c-Jun N-terminal kinase is crucially involved in renal tubulo-interstitial inflammation

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Nonstandard abbreviations:

<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CCR2</td>
<td>C-C motif chemokine receptor 2</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>DN</td>
<td>diabetic nephropathy</td>
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<td>eGFR</td>
<td>estimated glomerular filtration rate</td>
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<td>FGS</td>
<td>focal glomerulosclerosis</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
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<td>IF</td>
<td>interstitial fibrosis</td>
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<td>IL-1β</td>
<td>interleukin-1β</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>MAP kinase</td>
<td>mitogen activated protein kinase</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
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<td>MGP</td>
<td>membraneous glomerulopathy</td>
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<td>MME</td>
<td>mesangial matrix expansion</td>
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<td>mØ</td>
<td>macrophage accumulation</td>
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<td>MPGN</td>
<td>mesangioproliferative glomerulonephritis</td>
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<td>SMA</td>
<td>α-smooth muscle actin</td>
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<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<td>TGF-β</td>
<td>transforming growth factor-β</td>
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ABSTRACT

Chronic inflammation is a major outcome determinant in several renal disorders. Induction of MCP-1 (monocyte chemoattractant protein-1) expression in tubular epithelial cells importantly contributes to the recruitment of inflammatory cells from the circulation towards the damaged tubulo-interstitium. Since the MCP-1 gene contains several c-Jun binding sites, we hypothesized that the c-Jun N-terminal kinase (JNK) pathway regulates MCP-1 expression and, subsequently, tubulo-interstitial inflammation. This was investigated in cultured rat tubular epithelial cells (NRK-52E) and in the rat unilateral ischemia/reperfusion (I/R) model. In NRK-52E cells, the JNK inhibitor SP600125 reduced IL-1β-, TGF-β- or BSA-induced MCP-1 expression in a potent manner (up to 150-fold). In the rat I/R model, JNK activation was low in controls but induced in tubular cells from 30 min after I/R. The extent of JNK activation correlated with interstitial macrophage accumulation. Treatment with SP600125 (30 mg/kg/day i.p. for 4 days) reduced renal c-Jun activation, MCP-1, osteopontin and vimentin expression, and interstitial macrophage and T cell accumulation (all p<0.05). In human renal disease, we also found induction of JNK activation, which correlated strongly with interstitial macrophage accumulation, tubulointerstitial fibrosis, and renal function loss. In conclusion, these data indicate that the JNK pathway plays an important role in renal inflammation, at least partly through induction of MCP-1 gene expression in tubular epithelial cells.
INTRODUCTION

Renal inflammation, and in particular the accumulation of macrophages in the renal interstitium, plays an important role in the initiation and progression of kidney injury (Sean Eardley and Cockwell, 2005; van Goor H., et al., 1994). However, the mechanisms of interstitial macrophage attraction are only partly understood. Several studies indicate that the local expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) at sites of renal injury promotes macrophage adhesion and chemotaxis through ligation of the C-C motif chemokine receptor 2 (CCR2) (Furuichi, et al., 2003). Furthermore, MCP-1 and its receptor CCR2 are associated with a detrimental M1 macrophage response and experimental blockade of this pathway has been shown to reduce renal fibrosis (Kitagawa, et al., 2004). Moreover, studies in patients show that tubular MCP-1 is increased in progressive renal diseases (Grandaliano, et al., 1996), and that albuminuria is associated with MCP-1 and macrophage infiltration (Eardley, et al., 2006). Yet, the intracellular signaling pathways that are involved in MCP-1-mediated attraction of macrophages to the renal interstitium are largely unknown. Mitogen-activated protein (MAP) kinases are intracellular signal transduction molecules that are activated in response to several stimuli including oxidative stress and pro-inflammatory factors (de Borst, et al., 2006). MAP kinases modulate gene transcription, mainly through activation of transcription factors. Particularly the MAP kinase c-Jun N-terminal kinase (JNK) and its downstream transcription factor c-Jun have been associated with inflammation, for example in colitis (Assi, et al., 2006), asthma (Eynott, et al., 2003) and arthritis (Han, et al., 2001). Moreover, glomerular JNK activation has been demonstrated in experimental glomerulonephritis (Seto, et al., 1998). In addition, a recent study in the unilateral ureteral obstruction model showed that systemic JNK inhibition reduced renal fibrosis and tubular apoptosis (Ma, et al., 2007).
Given the central role of the JNK pathway in inflammatory processes, combined with the consistent finding that damaged tubular epithelial cells produce numerous inflammatory chemokines (such as MCP-1) and cytokines (such as IL-1, TNF-α), we hypothesized that JNK activation in tubular cells contributes to renal interstitial inflammation. Importantly, overexpression of c-Jun, a major transcription factor downstream of JNK, induces MCP-1 in endothelial cells (Wang, et al., 1999). We recently demonstrated that c-Jun inhibition decreases MCP-1 gene expression in human tubular epithelial cells (de Borst, et al., 2007a). Conversely, dominant-negative c-Jun gene therapy reduced myocardial MCP-1 expression in a model of cardiac hypertrophy (Kim-Mitsuyama, et al., 2006). Furthermore, the 5′-flanking region of the MCP-1 gene contains multiple AP-1 sites (Nakayama, et al., 2001), supporting the hypothesis that JNK regulates MCP-1 gene expression through the transcription factor c-Jun, which is an important part of the AP-1 complex.

We hypothesize that JNK regulates MCP-1 expression in renal disease and thereby interstitial damage. To substantiate this hypothesis, we first studied the effect of specific JNK inhibition on MCP-1 gene expression in cultured rat kidney tubular epithelial cells. Secondly, the pathophysiological role of JNK was investigated in the rat unilateral renal ischemia/reperfusion (I/R) model, which was chosen since it is characterized by tubular injury, tubular MCP-1 expression (Sung, et al., 2002), and interstitial macrophage accumulation. We studied the time course of renal JNK activation and administered the specific JNK inhibitor SP600125 (Bennett, et al., 2001;Bogoyevitch, et al., 2004) in this model to study effects on MCP-1 expression and influx of macrophages. The effect on T cell infiltration was also studied, given recent evidence on the involvement of T cells in renal ischemia/reperfusion injury (Burne, et al., 2001). Finally, we evaluated whether activated JNK is present in the kidney in human renal disorders, and if the degree of JNK activation correlates with the severity of (inflammatory) renal damage.
METHODS

Cell culture studies

Cell culture experiments were performed in NRK-52E rat tubular epithelial cells (American Type Culture Collection (ATCC), Manassas, VA, USA). Cells were grown in DMEM (Cambrex, Verviers, Belgium) containing 4.5 g/l glucose, supplemented with 2 mM L-glutamine, penicillin (100 U/ml) / streptomycin (100 μg/ml), and 5% fetal calf serum, in humidified air at 37 °C with 5% CO₂. Cells were seeded in 6-well plates until approximately 80% confluence; prior to each experiment cells were washed twice with HBSS and starved in serum-free medium for 24 h.

Cells were stimulated with human recombinant TGF-β1 (Roche Diagnostics, Almere, the Netherlands), recombinant rat IL-1β (R&D Systems, Abingdon, UK) or bovine serum albumin (BSA, Sigma, St. Louis, MO) under serum-free conditions. One hour before stimulation, cell were pre-incubated with either the JNK inhibitor SP600125 (Tocris), dissolved in DMSO, or vehicle (DMSO). Experiments were performed at least four times.

Rat renal ischemia/reperfusion studies

All experimental protocols for animal studies were approved by the Animal Ethics Committee of the University of Groningen. Activation of the JNK/c-Jun pathway was assessed in a rat model of unilateral renal ischemia/reperfusion (I/R). Normal male Wistar rats weighing 220-240 grams (Harlan, Horst, the Netherlands) were anaesthetized and the left renal artery and vein were clamped. After 45 min, clamps were removed and reperfusion of the kidney was observed before closing of the wound. Animals were sacrificed at 30 min, 90 min, 6 h, 24 h, 4 days, 9 days, 14 days and 21 days after reperfusion (n=6 per time point). Sham-operated animals (n=6) received the same surgical procedure except ischemia/reperfusion. Although
sham-operated animals at all time points may have been preferable, only one sham group, sacrificed at 6 hour after I/R, was used for ethical reasons. At sacrifice, kidneys were isolated after gently flushing with saline and preserved in 4 % formalin for preparation of paraffin-embedded sections.

Next, the effect of the specific JNK inhibitor SP600125 was evaluated in the rat unilateral I/R model. Wistar rats underwent I/R as described above. Rats were intraperitoneally injected with SP600125 (Tocris Bioscience, Bristol, UK, 30 mg/kg/day) dissolved in olive oil (n=9) or with vehicle alone (olive oil, n=8) at 2 h before I/R and at 24, 48, and 72 h after I/R. Animals were sacrificed at 4 d after I/R. Furthermore, two separate groups of rats (n=8 per group) underwent I/R and were similarly treated with SP600125 (30 mg/kg/day) or vehicle for the first 4 days following I/R, but were sacrificed at day 15 after I/R (no treatment was given from day 5 until sacrifice). Since daily injection of the JNK inhibitor for 15 days was considered unethical (and highly expensive), we selected this approach to study both early and later effects of treatment with the JNK inhibitor. Age-matched untreated Wistar rats were used to obtain reference values. Kidneys were isolated after gently flushing with saline and preserved in 4 % formalin for preparation of paraffin-embedded sections or snap-frozen in liquid nitrogen and stored at -80°C.

Patients

All procedures and use of anonymized tissue were performed according to Dutch national ethical guidelines. Renal biopsy specimens were obtained from 83 patients with various renal diseases, admitted to our hospital. Diagnoses were adjudged by a qualified pathologist, unrelated to the present study. Patients were selected to represent a variety of disorders: membranous glomerulopathy (n=8), membranoproliferative glomerulonephritis (n=6), focal glomerulosclerosis (n=8), IgA nephropathy (n=7), acute rejection (n=9), hypertension (n=6),
Wegener's granulomatosis (*n*=7), systemic lupus erythematosus (SLE, *n*=8), diabetic nephropathy (*n*=7) and minimal change disease (*n*=7). Unaffected parts of kidneys from patients with renal cell carcinoma, who were not on medication related to this disease, (*n*=8) were used as control specimens. Tissue was fixed in 4% paraformaldehyde and processed for paraffin embedding. Clinical parameters obtained at the time of biopsy were determined according to routine procedures.

**Realtime PCR**

Cultured cells were lysed and RNA was isolated using a Stratagene Minikit (Stratagene, La Jolla, CA). Rat renal cortex tissue was homogenized in lysis buffer and RNA was isolated using Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA). RNA content was measured by a nanodrop UV-detector (NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from similar amounts of RNA using Superscript III (Invitrogen, Carlsbad, CA). Primers were obtained from Sigma-Genosys (Haverhill, UK): monocyte chemoattractant protein-1 (5'-TCCTCCACCCTATGCAGGT-3' and 5'-TTCCCTATTGGGGTCAGCAC-3', 255 bp), procollagen-1α1 (5'-AGCCTGAGCCAGCAGATTGA-3' and 5'-CCAGGTTGCAGCCTTGGTTA-3', 145 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); 5'-CGCTGGTGCTGAGTATGTCG-3' and 5'-CTGTGGTCATGAGCCCTTCC-3', 179 bp. SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) was used as a fluorescent probe. The cDNA amplification was performed until 40 cycles. Finally, the threshold cycle number (Ct) was calculated for each gene and relative gene expressions were calculated after normalizing for the expression of the control gene GAPDH.
Antibodies

Primary antibodies specifically detecting phosphorylated JNK1/2/3, phosphorylated c-Jun (both Cell Signaling Technology), macrophages (rat: anti-ED1, human: anti-CD68; Serotec Ltd, Oxford, UK), osteopontin (anti-MPIIIB10, Developmental Studies Hydridoma Bank, Baltimore, MD) or vimentin (clone V9, DakoCytomation, Glostrup, Denmark) were used. For double-immunostaining, the following primary antibodies were used: anti-α-smooth muscle actin (SMA, clone 1A4, Sigma Chemical Co., St. Louis, MO, USA); anti-vimentin (DakoCytomation, Glostrup, Denmark); and anti-CD68. Binding was detected by incubation with horseradish peroxidase (HRP)-labeled polyclonals (Dakopatts, Glostrup, Denmark). For double-immunohistochemistry, alkaline phosphatase (AP)-labeled polyclonals (Dakopatts) were used.

Western Blot analysis

Frozen cortical tissue was lysed in ice-cold RIPA buffer (PBS containing 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS) with 10 µg/ml aprotinin, 1 mM orthovanadate and 10 mM NaF, placed on ice for 30 min, and homogenized. PMSF (10 mg/ml in isopropanol) was added, the lysate was placed on ice for 30 min and then centrifuged. Protein quantities were measured using pyrogallol red-molybdate. Similar amounts of lysate were separated on a 10% polyacrylamide gel and electroblotted onto a PVDF membrane. Blots were incubated for 60 min in blocking buffer (TBS-T (TBS, 0.1% Tween 20, pH 7.6) with 5% skimmed milk) and incubated overnight at 4 ºC with antibodies recognizing p-c-Jun or β-actin. Immunostaining was detected by incubation with HRP-conjugated secondary antibodies for 60 min. Blots were washed and membrane-bound antibodies were visualized using LumiGlo (Upstate, Charlottesville, VA).
**Immunohistochemistry**

Two-μm paraffin sections were dewaxed and antigen retrieval was achieved by incubation in 0.1 M Tris/HCl buffer (pH=9.0) overnight at 80°C. Endogenous peroxidase was blocked by incubation with 0.3% H₂O₂ in PBS for 30 min. Sections were incubated with the primary antibody (see above) for 60 min at room temperature. Binding was detected by sequential incubation with HRP-labellel secondary and tertiary antibodies, respectively, in the presence of 1% AB serum (Sigma). The peroxidase activity was visualised using 3,3’-diaminobenzidine tetrahydrochloride (DAB, DAKO) for 10 min; sections were counterstained with hematoxilin and mounted with Kaiser’s glycerin gelatin.

**Double-immunohistochemistry**

Paraffin sections were incubated with pJNK or pc-Jun antibodies for 60 min. Binding was detected by sequential incubation with appropriate secondary and tertiary horseradish peroxidase-labeled antibodies in the presence of 1% AB serum for 30 min; peroxidase activity was visualized using 3-amino-9-ethylcarbazole (AEC). The immunoreaction was stopped by incubation with 0.1 M glycine (pH=2.5) for 60 min. Then, sections were incubated for 60 min with the second primary antibody, followed by appropriate alkaline phosphatase (AP)-labeled antibodies. Endogenous AP was blocked with levamisole (Sigma). Specific AP activity was developed with Naphtol AS-MX phosphate and Fast Blue BB (Sigma).

**Quantification of immunostaining**

Since activated JNK (pJNK) was found in the nuclear and cytoplasmic compartments, we assessed glomerular and tubular pJNK by computerized morphometry (de Borst, et al., 2003). Per section, immunostaining within 30 glomeruli (excluding Bowman’s capsule) was measured and the mean pJNK staining per glomerulus was calculated. To quantify tubular
pJNK staining, thirty rectangular fields (magnification 200x) were selected and immunostaining was measured. Vascular and glomerular areas were excluded manually. The extent of tubulointerstitial osteopontin and vimentin immunostaining was determined similarly (de Borst, et al., 2007b).

Glomerular presence of the activated transcription factor c-Jun (pc-Jun), which was solely localized in the nucleus, was determined by blinded counting of pc-Jun-positive nuclei in glomerular cells within Bowman’s capsule (parietal epithelial cells not included). The mean glomerular pc-Jun score was calculated. Expression of nuclear pc-Jun in tubuli was blindly counted in square high-power (400x) fields, for 25 fields per biopsy, excluding glomerular and vascular areas. Numbers of pc-Jun+ cells per mm² were calculated. These quantification methods are similar to those used in previous studies on MAP kinase activation in human renal disease (Masaki, et al., 2004; Stambe, et al., 2004).

**Analysis of histopathological changes**

Renal biopsies, stained with hematoxylin and eosin, methanamine-silver, or periodic acid-Schiff (PAS), were blindly scored for glomerular mesangial matrix expansion (MME), focal glomerulosclerosis (FGS) and interstitial fibrosis by a qualified pathologist. MME was scored positive if broadening of mesangial areas was 2-3 times that of the mesangial width seen in glomeruli of control renal tissue. FGS was scored positive if collapse of capillary lumina, MME, hyalinosis and adhesion of the glomerular tuft to Bowman’s capsule were simultaneously present. Glomeruli were scored for MME and FGS as follows: unaffected glomeruli were scored as 0; if one glomerular quadrant was affected a score of 1 was given; two quadrants affected was scored as 2; 3 quadrants affected was scored as 3; if all quadrants were positive for MME or FGS, a score of 4 was given.
Interstitial fibrosis was scored positive when tubular atrophy and broadening of the peritubular compartment were simultaneously present. Scores of 0-4 were assigned: a score of 0 indicated no interstitial fibrosis, a score of 1 indicated 0-25% involvement of the total interstitial surface of the biopsy, a score of 2 indicated 25-50% involvement, a score of 3 indicated 50-75% involvement, and a score of 4 indicated 75-100% involvement.

**Statistical analysis**

Data are indicated as mean±standard error of the mean. Statistical differences between groups were calculated using the non-parametric Kruskal-Wallis test. Correlations were determined by Spearman's rank test for non-parametric variables. An alpha value of 0.05 was considered statistically significant. All analyses were performed using SPSS 12.0 (SPSS Inc, Chicago, USA).
RESULTS

**JNK inhibition reduces MCP-1 and procollagen-1α1 gene expression in NRK-52E cells**

In cultured rat tubular epithelial cells (NRK-52E), stimulation with IL-1β (20 ng/ml), TGF-β1 (10 ng/ml) or BSA (30 mg/ml) for 24 h potently induced MCP-1 expression (Figure 1A). MCP-1 gene expression also increased in response to TGF-β1 and BSA. Pre-incubation with the JNK inhibitor SP600125 (20 μM) strikingly reduced MCP-1 expression; IL-1β-induced MCP-1 induction was reduced 150-fold, and TGF-β1-induced MCP-1 expression was reduced to below control levels. Furthermore, the procollagen-1α1 gene was induced by IL-1β, TGF-β1 and BSA, and strongly reversed by SP600125 (Figure 1B). The JNK inhibitor alone reduced basal expression of MCP-1 and procollagen-1α1, however no increased cell death was observed in cells treated with SP600125 at the end of the experiment. The JNK inhibitor SP600125 dose-dependently reduced MCP-1 gene expression (Figure 1C).

**Time-course of JNK activation after unilateral renal ischemia/reperfusion (I/R)**

In the rat unilateral I/R model we found activation of JNK and the downstream transcription factor c-Jun within renal tubular epithelial cells, which displayed a biphasic pattern (Figure 2A). The JNK pathway was activated at 30 min after I/R; activation declined at 6 h, but increased even more after 24 h. Although pJNK was predominantly found in the nuclear compartment, in some cases the cytoplasm was positive as well. pJNK was mainly present in tubular epithelial cells, but also in some collecting duct cells, large arteries (within vascular smooth muscle cells and endothelial cells) and a limited number of glomerular and parietal glomerular epithelial cells (Figure 2B). The activated transcription factor c-Jun (pc-Jun), which is regulated by JNK, was present in the same cell types as pJNK, but restricted to the nuclear compartment. Both tubular pJNK staining and numbers of pc-Jun-positive cells
correlated with interstitial macrophage accumulation across all timepoints from 24 h on (Figure 3). Macrophages were mainly present in pJNK+ and pc-Jun+ areas.

**Effects of the JNK inhibitor SP600125 in the rat I/R model**

Next we studied the effects of systemic administration of the specific JNK inhibitor SP600125 on the development of renal damage in the I/R model. Treatment with either SP600125 (30 mg/kg/day i.p.) or vehicle was started 2 hrs before I/R and continued until 4 days after I/R. Two groups (SP600125 or vehicle, both n=8) were sacrificed at 4 days, and two similar groups at 15 days after I/R. Renal JNK activity, as represented by the amount of phosphorylated c-Jun in kidneys 4 days after I/R, was strongly induced compared with control renal tissue; this increase was significantly reduced by treatment with SP600125 (Figure 4). The JNK inhibitor reduced tubular injury, as indicated by a reduction in tubulointerstitial osteopontin expression at 15 days after I/R compared to vehicle-treated rats (Figure 5A). Moreover, tubular vimentin expression was significantly reduced by SP600125 at both time points (Figure 5B). In animals treated with vehicle, renal MCP-1 gene expression (Figure 6) was induced at 4 days after I/R and increased further at 15 days; SP600125 significantly reduced MCP-1 at 4 days (-40%, p<0.05), this blunting was even more pronounced at 15 days (-53%, p<0.001) after I/R compared to vehicle. Renal procollagen-1α1 gene expression levels showed a trend towards reduction in rats treated with SP600125: normal rats 1.00±0.1 (mean±standard error of the mean); I/R+vehicle after 4 days 1.17±0.2; I/R+SP600125 after 4 days 1.13±0.1; I/R+vehicle after 15 days 1.45±0.1 (p<0.05 vs normal); I/R+SP600125 after 15 days 1.27±0.1 (p=ns). SP600125 strongly reduced interstitial accumulation of macrophages (Figure 7A,B) and T cells (Figure 8A,B) at 4 days. Circulating white blood cell numbers were not affected by JNK inhibition (data not shown).
JNK activation in human renal disease

Activated JNK (phosphorylated JNK, pJNK) was studied in renal biopsies from patients with various renal disorders (Table 1). In human control renal tissue, activated JNK was found in limited numbers of tubular epithelial cells (Figure 9). Very weak pJNK staining was also present in a low number of glomerular cells and large arteries, in vascular smooth muscle cells and endothelial cells. In contrast, pJNK was induced in both glomerular and tubular epithelial cells in all human renal diseases studied here (membranous glomerulopathy, membranoproliferative glomerulonephritis, focal glomerulosclerosis (FGS), IgA nephropathy, acute rejection, hypertension, Wegener's granulomatosis, SLE, diabetic nephropathy and minimal change disease), although considerable variation in pJNK expression levels was present among the disorders. (Figures 9,10). In some diseases, e.g. diabetic nephropathy, FGS, IgA nephropathy, membranoproliferative glomerulonephritis, SLE and Wegener’s disease, glomerular JNK activation was more abundant than in hypertensive nephropathy, acute rejection and minimal change disease. Tubular JNK activation parallels glomerular JNK activation in a number of disorders, but was relatively low in IgA nephropathy, minimal change and SLE, and relatively high in acute rejection. The extent of tubular pJNK staining strongly correlated with interstitial macrophage accumulation (Figure 11A), loss of renal function (Figure 11B), and tubulointerstitial fibrosis (Figure 11C) across all diseases, but glomerular pJNK did not correlate significantly with proteinuria. Double-immunostaining for pJNK and CD68 revealed that pJNK was localized in tubular cells surrounded by areas of interstitial macrophage accumulation (Figure 11D). Glomerular JNK activation correlated with MME (r=0.324, p<0.01), FGS (0.368, p<0.01) and IF (0.415, p<0.01); the relationship between glomerular JNK activation and eGFR showed a trend to correlation (r=-0.204, p=0.2). In addition, tubular pJNK expression correlated with MME (r=0.256, p<0.05) and FGS (0.320, p<0.01). In human renal disease, pJNK was also present in collecting duct cells,
and in endothelial and vascular smooth muscle cells of arteries. Specificity of pJNK immunostaining was confirmed using a pJNK blocking peptide which abrogated immunostaining (Figure 9). Appropriate PBS controls were consistently negative.
DISCUSSION

This study indicates that the JNK signaling pathway is involved in renal tubulo-interstitial inflammation. Pharmacological blockade of JNK activation in cultured tubular epithelial cells strongly reduced MCP-1 gene expression, suggesting that the inflammatory effects of JNK signaling are at least in part mediated by MCP-1. Inhibition of the JNK pathway in the rat unilateral ischemia/reperfusion model reduced tubular injury, MCP-1 expression, and the interstitial accumulation of macrophage and T cells. Furthermore, the JNK pathway was strongly activated in human renal disease, in association with renal inflammation, interstitial fibrosis and decline of renal function.

In cultured tubular epithelial cells, we found that JNK modulates MCP-1 gene expression induced by IL-1β, TGF-β or BSA (Prakash, et al., 2006). This is in line with data from genetic studies, indicating binding sites for the JNK target molecule c-Jun in the flanking region of the MCP-1 gene (Nakayama, et al., 2001). Overexpression of c-Jun induces MCP-1 gene expression (Wang, et al., 1999). JNK is also involved in MCP-1 expression in other cell types including renal mesangial cells (Kawano, et al., 2003), and vascular smooth muscle cells (Chen, et al., 2004), but previous data on the involvement of JNK in MCP-1 expression in cultured human and mouse tubular epithelial cells have been conflicting (Sengul, et al., 2003; Tsuboi, et al., 2002).

In rat unilateral renal I/R injury, activated JNK and c-Jun were expressed by tubular epithelial cells already 30 minutes after I/R, in line with previous studies in uni- or bilateral ischemia/reperfusion (Park, et al., 2001). The extent of JNK activation in tubular cells correlated with interstitial macrophage accumulation, suggesting involvement in renal inflammation. The time course of JNK and c-Jun activation in the I/R model appears biphasic. Although difficult to interpret, we speculate that JNK/c-Jun activation within the first hours
after I/R is associated with the recruitment of macrophages. The second peak, which is sustained until at least 3 weeks after I/R, may be associated with the local inflammatory response or may, for example, be related to renal repair in response to I/R.

Intervention with the JNK inhibitor SP600125 attenuated renal c-Jun activation, demonstrating pharmacological efficacy of the inhibitor at the used dose. Specificity of SP600125 for JNK over, for example, ERK and p38 has been shown previously (Bennett, et al., 2001). Tubular expression of the chemoattractant osteopontin and the dedifferentiation marker vimentin was attenuated by the JNK inhibitor, indicating that JNK inhibition reduced tubular injury. JNK inhibition also reduced I/R damage in other organ systems (Ishii, et al., 2004; Uehara, et al., 2005). Osteopontin protein expression was decreased at 15 days, but not at 4 days after I/R injury. This suggests that JNK does not directly regulate osteopontin but rather provides renoprotection through other mechanisms, which results in downregulation of osteopontin at 15 days post-I/R. Another explanation may be that the time course of osteopontin expression differs among proximal and distal tubular cells (Persy, et al., 1999); JNK may preferentially reduce proximal tubular osteopontin. This could also explain the difference between osteopontin and vimentin expression in response to JNK inhibition. Our data, indicating a tubulo-interstitial protective effect of JNK inhibition, are in line with other studies showing that JNK mediates IL-1β-mediated tubular transdifferentiation in HK-2 human tubular epithelial cells (Zhang, et al., 2005). Moreover, in unilateral ureteral obstruction, JNK inhibition reduced tubulo-interstitial fibrosis (Ma, et al., 2007). In addition, JNK inhibition reduces apoptosis of tubular epithelial cells in I/R and in unilateral ureteral obstruction (Ma, et al., 2007; Wang, et al., 2007).

Along with inhibition of MCP-1 gene expression, treatment with SP600125 substantially reduced interstitial macrophage accumulation in post-ischemic kidneys. Importantly, upon treatment with SP600125 during the first four days, expression of the MCP-1 gene and
tubulointerstitial macrophage accumulation did not increase between day four and fifteen. Oppositely, in vehicle-treated rats, MCP-1 gene expression and macrophage accumulation were further increased at 15 days after I/R. These data, and the time course, support the relevance of the JNK pathway in the recruitment of macrophages, and hence in renal injury, during the first days after injury. In a previous study, bone marrow-derived macrophages were treated with SP600125 and injected in rats treated with anti-glomerular basement membrane serum (Ikezumi, et al., 2004). Surprisingly, in that model, SP600125 did not affect renal macrophage accumulation; however it strongly reduced proteinuria and glomerular cell proliferation. The fact that JNK inhibition in macrophages did not inhibit their influx (Ikezumi et al.) is in agreement with our findings indicating that JNK mediates MCP-1 production in cultured tubular epithelial cells. We could not detect activated JNK in renal macrophages, neither in the I/R model, nor in human renal disease. Of interest, SP600125 also reduced infiltration of T cells, which play an important role in ischemia/reperfusion injury (Burne, et al., 2001). JNK may regulate T cell influx either directly (possibly even through MCP-1 (Brown, et al., 2007)), or indirectly via effects on macrophages.

JNK inhibition resulted in a trend towards reduction of procollagen-1α1 mRNA expression in I/R, as opposed to the strong effects observed in cultured rat tubular epithelial cells. Procollagen production may be considered a marker for renal fibrosis, although collagen production is also considered crucial to the renal repair process. This could explain the difference between findings in cultured cells and in the I/R model: it is plausible that I/R-induced injury initiated collagen production as a repair mechanism (Cochrane, et al., 2005). Thus, it could be that JNK inhibition on the one hand reduced procollagen-1α1 expression in tubular epithelial cells (anti-fibrotic), but on the other hand promoted repair (and thus procollagen expression) in other cell types.
Across human renal disorders, we found strong correlations between tubular JNK activation and interstitial macrophage accumulation, interstitial fibrosis and reduced estimated GFR. These findings are in line with data from our previous study in which activation of the downstream transcription factor c-Jun strongly correlated with severity of renal disease (de Borst, et al., 2007a). Subtle but significant JNK activation was found in diseases with limited or no structural abnormalities such as minimal change disease, suggesting that JNK activation is an early event in renal injury. Alternatively, chronic exposure to albumin, which is known to cause injury to tubular cells (Caruso-Neves, et al., 2006), may activate the JNK pathway, explaining our results in patients with minimal change disease. Similarly, we also found subtle activation of the transcription factor c-Jun, a major target of JNK, in minimal change disease (de Borst, et al., 2007a). JNK activation did not significantly correlate with proteinuria; this may be explained by the diversity of the population (which was analyzed across diseases) and by the fact that many patients were on antiproteinuric treatment, possibly dissociating the amount of proteinuria from the ongoing intrarenal pathophysiological processes. In line with the latter assumption, in animal models dissociation between reduction of proteinuria and ongoing tubulo-interstitial damage has been demonstrated during antiproteinuric therapy (Hamming, et al., 2006). JNK activation was generally more prominent in tubular than in glomerular cells, moreover, tubular JNK activation was stronger associated with the severity of renal damage.

In conclusion, the current study suggests that JNK activation in tubular cells plays a role in tubulointerstitial inflammation in response to I/R injury. Given the central role of interstitial macrophage accumulation in renal pathology (Sean Eardley and Cockwell, 2005; van Goor H., et al., 1994) and the profound JNK activation in human renal disease, JNK inhibition may be a suitable strategy in renal diseases, especially those in which macrophages or T cells play a central role.
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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. JNK inhibition strongly reduces MCP-1 expression in cultured rat tubular epithelial cells in a dose-dependent manner

Effects of the JNK inhibitor SP600125 on gene expression in cultured rat tubular epithelial cells (NRK-52E). Cells were pre-incubated with SP600125 (20 μM) or vehicle (DMSO) for 1 h and then stimulated with IL-1β (20 ng/ml), bovine serum albumin (BSA, 30 mg/ml) or TGF-β (10 ng/ml) for 24 h. Expression of (A) MCP-1 or (B) procollagen-1α1 was determined by real-time PCR. JNK inhibition strongly reduced MCP-1 or procollagen-1α1 gene expression, which was induced by all stimuli.

(C) Dose response curve. NRK-52E cells were stimulated with IL-1β, after pre-incubation with medium, or increasing doses (as indicated) of the JNK inhibitor SP600125, respectively. MCP-1 gene expression was determined by real-time PCR. A dose-dependent effect of the JNK inhibitor on MCP-1 gene expression can be observed.

Figure 2. Time-course of tubular JNK and c-Jun activation in rat unilateral ischemia/reperfusion

(A) Tubular JNK and c-Jun activation at several timepoints after unilateral ischemia/reperfusion (I/R), quantified by counting pJNK or pc-Jun positive tubular epithelial cells per tubulointerstitial field following immunohistochemistry. Already at 30 min, activation of JNK and c-Jun is induced, then, their activities decline at 6 hours after I/R. 24 hours post-I/R, activities of both JNK and c-Jun are again strongly induced. Note the increased interstitial accumulation of macrophages (gray) from 24 hours after I/R, which parallels induction of tubular JNK and c-Jun activation. At all time points, pJNK and pcJun expression are significantly increased compared to sham (p<0.05). (B) Representative images
of renal pJNK (upper images) and pc-Jun (lower images) immunostaining at several time points after I/R. Note that pJNK is found in the nucleus and the cytoplasm, whereas the activated transcription factor pc-Jun is only expressed inside the nucleus.

Figure 3. Correlations between tubular JNK/c-Jun activation and interstitial macrophage accumulation

(A) Scatter diagram illustrating the correlations between interstitial macrophage accumulation and tubular pJNK (open squares) or pc-Jun (triangles) expression in the rat unilateral ischemia/reperfusion model. In this analysis, data from several time points ≥24 h after I/R, when the interstitial accumulation of macrophages is induced, were included. Interstitial macrophage accumulation correlated with tubular expression of pJNK and pc-Jun, respectively. (B) Double-immunohistochemistry identified pJNK (upper images, brown staining) or pc-Jun (lower images, brown) immunostaining in tubular epithelial cells within areas of interstitial macrophage accumulation (mø, blue). Magnifications: 200x (left images) and 400x (right images).

Figure 4. JNK inhibition reduces renal c-Jun activation in rat unilateral ischemia/reperfusion

Rats were treated with the specific JNK inhibitor SP600125 (30 mg/kg/day) from 2 h before induction of I/R until 4 days after I/R. The presence of phosphorylated c-Jun within renal lysates was determined by Western Blotting (representative bands are shown). Band immunostaining was quantified by densitometry. Values represent the mean band intensities of pc-Jun / β-actin measured in the same sample. In rats treated with vehicle, c-Jun phosphorylation was induced compared to normal kidney tissue, which was reversed by SP600125.
Figure 5. The JNK inhibitor SP600125 ameliorates tubular osteopontin and vimentin expression in the rat unilateral ischemia/reperfusion model

(A) Tubular expression of the chemoattractant osteopontin was induced in I/R rats. Animals treated with the JNK inhibitor SP600125 showed similar osteopontin expression compared to vehicle at 4 days after I/R. At 15 days after I/R, however, the expression of osteopontin was significantly reduced in the SP600125-treated animals, compared to vehicle-treated rats. The reduction between 4 and 15 days was significant in SP600125-treated rats (p=0.01), not in vehicle-treated rats (p=0.09).

(B) Expression of the tubular dedifferentiation marker vimentin is strongly induced in vehicle-treated rats after I/R in comparison with normal rats. JNK inhibition significantly reduced vimentin expression at both 4 and 15 days after induction of I/R.

Figure 6. JNK inhibition reduces renal MCP-1 gene expression in the rat unilateral ischemia/reperfusion model

Renal MCP-1 gene expression after I/R in rats treated with SP600125 or vehicle. Rats were treated for 4 days with SP600125 (30 mg/kg/day) or vehicle and were sacrificed at 4 or 15 days after I/R. Normal Wistar rats were used as controls. Renal MCP-1 gene expression was induced at 4 days and was increased further at 15 days after I/R in vehicle-treated rats; treatment with SP600125 strongly reduced renal MCP-1 gene expression at 4 days, moreover, MCP-1 remained stable at 15 days after I/R in rats treated with SP600125.
Figure 7. JNK inhibition reduces interstitial macrophage accumulation in the rat unilateral ischemia/reperfusion model

(A) Interstitial macrophage accumulation in the unilateral I/R model. In comparison with normal kidney tissue, interstitial macrophage accumulation was increased in rats treated with vehicle at 4 days after I/R and even more pronounced at 15 days. SP600125 reduced interstitial macrophage accumulation at 4 and (even stronger) at 15 days.

(B) Representative images of immunohistochemistry for the rat macrophage marker ED-1 (counterstained with period acid-Schiff), illustrating the striking reduction of interstitial macrophage accumulation in I/R rats treated with SP600125 (right images) compared with vehicle-treated rats (left images). Rats were sacrificed either 4 (upper images) or 15 (lower images) days after induction of I/R. Original magnification: 100x

Figure 8. JNK inhibition reduces interstitial T cell accumulation in the rat unilateral ischemia/reperfusion model

(A) Interstitial T cell accumulation in the unilateral I/R model. In comparison with normal kidney tissue, interstitial T cell accumulation was increased in rats treated with vehicle at 4 days after I/R and even more pronounced at 15 days. SP600125 reduced interstitial T cell accumulation at 4 and (even stronger) at 15 days.

(B) Representative images of immunohistochemistry for the rat T cell marker CD3 (counterstained with hematoxillin), which illustrates the reduction of interstitial T cell accumulation in I/R rats treated with SP600125 (right images) compared with vehicle-treated rats (left images). Rats were sacrificed either 4 (upper images) or 15 (lower images) days after induction of I/R. Original magnification: 100x
Figure 9. Phosphorylated JNK (pJNK) immunohistochemistry in human renal disease

Representative images of pJNK immunostaining (brown precipitate) in human renal disease. (A) In control renal tissue, pJNK was weakly expressed in a limited number of tubular epithelial cells only (arrows). (B) pJNK expression is induced in a patient with diabetic nephropathy (DN) in tubular epithelial cells, glomerular cells, and in vascular smooth muscle and endothelial cells. (C) In a patient with IgA nephropathy, pJNK was also strongly induced in tubular epithelial cells and glomerular cells. (D) Section of a patient with Wegener's disease showing strong tubular pJNK immunostaining. In addition, some interstitial cells are pJNK-positive, which was the case in a limited number of biopsies showing abundant tubular pJNK. (E) Serial section to Figure 1d, showing abrogation of immunostaining when the primary antibody was incubated with a pJNK blocking peptide before application to the section.

Figure 10. JNK activation levels in human renal disorders

Levels of renal JNK activation in various human renal disorders. Both in glomerular and tubular cells, pJNK expression is significantly increased in all renal disorders compared to controls, although there are considerable differences among the disorders. Tubular JNK activation parallels glomerular JNK activation in a number of disorders, but is relatively low in IgA nephropathy, minimal change and SLE, and relatively high in acute rejection.

Figure 11. Association between the extent of JNK activation and parameters of renal damage in human renal disease

(A) Strong correlation between tubular pJNK immunostaining correlated and interstitial macrophage accumulation in human renal disease. (B) Strong association between tubular pJNK expression and loss of estimated glomerular filtration rate (eGFR). Since the decay was
exponential, we transformed JNK activation to [1/(tubular pJNK)] on the y-axis. Nonlinear regression analysis revealed an \( r^2 \) of 0.449 indicating a very strong association between tubular pJNK expression and renal function loss. (C) Relationship between tubular pJNK expression and tubulointerstitial fibrosis (TIF). Data are presented as mean number of pJNK+ tubular cells per tubulointerstitial field, categorized by TIF score (ranging 0-4). (D) Double-immunohistochemistry for pJNK (brown) and macrophages (blue), illustrating that pJNK expression in tubular epithelial cells was mainly present in areas of macrophage accumulation. Additional macrophages surrounding pJNK+ cells may have been present but could not be visualized due to the 2D aspect of the image. Magnification 400x. Magnification ±800x. Renal disease patients are indicated as closed boxes, controls as open circles.
**TABLES**

**Table 1**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Age (years)</th>
<th>Gender (M/F)</th>
<th>Serum creatinine (μmol/l)</th>
<th>Proteinuria (g/d)</th>
<th>eGFR (ml/min)</th>
<th>IF</th>
<th>Interstitial mø</th>
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<tbody>
<tr>
<td>Control</td>
<td>60±5</td>
<td>4/4</td>
<td>91±8</td>
<td>–</td>
<td>72±8</td>
<td>0.1±0.1</td>
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<td>57±4</td>
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<td>4.5±1.1</td>
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<td>6.8±1.2</td>
<td>36±10</td>
<td>2.8±0.6</td>
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<td>173±52</td>
<td>3.8±0.9</td>
<td>76±8</td>
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<td>MGP</td>
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<td>6/2</td>
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<td>58±12</td>
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<td>Minimal change</td>
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<td>62±8</td>
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<tr>
<td>Acute rejection</td>
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Clinical and histopathological parameters of the studied patient population. DN = diabetic nephropathy, FGS = focal glomerulosclerosis, IgA = IgA nephropathy, MGP = membraneous glomerulopathy, MPGN = mesangioproliferative glomerulonephritis, SLE = systemic lupus erythematosus, eGFR = estimated glomerular filtration rate, IF = interstitial fibrosis, mø = macrophage accumulation.
Figure 2

A

Tubular pJNK/pc-Jun expression

Intersitial macrophages per tubulointerstitial field

B

30 min after I/R  
4 d after I/R  
21 d after I/R

pJNK

pc-Jun
Figure 3

A

Tubular cells expressing p-JNK or pc-Jun per tubulointerstitial field

Interstitial macrophages per tubulointerstitial field

- pJNK
  - $r = 0.533$
  - $p < 0.01$
  - $n = 25$

- pc-Jun
  - $r = 0.401$
  - $p < 0.05$
  - $n = 25$

B

Images showing distribution of pJNK and pc-Jun expression.

- pJNK/mø
- pc-Jun/mø
Figure 4

![Graph showing pc-Jun expression](image)

- Normal
- I/R + vehicle
- I/R + SP600125

pc-Jun expression (arbitrary units)

- p<0.01
- p<0.01

![Western blot images](image)

- pc-Jun
- β-actin

- Normal kidney
- I/R + vehicle
- I/R + SP600125
Figure 5

A

![Bar graph showing osteopontin protein expression](chart1)

- Normal
- Vehicle
- SP600125

4 days after I/R

15 days after I/R

Significance levels: p<0.01, p<0.001

B

![Bar graph showing vimentin protein expression](chart2)

- Normal
- Vehicle
- SP600125

4 days after I/R

15 days after I/R

Significance levels: p<0.05
Figure 7

A

![Bar graph showing Macrophages / tubulointerstitial field](image)

- **Normal**
- **Vehicle**
- **SP600125**

- 4 days after I/R
- 15 days after I/R

**p-values:**
- P < 0.01
- P = 0.01
- P = 0.001

B

![Images of kidney sections](image)

- 4d-vehicle
- 4d-SP600125
- 15d-vehicle
- 15d-SP600125
Figure 8

A

![Bar chart showing T cells per tubulointerstitial field for normal, vehicle, SP600125 at 4 days and 15 days after I/R.](image)

B

![Images of tissue samples stained for T cells, showing 4 days vehicle, 4 days SP600125, 15 days vehicle, 15 days SP600125.](image)
Figure 10

Glomerular pJNK
- Controls
- DN
- FGS
- Hypt
- IgA
- MGP
- MIN
- MPGN
- SLE
- AR
- Weg

Tubulointerstitial pJNK

all groups p ≤ 0.05 vs controls

Graph showing the expression levels of glomerular and tubulointerstitial pJNK in different conditions: Controls, DN, FGS, Hypt, IgA, MGP, MIN, MPGN, SLE, AR, Weg.
Figure 11

(A) pJNK expression vs interstitial macrophages

- $r^2 = 0.430$
- $p < 0.0001$
- $n = 64$

(B) $1/(pJNK)$ vs estimated GFR

- $r^2 = 0.440$ (non-linear regression)
- $p < 0.001$
- $n = 36$

(C) pJNK vs tubulointerstitial fibrosis

- $p < 0.01$

(D) Image of tubulointerstitial fibrosis

- Tubulointerstitial fibrosis score:
  - 0
  - 1
  - 2
  - 3
  - 4