The Acid Deoxyribonuclease of Neutrophils: A Possible Participant in Apoptosis-Associated Genome Destruction

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Human neutrophils are terminally differentiated cells that spontaneously undergo apoptosis in tissue culture. Apoptosis in these cells can be delayed by culture in the presence of granulocyte colony-stimulating factor or other inflammatory mediators. Neutrophils were found to contain an acid endonuclease that appeared to be responsible for the internucleosomal DNA cleavage that accompanies apoptosis. As measured by a plasmid nicking assay, this endonuclease had a molecular weight (M,.) of 35,000, a pH optimum of 5.5, and a threshold for activity of pH 6.6 to 6.8. It was weakly inhibited by divalent cations (Ca++, Mg++, and Zn++) and more strongly inhibited by aurintricarboxylic acid (an acid endonuclease that appeared to be responsible for the plasmid cleavage assays, consistent with internucleosomal DNA cleavage by the acid endonuclease. We have previously shown that neutrophils undergo acidification to a pH value as low as 6.0 during apoptosis; we suggest that this endonuclease may be responsible for the DNA cleavage seen in apoptotic neutrophils.

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MATERIALS AND METHODS

Isolation of neutrophils. After obtaining informed consent from the volunteer donors, blood was drawn from the donors. Neutrophils were isolated by dextran sedimentation, hypotonic lysis, and Ficoll centrifugation, as described previously.7

Preparation of nuclear extracts. Purified neutrophils were incubated with 2.7 mmol/L dithiopyridinothionophosphate (DFP) on ice for 20 minutes and then disrupted by hypotonic lysis in 1.5 mmol/L MgCl2/10 mmol/L Tris (pH 7.4). The nuclear pellet obtained after sedimentation at 2,000g for 2 minutes at 20°C was washed once with TMS (10 mmol/L Tris [pH 7.4]/1.5 mmol/L MgCl2/0.24 mol/L sucrose). To remove the remaining persistently adherent granules, nuclei were incubated on ice for 15 minutes in TMS containing 0.1% Triton X-100, were sedimented at 2,000g for 1 minute, and were washed three times at 20°C with TMS containing no detergent. The resulting nuclear pellet was resuspended in nuclear extract buffer (20 mmol/L Tris [pH 7.5]/0.4 mol/L NaCl/1.5 mmol/L MgCl2/0.1 mmol/L DTT/25% [vol/vol] glycerol) and incubated for 30 minutes at 4°C with gentle shaking. After sedimentation at 17,000g, the supernatant (nuclear extract) was stored at −70°C until use.

Plasmid DNA digestion assay. Substrate (supercoiled pCAT plasmid) was prepared by CsCl purification. One microliter of substrate (supercoiled plasmid DNA, in APT buffer (10 mmol/L sodium acetate/10 mmol/L potassium phosphate/10 mmol/L Tris) at pH 5.5 or as indicated) was added to the reaction volume was 50 µL. Each condition was tested in triplicate. The total reaction volume was 50 µL. Proteins in some extracts caused aggregation of plasmid DNA; therefore, at the end of the 1 hour of incubation, sodium dodecyl sulfate (SDS; 1%) and EDTA (10 mmol/L) were added. Samples were then assayed for nicking by measuring loss of supercoiling, using electrophoresis on a 1% agarose gel to separate supercoiled from nicked and linearized DNA and ethidium bromide staining to visualize the plasmids.

Fast protein liquid chromatography (FPLC) gel filtration. Nuclear or whole cell extracts were subjected to FPLC gel filtration on a Pharmacia (Uppsala, Sweden) Superdex 75 column (10 x 300 mm) in APT-saline buffer (10 mmol/L sodium acetate/10 mmol/L potassium phosphate/10 mmol/L Tris [pH 7.5]/100 mmol/L NaCl). Chromatography was performed at 0.4 mL/min, collecting 0.5 mL fractions. Fractions were assayed for endonuclease activity and the molecular weight inferred by comparison to elution profiles of molecular weight standards (Sigma, St Louis, MO).

DNA isolation from neutrophils. Neutrophils were cultured at 10° cells/mL in phosphate-buffered saline (PBS) with 0.1% glucose and 0.25% autologous plasma for the times designated. Harvested cells were washed twice in PBS and resuspended in lysis buffer (10 mmol/L Tris [pH 7.5]/10 mmol/L NaCl/20 mmol/L EDTA/1% SDS),
After adding proteinase K (0.5 mg/mL), the samples were incubated at 55°C for intervals ranging from 3 hours to overnight. DNA was extracted once with phenol:chloroform:isoamyl alcohol, (24:24:1) and once with chloroform:isoamyl alcohol, (24:1) and was then precipitated with 1/20 vol of 3 M sodium acetate and 2 vol of 100% ethanol at -20°C for 1 hour. The precipitated DNA was sedimented for 15 minutes at 17,000g and then resuspended in TE buffer (10 mM Tris and 1 mM EDTA). Five-microgram portions of the DNA from each sample were resolved by electrophoresis on a 2% agarose gel containing 0.5 μg/mL ethidium bromide.

In situ nick end labeling (ISEL). Cells were labeled using a modification of the method of Wijisman et al. Briefly, neutrophils were fixed for 5 minutes with 4% formaldehyde in PBS, air-dried on glass slides, and then rinsed and incubated with a reaction mix containing 0.05 mM/L biotin-14-dATP, 5 μM/L dTTP, 5 μM/L dGTP, and 5 μM/L dCTP in ISEL buffer (50 mM/L Tris [pH 7.5]/1 mM/L EDTA). Five-microgram portions of the DNA from each sample were resolved by electrophoresis on a 2% agarose gel containing 0.5 μg/mL ethidium bromide.

RESULTS

Properties of the neutrophil endonuclease. Nuclear extracts prepared from freshly purified neutrophils were assayed for their ability to cleave plasmid DNA under a variety of conditions (Fig 1). At neutral pH, neutrophils lacked endonuclease activity both in the presence and the absence of divalent cations. Under the conditions of the cell-free assay, activity began to appear as weak single-strand nicking at pH 6.4, becoming progressively more evident as the pH in the incubation mixtures was decreased (Fig 1, panels 1 and 2). This acidic endonuclease activity was independent of divalent cations, because it was seen in the absence of added divalent cations and in the presence of 10 mM/L EDTA (Fig 1, third panel). (The extent of digestion varied in the three experiments shown in Fig 1 because different preparations of endonuclease were used in each of the experiments.) With longer incubation times, higher enzyme concentrations, or lower pH, supercoiled plasmid DNA was digested into progressively smaller fragments. It is not clear whether this is due to multiple single-strand nicks or to double-strand breaks as well. Spleen acidic DNase is known to exhibit both types of activity.

This cation-independent acidic endonuclease activity was found to be very widely distributed. Cells in which this activity was sought included cardiomycocytes, HeLa cells, HL-60 cells, Jurkat cells, and C127 cells, a mammary epithelial line. Figure 2 shows that the acidic DNase was present in cardiomycocytes, C127 cells, and Jurkats, but that, unlike neutrophils, these cells also contained one or more cation-dependent DNases that were active at neutral pH. Acid endonuclease activity was also found in HL-60 cells and HeLa cells and showed a similar molecular weight (M,) and pH dependency (data not shown). In no cell type examined was the acidic endonuclease absent.

To determine if this endonuclease was similar to DNase II, it was tested for sensitivity to a panel of agents known to inhibit DNase II (Fig 3). Like DNase II, the endonuclease activity was inhibited by aurintricarboxylic acid (100 μg/mL) and N-bromosuccinimide (1 mM/L) but not by iodoacetamide (10 mM/L). Not surprisingly, the neutrophil enzyme was inactivated by boiling and by treatment with proteinase K (data not shown). However, it was moderately resistant to limited trypsinolysis.

To estimate the molecular weight of the DNase, nuclear extracts were subjected to FPLC gel filtration on a Superdex-75 column and column fractions were assayed as described above (Fig 4). Peak activity was detected in fractions corresponding to an estimated M, of 35,000. Furthermore, the M, was the same whether the chromatography was conducted at
The amount of endonuclease activity in different cell extracts was not affected by cycloheximide (Fig 6). This suggests that the activity observed at pH 5.5 was not due to the acid-induced conversion of a proenzyme to a catalytically active species but was an inherent property of the enzyme itself.

In neutrophils, the apoptosis program appears to be independent of both gene expression and protein synthesis, because it is activated by the protein synthesis inhibitor cycloheximide, as indicated by the loss of very high molecular weight material and the prominence of the nucleosomal ladder in DNA from cycloheximide-treated neutrophils as compared with DNA from neutrophils cultured for 24 hours without cycloheximide (Fig 5). Consistent with a role for the acidic endonuclease in apoptosis, the enzyme was present in freshly isolated neutrophils as well as in aged neutrophils, and its activity in these cells was not affected by cycloheximide (Fig 6).

Digestion of chromatin in whole cells depends on pH. To determine if the digestion of nuclear chromatin depended on pH, DNA was extracted and analyzed after overnight incubation of purified neutrophils at 37°C with the proton ionophore nigericin (I mM) in a high-potassium buffer (120 mmol/L KCl, 30 mmol/L potassium phosphate, 5 mmol/L glucose) adjusted to defined pH (Fig 7). No DNA fragmentation could be detected in neutrophils incubated at pH 7.4, even though extensive laddering would have been expected in neutrophils incubated overnight in the absence of nigericin. This finding suggests that clamping the intracellular pH at 7.4 prevented endonuclease activation. Progressively more internucleosomal cleavage was noted as the pH was decreased from 7.0 to 6.2. The observation that DNA cleavage was seen at pH 7.0 in whole cells but not in the cell-free assay could be explained by the greater duration of digestion in the whole cell experiment and by the finding that the intracellular pH in nigericin-treated cells analyzed by flow cytometry in pH 7.0 buffer was 7.0 ± 0.2 SD, indicating that 2% of the cells showed pH values less than 6.6, which is sufficient to permit some endonuclease activity. However, in cells analyzed in pH 7.4 buffer, an intracellular pH of 6.6 or lower occurred in less than 0.01% of the cells (mean 7.40 ± 0.15 SD), consistent with the absence of detectable DNA cleavage in these cells.

In a second series of experiments, neutrophils from two donors were incubated in the presence of nigericin at pH 6.2, 6.6, 7.0, or 7.4 for 30 and 60 minutes at 37°C. Portions of each of the neutrophil suspensions were then fixed, mounted on glass slides, and processed for in situ nick end labeling. Cells incubated at pH 5.8 and 6.2 were positive for nicked DNA, whereas those incubated at pH 6.6 and 7.0 were negative at both the 30- and 60-minute time points (data not shown). This finding showed again that DNA cleavage in neutrophils depended on a decrease in pH and that the pH threshold for DNA cleavage in whole neutrophils was similar to the threshold for DNA cleavage seen in the digestion of plasmids by nuclear extracts of neutrophils.

**DISCUSSION**

The present study and earlier work by Lamers et al indicate that the only DNase detectable in neutrophils is a cation-independent enzyme that is active only at pH values less than 6.6.* Its requirement for acid, its cation independence, its

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* The trace amount of calcium- and magnesium-dependent endonuclease activity that was detected in some neutrophil preparations probably represents contaminating activity from lymphocytes and monocytes.
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The very broad distribution of DNase II-like activity has been shown not only in the present study but in earlier work as well. DNase II activity has been isolated from several tissues of diverse ontogeny (liver, spleen, and gastric mucosa) as well as from a number of cell types and cell lines (HeLa, iHL-60, cardiomyocytes, and CHO cells). The breadth of its distribution suggests that DNase II is likely to play a general and very important role in biologic systems. We believe that this role involves the destruction of the genome in cells undergoing apoptosis.

Objections can be raised to this idea. The endonuclease generally regarded as responsible for DNA hydrolysis in apoptosis is a Ca²⁺- and Mg²⁺-dependent enzyme with a pH optimum in the vicinity of neutrality. However, a DNase with these properties could not be detected in neutrophils. Furthermore, in neutrophils, calcium influxes actually delay apoptosis, making a Ca²⁺ and Mg²⁺-dependent endonuclease an unlikely participant in apoptosis a priori, at least in these cells. Skepticism has also been expressed regarding the participation of DNase II in apoptosis because of the enzyme’s requirement for a pH well less than 7.0 for activity. However, Barry et al. showed that HL-60 cells undergoing apoptosis acidified their cytoplasm to a level compatible with the activity of enzymes of the DNase II class, and we obtained similar results with neutrophils. Finally, the unusual nature of the cleavage reaction catalyzed by the DNase II enzymes, a reaction that, as discussed above, produces DNA fragments phosphorylated on the 3’-end and not on the 5’-end, could make the repair of nicks introduced into the genome by these enzymes a difficult matter, thereby facilitating repair.

The FPLC gel filtration profile of the endonuclease. Column fractions were incubated with supercoiled plasmid DNA for 1 hour at 37°C at pH 5.5. Samples were resolved on a 1% agarose gel. Numbers denote fraction number. Arrows signify elution of molecular weight standards. Endonuclease activity is reflected by the loss of supercoiled plasmid DNA.

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Fig 4. FPLC gel filtration profile of the endonuclease. Column fractions were incubated with supercoiled plasmid DNA for 1 hour at 37°C at pH 5.5. Samples were resolved on a 1% agarose gel. Numbers denote fraction number. Arrows signify elution of molecular weight standards. Endonuclease activity is reflected by the loss of supercoiled plasmid DNA.

Fig 5. Effect of cycloheximide on DNA cleavage in neutrophils. DNA was extracted from neutrophils cultured for 24 hours without (lane 1) or with (lane 2) cycloheximide (100 µg/mL). DNA was extracted and resolved on 1.8% agarose gel.

Fig 6. Acid endonuclease activity from aged and from cycloheximide-treated neutrophils. Nuclear extracts were prepared from 24-hour cultures of control cells (lanes 1 through 4, left) and cells treated with cycloheximide (100 µg/mL; lanes 1 through 4, right). Ten-fold serial dilutions of these extracts were used in a plasmid digestion assay.
the genomic demolition that is an almost universal feature of cells undergoing apoptosis.

Brown et al\textsuperscript{15} have suggested that two endonucleases participate in apoptosis, one generating infrequent nicks or double-strand breaks and the second producing the nucleosomal fragments from the incised products of the first. Because neutrophils possess only the acidic endonuclease, both early nicking and later internucleosomal cleavage must be attributed to the activity of this enzyme. DNase II has been shown to possess both activities.\textsuperscript{9}

It could be postulated that the acidic endonuclease of neutrophils had nothing to do with apoptosis and that the neutrophil DNA was degraded by a different endonuclease that had been synthesized as part of the apoptosis program. However, nuclear extracts prepared from apoptotic neutrophils still contained only the acid endonuclease. The findings that DNA fragmentation could be prevented by clamping the intracellular pH greater than 7.0 and the recent reports that acidification accompanies apoptosis in neutrophils\textsuperscript{14} and other cells\textsuperscript{16} suggest that the acid endonuclease (DNase II) is responsible for the DNA fragmentation of apoptosis. The protein synthesis inhibitor cycloheximide\textsuperscript{17} precipitated apoptosis without causing a decrease in endonuclease activity. Both these observations argue against the de novo synthesis of another endonuclease (e.g., a Ca\textsuperscript{2+} - and Mg\textsuperscript{2+}-dependent DNase) during neutrophil apoptosis.

\textbf{REFERENCES}


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