An Evaluation of Renal Tubular DNA Laddering in Response to Oxygen Deprivation and Oxidant Injury

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ABSTRACT

It has recently been suggested that endonuclease activation and/or apoptosis, possibly triggered by oxidant stress, are important pathogenetic mechanisms in oxygen deprivation/reoxygenation-induced proximal tubular cell death. To explore this possibility, DNA "laddering," a characteristic feature of these processes, was sought in: (1) postischemic rat kidneys (25- or 40-min arterial clamping; 0, 1, 4, 8, 24, and 48 h and 6 days reflow); (2) posthypoxic isolated rat proximal tubular segments and (3) cultured human kidney proximal tubular cells (HK-2) subjected either to energy depletion plus Ca2+ overload (antimycin A plus 2-deoxyglucose plus Ca2+ ionophore A23187), or to H2O2-induced cell death. DNA was subsequently extracted, electrophoresed through agarose gels, and visualized with ethidium bromide or Southern blotting. To maximize ladder detection, DNA samples were also end-labeled with 32P dideoxyadenosine triphosphate with terminal deoxynucleotidyl transferase (ttd), followed by electrophoresis. None of the postischemic DNA samples demonstrated any laddering by either ethidium bromide staining or Southern analysis (apoptotic lymphocyte DNA was a positive control). However, trace laddering was apparent by the ttd technique, commencing at 1 h of reflow, peaking at 24 h, and resolving slowly thereafter. This finding correlated with the morphologic expression of tubular necrosis, not apoptosis. Hypoxia/reoxygenation caused proximal tubular segment death (44 to 64%), and HK-2 cells were slowly killed by both the H2O2 and the energy depletion/Ca2+-loading protocols. However, neither protocol induced ethidium bromide- or ttd-detectable DNA laddering. It was concluded that: (1) minimal DNA laddering develops postischemia, and this change is reliably detected only by the ttd method; (2) it correlates with the morphologic expression of tubular necrosis, not apoptosis; and (3) in vitro oxidative- and energy depletion-mediated proximal tubular cell death can be dissociated from DNA ladder formation.

Key Words: Apoptosis, endonuclease, hydrogen peroxide, ischemia, hypoxia

Apoptosis is an internally regulated program by which cells can be eliminated from tissues in a controlled fashion in response to a variety of stimuli (reviewed in References 1–5). It has been widely proposed as a mechanism for the elimination of cells during development, for the removal of senescent cells, and for the control of excessive proliferation during tissue regeneration. It may also be induced by a variety of disease processes (e.g., neurodegenerative disorders, radiochemotherapy, oxidative stress), by selected regulatory hormones (e.g., glucocorticoids, addition to lymphocytes), or by the withdrawal of specific trophins (e.g., interleukins, estrogen, testosterone) (1–6). Irrespective of the stimulus, strikingly similar morphologic changes result, most notably chromatin condensation and nucleolar degeneration, nuclear compaction, and decreased cell volume (3). Subsequently, the involved cells fragment into "apoptotic bodies" that are rapidly eliminated, either by phagocytosis or by in situ degeneration. From a pathogenetic standpoint, apoptosis is believed to differ markedly from ischemic or toxic cell death (7): the latter are externally, not internally, controlled; they usually affect large numbers of contiguous cells in a relatively nonspecific fashion; and early cell swelling, not condensation, results.

Although the precise mechanisms that mediate apoptosis are poorly defined, a characteristic feature of it is endonuclease activation, which results in the degradation of genomic DNA at internucleosomal linker regions. This process generates 180- to 185-base-pair fragments, which by agarose gel electrophoresis, yield a "laddered" DNA appearance (e.g., References 4 and 6). Because the morphologic changes of apoptosis are extremely transient and, thus, are easily missed, DNA "laddering" has become widely accepted as a marker of apoptotic cell death.

In 1992, Schumer et al. reported that DNA,
extracted from rat kidneys 12 to 48 h posts ischemia (5, 30, 45-min arterial clamping), generated characteristic apoptotic ladders, as assessed by agarose gel electrophoresis with ethidium bromide staining (8). On the basis of those observations, it has been suggested that apoptosis may be triggered by ischemia, potentially contributing to the development of posts ischemic tubular cell death and, hence, acute renal failure (ARF) [8,9]. To gain support for this intriguing possibility, our laboratory recently used a newly described histocytochemical technique (10) to ascertain whether DNA damage can be observed in situ in postischemic rat kidneys (11). This method uses formalin-fixed tissues that are incubated with terminal deoxynucleotidyl transferase (ttd) and biotinylated deoxyuridine, the latter being incorporated into the 3'OH ends of DNA, exposed with DNA breaks (10). Subsequently, the deoxyuridine signal is amplified with avidin peroxidase, allowing for its detection by conventional light microscopy. The results of that study indicated that DNA breaks can be found within postischemic rat kidneys; however, they appeared to be limited to those tubular cells that were already overtly necrotic, suggesting that the DNA changes were a result, rather than a mediator, of postischemic tubular cell death. Furthermore, although DNA breaks were observed in that study, in situ detection with the ttd method does not necessarily mean that characteristic apoptotic laddering had resulted. In other words, the DNA changes could have reflected nonspecific DNA degradation, rather than endonuclease-mediated "apoptotic" death.

As recently suggested by Bonventre (9), the documentation of apoptosis, or DNA laddering, in early postischemic renal tissues could substantially alter our current thinking about the mechanisms of posts ischemic ARF. Therefore, the goals of this study were to confirm the presence of laddered DNA in postischemic rat kidneys by a variety of methods and to further explore its pathogenetic significance with three in vitro models of proximal tubular cell death.

METHODS

In Vivo Ischemia/Reperfusion Experiments

Male Sprague Dawley rats (175 to 275 g) were anesthetized with pentobarbital (30 to 40 mg/kg ip), a midline laparotomy was performed, and the renal arteries were then occluded with smooth vascular clamps. To create different degrees of ischemic tissue damage, the left and right kidneys of each rat were occluded for 25 and 40 min, respectively. After the ischemic insults were completed, the kidneys were either immediately removed without allowing reperfusion or variable lengths of reflow were permitted (1, 4, 8, 24, 48 h or 6 days; N = two to four rats for each time frame). Rats undergoing 8 h or more of reperfusion were allowed to recover from anesthesia, with free food and water access being provided. At the appropriate reperfusion time, the kidneys were resected, and DNA was isolated and analyzed with ethidium bromide-stained agarose gels. Southern analysis, and/or the ttd method, as described below. DNA extracted from normal rat kidneys during each tissue process-
8-, 24-, and 48-h incubations, the cell viability of the control and challenged cells was determined in representative wells by vital dye exclusion (the percentage of cells with nuclei that stained positive within 5 min of 50 μg/ml ethidium bromide addition; total cell numbers were determined by counterstaining with 15 μg/ml acridine orange in India ink). The cells in the remaining wells were mechanically detached from the plastic and centrifuged; the DNA was isolated and analyzed with ethidium bromide-stained gels and the tdt method, as described below.

**H₂O₂-Induced Oxidant Stress of HK-2 Cells: Evaluation of DNA Fragmentation**

Because postischemic tubular necrosis has been reported to be mediated, in part, by iron-dependent oxidant stress (e.g., References 15 and 16) and because oxidant stress can clearly trigger DNA damage (e.g., References 6 and 17), the following experiments were undertaken to ascertain whether the iron-dependent oxidative death of HK-2 cells correlates with endonuclease-mediated double-stranded DNA breaks. To this end, the effects of H₂O₂ on HK-2 cell viability and DNA ladder formation were assessed, because it has previously been shown that H₂O₂ induces HK-2 cell death via an iron-dependent mechanism (14). Plates of HK-2 cells were prepared as noted above, and then, they were incubated under control conditions or in the presence of 5 mM H₂O₂ for 8, 24, or 48 h. After the completion of each incubation time, percent cell viability was assessed in control and H₂O₂-exposed wells by ethidium bromide staining, as denoted above. Cells in the remaining wells were used to harvest DNA samples for subsequent electrophoretic analysis, as detailed below.

**DNA Isolation and Analysis**

Total DNA was isolated from whole kidney tissues, PTS, and HK-2 cells by the technique of Tilly and Hsueh (18). In brief, the tissues were snap frozen in liquid nitrogen and stored at −80°C. Subsequently, they were suspended in 4 vol of a homogenizing buffer (0.1M NaCl, 0.2 M sucrose, 10 mM EDTA, 0.1 M Tris; pH 8.0) and homogenized with a Polytron tissue dispensor. One-tenth volume of 10% sodium dodecyl sulfate was added and incubated at 65°C for 30 min. One-third volume of 4 M potassium acetate was added, the homogenate was mixed and incubated for 1 h at 4°C, and the cellular debris and protein precipitates were pelleted by centrifugation (5,000 × g for 10 min; 4°C). The supernatant was extracted two times with 2 vol of phenol:chloroform (1:1), followed by reextraction with 2 vol of chloroform. The aqueous phase was collected; the DNA was precipitated with the addition of 3 vol of chilled ethanol and then held at −80°C for more than 1 h. The DNA was pelleted by microfuging (14,000 rpm × 30 min; 4°C); it was then washed with 70% ethanol and air dried for 10 min. The material was resuspended in 50 μl of TE (10 mM Tris [pH 8.0], 1 mM EDTA) and incubated at 37°C for 1 h with 1 μl of DNAase-free RNase. The phenol:chloroform extractions were then repeated. Finally, the DNA was precipitated with ethanol and solubilized in 25 μl of one-tenth strength TE. DNA concentrations were determined by absorbance at 260 nm.

DNA samples (either 2 or 5 μg) were electrophoresed through 1.6% agarose containing ethidium bromide (19), with laddered phage DNA (220 to 12,200 base pairs) being used as molecular weight markers. Gels containing samples from normal kidneys, postischemic kidneys (8, 24, or 48 h or 6 days after 25 or 40 min of ischemia), the PTS, and the apoptotic lymphocytes were also analyzed by Southern analysis in an effort to optimize DNA ladder detection. These gels (N = 2 each) were transferred to nylon and subjected to blotting hybridization under low-stringency conditions with 32P-labeled rat or human genomic DNA probes (20). They were exposed for 3 days on X-OMT AR-F5 film (Kodak, Rochester, NY), with the use of an intensifying screen to maximize ladder detection.

The tdt method was used to analyze at least two DNA samples obtained from each of the following protocols: normal and ischemic/postischemic kidneys (25 and 40 min of ischemia/no reflow; or 1, 4, or 24 h or 6 days of reflow); three complete sets of PTS allquots; and all HK-2 cell experiments. Approximately 2 μg of purified DNA was quantitatively end-labeled with 32P dideoxyadenosine triphosphate (Amersham, Arlington Heights, IL), with tdt (Boehringer Mannheim, Indianapolis, IN), according to the manufacturer’s instructions. Subsequently, the labeled DNA samples were electrophoresed through 2% agarose gels, dried under a vacuum, and autoradiographed.

**RESULTS AND DISCUSSION**

Irrespective of the length of ischemia (25 or 40 min) or reperfusion (0 min to 6 days), none of the kidney samples obtained from the in vitro experiments appeared degraded or generated apoptotic ladders, as assessed by the ethidium bromide-stained gels (Figure 1, top). This indicates that, at the very most, only

![Figure 1](image-url)

Figure 1. (A and C) Ethidium bromide-stained gels containing DNA from control kidneys, from kidneys that were 8, 24, or 48 h or 6 days postischemia, and from PTS subjected to continuous oxygenation (oxy) or hypoxia/reoxygenation (hyp) injury. None of these samples demonstrated laddered DNA. In contrast, DNA from dexamethasone-treated 284 lymphocytes generated a characteristic ladder pattern, serving as a positive control. M, molecular weight markers (range, 220 to 12,200 base pairs). (B and D) Southern analysis of the above positioned gels. Slight DNA degradation is seen in one sample (24 h post-25 min of ischemia), but no laddering was apparent. In contrast, laddering was seen in the samples obtained from dexamethasone-treated 284 lymphocytes.
trace amounts of laddered DNA develop in the aftermath of ischemic renal injury. These negative ethidium bromide results cannot be readily ascribed to a technical artifact because laddering was observed with this method when DNA samples from apoptotic lymphocytes were studied (Figure 1, top). Of note, Schumer et al. previously reported extensive DNA laddering in posts ischemic rat kidneys using ethidium bromide-stained gels (8). Thus, our findings stand in contrast to their results. The reason for this discrepancy remains unknown. However the fact that they found prominent laddering even in kidneys subjected to as little as 5 min of ischemia and that ≥25 min of ischemia is generally required to induce any in vivo tubular cell death raises a question as to whether those results predominantly represented in vivo, versus ex vivo, DNA degradation.

In a further attempt to document DNA laddering in the aftermath of posts ischemic kidney injury, DNA from our in vivo experiments were also subjected to Southern analysis. Again, no clear laddering was detected, even by this method (Figure 1, bottom). In contrast, DNA extracted from apoptotic lymphocytes did generate ladders by this technique, even when the sample applied to the gel was reduced from 5 to 0.2 µg of DNA. The fact that no laddering was seen when this reduced amount of DNA was analyzed with ethidium bromide-stained gels confirmed the greater sensitivity of the Southern technique. Thus, the results with Southern analysis substantiated the ethidium bromide results, indicating that, at most, only trivial amounts of laddered DNA develop post-renal ischemia.

Given the completely negative results with ethidium bromide-stained gels and Southern analyses, the even more sensitive tdt method was used to define whether any laddered DNA existed within our posts ischemic kidney samples. The fact that we had previously documented in situ posts ischemic DNA damage by this technique (11) suggested to us that it should have sufficient sensitivity to prove whether or not that damage reflected laddered DNA. Neither the normal kidney samples nor those extracted from kidneys subjected to ischemia/no reflow demonstrated any fragmented DNA. However, by 1 h of reflow, trace laddering was seen in some of the samples, and the amount increased progressively over the ensuing 23 h (Figure 2). By 6 days of reflow, a time correlating with renal functional recovery (e.g., References 11 and 21), the vast majority of the tdt-detectable DNA damage had disappeared. Finally, the extent of laddering seemed to correlate with the severity of ischemic injury, because at 4 h and 24 h and 6 days of reflow, the changes were noticeably greater in samples extracted from kidneys that had undergone the 40 min versus the 25 min, ischemic insult. Thus, the tdt experiments indicated that: (1) laddered DNA does, in fact, develop in posts ischemic rat kidneys; (2) the amount generated is exceedingly small, because only the tdt method could detect it; (3) it commences during early reperfusion (1 to 4 h), increases progressively over the first 24 h, and resolves slowly thereafter; and (4) its extent appears to reflect the amount of posts ischemic tissue damage.

To discern the temporal relationship between tdt-detectable laddering and the onset of tubular morphologic injury, renal histologic sections were examined from 1 to 24 h post ischemia. Proximal tubular cell injury was clearly discernible by 1 h of reflow (brush border membrane blebbing and shedding), and overt tubular necrosis was first seen at 4 h of reflow, reaching its maximum 20 h later. Thus, the onset and extent of the laddering appeared to reflect the degree of tubular injury, suggesting that it may have been a secondary consequence of that injury, rather than being a primary mediator of it. Indeed, we reached this same conclusion in our former study of the in situ tdt analysis of posts ischemic kidneys (11). In that study, tdt-detectable DNA fragmentation was seen only
cells that were overtly necrotic, and not in their morphologically intact counterparts, suggesting that the DNA changes occurred as a secondary response to lethal cell damage. Of note, none of the 1- to 24-h postischemic kidney sections demonstrated clear morphologic evidence of apoptosis. As we previously reported, apoptotic cells can be observed within postischemic kidneys (11). However, they appear predominantly during the late recovery period (5 to 6 days postischemia), they are very infrequent and largely confined to occasional nodules of regenerating epithelia, and they undoubtedly reflect a pathway for the elimination of redundant cells, produced as part of the regenerative response (11). Thus, although apoptosis may be an important event in posts ischemic ARF, it seems more likely that its dominant role is during the recovery, rather than the initiation, phase of injury.

To further gauge the temporal relationship between DNA laddering and the loss of tubular cell viability (thereby assessing a possible cause and effect relationship), the isolated PTS experiments were performed. Of note, because the loss of cell viability in this system can be precisely timed by LDH release, these experiments should permit a precise determination of whether DNA laddening precedes, or results from, tubular cell death. Those studies revealed the following noteworthy results: (1) clear DNA ladders could not be documented in oxygenated or hypoxic/posthypoxic PTS aliquots when assessed by ethidium bromide staining or Southern analysis (Figure 1); (2) tdt-detectable laddering was observed in all continuously oxygenated PTS (Figure 3), undoubtedly reflecting DNA damage sustained during the tubular isolation process. (Of note, we have documented this same finding in PTS isolated in another laboratory [T. J. Burke, Denver, CO], confirming that this isolation damage is not unique to our laboratory); and (3) hypoxia/reoxygenation induced 44 to 64% tubular cell death (LDH release), and despite this, there was absolutely no increase in the amount of tdt-detectable laddered DNA over that seen in the oxygenated controls (10 to 15% LDH release) (Figure 3). Thus, these results clearly demonstrate that hypoxia/reoxygenation–induced tubular cell death can occur without developing double-stranded DNA breaks.

It should be noted that interpretation of the above PTS results is somewhat complicated by the fact that baseline tdt-detectable DNA breaks were observed even in those tubules not subjected to hypoxic injury. Because of this, it was not possible to completely dissociate hypoxia/reoxygenation cell death from laddered DNA. Thus, we next studied the evolution of energy depletion–induced proximal tubular cell death in HK-2 cells, because no background DNA damage exists in this cell culture system. To this end, HK-2 cells were incubated with a mitochondrial respiratory inhibitor (antimycin A) plus an inhibitor of glucose uptake (2-deoxyglucose), a protocol shown in pilot experiments to induce ~90% ATP depletion. However, because lethal cell injury does not result from this degree of energy depletion alone, a superimposed Ca\(^{2+}\) ionophore challenge was used (22), which only causes HK-2 cell death when they are in an energy-depleted state. As shown in Figure 4, this combined challenge induced a time-dependent model of HK-2 cell death. However, at no time during the course of the experiments did DNA laddering result (Figure 5). Thus, these findings completely support the conclusions from the isolated tubule experiments by proving that energy depletion/Ca\(^{2+}\) overload–mediated cell

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**Figure 3.** tdt analysis of isolated PTS DNA subjected to continuous oxygenation (oxy), hypoxia (hyp), or hypoxia/reoxygenation injury (hyp/reoxy). tdt-detectable ladders were apparent in all oxygenated aliquots, but the amount of the laddering was not increased in response to hypoxia/reoxygenation.

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**Figure 4.** Time course of HK-2 cell death in the H\(_2\)O\(_2\) (8, 24, and 48 h) and the energy depletion + Ca\(^{2+}\) ionophore (1, 4, 8, 24, and 48 h) experiments. Cell viability was assessed by vital dye (ethidium bromide) exclusion (see Text). Note the gradual onset of cell death, reaching ~100% at 24 h. Despite this slow evolution of injury, no DNA laddering became apparent (see Figures 5 and 6).

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**Figure 5.** tdt analysis of normal HK-2 cells and HK-2 cells subjected to energy depletion + Ca\(^{2+}\) ionophore treatment. No DNA laddering developed at any time during the course of these experiments. C, control incubation with K-SFM.
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(2) technique is likely to be required; and
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mment tdt-detectable DNA breaks during early vascular
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reason for addressing this issue is that we did docu-
many-dependent oxidant tissue stress
assary for inducing laddered DNA.
in vito results. To this end, HK-2 cells were incubated with 5 mM H2O2, which induced gradual
cell death over the course of the experiment (Figure 4). However, absolutely no DNA laddering resulted (Fig-
branched bands of intact DNA near the top of the gel). Despite this, no DNA laddering were detected.
death can, indeed, occur in the absence of endonucle-
ase-mediated double-stranded DNA breaks.

The final goal of this study was to assess whether H2O2-induced/iron-dependent HK-2 cell killing oc-
curs via an endonuclease-dependent pathway. The reason for addressing this issue is that we did docu-
ment tdt-detectable DNA breaks during early vascular reperfusion (Figure 1; Reference 11), a time of pur-
ported critical iron-dependent oxidant tissue stress (15,16). Thus, had we been able to document HK-2
DNA laddering with oxidant injury, this would provide a possible pathogenetic insight into both our past (1)
and these in vito results. To this end, HK-2 cells were incubated with 5 mM H2O2, which induced gradual

tube cell line (6). Thus, our HK-2 results appear discordant with those data. Although the reason for this discrepancy remains
unknown, it indicates that pathways of H2O2-induced injury may vary considerably, depending on the cell
lined. Thus, results obtained with one should be interpreted with caution. It should be noted, however, that
HK-2 cells are not completely resistant to apoptotic/endonuclease-mediated cell death, because tdt-
detectable ladders do develop when they are mainta-
ined under conditions of growth factor and nutrient
withdrawal (unpublished data from this laboratory). Thus, the negative results with our H2O2 and energy
depletion/cell Ca2+-loading experiments cannot simply be ascribed to an absence of the pathways neces-
sary for inducing laddered DNA.

In conclusion, this study indicates that: (1) trace
amounts of DNA laddering can be documented in the
aftermath of in vitro ischemic renal injury. However,
the amount generated appears to be far too low to
permit reliable detection by routine ethidium bro-
mide-stained agarose gels or even Southern analysis.
Thus, if one wishes to document DNA laddering in
postischemic kidneys, the highly sensitive tdt tech-
nique is likely to be required; and (2) despite the appearance of tdt-detectable postischemic DNA lad-
ders, the pathogenetic significance of this finding
remains open to interpretation. This is because the
bulk of the DNA damage appears concomitantly with
the morphologic appearance of necrosis, rather than
proceeding it (suggesting that it may be a secondary,
rather than being a primary event), and because the in vitro studies presented here demonstrate that both
energy depletion/Ca2+ overload- and oxidant-trig-
gered proximal tubular cell death can develop in the
absence of laddered DNA. This does not mean that
DNA damage is irrelevant to these forms of injury.
However, we do believe that double-stranded DNA
breaks are not necessarily critical to the initiation of
them in all circumstances, as demonstrated by this report.

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