JNK signalling in human and experimental renal ischaemia/reperfusion injury

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Abstract

Background. Ischaemia/reperfusion (I/R) is an important factor in delayed graft function in renal transplantation and is a determinant of long-term graft outcome. This study examined the role of c-Jun N-terminal kinase (JNK) signalling in human and experimental renal I/R injury.

Methods. Biopsies obtained 15–20 min after reperfusion of human renal allografts were examined for JNK signalling by immunostaining for phospho-c-Jun. To examine the pathologic role of JNK signalling, a selective JNK inhibitor (CC-401) was administered to rats before or after the induction of a 30-min period of bilateral renal ischaemia followed by reperfusion. Renal function and tubular damage were analysed.

Results. Substantial JNK activation was evident in tubular epithelial cells in kidneys from deceased donors (n = 30) which was less prominent in kidneys from live donors (n = 7) (44.6 ± 24.8% vs 29.1 ± 20% p-c-Jun+, respectively; P < 0.05), whereas biopsies of thin basement membrane disease exhibited little, or no, p-c-Jun staining. The degree of p-c-Jun staining correlated with ischaemic time in deceased donor allografts, but not with graft function. Administration of CC-401 to rats prior to bilateral renal I/R prevented acute renal failure and largely prevented tubular damage, leucocyte infiltration and upregulation of pro-inflammatory molecules. However, delaying CC-401 treatment until 1 h after reperfusion (after the peak of JNK activation) had no protective effect.

Conclusions. We have identified acute activation of the JNK signalling pathway following I/R in human kidney allografts. Experimental studies indicate that blockade of JNK signalling, commenced prior to this activation, can prevent acute tubular necrosis and renal dysfunction secondary to I/R injury.

Keywords: apoptosis; c-Jun; ischaemia/reperfusion; kidney transplant; macrophage

Introduction

Ischaemia/reperfusion (I/R) injury is a significant clinical problem that occurs in a variety of diverse scenarios including kidney transplantation. It is an important cause...
of delayed graft function, and it increases allograft immunogenicity [1]. The pathogenesis of I/R injury is complex and involves a number of pathogenic mechanisms that result in acute tubular necrosis and renal dysfunction [2,3]. These include adenosine triphosphate (ATP) depletion, generation of reactive species, leucocyte infiltration, production of pro-inflammatory mediators and induction of tubular apoptosis [2,4]. However, we have little in the way of specifically targeted therapies to mitigate the effects of I/R injury in renal transplantation.

C-Jun N-terminal kinase (JNK) is a stress-activated protein kinase which can be induced by various stimuli, including reactive oxygen species, pro-inflammatory cytokines, osmotic and mechanical stress, and Toll-like receptor activation [5]. These stimuli induce a cascade of events involving upstream kinases that result in phosphorylation of the JNK activation motif. Activated JNK can then phosphorylate specific proteins including transcription factors, resulting in modulation of gene transcription and cellular responses such as production of pro-inflammatory molecules, cell differentiation, cell proliferation and apoptosis [5]. The best characterized JNK target/substrate is phosphorylation of the amino terminus of c-Jun at Ser63 and Ser73.

In the context of renal injury, JNK signalling is induced by different insults, including I/R, ureteric ligation, immune cell-mediated injury and hyperglycaemia [6,7]. Immunostaining of biopsies has shown JNK activation in various glomerulonephritides [8]. Administration of a selective JNK inhibitor, CC-401, has been shown to suppress inflammation in rat anti-glomerular basement membrane disease and to suppress tubular apoptosis and interstitial fibrosis in the obstructed kidney [9,10]. Given that immune cells and apoptosis are important mechanisms of renal injury following I/R, this provides a rationale for targeting JNK signalling in I/R injury. However, we do not know whether activation of JNK signalling occurs in the setting of human renal transplantation.

The aims of this study were to examine whether there is acute induction of the JNK signalling pathway in human renal transplantation, and to examine the therapeutic potential of JNK inhibition in a rat model of I/R injury.

**Materials and methods**

**Human transplant biopsies**

Baseline human renal allograft biopsies were performed as part of our routine protocol 15–20 min after reperfusion. Patient demographics are summarized in Table 1. Biopsies from three subjects with thin basement membrane disease (TBMD) were used as controls. Studies were approved by the Research and Ethics Committee at our institution and comply with ethical standards outlined in the 2000 revision of the Helsinki Declaration.

### Antibodies

Immunohistochemistry was performed using: rabbit anti- phospho-JNK (Tyr183/Tyr185), rabbit anti-phospho-c-Jun(Ser63), phospho-p38 (Thr180/Tyr182) and rabbit anti-phospho-ERK1/2 (Thr202/Thr204) (all from Cell Signaling, San Diego, CA, USA); mouse anti-rat CD68 recognizing monocytes/macrophages (ED1, Serotec, Oxford, UK); mouse anti-rat neutrophil (RP1, Becton Dickinson, San Diego, CA); mouse anti-rat T-cell receptor (R73, Serotec); mouse anti-rat ICAM-1 (1A29, Serotec); and mouse anti-rat CD44 (OX-50) [11]. Secondary antibodies used were HRP-conjugated sheep anti-rabbit or rabbit anti-mouse immunoglobulin G (IgG) and rabbit or mouse peroxidase-conjugated anti-peroxidase complexes (PAP).

### CC-401

The selective JNK inhibitor, CC-401, was synthesized and provided by Celgene, San Diego, CA, USA. CC-401 has previously been described in detail [9,10]. CC-401 inhibits JNK signalling by competitive and reversible binding to the adenosine triphosphate-binding site in the active, phosphorylated, form of JNK, which prevents JNK from phosphorylating its downstream targets, including Ser63 in the amino-terminal activation domain of c-Jun.

### Rat model of I/R injury

Male Sprague–Dawley rats (150–180 g) were placed on a heating pad to maintain body temperature at 37°C and anaesthetised with ketamine/xylazine. Following abdominal incision, both renal pedicles were clamped using non-traumatic vascular clamps. The abdomen was temporarily sutured to reduce fluid and heat loss. After 30 min of ischaemia, clamps were removed, kidneys were reperfused and animals were fully sutured. In the first experiment, 12 rats underwent I/R surgery and were treated with CC-401 (20 mg/kg) given as a single i.v. injection 15 min before surgery and then by oral gavage (200 mg/kg) at 4 and 22 h after reperfusion. Animals were sacrificed 24 h after reperfusion. As controls, a further 12 rats underwent surgery and received vehicle (50 mM citric solution, pH 5.0) following the same schedule. A third group underwent sham surgery in which renal pedicles were identified but not clamped. To investigate early events following reperfusion, groups of 10 rats were given an intravenous injection of CC-401, or vehicle alone, 15 min before surgery, and killed 30 min post-reperfusion. As a control, a group of 10 sham-operated rats was killed at 30 min post-reperfusion. In the second experiment, 10 rats underwent I/R surgery and were treated with CC-401 after surgery (i.v. bolus 1 h post-reperfusion and two oral doses 6 and 21 h after reperfusion) before sacrifice at 24 h. As controls, groups of 10 rats underwent I/R surgery and received vehicle or saline following the CC-401 schedule. Blood samples were collected at sacrifice, and serum creatinine and urea were analysed (Biochemistry Department, Monash Medical Centre). Our institution’s Animal Ethics Committee approved all animal procedures.

<table>
<thead>
<tr>
<th>Donor type</th>
<th>Number</th>
<th>Donor age (years)</th>
<th>Ischaemic time (h)</th>
<th>Delayed graft function (incidents/total)</th>
<th>Acute rejection (incidents/total)</th>
<th>Days to serum creatinine &lt;200 μmol/L (days)</th>
<th>Creatinine at 12 months post-transplant (μmol/L)</th>
</tr>
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<tbody>
<tr>
<td>All</td>
<td>37</td>
<td>43.9 ± 18.1</td>
<td>10 ± 5.0</td>
<td>3/37</td>
<td>12/37</td>
<td>8.5 ± 10.0</td>
<td>140.5 ± 53.2</td>
</tr>
<tr>
<td>Deceased</td>
<td>30</td>
<td>42.3 ± 19.6</td>
<td>12.1 ± 2.8</td>
<td>3/30</td>
<td>11/30</td>
<td>7.3 ± 9.3</td>
<td>137.0 ± 57.1</td>
</tr>
<tr>
<td>Living</td>
<td>7</td>
<td>49.3 ± 7.8</td>
<td>1 ± 0</td>
<td>0/7</td>
<td>1/7</td>
<td>1.9 ± 0.9</td>
<td>153.6 ± 35.2</td>
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Delayed graft function was defined as a need for dialysis following transplantation. Acute rejection was biopsy proven. Cases reported above include humoral rejection (three cases) and Banff class 1a and 1b rejection detected in clinically indicated as well as protocol biopsies by 3 months post-transplant. All patients were biopsied within this 3-month period. Cases of borderline rejection are not included (three deceased and two living). Rejection occurring beyond 3 months post-transplant is also not included (three cases; all occurring >200 days post-transplant with normal protocol biopsies at 3 months).
Renal histology

One hundred intersections were examined on coded periodic acid–Schiff (PAS)-stained paraffin sections of the left kidney of each animal by a renal pathologist. A score of 0–3 was given for each profile as follows: normal histology (0); tubular swelling, loss of brush border and condensation with up to one-third nuclear loss (1); between one- and two-thirds nuclear loss (2); more than two-thirds nuclear loss (3). Scores were added to give a maximum score of 300.

Immunocytochemistry

In human formalin-fixed human biopsies, immunoperoxidase staining for p-JNK and p-c-Jun Ser63 was performed on 4-μm sections after antigen retrieval (microwave heating in 0.1 M sodium citrate pH 6.0 for 12 min) followed by a three-layer avidin–biotin peroxidase complex (ABC) staining method [10]. The percentage of tubules containing p-c-Jun-stained cells were counted in the cortex and expressed as a percentage of tubules.

In rat kidneys, p-JNK and p-c-Jun, pp38 and p-ERK immunostaining was performed as above, and the number of p-c-Jun-stained tubular cells was counted in the cortex and outer medulla. Immunostaining using ED1 was performed on 4-μm sections of methyl Carn–fixed sections followed by a three-layer PAP staining method. Immunostaining with R73, RPI, 1A29 and OX-50 antibodies used frozen sections of tissue fixed in 2% paraformaldehyde for 10 min at room temperature. Endogenous peroxidase activity was blocked by incubation in 3% H2O2 in PBS for 10 min. After treatment with equilibration buffer, TdT enzyme was added for 60 min at 37°C and incubated with anti-digoxigenin antibody conjugated to a peroxidase reporter molecule for 30 min. Sections were washed, developed in 3,3′-diaminobenzidine hydrochloride and counterstained with haematoxylin. The number of TUNEL-positive apoptotic cells was counted in the cortex and outer medulla and expressed as cells per square millimetre. Counting was performed on blinded slides.

Apoptosis assay

Apoptotic cells in kidneys were determined using ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore-Chemicon, MA, USA). Four-micrometre formalin-fixed paraffin sections were treated with 20 μg/ml proteinase-K (Promega, WI, USA) for 10 min at room temperature. Micrometre formalin-fixed paraffin sections were treated with 20 μg/ml proteinase-K for 10 min. The nuclei were stained with haematoxylin. The number of TUNEL-positive apoptotic cells was counted in the renal cortex and outer medulla and expressed as cells per square millimetre. Counting was performed on blinded slides.

Real-time RT-PCR

Rat kidney cross-section samples were snap frozen in liquid nitrogen and stored at −80°C until RNA was extracted using a RiboPure RNA isolation kit (Ambion, Austin, TX, USA). cDNA was prepared from total RNA by reverse transcription using random hexamer primers and Superscript II (Invitrogen, Carlsbad, CA, USA). PCR involved thermal cycling conditions with the Rotor-Gene 3000 system (Corbett Research, Sydney, NSW, Australia): 37°C, 10 min; 95°C, 5 min; then 50 cycles of 95°C, 15 s; 60°C, 20 s; 68°C, 20 s. Primer pairs and probes used were: KIM-I (Forward: TAA ACC AGA AAT GCC CAC AAG; Reverse: TGT TGG AGT AGA GGT GGA GA; Probe: CAA CAA GAC CCA CAA C), TNFα (Forward: CTT TTC TAC TCC CAG GAT CTC T; Reverse: TCT CCT GTG CTT AGG AAA TGG CAA A; Probe: TCA CCC ACG CCG TCA G), MMP-12 (Forward: GTC ACA ACA GGT GGA GAA; Reverse: GCC CAC ATG GAA GAA ATT GGA; Probe: AGT CCA GCC AAC AAC A), and iNOS (Forward: TTC ATG GTC AAT GCT TCC TAC CAA G; Reverse: TGT GGT GTG CTT CTT GTA GAA TTA; Probe: GAA GGA AGA AAA GGA CA), and EGR1 (Forward: ATG GAT GAA GAG AAG AGG; Reverse: ATG GGT AGG AAG AGG AAG; Probe: GAA GGA CAA GAA AGC AGA). The relative amount of mRNA was calculated using the comparative Ct (ΔCt) method. All amplicons were normalized against 18S RNA levels, determined in parallel as an internal control (Applied Biosystems).

Statistics

Data are shown as mean ± SD. Results were analysed using parametric ANOVA with post hoc analysis using Bonferroni’s post-test for multiple comparisons. Linear regression was used to explore the relationship between JNK activation and clinical variables in human kidney transplantation. Analyses were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA) and Intercooled Stata 10.1 (Statacorp, College Station, TX, USA).

Results

JNK activation in human kidney transplantation

In contrast to the essentially normal histology of TBMD, biopsies taken shortly after reperfusion of renal transplants showed acute tubular damage with tubular swelling and sloughing of cells into the tubular lumen (Figure 1A and D). Immunostaining for p-JNK was limited to some collecting ducts in TBMD (Figure 1B), while very little p-c-Jun staining was detected (Figure 1C). Transplant biopsies showed profound JNK activation with p-JNK staining prominent in the cytoplasm and nuclei of many cells in different parts of the tubule (Figure 1E). The p-JNK staining pattern was focal and patchy, being mainly restricted to tubular epithelial cells. Many tubular epithelial cells with nuclear p-c-Jun staining were evident in renal transplants, having a similar distribution to that of p-JNK staining (Figure 1F), and demonstrating acute induction of JNK signalling following reperfusion of ischaemic allografts.

Allografts from deceased donors had a significantly higher percentage of tubules exhibiting p-c-Jun immunostaining compared to allografts from live donors (Figure 1G), although there was wide variation in the number of p-c-Jun + tubules within each group. A significant correlation between the percentage of p-c-Jun + tubules and ischaemia time was evident when considering all transplant patients as a single group (R² = 0.28, P = 0.001), and within the deceased donor group (R² = 0.19, P = 0.02) (Figure 1H). However, no other correlations were identified for p-c-Jun + tubules and other parameters, including donor age, donor sex, incidence of allograft rejection and renal function at Day 3 and 7 or at Month 1, 3 and 12 post-transplant.

Acute JNK activation in rat renal I/R injury

To examine the pathological importance of this early induction of JNK signalling in the reperfused ischaemic kidney, we used a rat model of bilateral I/R injury. A time-course study identified a dramatic increase in p-JNK 5 min after reperfusion, although p-JNK was still elevated 24 h post-reperfusion (Figure 2A). This induction of JNK phosphorylation was not evident 30 min after sham surgery (data not shown), while immunohistochemistry showed little or no p-c-Jun staining at 30 min or 24 h following sham operations (Figures 2B and 3D), demonstrating that neither the anaesthetic nor the surgical procedure activated this stress-responsive signalling pathway.

The sham groups showed p-JNK staining in some collecting ducts and little or no p-c-Jun staining (Figures 2B and 3A and D), consistent with previous studies in normal rat kidney [10], and the staining pattern seen in human TBMD. Immunostaining identified many p-c-Jun + tubular epithelial cells in the outer medulla and cortex at 30 min after I/R injury (Figure 2B), consistent with the marked increase in
JNK activity seen by western blotting (Figure 2A). Although less prominent, an increase in tubular staining for p-c-Jun and p-JNK was still evident at 24 h after I/R injury (Figure 3).

JNK inhibition in rat I/R injury

To target this early induction of JNK signalling, we administered the JNK inhibitor, CC-401, beginning 15 min before the induction of ischaemia and gave two further doses before animals were killed 24 h post-reperfusion, or animals were killed 30 min post-reperfusion having received a single dose of CC-401. As CC-401 inhibits p-JNK from phosphorylating its downstream targets, we used p-c-Jun staining as a read-out of JNK activity.

CC-401 treatment substantially reduced JNK activation as shown by the marked reduction in the number of p-c-Jun + cells at 30 min and 24 h post-reperfusion (Figures 2 and 3), and this was accompanied by a reduction in p-JNK staining (Figure 3C). Specificity of CC-401 treatment for inhibition of the JNK signalling pathway was shown by the lack of effect of the drug on the marked tubular activation of the two most closely related signalling pathways, p38 and ERK, at 30 min after I/R injury (Figure 2B).
JNK blockade prevents renal dysfunction and tubular damage in rat I/R injury

At 30 min post-reperfusion, vehicle-treated animals showed little histologic evidence of tubular damage on PAS-stained sections, and serum creatinine remained normal (data not shown). To examine early changes in the kidney following I/R injury which precede gross histologic damage, we quantified mRNA levels of selected genes. Upregulation of kidney injury molecule-1 (KIM-1), early growth response 1 (EGR1) and the pro-inflammatory cytokine TNF-α are well-characterized early responses to I/R injury [12–14], with gene transcription of EGR1 and TNF-α known to be inducible via the JNK signalling pathway [15,16].

Fig. 2. JNK activation at 30 min post-renal ischaemia/reperfusion (I/R) injury in the rat. (A) Western blot showing p-JNK in the kidney in normal rats and at different times after I/R injury. (B) Immunostaining for p-c-Jun, p-p38 and p-ERK in renal tissue at 30 min post-reperfusion from rats treated with CC-401 or vehicle, and sham control. Quantification of the number of p-c-Jun ± tubular cells in: (C) the cortex and (D) the outer medulla. Real-time RT-PCR analysis of kidney RNA at 30 min post-reperfusion for the relative mRNA expression of: (E) EGR1, (F) TNF-α and (G) KIM-1.

Fig. 3. JNK activation at 24 h post-renal ischaemia/reperfusion (I/R) injury in the rat. (A) Immunostaining for p-JNK (A–C) and p-c-Jun (D–F) in renal tissue from rats 24 h after undergoing: (A, D) sham surgery, (B, E) I/R injury with vehicle treatment and (C, F) I/R injury with CC-401 treatment. Quantification of the number of p-c-Jun + tubular cells in: (G) the cortex and (H) the outer medulla.
icant upregulation of each of these mRNA species was seen in vehicle-treated rats at 30 min post-reperfusion. However, mRNA levels for each of these molecules was substantially reduced by CC-401 treatment (Figure 2E–F), indicating protection from the early stages of tubular damage.

Vehicle-treated rats showed a significant increase in serum creatinine levels 24 h after I/R that was prevented by CC-401 treatment (Figure 4B). Similarly, the increase in serum urea seen in vehicle-treated I/R over that in the sham group (16.8 ± 14.4 vs 4.3 ± 0.7 mmol/L, respectively; \( P < 0.01 \)) was prevented by CC-401 treatment (5.7 ± 1.3 mmol/L; \( P < 0.01 \) vs vehicle).

Tubular damage featuring loss of brush border, swelling, condensation with nuclear loss and intratubular cell detachment was evident in vehicle-treated animals 24 h after I/R (Figure 4A and C). In contrast, CC-401-treated animals showed well-preserved tubular structure 24 h after I/R with only mild damage seen in some tubules (Figure 4C and D). An 11-fold increase in the number of tubular apoptotic cells was evident in vehicle-treated I/R, as assessed by TUNEL staining combined with characteristic nuclear morphological changes. This was reduced by 60% with CC-401 treatment (Figure 4E). As an additional marker of tubular damage, we measured KIM-1 mRNA levels 24 h after I/R (Figure 2E). The dramatic upregulation of KIM-1 mRNA seen in vehicle-treated rats was reduced by 80% with CC-401 treatment (Figure 2E). We also analysed tubular expression of leucocyte adhesion molecules that are upregulated during tubulointerstitial inflammation. A pronounced upregulation of tubular CD44 and ICAM-1 expression was evident in vehicle-treated I/R, which was largely prevented by CC-401 treatment (Figure 2E–F), consistent with the reduction in other parameters of tubular damage seen with drug treatment.

**JNK blockade prevents renal leucocyte infiltration and upregulation of inflammatory mediators in rat I/R injury**

A significant infiltrate of ED1 + macrophages and R73 + T cells was evident in vehicle-treated rats at 24 h after I/R, while the increase in numbers of RP1 + neutrophils just failed to reach statistical significance (Figure 5C–E). CC-401 treatment of I/R injury significantly reduced the macrophage infiltrate in the outer medulla, although this was still increased above sham (Figure 5C). CC-401 treatment also prevented the T-cell infiltrate in the cortex and outer medulla (Figure 5D).

Inducible nitric oxide synthase (iNOS) is a major product of classically activated macrophages, implicated in promoting damage in response to renal I/R [17], while MMP-12 is a relatively macrophage-specific product, which is also upregulated in classically activated macrophages, and causes injury in a macrophage-dependent model of kidney disease [18]. Consistent with the significant macrophage infiltrate seen in vehicle-treated 24-h I/R, we identified a marked increase in iNOS and MMP-12 mRNA levels in whole kidney tissue (Figure 5F and G). While CC-401 treatment of I/R only partially reduced the macrophage infiltrate, drug treatment was highly effective in preventing upregulation of iNOS and MMP-12 mRNA expression (Figure 5F and G). TNF-α has been implicated in causing tissue damage following I/R injury [19]. CC-401 treatment of I/R injury suppressed the significant increase in TNF-α mRNA seen in the vehicle-treated group (Figure 5H).

**Fig. 4.** JNK blockade improves renal function and suppresses tubular injury in ischaemia/reperfusion (I/R) injury. Groups of vehicle- and CC-401-treated rats underwent I/R surgery and were killed 24 h later, with a control group undergoing sham surgery. (A) PAS staining showing acute tubular necrosis in vehicle-treated I/R, which is largely prevented with CC-401 treatment (D). (B) Renal function measured by serum creatinine levels. (C) Graph quantifying tubular damage assessed on PAS-stained sections. (E) Quantification of the number of apoptotic tubular epithelial cells using TUNEL staining. (F) Relative quantification of mRNA levels for KIM-1, a marker of tubular injury, using real-time RT–PCR.
CC-401 treatment beginning after the peak of JNK signalling fails to protect from renal I/R injury

The data in Figures 1–3 illustrate the dramatic activation of JNK signalling shortly after reperfusion of the ischaemic kidney. While JNK signalling is still increased 24 h after reperfusion, the peak of JNK signalling occurs around 30 min post-reperfusion. Given the effectiveness of CC-401 treatment begun prior to induction of I/R injury, we sought to determine whether delayed CC-401 administration could still provide protection. CC-401 treatment beginning 1 h post-reperfusion did not prevent the induction of renal dysfunction or tubular damage compared to vehicle- or saline-treated control groups (Figure 6).

Discussion

This study examined the clinical and pathological significance of acute activation of the JNK signalling pathway in renal I/R injury. Using human renal allograft biopsies performed on the operating table, we have, for the first time,
demonstrated acute activation of JNK signalling following ischaemia and reperfusion. There was a significantly higher level of JNK activation in deceased donor compared to live donor allografts, suggesting that higher JNK activation reflects greater ischaemic damage. Furthermore, the degree of JNK activation within the deceased donor group correlated with the duration of allograft ischaemia. However, other factors clearly affect JNK activation since there was still considerable heterogeneity in JNK activation within the deceased donor allografts in which ischaemic times were consistently short. The lack of correlation between the degree of JNK activation and short-term and long-term graft function suggests that multiple factors, of both donor and recipient origin, are likely to be involved in determining overall graft function. Additionally, the study group was small, and this possibly limited the ability to identify such a relationship.

These clinical findings provide relevance to the studies of JNK signalling in experimental models of renal I/R injury. Consistent with previous studies [7,20], we identified profound induction of JNK signalling shortly after reperfusion in a rat model of I/R injury. One study previously described JNK activation in acute tubular necrosis post-transplantation [21], with biopsies performed for delayed graft function between Day 8–71 post-transplantation. An increase in p-JNK staining was noted particularly in tubules and interstitial cells [21], suggesting involvement of JNK activation in both acute cellular rejection and acute tubular necrosis.

The mechanism underlying this acute and dramatic induction of the stress-responsive JNK signalling is not entirely clear, but there is evidence suggesting that production of reactive oxygen species during the reperfusion process may be an important factor [22,23]. There is general agreement that blockade of JNK activation reduces the induction of apoptosis during brain and cardiac I/R injury [24–26]; however, there are conflicting reports as to the beneficial or detrimental effects of JNK blockade in liver I/R injury [27,28]. One study has reported a beneficial effect of the JNK inhibitor, SP600125, in renal I/R injury [29]. Administration of SP600125 prior to ischaemia reduced JNK activation and decreased apoptosis of tubular epithelial cells, although the pro-inflammatory role of JNK signalling was not examined.

In the current study, we have demonstrated that CC-401, a selective and soluble JNK inhibitor, is effective in preventing tubular damage and renal dysfunction when administered prior to I/R injury. Delaying CC-401 administration until 1 h post-reperfusion was ineffective. Thus, inhibition of the peak signalling event is critical for therapeutic effect and demonstrates that JNK inhibition could potentially be used in renal transplant recipients where prophylactic treatment (or treatment of the donor) is feasible, but that JNK inhibition is not likely to be feasible in situations of unanticipated acute tubular necrosis secondary to an individual ischaemic episode.

There are several potential mechanisms by which JNK signalling may contribute to acute tubular damage and renal dysfunction following acute I/R injury. Firstly, JNK signalling has a potent pro-inflammatory action. JNK signalling induces activation of the AP-1 transcription factor which promotes transcription of several genes encoding pro-inflammatory mediators, including TNF-α. JNK blockade suppressed upregulation of TNF-α mRNA levels at both 30 min and 24 h following I/R, and this may account for many of the protective effects of CC-401 treatment. TNF-α can induce renal injury in several ways including leucocyte recruitment via induction of adhesion molecules and chemokines by intrinsic renal cells, direct activation of infiltrating leucocytes, and induction of tubular cell apoptosis [19]. The ability of JNK blockade to prevent upregulation of tubular CD44 and ICAM-1 expression, to reduce leucocyte infiltration and to decrease tubular cell apoptosis would be consistent with inhibition of TNF-α production following I/R. Indeed, ICAM-1 gene-deficient mice show protection from leucocyte infiltration and renal dysfunction following I/R [30]. Similarly, upregulation of CD44 by peritubular capillaries has been shown to promote leucocyte infiltration and renal dysfunction following I/R [31].

A second mechanism by which JNK signalling may contribute to renal I/R injury is through the activation of resident and infiltrating macrophages. Recent studies have identified a pathologic role for monocytes/macrophages in renal I/R injury [32,33]. The marked upregulation of iNOS and MMP-12 mRNA levels seen in vehicle-treated I/R is consistent with a pro-inflammatory phenotype (classical activation or M1) of the macrophage population. The suppression of acute tubular necrosis and renal dysfunction in renal I/R seen with CC-401 treatment may be, in part, due to blockade of the macrophage pro-inflammatory response. There is a significant reduction in the degree of
renal dysfunction and tubular damage following I/R injury in iNOS gene-deficient mice [34]. In vitro studies have shown that JNK signalling plays a direct role in the upregulation of iNOS and MMP-12 expression in macrophages [16,35], while recent studies in experimental crescentic glomerulonephritis have demonstrated that JNK blockade can suppress TNF-α, iNOS and MMP-12 expression and protect against progressive renal damage and dysfunction [35]. It is not known whether MMP-12 is directly involved in renal I/R injury; however, macrophage production of MMP-12 has been shown to cause glomerular injury and proteinuria in crescentic glomerulonephritis [18], and to cause lung damage, in part through the induction of TNF-α in macrophages, in experimental emphysema [36].

A third mechanism by which JNK signalling could promote renal I/R injury may be through the recruitment and activation of T cells. Several studies have described the role of T cells in causing renal I/R injury [37,38], presumably as part of an innate immune response rather than an antigen-specific immune response. The primary effect of JNK blockade was in reducing T-cell infiltration which is likely to be an indirect effect through preventing upregulation or activation of leucocyte adhesion molecules, as illustrated by the effects on ICAM-1 and CD44 expression. However, there is also evidence that JNK signalling plays a regulatory role in the primary T-cell response [39], which could represent a direct mechanism targeted by JNK blockade in I/R injury.

Finally, the JNK signalling pathway has a well-described role in the induction of apoptosis in a wide range of cell types [5]. Although the major mechanism of cell death following I/R injury is necrosis, a substantial induction of apoptosis is evident, and pharmacological and genetic approaches to inhibit caspase-1 and caspase-3 activation can prevent in vivo can be prevented in vivo by CC-401 treatment or using JNK1 deletion [10]. In addition, the induction of apoptosis in cultured tubular epithelial cells by reactive oxygen species is dependent upon JNK signalling [10], which is thought to be an important mechanism for the induction of tubular cell apoptosis following I/R. Therefore, JNK blockade may have directly suppressed tubular apoptosis following I/R injury. However, one other interpretation is that JNK blockade was effective in reducing the initial damage which leads to tubular apoptosis. This possibility is supported by the finding that CC-401 suppressed the early upregulation of the markers of tubular damage, EGR1 and KIM-1, at 30 min post-reperfusion.

In conclusion, we have identified acute activation of the JNK signalling pathway following I/R in human renal allografts. This induction of JNK signalling was related to the ischaemic time, although no relationship to subsequent allograft function was identified. Experimental studies demonstrate that administration of a JNK inhibitor prior to, but not after, the induction of renal I/R is effective in preventing subsequent tubular damage and renal dysfunction.

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Conflict of interest statement. None declared.

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Depletion of kidney CD11c<sup>+</sup> F4/80<sup>+</sup> cells impairs the recovery process in ischaemia/reperfusion-induced acute kidney injury

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Abstract

**Background.** Recent studies provided evidence of the potential role of CD11c<sup>+</sup> F4/80<sup>+</sup> dendritic subset in mediating injury and repair. The purpose of this study was to examine the role of kidney CD11c<sup>+</sup> F4/80<sup>+</sup> dendritic subset in the recovery phase of ischaemia/reperfusion injury (IRI).

**Methods.** Following ischaemia/reperfusion (I/R), liposome clonodrate or phosphate buffered saline (PBS) was administered, and on day 7 biochemical and histologic kidney damage was assessed. Activation and depletion of CD11c<sup>+</sup> F4/80<sup>+</sup> dendritic subset were confirmed by flow cytometry. Isolation of kidney CD11c<sup>+</sup> cells on days 1 and 7 with in vitro culture for measuring cytokines was performed to define functional characteristics of these cells, and adoptive transfer of CD11c<sup>+</sup> cells was also done.