pH-dependent DNA cleavage in permeabilized human fibroblasts

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In several cell types, apoptosis is associated with intracellular acidification and activation of a pH-dependent endonuclease. We have examined the effect of acidic pH on the DNA of permeabilized human fibroblasts, and observed cleavage of DNA into high-molecular-mass fragments. This pH-dependent DNA breakage was modulated by temperature, the presence of histones and diethyl pyrocarbonate. Superoxide dismutase and chelators with high affinity for Cu prevented DNA fragmentation, whereas catalase, DMSO and Desferal (desferrioxamine mesylate) offered no protection. Fragmentation of DNA into high-molecular-mass fragments, which is occasionally observed as an early phase of apoptosis, is thought to result from the activation of endonuclease(s). Our results suggest that such fragmentation also occurs through induction of copper-mediated site-specific DNA damage that is enhanced by intracellular acidification.

INTRODUCTION

Apoptosis and necrosis are two distinct forms of cell death. During necrosis, a pathological form of cell death, the acutely traumatized cells swell and lyse, and the released cytoplasmic contents often trigger an inflammatory response. In contrast, apoptosis generally implies a genetically controlled cellular response to a signal or to stress. The morphological criteria of apoptosis, as described by Kerr et al. [1], include chromatin condensation at the nuclear membrane, cell shrinkage and convulsions of the cell surface. The cell ultimately disassembles into membrane-enclosed apoptotic bodies, which are rapidly phagocytosed by neighbouring cells and macrophages. Hence, in contrast to necrosis, inflammation is avoided.

During the past 5 years it has become evident that apoptosis, a physiological form of cell death, is essential for normal development and for the control of tissue homoeostasis [2]. Disturbances in the regulation of apoptosis may be involved in the pathogenesis of a variety of human diseases [3]. Apoptosis can be activated by a broad range of internal and external stimuli, which include changes in cytokine or growth factor levels [4], hormonal signals [5], as well as cell injury causd by many cytotoxic agents [6]. The distinct signalling pathways, which seem to converge into an evolutionarily well conserved phase of final cell collapse, have been extensively studied. Relatively less is known about the effector molecules involved in the actual execution of cell disassembly [7]. For instance, no consensus has been reached as yet about the nature of the endonuclease responsible for DNA cleavage, although several candidates have been proposed (for a review, see [8]).

The presumed role of reactive oxygen species (ROS) in the induction as well as the execution of apoptosis is another extensively discussed topic [9–11]. Small amounts of ROS, including hydroxyl radicals, superoxide anions and hydrogen peroxide, are constantly generated in aerobic organisms in response to both external and internal stimuli [12]. Low concentrations of ROS may be beneficial or even indispensable in processes such as intracellular messaging and defence against micro-organisms. In contrast, high doses and/or inadequate removal of ROS result in oxidative stress, which in turn may cause severe metabolic dysfunctions and damage to biological macromolecules [13]. It has been shown that both types of cell death, i.e. apoptosis as well as necrosis, may be induced by ROS, depending on the level of exposure [14].

An interesting aspect of apoptosis is its association with intracellular acidification in several cell lines [15–17]. In all cases, fragmentation of DNA into oligonucleosomes by a pH-dependent endonuclease, identified as DNAse II [18], was observed. The observed pH drop was about 0.9 unit compared with non-apoptotic cells, and was suggested to be caused by impaired function of the Na^+/H^+ antiporter of mitochondria [16]. Acidic-pH-induced strand breakage (pH-dependent DNA breakage; PDDB) in permeabilized human fibroblasts, with a maximum effect at pH 6.25, has been reported [19], and activation of unknown endonuclease(s) was suggested to be the factor responsible. The experiments presented herein were aimed at investigating the mechanisms behind PDDB. The model system of permeabilized human fibroblasts was chosen because of the low background of DNA breakage and the structural similarity to intact chromatin. Here we report that an acidic milieu by itself causes considerable DNA breakage, apparently due to enhanced production of ROS mediated by transition-metal ions.

MATERIALS AND METHODS

Reagents

All chemicals and enzymes were obtained from Sigma Chemical Co., except for diethyl pyrocarbonate (Aldrich Chemie), 1,10-phenanthroline (Fluka Chemie AG), Proteinase K (Boehringer Mannheim) and molecular biology grade phenol (IBI). One unit of catalase decomposes 1 μmol of H_2O_2/min at pH 7.0 at 25 °C under the reaction conditions given in the Sigma catalogue. Units of superoxide dismutase (SOD) were as defined by the

Abbreviations used: DAPI, 4,6-diaminido-2-phenylindole; Desferal, desferrioxamine mesylate; PDDB, pH-dependent DNA breakage; ROS, reactive oxygen species; SOD, superoxide dismutase.

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cytochrome *c* assay [20]. Ultrapure water (18 MΩ) was used in all buffers.

Maintenance and pH treatment of VH-10 human fibroblasts

Human diploid VH-10 fibroblasts were seeded into 24-well Nunclon® Multidishes (Nunc) and grown in Dulbecco's modified Eagle's medium with 10 % foetal calf serum (ICN Flow) at 5 % CO_2 and 37 °C in a humidified incubator. The medium contained 3.4×10^4 Bq/ml [*methyl*-^{14}C]thymidine (Du Pont NEN) for *in vivo* labelling of the cellular DNA. Confluent cultures (10–15 passages; 5–7 days after seeding) were utilized for experiments, except where otherwise stated.

The cells were washed with ice-cold PBS (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and permeabilized for 10 min on ice with a solution of 0.5 % Triton X-100 in 0.004 M Tris/HCl, pH 7.4. The resulting nuclear monolayers [21] consist of compact chromatin within residual cellular structures attached to the substratum. To prepare nucleoid monolayers [22], which contain supercoiled DNA without histones, 2 M NaCl was included in the permeabilization solution.

The chromatin substrates were gently washed with 2×1 ml of ice-cold PBS and covered with 0.5 ml of 100 mM citrate/phosphate buffer of the pH to be tested. The plates were kept at 4 °C or 37 °C for either 10 min or 2 h, depending on the experiment. When used, enzymes or other reagents were added in citrate/phosphate buffer. After treatment, the substrates were washed with 2×1 ml of ice-cold PBS. DNA strand breaks were determined by alkali unwinding and hydroxyapatite chromatography, as described previously [23], except that 0.03 M NaOH was used for unwinding. The *k* factor [23] was found to be −40 for these cells under these unwinding conditions.

In some experiments, pH-challenged nucleoid monolayers were subjected to a nick translation assay, which was carried out essentially as described previously [24]. Briefly, the nucleoid monolayers were, immediately after pH challenge, covered by nick translation assay reaction mixture {66 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 μg/ml BSA, 20 μmol of each of dATP, dGTP and dTTP, 1.66 pmol (3.7×10^4 Bq) of [α-^{35}S]dCTP and 12 units of DNA polymerase I from *Escherichia coli*} and nick-translated for 15 min at 15 °C. After the assay, the samples were transferred on to GF/C filters and rinsed extensively, and the amount of radioactivity retained on the filters was measured by liquid scintillation counting. The number of corresponding strand breaks was calculated as described [24].

Induction of apoptosis in VH-10 fibroblasts by etoposide treatment

VH-10 fibroblasts released from confluence by trypsinization were seeded at low density into 75 cm^2 flasks (Nunc) for detection of oligonucleosomal DNA fragmentation, and into microslide culture chambers (Bellco Glass Inc.) for detection of morphological changes by 4,6-diaminido-2-phenylindole (DAPI) staining. At 5 h after seeding, etoposide (VP-16) was added to the culture medium at a final concentration of 10 or 20 μM and the cells were incubated for an additional 4 days. Formaldehyde-fixed samples of microslide-attached fibroblasts were prepared every 24 h during the incubation period. The samples were stained with DAPI (0.5 μg/ml) for 10 min and the cells were examined for morphological changes characteristic of apoptosis, such as chromatin condensation and cell fragmentation.

Agarose-gel electrophoresis

After 4 days of treatment with various concentrations of etoposide, DNA from approx. 4×10^6 cells was isolated as described [25]. The samples were subjected to electrophoresis in 1.8 % agarose at a constant voltage of 1 V/cm for 4 h. DNA ladder formation, considered to be a biochemical hallmark of apoptosis, was revealed by ethidium bromide staining and UV illumination.

RESULTS AND DISCUSSION

Complex pattern of PDDB

Nuclear monolayers of human VH-10 fibroblasts were challenged with citrate/phosphate buffer at various pH values. Incubation for 10 min at 4 °C resulted in a significant increase in the number of DNA strand breaks in the pH range 4.5–6.5 when compared with control treatment at pH 7 (Figure 1A). PDDB reached a maximum at pH 6.2, with about 20000 strand breaks per genome. Increasing the incubation temperature to 37 °C did not result in further increases in DNA fragmentation (Figure 1A), probably due to the presence of histones. Indeed, the protective effect of histones was confirmed by a subsequent experiment. When histones were stripped off from the DNA, there was a marked increase in DNA breakage induced by pH challenge of nucleoid monolayers at 37 °C compared with that at 4 °C (Figure 1B).

To examine the nature of pH-induced DNA breaks we used the nick translation assay, which is based on the incorporation of

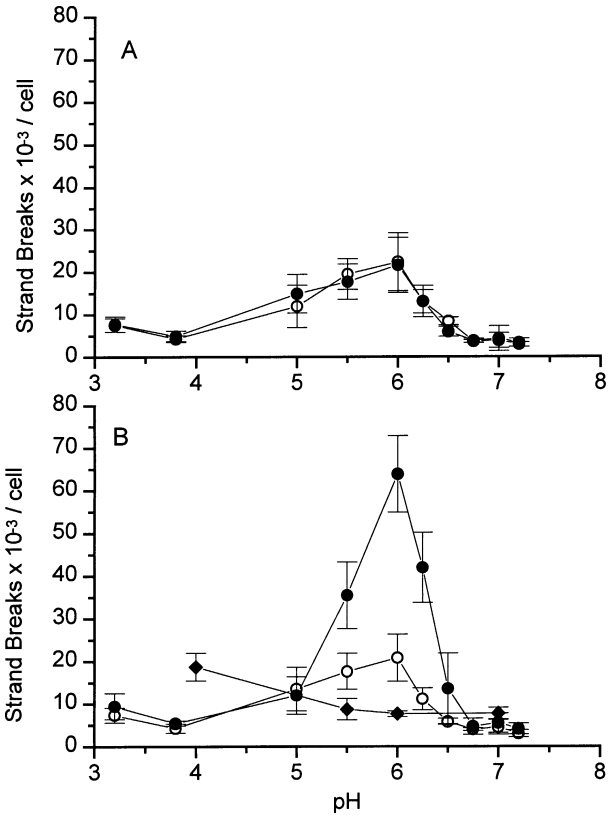

Figure 1 Induction of PDDB in nuclear (A) and nucleoid (B) monolayers

The substrates were incubated at 4 °C (○) or 37 °C (●). The influence of diethyl pyrocarbonate (◆) on PDDB is also shown in (**B**). The values are means ± S.D. ($n = 6$).

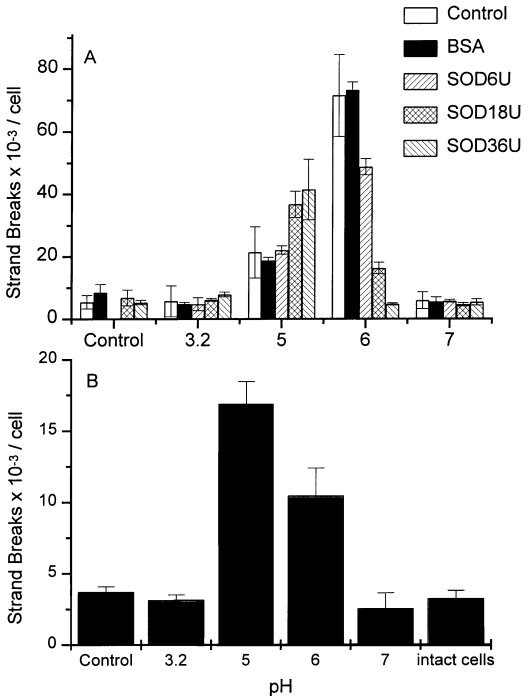

Figure 2 (A) Inhibition of PDDB in nucleoid monolayers by SOD, and (B) strand breaks in nucleoid monolayers exposed to acidic pH as detected by nick translation assays

In (**A**), SOD was replaced by BSA as a negative control; 6U, 18U and 36U denote 6, 18 and 36 units respectively. The values are means ± S.D. ($n = 9$ in **A**; $n = 6$ in **B**).

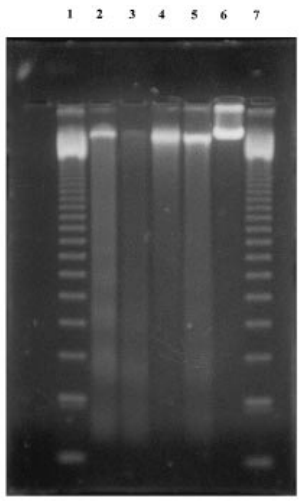

Figure 3 Internucleosomal cleavage of DNA in VH-10 fibroblasts exposed to etoposide

Lanes 1 and 7, 123 bp ladder; lanes 2 and 3, DNA from etoposide-treated fibroblasts (20 μg/ml of medium) isolated on days 3 and 4 of treatment respectively; lanes 4 and 5, as for lanes 3 and 4, except that the concentration of etoposide used was 10 μg/ml; lane 6, control cells.

a radiolabel into DNA single-strand breaks (double-strand breaks are labelled to a negligible extent by this assay). The maximum number of single-strand breaks, as detected by nick translation assays, occurred at pH 5 (Figure 2B). A considerably smaller number of single-strand breaks (detectable by the nick translation assay) was observed at pH 6, although the highest number of strand breaks occurred at this pH value when measured by alkali unwinding. This observation may be explained by the presence of both single- and double-strand breaks. Single-strand breaks are indicative of attack on the DNA by a physical agent, such as hydroxyl radicals. The presence of double-strand breaks could have two possible explanations. They may have been induced either by endonucleolytic cleavage of DNA or, when present at a higher level, by the juxtaposition of neighbouring single-strand breaks that could lead to a significant number of double-strand breaks (characteristic of endonucleolytic DNA cleavage), which are virtually undetectable by the nick translation assay. Although the presence of endonucleases in the chromatin substrates used was unlikely, due to the removal of the majority of the proteins, we examined both of these possibilities in further experiments.

Investigation of endonuclease activity

Diethyl pyrocarbonate, a general inhibitor of endonucleases, had an almost complete inhibitory effect on PDDB in the pH range examined (Figure 1B). In order to test whether VH-10 fibroblasts indeed possess an apoptosis-associated endonuclease activity, exponential-phase as well as contact-inhibited, quiescent, cells were treated with the anti-cancer drug etoposide (VP-16). Etoposide has previously been shown to induce apoptosis accompanied by oligonucleosomal fragmentation of DNA in several cell lines [6,15]. Indeed, continuous treatment of exponential-phase VH-10 fibroblasts with etoposide resulted in extensive apoptosis. After several hours of etoposide treatment, morphological changes such as chromatin condensation and cell shrinkage, which are characteristic of the apoptotic mode of cell death, were revealed in the cells by DAPI staining. The typical DNA ladder appeared on day 3 of treatment, upon electrophoresis of the isolated DNA (Figure 3). The data indicate that VH-10 fibroblasts contain an endonuclease activity which is able to fragment the cellular DNA into oligonucleosomes upon exposure to a cytotoxic agent.

Apoptosis is frequently associated with the activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease [5], and so we tested whether PDDB could be modulated by the presence of these cations or their chelators. No significant changes in PDDB were observed after incubation of chromatin substrates with bivalent cations, alone or in combination, at millimolar concentrations (Table 1). Conventional electrophoresis of DNA isolated from permeabilized pH- and bivalent cation-challenged fibroblasts did not reveal the characteristic DNA ladder (results not shown), even with an extended incubation time of 2 h. Similar concentrations of these ions have been reported to induce significant oligonucleosomal fragmentation after 90 min upon incubation of isolated rat liver nuclei [26]. The presence of the Mg^{2+} and Ca^{2+} chelators EDTA and EGTA during the pH challenge of permeabilized cells had little or no effect on PDDB (Table 1); the observed slight fluctuations may be attributed to changes in the chromatin conformation due to the depletion of bivalent cations [27].

In summary, our investigations of the presumed involvement of endonucleases in PDDB yielded contradictory results. Although the inhibitory effect of diethyl pyrocarbonate, the temperature-dependence of PDDB, the protective effect of histones and the results of the nick translation assays all implicated a possible endonuclease activity, the results from the chelator/bivalent-cation experiments argued strongly against endonucleolytic cleavage of DNA as being the mechanism responsible

Table 1 Modifiers of PDDB

The values shown represent PDDB as a percentage of the control (PDDB induced in nucleoid monolayer by citrate/phosphate buffer, pH 6.0), and are means ± S.D. ($n = 6–9$). N.T., not tested.

Modifier	Concentration (mM)	PDDB at pH 6 (% of control) 4 °C	37 °C
EDTA	0.1	N.T.	102.4 ± 11.3
EGTA	0.1	N.T.	98.1 ± 4.4
EDTA + EGTA	0.1 each	N.T.	101.9 ± 8.9
Ca^{2+}	2.0	N.T.	96.1 ± 5.7
Mg^{2+}	2.0	N.T.	103.9 ± 11.8
$Ca^{2+} + Mg^{2+}$	2.0 each	N.T.	94.5 ± 7.6
Desferal	0.01	102.5 ± 6.9	97.7 ± 8.0
Desferal*	0.01	143.7 ± 11.3	151.4 ± 2.5
1,10-Phenanthroline	0.1	26.7 ± 5.9	58.4 ± 6.5
Diethyldithiocarbamate	0.01	37.7 ± 6.9	44.9 ± 4.2
Quin2	0.1	40.2 ± 9.3	55.9 ± 7.7
H_2O_2	100	96.4 ± 8.9	103.4 ± 10.9
Catalase	1000 units	N.T.	97.4 ± 12.4
DMSO	0.1	87.1 ± 8.4	71.8 ± 12

* Desferal included in permeabilizing solution.

for PDDB. Hence we had to consider whether PDDB may result from the attack of ROS, such as hydroxyl radicals, on DNA. Indeed, the Fenton reaction can be modulated by pH and temperature [28], offering an explanation for the increase in PDDB caused by an increase in temperature. In addition, histones are potent scavengers of hydroxyl radicals [29], which may account for their protective effect against PDDB. Finally, one has to bear in mind that, at neutral or slightly acidic pH, diethyl pyrocarbonate will modify and possibly inactivate any proteins by modification of their histidine residues [30]. Although our intention in the use of diethyl pyrocarbonate was as an endonuclease inhibitor, its inhibitory effect on PDDB could be exerted through inactivation of residual ROS-producing systems in the chromatin substrates used.

Role of ROS in PDDB

PDDB has been observed after a short incubation at 4 °C; this implies the occurrence of a Fenton-type reaction. Although the mild permeabilization procedure used by us removes the majority of the cytoplasmic contents [29], remnants of cellular organelles are likely to be trapped in the permeabilized fibroblasts. Mitochondria, the endoplasmic reticulum and the nuclear membrane are all known to be sites of superoxide generation [32]. Superoxide radicals can react with traces of transition metals (Cu^{2+}, Fe^{2+}), bound to DNA or proteins, to produce H_2O_2 and hydroxyl radicals [33]. Moreover, the rate of superoxide dismutation is greatly enhanced at acidic pH [31,33] compared with that at the pH of most body tissues and fluids. Indeed, SOD was able to completely suppress PDDB at pH 6 (Figure 2A). The ineffectiveness of SOD at pH 5 is in accordance with its reported pH optimum range of 5.3–9.2 [34]. However, the complete inhibition of PDDB by SOD at pH 6 suggests that superoxide anions are major ROS in the chromatin substrates used, and this could be considered to be the strongest argument against the supposed activation of endonuclease(s).

If the metal catalysts of hydroxyl radical production are bound to DNA or DNA-associated proteins, the radicals generated, owing to their very short life-span and high reactivity, will immediately attack the DNA in a so-called 'site-specific' manner [33]. Such site-specific damage is suggested by the fact that the presence of histones had no effect on PDDB at 4 °C when compared with nucleoid monolayers (Figure 1A). This observation is plausible if the metal ion is bound either to nuclear-matrix-associated DNA or to the proteins of the same matrix. These regions of DNA are suggested to be transcriptionally active [35] and therefore to be of a more open conformation than the bulk DNA. Such differences in DNA conformation are suggested to be the reason for the observed higher susceptibility of matrix-associated DNA to damage by ionizing radiation [36].

We tested several metal chelators with affinity for iron or copper (metals known to be associated with DNA) for their ability to suppress PDDB (Table 1). Chelators with high affinity for Cu^{2+}, such as quin2, 1,10-phenanthroline and diethyl dithiocarbamate, strongly suppressed PDDB at 4 °C, whereas their protective effect was less pronounced at 37 °C (Table 1). On the other hand, Desferal (desferrioxamine mesylate), a strong chelator of Fe^{2+}, had no effect on PDDB (Table 1). In some experiments we added Desferal to the permeabilization buffer and observed a marked rise in PDDB (Table 1) compared with experiments in which Desferal was added only to the citrate/phosphate buffer. It has been reported previously that the Desferal–copper complex (but not Desferal–iron) has DNA-cleavage activity [37], which may explain our observations.

Fenton-type chemistry was becoming evident as the mechanism responsible for PDDB, since superoxide radicals and Cu^{2+} played a major role. Thus we tested whether modulation of the presumed third participant, H_2O_2, could affect the observed phenomena. The presence of H_2O_2 at a concentration of up to 100 mM during pH exposure did not result in additional DNA breakage (Table 1). This observation alone could be explained by the limited availability of the other components of Fenton reaction. Moreover, it has been shown previously that H_2O_2 is essential for iron- but not copper-induced DNA cleavage [38] when a reducing agent such as ascorbate or superoxide anion is present. This seems to be the case in our experiments too, as catalase was not able to suppress PDDB (Table 1). At pH 7.4, the same concentration of catalase (1000 units/ml) as used in our experiment was sufficient to completely inhibit DNA breakage induced either by the superoxide-generating xanthine/xanthine oxidase system at pH 7.4 or by the Cu^{2+}/H_2O_2/ascorbic acid system [39]. Thus it can be concluded that H_2O_2 is not involved in PDDB.

We presumed that the most probable effector molecules of PDDB were hydroxyl radicals, the highly reactive product of the Fenton reaction. To our surprise, DMSO (a scavenger of hydroxyl radicals) had only a very slight inhibitory effect on PDDB (Table 1). The inability of DMSO to affect PDDB may depend on the formation of copper–peroxyl complexes rather than hydroxyl radicals. Such an alternative mechanism for a copper-mediated Fenton reaction has been suggested [40]. On the other hand, there are other explanations for the inability of a scavenger to interfere with hydroxyl radical-mediated DNA damage [33]. It has been noted that, due to the site-specific nature of the reaction of the hydroxyl radicals with the DNA, it is very difficult for any trapping molecule to intercept them [39].

In summary, we have observed acidic-pH-induced DNA fragmentation in permeabilized human fibroblasts. Two possible candidate mechanisms behind this PDDB, namely activation of a pH-dependent endonuclease and DNA damage caused by ROS, have been explored. VH-10 fibroblasts contain an apoptosis-associated endonuclease activity, as their DNA is fragmented into oligonucleosomes upon exposure to etoposide; however, this does not seem to be involved in PDDB. Although

activation of a pH-dependent nuclease(s), still present in the nucleus of permeabilized fibroblasts, cannot be excluded, PDDB seems rather to be associated with enhancement of a copper-driven Fenton reaction by acidic conditions. Our results are supported by data from other studies [41–43]. For example, a copper-mediated Fenton reaction has been implicated in the induction of apoptosis in rat thymocytes [41]. Another study reported a role for endogenous DNA-associated Cu^{2+} ions in the induction of intranucleosomal fragmentation in isolated rat liver nuclei [42]. Perhaps most significantly, a study has shown the non-random distribution of copper-induced double-strand breaks in isolated V79 nuclei [43]. The observed production of high-molecular-mass DNA fragments was resistant to DMSO, but markedly inhibited by SOD. In our study, assuming a random distribution of strand breaks, the calculated mean sizes of DNA fragments produced by PDDB in nuclear and nucleoid monolayers are 300 and 80 kbp respectively. DNA fragments of similar sizes have been observed previously during the early stages of apoptosis in several cell lines [44] and proposed to result from endonucleolytic cleavage. We suggest that, in some cases, copper-mediated PDDB might be responsible for the production of high-molecular-mass DNA fragments during the early phases of apoptosis and/or necrosis.

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