Induction of Apoptosis in Ischemia-Reperfusion Model of Mouse Kidney: Possible Involvement of Fas

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Abstract. Although ischemia-reperfusion of mouse kidney is known to cause severe renal failure due to tubular cell death, the exact cellular mechanism responsible for this phenomenon is not clear. To investigate the spatial and temporal development of renal cell death and the role of Fas/APO-1/CD95 (Fas) in this process, the left renal vessels were occluded in a group of mice for 30, 60, or 120 min followed by reperfusion for 24 h ($n = 4$ for each group). Analysis of the isolated DNA in agarose-gel electrophoresis revealed a typical ladder pattern of bands consisting of multiples of 180 to 200 bp, considered the hallmark of apoptosis. The intensity of the bands increased proportionately with the duration of ischemia. Histochemical analysis using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling showed the presence of nuclei with DNA double-strand breaks specifically in distal renal tubules of the outer medulla. The presence of apoptosis was also confirmed by electron microscopy. Analysis of total RNA by Northern blotting revealed one appropriate-sized band for Fas mRNA in the normal kidney, which intensified in the ischemia-reperfused kidney. Moreover, nonradioactive in situ hybridization revealed that distal renal tubular epithelial cells were positive for Fas mRNA in the outer medulla. Fas antigen was also localized to the renal tubular epithelial cells of the outer medulla by immunohistochemistry. The number of apoptotic cells in the ischemia-reperfusion kidney of the lpr/lpr mouse was low. These findings strongly indicate that ischemia-reperfusion of the kidney induces apoptosis of a specific area of tubular epithelial cells in the outer medulla through the Fas system. (J Am Soc Nephrol 9: 620–631, 1998)

Ischemia-reperfusion causes failure of organ function of a severity proportional to the duration of ischemia (1–3). In the kidney, ischemia-reperfusion (4), hypoxia (5), and renal artery stenosis (6) may cause renal tubular atrophy. Although renal tissue damage is well documented to be due to cell death, the cellular mechanisms underlying cell death have been discussed in only a limited number of studies (1–3). Recent studies have suggested that apoptosis, originally described by Kerr et al. (7), is probably responsible for renal tubular atrophy induced by mild renal artery stenosis (6) and ischemia-reperfusion (8), as well as other types of renal failure, including hydronephrosis (9,10), radiation nephropathy (11), and analgesic nephropathy (12). However, details of the spatial and temporal appearance of apoptosis in the damaged kidneys and the molecular entities engaged in the induction of apoptosis are not clear. To demonstrate the presence of apoptosis, the current notion requires examination of various histologic and biochemical parameters (13), including the condensation of nuclear and cytoplasmic components, the formation of apoptotic bodies, and internucleosomal DNA fragmentation (14).

Fas/APO-1/CD95 (15), a 45-kD transmembranous glycoprotein, is a member of the tumor necrosis factor/nerve growth factor receptor family and mediates apoptosis possibly via activation of interleukin-1β converting enzyme (ICE)/Ced-3-related protease (16) in a variety of cells, including activated T cells (17), hepatocytes (18,19), vaginal epithelial cells (20), and ovarian granulosa cells (21). A 31-kD type II transmembranous protein has been identified as the Fas ligand (22), which is known to initiate apoptosis in activated T lymphocytes by binding to Fas expressed on these cells (23). Apart from the immune system, mouse hepatocytes, which normally express Fas, undergo apoptosis when injected with anti-Fas antibody (18). We have also found that the Fas-Fas ligand system mediates the induction of granulosa cell apoptosis in ovarian follicle atresia (21), as well as abnormal acceleration of intestinal epithelial cell apoptosis in ulcerative colitis (24). These findings indicate that the Fas system plays an important role in physiologic and pathologic tissue destruction of adult organisms as a mediator of apoptotic cell death.
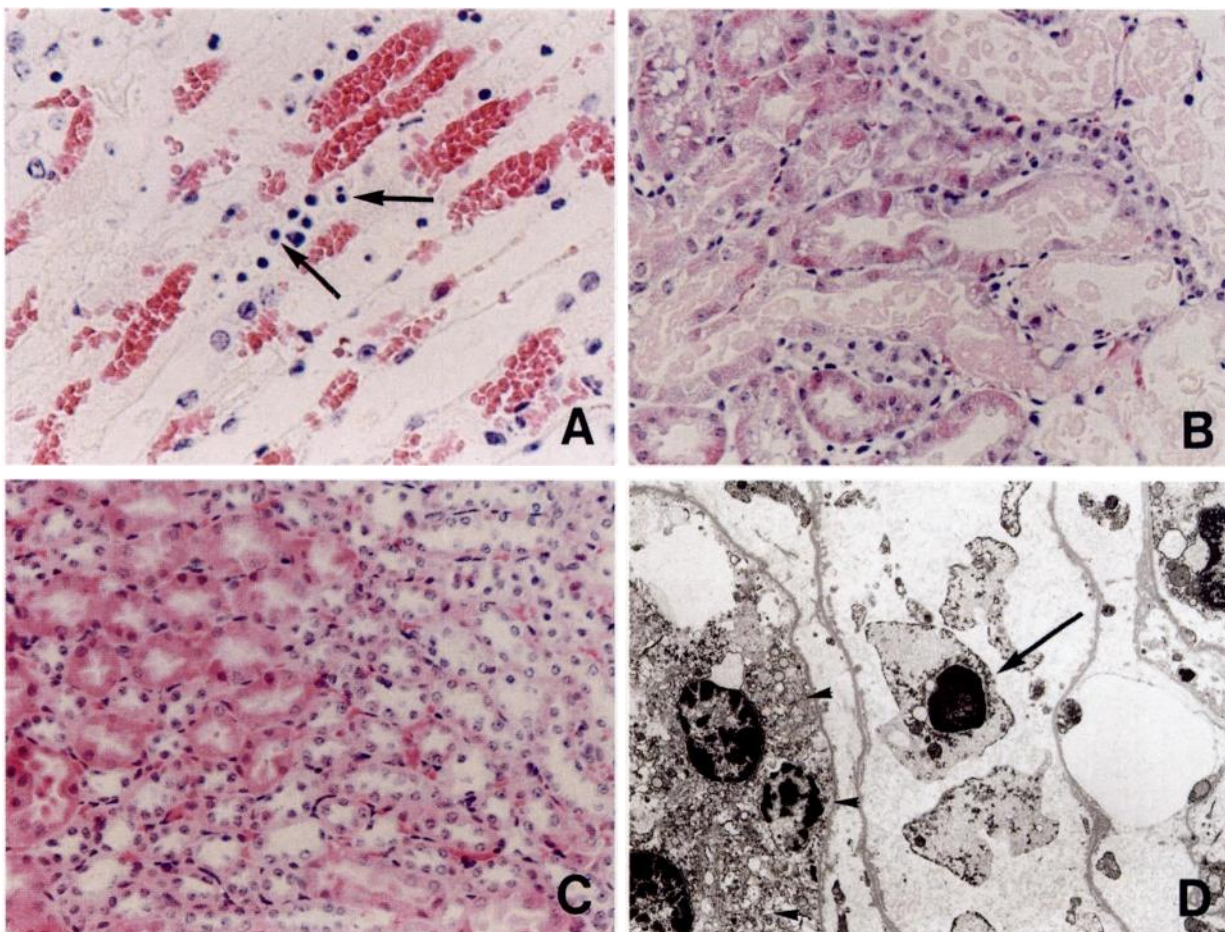
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Figure 1. Morphology of ischemia-reperfusion mouse kidney. (A through C) Photomicrographs of hematoxylin-eosin-stained sections. (A) A section from 120-min ischemia kidney showing apoptotic cells (arrow) in the tubular lumen of Henle's loop at the inner stripe. (B) Extensive necrotic changes in the proximal tubules at the outer stripe of a section from 120-min ischemia kidney. (C) A section from sham-operated kidney showing no morphologic changes in the outer medulla. (D) Electron micrograph of 60-min ischemia kidney. Arrow, apoptotic tubular epithelial cell; arrowhead, necrotic tubular epithelial cell. Magnification: ×400 in A; ×200 in B and C; ×2800 in D.

In the present study, we initially evaluated the presence of apoptosis in the ischemia-reperfusion kidney of the mouse, using electron microscopy to detect apoptosis-specific morphologic changes and agarose-gel electrophoresis of DNA to confirm a ladder of bands with multiples of 180 to 200 bp for the presence of internucleosomal DNA fragmentation. We also used the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method to identify cells with DNA double-strand breaks. In the second part of the study, we investigated the involvement of Fas expression in inducing apoptosis at the mRNA and protein level, using Northern blotting, *in situ* hybridization, and immunohistochemistry with polyclonal antibody against mouse Fas synthetic peptide, respectively. Our results confirmed that ischemia-reperfusion of the mouse kidney induces apoptosis of renal tubular cells in the outer medulla and revealed that the appearance of apoptosis correlates well with the presence of Fas antigen and Fas mRNA in the cells, spatially and temporally. Our findings indicate the possible involvement of the Fas system in the induction of apoptosis of renal tubular cells by ischemia-reperfusion.

Materials and Methods
Reagents

Paraformaldehyde (PFA) was purchased from Merck (Darmstadt, Germany), OCT compound from Tissue-Tek (Miles, Inc., Elkhart, IN), 3,3'-diaminobenzidine-4 HCl (DAB) from Dojin Chemical Co. (Kumamoto, Japan), λ phage DNA from Takara Co. (Tokyo, Japan), and formamide from Nacarai (Kyoto, Japan). Bovine serum albumin (BSA), DNase I, proteinase K, yeast tRNA, salmon testis DNA, dextran sulfate, Ficoll-400, polyvinylpyrrolidone, and Brij 35 were purchased from Sigma Chemical Co. (St. Louis, MO). Digoxigenin (Dig)-11-dUTP and terminal deoxynucleotidyl transferase (TdT) were purchased from Boehringer Mannheim (Mannheim, Germany). All other reagents used in this study were from Wako Pure Chemicals (Osaka, Japan) of analytical grade.

Antibodies

Horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-(thymine-thymine (T-T) dimer) antibody (1:80) was obtained from Kyowa Medex (Tokyo, Japan). Anti-Fas serum (1:800) was prepared by immunization of rabbits against synthetic oligopeptides corresponding to the intracellular domain (amino acids 292 to 306) of mouse Fas (25), as described previously (26). HRP-conjugated goat
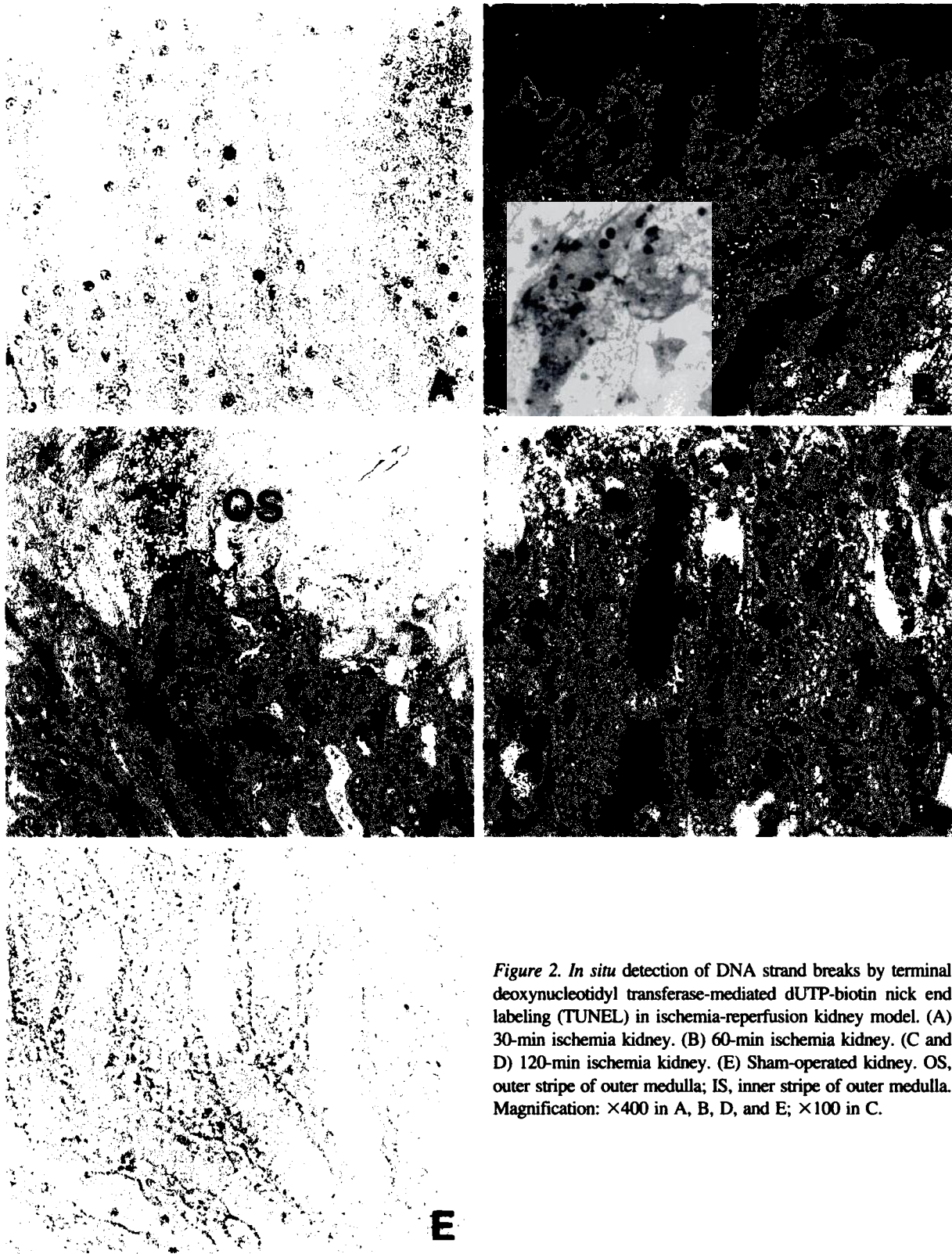
Figure 2. In situ detection of DNA strand breaks by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) in ischemia-reperfusion kidney model. (A) 30-min ischemia kidney. (B) 60-min ischemia kidney. (C and D) 120-min ischemia kidney. (E) Sham-operated kidney. OS, outer stripe of outer medulla; IS, inner stripe of outer medulla. Magnification: ×400 in A, B, D, and E; ×100 in C.

anti-rabbit IgG F(ab')$_2$ (1:800) was purchased from Medical and Biological Laboratory (Nagoya, Japan). HRP-conjugated sheep anti-Dig (1:100) was from Boehringer Mannheim. PC10 (mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody) (1:80) was from Dakopatts (Glostrup, Denmark).

Animals

Male ICR mice (5 to 6 wk old) weighing 30 to 40 g were used. In some experiments, we also used male *lpr/lpr* B6 mice (5 to 6 wk old) (a kind gift from Dr. M. Nose, The First Department of Pathology, Tohoku University School of Medicine), characterized with a low
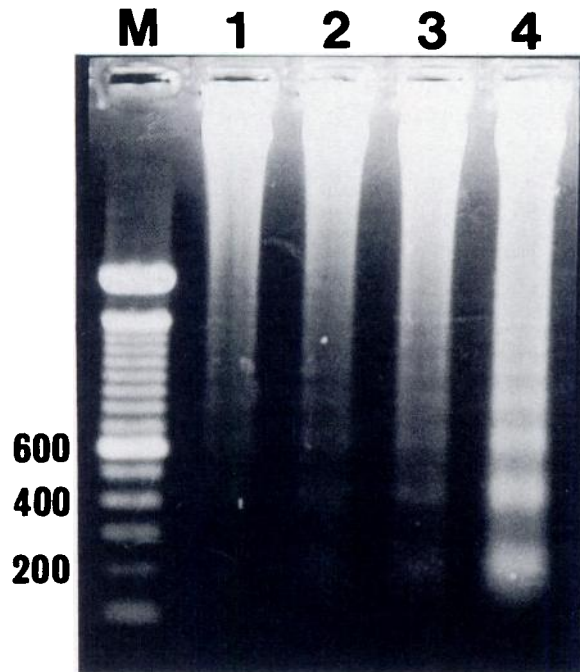
Figure 3. Agarose gel electrophoresis of DNA extracted from isch-emia-reperfusion kidney. Five micrograms of DNA were applied to each lane. Lane 1, sham operation; lane 2, 30-min ischemia; lane 3, 60-min ischemia; lane 4, 120-min ischemia. M, molecular marker.

level of Fas expression by insertion of retroviral transposon into the Fas gene (27), as well as male wild-type B6 mice (5 to 6 wk old). Each experimental group consisted of four mice. The experimental protocol was approved by the Animal Ethics Review committees of our insti-tutions. Under anesthesia with intraperitoneal injection of pentobar-bital (40 mg/kg body wt), an abdominal incision was made and the renal vessels on both sides were identified. The renal artery and vein of the left kidney were occluded with a vascular clamp for 30, 60, or 120 min. The clamp was then removed and the organ was allowed to reperfuse. One day after reperfusion, the animals were anesthetized again and the renal tissues were removed. The right kidney was used as a control in each animal. Sham operation was performed in a similar manner, except for clamping the renal vessels.

Tissue Preparation

The renal tissues were first cut transversely into two pieces, then further cross-sectioned to recognize the anatomical details, and di-vided into four groups. In the first group, the tissues were quickly frozen in liquid nitrogen and later used for the extraction of DNA or RNA. Tissue samples from the second group were embedded in OCT compound and quickly frozen in ethanol/dry ice. Tissue samples from the third group were fixed in 4% PFA in phosphate-buffered saline (PBS), pH 7.4, at 4°C overnight, and embedded in paraffin using standard procedures. The sections were later stained with hematoxy-lin-eosin (HE) and used for histologic evaluation of tissue damage. For electron microscopy, the remaining pieces were fixed in 2.5% glutaraldehyde/2% PFA in 0.075 M sodium cacodylate, pH 7.4, for 4 h. The tissues were then post-fixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h and embedded in Epon 812. The

sections were stained with uranyl acetate and lead nitrate, and exam-ined under a JEOL-1010 transmission electron microscope.

Electrophoretic Analysis of Extracted DNA

Total DNA was extracted from the frozen tissue samples using DNA extractor WB kit (Wako Pure Chemicals, Osaka, Japan). The concentration of DNA was measured spectrophotometrically at 260 nm. Aliquots of DNA samples (5 μg/lane) were separated on a 1.5% agarose gel and stained with ethidium bromide.

In Situ Detection of DNA Strand Breaks

To identify nuclei with DNA strand breaks at a cellular level, TUNEL was performed according to the method of Gavrieli *et al.* (28), with a slight modification. Briefly, paraffin-embedded sections (5 to 6 μm) were cut onto silane-coated glass slides and dewaxed in a routine manner. After washing with PBS, the sections were treated with 1.0 μg/ml proteinase K in PBS at 37°C for 15 min. The sections were then rinsed once with deionized distilled water and incubated with TdT buffer (25 mM Tris-HCl buffer, pH 6.6, containing 0.2 M potassium cacodylate and 0.25 mg/ml BSA) alone at room tempera-ture (RT) for 30 min. After incubation, the slides were reacted with 0.1 U/μl TdT dissolved in TdT buffer supplemented with 1.0 nM Dig-11-dUTP, 20 μM dATP, 1.5 mM $CoCl_2$, and 0.1 mM dithiothre-itol at 37°C for 3 h. The reaction was terminated by washing with 50 mM Tris-HCl buffer, pH 7.4, for 15 min. Endogenous peroxidase activity was inhibited by immersing the slides in 0.3% H_2O_2 in methanol at RT for 15 min. The signals were detected immunohisto-chemically with HRP-conjugated sheep anti-Dig antibody, as de-scribed previously (29).

DNA Probes

Mouse Fas cDNA (0.9-kb *BamI/Hind*III fragment) (30) was labeled with ^{32}P by random priming and used as a hybridization probe for Northern blot analysis of Fas mRNA. As for probes used in *in situ* hybridization, the same cDNA was irradiated with ultraviolet light at a dose of 10 kJ/m² to form T-T dimers and fragmented by a partial digestion with DNase I, as described previously (31). As a negative control probe, λ phage DNA was treated in a similar manner.

Northern Blot Analysis of Fas mRNA Expression

Total RNA was extracted from the frozen kidney tissue samples according to the method of Chomczynski and Sacchi (32). For this purpose, 20 μg of total RNA was separated on agarose gel containing formaldehyde, transferred onto a nitrocellulose membrane, and hy-bridized with the probe under standard conditions (33). The hybrid-ized filters were exposed to x-ray film. As a positive control, we used total RNA from a normal mouse liver.

In Situ Hybridization

The procedures, sensitivity, and reliability of *in situ* hybridization using T-T dimerized cDNA probe were described previously in detail (31,34). The pretreatment protocol, including proteinase K digestion, was optimized by using *in situ* hybridization of 28S rRNA, as de-scribed previously (35). Briefly, the paraffin-embedded sections (5 to 6 μm) were cut onto silane-coated glass slides, deparaffinized, and rehydrated with serial ethanol and PBS. After inactivation of endog-
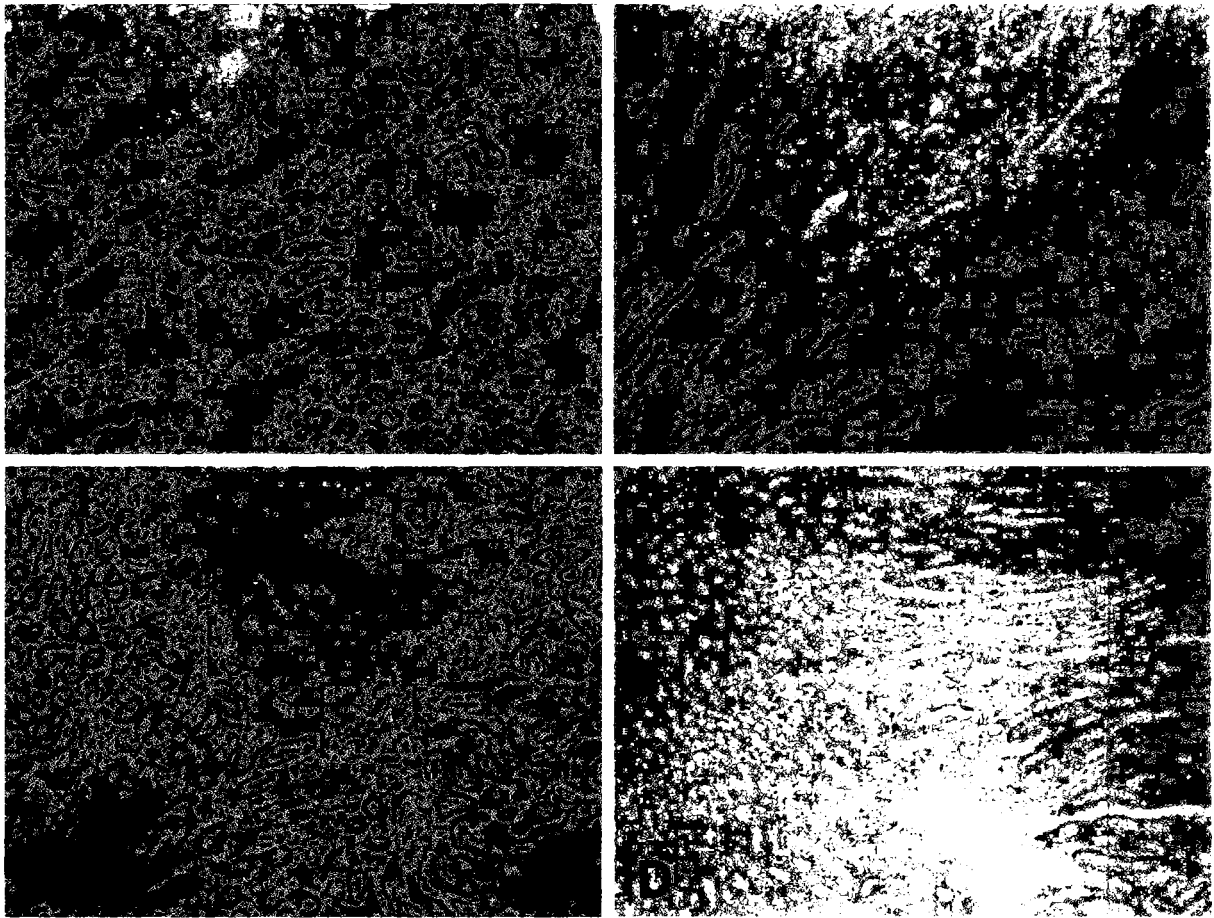
Figure 4. Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) in paraffin sections from the ischemia-reperfusion kidney. (A and B) Sections from 120-min ischemia kidney. Note the presence of several PCNA-positive cells at the proximal tubules in the cortex and outer stripe (A), but not in the inner stripe (B). (C and D) Sections from the sham-operated kidney. Note a few PCNA-positive cells in the cortex and outer stripe (C), as well as inner stripe (D). Magnification, ×75.

enous peroxidase with 0.3% H_2O_2 in methanol (RT, 15 min), the slides were treated with 0.2N HCl (RT, 20 min) and 5 μg/ml protein-ase K (37°C, 15 min), successively. Then, the sections were post-fixed with 4% PFA in PBS and immersed in 40% deionized formamide in 4× SSC (SSC; 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) for 30 min until hybridized. Hybridization was performed using 2 μg/ml T-T dimerized Fas cDNA dissolved in 10 mM Tris-HCl, pH 7.4, containing 0.6 M NaCl, 1.0 mM ethylenediaminetetra-acetic acid, 40% deionized formamide, 1× Denhardt's solution, 250 μg/ml yeast tRNA, 125 μg/ml salmon testis DNA, and 10% dextran sulfate at 37°C for 15 to 17 h. After appropriate washing with 50% formamide in 0.5× SSC at 37°C, the sections were reacted with HRP-mouse anti-(T-T dimer) antibody, and the sites of HRP were visualized with DAB, H_2O_2, Co^{2+}, and Ni^{2+} (21). To verify the specificity of signals, T-T dimerized λ phage DNA was hybridized with the adjacent sections as a negative control probe in every experimental run.

Immunohistochemistry

Localization of Fas antigen was examined immunohistochemically using the rabbit anti-Fas serum (anti-P4), which was raised against a synthetic peptide of mouse Fas and characterized previously (21). The procedures for immunohistochemistry of Fas antigen were fully op-timized, as described recently by our group (26). Briefly, the paraffin samples were cut into 5- to 6-μm-thick sections, placed onto silane-

coated glass slides, deparaffinized, and rehydrated. After inactivation of endogenous peroxidase with 0.3% H_2O_2 in methanol (RT, 15 min), the slides were preincubated with 5% BSA in PBS for 60 min to block nonspecific reaction with the antiserum. Then, the sections were reacted with anti-Fas serum (1:800) for 2 h, washed with 0.075% Brij 35 in PBS, and reacted with HRP-goat anti-(rabbit IgG) F(ab')$_2$ (1:200) for 1 h. After washing with 0.075% Brij 35 in PBS, the sites of HRP were visualized with DAB and H_2O_2. As a negative control, normal rabbit serum was used at the same dilution. PCNA staining was performed according to the protocol described by Hashimoto *et al.* (36).

Quantitative Analysis of Apoptotic Cells

Sixteen fields of inner stripe of the outer medulla, which were observed under a microscope (magnification, ×400), were randomly selected in each section to count the number of HE-stained and TUNEL-positive apoptotic cells. The number of apoptotic cells was expressed per 1000 of the total tubular cells in each section.

Statistical Analyses

We performed ANOVA followed by Scheffe's correction to compare the number of apoptotic or TUNEL-positive cells in each experimental condition. Statistical significance was defined as $P < 0.05$.
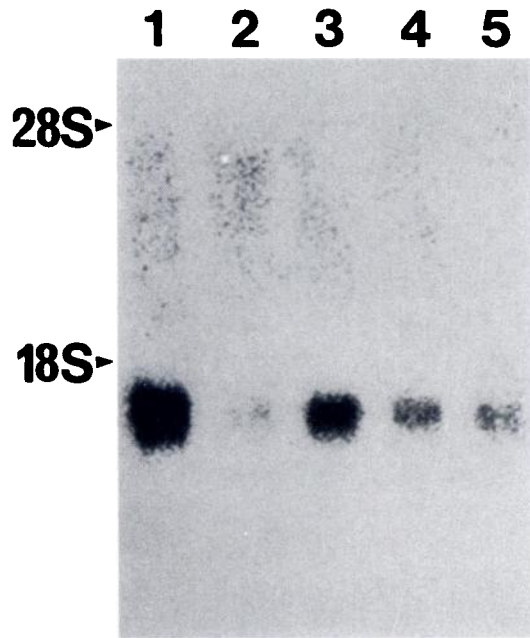
1   2   3   4   5

28S▸

18S▸

Figure 5. Northern blot of mouse Fas mRNA. Twenty micrograms of total RNA prepared from ischemia-reperfusion kidney were hybridized with ^{32}P-labeled Fas cDNA probe. Fas mRNA was already detected in sham-operated specimen (lane 2). The intensity of Fas mRNA was maximum at 30-min ischemia kidney (lane 3) and decreased gradually in 60-min (lane 4) and 120-min (lane 5) ischemia kidney. Normal mouse liver tissue was used as a positive control (lane 1).

Results

Morphologic, Histochemical, and Biochemical Evidence of Apoptotic Cell Death

The most dramatic histologic changes in the ischemia-reperfusion kidney were found in the outer medulla, where apoptotic cells in the tubular epithelium and lumen were detected in the thick ascending limb of Henle's loop (Figure 1A). Characteristically, the number of apoptotic cells increased with the duration of ischemia. The features of apoptosis resembled those observed in the differentiation of the loop of Henle (37). On the other hand, ischemia/reperfusion resulted in necrosis of the proximal tubules of the outer medulla, and the number of necrotic cells was proportionate with the duration of ischemia (Figure 1B). The histologic features of sham-operated left kidneys and right control kidneys were essentially not different from those of a normal kidney, regardless of the duration of ischemia of the left kidneys (Figure 1C). The presence of apoptotic tubular cells, characterized by condensation of chromatin to nuclear periphery, was also confirmed in the outer medulla of the ischemia-reperfusion kidney by electron microscopic examination (Figure 1D). Necrotic proximal tubular cells, identified by the presence of disrupted intracellular organelles and karyolysis, were also observed in the outer medulla.

To identify cells with DNA strand breaks at an individual cell level, we carried out TUNEL staining using paraffin-embedded sections. A few nuclei of the distal tubular epithelial cells in the outer medulla were stained after 30 min of ischemia (Figure 2A), but the number of TUNEL-positive nuclei increased with prolonged ischemia (Figure 2B) and reached a maximum in kidneys subjected to 120 min of ischemia (Figure 2D). In these sections, not only were the nuclei of cells present within the tubular lumen TUNEL-positive, but nuclei of "non-dropped" tubular epithelial cells were also TUNEL-positive. In contrast, only a few positive nuclei were observed in the cortex and the inner medulla in all specimens (Figure 2C), and no positive cells were detected in the proximal tubules. When sections from sham-operated and right control kidneys were examined by TUNEL, no positive cells were observed (Figure 2E). Furthermore, when TdT was omitted from the reaction mixture of TUNEL, the sections were essentially blank in all sections (data not shown).

The increase in TUNEL-positive cells after ischemia-reperfusion paralleled the appearance of DNA ladder. The latter consisted of multiples of 180 to 200 bp in agarose gel-electrophoresis of DNA, a biochemical hallmark of apoptosis (38). As shown in Figure 3, a faint but visible ladder was observed 30 min after ischemia, and the intensity of the ladder increased with prolongation of ischemia. In contrast, no ladder formation was detected in DNA obtained from the sham-operated kidney. These results confirmed the work of other investigators (8,39).

Proliferative Activity Assessed by PCNA Staining

We have recently shown that cells undergoing replicative DNA synthesis can also be detected by TUNEL staining (40). However, as shown in Figure 4, the distribution of PCNA-positive cells was highly restricted to the proximal renal tubules in both the cortex and outer stripes of the outer medulla in the ischemia-reperfusion kidney, whereas only a few positive cells were observed in the inner stripes. In the sham-operated kidney, only a few positive cells were observed in the cortex and outer medulla. Therefore, the majority of TUNEL-positive nuclei were not related to replicative DNA synthesis.

Involvement of Fas in Renal Tubular Apoptosis

We also investigated the involvement of Fas in renal tubular apoptosis in the ischemia-reperfusion kidney model by analyzing total RNA of the frozen sections for Fas mRNA expression using Northern blotting. As shown in Figure 5, a Fas mRNA band of 2.1 kb was barely detectable in the sham-operated kidney. However, the amount of Fas transcript was high in the ischemic kidney, reaching a peak at 30 min of ischemia, but the level diminished gradually thereafter. Total RNA from normal mouse liver was similarly processed, and a band of 2.1 kb was detected.

In the next step, we performed *in situ* hybridization of Fas mRNA to identify Fas mRNA-positive cells in the ischemia-reperfusion kidneys, as shown in Figure 6. To verify the specificity of signals, we examined the sham-operated kidneys and right control kidneys, which showed no staining. Moreover, when T-T dimerized λ phage DNA was used as a control probe, no signal was detected (Figure 6A). When the sections from ischemia-reperfusion kidneys were hybridized with Fas probe, Fas mRNA was found in the distal tubules at the outer
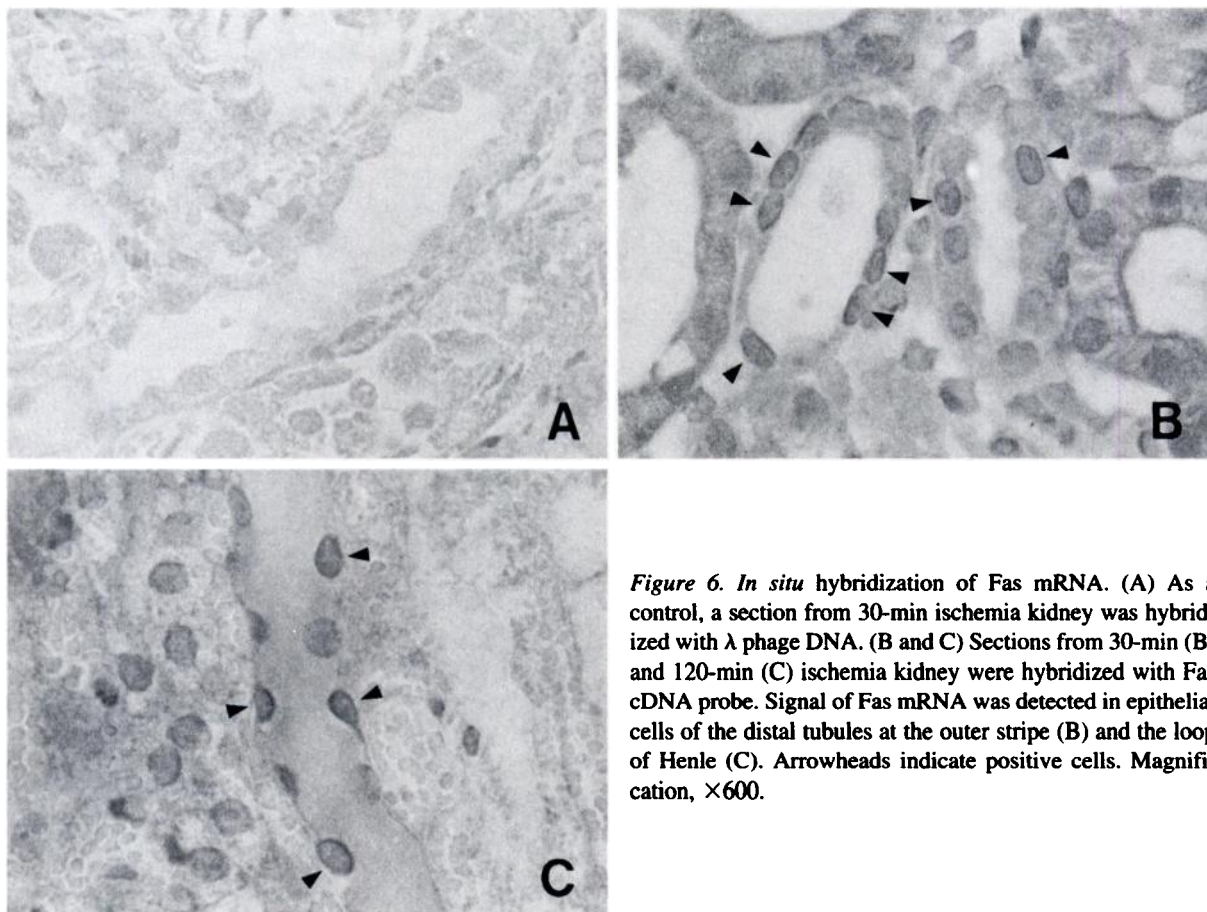
Figure 6. In situ hybridization of Fas mRNA. (A) As a control, a section from 30-min ischemia kidney was hybridized with λ phage DNA. (B and C) Sections from 30-min (B) and 120-min (C) ischemia kidney were hybridized with Fas cDNA probe. Signal of Fas mRNA was detected in epithelial cells of the distal tubules at the outer stripe (B) and the loop of Henle (C). Arrowheads indicate positive cells. Magnification, ×600.

stripes, but not in the proximal tubules in the outer medulla (Figure 6B). In the inner stripes of the outer medulla, the cells of loop of Henle were positive to Fas mRNA (Figure 6C). The number of positive cells reached a maximum in kidneys exposed to 30 min of ischemia but the number decreased thereafter, whereas the signal intensity of individual positive cells did not change, regardless of the duration of ischemia. In addition, the distal, but not proximal, tubules in the medullary ray of the cortex were positive for Fas transcript (data not shown).

We also examined the expression of Fas antigen immunohistochemically using rabbit anti-mouse Fas serum raised against the synthetic peptide corresponding to a part of mouse Fas (21). As shown in Figure 7, the distal tubules of the cortex of kidneys exposed to 30-min of ischemia were stained for Fas antigen (Figure 7A). The distal tubules in the outer medulla were positive (Figure 7, B and C). Similar to Fas mRNA, the number of cells positive to Fas antigen was highest in kidneys exposed to 30 min of ischemia but then decreased, whereas the intensity of Fas staining was almost the same in Fas-positive cells, regardless of the duration of ischemia (Figure 7, D and E). Sections from the sham-operated kidneys did not stain with the antiserum (Figure 7F). When sections from the ischemia-reperfusion kidneys were reacted with normal rabbit serum instead of the anti-Fas serum, no positive cells were observed (data not shown).

To provide other evidence for the involvement of Fas in

renal tubular apoptosis, we conducted similar experiments using B6 *lpr/lpr* mice kidneys. When the mice were subjected to 120 min of ischemia, only a few apoptotic cells (Figure 8A) and TUNEL-positive cells (Figure 8C) were detected at the inner stripe of the outer medulla in this strain. In contrast, both apoptotic cells (Figure 8B) and TUNEL-positive nuclei (Figure 8D) were abundant in wild-type B6 mouse ischemia-reperfusion kidneys, as in the case of ICR mice.

Quantitative Analysis of TUNEL-Positive Cells

We also counted the number of TUNEL-positive tubular cells in the inner stripe of outer medulla in the ischemia-reperfusion kidneys, and compared the number of HE-stained cells with morphologic features of apoptosis. As shown in Figure 9A, the number of apoptotic cells was significantly higher in ICR and wild-type B6 mice, but not in B6 *lpr/lpr* mice, after ischemia-reperfusion. The number of TUNEL-positive cells was significantly high in ischemia-reperfusion sections (Figure 9B) and was significantly higher than that of cells with morphologic features of apoptosis. However, there was essentially no difference in the response to ischemia-reperfusion between ICR and wild-type B6 mice.

Discussion

The major findings of the present study were the detection of apoptosis predominantly in the distal renal tubules of ischemia-
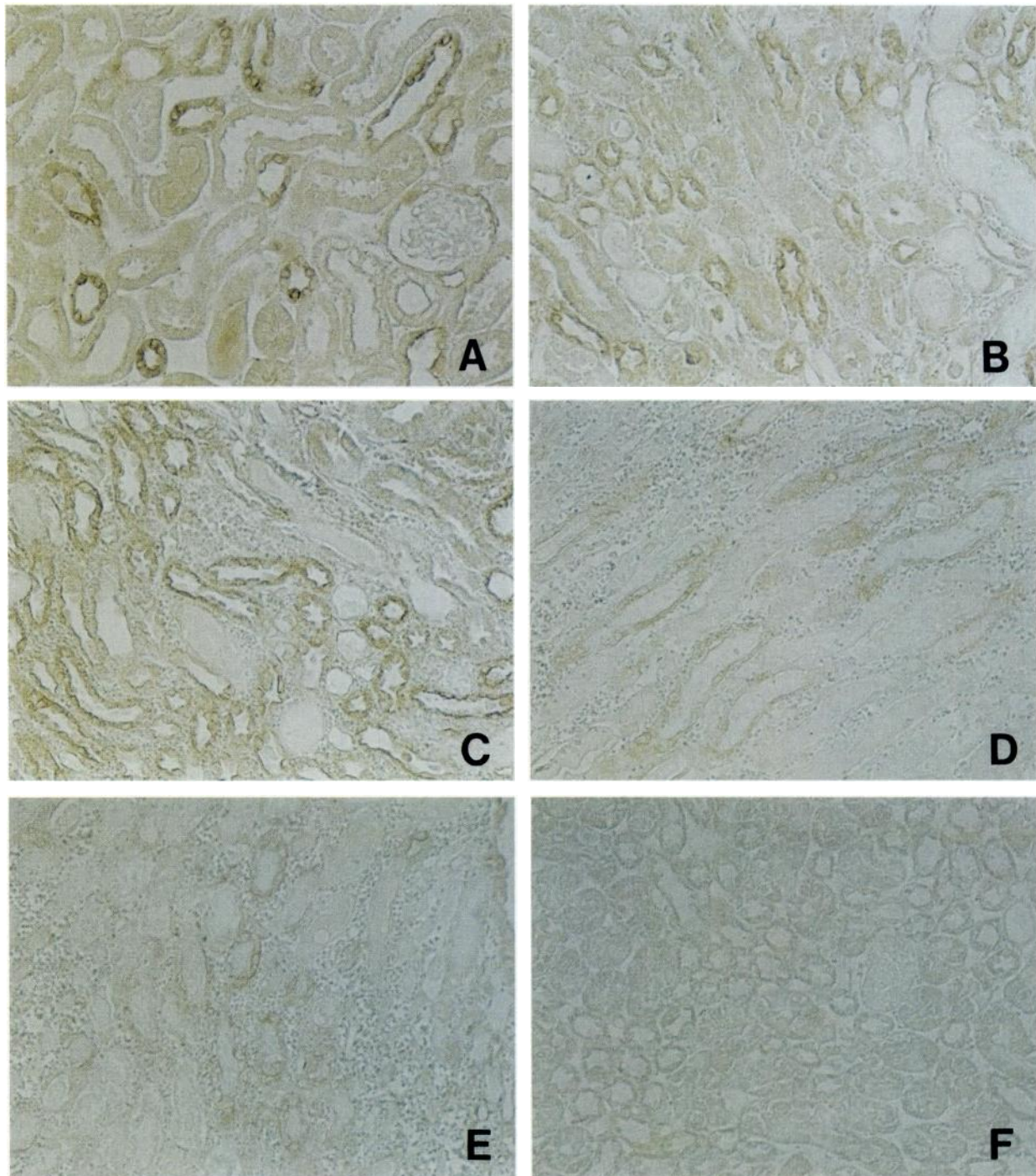
Figure 7. Immunohistochemical detection of Fas in ischemia-reperfusion kidney. (A) Cortex; (B) outer stripe of outer medulla; (C) inner stripe of outer medulla from 30-min ischemia kidney. In all parts, the distal tubules were stained with anti-Fas (P4) antibody. In contrast, glomeruli and proximal tubules were Fas-negative. (D and E) Inner stripe from 60-min (D) and 120-min (E) ischemia kidney. Note the small decrease in Fas-positive distal tubules relative to the 30-min ischemia kidney. (F) Inner stripe of outer medulla from sham-operated specimen. Note a lack of Fas-positive tubules. Magnification, ×200.

reperfusion mouse kidney and identification of Fas as a possible mediator of this apoptotic process. These findings support and extend earlier results showing apoptosis in the renal tubules of kidneys with mild renal ischemia (6,10) and transient complete ischemia (8). In these early studies, however, the exact sites of apoptosis were not determined.

Apoptosis was initially defined by Kerr *et al.* (7) based on morphologic features. Further examination of the apoptotic process revealed that it is often associated with internucleosomal fragmentation of genomic DNA, a process that can be recognized as a ladder formation in gel-electrophoresis of the DNA (38). Although the occurrence of the ladder was accepted
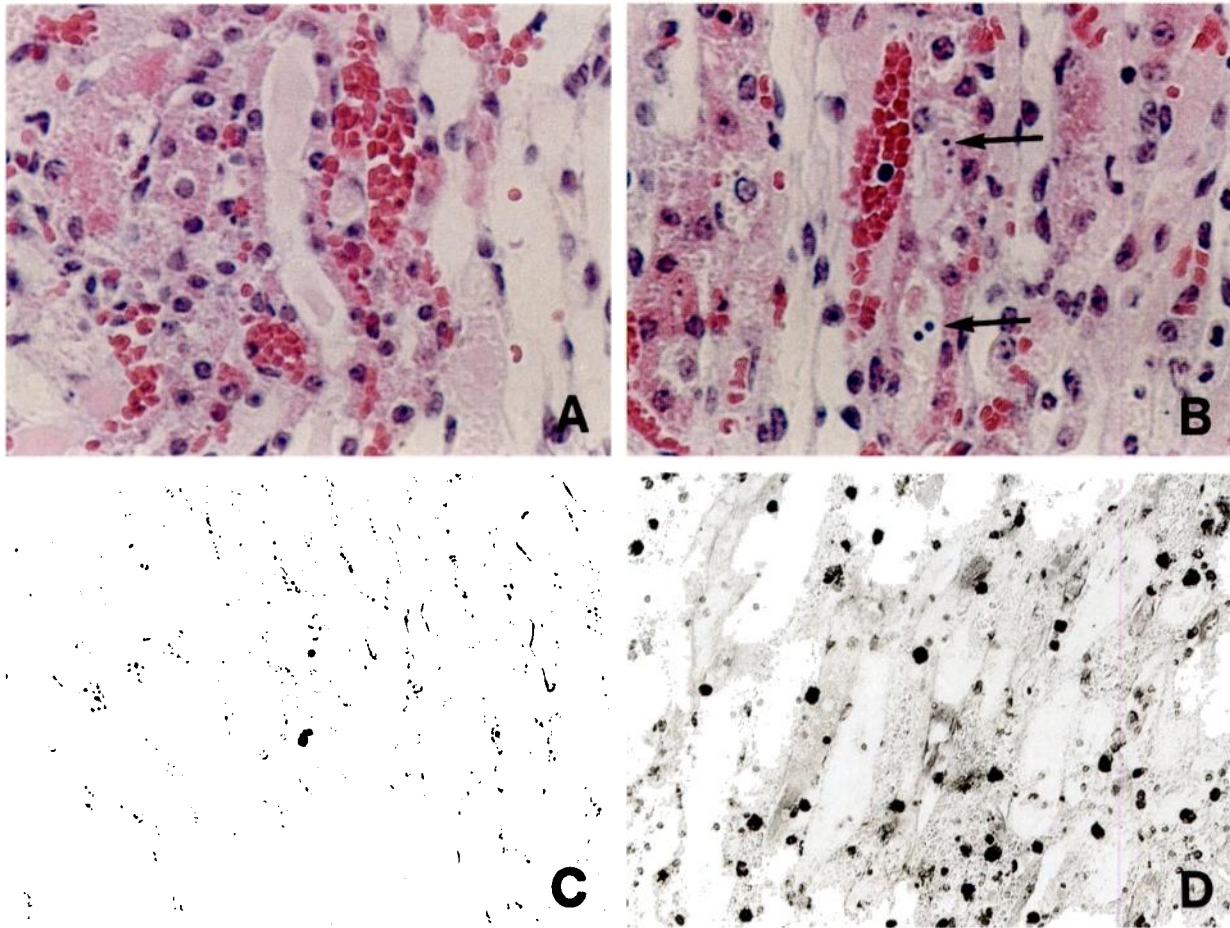
Figure 8. Morphology and *in situ* detection of DNA strand breaks by TUNEL in ischemia-reperfusion kidney model of B6 *lpr/lpr* mice as well as its wild type. (A and C) Sections from *lpr/lpr* B6 mouse, subjected to 120-min ischemia. Note the presence of only a few apoptotic (A) and TUNEL-positive (C) cells. (B and D) Sections from the wild-type B6 mouse, subjected to 120-min ischemia, showing several apoptotic (B, arrow) and TUNEL-positive (D) cells at the inner stripe. Magnification: ×500 in A and B; ×250 in C and D.

as a marker of apoptosis in most cases, several exceptions to this rule have been recently described (41). Histochemical detection of double-stranded DNA breaks by TUNEL has been widely used as a simple method to identify apoptotic nuclei. Again, however, TUNEL staining alone cannot demonstrate the presence of apoptotic cell death because the method can detect single-stranded DNA breaks as well (39). Therefore, various lines of indirect evidence should be used to demonstrate the presence of apoptosis. In this study, we used a number of currently available methods to demonstrate the presence of apoptosis and verified the occurrence of apoptotic cell death in distal renal tubules after ischemia-reperfusion of kidney.

Our results indicated that the proportion of TUNEL-positive cells was higher than that of cells with morphologic features of apoptosis. This should not be surprising because it has been widely accepted that DNA fragmentation precedes the apoptotic morphologic changes (28). Moreover, it should be noted that in the hypoxia/reoxygenation injury of cultured tubular cells, the presence of DNA fragmentation may not be associated with morphologic features of apoptosis (42).

Interestingly, the response of renal tubules to ischemia-

reperfusion varied from one section to another and depended on the anatomic location of these tubules; PCNA-positive cells were found only in the proximal tubules of the cortex and outer medulla, whereas the distal tubules, but not the proximal tubules, were positive for Fas mRNA and protein. Again, as described in the present study, apoptosis occurred in the distal tubules in response to the ischemia-reperfusion, whereas no such process was observed in the proximal tubules. In contrast, necrosis was evident in the proximal tubules, an effect similar to that seen in oxygen deprivation (43). These heterogenous responses to ischemia-reperfusion by different nephron segments may be explained by the marked differences in the response to cellular energy depletion between the thick ascending limb and proximal tubules (44).

Concerning the fate of apoptotic cells in the distal tubule, they were mainly extruded into the tubular lumen, whereas some cells seemed to be phagocytosed because apoptotic bodies were detected in the neighboring cells (data not shown). After the loss of cells by apoptosis, the stripped part of distal tubule was not covered by any cells, which might result in prolonged tubular dysfunction.

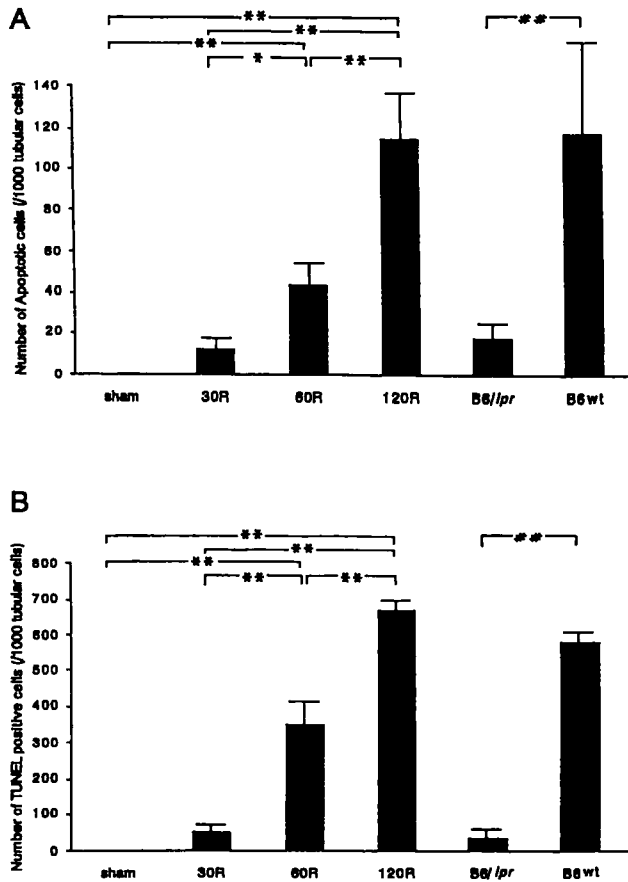Fas/APO-1/CD95 is a prominent molecule discovered by

Figure 9. Quantitative analysis of renal tubular apoptosis after ischemia-reperfusion. (A) Number of apoptotic cells determined in hematoxylin-eosin-stained sections. (B) Number of TUNEL-positive cells. The cell number is expressed per 1000 of total tubular cells. sham, sham-operated ICR mice; 30R, 60R, and 120R, ICR mice subjected to 30-, 60-, and 120-min ischemia followed by 24-h reperfusion, respectively. B6/lpr and B6wt, lpr/lpr and wild-type B6 mice subjected to 120-min ischemia followed by 24-h reperfusion. Values are expressed as mean ± SD. *$P < 0.05$; **$P < 0.01$ compared with ICR mice; ##$P < 0.01$ compared with B6 wild-type mice.

Yonehara et al. (45) and Trauth et al. (17) as a death factor in immune and tumor cells, respectively. Although the role of Fas in the induction of apoptosis has already been established in the immune system (46), little is known about the expression and function of Fas outside the immune system, and the information that is available is somewhat controversial (30,47). Very recently, however, Fas-dependent induction of apoptotic cell death has been noted in some organs, including the liver (18), vagina (20), ovary (21), and colon with ulcerative colitis (24). To our knowledge, the present study showed for the first time the induction of Fas expression in response to renal ischemia-reperfusion and the possible involvement of Fas in the induction of renal tubular apoptosis in the mouse kidney.

In this study, we used lpr/lpr mice to provide firm evidence for the involvement of Fas in the induction of renal tubular apoptosis by ischemia-reperfusion. Because the lpr/lpr mouse is known to suffer from various disorders that vary according to the degree of immunologic abnormality (27), it is possible

that certain survival cytokines secreted by infiltrating leukocytes may inhibit the induction of renal tubular apoptosis, together with lack of Fas action.

As a natural ligand of Fas, Fas ligand was identified and its transcript was shown to be expressed in normal kidney (22). To understand the difference in the induction of apoptosis between cortical distal tubules and medullary distal tubules, both of which became positive to Fas antigen after ischemia-reperfusion, analysis of Fas ligand distribution may be necessary. Unfortunately, however, we were unable in the present study to obtain consistent results of Fas ligand expression (data not shown). More extensive studies of Fas ligand expression in ischemia-reperfusion kidney are now under way in our laboratory.

A brief episode of renal ischemia increases the expression of the immediate-early genes such as c-fos and c-jun (48) and reduces the expression of prepro-epidermal growth factor (49) in the distal tubule. It is possible that these changes in transcriptional regulation may be part of the stress response. However, the increased expression of c-Fos and c-Jun do not seem to be associated with the regenerative DNA synthesis, a process restricted to the proximal tubule (48). Although ischemia-reperfusion induces heat shock proteins in various tissues including the kidney (50), these proteins fail to prevent renal tubular injury (51). Thus, Fas antigen can be induced in the renal tubule as part of the stress response to ischemia-reperfusion, and it may induce renal tubular apoptosis by itself or in concert with the immediate-early gene products, as well as stress proteins through unknown mechanisms.

What is the functional significance of apoptosis of distal tubules? We believe that the development of apoptosis in renal cells may exacerbate the severity of the ischemic insult since apoptosis of the distal tubules is not followed by active renal cell regeneration, in sharp contrast to necrosis of the proximal tubules. By extending this analogy to the lpr/lpr mice lacking Fas expression, this strain may develop only a mild form of renal tubular damage in response to ischemia-reperfusion.

Studies examining the underlying mechanisms of renal tubular apoptosis are important to design strategies to modulate renal injury by manipulating the expression of one or more specific genes, since apoptosis is thought to be genetically regulated. Clinical studies indicate that a variable degree of ischemia occurs in the transplanted kidney during the period between procurement from the donor and subsequent implantation in the recipient and that the major site of postischemic injury is the renal tubules in humans (52,53). Thus, the present results indicate that manipulation of the expression and/or function of Fas during kidney transplantation may represent a new strategy useful for the prevention or treatment of ischemic renal injury.

Acknowledgment

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