α-MSH decreases apoptosis in ischaemic acute renal failure in rats: possible mechanism of this beneficial effect

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Abstract

Background. Apoptosis frequently occurs in acute renal injury but the molecular mechanisms responsible for this distinct form of cell death are largely unknown. Fas belongs to the tumour necrosis factor (TNF)/nerve growth factor superfamily and engagement by Fas ligand induces apoptosis in various epithelial cells. To investigate the role of apoptosis and associated mechanisms, we examined the occurrence of apoptosis and Fas and Fas ligand expression, and the therapeutic effect of α-melanocyte-stimulating hormone (α-MSH), a potent anti-inflammatory cytokine in an ischaemic acute renal failure (ARF) rat model. We also examined neutrophil infiltration together with intercellular adhesion molecule-1 (ICAM-1) expression because of their possible involvement in apoptosis due to their ability to release various inflammatory cytokines and reactive oxygen species.

Methods. After unilateral nephrectomy in female Sprague–Dawley rats, the renal artery of the contralateral kidney was clamped for 40 min and reperfused. α-MSH or vehicle was injected intraperitoneally immediately after reperfusion and at 1, 6, or 24 h after reperfusion. The expression of Fas and Fas ligand was studied by western blot analysis and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) method, and neutrophil infiltration by naphthol AS-D chloracetate staining. The degree of apoptosis, neutrophil infiltration, and Fas and Fas ligand, and ICAM-1 expression, as well as biochemical and histological data were compared between the α-MSH and the vehicle-treated groups.

Results. Intraperitoneally administered α-MSH significantly reduced renal injury, measured by blood urea nitrogen (BUN) and creatinine and by the degree of tubular necrosis (109.6 ± 7.1/54.7 ± 3.1 mg/dl for BUN, and 1.6 ± 0.2/1.03 ± 0.06 mg/dl for creatinine 24 h after ischaemia) (5.4 ± 0.8/2.6 ± 0.3 for injury score 24 h after ischaemia). Ischaemia caused an increase in Fas and Fas ligand expression and was accompanied by morphological evidence of apoptosis. α-MSH significantly reduced the degree of apoptosis, as well as Fas and Fas ligand expression (mean apoptotic cell number, 41.7 ± 3.5/14.2 ± 2.2 per × 200 field at 24 h after ischaemia. Fas protein expression: sham, 1409 ± 159 DI (densitometric index); vehicle/α-MSH, 2818 ± 635/1306 ± 321 DI at 24 h and 5542 ± 799/2867 ± 455 DI at 72 h after ischaemia. Fas ligand protein expression: sham, 1221 ± 181 DI; vehicle/α-MSH, 2590 ± 85/1279 ± 169 DI at 4 h, 4376 ± 268/2432 ± 369 DI at 24 h and 5200 ± 648/2253.7 ± 1104 DI at 72 h after ischaemia). Neutrophil infiltration and ICAM-1 expression were also significantly reduced in α-MSH group (neutrophil infiltration: vehicle/α-MSH, 5.05 ± 1.8/1.59 ± 0.4) (ICAM-1 expression, vehicle/α-MSH 0.46 ± 0.21/0.29 ± 0.19).

Conclusion. These results suggest that apoptosis clearly contributes to tubular cell loss in ischaemia/reperfusion (I/R) injury possibly by neutrophil-mediated pathways or an increase in Fas–Fas ligand expression. The observed beneficial effect of α-MSH could be related to these mechanisms.

Keywords: acute renal failure; apoptosis; ICAM-1; α-MSH; Fas; neutrophil

Introduction

Acute renal failure (ARF) that results from ischaemic or toxic insults to the kidney is usually referred to ‘acute tubular necrosis’ (ATN) pathologically, and loss of functional tubular epithelial cells is a major contributing factor to renal dysfunction in ARF. But
it is something of a misnomer because frank necrosis of tubular cells is found in only a few segments in human biopsy specimens and in experimental animal models [1,2]. This suggests that mechanisms other than necrosis may contribute to renal dysfunction in ARF.

Apoptosis, a particular form of cell death, has been frequently observed in kidney ischaemia/reperfusion (I/R) injury animal model and human ATN [3,4] and is thought to be an important mechanism of renal dysfunction in ARF. During the last decade, there has been much progress in understanding the role of apoptosis and also its triggering factors. Relative deficiencies in survival signals, various cytotoxic stimuli like reactive oxygen species, nitric oxide (NO) and receptor-mediated mechanisms can trigger apoptosis in the setting of ARF [5].

Fas, a 45-kDa transmembrane glycoprotein, is the best characterized receptor that can trigger apoptosis in various cells [6,7], and is also expressed in renal tubular epithelial cells [8]. The ligand for Fas belongs to the tumour necrosis factor (TNF) family and the Fas–Fas ligand system plays an important role in B and T lymphocyte development and maturation and in T cell cytotoxicity [9]. Recently Lorz et al. [10] demonstrated that Fas ligand is also expressed in normal renal tubular epithelial cells, suggesting that Fas–Fas ligand system may have some role in renal tubular cell biology. Although evidence for the involvement of this receptor mediated apoptosis in ARF is still lacking, recent reports by Feldenberg et al. [11] suggested that a Fas-mediated pathway may play a critical role in ischaemia, by demonstrating that increased apoptosis of MDCK cells was accompanied by increased Fas protein expression with partial ATP depletion. In addition, Ortiz-Arduan et al. [12] reported that lipopolysaccharide (LPS) can induce Fas and Fas ligand transcripts in cultured murine renal cells and suggested that they may play some role in endotoxaemia-induced ARF. These results suggest that Fas–Fas ligand interaction-induced apoptosis can be a major contributing factor to apoptosis in ARF.

α-Melanocyte stimulating hormone (α-MSH) is a pro-opiomelanocortin derivative and is an endogenous cytokine that suppresses the inflammation in various animal models by way of its inhibitory action on proinflammatory cytokines and chemo-attractive chemokines [13,14]. Recently Chiao et al. [15] reported the beneficial effects of α-MSH in ischaemic ARF rat models. The mechanisms of action of α-MSH in I/R injury are thought to be its inhibitory effect on neutrophil accumulation by down-regulating neutrophil chemokine and adhesion molecules like ICAM-1, as well as on maladaptive cytotoxic response during reperfusion period mediated by excessive production of NO [15,16].

Because activated neutrophils release various enzymes, inflammatory cytokines, and cytotoxic reactive oxygen species, and because these are also well-known apoptosis-inducing factors, we hypothesized that beneficial effects of α-MSH could be partly due to its possible inhibitory effect on apoptotic cell death.

In this study we examined the effects of α-MSH on renal injury and also on the occurrence of apoptosis in a model of ischaemic ARF. The degree of renal damage was assessed by biochemical and histological studies and apoptosis was examined by the terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick-end labelling (TUNEL) method. ICAM-1 expression and neutrophil infiltration into injured tissue were also examined. Finally, to clarify the effect of α-MSH on the Fas system, we examined Fas and Fas ligand expression in our rat model of ischaemic ARF.

Subjects and methods

Experimental design

Adult female Sprague-Dawley rats (National Institute of Health, Seoul, Korea) weighing 250–300 g were housed with free access to water and food. After anaesthesia with an intraperitoneal injection of 100 mg/kg ketamine, bilateral flank incisions were made. After left nephrectomy, the right renal artery was clamped using an atraumatic vascular clamp for 40 min. Animals were divided into three groups: ischaemia/vehicle, ischaemia/α-MSH, and sham group, and each group consisted of five rats. α-MSH, 50 μg, and the same volume of phosphate-buffered saline (PBS) (vehicle) was given intraperitoneally at the end of ischaemia, 1, 6 and 24 h after reperfusion, and then every 24 h thereafter. The sham group received the same surgical procedure except renal artery clamping and were sacrificed 24 h after the operation. Rats were sacrificed at 4, 24 and 72 h after reperfusion and blood samples were drawn by intracardiac puncture and tissues were snap-frozen in liquid nitrogen and stored at −80°C.

Biochemical analysis

BUN and plasma creatinine levels were evaluated using Hitachi 747 automatic analyser.

RNA extraction and semiquantitative reverse transcription polymerase chain reaction (RT-PCR) for Fas

Renal tissue was finely minced with a razor blade on ice and then homogenized in Trizol reagent (Gibco BRL, Grand Island, NY, USA). RNA extraction was performed according to the manufacturer’s protocol. After resuspension in a Tris-EDTA buffer, RNA concentrations were determined using spectrophotometric readings at absorbance 260 nm. One micromgram of RNA was reverse transcribed at 42°C for 60 min in the presence of 5 × first-strand buffer, 10 mmol/l dNTP, 20 U Rnasin, and 500 U of Moloney murine leukaemia virus reverse transcriptase (Superscripts, Gibco BRL) in a 25-μl reaction volume. First-strand cDNA (1 μl) was amplified using 2.5 U Taq polymerase (Perkin Elmer, Foster city, CA, USA) in a 50-μl reaction volume containing 0.4 μmol/l primer pair, 200 μmol/l dNTP, 10 mmol/l...
Tris–HCl, 1.5 mmol/l MgCl$_2$, and 50 mmol/l KCl. The sequences of primer for rat Fas were: sense primer, 5'-GACCCCGAACTACCAAGTGA-3', antisense primer, 5'-CTGTTGTTGCGTGTCTTTGG-3' and for rat ribosomal protein L-19, as an internal standard, sense primer, 5'-AGCCTGCTGACTGCTACTCC-3' and antisense primer, 5'-TTTCATCTCTAGCCTGAGG-3'. The amplifying conditions were 36 cycles of the following: denaturation for 60 s at 94°C, annealing for 60 s at 58°C, and extension for 60 s at 72°C. The PCR products were analysed in a 1% agarose gel stained with ethidium bromide and band densities were estimated using a Digital Imaging & Analysis System (Alpha Innotech Corp., San Leandro, CA, USA). The ratio of Fas PCR product to L-19 were compared.

**Northern blot analysis**

Samples of total RNA (40 μg) were fractionated by electrophoresis on 1% agarose-formaldehyde gel and transferred to a nylon membrane. The equality of RNA samples was substantiated by UV illumination of ethidium bromide. After fixation in a UV cross-linker, membranes were pre-hybridized at 43°C in a Prehyb/Hyb buffer (Ambion, Austin, TX, USA) for 3 h and then hybridized with [32P]CTP labelled cDNA clones. The hybridized membranes were washed twice in 1x SSC and 0.1% SDS at 43°C, and subjected to autoradiography at ~70°C. The band intensities were compared using the Digital Imaging & Analysis System (Alpha Innotech Corp., San Leandro, CA, USA). Loading of RNA was normalized by rehybridizing with L-19. 0.4 kb portions of rat ICAM-1 and as an internal standard, 0.35 kb rat L-19 cDNA were generated by PCR and cloning (TA cloning kit, Invitrogen, San Diego, CA, USA) and cloned products were confirmed by sequencing.

**Western blot analysis**

Rat kidney tissue was minced in the presence of 50 mmol/l Tris, 0.1% NP-40, 0.2 mol/l NaCl, 1 mmol/l EDTA, 50 μg/ml PMSF, 1 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. The resultant pellet was subjected to homogenization. The supernatant was collected and saved. The protein content was measured by absorbance at 540 nm using a BCA protein assay kit (Pierce, Rockford, IL, USA). The samples were initially analysed by 10% SDS–polyacrylamide gel electrophoresis and Coomassie stained. The aliquots of each sample containing 25 μg of protein were resolved using a 10% SDS–polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. After incubation in a blocking solution (5% non-fat dry milk in PBST (0.05% Tween 20 in PBS)) at room temperature for 1 h, the membranes were subjected to repeated washing in PBST (4 × 10 min) and then incubated with primary antibodies in blocking solution at 4°C overnight. After repeated washing, membranes were reacted with a secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. The primary antibodies used were: (i) 1:1000 goat anti-Fas IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and (ii) 1:2000 mouse anti Fas ligand IgG (Transduction Laboratories, Lexington, KY, USA). After washing in PBST (8 × 10 min), the membranes were visualized using enhanced chemiluminescence (ECL: Amersham, Arlington Heights, IL, USA) and exposed to Kodak XAR5 film. The band intensities were compared using the Digital Imaging & Analysis System (Alpha Innotech Corp., San Leandro, CA, USA).

**Histological examination**

Ten per cent formalin-fixed and paraffin-embedded kidney tissues were stained with haematoxylin and eosin (H&E, 3 μmol/l), periodic acid–Schiff (PAS) and naphthol AS-D chloroacetate esterase (Sigma Chemical Co., St Louis, MO, USA). The severities of renal injury were quantified using histological scoring system that we developed (Table 1). The number of tubular cell necrosis, intratubular cast formation, apoptosis, and brush-border changes in the outer medulla were examined in 20 randomly selected 200 × field sections and according to the scoring system, a mean of the total score was compared between the groups. Neutrophil infiltration was also quantitatively measured by counting 20 randomly selected ×400 field sections in the outer medulla, and the mean number of infiltrated cells was compared between the groups.

**In-situ detection of DNA strand breaks**

To identify nuclei with DNA strand breaks, the TUNEL method using Apop tagTM (Oncor Inc., Gaithersburg, MD, USA) was used. Briefly, paraffin-embedded sections were deparaffinized in xylene for 5 min and rehydrated through graded concentrations of ethanol. After washing with PBS twice for 5 min, the sections were treated with 1.0 μg/ml proteinase K in PBS at 37°C for 15 min and washed with deionized water for 10 min. To inactive endogenous peroxidase, the tissue sections were incubated in 2% H$_2$O$_2$ at 37°C for 15 min and then rinsed with deionized water for 10 min. The slides were then incubated with a TdT buffer (25 mmol/l Tris–HCl buffer, pH 6.6, 0.2 mol/l potassium cacodylate, and 0.25 mg/ml BSA) at room temperature for 30 min, and after the incubation the slides were reacted with 0.1 U/μl TdT dissolved in a TdT buffer supplemented with 1.0 mmol/l digoxigenin-dUTP in a humid chamber at 37°C for 1 h. The signals were detected immunohistochemically with a horseradish peroxidase-conjugated sheep anti-Dig antibody. Quantitative measurement of apoptotic cells was done by examining the 10 randomly selected ×200 fields in the outer medulla and counting the mean number of apoptotic cells per field.

**Statistical analysis**

Results were presented as means±SEM. Comparison between the ischaemia/vehicle group and ischaemia/z-MSH group was done using an independent sample t-test and a P value < 0.05 was considered to be statistically significant.

**Table 1. Histological scoring system**

<table>
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Results

Biochemical data

BUN and plasma creatinine levels started to rise in both vehicle and α-MSH groups at 4 h after reperfusion and peaked at 24 h. In the α-MSH group, BUN and creatinine levels at 24 h after reperfusion were significantly lower than those of vehicle group (109.6 ± 7.1/54.7 ± 3.1 mg/dl for BUN, 1.6 ± 0.2/1.03 ± 0.06 mg/dl for creatinine) (P = 0.002, P = 0.04) (Figure 1).

Histology

Kidneys were processed for histological examination 24 and 72 h after reperfusion, and in the vehicle-treated ischaemia group the most severe and pronounced injuries were seen in the outer stripes of outer medulla at 24 h after reperfusion, with typical ‘acute tubular necrosis’ pattern; namely, widespread tubulocell necrosis, intratubular cast formation and flattening of brush borders. However, in the α-MSH treated group, these areas of tubular necrosis and tubule obstruction with casts were more focal and mild (Figure 2). In the histological grading system, the mean score of injury in the vehicle group 24 h after reperfusion was 5.4 ± 0.8 and in the α-MSH group, 2.6 ± 0.3 (P = 0.008) (Figure 2).

Mean number of neutrophils infiltrated in ×400 field at 24 h increased from 0.18 ± 0.06/field in the sham group to 5.05 ± 1.79 in vehicle group, but in the α-MSH group, neutrophil infiltration decreased significantly to 1.59 ± 0.39 (P = 0.009) (Figure 3).

Detection of apoptosis

TUNEL-positive apoptotic cells were observed as early as 4 h after reperfusion and the number of apoptotic cells peaked 24 h after renal ischaemia. There were more apoptotic cells in the outer medulla than in the cortex and most apoptotic cells were detached from tubular basement membrane and located in the tubular lumen (Figure 4). In the α-MSH group, the mean number of apoptotic cells in the outer medulla 24 h after reperfusion decreased significantly compared with the vehicle group (41.7 ± 3.5/14.2 ± 2.2 per ×200 field) (P = 0.004) (Figure 4).

Effects of α-MSH on ICAM-1 mRNA expression

ICAM-1 mRNA expression showed a decrease in the α-MSH group at 24 h reperfusion (0.46 ± 0.21/0.29 ± 0.19) and significantly decreased at 72 h reperfusion (0.49 ± 0.01 and 0.31 ± 0.17) (P = 0.11 and P = 0.04 respectively) (Figure 5).

Fas mRNA expression in ischaemia/reperfusion injury and effects of α-MSH on Fas expression

Fas mRNA expression was examined using a semi-quantitative RT-PCR technique. Fas/L-19 ratio increased from baseline values of 0.86 ± 0.2 in the sham-operated group to 1.45 ± 0.4 and 1.14 ± 0.2 at 4 and 24 h after reperfusion. α-MSH decreased Fas/L-19 ratio to 1.18 ± 0.1 and 0.62 ± 0.1 respectively (Figure 6).

Fas and Fas ligand protein expression in ischaemia/reperfusion injury and effects of α-MSH on Fas and Fas ligand protein expression

Fas protein expression was detectable at low levels in the sham-operated group (1409 ± 159 DI) and increased in the vehicle group to 2818 ± 635 DI at 24 h, and further increased to 5542 ± 799 DI at 72 h after reperfusion. α-MSH markedly inhibited Fas protein expressions to 1306 ± 321 DI and 2867 ± 455 DI at 24 and 72 h respectively (P = 0.07 and 0.047) (Figure 7).

Fas ligand protein expression was also detectable at low levels in the sham-operated group and increased in the vehicle group at 4 h and further increased at 24 and 72 h after reperfusion (1221 ± 181 DI for
Fig. 2. Effect of α-MSH on renal histology in kidney I/R injury. (A) Vehicle group. (B) α-MSH group. In the vehicle group, multifocal areas of ATN with desquamation of epithelial cells and cytoplasmic casts in the lumen are noted. In the α-MSH group, only a focal area is affected by mild tubular changes with loss of brush borders and flattening of tubular cells. (PAS stain, outer medulla, ×200). (C) Quantitation by histological grading system. The severity of injury peaked at 24 h after reperfusion, and in the α-MSH group the severity of injury decreased significantly. Data are expressed as mean ± SEM. *P<0.05 compared with vehicle group.

Fig. 3. Effect of α-MSH on neutrophil infiltration in kidney I/R injury. (A) Vehicle group. (B) α-MSH group. Occasional neutrophil infiltrations are noted at peritubular space in outer medulla in vehicle group (arrow). (Naphthol AS-D chloracetate stain, ×200). (C) Quantitation of neutrophil infiltration. Neutrophil infiltration significantly decreased in α-MSH group. Data are expressed as mean ± SEM. *P<0.05 compared with vehicle group. at 24 h, and 2253.7 ± 1104 DI at 72 h respectively (P = 0.002, P = 0.013 and P = 0.06) (Figure 8).

Discussion

Cell death from apoptosis is a well-recognized phenomenon in various renal diseases as well as in early renal development [17,18]. Apoptotic cell death is
**Fig. 4.** Effect of α-MSH on apoptosis in kidney I/R injury. (A) Vehicle group. (B) α-MSH group. Many TUNEL-positive apoptotic cells are noted in tubular lumen at 24 h after reperfusion in vehicle group. In α-MSH group, occasional apoptotic cells are noted demonstrating the lesser degree of apoptosis (In situ end labelling, outer medulla, ×200). (C) Mean number of apoptotic cells. Compared to the sham group in which there were very few apoptotic cells, the number of apoptotic cells increased at 4 h after reperfusion and peaked at 24 h. In the α-MSH group, apoptosis decreased significantly at 24 h after reperfusion. Data are expressed as mean ± SEM. *P < 0.05 compared with vehicle group.

**Fig. 5.** Northern blot of ICAM-1 in kidney I/R injury. Compared with vehicle group, ICAM-1 mRNA expression decreased in the α-MSH group. Data are expressed as mean ± SEM. *P < 0.05 compared with vehicle group.

**Fig. 6.** Semi-quantitative RT-PCR of Fas in kidney I/R injury. Fas mRNA expression started to increase at 4 h after reperfusion and had increased further at 24 h. Compared to vehicle group, Fas mRNA expression decreased significantly in the α-MSH group. Data are expressed as mean ± SEM. *P < 0.05 compared with vehicle group.
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Fig. 7. Western blot of Fas in kidney I/R injury. Fas protein was basally expressed in the sham group and increased in vehicle group. In the z-MSH group, Fas expression decreased significantly. Data are expressed as mean±SEM. *P<0.05 compared with vehicle group.

Fig. 8. Western blot of Fas ligand in kidney I/R injury. Fas ligand protein was detectable in the sham group and increased in the vehicle group. In the z-MSH group, Fas ligand expression decreased significantly. Data are expressed as mean±SEM. *P<0.05 compared with vehicle group.

frequently observed in ATN biopsy specimens and in I/R injury animal models, and this might play a pathogenetic role in renal dysfunction in ARF [1,2]. There are multiple factors that are known to induce tubular cell apoptosis and those factors can be divided into several categories [5]. First, lack of survival signals from relative deficiencies in soluble growth factors and from loss of normal cell–cell and cell–matrix interactions; second, cytotoxic stimuli such as increased calcium, reactive oxygen species and numerous nephrotoxic drugs; and third, various receptor-mediated mechanisms. Of the receptor-mediated mechanisms, Fas/APO-1/CD95, members of the TNF receptor/nerve growth factor receptor superfamily have recently been implicated in various disease pathogenesis [7,12,19]. Fas and Fas ligand system have been known to be linked to lymphocyte maturation and lymphocyte-mediated disease, and these were originally believed to be restricted primarily to activated T cells and immune privileged tissues [9]. But recent studies demonstrated that Fas and Fas ligand are basally expressed in many epithelial cells and their upregulation is responsible for cell losses in various disease processes [7,10,12,19].

Feldenberg et al. [11] reported that partial ATP depletion induced apoptosis in MDCK cells and it was accompanied by Fas upregulation. Nogae et al. [3] also suggested possible involvement of Fas in ischaemia/reperfusion models of mouse kidneys by demonstrating increased Fas mRNA expression.

Fas ligand, a 40-kDa type II transmembrane protein, was thought to be primarily restricted to immune privileged sites, but is reported to be constitutively expressed in kidney tubular cells and upregulated upon TNF-α stimulation, with a resultant increase in apoptosis [10,12,19]. Ortiz-Arduan et al. [12] demonstrated increased Fas and Fas ligand expression accompanied by morphological evidence of apoptosis in LPS-stimulated renal cells as well as in endotoxaemia rat models induced by LPS injection. All these results suggest that Fas and Fas ligand upregulation-induced apoptosis can be an another pathogenetic mechanism of renal dysfunction in ischaemic ARF. However, Fas dependent apoptosis has not always been demonstrated evenly in all experiments, Boonstra et al. [8] reported that despite constitutive expressions of Fas on tubular epithelial cells, they appeared to be resistant to Fas-mediated apoptosis when treated with a stimulatory anti-Fas antibody. So whether Fas-mediated apoptosis of tubular cells contributes to tubular cell loss or to renal dysfunction in ARF still remains unclear.

In this study, apoptosis, confirmed by DNA fragmentation using the TUNEL method, was observed mainly in the outer medulla, the most susceptible area in ischaemia, as early as 4 h after ischaemia, peaking at 24 h. Most apoptotic cells were detached from the tubular basement membrane and found in the tubular lumen, indicating that most apoptotic cells are tubular cells rather than infiltrated inflammatory cells in the interstitium. At 72 h after ischaemia, there were few
apoptotic cells, and this was accompanied by frequent mitosis of tubular cells, suggesting that in ischaemic ARF, apoptosis observed in the first 3 days after I/R injury is responsible for tubular cell loss, not regeneration activity.

Recently neutrophils recruited during reperfusion have been implicated as mediators of renal parenchymal injury, and because of their ability to release a variety of toxic materials such as oxygen free radicals and inflammatory cytokines, infiltrated neutrophils can induce tubular-cell apoptosis [20]. In this study we demonstrated an approximately 10-fold increase in neutrophil infiltration compared to the sham group. Most neutrophils were found at the peritubular space in the outer medullary area in the vehicle group compared with the sham group, and this increase in neutrophil infiltration could be associated with increased tubular-cell apoptosis.

But we also demonstrated a significant increase in renal tissue Fas mRNA and protein expression in the vehicle group, which persisted even at 72 h after reperfusion. Ischaemia also induced an increase in Fas ligand expression and this effect persisted at 24 and 72 h after reperfusion as well.

The observation that tubular cell apoptosis in ischaemic ARF was accompanied by increased Fas and Fas ligand expression suggests that Fas–Fas ligand upregulation-induced apoptotic cell death could also play some role in renal dysfunction in ARF. This is consistent with the observations by Feldenberg et al. [11] and Ortiz-Arduan et al. [12], but the exact mechanisms of Fas and Fas ligand upregulation in ischaemic ARF remains undefined.

Direct upregulation by ischaemia itself or induction by pro-inflammatory cytokines such as TNF-α or interleukin-1β (IL-1β) produced in the reperfusion period might be responsible for them [21]. TNF-α in I/R injury can induce various adhesion molecules, like ICAM-1, E-selectin, and P-selectin on endothelial cells and continue the cascade of events that increase cell adherence, leukocyte activation, and inflammatory processes in injured tissues [22]. In addition, local production of TNF-α after ischaemic injury has been reported to be associated with DNA fragmentation, and treatment of anti-TNF monoclonal antibody reduced the degree of DNA fragmentation as well as the degree of biochemical and histological renal damage, suggesting that TNF-α is a major factor not only in inflammation but also in inducing apoptosis in ischaemic ARF [21,23]. In this study, the discrepancy between the peak occurrence of apoptosis at 24 h and that of Fas and Fas ligand expression at 72 h, when apoptosis was hardly found in this study, also favours the suggestion that increased Fas and Fas ligand expression might be just an epiphenomenon secondary to increased inflammatory cytokine, like TNF-α, playing a relatively minor role in apoptosis of tubular cells in I/R injury.

The observation by Donnahoo et al. [24] that early renal tissue TNF-α expression contributes to neutrophil infiltration in I/R injury also supports this, and we can suggest that neutrophil-mediated or other cytokine-mediated pathway is more important than increased Fas and Fas ligand expression in tubular cell apoptosis. However, to define the exact role of an individual pathway in inducing apoptosis, the temporal relationship between the expression of various cytokines, Fas and Fas ligand expression and apoptosis should be examined as well as the effects of antagonistic Fas monoclonal antibodies.

α-MSH, an endogenous anti-inflammatory cytokine, has been known to reduce cellular infiltrations in inflammatory conditions such as various models of inflammation and liver injury from septic shock [13,14]. In LPS-induced liver inflammation, α-MSH prevented liver damage, and its suggested mechanisms of action included an inhibition of pro-inflammatory cytokine (TNF-α) and chemoattractant chemokine (KC/IL-8 or monocyte chemoattractant protein-1) gene expression, with resultant inhibition of hepatic neutrophil infiltrations, in addition to inhibition of systemic NO production [13]. Recently α-MSH has proved beneficial in ischaemic ARF [15]. Chiao et al. [15,16] demonstrated that α-MSH could attenuate I/R injury in bilateral 40-min renal artery clamping murine models through its inhibitory actions on the mouse chemokine KC and ICAM-1 message, and on the induction of iNOS with a resultant decrease in peroxynitrate production [14–16].

In this study we could also demonstrate that intraperitoneally administered α-MSH inhibited the increase in the BUN and plasma creatinine levels 24 h after reperfusion and also the degree of histological damage. ICAM-1 message and polymorphonuclear cell (PMN) infiltrations were significantly reduced in α-MSH-treated groups. Additionally, α-MSH significantly decreased apoptosis in the outer medulla at 4 and 24 h after ischaemia with concomitant decreases in Fas and Fas ligand expression. The effects of α-MSH on apoptosis and the expression of Fas and Fas ligand has not been studied previously, but these data suggest that α-MSH may decrease apoptosis via at least two distinct mechanisms: (i) by inhibiting neutrophil- and inflammatory cytokine-mediated pathways, and (ii) by direct inhibitory effect on renal tissue Fas and Fas ligand expression. Although early renal tissue TNF-α and neutrophil infiltration increase tubular-cell apoptosis and the inhibitory action of α-MSH on the expression of TNF-α, neutrophil chemokines, and adhesion molecules, as demonstrated by Chiao et al. [13,15], whether α-MSH has direct inhibitory effect on Fas and Fas ligand has not been clarified; this needs in vitro experiments to exclude neutrophil and cytokine effects.

In addition, our data do not exclude the possibility that tubular-cell apoptosis occurred by Fas-independent mechanisms, and that Fas and Fas ligand up-regulation was mediating subsequent events such as the removal of damaged tubular cells. But Fas and Fas ligand up-regulation beginning as early as 4 h after reperfusion, together with apoptosis and
significant inhibition of their expression, as well as apoptosis in the \( \alpha \)-MSH group, can suggest that increased Fas and Fas ligand expression may partially mediate tubular-cell losses and subsequent renal dysfunction in ARF.

Future availability of soluble Fas or recombinant Fas ligand will help in identifying their exact role in inducing apoptosis and also the effect of \( \alpha \)-MSH on apoptosis and Fas and Fas ligand expressions. Additionally, the therapeutic trial of \( \alpha \)-MSH in conditions in which apoptotic cell death is predominant, such as drug-induced nephrotoxicity or ureteral obstruction, will be useful.

Our study suggests that tubular-cell apoptosis may play some role in renal dysfunction in ischaemic ARF, and the beneficial effects of \( \alpha \)-MSH are partially related to the inhibitory action on apoptosis. The suggested mechanisms of \( \alpha \)-MSH in decreasing apoptosis may include its inhibitory effect on neutrophil infiltration and inflammatory cytokine production. However, its direct effect on renal tissue Fas and Fas ligand system may also be partially responsible for the beneficial effect on renal dysfunction in ischaemic ARF.

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