Compared with TMD (a; which was very similar to normal human kidney), there was a dramatic increase in glomerular p-p38 immunostaining (brown) in biopsy-proven glomerulonephritis, including minimal change disease (MCD; b), membranous glomerulonephritis (c), IgA nephropathy (d), and ANCA-positive pauci-immune crescentic glomerulonephritis (e). Many p-p38-positive cells (brown) were seen in a fibrocellular crescent (e, arrowheads). Incubation of the p-p38 antibody with the p-p38 peptide before the staining protocol abrogated the nuclear staining pattern in glomeruli and tubules of IgA nephropathy (f). Magnification, ×250.
FSGS, some cases of IgA nephropathy, SLE WHO class III/IV glomerulonephritis, and ANCA-positive pauci-immune vasculitis (Figure 6f). Tubular p38 MAPK activation was prominent in areas with tubular damage and infiltration of both macrophages and T cells (data not shown).

**Correlation of p38 MAPK Activation with Clinical Parameters in Human Glomerulonephritis**

The clinical parameters of patients with glomerulonephritis are shown in Table 1. Renal function inversely correlated with the number of glomerular, tubular, and interstitial p-p38–positive cells in proliferative glomerulonephritis but not nonproliferative glomerulonephritis (Table 3). Twenty-four-hour urinary protein excretion correlated strongly with the number of glomerular p-p38–positive cells in nonproliferative glomerulonephritis and with tubular and interstitial p-p38–positive cells in nonproliferative and proliferative glomerulonephritis (Table 3). To determine whether proteinuria in nonproliferative glomerulonephritis was related to p38 activation within podocytes, we counted the number of positive p-p38 cells double stained for the cytoplasmic podocyte marker podocalyxin. There was a significant increase in the number of p-p38–positive podocytes in MCD, membranous nephropathy, and FSGS (Figure 7). In these nonproliferative glomerulonephritides, proteinuria correlated with the number of p-p38–positive podocytes ($r^2 = 0.251, P < 0.002$). Hematuria correlated with the number of glomerular and interstitial p-p38–positive cells in all glomerulonephritides, and the erythrocyte sedimentation rate correlated with the number of glomerular, tubular, and interstitial p-p38–positive cells in all glomerulonephritides.

**Correlation of p38 MAPK Activation with Histologic Parameters of Renal Injury in Human Glomerulonephritis**

The histologic parameters of renal injury are described in Table 2. In human glomerulonephritis, there was a significant correlation between the number of p-p38–positive glomerular cells and the percentage of glomeruli with proliferative and or necrotic segmental lesions (Figure 8). This relationship was also maintained after analysis of the proliferative glomerulonephritides alone (Spearman $r = 0.423, P = 0.003$). There was
no correlation, however, between the number of p-p38–positive glomerular cells and the percentage of glomeruli with segmental sclerosis. For the assessment of interstitial damage, only three patients were graded as having severe interstitial inflammation with a score of 3, and only two patients graded as having severe interstitial fibrosis with a score of 3. Given these small numbers, therefore, the analysis was performed using the combined score of 2 and/or 3 for interstitial inflammation and fibrosis. The number of p-p38–positive interstitial cells correlated with the degree of interstitial inflammation but not with interstitial fibrosis (Figure 9, a and b). The number of p-p38–positive tubular cells did not correlate with the degree of either interstitial inflammation or fibrosis (Figure 9, c and d).

Discussion

In this study, activation of the p38 MAPK pathway was identified in glomeruli and some tubules in normal human kidney and TMD. Within glomeruli, p-p38 is localized to podocytes and to parietal epithelial cells of Bowman’s capsule. The stimulus for p38 activation in podocytes of normal human kidney or TMD is unclear. The degree of p38 activation seen in podocytes in normal kidney suggests an in vivo role for p38 activation in normal podocyte physiology. Nephrin, a transmembrane protein of podocytes, is an integral part of the slit diaphragm (30), and overexpression of nephrin in a human embryonic kidney cell line deficient in nephrin results in p38 activation, suggesting a potential role for p38 signaling in maintaining normal podocyte function (31). This raises the question of what impact p38 MAPK inhibition may have on normal renal function. However, the successful use of p38 MAPK inhibitors in the treatment of human inflammatory bowel disease (32) and in phase II clinical studies in the treatment of rheumatoid arthritis (33) and neurodegenerative diseases (34) suggests that p38 blockade has no major deleterious impact on normal renal function or physiology.

One previous study of human renal tissue reported approximately one p-p38–positive cell per glomerular cross-section within glomeruli of TMD and MCD and approximately four p-p38–positive cells per glomerular cross-section within glomeruli of crescentic glomerulonephritis, predominantly localized to the crescent (35). These results contrast with the findings in the current study. This disparity is most likely due to differences in the immunostaining protocol. Specifically, Sakai et al. (35) did not use an antigen retrieval technique before immunostaining. In developing the immunostaining protocol for p-p38, we found that microwave oven treatment of the tissue was essential for antigen retrieval, with only a very weak signal in a few cells seen in the absence of this treatment. Furthermore, we were able to demonstrate specificity of immunostaining by abrogation of the staining pattern after incubation of the anti–p-p38 antibody with the phosphorylated p38 peptide. Our results are in accordance with p38 activation in normal rat kidney and in rat anti–glomerular basement membrane disease in which there was an excellent correlation between Western blotting and immunostaining for p-p38 (23).

This study provides the first demonstration of p38 activation within human podocytes in diseased kidney. The role of p38 podocyte activation in human glomerulonephritis is unclear. Podocyte damage is associated with proteinuria (30), and in this study, podocyte p38 activation correlated with proteinuria in nonproliferative glomerulonephritis, suggesting that p38 MAPK activation promotes the development of proteinuria. Alternatively, exposure of podocytes to altered proteins and lipids in nephrotic syndrome may result in podocyte p38 MAPK activation. The similar numbers of p-p38–positive podocytes in different proteinuric renal diseases, with presumably different podocyte insults, supports this alternative explanation. Although nephrin has been associated with p38 activation (31), the reduction in nephrin seen in many forms of proteinuric glomerulonephritides argues against a major role for this molecule as a stimulus for p38 activation in nonproliferative glomerulonephritis. In addition, in human and experimental glomerulonephritis, podocytes are an important source of IL-1 (4, 5), and the increase in podocyte p38 MAPK activation may be related to podocyte cytokine production. TNF-β–induced podocyte apoptosis has been associated, in vitro, with p38 MAPK activation (36). The increased number of podocytes positive for p38 activation demonstrated in MCD, a reversible condition not typically associated with podocyte cell loss, suggests that this pathway does not play a major role in podocyte cell death in human glomerulonephritis.
Table 3. Correlation of glomerular, tubular, and interstitial p-p38 immunostaining with clinical parameters in human glomerulonephritis

<table>
<thead>
<tr>
<th></th>
<th>Creatinine Clearance</th>
<th>24-Hour Urinary Protein</th>
<th>Hematuria All</th>
<th>ESR All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>NP</td>
<td>P</td>
<td>All</td>
</tr>
<tr>
<td>Glomerular p-p38</td>
<td>0.141&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.061</td>
<td>0.159&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.012</td>
</tr>
<tr>
<td>Tubular p-p38</td>
<td>0.116&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.018</td>
<td>0.192&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.198&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interstitial p-p38</td>
<td>0.189&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.003</td>
<td>0.356&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.213&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The number of glomerular p-p38–, tubular p-p38–, and interstitial p-p38–positive cells was compared with creatinine clearance, proteinuria, hematuria, and ESR using the Pearson single correlation coefficient in all glomerulonephritides (All), nonproliferative glomerulonephritis (NP), and proliferative glomerulonephritis (P). Values are given as r<sup>2</sup>; <sup>b</sup>P < 0.05, <sup>c</sup>P < 0.01, <sup>d</sup>P < 0.0001 compared with normal.

Figure 8. Correlation of the number of p-p38–positive glomerular cells with the percentage of glomeruli with segmental proliferative or necrotic lesions in human glomerulonephritis. The Spearman single correlation coefficient was used to compare the mean number of p-p38–positive glomerular cells for each patient (excluding normal and TMD) with the percentage of glomeruli demonstrating segmental (or global) proliferative or necrotic lesions.

Figure 9. Correlation of the number of p-p38–positive interstitial and renal tubular cells with the degree of interstitial inflammation and interstitial fibrosis in human glomerulonephritis. Box graph representation (median, 25th and 75th centiles ± range) of the number of interstitial (a and b) and tubular (c and d) p-p38–positive cells in human glomerulonephritis for the degree of interstitial inflammation (a and c) and interstitial fibrosis (b and d) graded as 0 (0 to 5% by area), 1 (6 to 20% by area), and 2 to 3 (>20% by area). Statistical significance was determined using the Kruskal-Wallis test. All patients were included in the analysis.

In vitro studies suggest that p38 activation plays an important role in the endothelial response to inflammatory stimuli, such as in the production of chemokines (37–39). Our findings of endothelial p38 activation in inflamed glomeruli in vivo provide human relevance to these in vitro findings. Similarly, we have identified p38 activation in macrophages and neutrophils in the diseased human kidney, cells known to play an important role in disease pathogenesis (1, 2). These results are consistent with in vitro studies, which have identified p38-dependent proinflammatory responses in these cell types (7, 14, 16, 40–42). Unexpected, p38 activation was not seen in infiltrating T cells. In vitro studies have suggested an important role for the p38 MAPK pathway in lymphocyte activation (15). Although autophosphorylation of the p38 kinase has been demonstrated (43), sustained activation of the p38 pathway is dependent on the continued presence of the stress or stimulus. It is possible that either p38 activation was no longer present within infiltrating T cells as a result of the absence of a stimulus at the time of the biopsy or, alternatively, p38 activation is not important for lymphocyte activation in human glomerulonephritis.

p38 activation occurs in a small number of tubular cells in normal kidney and TMD, with a marked increase in the number of p-p38–positive tubular cells in proliferative glomerulonephritides, in addition to MCD. p38 activation of renal tubular cells in vitro is associated with the production of inflammatory mediators (18, 44). The increase in the number of tubular p-p38–positive cells in proliferative glomerulonephritis may relate to tubular cell activation as a consequence of the interstitial inflammatory infiltrate that accompanies the disease process. However, the tubular activation seen in MCD, IgA nephropathy, and postinfectious glomerulonephritis is not accompanied by a prominent interstitial infiltrate, and tubular p38 activation does not correlate with the degree of interstitial...
infiltration. An alternative explanation is that elements within the tubular fluid contribute to tubular cell p38 activation. Overall, protein excretion correlates with tubular p-p38 immunostaining in both proliferative and nonproliferative glomerulonephritis, suggesting that albumin or other urinary factors, directly or indirectly, induce tubular p38 activation. Although there was a marked increased in tubular p38 activation in MCD, this was not significantly increased beyond normal in other proteinuric conditions such as membranous nephropathy or primary FSGS. Furthermore, there was no statistical difference in proteinuria between MCD and primary FSGS, suggesting that tubular p38 activation in MCD may relate to factors other than proteinuria and intrinsic to MCD pathology. There is currently no in vitro or in vivo evidence suggesting that albumin is a direct stimulus to renal tubular cell p38 activation. Urinary red blood cells excretion does not correlate with tubular p-p38 immunostaining in either proliferative or nonproliferative glomerulonephritis and thus is unlikely to contribute directly to tubular p38 activation. Osmotic stress is a potent stimulus for p38 activation (8, 45–47) and may contribute to tubular p38 activation in proteinuric glomerulonephritides.

An increase in interstitial p38 activation was seen in SLE WHO class III/IV glomerulonephritis and ANCA-positive pauci-immune crescentic glomerulonephritis and largely relates to the infiltration of p-p38–positive macrophages. Not surprising, therefore, interstitial p38 activation correlates with the degree of interstitial inflammation. p38 activation was also present in fibroblast-like cells within fibrocellular crescents and in interstitial myofibroblasts. However, interstitial p38 activation did not correlate with interstitial fibrosis/tubular atrophy, possibly as there are few myofibroblasts present within areas of well-established fibrosis. p38 activation within myofibroblasts has been shown to be important in collagen IV and fibronectin production in vitro (48–52), but this is the first demonstration of p38 activation in myofibroblasts within sclerotic lesions of diseased human kidney.

In summary, this study has identified p38 activation in normal human kidney and a marked increase in p38 activation in many forms of glomerulonephritis. The correlation of p38 activation with clinical and histologic parameters argues that this pathway plays an important role in disease pathogenesis. Furthermore, localization of p38 activation to individual cell types suggests that this pathway may play a pivotal role in promoting both renal inflammation and fibrosis. Thus, blockade of p38 MAPK may provide a novel approach in the treatment of human glomerulonephritis.

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References


