Activation and cellular localization of the p38 and JNK MAPK pathways in rat crescentic glomerulonephritis

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Background. The p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) are intracellular signal transduction pathways involved in the production of inflammatory mediators. Little, however, is known about the contribution of these pathways to renal inflammation, nor the cell types in which these pathways are activated within normal and inflamed kidneys. The aim of this study was therefore to delineate the pattern and cellular localization of p38 and JNK activation in normal rat kidney and rat acute and chronic inflammatory renal disease.

Methods. Normal male Sprague-Dawley rats and groups of rats given accelerated anti-glomerular basement membrane (GBM) disease were killed at 3 hours, day 1, day 7, or day 28 and examined for p38 and JNK pathway activation by Western blotting and immunolocalization of the phosphorylated p38 (p-p38) and JNK (p-JNK) kinases.

Results. In terms of glomerular MAPK activation, Western blotting identified the presence of both p-p38 and p-JNK in normal glomeruli, localized by immunohistochemistry to podocytes and epithelial cells of Bowman’s capsule. In anti-GBM disease, Western blotting showed that p38 activation peaked at 3 hours and remained elevated above normal throughout the disease timecourse. JNK activation (via the 54 kD isoform) likewise increased at 3 hours of anti-GBM disease and remained elevated throughout disease. At 3 hours, p-p38, but not p-JNK, was localized to neutrophils and glomerular endothelial cells. p-JNK was localized to glomerular endothelial cells at day 7. Macrophages, lymphocytes, activated podocytes, and myofibroblasts were positive for both p-p38 and p-JNK. In terms of tubular MAPK activation, Western blotting identified p38 and JNK activation in tubules of normal kidney. Immunostaining showed that most cortical tubules contained some p-p38 and p-JNK stained cells. There was a significant increase in tubular p38 activation at 3 hours of anti-GBM disease, followed by increased JNK activation of the 54 kD isoform from day 7 onward, and the 46 kD isoform at day 28. Immunostaining of diseased tissue localized p-p38 and p-JNK to virtually all cortical tubular cells.

Key words: p38, JNK, glomerulonephritis, podocyte, myofibroblast, immunohistochemistry.

Conclusion. The p38 and JNK MAPK pathways are activated in glomeruli and tubules of normal kidney. In acute anti-GBM disease, there was an increase in p38 activation within glomerular endothelial cells and with infiltrating neutrophils, suggesting an important role for p38 MAPK in acute inflammation. In progressive anti-GBM disease, p38 and JNK activation in podocytes, glomerular endothelial cells, infiltrating macrophages, T cells, and myofibroblasts suggests that both the p38 and JNK MAPK pathways are important in chronic inflammation and fibrosis. Blockade of these pathways may therefore be potentially therapeutic in the treatment of acute and chronic renal inflammation.

Crescentic glomerulonephritis is characterized by acute renal inflammation, an aggressive condition resulting in tissue destruction and a loss of renal function, leading to end-stage renal failure. Leukocyte infiltration and activation of intrinsic renal cells are prominent in crescentic disease, with consequent production of various inflammatory mediators, including cytokines and chemokines [1].

The p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) pathways are well-described intracellular signal transduction pathways critical to cytokine production. These pathways are involved in the transduction of a cell surface signal to the nucleus resulting in an alteration in the pattern of gene transcription and the production of inflammatory mediators [2–4]. Both pathways are activated by stressors such as interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α) binding to their surface receptors, which leads via G-coupled and G-independent mechanisms to a rapid cascade of sequential kinase phosphorylation resulting in dual phosphorylation of the Tyr-X-Thr motif of the p38 and JNK kinases [5]. Once phosphorylated, p38 and JNK translocate to the nucleus and activate a variety of transcription factors by phosphorylation [6–9].

Activation of these pathways is important in development of the proinflammatory response, as inhibition of either of these pathways has been shown to suppress a number of inflammatory conditions. p38 blockade is effective in the treatment of human endotoxemia and rat
models of acute lung injury and arthritis [10–12]. JNK inhibition has recently been shown to be an effective treatment in a rat model of arthritis [13]. Although considered central to the inflammatory process, the role of p38 and JNK in induction and propagation of renal injury is not well understood. Little is known of the relationship between pathway activation and the onset of the insult, the relative contributions of these pathways to the early and late inflammatory response in any inflammatory disease and the cell types, both intrinsic and infiltrative, in which these pathways become activated in situ. We therefore examined the activation and cell specific localization of the p38 and JNK MAPKs in a rat model of aggressive inflammatory kidney disease, anti-glomerular basement membrane (GBM) disease. This is the first study to compare and contrast the temporal pattern of activation of the p38 and JNK MAPKs in the inflamed kidney and to localize the pathways to individual cell types including intrinsic renal cells and infiltrating leukocytes.

**METHODS**

**Rat model of crescentic glomerulonephritis**

Accelerated anti-GBM glomerulonephritis (anti-GBM disease) was induced in inbred male Sprague-Dawley rats (140 to 180 g) (Monash Animal Services, Melbourne, Australia) as described previously [14]. Briefly, rats were immunized by subcutaneous injection of 5 mg of sheep IgG in Freund's complete adjuvant followed 7 days later (day 0) by an intravenous injection of 5 mL/kg body weight of sheep anti-GBM serum. Groups of 10 animals were killed at 3 hours, 24 hours, 7 days, or 28 days following anti-GBM serum injection. Animals were housed in metabolic cages for 24 hours in order to collect urine at days 0 to 1, 6 to 7, and 27 to 28. All animals were bled when killed. Tissues were fixed for 4 hours in 4% formalin or 3 hours in 2% paraformaldehyde-lysine-periodate (PLP) and processed for histopathology analysis. Serum and urine creatinine were measured using a Dupont ARL analyzer and urinary protein estimations (in-house benzasthonium chloride method) were performed by the Department of Biochemistry, Monash Medical Centre. Animal experiments were approved by the Monash Medical Centre Animal Ethics Committee.

**Antibodies**

The following mouse monoclonal antibodies were used in this study: anti-phospho p38 (p-p38) (Sigma-Aldrich, St Louis, MO, USA # M1877) raised against the dual phosphorylated p38 peptide and recognizing all the phosphorylated p38 isoforms; anti-phospho JNK (p-JNK) (Sigma-Aldrich, # J4750) raised against the dual phosphorylated JNK peptide and recognizing both the phosphorylated 46 kD and 54 kD isoforms; anti-CD68 recognizing rat macrophages (Serotec, Oxford UK); anti-α1-tubulin (Sigma-Aldrich); antidesmin recognizing activated or injured podocytes [15–18] (Dako, Glostrup, Denmark, # M0760); 1A4, anti-α-smooth muscle actin (Sigma-Aldrich); RP-1 recognizing the RP-1 antigen on rat neutrophils (Becton Dickinson, San Diego, CA, USA); RECA-1 recognizing rat endothelial cells (Serotec) and W3/13 (anti-CD43) recognizing T lymphocytes and granulocytes [19]. Also used was a rabbit antitrat podocalyxin antibody recognizing rat podocytes, a kind gift from Professor Kerjaschki [20]. Horseradish peroxidase and alkaline phosphatase–conjugated goat anti-mouse (or rabbit) IgG, and peroxidase-conjugated mouse (or rabbit) antiperoxidase complexes (PAP) and alkaline phosphatase–conjugated mouse antialkaline phosphatase complexes (APAAP) were purchased from Dako. p-p38 and p-JNK peptides were kindly provided by Cell Signaling Technology (Beverly, MA, USA).

**Western blot analysis**

At sacrifice, the right kidney from each animal was dissected and placed immediately into ice cold phosphate-buffered saline (PBS) and sieved sequentially through 250 μm, 150 μm, and 75 μm mesh to obtain glomerular and tubular fractions. The purity of separation was visually determined to be greater than 95% for glomeruli and greater than 98% for tubular fragments by light microscopy. The fractions were centrifuged and resuspended in 0.5 or 1.0 mL sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 5 minutes vortexing every 2 minutes and heated to 100°C for 5 minutes. The samples were centrifuged at 14,000 rpm for 5 minutes and the supernatant was stored at −80°C. Protein estimations were performed using a Bradford assay (Pierce, Rockford, IL, USA). Glomerular and tubular protein was loaded at 80 μg per well and separated on a 12.5% SDS-PAGE gel. Gels were electroblotted onto a polyvinylidene difluoride (PVDF) membrane, incubated for 4 hours in 20 mL of blocking buffer (PBS, 5% skim milk), washed three times in wash buffer (PBS, 0.05% Tween 20, pH 7.6), and incubated with either anti-p-p38 (1 ug/mL) or anti-p-JNK (2 μg/mL) in 5% bovine serum albumin (BSA) in wash buffer overnight at 4°C. Blots were washed three times and incubated with horseradish peroxidase–conjugated goat antimouse IgG [1:10,000 in 10% normal rat serum, 1% normal sheep serum, and 1% fetal calf serum (FCS)] for 2 hours at room temperature. Membranes were washed three times and the membrane-bound antibody detected by Supersignal West Pico chemiluminescent substrate (Pierce) and captured on x-ray film. To determine the equivalence of protein loading, membranes were run in parallel with a standard molecular weight marker.
were stripped using ×1 stripping buffer (Chemicon International, Temecula, CA, USA), blocked with 20 mL of blocking buffer for 4 hours, then probed with anti-α1-tubulin (1:5000) in 5% BSA in wash buffer overnight. Membranes were washed three times and incubated with horseradish peroxidase–conjugated goat antimouse IgG, washed in wash buffer three times, and developed with chemiluminescence (Pierce) and captured on x-ray film. Densitometry analysis was performed using a Gel Pro analyzer program (Media Cybernetics, Silver Springs, MD, USA).

Immunohistochemistry

For p-p38 immunostaining, slices of the left kidney were fixed in 4% buffered formalin, embedded in paraffin, 4μm sections cut and dewaxed in histosol and rehydrated. For p-JNK immunostaining, slices of the left kidney were fixed in PLP, 22-oxacalcitriol (OCT) embedded and 4 μm sections cut. Two-color immunohistochemical staining was performed as described previously [21]. For both p-p38 and p-JNK immunostaining, the sections were microwave oven heated in Dako microwave buffer (Dako, # S1700) for 10 minutes, allowed to cool, washed in PBS, and blocked with 10% normal sheep serum, 10% FCS in PBS for 30 minutes at room temperature, and incubated overnight at 4°C with either anti-p-p38 or anti-p-JNK at 5 μg/mL in 10% normal rat serum, 1% BSA in PBS. Sections were subsequently washed once in PBS, endogenous peroxidase inactivated in 1% H2O2 in methanol for 20 minutes, incubated with horseradish peroxidase–conjugated goat antimouse IgG followed by mouse PAP and developed with 3,3-diamenobenzidine to produce a brown color. When double labeling, the sections were microwave oven heated, blocked with 10% normal sheep serum and 10% FCS in PBS and incubated with anti-CD68 at 1 μg/mL, RP-1 (1:100), W3/13 (1:500), antidesmin (1:100), antimouse (or rabbit) IgG followed by mouse PAP and developed with Vector SG (Vector Laboratories, Burlingame, CA, USA) to produce a gray color. Sections were either counterstained with periodic acid-Schiff (PAS) reagent or left uncounterstained and mounted with glycerol medium.

In addition to replacement of the primary antibodies with irrelevant isotype-matched control antibodies in the staining protocol, specificity of p-p38 and p-JNK immunostaining was demonstrated by incubating the anti-p-p38 antibody or anti-p-JNK antibody with a tenfold molar excess of the p-p38 or p-JNK peptides for 30 minutes at room temperature prior to application to tissue sections. Incubation of the p-p38 antibody with the p-p38, but not the p-JNK, peptide abolished the p-p38 staining pattern. Incubation of the p-JNK antibody with the p-JNK, but not the p38 peptide, abolished the p-JNK staining pattern. Quantitation of p-p38 and p-JNK immunostaining in RECA-1–positive endothelial cells and podocalyxin-positive podocytes was performed by counting the number of double stained cells on 20 glomerular cross-sections (gcs) per animal on blinded slides.

Statistical analysis

Data are presented as mean ± 1 SD. Comparisons were made between groups of animals by the analysis of variance (ANOVA), using the Bonferroni correction for multiple comparisons (GraphPad Software, San Diego, CA, USA).

RESULTS

Characterization of rat anti-GBM glomerulonephritis

Administration of anti-GBM serum to primed rats resulted in a severe glomerulonephritis in which animals developed heavy proteinuria (Fig. 1A) and a loss of renal function (Fig. 1B). Renal histology showed the development of crescentic glomerulonephritis with 32% of glomeruli exhibiting crescents on day 28 (Fig. 1). Hypercellularity, leucocyte infiltration, glomerular PAS deposits, and tubular damage were also evident.

p-p38 and JNK activation in normal rat kidney

Western blotting of normal rat kidney demonstrated activation of the p38 MAPK pathway in both glomeruli and tubules (Fig. 2A and B). p-p38 immunostaining was present in normal glomeruli in a nuclear pattern and was localized mainly to podocytes and parietal epithelial cells of Bowman’s capsule (Figs. 3A and 4A), although endothelial cells were also occasionally stained (data not shown). Most cortical tubular cross-sections showed the presence of one or more cells stained for p-p38 (Fig. 5A). The JNK MAPK pathway was also activated within both glomeruli and tubules of the normal rat kidney, as demonstrated by Western blotting, with activation of the 46 kD isoform (pJNK46) predominant over the 54 kD isoform (pJNK54) (Fig. 2C and D). p-JNK immunostaining was present within glomeruli in a nuclear distribution and was localized mainly to podocytes and parietal epithelial cells.
of Bowman’s capsule (Figs. 3B and 4B). p-JNK immunostaining was also present in many tubular cells within the cortex (Fig. 5B).

**Glomerular p38 and JNK activation in rat anti-GBM disease**

By Western blotting, there was a peak ninefold increase in glomerular p-p38 at 3 hours after induction of the disease with a two- to threefold increase in p-p38 compared to normal at days 1, 7, and 28 (Fig. 2A). Glomerular activation of the JNK pathway also occurred early, as a consequence of phosphorylation of the 54 kD isoform with an 18-fold increase at 3 hours. However, unlike p-p38, there was an increase in phosphorylation with disease progression with a 32-fold increase of p-JNK54 above normal at day 28. Glomerular activation of the JNK MAPK pathway is almost exclusively via phosphorylation of the 54 kD isoform; phosphorylation of the 46 kD isoform remained unchanged throughout the time-course of the disease (Fig. 2C).

Within 3 hours of induction of anti-GBM disease, there was a substantial increase in glomerular p-p38 immunostaining, consistent with Western blot data. There was approximately a fivefold increase in the number of glomerular p-p38–positive cells (Figs. 3C and 6A), which remained elevated at both day 7 and day 28 (Figs. 3E and 6A). The number of glomerular p-JNK–positive cells was not increased at 3 hours of the disease, inconsistent with the Western blot data; however, there was a progressive increase in the number of p-JNK–positive cells from day 1 following induction of the disease, such that there was a fourfold increase by day 28 (Figs. 3F and 6B).

**Specificity of p-p38 and p JNK immunostaining**

Antibody specificity was determined by incubating the antibodies with either the p-p38 and p-JNK peptides prior to immunostaining of tissue. Incubation of the anti-p-p38 antibody with the p-p38 peptide (Fig. 3G), but not the p-JNK peptide (not shown), abrogated p-p38 staining. Likewise, incubation of the anti-p-JNK antibody with the p-JNK peptide (Fig. 3H), but not the p-p38 peptide (not shown) abrogated p-JNK staining.

**Inflammatory cells: p-p38 and p-JNK immunolocalization**

The presence of p-p38 and p-JNK within infiltrating inflammatory cells was examined. Virtually all neutrophils, the predominant inflammatory cell within the glomerulus at 3 hours, exhibited nuclear p-p38 (Fig. 7A), but not p-JNK (Fig. 7B), staining suggesting that the early peak of p-p38 identified by Western blotting may be in part as a result of extensive neutrophil infiltration. Extensive glomerular and interstitial macrophage infiltration occurs with progression of the disease. Both p-p38 and p-JNK immunostaining was present within macrophages (Fig. 7C and D), infiltrating the interstitium, glomerular tuft and fibrocellular crescents (data not shown). In order to determine whether macrophages express p-p38 or
Fig. 2. Expression of phospho p38 (p-p38) and phospho JNK (p-JNK) in glomeruli and tubules in rat crescentic glomerulonephritis. Glomeruli (A and C) and tubules (B and D) were isolated from normal rats and from rats at 3 hours, 1 day, 7 days, or 28 days following the induction of anti-glomerular basement membrane (GBM) disease and probed for p-p38 (A and B), and p-JNK (C and D) by Western blotting. Blots were stripped and probed for α1-tubulin as a loading control. Two representative animals are shown at each time point. Graphs show densiometric analysis (mean ± 1 SD, groups of ten animals) of the ratio of p-p38 (A and B) or p-JNK (C and D) to α1-tubulin compared to normal animals (assigned a p-p38 or p-JNK to α1-tubulin ratio of 1). *P < 0.05; **P < 0.001 vs. normal.

p-JNK during early recruitment, we examined macrophage infiltration at 3 hours. Dual staining of macrophages with both p-38 and p-JNK suggests activation of macrophages throughout the disease course occurs via both stress-activated pathways (data not shown). In addition, both interstitial and glomerular lymphocytes at day 28 were positive for p-p38 and p-JNK immunostaining (Fig. 7E and F).
Fig. 3. Localization by immunohistochemistry of phosphorylated p38 (p-p38) and phosphorylated JNK (p-JNK) in normal and diseased glomeruli.

Normal rat glomeruli demonstrated nuclear staining of p-p38 (A) in many glomerular cells, including podocyte-like cells (arrowheads), in addition to parietal epithelial cells of Bowman’s capsule (arrow). Within 3 hours of intravenous injection of nephrotoxic serum, there is a substantial increase in p-p38 (C), which remained elevated at day 28 (E). Glomerular and tubular p-p38 (G) immunostaining of tissue of day 28 anti-glomerular basement membrane (GBM) disease was abrogated following incubation of the anti-p-p38 antibody with the p-p38 peptide. Normal rat glomeruli
Fig. 4. Immunolocalization of phosphorylated p38 (p-p38) and phosphorylated JNK (p-JNK) to podocytes in normal rat glomeruli. Normal rat glomeruli were immunostained for podocytes (gray) using a podocalyxin marker. Podocyte nuclei were positive for (A) p-p38 (brown, arrowheads) and (B) p-JNK (brown, arrowheads) (magnification ×1200).

Fig. 5. Localization by immunohistochemistry of phosphorylated p38 (p-p38) and phosphorylated JNK (p-JNK) in normal and diseased tubules. In normal cortex (A) most tubular cross-sections showed p-p38 immunostaining (brown) of one of more epithelial cells. p-JNK was also present in many tubular cells (B) of normal cortex. At 3 hours of anti-glomerular basement membrane (GBM) disease, there was an increase in p-p38 staining of most cortical tubular cells (C, brown), which persisted to day 28 of disease (E, brown). In contrast, there was no increase in tubular immunostaining of p-JNK at 3 hours of anti-GBM disease (D, brown); however, virtually all tubular cells were positive at day 28 of disease (F, brown) [magnification (A to D) ×400; (E and F) ×250].

demonstrated nuclear staining of p-JNK (B) in podocyte-like cells (arrowheads) and parietal epithelial cells of Bowman’s capsule (arrow). At 3 hours anti-GBM disease, there was no apparent increase in glomerular p-JNK immunostaining (D). However p-JNK staining was increased 1 day following induction of anti-GBM disease, and at day 28 (F). Glomerular and tubular p-JNK (H) immunostaining of tissue of day 28 anti-GBM disease was abrogated following incubation of the anti-p-JNK antibody with the p-JNK peptide [magnification (A to F) ×400; (G and H) ×250].
Intrinsic glomerular cells: p-p38 and p-JNK immunolocalization

In normal glomeruli, occasional endothelial cells were positive for both p-p38 and p-JNK (data not shown); however, at 3 hours following induction of disease, endothelial p-p38 (Figs. 8A and 9A), but not p-JNK immunostaining was substantially increased and remained elevated at all subsequent time points. By day 7, however, endothelial p-JNK immunostaining was increased (Figs. 8B and 9A) and remained elevated at day 28. In addition, there was an increase in the number of both p-p38 and p-JNK–positive podocytes during the development of anti-GBM disease (Fig. 9). An increase in podocytic desmin staining is a well-recognized marker of injured or activated podocytes [15–18]. Double immunohistochemistry identified that many injured or activated podocytes (desmin-positive cells) were positive for p-p38 and p-JNK (Fig. 8C and D).

Myofibroblasts express p-p38 and p-JNK

Interstitial and glomerular fibrosis is a feature of chronic anti-GBM disease at day 28. Both p-p38 and p-JNK were localized to glomerular (including within fibrocellular crescents) and interstitial myofibroblasts, by double immunohistochemical staining for α-smooth muscle actin, a cytoplasmic marker of myofibroblasts (Fig. 8E).

Tubular p38 and JNK activation in rat anti-GBM disease

Western blotting demonstrated an early four- to sixfold increase in tubular p-p38 at 3 hours anti-GBM disease and this persisted with progression of the disease (Fig. 2B). In contrast, there was no increase in activation of the JNK MAPK pathway until day 7 of anti-GBM disease. This was due to increased p-JNK54 at day 7, and a threefold and sixfold increase of p-JNK46 and p-JNK54, respectively, at day 28 (Fig. 2D).

Immunostaining identified a substantial increase in p-p38 immunostaining in cortical tubules at 3 hours of anti-GBM disease, and this remained evident throughout the disease time course (Fig. 5C and E). Tubular p-JNK immunostaining was not different to normal at 3 hours or day 1 of anti-GBM disease, but was substantially increased in all tubules (both well preserved and damaged) on days 7 and 28 (Fig. 5D and F).

DISCUSSION

Activation of the p38 and JNK pathways has been described previously in glomerular lysates of acute rat anti-GBM disease [22, 23]. However, no studies to date have compared the activation of these MAPK pathways in glomeruli versus tubules, nor clearly identified the cell types in which these pathways are activated in normal or diseased kidney. We have confirmed activation of p-p38 in acute anti-GBM disease and identified both similarities and differences with regard to the activation of the p38 and JNK pathways with progression of the disease in both the glomerular and tubulointerstitial compartments.

In normal rat kidney, p38 and JNK MAPK pathway activation is present within podocytes, parietal cells of Bowman’s capsule, and most tubules of the cortex. JNK activation of the 46 kD isoform predominated over activation of the 54 kD isoform in both glomeruli and tubules. The physiologic role for these pathways in normal rat kidney is not understood and the triggers for dual pathway activation in podocytes and cortical tubules are unknown, but may be related to their functions of selective filtration and solute reabsorption, respectively. Therefore, blockade of the p38 or JNK MAPK pathways could potentially
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Fig. 7. Immunolocalization of phosphorylated p38 (p-p38) and phosphorylated JNK (p-JNK) to infiltrating leukocytes in rat crescentic glomerulonephritis. At 3 hours following induction of disease, infiltrating neutrophils (A and B; blue) express p-p38 (A, brown; arrowheads) but not p-JNK (B, brown; arrowheads). Infiltrating macrophages (C and D; blue) and T lymphocytes (E and F; blue) at day 28 anti-GBM disease express both p-p38 (C and E; brown; arrowheads) and p-JNK (D and F; brown; arrowheads) (magnification ×800).

result in deleterious changes in normal renal function. Previous studies have found p-JNK staining of podocytes and distal tubules in normal rat kidney [24] and p-p38 staining in glomeruli in rat crescentic glomerulonephritis [25]. However, the p-p38 and p-JNK staining pattern in these studies is non-nuclear. We demonstrated the presence of the activated kinases within nuclei of these cell types in normal and diseased kidney. The nucleus is the expected location of p38 and JNK after phosphorylation and we confirmed the specificity of immunostaining by the use of blocking phospho peptides. The reason for the difference in staining pattern with these studies is not clear, but may relate to fixation conditions or the immunohistochemistry techniques used.

The p38 MAPK pathway was activated within both glomeruli and tubules in the acute phase of anti-GBM disease and remained activated throughout the disease course. The JNK MAPK pathway was also activated in glomeruli in acute anti-GBM disease and thereafter increased progressively; however, in contrast to the p38 pathway, JNK activation was not increased in tubules until late in the course of disease. These temporal differences with respect to activation in glomeruli and tubules raise the possibility that p38 MAPK is important as an early or acute inflammatory signal transduction pathway and that JNK MAPK is important in late or chronic inflammation.

Although we could demonstrate an increase in glomerular JNK activation at 3 hours by Western blotting, there was no apparent increase in the number of glomerular cells stained for p-JNK at this time point. Since there was no change in tubular JNK activation at 3 hours, the increase in glomerular JNK activation seen by Western blotting could not be due to tubular contamination (<5%) of the glomerular preparations. The

Fig. 8. Immunolocalization of phosphorylated p38 (p-p38) and phosphorylated JNK (p-JNK) to endothelial cells, podocytes, and myofibroblasts in rat anti-glomerular basement membrane (GBM) disease. Endothelial cells (A and B; gray) were immunostained for (A) p-p38 (brown; arrowheads) at 3 hours and (B) p-JNK (brown; arrowheads) at day 7 following induction of anti-GBM disease. Activated podocytes identified by desmin staining (C and D; blue) express both (C) p-p38 (brown; arrowheads) and (D) p-JNK (brown; arrowheads) at 28 days following induction of anti-GBM disease. Myofibroblasts identified by α-smooth muscle actin staining (E and F; gray) are immunostained for (E) p-p38 (brown; arrowheads) as well as (F) p-JNK (brown; arrowheads) at day 28 anti-GBM disease (magnification ×1000).

The discrepancy may be explained by a marked increase in JNK activation within the cells that already have some level of JNK activity in the normal glomerulus. Since the immunostaining technique uses substantial signal amplification, it is a semiquantitative rather than a quantitative technique and as such the signal generated is not proportional to the amount of antigen present.

In acute anti-GBM disease, the rapid increase in glomerular p-p38 seen at 3 hours was due to the activation of the pathway within podocytes, endothelial cells, infiltrating neutrophils, and tubular cells, whereas JNK activation was confined to podocytes. Podocytes become activated, either as a direct consequence of the initial glomerular insult or as a result of the inflammatory cytokine milieu produced by infiltrating neutrophils and macrophages. Podocytes themselves are an important source of IL-1 generation in rat crescentic nephritis [26–29]; however, the intracellular signaling mechanisms leading to podocyte activation are not understood. Activation of the p38 MAPK pathway in podocytes has been linked with apoptosis, but is not known to occur in resting podocytes [30]. This is the first demonstration of p38 and JNK activation within podocytes in diseased kidney.

p38 and JNK MAPK activation have previously been shown to be important in endothelial cell expression of cytokines and adhesion molecules in vitro [31–35]. We have demonstrated in an in vivo inflammatory model acute endothelial p38 activation and late or chronic endothelial JNK activation. Furthermore, activation of the p38, but not the JNK, MAPK was seen within neutrophils at this early time point. This differential staining pattern suggests that neutrophils become activated at least in part via the p38 but not the JNK MAPK pathway. This is consistent with in vitro studies that have demonstrated the importance of p38 activation to neutrophil functions such as chemotaxis, superoxide generation, cytokine production, adhesion, and degranulation [36–39] and that cytokines, chemotactic factors, and mitogens fail to stimulate JNK pathway activation within neutrophils, despite the presence of the JNK kinase [40, 41]. Our findings support a role for p38, but not JNK activation in acute renal inflammation. This is consistent with a study using a novel immunosuppressant drug with activity against the p38 pathway, which inhibited acute renal injury in the Wistar Kyoto (WKY) model of anti-GBM disease [42].

In chronic anti-GBM disease, p-p38 and JNK activation occurred in intrinsic glomerular cells such as activated podocytes and endothelial cells in addition to infiltrating macrophages, lymphocytes, and myofibroblasts. p-p38 and p-JNK colocalize to macrophages and lymphocytes within the glomerular tuft, glomerular crescent, and interstitium. One previous report has localized nuclear p-p38 to glomeruli in human crescentic glomerulonephritis, although the cell types stained positive for p-p38 were not described [43]. Activation of the p38 and JNK kinases has been demonstrated in vitro to be critical for macrophage and lymphocyte activation, and the production of proinflammatory cytokines [44–52]. Our study confirms p38 and JNK MAPK activation in these cell types in an in vivo inflammatory model. The presence of p38 and JNK MAPK activation in myofibroblasts during the sclerotic phase of the disease supports recent in vitro evidence that transforming growth factor-β (TGF-β1) induced fibrosis is in part dependent on activation of these pathways [53–56], and treatment with a p38 inhibitor has been shown to reduce fibrosis in a rat model of toxic lung injury [11]. Dual staining of macrophages, T cells, and myofibroblasts in progressive disease suggests a role for...
both pathways in chronic inflammation and fibrosis, and the propagation of renal injury.

This study has identified differential tubular activation of the p38 and JNK pathways at different stages of the inflammatory process. Tubular activation of p38 MAPK is increased in the acute phase of disease and involves all cortical tubular segments. In contrast, the increase in tubular JNK pathway activation occurs later in the disease, with a detectable increase in phosphorylation at days 7 and 28 anti-GBM disease, with all cortical tubular segments involved. Tubular epithelial cells are known to produce a variety of inflammatory mediators when stimulated and contribute to the development of interstitial injury. In vitro studies of human proximal convoluted tubular cells have demonstrated that IL-6 and TNF-α production is p38 dependent [57–59]. Furthermore, in this same cell type, arachidonic acid has been shown to stimulate phosphorylation of both the JNK and p38 MAPK and hyperglycaemia can induce phosphorylation of p38 and stimulate production and secretion of angiotensinogen in vitro [60–62]. Differential activation of these pathways in tubular cells within an in vivo inflammatory model has not been previously described and no studies to date have identified a role for these signal transduction pathways in tubular cells within renal disease.

We have demonstrated activation of the p38 and JNK MAPK pathways in both glomeruli and tubules within normal kidney and in accelerated crescentic glomerulonephritis. Some of the differences in activation of the pathways relate to the changes in the nature of the infiltrating leukocytes involved in renal injury, as well as temporal differences in activation in endothelial and tubular cells. Our findings suggest that the p38 pathway may be important in the acute phase of anti-GBM disease, whereas both the p38 and JNK pathways may be important in chronic anti-GBM disease. Inhibition of these stress activated pathways, therefore, has the potential to affect different stages of the disease process, and single or dual blockade of the p38 and the JNK MAPK pathways becomes an attractive treatment option for inflammatory kidney disease.

ACKNOWLEDGMENTS

This research was supported by grants from the Australian Kidney Foundation and the National Health and Medical Research Council of Australia.

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