Mitochondrial Pathway Is Responsible for Aging-Related Increase of Tubular Cell Apoptosis in Renal Ischemia/Reperfusion Injury

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Aging-related changes of tubular cell apoptosis and its mechanisms in renal ischemia/reperfusion (I/R) injury are unclear. In the present study, aged (27-month-old) and young (3-month-old) Wistar rats were used to investigate aging-related tubular cell apoptosis in the setting of renal I/R injury. The renal I/R model was induced by clamping bilateral renal arteries for 30 minutes followed by reperfusion for 18 hours. Cyclosporine A (CsA, 2 mg/kg) or mycophenolate mofetil (MMF, 20 mg/kg/d) was used before ischemia. Age-matched sham-operated rats served as controls. We found that tubular cell apoptosis increased more significantly in aged rats than in young rats after renal I/R. More pronounced increases of Bax/Bcl-2 ratio, cytosolic cytochrome c, and caspase-9, which are involved in mitochondria-mediated apoptosis, were found in aged rats than in young rats, and were associated with a more pronounced decrease in superoxide dismutase activity and increase of malondialdehyde content. However, increases of tumor necrosis factor-α and caspase-8, two components of death receptor-mediated apoptosis, showed no aging-related differences. Interfering mitochondria and death receptor pathways with CsA and MMF, respectively, reduced the apoptosis in both age groups, whereas CsA was more effective in aged rats. Our results have demonstrated that there was an aging-related increase of tubular cell apoptosis in the renal I/R model, which may be, at least partly, due to an enhanced mitochondrial pathway resulting possibly from increased oxidative stress.

ACUTE renal failure (ARF) secondary to ischemia/reperfusion (I/R) injury is a frequent clinical problem associated with high morbidity and mortality (1,2). Susceptibility to ARF in elderly people is more common given the underlying compromise of renal function with aging (3).

Traditionally, tubular cell death in ARF was attributed to necrosis, but recent studies demonstrated that apoptosis accounts for a significant component of renal dysfunction in ARF (4–7). A brief period of ischemia induced remarkable apoptotic tubular cell death (8).

Dysregulation of apoptosis is also associated with the aging process (9–12). Aged rats exhibited enhanced tubular cell apoptosis compared with young ones in physiological conditions (13). However, whether apoptosis is increased in aged animals with renal I/R injury is yet unknown.

Two major caspase-dependent pathways have been described to regulate apoptosis (4). First is the mitochondrial (intrinsic) pathway, in which mitochondria play a central role (14). Mitochondria participate in apoptosis by opening the mitochondrial permeability transition pore (PTP) and releasing apoptogenic proteins, mainly cytochrome c. Release of cytochrome c facilitates activation of caspase-9 (4,15). Oxidative stress is known to trigger PTP. Bcl-2 family members, such as Bcl-2 (an anti-apoptotic protein) and Bax (a pro-apoptotic protein) tightly regulate the mitochondrial PTP (4,16). Cyclosporine A (CsA) is an inhibitor of mitochondrial PTP, and it prevented apoptosis in many cell types (17–21). The second is the death receptor (extrinsic) pathway, which is initiated by binding of extracellular ligands (tumor necrosis factor-α [TNF-α], Fas ligand) to their cell-surface receptors, leading to caspase-8 activation (4,22). Mycophenolate mofetil (MMF) interfered with the extrinsic pathway by inhibiting the expression of TNF-α (23). Pretreatment with CsA or MMF protected against renal I/R injury (24,25). In both the intrinsic and extrinsic pathways, activation of downstream effector caspases (of which caspase-3 is the most important) plays an indispensable role.

It is reported that both intrinsic and extrinsic pathways participate in renal I/R injury-induced tubular cell apoptosis in young animals (4). Aging-related apoptotic pathway(s) are currently unclear. Higami and colleagues (26) reported that Fas messenger RNA (mRNA) levels increased with aging in rat liver, proposing that Fas-mediated apoptosis might be the underlying mechanism for increased susceptibility of aged liver cells to apoptosis. Zhang and colleagues (9) found an increase in the activity of mitochondrion pathway of apoptosis in aged rats’ liver, indicating a potential role for the mitochondria in the aging-dependent increase of apoptosis. Information in the literature on renal I/R injury in aged animals is scarce. In the present study, we
examined aging-related differences in tubular cell apoptosis as well as caspase-dependent intrinsic and extrinsic pathways in a rat model of renal I/R injury. Then we performed experiments to interfere with the intrinsic and extrinsic pathways using CsA or MMF, respectively, and tried to explore the differences of apoptosis degree and responsible pathway(s) in the model of renal I/R injury of rats at different ages.

**METHODS**

**Care and Use of Laboratory Animals**
Animal experiments were performed in accordance with the regulations for the care and use of laboratory animals, and were approved by the local authorities. Young adult (3-month-old) and aged (27-month-old) male Wistar rats were housed at 21°C on a 12-hour light/dark cycle, and were allowed free access to food and water.

**Establishment of Animal Model**
Young and aged male Wistar rats were divided into four groups (sham, I/R, I/R + CsA and I/R + MMF), respectively, based on randomized block design. Rats were anesthetized with intraperitoneally administered pentobarbital (50 mg/kg), and body temperature was maintained between 36°C and 38°C. Rats (except those in the sham group) were subjected to 30 minutes of renal ischemia by clamping bilateral renal arteries with nontraumatic vascular clamps. Sham operation was performed in the same way except clamping the arteries. Rats in aged and young I/R + CsA groups were pretreated with CsA (2 mg/kg, administered intraperitoneally; Sandimmun; Novartis, Basel, Switzerland), a mitochondrial PTP antagonist, 15 minutes before renal ischemia. Rats in aged and young I/R + MMF groups received MMF (20 mg/kg/d, administered by gavage; Roche Laboratories, Nutley, NJ), a drug interfering with the extrinsic pathway, 2 days before ischemia and maintained during the entire study. The use of CsA or MMF was based on our preliminary studies, which indicated that both intrinsic and extrinsic pathways participated in renal I/R injury-induced tubular cell apoptosis, and that there were significant differences in the extent of different apoptotic pathways between the aged and young rats. Doses of CsA and MMF were selected based on our preliminary studies and the results of others (24,25). Rats were killed at 18 hours of reperfusion. Blood and both kidneys were harvested for further analysis.

**Assessment of Renal Function**
Renal function was assessed by measuring serum creatinine concentrations with a commercially available colorimetric method (Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R. China).

**Histological Examinations**
Kidney slices were fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 3 μm, and stained with periodic acid-Schiff (PAS). Tubulointerstitial tissue damage was evaluated using a semiquantitative scoring system, as previously described (27). The different histologic parameters, such as cell swelling, nuclear pyknosis, cell lysis, loss of proximal tubules brush border, cast formation, and interstitial infiltration in the outer medulla were examined in 20 randomly selected ×200 field sections and, according to the scoring system, a mean of all the scores was used for comparisons between the groups. Morphologic assessment was performed by an experienced renal pathologist, who was unaware of the treatment each animal had received.

**In Situ Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End-Labeling Assays**
Terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) staining was used to detect DNA fragmentation in apoptosis. Fixed rat kidney sections were deparaffinized in xylene and rehydrated through a graded ethanol series to water. TUNEL staining (brown) was performed with a commercially available in situ cell death detection kit (Promega, Madison, WI), according to the instructions provided by the manufacturer. For observation of the total number of cells in the field, kidney sections were also stained with hematoxylin (blue). TUNEL-positive nuclei were expressed as a percentage of total nuclei per field. Six to eight outer medulla and inner medulla fields per section and two to three sections per kidney were examined in each experiment (28).

**Apoptosis-Induced DNA Laddering**
Renal apoptosis was also demonstrated by DNA laddering (intranucleosomal DNA fragmentation). The extracted DNA was subjected to electrophoresis at 100 V through a 2.0% agarose gel in Tris–acetate–EDTA buffer. The gel was stained with ethidium bromide and photographed with ultraviolet illumination.

**Superoxide Dismutase Activity and Malondialdehyde Content**
For measurement of superoxide dismutase (SOD) activity and products of lipid peroxidation malondialdehyde (MDA), rat kidneys were homogenized in ice-cold 20 mM Tris–HCl buffer (pH 7.4). SOD activity and MDA content were determined with commercially available kits, respectively, according to the manufacturer’s instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R. China).

**Northern Blot Analysis for TNF-α, Bax, and Bcl-2 Expression**
Total renal RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were determined on the basis of spectrophotometric absorbance at 260/280 nm, and total RNA (25 μg) was subjected to electrophoresis on 1% agarose-formaldehyde gels and transferred to a nylon membrane. After fixation in an ultraviolet cross-linker, membranes were prehybridized at 42°C in a Prehyb/Hyb buffer (Ambion Institute, Austin, TX) for 2 hours and then hybridized with 32P-labeled complementary DNA (cDNA) clones for 16 hours. The band intensities were compared with 28S RNA to confirm equal RNA input.
Western Blot Analysis for Cytosolic Cytochrome c and Caspase-3, 8, and 9 Expressions

Protein isolation from kidney tissue was performed as reported (29). Briefly, for caspase-3, -8, and -9 protein isolations, frozen kidney tissue was homogenized in radioimmunoprecipitation (RIPA). The homogenates were centrifuged at 12,000 g for 10 minutes at 4°C, and the supernatants were collected.

For cytosolic protein isolation, kidney tissue was lysed in ice-cold buffer (20 mM HEPES-KOH, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, digitonin at 500 µg/ml, 0.1 mM PMSF, aprotinin at 2 µg/ml, leupeptin at 10 µg/ml, and pepstatin at 5 µg/ml). Lysates were centrifuged at 750 g for 10 minutes at 4°C, and then at 8000 g for 20 minutes at 4°C. The supernatant was centrifuged again at 100,000 g for 60 minutes at 4°C to remove any mitochondrial contamination.

The total and cytosolic fractions were denatured in sample buffer at 100°C for 5 minutes. Protein (60 µg) was subjected to electrophoresis on sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes, and blocked with 5% nonfat milk in TBST (Tris-buffered saline plus Tween 20) buffer overnight at 4°C. Membranes were then incubated with rabbit polyclonal anti-caspase-3, rabbit polyclonal anti-caspase-8, rabbit polyclonal anti-caspase-9, or rabbit polyclonal anti-cytochrome c (all 1:200; all obtained from Santa Cruz Biotechnology, Santa Cruz, CA). Blots were washed with TBST buffer and subsequently incubated with peroxidase-conjugated AffiniPure goat anti-rabbit immunoglobulin G (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were washed with TBST buffer and developed with enhanced chemiluminescence (ECL) reagents (Santa Cruz Biotechnology). Rat polyclonal anti-β-Actin antibody (1:100; Santa Cruz Biotechnology) was used as the control for each sample.

Statistical Analysis

Results were presented as means ± standard deviation. The data were analyzed with one-way analysis of variance (ANOVA) plus the post hoc multiple-comparison test, for comparisons of mean values among multiple treatment groups within the same age group. Student’s t test was used for comparisons between two age groups receiving the same treatment. A p value < .05 was considered statistically significant. Renal I/R injury-induced increase in a variable was defined as follows: (I/R group–age-matched sham group)/age-matched sham group. The percentage of decrease of a value in treated groups was calculated as follows: (age-matched I/R group – treated group)/(age-matched I/R group – age-matched sham group) × 100%.

RESULTS

Renal Function

There was a higher baseline of serum creatinine level in aged rats than in young rats (p < .05). Both aged and young rats subjected to renal I/R injury appeared with markedly increased serum creatinine, compared with age-matched sham controls (p < .05). The increase was more pronounced in aged rats than in young ones (1.75 ± 0.42 fold versus 0.88 ± 0.16 fold, p < .05). Pretreatment with CsA or MMF demonstrated significantly improved renal function in both aged and young rats, but CsA and MMF had different protective effects on aged and young rats. CsA was more effective in aged rats than in the young ones (creatinine decreased by 42.76 ± 7.82% vs 18.18 ± 4.27%, p < .05) (Table 1).

Renal Histology

Young sham rats showed normal renal histology (Figure 1A). Kidneys of aged sham rats exhibited significant morphology changes (Figure 1E). Glomeruli displayed various degrees of glomerulosclerosis, mesangial matrix expansion, and thickening of the glomerular capillary basement membrane. Pronounced tubulointerstitial injury was also seen, with some areas displaying tubular dilation, intratubular cast formation, tubular atrophy, thickening and splitting of tubular basement membranes, widening of the interstitium with fibrosis, and loss of focal peritubular capillaries. Both aged and young rats subjected to I/R injury showed worsened renal histology changes (Figure 1, F and H). These included a loss of brush-border membranes, tubular dilatation, flattened tubular epithelium, cast formation, luminal debris, and an interstitial infiltration. Kidney morphology changes were more severe in aged rats than in young rats (p < .05). Both CsA and MMF improved renal morphology in aged (Figure 2, G and H) and young rats (Figure 1, C and D) (p < .05).

The Severity of Apoptosis

TUNEL assay.—Aged sham rats (Figure 2E) showed more TUNEL-positive cells than did young sham rats (Figure 2A) (1.53 ± 0.24% vs 0.99 ± 0.17%, p < .05). Both aged (Figure 2F) and young rats (Figure 2B) subjected to I/R injury displayed extensive TUNEL-positive staining (aged I/R: 39.52 ± 8.97%, p < .05 vs aged sham; young I/R: 20.14 ± 3.70%, p < .05 vs young sham). TUNEL-positive cells increased more significantly in aged rats than in young ones subjected to the same stress (25.70 ± 4.99 fold vs 19.61 ± 2.15 fold, p < .05). Pretreatment with CsA or MMF decreased the number of TUNEL-positive cells in both aged (Figure 2, G and H) and young rats (Figure 2, C and D) (aged I/R + CsA: 22.28 ± 4.72%, p < .05 vs aged I/R; aged I/R + MMF: 27.94 ± 3.18%, p < .05 vs aged I/R; young I/R + CsA: 14.61 ± 3.39%, p < .05 vs young I/R; young I/R + MMF: 6.92 ± 1.54%, p < .05 vs young I/R).
young I/R + MMF: 11.94 ± 2.46%, p < .05 vs young I/R). But CsA and MMF had different protective effects on aged and young rats. CsA was more effective in aged rats than in the young ones (TUNEL-positive cells decreased by 45.34 ± 8.04% vs 29.07 ± 4.87%, p < .05) (Figure 2I).

Apoptosis was most evident in the corticomedullary junction and medullary area, with occasional occurrence in the cortex. Apoptosis was predominantly localized to distal tubular cells and ascending limb of Henle’s loop, and most apoptotic cells detached from tubular basement membrane and located in the tubular lumen. Apoptotic proximal tubular cells were also noted.

**DNA laddering.**—Genomic DNA isolated from kidneys of aged and young sham controls did not exhibit DNA laddering. In contrast, laddering was present in DNA isolated from kidneys of aged and young rats subjected to I/R injury. Pretreatment with CsA or MMF reduced the DNA laddering pattern in both aged and young rats (Figure 3).

**Bax and Bcl-2 mRNA Expressions**

Aged sham rats displayed higher Bax and Bcl-2 mRNA levels than did the young sham rats (Bax: 0.17 ± 0.04 vs 0.09 ± 0.02, p < .05; Bcl-2: 0.26 ± 0.07 vs 0.15 ± 0.02, p < .05). Renal I/R injury significantly up-regulated Bax and Bcl-2 mRNA expressions in both aged and young rats (Bax: aged I/R: 1.22 ± 0.37, p < .05 vs aged sham, young I/R: 0.52 ± 0.15, p < .05 vs young sham; Bcl-2: aged I/R: 0.69 ± 0.14, p < .05 vs aged sham, young I/R: 0.51 ± 0.09, p < .05 vs young sham). Bax was up-regulated more significantly in aged rats than in the young ones, whereas Bcl-2 up-regulation in aged rats was not as significant as in the young ones (Bax: aged I/R: 6.22 ± 1.38 fold vs 4.73 ± 1.05 fold, p < .05; Bcl-2: 1.67 ± 0.41 fold vs 2.40 ± 0.75 fold, p < .05). Thus, the ratio of Bax to Bcl-2 increased in aged rats compared with young rats (1.29 ± 0.42 fold vs 0.68 ± 0.17 fold, p < .05). Neither CsA nor MMF had any influence on Bax and Bcl-2 expressions in aged and young rats (Figure 4).

**SOD Activity and MDA Content**

Aged rats showed decreased SOD activities and increased MDA values at baseline compared with young ones (p < .05). Renal I/R injury significantly decreased SOD activities and increased MDA contents in both aged and young rats (p < .05). The changes were greater in aged rats than in young rats (SOD decreased by 0.62 ± 0.13 fold vs 0.41 ± 0.12 fold, p < .05; MDA increased by 1.49 ± 0.35 fold vs
0.84 ± 0.26 fold, $p < .05$). CsA or MMF had little influence on SOD activity and MDA content (Table 2).

**Cytosolic Cytochrome c Protein Expression**

Aged sham rats had a higher cytosolic cytochrome c protein level than did young sham rats (0.31 ± 0.06 vs 0.18 ± 0.04). Renal I/R injury significantly up-regulated cytosolic cytochrome c protein expression in kidneys of both aged and young rats (aged I/R: 1.09 ± 0.14, $p < .05$ vs aged sham; young I/R: 0.48 ± 0.07, $p < .05$ vs young sham). The up-regulation was more pronounced in aged rats than in young ones (2.52 ± 0.67 fold vs 1.59 ± 0.42 fold, $p < .05$). CsA inhibited the up-regulation in both aged and young rats. MMF had little effect on cytosolic cytochrome c protein expression (Figure 5).

**TNF-α mRNA Expression**

There was no significant difference in TNF-α mRNA expression between aged and young rats at baseline ($p > .05$). Renal I/R injury significantly up-regulated TNF-α
expression in the kidneys of both aged and young rats (p < .05). TNF-α mRNA expression showed no significant differences between the two I/R groups (p > .05). MMF down-regulated TNF-α mRNA expression in both aged and young rats (p > .05). CsA showed little influence on TNF-α mRNA expression (Figure 6).

Caspase-8 Protein Expression
Aged and young sham rats showed no difference in active caspase-8 expression (p > .05). Active caspase-8 expression was significantly increased in aged and young rats after I/R injury. There were no significant differences between the two I/R groups (p > .05). MMF reduced active caspase-8 expression in both aged and young rats (p > .05). CsA had little influence on active caspase-8 (Figure 7).

Caspase-9 and Protein Expression
Aged sham rats showed increased active caspase-9 expression compared with young sham rats (0.31 ± 0.06 vs 0.18 ± 0.04, p < .05). Renal I/R injury significantly up-regulated active caspase-9 expression in kidneys of both aged and young rats (aged I/R: 1.09 ± 0.24, p < .05 vs aged sham, young I/R: 0.47 ± 0.08, p < .05 vs young sham). The up-regulation was more pronounced in aged rats than in young ones (2.56 ± 0.61 fold vs 1.62 ± 0.48 fold, p < .05). CsA inhibited up-regulation in both aged and young rats. MMF had no effects on active caspase-9 expression (Figure 8).

Table 2. Superoxide Dismutase Activity and Malondialdehyde Content in Aged and Young Rats (Means ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD Activity (U/mg Protein)</th>
<th>MDA Content (mmol/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Aged</td>
</tr>
<tr>
<td>Sham group</td>
<td>44.87 ± 5.94</td>
<td>37.94 ± 4.84</td>
</tr>
<tr>
<td>I/R group</td>
<td>26.51 ± 6.13*</td>
<td>13.69 ± 2.92*</td>
</tr>
<tr>
<td>I/R + CsA group</td>
<td>27.29 ± 5.49</td>
<td>13.35 ± 2.16</td>
</tr>
<tr>
<td>I/R + MMF group</td>
<td>28.88 ± 6.34</td>
<td>14.07 ± 3.59</td>
</tr>
</tbody>
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Note: *p < .05 versus age-matched sham group.
\[^{1}\]p < .05 versus corresponding young group.
SOD = superoxide dismutase; MDA = malondialdehyde; SD = standard deviation; I/R = ischemia/reperfusion; CsA = cyclosporine A; MMF = mycophenolate mofetil.
The expression of active caspase-3 in aged rats was higher than that of young ones at baseline (0.14 ± 0.04 vs 0.07 ± 0.02, \( p < 0.05 \)). Active caspase-3 expression was significantly up-regulated in aged and young ones after I/R injury (aged I/R: 1.12 ± 0.31, \( p < 0.05 \) vs aged sham, young I/R: 0.48 ± 0.12, \( p < 0.05 \) vs young sham). The up-regulation was more pronounced in aged rats than in the young ones (6.79 ± 2.16 fold vs 5.47 ± 0.87 fold, \( p < 0.05 \)). Pretreatment with CsA and MMF significantly reduced active caspase-3 level in both aged and young rats. CsA was more effective in aged rats than in the young ones (decreased by 60.39 ± 9.62% vs 48.38 ± 8.21%, \( p < 0.05 \)) (Figure 5).

**DISCUSSION**

Nearly every organ or tissue of the body undergoes aging-related restructuring, leading to a general decline of physiological function. Like other organs, the kidneys undergo involutional changes with age (30). In the present study, the kidneys of aged sham rats exhibited significant morphology changes. At the same time, renal function of aged rats declined. Whether apoptosis plays a role in this process is currently unclear. But a potential role of apoptosis in aging has drawn increasing attention in recent years. An increase in the number of apoptotic cells has been observed in a variety of tissues and cell types (9–11). Thomas and colleagues (13) reported that tubular cell apoptosis is enhanced in aged rats. Here we found that, in addition to the structural and functional changes, there were more apoptotic tubular cells in the kidneys of aged rats, indicating apoptosis may contribute to these changes in physiological conditions.

Aging-related changes in physiological or metabolic parameters may not be evident under basal conditions; however, when an organ is under stress, these changes contribute to organ dysfunction. Therefore, the study of aging-related diseases must include the investigation of both baseline and challenged states (31). Ischemic ARF is a common problem in elderly people, and morbidity and mortality increase with advanced age. In the present study, a rat model of renal I/R injury was used to investigate the effects of aging on the alteration of renal dysfunction. Loss of functioning tubular cells by apoptosis or necrosis is
a major contributing factor to renal dysfunction in I/R injury, and tubular cell apoptosis has recently drawn close attention (7). We chose 30 minutes as the duration of ischemia, because in this duration of ischemic time, apoptosis was the main cell death mode contributing to ARF. Our results indicated that aged rats exhibited more pronounced tubular cell apoptosis in the injury than do the young ones. Meanwhile, renal I/R injury induced more severe renal dysfunction and morphology changes in aged rats, suggesting that enhanced tubular cell apoptosis may play a role in the increased susceptibility of the aged rats to renal I/R injury.

Next, we explored the pathway(s) responsible for enhanced tubular cell apoptosis in aged rats in the setting of renal I/R injury. It is reported that caspase-dependent intrinsic and extrinsic pathways were both activated during renal I/R injury (4). Mitochondria play a central role in the intrinsic pathway, and participate in apoptosis by opening the PTP. The pore is formed from a complex of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase, and cyclophilin-D (CyP-D) at contact sites between the mitochondrial outer and inner membranes (32). Bcl-2 family members interact with the VDAC and regulate the opening of mitochondrial PTP (16,33). We examined the mRNA expressions of Bcl-2 family members, which have either pro-apoptotic (Bax) or anti-apoptotic (Bcl-2) activity (34). The results demonstrated that renal I/R injury significantly up-regulated mRNA expressions of Bax and Bcl-2 in both aged and young rats. Bax was up-regulated more significantly in the aged rats than in the young ones, whereas Bcl-2 up-regulation in aged rats was not as significant as in the young ones. Thus, the ratio of Bax to Bcl-2 increased in aged rats compared with young rats. The elevation of the Bax/Bcl-2 ratio indicated that the mitochondrial pathway was enhanced in aged rats.

We evaluated the causes of the enhanced activation of mitochondrial pathway. It is well known that mitochondria are not only the source of energy but also the major source of reactive oxygen species (ROS) (35). It has been reported that mitochondrial function and morphology were impaired upon aging, as demonstrated by a decline in the membrane potential and a reduction in SOD (36). These make

Figure 7. Caspase-8 protein expression in aged and young rats. A, Representative 20 kD active caspase-8 expression measured by western blot. B, Densitometric quantifications of band intensities from western blot for 20 kD active caspase-8/β-Actin. Aged (Lane 5) and young sham (Lane 1) rats showed little difference in active caspase-8 level. Renal ischemia/reperfusion (I/R) injury significantly increased active caspase-8 in both aged (Lane 6) and young (Lane 2) rats. The increase showed no aged-related differences. Mycophenolate mofetil (MMF) reduced active caspase-8 in both aged (Lane 8) and young (Lane 4) rats. Cyclosporine A (CsA) had little effect on active caspase-8 expression. *p < .05 versus age-matched sham group; †p < .05 versus age-matched I/R group. Data in bar graphs are means ± standard deviation.

Figure 8. Caspase-9 protein expression in aged and young rats. A, Representative 35 kD active caspase-9 expression measured by western blot. B, Densitometric quantifications of band intensities from western blot of 35 kD active caspase-9/β-Actin. Aged sham rats (Lane 5) had a higher active caspase-9 level than did young sham rats (Lane 1). Renal ischemia/reperfusion (I/R) injury significantly increased active caspase-9 in both aged (Lane 6) and young (Lane 2) rats. The increase was more marked in aged rats than in young ones (2.56 ± 0.61 fold vs 1.62 ± 0.48 fold, p < .05). Cyclosporine A (CsA) reduced active caspase-9 in both aged (Lane 7) and young rats (Lane 3). Mycophenolate mofetil (MMF) had little effect on active caspase-9 expression. *p < .05 versus age-matched sham group; †p < .05 versus corresponding young group; ‡p < .05 versus age-matched I/R group. Data in bar graphs are means ± standard deviation.
oxidative stress accumulate with aging and render the mitochondria of the aged animals more susceptible to oxidative injury (18). We measured the kidney levels of SOD activity as well as a marker of lipid peroxidation, MDA. The results indicated that renal I/R injury decreased SOD activities and increased MDA contents in both aged and young animals. But the changes were more pronounced in aged rats, indicating that mitochondrial dysfunction occurred during the injury, which was more severe in the aged rats than in the young ones.

Although a small amount of ROS is important in regulating cell proliferation and differentiation, a large amount of ROS induces deleterious damage and apoptosis (37). Oxidative stress is a potent inducer of the opening of mitochondrial PTP. The activation of mitochondrial PTP in aged mouse brain and liver was enhanced (18). We also observed that, with the increase of MDA content, the expressions of cytosolic cytochrome c and caspase-9 isolated from kidneys of aged and young rats subjected to I/R injury were up-regulated, while the up-regulation was more pronounced in the aged rats than in the young ones, indicating that the intrinsic pathway participated in tubular cell apoptosis in both aged and young rats, but was enhanced in the aged rats compared with the young ones after the stress.

TNF-α has been implicated in renal I/R injury-induced apoptotic tubular cell death. The binding of TNF-α to its receptor (TNFR-1) leads to caspase-8 activation (4,22). Our results displayed an up-regulation of TNF-α and caspase-8 expressions in aged and young rats with renal I/R injury. There were no significant differences in the extent of TNF-α and caspase-8 up-regulation between the two age groups, suggesting that the extrinsic pathway participated in renal I/R injury induced apoptosis, and that there was no age-related difference in this pathway.

To confirm our findings, we performed experiments to interfere with the intrinsic and extrinsic pathways of apoptosis, respectively. CsA is an inhibitor of mitochondrial PTP. CsA blocks PTP opening by binding to CyP-D (32). Although CsA is nephrotoxic at a high dose and can induce tubular cell apoptosis (38,39), at a low dose it can protect renal function and inhibit tubular cell apoptosis after I/R injury (24). MMF is a powerful immunosuppressant that inhibits the proliferation of lymphocytes. It has been reported that it also inhibited the expression of TNF-α (23). MMF decreased tubular cell apoptosis in patients with renal transplantation (40). Our results indicated that both CsA and MMF attenuated renal I/R injury-induced tubular cell apoptosis in aged and young rats. CsA significantly inhibited the up-regulations of cytosolic cytochrome c, caspase-9, and caspase-3 in both age groups, but had no effects on Bax and Bcl-2 expressions, SOD activity, or MDA content. This finding may be due to the fact that both CsA and Bcl-2 family members regulate the mitochondrial PTP through binding to different components of PTP, and that SOD activity and MDA content were not the causes instead of the results of mitochondrial PTP opening. MMF markedly decreased the expressions of TNF-α, caspase-8, and caspase-3 in both age groups. CsA and MMF had different protective effects on aged rats and young rats. CsA exerted stronger effects on aged rats than on young ones, as the decrease of apoptotic tubular cells and down-regulation of caspase-3 were more pronounced in aged rats than in young ones, further indicating that the activation of intrinsic pathway was enhanced in the aged rats.

Summary

Our findings have demonstrated that there was an age-related increase of tubular cell apoptosis in the stress of renal I/R injury, which may be, at least partly, due to the enhanced mitochondrial pathway resulting possibly from increased oxidative stress.

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