c-Jun NH₂-Terminal Kinase Is Crucially Involved in Renal Tubulo-Interstitial Inflammation

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ABSTRACT

Chronic inflammation is a major outcome determinant in several renal disorders. Induction of monocyte chemoattractant protein (MCP)-1 expression in tubular epithelial cells contributes importantly to the recruitment of inflammatory cells from the circulation toward the damaged tubulo-interstitium. Because the MCP-1 gene contains several c-Jun binding sites, we hypothesized that the c-Jun NH₂-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 (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. 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 in part through induction of MCP-1 gene expression in tubular epithelial cells.

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 (Van Goor et al., 1994; Sean Eardley and Cockwell, 2005). However, the mechanisms of interstitial macrophage attraction are only partially understood. Several studies indicate that the local expression of monocyte chemoattractant protein (MCP)-1/CCL2 at sites of renal injury promotes macrophage adhesion and chemotaxis through ligation of the C-C motif chemokine receptor 2 (Furuichi et al., 2003). Furthermore, MCP-1 and its receptor chemokine receptor 2 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 proinflammatory factors (de Borst et al., 2006). MAP kinases modulate gene transcription, mainly through activation of transcription factors. In particular, the MAP kinase c-Jun NH₂-terminal kinase (JNK) and its downstream transcription factor

ABBREVIATIONS: MCP, monocyte chemoattractant protein; MAP, mitogen-activated protein; JNK, c-Jun NH₂-terminal kinase; IL, interleukin; I/R, ischemia/reperfusion; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one-1,9-pyrazoloanthrone; TGF, transforming growth factor; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; SLE, systemic lupus erythematosus; PCR, polymerase chain reaction; HRP, horseradish peroxidase; AP, alkaline phosphatase; PBS, phosphate-buffered saline; pc-Jun, activated (phosphorylated) c-Jun; pJNK, activated (phosphorylated) c-Jun NH₂-terminal kinase; MME, mesangial matrix expansion; FGS, focal glomerulosclerosis.
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 and tumor necrosis factor-
), we hypothesized that JNK activation in tubular cells contributes to renal interstitial inflammation. It is important to note that overexpression of c-Jun, a major transcription factor downstream of JNK, induces MCP-1 in endothelial 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-Mitsuayama et al., 2006). Furthermore, the 5′-flanking region of the MCP-1 gene contains multiple activator protein-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 activator protein-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. Second, the pathophysiological role of JNK was investigated in the rat unilateral renal ischemia/reperfusion (I/R) model, which was chosen because 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 (Burren et al., 2001). Finally, we evaluated whether activated JNK is present in the kidney in human renal disorders and whether the degree of JNK activation correlates with the severity of (inflammatory) renal damage.

**Materials and Methods**

**Cell Culture Studies.** Cell culture experiments were performed in NRK-52E rat tubular epithelial cells (American Type Culture Collection, Manassas, VA). Cells were grown in Dulbecco’s modified Eagle’s medium (Lonza Verviers SPRL, 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 six-well plates until approximately 80% confluence; before each experiment, cells were washed twice with Hank’s balanced salt solution 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-Aldrich, St. Louis, MO) under serum-free conditions. One hour before stimulation, cell were preincubated with either the JNK inhibitor SP600125 (Tocris Bioscience, Bristol, UK), 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 (Groningen, The Netherlands). Activation of the JNK/c-Jun pathway was assessed in a rat model of unilateral renal I/R. Normal male Wistar rats weighing 220 to 240 g (Harlan, Horst, The Netherlands) were anesthetized, and the left renal artery and vein were clamped. After 45 min, clamps were removed and 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/group/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 h 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 (30 mg/kg/day) (Tocris Bioscience) 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 days after I/R. Furthermore, two separate groups of rats (n = 8/group) underwent I/R and were similarly treated with SP600125 (30 mg/kg/day) or vehicle for the first 4 days after I/R but were sacrificed at day 15 after I/R (no treatment was given from day 5 until sacrifice). Because 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.

**Real-Time PCR.** Cultured cells were lysed and RNA was isolated using a Stratagene Mini Kit (Stratagene, La Jolla, CA). Rat renal cortex RNA was isolated using Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA). A 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) and cDNA was reverse transcribed into cDNA using a Stratagene Mini Kit (Stratagene, La Jolla, CA). Rat renal cortex tissue was homogenized in lysis buffer and RNA was isolated using a Qiagen Mini Kit (Qiagen, Hilden, Germany) and preserved in 4% formalin for preparation of paraffin-embedded sections or snap-frozen in liquid nitrogen and stored at −80°C.

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Finally, the threshold cycle number was calculated for each
gene, and relative gene expressions were calculated after normalizing for the expression of the control gene glyceraldehyde-3-phosphate dehydrogenase.

**Antibodies.** Primary antibodies specifically detecting phosphorylated JNK1/2/3, phosphorylated c-Jun (both from Cell Signaling Technology Inc., Danvers, MA); macrophages (rat, anti-ED1; human, anti-CD68; Serotec, Oxford, UK); osteopontin (anti-MPIIIB10; Developmental Studies Hybridoma Bank, Baltimore, MD); or vimentin (clone V9, DakoCytomation, Glostrup, Denmark) were used. For double immunostaining, the following primary antibodies were used: anti-a-smooth muscle actin (clone 1A4; Sigma-Aldrich); anti-vimentin (Dako Denmark A/S, Glostrup, Denmark); and anti-CD68. Binding was detected by incubation with horseradish peroxidase (HRP)-labeled polyclonal antibodies (Dakopatts, Glostrup, Denmark). For double immunohistochemistry, alkaline phosphatase (AP)-labeled polyclonal antibodies (Dakopatts) were used.

**Western Blot Analysis.** Frozen cortical tissue was lysed in ice-cold radioimmunoprecipitation assay 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. Phenylmethylsulfonyl fluoride (10 mg/ml in isopropanol) was added, and 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 polyvinylidene difluoride membrane. Blots were incubated for 60 min in blocking buffer (Tris-buffered saline and 0.1% Tween 20, pH 7.6, with 5% skimmed milk) and incubated overnight at 4°C with antibodies recognizing activated (phosphorylated) c-Jun (pc-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 reagent (Millipore, Billerica, MA).

**Immunohistochemistry.** Two-micrometer 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-labeled secondary and tertiary antibodies, respectively, in the presence of 1% AB serum (Sigma-Aldrich). The peroxidase activity was visualized using 3,3′-diaminobenzidine tetrahydrochloride (Dako Denmark A/S) for 10 min; sections were counterstained with hematoxylin and mounted with Kaiser’s glycercin gelatin.

**Double Immunohistochemistry.** Paraffin sections were incubated with activated (phosphorylated) JNK (pJNK) or pc-Jun antibodies for 60 min. Binding was detected by sequential incubation

![Fig. 1](https://example.com/fig1.png)

**Fig. 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 preincubated with SP600125 (20 µM) or vehicle (DMSO) for 1 h and then stimulated with IL-1β (20 ng/ml), BSA (30 mg/ml), or TGF-β (10 ng/ml) for 24 h. Expression of MCP-1 (A) or procollagen-1α1 (B) 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 preincubation 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.
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. 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 AP-labeled antibodies. Endogenous AP was blocked with levamisole (Sigma-Aldrich). Specific AP activity was developed with Naphtol AS-MX phosphate and Fast Blue BB (Sigma-Aldrich).

Quantification of Immunostaining. Because 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, 30 rectangular fields (magnification 200×/H11003) 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 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 (400×) fields, for 25 fields per biopsy, excluding glomerular and vascular areas. Numbers of pc-Jun+ cells per square millimeter 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, methenamine-silver, or periodic acid-Schiff, 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 two to three 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; three quadrants affected was scored as 3; and if all quadrants were positive for MME or FGS, a score of 4 was given.

**Fig. 2.** Time course of tubular JNK and c-Jun activation in rat unilateral ischemia/reperfusion. A, tubular JNK and c-Jun activation at several time points after unilateral I/R, quantified by counting pJNK- or pc-Jun-positive tubular epithelial cells per tubulointerstitial field after immunohistochemistry. Already at 30 min, activation of JNK and c-Jun is induced, and then their activities decline at 6 h after I/R. Twenty-four 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 h after I/R, which parallels induction of tubular JNK and c-Jun activation. At all time points, pJNK and pc-Jun expression are significantly increased compared with sham (p < 0.05). B, representative images of renal pJNK (top images) and pc-Jun (bottom 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.

**Fig. 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. Intestinal macrophage accumulation correlated with tubular expression of pJNK and pc-Jun, respectively. B, double immunohistochemistry identified pJNK (top images, brown staining) or pc-Jun (bottom images, brown) immunostaining in tubular epithelial cells within areas of interstitial macrophage accumulation (mø; blue). Magnifications, 200× (left images) and 400× (right images).
Interstitial fibrosis was scored positive when tubular atrophy and broadening of the peritubular compartment were simultaneously present. Scores of 0 to 4 were assigned: a score of 0 indicated no interstitial fibrosis, a score of 1 indicated 0 to 25% involvement of the total interstitial surface of the biopsy, a score of 2 indicated 25 to 50% involvement, a score of 3 indicated 50 to 75% involvement, and a score of 4 indicated 75 to 100% involvement.

**Statistical Analysis.** Data are indicated as mean ± S.E.M. Statistical differences between groups were calculated using the non-parametric Kruskal-Wallis test. Correlations were determined by Spearman's rank test for nonparametric variables. An α value of 0.05 was considered statistically significant. All analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL).

**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 (Fig. 1A). MCP-1 gene expression also increased in response to TGF-β1 and BSA. Preincubation 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 (Fig. 1B). The JNK inhibitor alone induced 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 (Fig. 1C).

**Time Course of JNK Activation after Unilateral Renal 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 (Fig. 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 it also was present 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 (Fig. 2B). p-c-Jun, which is regulated by JNK, was present in the same cell types as pJNK, but it was restricted to the nuclear compartment. Both tubular pJNK staining and numbers of pc-Jun-positive cells correlated with interstitial macrophage accumulation across all time points from 24 h on (Fig. 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 h before I/R and continued until 4 days after I/R. Two patterns emerged (Fig. 4A). SP600125 treatment significantly reduced renal c-Jun activation in rat unilateral ischemia/reperfusion (I/R) model. The presence of phosphorylated c-Jun within renal lysates was simultaneously assessed with normal kidney tissue, which was reversed by SP600125.

**Fig. 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 with normal kidney tissue, which was reversed by SP600125.

**Fig. 5.** 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 with 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 with vehicle-treated rats. The reduction between 4 days and 15 days was significant in SP600125-treated rats (p = 0.01) but 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.
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 (Fig. 4). The JNK inhibitor reduced tubular injury, as indicated by a reduction in tubulointerstitial osteopontin expression at 15 days after I/R compared with vehicle-treated rats (Fig. 5A). Moreover, tubular vimentin expression was significantly reduced by SP600125 at both time points (Fig. 5B). In animals treated with vehicle, renal MCP-1 gene expression (Fig. 6) was induced at 4 days after I/R and increased further at 15 days; SP600125 significantly reduced MCP-1 gene expression at 4 days; moreover, MCP-1 remained stable at 15 days after I/R in rats treated with SP600125.

Fig. 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.

JNK Activation in Human Renal Disease. 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 (Fig. 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, 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 (Figs. 9 and 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 (Fig. 11A), loss of renal function (Fig. 11B), and tubulointerstitial fibrosis (Fig. 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

Fig. 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) 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 days (top images) or 15 days (bottom images) after induction of I/R. Original magnification, 100×.
accumulation (Fig. 11D). Glomerular JNK activation correlated with MME (r = 0.324, p < 0.01), FGS (0.368, p < 0.01), and interstitial fibrosis (r = 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 that abrogated immunostaining (Fig. 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 (Tsuboi et al., 2002; Sengul et al., 2003).

In rat unilateral renal I/R injury, activated JNK and c-Jun were expressed by tubular epithelial cells already 30 min after I/R, in line with previous studies in unilateral ischemia/reperfusion (Park et al., 2001). The extent of JNK activation in tubular cells correlated with interstitial macrophage accumulation, suggesting involvement in renal inflam-

![Fig. 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 days and (even stronger) at 15 days. B, representative images of immunohistochemistry for the rat T-cell marker CD3 (counterstained with hematoxylin), 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 days (top images) or 15 days (bottom images) after induction of I/R. Original magnification, 100×.](image-url)

**TABLE 1**

Clinical and histopathological parameters of the studied patient population

<table>
<thead>
<tr>
<th>Disease</th>
<th>Age</th>
<th>Gender</th>
<th>Serum Creatinine</th>
<th>Proteinuria</th>
<th>eGFR</th>
<th>IF</th>
<th>Interstitial mes</th>
<th>yr</th>
<th>µmol/l</th>
<th>g/d</th>
<th>ml/min</th>
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<td>60±5</td>
<td>4/4</td>
<td>91±8</td>
<td>4.5±1.1</td>
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<td>90±5</td>
<td>50±20</td>
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<td>3/4</td>
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<td>6.8±1.2</td>
<td>36±10</td>
<td>2.8±0.6</td>
<td>49±6</td>
<td>11</td>
<td>90±5</td>
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<td>291±83</td>
<td>1.7±0.9</td>
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<td>2.8±0.6</td>
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<td>76±8</td>
<td>1.5±0.2</td>
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<td>5/2</td>
<td>173±52</td>
<td>9.9±2.3</td>
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<td>1.0±0.4</td>
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<td>SLE</td>
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<td>1/7</td>
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<td>5/4</td>
<td>342±110</td>
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<td>5/2</td>
<td>395±95</td>
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DN, diabetic nephropathy; IF, interstitial fibrosis; ms, macrophage accumulation; MGP, membranous glomerulopathy; MPGN, mesangio proliferative glomerulonephritis.

Values are male/female.
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, extracellular signal-regulated kinase 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 down-regulation 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 posts ischemic kidneys. It is important to note that upon treatment with SP600125 during the first 4 days, expression of the MCP-1 gene and tubulointerstitial macrophage accumulation did not increase between day 4 and day 15. In contrast, 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 were treated with antiglomerular basement membrane serum (Ikezumi et al., 2004). It is surprising that, in that model, SP600125 did not affect renal macrophage accumulation; however, it strongly reduced proteinuria and glomerular cell proliferation. 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. It is interesting that 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 toward reduction of procollagen-Iα1 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 consid-
ered 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-1 expression in tubular epithelial cells (antifibrotic) 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 eGFR. 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 many patients being 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 tubulo-interstitial inflammation in response to I/R injury. Given the central role of

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**Fig. 11.** Association between the extent of JNK activation and parameters of renal damage in human renal disease. A, strong correlation between tubular pJNK immunostaining and interstitial macrophage accumulation in human renal disease. B, strong association between tubular pJNK expression and loss of eGFR. Because the decay was exponential, we transformed JNK activation to 1/(tubular pJNK) on the y-axis. Nonlinear regression analysis revealed an r² 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. Data are presented as mean number of pJNK+ tubular cells per tubulointerstitial field, categorized by tubulointerstitial fibrosis score (range, 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 two-dimensional aspect of the image. Magnification, 400×. Magnification ± 800×. Renal disease patients are indicated as closed squares and controls as open circles.
interstitial macrophage accumulation in renal pathology (Van Goor et al., 1994; Sean Eardley and Cockwell, 2005) 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


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