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Technical Advance

Internucleosomal DNA Cleavage Triggered by Plasma Membrane Damage during Necrotic Cell Death

Involvement of Serine but Not Cysteine Proteases

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Autolytic DNA breakdown, detected as smears in electrophoretic gels, is a late event in necrosis. On the other hand, internucleosomal DNA cleavage, visualized as ladders, is thought to be a hallmark of apoptosis. We now report that this specific form of DNA fragmentation also occurs during necrosis and is an early event but appears to be triggered by proteolytic mechanisms significantly different from those documented in apoptosis. Treatment of MDCK cells with a mitochondrial uncoupler and a Ca^{2+} ionophore led to ATP depletion, necrotic morphology, and progressive fragmentation of DNA in an internucleosomal or ladder pattern. DNA breakdown was immediately preceded by increased permeability of the plasma membrane to macromolecules. Provision of glycine along with the noxious agents did not modify the extent of ATP depletion, but prevented plasma membrane damage. This was accompanied by complete inhibition of DNA fragmentation. Internucleosomal DNA cleavage was observed also during necrosis after rapid permeabilization of plasma membranes by detergents or streptolysin-O in hepatocytes, thymocytes, and P19, Jurkat, and MDCK cells. DNA fragmentation associated with necrosis was Ca^{2+}/Mg^{2+} dependent, was suppressed by endonuclease inhibitors, and was abolished by serine protease inhibitors but not by inhibitors of interleukin-1β converting enzyme (ICE)-related proteases or caspases. Moreover, unlike apoptosis, it was not accompanied by caspase-mediated proteolysis. On the other hand, the cleavage-site-directed chymotryptic inhibitor *N*-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK) suppressed DNA fragmentation not only in necrotic cells but also during Fas-mediated apoptosis, without inhibiting caspase-related proteolysis. The results suggest a novel pathway of endonuclease activation during necrosis not involving the participation of caspases. In addition, they indicate that techniques based on double-strand DNA breaks may not reliably differentiate between apoptosis and necrosis. *(Am J Pathol 1997, 151:1205–1213)*

Necrosis and apoptosis are two major forms of cell death, which are distinguished from each other morphologically and biochemically.[1,2] Apoptosis is usually associated with internucleosomal cleavage of DNA, recognized as ladders in agarose gels after electrophoresis.[3–5] Early observations indicated that DNA laddering occurs during apoptosis but not necrosis.[6–8] More recently, the requirement of internucleosomal DNA cleavage in the apoptotic process has been questioned.[9] This form of DNA breakdown was dispensable in some apoptotic models,[10–12] and moreover, DNA ladders have been shown to occur in cells without apoptotic morphology.[13–16] Nevertheless, internucleosomal DNA cleavage continues to be regarded as an important event in programmed cell death,

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and DNA laddering is frequently used to characterize apoptosis (for a review see Ref.5).

During studies on the pathogenesis of necrosis caused by ATP depletion, we observed DNA degradation, and electrophoretic analysis showed DNA ladders indistinguishable from those caused by internucleosomal DNA cleavage in apoptotic cells. Additional investigation showed that this type of DNA fragmentation occurred soon after the loss of plasma membrane integrity. Glycine, an agent that prevents plasma membrane damage during ATP depletion,[17–23] completely inhibited the fragmentation of DNA. However, when the plasma membrane of such glycine-protected cells was permeabilized by detergents, DNA fragmentation occurred soon thereafter, even in the presence of glycine. Because DNA laddering appeared to be coupled to plasma membrane damage, we tested in several types of cells the effects of streptolysin-O (SLO) and saponin, agents that rapidly induce necrosis by plasma membrane permeabilization. The results show that internucleosomal DNA cleavage occurs ubiquitously after the loss of plasma membrane integrity and development of necrotic morphology. Furthermore, serine proteases, but not cysteine proteases, appear to participate in necrotic DNA laddering, suggesting the existence of diverse pathways of endonuclease activation during cell death. Thus internucleosomal cleavage may be a common form of DNA damage that occurs during necrosis as well as apoptosis. DNA ladders and cytochemical stains based on double-strand breaks may not be reliable markers for specific forms of cell death.

Materials and Methods

Materials

Ionomycin was from Calbiochem-Novabiochem International (La Jolla, CA). SLO was purchased from Wellcome Diagnostics (Dartford, UK). [^{3}H]Thymidine was obtained from NEN Life Science Products (Boston, MA). Anti-Fas monoclonal antibody was from Medical and Biological Laboratories (Nagoya, Japan). C-2–10 mouse monoclonal anti-poly(ADP-ribose) polymerase antibody was supplied by Biomol Research Laboratories (Plymouth Meeting, PA). Z-Val-Ala-Asp$_{(OMe)}$-CH_2F (Z-VAD.FMK) and Z-Asp$_{(OMe)}$-Glu$_{(OMe)}$-Val-Asp$_{(OMe)}$-CH_2F (Z-DEVD-.FMK) were purchased from Enzyme System Products (Dublin, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO)

ATP Depletion

Madin-Darby canine kidney (MDCK) cells were depleted of ATP by incubation in glucose-free Krebs-Ringer bicarbonate solution (KRB) containing 15 μmol/L carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), a mitochondrial uncoupler. Free Ca^{2+} in KRB was 1.25 mmol/L or was adjusted to 100 nmol/L with 2.25 mmol/L EGTA,[24] and 5 μmol/L ionomycin, a Ca^{2+} ionophore, was also included so that intracellular Ca^{2+} rose to high concentrations or did not increase beyond 100 nmol/L.[25] Experiments were done with or without the addition of 5 mmol/L glycine and/or 4% sucrose, a membrane impermeant osmolyte, to the incubation medium. Plasma membrane integrity was monitored by measuring the release of intracellular lactate dehydrogenase (LDH) into the incubation medium.[26]

Cell Permeabilization by Detergents or Streptolysin-O

Cells were incubated in KRB containing 0.2 mg/ml saponin or 0.1% Triton X-100 at 37°C. In other experiments, SLO, a pore-forming toxin, was used to permeabilize plasma membranes selectively, using a protocol modified from Miller and Moore.[27] Briefly, cells were preincubated in phosphate-buffered saline containing 1 U/ml activated SLO at 4°C for 30 minutes, transferred to KRB containing 100 nmol/L free Ca^{2+} without SLO, and incubated at 37°C.

Analysis of DNA Fragmentation

DNA fragments released from 2×10^6 cells were extracted and separated by electrophoresis in agarose gels according to Arends et al.[4] After experiments, cells were lysed in a hypotonic buffer containing 0.5% Triton X-100, 10 mmol/L Tris, and 20 mmol/L EDTA, pH 7.4. Cell lysate was then centrifuged at 14,000 × *g* for 20 minutes. The resultant supernatant was treated with proteinase K and RNase A and was then extracted with phenol/chloroform (1:1). In some experiments, the phenol/chloroform step was omitted, with identical results. DNA fragments were precipitated with 67% ethanol, 0.5 mol/L NaCl at −20°C for 18 hours and resuspended in 10 mmol/L Tris/HCl, 1 mmol/L EDTA, pH 8.0, before 1.5% agarose gel electrophoresis. Quantitation of DNA fragmentation was done by a method modified from Duke et al.[7] Briefly, 10^6 cells were labeled overnight with [^{3}H]thymidine (2.5 μCi/ml). After experiments, the incubation medium was saved. Cells were lysed as before and the lysate was centrifuged. DNA fragments in the incubation medium and lysate supernatants were precipitated with ethanol. Precipitates were dissolved in 10 mmol/L Tris with 1 mmol/L EDTA and counted for radioactivity by scintillation spectrometry. Specific DNA fragmentation was calculated as described.[7]

Detection of Poly(ADP-Ribose) Polymerase

Poly(ADP-ribose) polymerase (PARP) was detected by immunoblotting.[28] At the end of experimental incubation, protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 100 μmol/L 3,4-dichloroisocoumarin (DCI), 10 μg/ml aprotinin, and 5 μg/ml leupeptin) were added. Cells were then collected and dissolved in 9 mol/L urea/1% sodium dodecyl sulfate (SDS)/5% β-mercaptoethanol. Cell proteins were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with C-2-10, a mouse monoclonal antibody recognizing an epitope

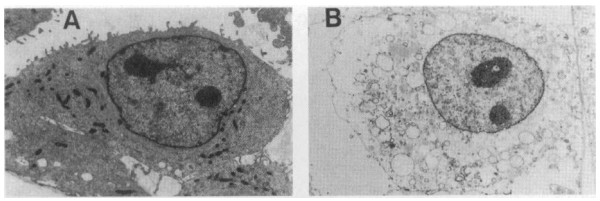

Figure 1. Electron microscopy of MDCK cells. MDCK cells were incubated for 3 hours in KRB with 100 nmol/L free Ca^{2+}, without further addition (**A**, time control) or with 15 μmol/L CCCP and 5 μmol/L ionomycin (**B**, ATP-depleted cells). LDH released from cells into the incubation medium was 2 and 94% for **A** and **B**, respectively. The cells were then fixed and processed for electron microscopy.

located at the carboxyl end of the DNA-binding domain of PARP.[29]

Morphological Observations

Cell morphology during the experimental incubation was followed by phase contrast microscopy. Cells were also fixed in 2% glutaraldehyde with 50 mmol/L lysine, 50 meq of Na, and 100 meq of cacodylic acid and processed for electron microscopy as described.[30] Nuclear morphology was also monitored by staining with Hoechst 33342.[12]

Results

Necrotic DNA Laddering in ATP-Depleted Cells

MDCK cells became necrotic during 3 hours of incubation in 100 nmol/L Ca^{2+} KRB containing CCCP and ionomycin. As shown in Figure 1, the affected cells had swollen and empty cell bodies with disrupted organelles. This was accompanied by increased permeability of the plasma membranes to LDH, a 136-kd protein. Nuclear chromatin was dispersed, unlike the condensation and fragmentation seen in apoptosis (Figure 1). Absence of nuclear fragmentation in necrotic cells was confirmed by fluorescence microscopy after staining with Hoechst 33342 (not shown).

Death of ATP-depleted cells was accompanied by progressively increasing breakdown of DNA, indicated by greater recovery of radioactive DNA fragments from [^{3}H]thymidine-labeled cells (Figure 2). Unexpectedly, agarose gel electrophoresis of necrotic DNA displayed a ladder-like pattern, consisting of ~180-bp DNA multimers identical to those observed in apoptosis (Figure 3A). DNA laddering, which was first noted at 2 hours and increased progressively with incubation time, was preceded by release of LDH from cells into the incubation medium (Figure 3A). As these findings suggested that cleavage of DNA was a secondary event that followed plasma membrane damage and loss of cellular solutes, we tested the effects of glycine on DNA fragmentation. Glycine prevents plasma membrane damage but not the ATP depletion that is induced in cells by hypoxia or other inhibitors of mitochondrial function.[17–23,25,26] As shown in Figures 2 and 3A (lane 7), not only LDH release but also DNA fragmentation was totally blocked by glycine.

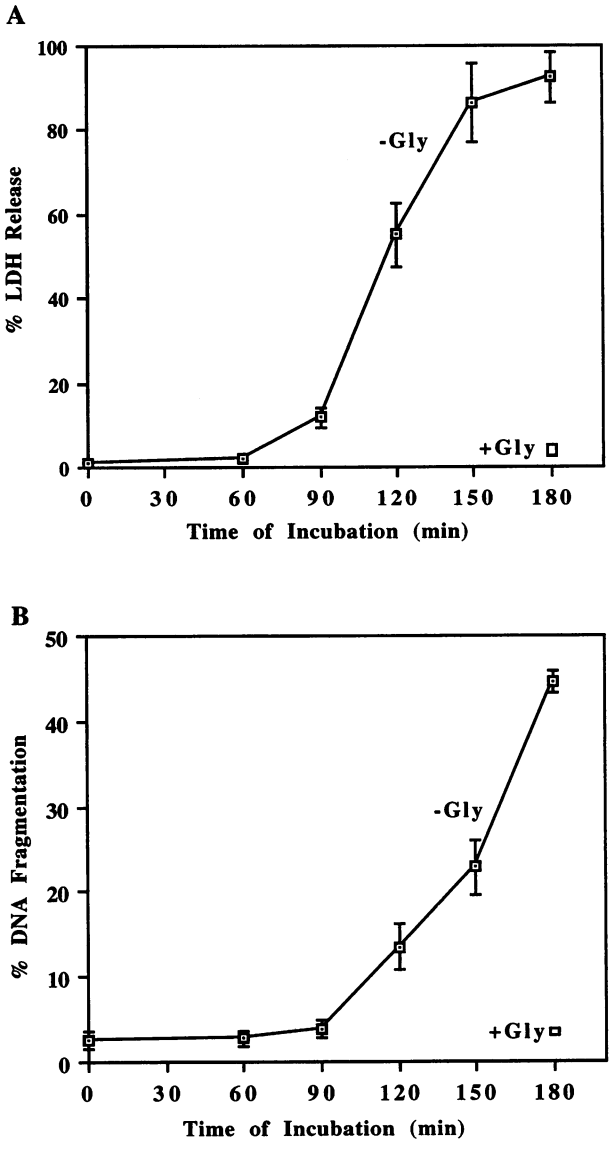

Figure 2. Quantitation of DNA fragmentation during ATP depletion. MDCK cells were prelabeled with [^{3}H]thymidine and then subjected to ATP depletion in KRB with 100 nmol/L free Ca^{2+} containing CCCP and ionomycin, without glycine (−Gly) or with 5 mmol/L glycine (+Gly). At the end of incubation, free LDH in the medium was measured (**A**). DNA fragments released from cells were counted for radioactivity and expressed as a percentage of label in whole cells (**B**).

Additional support for the role of membrane permeabilization in necrotic DNA laddering was provided by results displayed in Figure 3B. When large increases of cellular Ca^{2+} were induced in ATP-depleted cells by treatment with ionomycin in 1.25 mmol/L Ca^{2+} KRB, cell injury was accelerated (compare Figure 3, A and B). Release of LDH from cells into the medium was first observed after 30 minutes of incubation and increased thereafter to become maximal by 90 to 120 minutes. Morphologically, these cells were markedly swollen and showed features of necrosis by electron microscopy (not shown). This was accompanied by striking fragmentation of DNA in the characteristic ladder pattern, increasing in

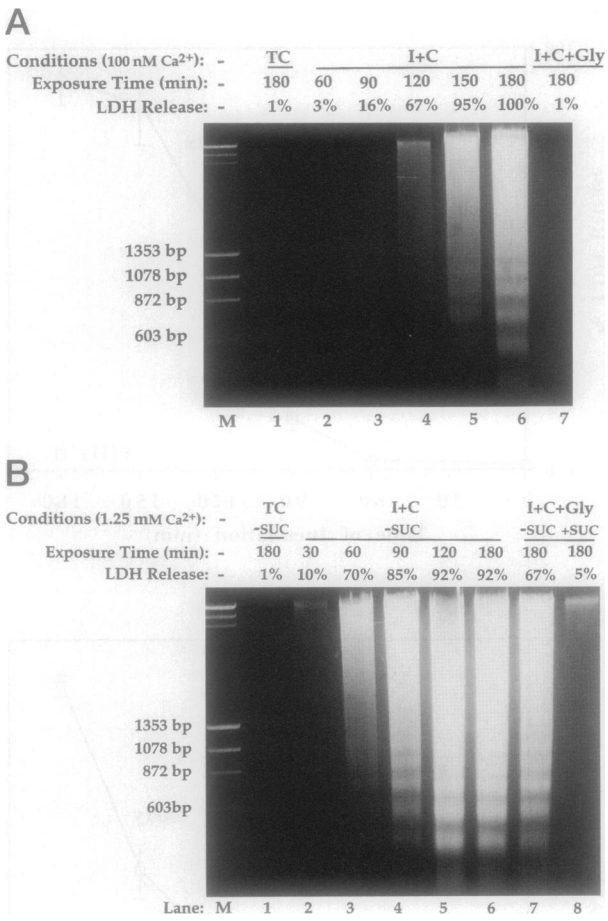

Figure 3. Internucleosomal DNA cleavage in ATP-depleted cells. **A:** MDCK cells were incubated in KRB with 100 nmol/L free Ca^{2+} without further additions (TC), with ionomycin and CCCP alone (I+C), or with ionomycin and CCCP in the presence of 5 mmol/L glycine (I+C+Gly). **B:** MDCK cells were incubated under conditions described in **A**, except that free Ca^{2+} concentration in the medium was 1.25 mmol/L. Separate groups of cells were incubated without sucrose (−SUC) or with 4% sucrose (+SUC) in the incubation medium to prevent swelling of the cells. Free LDH in the medium (shown at the top of each lane) and DNA fragments were analyzed as described. **Lane M**, DNA molecular size standards.

severity with progressive release of LDH (Figure 3B, lanes 2 to 6). Marked swelling and accelerated damage were observed also in similarly incubated cells provided with glycine (LDH release, 67%). This suggested that the plasma membrane protective actions of the amino acid had been overwhelmed by Ca^{2+}-triggered events. Such cells showed typical DNA ladders despite the presence of glycine (Figure 3B, lane 7). That the loss of plasma membrane protection was due to severe cell swelling and stretch trauma was demonstrated by the ability of sucrose, an impermeant solute, to prevent swelling and restore the ability of glycine to maintain membrane integrity (LDH release, 5%). Correspondingly, DNA breakdown in these cells was remarkably inhibited (Figure 3B, lane 8). Without glycine, sucrose was unable to ameliorate either LDH release or DNA fragmentation (not shown).

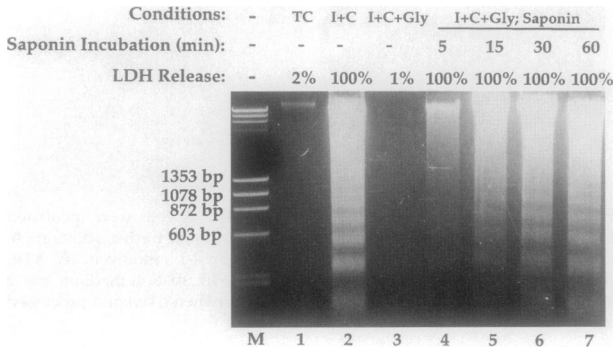

Figure 4. Internucleosomal DNA cleavage induced by saponin permeabilization of ATP-depleted cells. MDCK cells were incubated for 180 minutes in KRB containing 100 nmol/L Ca^{2+} without further addition (TC), with ionomycin and CCCP alone (I+C), or with ionomycin and CCCP in the presence of 5 mmol/L glycine (I+C+Gly). Separate groups of cells were incubated with ionomycin, CCCP, and 5 mmol/L glycine for 3 hours exactly as those shown in **lane 3** and were then permeabilized with 0.2 mg/ml saponin (I+C+G; Saponin) for indicated periods (5 to 60 minutes). LDH released into the medium (shown at the top of each lane) and DNA fragments were analyzed as described. **Lane M**, DNA molecular size standards.

Necrotic DNA Laddering Is Not Energy Dependent but Is Coupled to Plasma Membrane Damage

Fundamentally distinct from necrosis, apoptosis is an active energy-dependent process and in many cases needs new protein synthesis.[31,32] It could be argued that necrotic DNA laddering is triggered by energy-dependent events that occur early during cell injury when ATP is still available. To investigate this, we depleted MDCK cells of ATP with CCCP in 100 nmol/L Ca^{2+} medium in the presence of glycine and then permeabilized their plasma membranes with the detergent saponin. After 3 hours of treatment with CCCP, cell ATP fell to 0.0075% of control (not shown). At this time, they had been fully protected against LDH release and DNA fragmentation, unlike similarly incubated cells without glycine (1% LDH release with glycine, Figure 4, lane 3; 100% LDH release without glycine, Figure 4, lane 2). However, within 5 minutes after the addition of saponin, intracellular LDH was completely released, after which there was progressively increasing fragmentation of DNA in the ladder pattern, becoming maximal by 30 minutes (Figure 4, lanes 4 to 7).

DNA Laddering Induced by Cell Permeabilization without Previous ATP Depletion

The data presented above suggested that increased permeability of the plasma membrane played a decisive role in necrotic DNA laddering. This consideration prompted us to investigate whether rapid induction of necrosis by plasma membrane permeabilization without previous ATP depletion would also lead to the formation of DNA ladders. For this purpose, we used saponin and SLO, a bacterial toxin. Saponin preferentially binds plasma membrane cholesterol, forms micelles, and induces permeability defects. SLO causes necrosis by forming po-

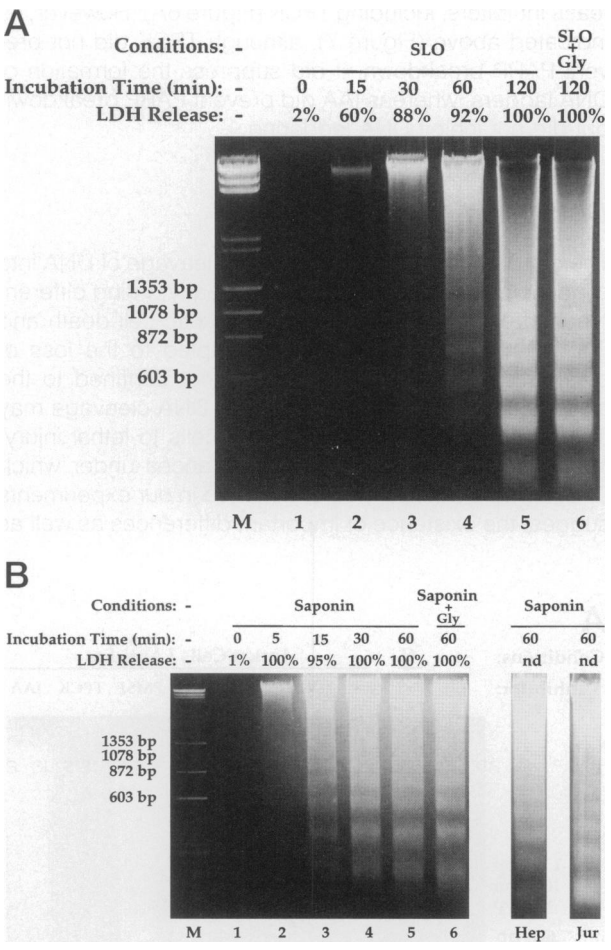

Figure 5. Internucleosomal DNA cleavage induced by cell permeabilization with streptolysin-O (**A**) or saponin (**B**). **A:** MDCK cells were exposed to streptolysin-O at 4°C, transferred to KRB containing 100 nmol/L free Ca^{2+}, and then incubated at 37°C for 0 to 120 minutes, without glycine (SLO) or with 5 mmol/L glycine (SLO+Gly). **B:** MDCK cells (**lanes 1** to **6**), hepatocytes (Hep), and Jurkat cells (Jur) were incubated in 100 nmol/L free Ca^{2+} KRB containing 0.2 mg/ml saponin for 0 to 60 minutes. MDCK cells were exposed to saponin without glycine (Saponin) or with 5 mmol/L glycine (Saponin+Gly). Hepatocytes were isolated from rat livers by collagenase perfusion (Ref. 50). Free LDH in the medium (shown on the top of each lane; nd, LDH release was not determined) and DNA fragments were analyzed as described. **Lane M**, DNA molecular size standards.

rous channels (diameter, ~20 to 30 nm) analogous to those produced by complement.[27] Treatment with saponin and SLO resulted in necrotic morphology (not shown) and complete release of intracellular LDH within a few minutes, followed by progressively increasing fragmentation of DNA in the typical ladder pattern (Figure 5, A and B). As expected, glycine could not prevent the induction of membrane permeabilization by either the pore-forming toxin SLO or the detergent saponin; neither could it inhibit the formation of DNA ladders (Figure 5, A and B, lane 6).

Our results showing the formation of DNA ladders in necrotic cells appear to be at variance with earlier reports.[7,8] The difference might lie in the diversity of cells examined. Therefore, we analyzed DNA from a variety of cells after detergent lysis. Without exception, permeabilization of the plasma membrane resulted in DNA laddering in a number of different cell types tested, including renal epithelial (MDCK), embryonic carcinoma (P19), and lymphocytic (Jurkat) cell cultures as well as freshly isolated mouse thymocytes and rat hepatocytes, with thymocytes showing relatively lower laddering potency. DNA ladders formed in saponin-lysed rat hepatocytes and Jurkat cells are displayed in Figure 5B.

Necrotic DNA Laddering Is Ca^{2+}/Mg^{2+} Dependent and Is Blocked by Endonuclease Inhibitors

Activation of Ca^{2+}/Mg^{2+}-dependent endonucleases has been proposed to be responsible for DNA fragmentation during apoptosis.[5] Similar Ca^{2+}/Mg^{2+} dependence was revealed for necrotic DNA laddering (Figure 6A). The formation of DNA ladders was partially blocked by omission of Mg^{2+} (Figure 6, lane 3) and completely inhibited by removing both Ca^{2+} and Mg^{2+} (lane 2) whereas omission of Ca^{2+} alone was without effect, suggesting that Mg^{2+} might be sufficient by itself as a cofactor for the endonucleases. Necrotic DNA laddering was markedly suppressed by compounds known to inhibit apoptotic endonuclease(s): Zn^{2+}, Evans blue, and aurintricarboxylic acid but not its analogue fuchsin acid (Figure 6B). These results suggest that endonucleases involved in internucleosomal DNA cleavage during necrosis and apoptosis are at least related, if not identical.

Involvement of Serine Proteases but Not Cysteine Proteases in Necrotic DNA Laddering

The ICE family of cysteine proteases or caspases play important roles in apoptosis and may trigger biochemical events that eventually result in endonuclease activation.[32–36] Cleavage-site-directed inhibitors of caspases, namely, Z-VAD.FMK and Z-DEVD.FMK, completely blocked Fas-antibody-triggered apoptotic morphology (not shown) as well as DNA laddering in Jurkat cells[37] (Figure 7A, lanes 3 and 4). However, neither compound inhibited necrotic DNA laddering in saponin-treated MDCK cells (Figure 7B, lanes 7 and 8), even at much higher concentrations (not shown). When Jurkat cells were exposed to Fas antibodies in the presence of iodoacetic acid (IAA), a general inhibitor of cysteine proteases, they developed necrotic, rather than apoptotic morphology (not shown). This was accompanied by DNA laddering (Figure 7A, lane 8). Likewise, IAA failed to inhibit saponin-induced necrotic DNA laddering in MDCK cells (Figure 7B, lane 6). On the other hand, active site inhibitors of serine proteases, DCI and PMSF, had no effect on apoptotic DNA laddering (Figure 7A, lanes 5 and 6) but markedly suppressed the formation of necrotic ladders (Figure 7B, lanes 3, 4, and 9). Of interest, the cleavage-site-directed chymotryptic protease inhibitor, *N*-tosyl-L-phenylalanylchloromethyl ketone (TPCK), blocked apoptotic as well as necrotic DNA laddering (Figure 7A, lane 7, amd 7B, lane 5).

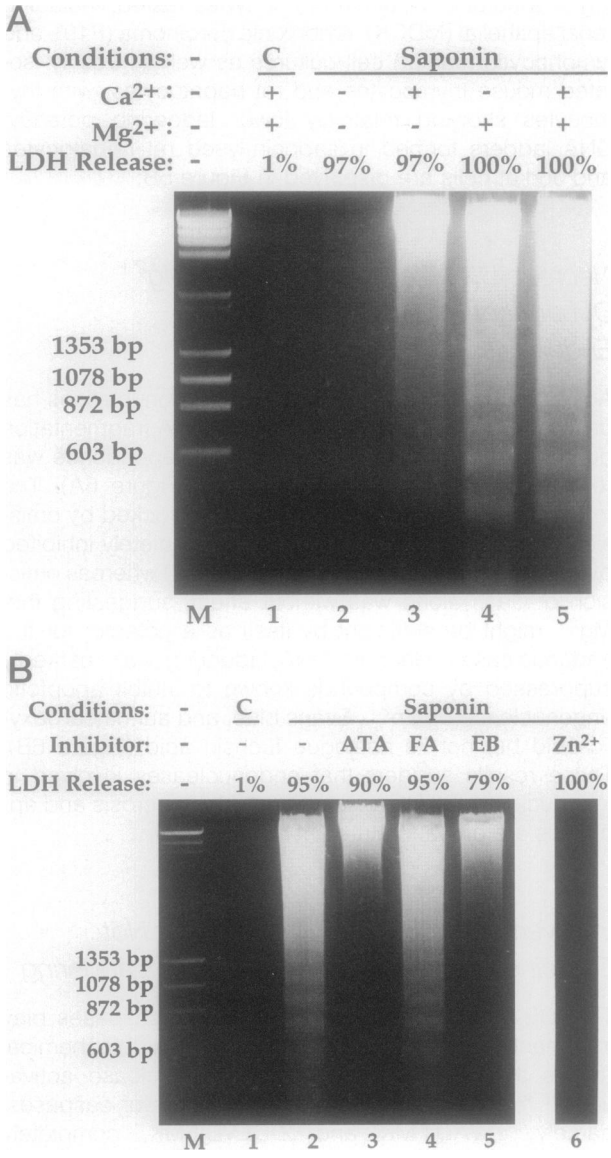

Figure 6. Internucleosomal DNA cleavage in saponin-permeabilized cells. **A:** Ca^{2+}, Mg^{2+} dependence. MDCK cells were permeabilized by 60 minutes of incubation in KRB containing 0.2 mg/ml saponin, with (+) or without (−) 1.25 mmol/L Ca^{2+} or 1 mmol/L Mg^{2+}. When Ca^{2+} or Mg^{2+} was omitted, 5 mmol/L EGTA or 1 mmol/L EDTA was included in the buffer. EDTA alone was sufficient to chelate both Ca^{2+} and Mg^{2+} and inhibit DNA laddering (not shown). **B:** Effects of endonuclease inhibitors. MDCK cells were permeabilized by 60 minutes of incubation in KRB containing 0.2 mg/ml saponin alone or saponin in the presence of 50 μmol/L aurintricarboxylic acid (ATA), 100 μmol/L fuchsin acid (FA), 10 μg/ml Evans blue (EB), or 50 μmol/L $ZnCl_2$ (Zn^{2+}). Free LDH in the medium (shown at the top of each lane) and DNA fragments were analyzed as described. C, control without exposure to saponin; **lane M**, DNA molecular size standards.

To further clarify the role of proteases in DNA laddering, we examined the degradation of PARP, an endogenous substrate of caspases.[28] In apoptotic Jurkat cells, PARP was specifically cleaved, releasing fragments of M_r ~85,000 (Figure 8A, lane 2). However, PARP remained intact during necrosis due to ATP depletion or saponin permeabilization in MDCK cells (Figure 8B). Apoptotic PARP degradation was completely abolished by cysteine protease inhibitors, including IAA, but not by serine protease inhibitors, including TPCK (Figure 8A). However, as indicated above (Figure 7), although TPCK did not prevent PARP breakdown, it did suppress the formation of DNA ladders, whereas IAA did prevent PARP breakdown but did not inhibit DNA laddering.

Discussion

The results of our studies show that cleavage of DNA into oligonucleosomal fragments occurs early during different types of experimentally induced necrotic cell death and that necrotic DNA laddering is coupled to the loss of plasma membrane integrity. Being not confined to the apoptotic process, internucleosomal DNA cleavage may thus be a generalized response of cells to lethal injury. Moreover, the biochemical circumstances under which DNA ladders were formed or inhibited in our experiments suggest the existence of important differences as well as

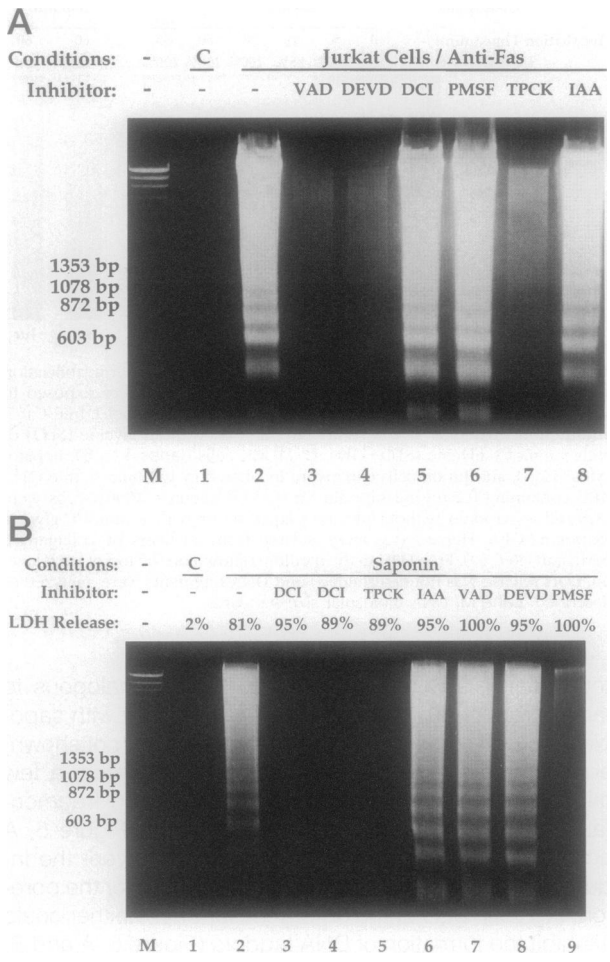

Figure 7. Effect of protease inhibitors on internucleosomal DNA cleavage during apoptosis (**A**) and necrosis (**B**). After 20 minutes of preincubation without or with 10 μmol/L Z-VAD-FMK, 14 μmol/L Z-DEVD-FMK, 100 μmol/L DCI, 1 mmol/L PMSF, 100 μmol/L TPCK, or 1 mmol/L IAA, 100 ng/ml Fas antibody was added to Jurkat cells for 5 hours to induce apoptosis (**A**), and 0.2 mg/ml saponin was added to MDCK cells for 1 hour to induce necrosis (**B**). Free LDH in the medium (shown at the top of each lane) and DNA fragments were analyzed as described. C, control without exposure to Fas antibodies or saponin; **lane M**, DNA molecular size standards.

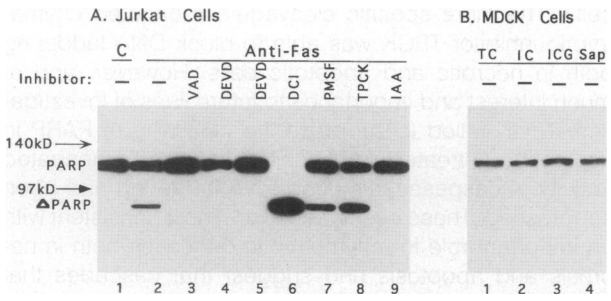

Figure 8. Cleavage of PARP in apoptotic (**A**) and necrotic (**B**) cells. **A:** Preincubation with protease inhibitors and induction of apoptosis in Jurkat cells were accomplished as described in Figure 7. **B:** MDCK cells were incubated for 1 hour in KRB containing 0.2 mg/ml saponin (Sap) or for 3 hours in KRB without further addition (TC), with ionomycin and CCCP alone (IC), or with ionomycin and CCCP in the presence of 5 mmol/L glycine (ICG). Cells were dissolved for SDS-polyacrylamide gel electrophoresis in 9 mol/L urea/1% SDS/5% β-mercaptoethanol. Proteolytic cleavage of PARP to a ~n 85-kd fragment was detected by immunoblotting with antibody C-2–10.

overlaps between proteolytic events required for the activation of the responsible endonuclease(s) during necrosis and a well characterized form of apoptosis. Unlike in apoptosis, caspases do not appear to play a role during necrosis; on the other hand, a serine protease sensitive to TPCK seems to be involved in both.

Our findings may be relevant to the emerging controversy regarding the significance of internucleosomal DNA cleavage and staining by cytochemical techniques such as the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay to detect strand breaks of DNA during cell death.[38–43] These uncertainties are particularly applicable to the analysis of cell death *in vivo*, because injury events are likely to be asynchronous, and tissues are often analyzed long after the death events have occurred. Thus, depending on the disease process, homogeneity of the cell populations involved, synchrony of injury, and time of sampling, the tissues analyzed may present appearances that reflect early events, terminal autolytic changes, or a confusing superimposition of late autolysis on early phenomena.

Our conclusions regarding necrotic DNA laddering depend critically on the inference that cells subjected to various noxious agents did not go through an apoptotic phase of injury before progressing on to necrosis. Several considerations indicate that this assumption is valid. Apoptosis is characterized by a morphology that is readily distinguished from that of necrosis: shrunken and blebbed cell bodies with condensed and fragmented nuclei,[1,2] as opposed to the swollen, lysed cells depicted in Figure 1.

Apoptotic features were observed routinely in Jurkat cells exposed to Fas antibodies. Cells exposed to mitochondrial uncoupler and Ca^{2+} ionophore, or agents that permeabilized the plasma membrane, typically displayed the classical features of necrosis, and apoptosis could be excluded by phase contrast and electron microscopy and staining with the DNA-reactive dye Hoechst 33342. Moreover, microscopic examination showed that cells exposed to mitochondrial uncoupler and Ca^{2+} ionophore did not develop apoptotic features at any time during entire period of treatment, including the early stages. On the other hand, with the onset of plasma membrane breakdown, shown by leakage of LDH, the cells rapidly developed necrotic morphology, after which DNA ladders were seen. In addition, the rapidity of membrane permeabilization in cells exposed to SLO and detergents precluded the occurrence of apoptosis. Cells permeabilized in this manner developed the morphological features of necrosis rapidly, but DNA laddering took 15 to 30 minutes to be fully manifest.

To further verify that plasma membrane damage but not an apoptotic process was required for necrotic DNA laddering, separate groups of cells were maximally depleted of ATP for prolonged periods under conditions that preserved membrane integrity, ie, in the presence of glycine. These cells should have been incapable of apoptosis, which is an active, energy-dependent process.[31,32] As expected, cells in this group did not exhibit apoptotic morphology or DNA cleavage as long as plasma membranes were intact but became necrotic within minutes of treatment with the permeabilizing agent saponin. Only after membrane permeabilization was complete were DNA ladders evident. These results and other observations indicated that the inhibitory actions of glycine on DNA ladder formation were only indirect and depended strictly on the known membrane protective effects of the amino acid.[17–23,25,26] Previous studies have shown that development of membrane defects during ATP depletion is related to loss of cell-associated glycine, an amino acid that is accumulated by energy-dependent transport. Restoration of glycine by adding the amino acid exogenously prevents membrane damage, although ATP remains maximally depleted.[44] That prevention by glycine of DNA laddering was strictly dependent on its membrane-protective actions was also shown by its inability to inhibit DNA fragmentation in SLO- or detergent-treated cells, in which plasma membranes were permeabilized regardless of the presence of the amino acid. The decisive role played by plasma membrane damage in necrotic DNA laddering was further established by results displayed in Figure 3B. When intracellular Ca^{2+} was allowed to increase uncontrollably during ATP depletion by incubation with ionophore in 1.25 mmol/L Ca^{2+} medium, extreme swelling of cells occurred, mechanically damaging the plasma membranes despite the presence of glycine; these cells showed typical DNA ladders. On the other hand, if cell swelling had been attenuated by the cell-impermeant solute sucrose, plasma membrane integrity was preserved, as long as glycine was also present. Concomitantly, DNA laddering was also prevented.

Our data appear to be at variance with older studies that failed to reveal DNA fragmentation in necrotic cells.[6–8] We could not attribute the apparent conflict to uniquely different responses by dissimilar cells, as our observations on a variety of cell types suggest that necrotic DNA laddering could be a general phenomenon. Possible reasons for the discrepancy might include differences in the conditions of incubation and composition of media. Furthermore, physical methods that have been used to disrupt cells such as heating and freeze-thaw cycles[7,8] have the potential to inactivate critical enzymes

required for the formation of DNA ladders. Another possible explanation is the masking of DNA ladders by less specific forms of DNA breakdown. DNA smearing is commonly observed in gels after electrophoresis of extracts from necrotic cells and tissues, and is in all likelihood attributable to late postmortem autolytic processes, which are superimposed on the more specific early necrotic events. On the other hand, scattered observations in the literature do indicate that necrotic cells develop DNA ladders,[13–15] but only in one instance have the changes been documented under circumstances where apoptosis has been rigorously excluded and necrosis authenticated by electron microscopy.[13] As indicated earlier, failure to observe DNA ladders in necrotic tissues may be related to the time of sampling and the presence of a majority of cells with intact plasma membranes, which would tend to dilute a positive signal from lysed cells.

The observations reported here may be relevant to internucleosomal DNA cleavage during apoptosis as well. It is generally believed that DNA fragmentation in apoptosis occurs before loss of plasma membrane integrity, but the asynchrony of apoptosis in most model systems has made it difficult to precisely determine the temporal relationship between increased plasma membrane permeability and internucleosomal DNA cleavage. Moreover, plasma membrane damage has been evaluated only approximately in a number of studies of apoptosis, such as by trypan blue staining.[45,46] Using more sensitive and accurate techniques, Ormerod et al[47] and Lizard et al[48] showed recently that plasma membrane permeability becomes increased during the early stages of apoptosis, preceding the condensation and fragmentation of cell nuclei.

Our results suggest that internucleosomal DNA cleavage during necrosis is accomplished by Ca^{2+}/Mg^{2+}-dependent endonuclease(s) with properties similar to those involved in apoptosis. Thus, necrotic DNA laddering was inhibited by Evans blue, Zn^{2+}, and aurintricarboxylic acid but not by its analogue fuchsin acid, even at high concentrations. Of great interest, the mechanism leading to the activation of endonucleases in necrotic cells appears to be significantly different from that seen in apoptosis. In agreement with published data,[37] inhibitors of caspases suppressed not only the cleavage of PARP into 85-kd fragments but also DNA laddering in Fas-antibody-treated Jurkat cells. Concomitantly, morphological changes of apoptosis were prevented. However, DNA laddering could be dissociated from caspase activation under some conditions in our studies. The nonspecific cysteine protease inhibitor IAA inhibited the cleavage of PARP in Fas-antibody-treated Jurkat cells, as might be expected, but in accordance with its known actions as a sulfhydryl-alkylating agent caused plasma membrane damage and necrosis defined by morphological criteria, and these changes were accompanied by DNA laddering. Importantly, PARP was not cleaved during necrosis, and caspase inhibitors were unable to prevent necrotic DNA laddering. On the other hand, the general serine protease inhibitors PMSF and DCI suppressed DNA laddering in necrotic but not apoptotic cells. The more specific cleavage-site-directed chymotryptic inhibitor TPCK was able to block DNA laddering both in necrotic and apoptotic cells. However, and of much interest and importance to future lines of investigation, TPCK failed to suppress the cleavage of PARP in Fas-antibody-treated Jurkat cells, showing the unabated activity of caspases, although DNA laddering had been suppressed. These observations are most consistent with an important role for chymotryptic proteases both in necrosis and apoptosis and suggest that cascades that lead to endonuclease activation in necrosis and apoptosis might have overlapping features at least in this respect, with important differences in upstream events. Proteolytic triggers for endonuclease activation in apoptosis are continuing to receive critical attention, and enzymes of the cysteine protease and serine protease families have been characterized, including a 24-kd TPCK-sensitive enzyme.[49] However, corresponding enzymatic events in necrosis remain to be studied. Our data provide clear-cut evidence for the tight coupling between plasma membrane damage and endonuclease activation during necrosis and point to the requirement for future characterization of the TPCK-sensitive protease(s) involved in necrotic as well as apoptotic DNA laddering.

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