Role of inflammation in the pathogenesis of cardiorenal syndrome in a rat myocardial infarction model

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ABSTRACT

Background. Cardiorenal syndrome is now frequently recognized, and the combined dysfunction of heart and kidney increases morbidity and mortality. This study aimed to investigate possible mechanisms that underlie renal damage following heart dysfunction using a rat myocardial infarction model, focusing on the inflammatory pathway.

Methods. Rats were randomized into four groups: normal, volume depletion, sham operation and myocardial infarction (MI). MI was induced by the ligation of the left coronary artery and a volume depletion model was produced by low-salt diet and furosemide injection. Biochemical, histological and flow cytometric analyses were performed at 3 days and 4 and 8 weeks after MI.

Results. On Day 3 following MI, the development of subclinical acute kidney injury was identified through significantly increased serum and urine neutrophil gelatinase-associated lipocalin level. We detected the increase of activated monocytes (CC chemokine receptor 2+ ED-1+) in peripheral blood, along with the infiltration of ED-1+ macrophages and the increment of nuclear p65 in the kidney of MI rats, suggesting the contribution of nuclear factor-kappa B-mediated inflammation in the development of Type 1 cardiorenal syndrome (CRS). The inflammatory cytokines, interleukin-6 and tumour necrosis factor-α (TNF-α) mRNA expression, as well as microvascular endothelial permeability and tubular cell apoptosis, significantly increased in the kidneys of MI rats. At 4 and 8 weeks after MI, tubular cell apoptosis, ED-1+ macrophage infiltration and interstitial fibrosis increased in MI rats, and these chronic changes were significantly mitigated by systemic monocyte/macrophage depletion using liposome clodronate.

Conclusion. This study identifies the possible important role of inflammatory response as a mediator of heart–kidney cross-talk in CRS.

INTRODUCTION

Heart and kidney dysfunction are frequently combined in hospitalized patients, leading to increased morbidity and mortality. Dysfunction of one organ often leads to that of another organ, with more pronounced damage in both. The clinical entity with regard to heart–kidney crosstalk was recently redefined as cardiorenal syndrome (CRS) according to the time frame and order of occurrence [1]. Heart and kidney diseases share many risk factors and the prevalence of CRS is increasing. In a retrospective study of 438 patients diagnosed with CRS, Type 1 CRS was most frequent (48.2%) followed by Type 2 CRS (21.9%) [2].
The pathophysiology of CRS is complex and involves haemodynamic alteration, pathological compensatory neurohormonal activation, oxidative stress, immune activation/inflammation, and even exogenous medications such as diuretics or therapeutic procedures [3]. Reduced cardiovascular mortality by angiotensin-converting enzyme inhibitor or angiotensin receptor blocker in chronic kidney disease (CKD) patients is strong evidence that the renin–angiotensin–aldosterone system plays an important role in heart–kidney crosstalk.

Although more than one pathophysiological process might be operative, we focused here on immune/inflammation-mediated mechanism in the development and progression of Type 1 and Type 2 CRS.

SUBJECTS AND METHODS

Animals and experimental protocols

Male Sprague–Dawley rats (180–230 g, n = 80; Orient, Seoul, Korea) were used, and the study protocol was approved by Korea University Institutional Animal Care and Use Committee (No. KUIACUC-20120106-1). Rats were fed standard rat chow and water ad libitum. For the study of Type 1 CRS, rats were divided into four groups: normal, volume depletion, sham and myocardial infarction (MI). MI was induced by the ligation of the left anterior descending (LAD) coronary artery under anaesthesia with 3% isoflurane and 0.3 L/min oxygen after intratracheal intubation. Rapid ischaemic colour change of the myocardium was identified, and prophylactic antibiotic (cefazolin 500 mg/kg, i.m.) was injected immediately after the procedure. The sham operation was performed in a similar manner, except for ligation of the coronary artery. Heart function by echocardiography and systolic blood pressure (BP) using the tail-cuff method in conscious rats were examined at baseline and 1 day after the operation (MI and sham). Volume depletion was induced by furosemide injection (20 mg/kg, i.p.) for 2 days with low-salt diet (0.3% NaCl), and systolic BP was measured on Day 3, 1 day after the last furosemide injection. Urine was collected before sacrifice on Day 3 to examine the fractional excretion of urea nitrogen (FEUN). Blood, liver and kidneys were collected from all groups for various molecular and histological examinations.

For the study of Type 2 CRS, rats with MI and sham operation were followed over three time points: 2, 4 and 8 weeks. Urine collection and blood sampling were performed at all time points, and rats were sacrificed Weeks 4 and 8. Kidneys were processed for molecular and histological examinations.

Blood and urine biochemical analysis

We measured blood and urine urea nitrogen by the urease method with glutamate dehydrogenase, plasma and urine creatinine by Jaffé reaction, plasma alanine aminotransferase (ALT) by the UV method without P5P and urine albumin by turbid immunoassay (Beckman Coulter, Brea, CA, USA).

Determination of plasma and urine neutrophil gelatinase-associated lipocalin

Plasma and urine samples were collected to measure neutrophil gelatinase-associated lipocalin (NGAL), on Day 3 and Weeks 2, 4 and 8 post-MI. NGAL was measured using a rat NGAL enzyme-linked immunosorbent assay (ELISA) kit (BioPorto, Gentofte, Denmark).

Depletion of monocytes/macrophages with liposome clodronate

Clodronate was purchased from Sigma-Aldrich (St. Louis, MO, USA), and liposome clodronate was prepared according to methods described previously [4]. One millilitre of liposome clodronate was injected through the internal jugular vein under intraperitoneal anaesthesia (ketamine 100 mg/kg and xylazine 12.5 mg/kg), starting on Day 7 of MI and then repeatedly injected every 7 days. MI rats with liposome clodronate administration were sacrificed at 4 and 8 weeks. Our laboratory previously reported the systemic effect of liposome clodronate in rats [5], and it was again confirmed by showing the depletion of circulating ED-1+ monocytes by flow cytometry (Supplementary data S1).

Echocardiographic examination

Echocardiography was performed at baseline, Day 1 and Week 4 in lightly anaesthetized animals (ketamine 40 mg/kg and xylazine 5 mg/kg, i.p.). Vivid 7 (GE Vingmed, Horten, Norway) and a 10-MHz linear probe were used to measure fractional shortening as described previously [6].

Determination of renal microvascular endothelial permeability

Microvascular endothelial permeability in the kidney was assessed by quantifying extravasation of Evans Blue dye (EBD). EBD (Sigma-Aldrich; 20 mg/kg) was injected intravenously, and 1 h later, rats were perfused with phosphate-buffered saline (PBS) and sacrificed. Following dehydration at 60°C for 2 days, kidneys were weighed and EBD that leaked into the kidney interstitium was extracted and measured as previously described with some modification [7]. An EBD concentration was determined spectrophotometrically at 620 nm and expressed as fold difference compared with the mean value of sham.

Detection of circulating monocytes by flow cytometry

On Day 3 following MI, 200 μL of whole blood was obtained in heparinized tubes for flow cytometric determination of circulating activated monocytes. Blood samples were incubated with fluorescein isothiocyanate-conjugated anti-rat CD68 (ED-1; Serotec, NC, USA) and allophycocyanin-conjugated anti-rat CC chemokine receptor 2 (CCR2; R&D Systems, Minneapolis, MN, USA) for 15 min at room temperature, and flow cytometric detection of CCR2+ ED-1+ activated circulating monocytes was performed using a FACS Caliber apparatus (BD Biosciences, San Jose, CA, USA).
Western blot analysis

Kidney nuclear and cytosolic proteins were separately extracted (NE-PER® nuclear and cytoplasmic extraction reagents; Thermo Scientific, Rockford, IL, USA), and the protein concentration was determined by measuring absorbance at 540 nm using a BCA protein assay kit (Thermo Scientific). Equal amounts of proteins were loaded into wells of 10% sodium dodecyl sulphate–polyacrylamide for gel electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto polyvinylidene difluoride membranes. After blocking with 5% non-fat dry milk in PBST (0.05% Tween 20 in PBS) at room temperature for 1 h, the membrane was subjected to repeated washings in PBST. For determination of the kidney tissue expression of nuclear factor-kappa B (NF-κB), western blot analysis for nuclear p65 and cytosolic inhibitory kappa B (IκB) was performed (primary antibodies 1:500, Cell Signaling Technology, Danvers, MA, USA; secondary anti-rabbit IgG, 1:1000, Santa Cruz Biotechnology, Dallas, TX, USA). The equivalent loading of proteins in each well was confirmed by lamin B1 (1:200, Abcam; secondary anti-rabbit IgG, 1:1000) and β-actin (1:500, Santa Cruz Biotechnology; secondary anti-rabbit IgG, 1:1000) for nuclear p65 and cytosolic IκB, respectively. The band intensities were quantified using Image J program (National Institutes of Health).

Quantitation of cytokine mRNA expression

Kidney total RNA was extracted using TRIzol reagent (Life Technologies, Foster, CA, USA). Complementary DNA was synthesized by reverse transcription and amplified using

![Figure 1](image-url)

**Figure 1**: MI provokes subclinical AKI compared with a sham or volume depletion model. Urine (A) and plasma (B) NGAL increased significantly in MI rats. Urine NGAL (μg/g Cr); sham 672.83 ± 39.83, VD 1167.66 ± 95.36 and MI 3290.52 ± 586.08. Plasma NGAL (μg/L); sham 106.86 ± 25.30, VD 139.95 ± 28.17, MI 454.89 ± 96.44. (C) Representative images of TUNEL-stained kidney tissue sections (×200). The mean value of TUNEL-positive cells in each group was compared. Significantly increased tubular cell apoptosis was observed in kidneys of MI rats. N = 5–8 per group. VD indicates volume depletion. *P < 0.05, **P < 0.01.
sequence-specific primer sets for rat tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6; Applied Biosystems, Foster, CA, USA) with iQ™ Supermix (Bio-Rad Laboratories). 18S rRNA was used as an internal control and fold increase compared with sham kidneys were calculated in both the volume depletion and MI group.

Histological examination
Formalin-fixed, paraffin-embedded kidney and liver were used for histological analysis. The sections of liver tissues were stained with periodic acid-Schiff (PAS). The sections of kidney tissues were stained with PAS, terminal deoxynucleotidyl transferase nick-end labelling (TUNEL; ApopTag Peroxidase In Situ Apoptosis Detection Kit, Millipore Corporation, Billerica, MA, USA), ED-1 and naphthol AS-D chloroacetate esterase (Sigma-Aldrich). The number of TUNEL-positive apoptotic cells, ED-1+ macrophages or esterase-positive neutrophils was quantified by counting 15–20 randomly selected ×200 fields in the cortex-to-corticomedullary region (glomeruli excluded), and the mean number of infiltrated cells was compared between the groups. In the kidney section from each group at 4 and 8 weeks, interstitial fibrosis was assessed by Masson’s Trichrome (MT) stain and immunohistochemical stain for TGF-β1 (Santa Cruz Biotechnology). For quantitative analysis, 10 random non-overlapping fields from the cortex-to-corticomedullary region from each section were captured using a ×40 magnification lens (BX51; Olympus, Tokyo, Japan). Blue on MT-stained sections or brown on TGF-β1-stained sections were selected for their intensity. The calculation of the proportional area (glomeruli excluded) was determined using image analysis (Leica Application Suite version 4.0, Leica Microsystems, Switzerland).

FIGURE 2: Systolic tail-cuff BP. Systolic BP was measured in conscious rats using the tail-cuff method; sham 125.54 ± 1.97, volume depletion 113.19 ± 1.80 and MI 114.08 ± 0.74 (mmHg). There was no difference between MI and volume-depleted rats (P = 0.69). *P < 0.05.

FIGURE 3: MI does not provoke liver injury. (A) Plasma ALT level did not increase in MI rats compared with sham; sham 29.40 ± 3.12 versus MI 28.00 ± 1.84 (IU/L), P = 0.71. (B) Representative images of PAS-stained liver tissue sections (×100). Liver cell necrosis was not found, and the infiltration of inflammatory cells was hardly seen in both sham and MI groups. N = 3 per group.
Statistical analysis

All data are expressed as mean ± standard error of mean (SEM) and were analysed by the Kruskall–Wallis test, followed by the Mann–Whitney U-test using SPSS 19 (SPSS, Chicago, IL, USA). A P-value of <0.05 was considered statistically significant.

RESULTS

Establishment of rat model of MI and volume depletion

Fractional shortening decreased significantly after LAD ligation (67.83 ± 0.03 versus 36.77 ± 2.92, baseline versus Day 1, P < 0.001) and remained decreased at Week 4 (54.75 ± 1.13 versus 42.58 ± 1.23, sham versus MI, P = 0.002). The establishment of the volume depletion model was confirmed by a significant decrease of FEUN in rats treated by low-salt diet and furosemide (0.64 ± 0.04 versus 0.45 ± 0.05, normal versus volume depletion, P = 0.036). A volume depletion model showed a significant decrease of body weight (+2.56 ± 1.28 versus −10.24 ± 1.19%, normal versus volume depletion on Day 3, P = 0.001).

Type 1 cardiorenal syndrome

MI-induced subclinical acute kidney injury. Rats were sacrificed on Day 3 post-surgery. Blood urea nitrogen (BUN), plasma creatinine level and PAS-stained kidney histology were not significantly different among the sham, volume depletion and MI groups (data not shown). However, urine and plasma NGAL levels and the number of TUNEL-positive apoptotic tubular cells increased significantly only in the MI group compared with the sham or volume-depleted groups, suggesting that MI provoked subclinical acute kidney injury (AKI; Figure 1A–C). Volume-depleted rats also showed slightly, but significantly elevated urine NGAL level (Figure 1A). However, plasma NGAL or tubular cell apoptosis did not increase (Figure 1B and C).

MI-induced subclinical AKI is caused by an exclusive heart–kidney crosstalk, but not by hypotension. To exclude the possibility that subclinical kidney injury following MI might

![Figure 4: MI provokes systemic and kidney inflammation. (A) Flow cytometric detection of CCR2+ ED-1+ cells in peripheral blood. The percentage of CCR2+ ED-1+ activated monocytes in peripheral blood increased significantly in MI rats. (B) Representative images of ED-1-stained kidney tissue sections (×200). The mean value of infiltrated ED-1+ cells in each group was compared. ED-1+ cell infiltration increased significantly in the kidneys of MI rats. N = 5–8 per group. (C) Quantitative RT-PCR of IL-6 and TNF-α in kidney tissue. Relative abundance of cytokines were expressed as fold difference compared with sham; IL-6, VD 2.97 ± 1.07, MI 4.75 ± 2.38; TNF-α, VD 1.47 ± 0.24, MI 1.95 ± 0.27. The kidneys of MI rats showed significantly increased inflammatory cytokine mRNA expression compared with sham. (D) Western blot analysis of nuclear p65 and cytosolic IκB in kidneys. Loading controls for nuclear and cytoplasmic proteins were lamin B1 and β-actin, respectively. Nuclear p65 increased and cytosolic IκB decreased in post-MI kidneys. N = 5–8 per group. VD indicates volume depletion. *P < 0.05, **P < 0.01. †P < 0.05 versus sham.](http://example.com/figure4)

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be simply caused by systemic hypotension, we measured BP and also examined functional and histological changes of the liver, another hypotension sensitive organ. Systolic BP significantly decreased in both volume-depleted and Day 1 post-MI groups compared with sham. However, there was no difference in BP between MI and volume-depleted groups (Figure 2). Neither plasma ALT nor PAS-stained liver tissue on Day 3 post-MI showed evidence of liver damage compared with sham (Figure 3A and B). These data could suggest that (1) volume-depleted rats can serve as an appropriate control with

**FIGURE 4:** Continued
comparable degree of haemodynamic compromise and (2) kidney injury observed on Day 3 following MI is likely to be caused by exclusive heart–kidney crosstalk, but not by MI-induced systemic hypotension.

**MI-induced subclinical AKI is characterized by inflammation and microvascular endothelial damage.** Following MI, the number of circulating CCR2+ activated monocytes increased in peripheral blood, and this was accompanied by significantly increased kidney infiltration of ED-1+ macrophages (Figure 4A and B). In contrast, esterase-positive neutrophil infiltration was hardly detected in all three groups (data not shown). Kidney tissue TNF-α and IL-6 mRNA expression also increased significantly post-MI, while the volume-depleted kidney showed no significant difference compared with sham (Figure 4C). In western blot analysis of the NF-κB pathway, an increase of nuclear p65 and decrease of cytosolic IκB in post-MI kidneys were observed, suggesting the possible contribution of the NF-κB-mediated inflammatory pathway in the development of subclinical AKI following MI (Figure 4D). In addition, we also observed that kidney microvascular endothelial permeability, assessed by EBD extravasation, increased significantly in post-MI kidneys (Figure 5).

**Type 2 cardiorenal syndrome**

**MI-induced Type 2 CRS.** Along with persistently decreased fractional shortening, a heart-to-body weight ratio in MI rats increased significantly compared with sham at Weeks 4 and 8 post-MI (Figure 6). To demonstrate chronic renal damage, we assessed the degree of interstitial fibrosis by MT stain and immunohistochemical stain for TGF-β1 in the kidney. MT-stained area, mainly located in the corticomedullary region, was significantly and progressively increased in MI rats from 4th week; area proportion (%), 0.23 ± 0.06 versus 0.44 ± 0.05 at Week 4, P = 0.033; 0.29 ± 0.01 versus 0.81 ± 0.11 at Week 8, P = 0.019, sham versus MI (Figure 7A). TGF-β1-stained area was also increased in MI rats, but it was statistically not significant; area proportion (%), 0.05 ± 0.01 versus 0.14 ± 0.03 at Week 4, P = 0.083; 0.13 ± 0.02 versus 0.59 ± 0.18 at Week 8, P = 0.087, sham versus MI (Figure 7A). In addition, the number of TUNEL-positive apoptotic tubular cells consistently and significantly increased until Week 8 following MI; 4.63 ± 0.72 versus 10.34 ± 1.47 cells/HPF at Week 4, P = 0.015; 0.13 ± 0.02 versus 0.59 ± 0.18 at Week 8, P < 0.001, sham versus MI (Figure 7B). While urinary albumin was not detected in sham or Day 3 post-MI, albumin excretion increased at Weeks 2, 4, and 8 post-MI: albumin/creatinine ratio (ACR, mg/mg Cr); 8.72 ± 1.79, 7.32 ± 0.97 and 10.56 ± 3.09; Weeks 2, 4 and 8, respectively (Figure 8). Urinary NGAL excretion (μg/g Cr) was still increased at Week 2 post-MI, but the difference gradually disappeared by Week 8 compared with sham (Figure 8). BUN or plasma creatinine did not increase until Week 8 (data not shown).

**Role of monocytes/macrophages in the pathogenesis of Type 2 CRS.** At Weeks 4 and 8 post-MI, the number of kidney infiltrating ED-1+ macrophages was significantly increased; 3.66 ± 0.75 versus 16.94 ± 1.76 cells/HPF at Week 4, P < 0.001; 3.51 ± 0.49 versus 12.53 ± 0.92 cells/HPF at Week 8, P < 0.001; sham versus MI (Figure 9A). Interestingly, the fibrotic area was in close proximity with ED-1+ macrophages (Figure 9B). To provide a better insight into the causal relationship between macrophage infiltration and renal fibrosis in Type 2 CRS, we compared the degree of renal fibrosis in MI rats treated with or without liposome clodronate, a systemic macrophage depletor. Administration of liposome clodronate, as expected, resulted in significant depletion of peripheral blood ED-1+ monocytes (Supplementary data S1). Injection of liposome clodronate was started on Day 7 post-MI and repeated every week until sacrifice. We observed that the fractional shortening of liposome clodronate-treated MI rats at Week 4 was comparable with that of MI rats without liposome clodronate administration (44.40 ± 1.36 versus 42.58 ± 1.23, P = 0.397, Figure 9C). The cross-sectional images of hearts
from both MI and liposome clodronate-treated MI rats at Week 4 are also presented as Supplementary data S2. Kidneys from liposome clodronate-treated MI rats showed significantly decreased ED-1+ macrophage infiltration and decreased fibrosis, suggesting the important causative role of monocytes/macrophages in the development of renal fibrosis in Type 2 CRS (Figure 9D). Furthermore, we also observed that increased TUNEL-positive tubular cells in post-MI kidneys were significantly decreased by liposome clodronate treatment (Figure 9D).

**Figure 7:** Development of CKD following MI. (A) Representative images of MT stained and TGF-β1 stained kidney tissue sections at Week 8 (×100) of sham and MI. Interstitial fibrosis increased in post-MI kidneys. (B) Representative images of TUNEL-stained kidney tissue sections (×100) of sham and MI. Tubular cell apoptosis increased in post-MI kidneys.
In this study using the rat MI model, we demonstrated the three novel findings. First, MI induced subclinical AKI, which was detected only by increases in the tubular damage marker, NGAL, and tubular cell apoptosis, consistent with the establishment of Type 1 CRS. Secondly, macrophage infiltration and inflammatory cytokine expression possibly mediated by the NF-κB pathway, and microvascular endothelial damage increased significantly in kidneys at 3 days post-MI, suggesting the important contribution of inflammation in the pathogenesis of Type 1 CRS. Thirdly, these changes ultimately led to renal interstitial fibrosis along with chronically decreased heart function (Type 2 CRS). We also confirmed the critical role of monocytes/macrophages in the development and progression of renal fibrosis in Type 2 CRS by showing the attenuation of renal fibrosis through systemic depletion of monocytes/macrophages.

Type 1 CRS is characterized by acute worsening of heart function leading to AKI. Worsening of heart function may originate from acute decompensated heart failure, acute coronary syndrome or cardiogenic shock. In this study, we created a rat model of acute coronary syndrome by permanent ligation of the LAD coronary artery, which resulted in significantly decreased fractional shortening of the left ventricle. We also made a volume depletion model as a control to this MI model, because haemodynamic alteration or neurohormonal activation resulting from reduced effective circulating volume has, until recently, been thought to be the predominant pathogenetic mechanism in Type 1 CRS.

In our rat model, MI provoked only subclinical AKI, which is diagnosed by the elevation of the tubular damage biomarker, NGAL and increased tubular cell apoptosis. The concept of subclinical AKI has recently emerged as a novel entity as the role of biomarkers has become better established. Recently, new diagnostic criteria of AKI that utilizes not only glomerular filtration rate/urine output, but also tubular damage markers, have been proposed [8]. Patients with subclinical AKI, who might not have been recognized before the biomarker era, are now known to be at an increased risk of progression to clinical AKI and, more importantly, are at an increased risk of adverse outcomes. A recent analysis of 10 different studies involving 2322 critically ill patients, predominantly with CRS, demonstrated that patients with NGAL+/serum Cr− were also at an increased risk of death or the need for renal replacement therapy [9]. Therefore, by measuring the plasma or urine NGAL level in MI patients, we can identify patients who are likely to benefit from intensive monitoring and precaution for further damage possibly from the use of diuretics or contrast. Presently, MI rats showed significantly elevated levels of plasma and urinary NGAL compared with rats in the sham or volume depletion groups. This is comparable with a recent report that showed the usefulness of urinary NGAL in distinguishing pre-renal from intrinsic renal failure in hospitalized AKI patients [10] and also supports our hypothesis that pre-renal azotaemia resulting from reduced effective circulating volume is not the dominant mechanism in Type 1 CRS. However, we also observed that urinary NGAL level increased significantly in volume-depleted rats compared with the sham group, with significantly lesser degree than MI rats. Although the significance of this finding is not clear in this study, recent studies have also showed that the urinary biomarker of AKI increases slightly in pre-renal AKI [11, 12].

Although a possible contribution of immune-mediated damage/inflammation to pathogenesis of Type 1 CRS has been postulated, direct evidence is still lacking. With a very small number of patients, a recent report raised the possibility that immune alteration or inflammation plays a critical role, by showing the defective regulation of monocyte apoptosis by plasma from patients with Type 1 CRS [13].

In this study, we first identified the increase of CCR2+ activated monocytes in peripheral blood along with significant macrophage infiltration in kidneys as early as 3 days after MI. The role of macrophages in the pathogenesis of other types of AKI such as ischaemia/reperfusion injury (IRI) has already been demonstrated [5]. Transmigrating from the circulation via
FIGURE 9: Role of monocytes/macrophages in the development of CKD following MI. (A) Representative images of ED-1 stained kidney tissue sections at Weeks 4 and 8 post-MI (×100). ED-1+ cell infiltration increased in post-MI kidneys. (B) Representative images of MT stained (left) and ED-1 stained (right) kidney tissue sections at Week 8 post-MI (×200). The MT-stained area overlapped with the area infiltrated by ED-1+ cells. (C) Fractional shortening at Week 4 post-MI with or without liposome clodronate treatment was compared, and there was no significant difference (P = 0.397). (D) The mean value of ED-1 or TUNEL-positive cells and the percentage of MT or TGF-β1-stained area in sham, MI and liposome clodronate-treated rats were compared. ED-1+ cell infiltration, tubular cell apoptosis and interstitial fibrosis (MT stain) increased significantly in post-MI kidneys, but these changes were decreased by liposome clodronate treatment. N = 5–8 per group. LC indicates liposome clodronate. P < 0.05, **P < 0.001 versus sham and LC, †P < 0.05 versus sham.
MCP-1/CCR2 signalling, ED-1⁺ macrophages play an important role in kidney injury by producing various proinflammatory cytokines. The observations that depletion of these cells has a renoprotective effect with decreased tissue cytokine levels and also that adoptive transfer of macrophages partially re-establishes post-ischaemic kidney injury strongly suggest the critical pathogenetic role of macrophages in IRI. Likewise, infiltration of macrophages into kidneys following MI is likely to contribute to kidney injury in our study. Although depletion strategy using liposome clodronate might be helpful in clarifying the direct causative role of monocytes/macrophages in MI-induced subclinical AKI, the possibility that depleting these cells before MI is likely to reduce initial infarct size precluded testing this strategy in assessing mechanisms underlying heart–kidney crosstalk in the development of Type 1 CRS.

Here, we also observed that kidney TNF-α and IL-6 expression increased significantly as well as tubular cell apoptosis, compared with sham and/or volume depletion model, and more interestingly, all these changes were associated with an increase in nuclear p65 and depletion of cytosolic IκB, suggesting that activation of the NF-κB pathway might be responsible for kidney inflammation in Type 1 CRS.

Considering that the role of tubular cell apoptosis in ischaemic or nephrotoxic AKI has been well known, cellular ATP depletion resulting from ischaemia caused by reduced effective circulating volume with a decreased ejection fraction following MI, production of proinflammatory cytokines, and oxidative stress can be the factors leading to increased tubular cell apoptosis following MI and likely to contribute to pathogenesis of Type 1 CRS.

In addition, we also assessed microvascular endothelial damage. Microvascular endothelial injury characterized by endothelial cell apoptosis, alteration of actin cytoskeleton or increased expression of leukocyte adhesion molecules has been known to be important in the initiation phase of IRI and by facilitating leukocyte transmigration, it substantially contributes to tissue inflammation. Therefore, increased kidney microvascular endothelial permeability possibly due to proinflammatory cytokines or generated oxygen-free radical following MI in our study is also thought to mediate kidney inflammation/injury.

However, due to the possibility that kidney injury observed in our study might result mainly from ischaemia provoked by MI-induced hypotension, we made the volume depletion model as a control and measured BP. Systolic tail-cuff BP decreased similarly in both MI and volume depletion groups, indicating that it was immune activation but not systemic hypotension that mediated kidney injury. In addition, we can also confirm the presence of exclusive heart–kidney inflammatory axis by demonstrating that another hypotension sensitive organ, liver, showed no evidence of functional and histological injury.

Although there was no functional change in the kidney, we could demonstrate the elevation of tubular damage biomarker

![Figure 9: Continued](image-url)
NGAL following acute MI. To our best knowledge, this is the first report that convincingly demonstrated the possible contribution of NF-κB-mediated inflammation, apoptosis and endothelial damage in the pathogenesis of Type 1 CRS. And as already mentioned, these results can also suggest that the long-held belief that AKI in Type 1 CRS is simply a fluid responsive prerenal azotaemia might be wrong.

Apart from Type 1 CRS, we also assessed whether MI provoked CKD; Type 2 CRS. This syndrome is characterized by chronic abnormalities of heart function such as chronic left ventricular dysfunction, atrial fibrillation or chronic ischaemic heart disease, leading to CKD. At Weeks 4 and 8 following MI, the development of kidney interstitial fibrosis was evident. As in Type 1 CRS, there was no significant functional renal change assessed by plasma creatinine in this model, but urinary albumin excretion increased from 2 weeks post-MI. We might need a longer term follow-up period to detect significant functional change. However, ED-1+ macrophage infiltration was still increased at Weeks 4 and 8 post-MI and was associated with interstitial fibrosis and increased TGF-β1 expression. More interestingly, the area of macrophage infiltration and fibrosis substantially overlapped, suggesting the critical role of macrophages in the development of fibrosis. Similar findings that showed the activation of Smad2–TGF-β pathway and fibrosis with a longer term follow-up in a rat MI model were recently demonstrated [14]. Macrophages play a critical role in the development of renal fibrosis following IRI, unilateral ureteral obstruction or in a chronic cyclosporine A nephrotoxicity model [15–17]. In these chronic fibrosing animal models, macrophages are thought to release profibrotic cytokine TGF-β, and depletion of monocytes/macrophages using liposome clodronate reduces the number of infiltrating macrophages and the degree of fibrosis. In our study, we also depleted monocytes/macrophages systematically, but injection of liposome clodronate was delayed until 1 week after MI due to the possibility that macrophage depletion could attenuate the degree of initial myocardial injury. Decreased renal fibrosis and decreased tubular cell apoptosis in liposome clodronate-treated MI rats was evident. Although immune/inflammation has been postulated as possible mechanism of CRS, this is the first report that provides direct and convincing evidence that monocyte-/macrophage-mediated inflammation plays a critical role in the progressive renal damage following MI. The findings suggest that targeting the inflammation–fibrosis pathway might be another potential therapeutic strategy in preventing renal damage in patients suffering from heart diseases.

In conclusion, this study identifies a possibly important role of monocytic inflammatory responses as a mediator of heart–kidney crosstalk in CRS. Further studies elucidating underlying pathophysiology are warranted to develop strategies to interrupt the harmful interaction between these organs and ultimately to improve patient outcome.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://ndt.oxfordjournals.org.

**CONFLICT OF INTEREST STATEMENT**

None declared. The results presented in this paper have not been published previously in whole or part, except in abstract format.

**REFERENCES**


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