Specific Proteolytic Cleavage of Poly(ADP-ribose) Polymerase: An Early Marker of Chemotherapy-induced Apoptosis

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ABSTRACT

Apoptosis is a morphologically and biochemically distinct form of cell death that occurs under a variety of physiological and pathological conditions. In the present study, the proteolytic cleavage of poly(ADP-ribose) polymerase (pADPRP) during the course of chemotherapy-induced apoptosis was examined. Treatment of HL-60 human leukemia cells with the topoisomerase II-directed antitumor agent etoposide resulted in morphological changes characteristic of apoptosis. Endonucleolytic degradation of DNA to generate nucleosomal fragments occurred simultaneously. Western blotting with epitope-specific monoclonal and polyclonal antibodies revealed that these characteristic apoptotic changes were accompanied by early, quantitative cleavage of the M$_r$ 116,000 pADPRP polypeptide to an M$_r$ ~25,000 fragment containing the amino-terminal DNA-binding domain of pADPRP and an M$_r$ ~85,000 fragment containing the automodification and catalytic domains. Activity blotting revealed that the M$_r$ ~85,000 fragment retained basal pADPRP activity but was not activated during morphogenesis (reviewed in Refs. 1 and 2), after withdrawal of trophic hormones (7-11), and after exposure of cells to other enzymes (56). To add to the confusion, other inhibitors of other enzymes (56). To add to the confusion, other inhibitors of

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INTRODUCTION

The term apoptosis denotes a distinctive sequence of morphological changes observed in certain cells as they die (reviewed in Refs. 1-6). In the nucleus, these morphological changes include condensation of the nuclear contents into clumps of heterochromatin adjacent to the nuclear envelope, then nuclear fragmentation, and finally packaging of the nuclear fragments into multiple membrane-enclosed apoptotic bodies. These morphological changes have been observed in cells that die during morphogenesis (reviewed in Refs. 1 and 2), after withdrawal of trophic hormones (7-11), and after exposure of cells to chemotherapeutic agents (1, 12-15), γ-irradiation, or cytotoxic lymphocytes (reviewed in Refs. 1, 3, 6, and 16). Conversely, it has been observed that treatment of cells with tumor-promoting phorbol esters (17-19) or overexpression of the bcl-2 oncogene (20-23) can inhibit the events leading to apoptosis in some cell systems. Understanding apoptotic cell death has, therefore, assumed an increasingly important role in the fields of experimental oncology and developmental therapeutics.

The morphological alterations of apoptosis are accompanied by a variety of biochemical changes. Elevations in cytosolic free calcium (reviewed in Ref. 24) and cytosolic hydrogen ion (25) are followed by internucleosomal DNA degradation (reviewed in Refs. 5, 16, 26, and 27) and sharp decreases in cellular NAD levels (28-31). These changes typically occur at the time that cells lose their proliferative capacity but before cells become permeable to dyes such as propidium iodide or trypan blue.

Although previous experiments have focused extensively on the activation of an endonuclease that catalyzes internucleosomal DNA degradation (reviewed in Refs. 5, 16, 26, and 27; see also 32-34), activation of the nuclear enzyme pADPRP is also thought to play a role in the biochemical changes that accompany apoptosis (reviewed in Refs. 35-38). pADPRP is a Mr 116,000 polypeptide that is present in ~10$^6$ copies/cell in somatic cells (39, 40). This enzyme converts NAD to nicotinamide and protein-linked ADP-ribose polymers (reviewed in Refs. 41 and 42). Results of studies of endogenous ADP-ribose polymer levels suggest that the polymerase is relatively inactive in unperturbed tissue culture cells (43-45). Polymer levels increase markedly after treatment of cells with agents that induce DNA single-strand and double-strand breaks (43-45), presumably as a consequence of pADPRP activation (46, 47) that results from a conformational change when the enzyme binds to damaged DNA (48) through one of its two zinc fingers (reviewed in Ref. 49). Activated pADPRP catalyzes the poly(ADP-ribose)ylation of a variety of nuclear polypeptides, but most of the polymer chains are bound to pADPRP itself (reviewed in Refs. 41 and 42). It has recently been shown in human cell extracts that this autophosphorylation of pADPRP facilitates release of the protein from damaged DNA and permits access of DNA repair enzymes to DNA single-strand breaks blocked by unmodified pADPRP (50).

Several investigators (29-31, 51) have proposed that the activation of pADPRP by DNA strand breaks contributes to the consumption of NAD that occurs in cells undergoing apoptosis. On the other hand, Nelipovich et al. (52) have reported that pADPRP activity actually decreases during the course of radiation-induced apoptosis in rat thymocytes. Additional studies using enzyme inhibitors have failed to clarify the role of pADPRP in apoptosis. Treatment with millimolar concentrations of 3-AB, an inhibitor of NAD-requiring enzymes, decreases apoptosis in certain cell systems (30, 51, 53-55). These results have been interpreted as indicating that activation of pADPRP contributes to apoptosis. More recent studies, however, indicate that the 3-AB concentrations utilized in these studies could potentially inhibit other enzymes (56). To add to the confusion, other inhibitors of...
pADPRp have been observed to provoke apoptosis, leading to the hypothesis that pADPRp activity normally protects the cell from apoptosis (57). Thus, the role of pADPRp in cells that undergo apoptosis is unclear.

It has been generally accepted that the macromolecular degradation in cells undergoing apoptosis is initially limited to internucleosomal DNA degradation. A previous study from this laboratory, however, revealed that internucleosomal degradation of DNA in etoposide-treated HL-60 human leukemia cells was accompanied by the early, quantitative cleavage of the M, 116,000 pADPRp polypeptide to a relatively stable M, ~85,000 fragment (58). A similar cleavage of pADPRp was observed in Mol04 cells undergoing purineless cell death after treatment with the glycinemide ribonucleotide transformylase inhibitor 5-deazaacyclotetrahydrofolate (59). It was unclear whether this cleavage of pADPRp was limited to human leukemia cell lines treated with a select group of agents or whether it was a general phenomenon occurring in a wide variety of cell types undergoing chemotherapy-induced apoptosis. Moreover, the effect of this cleavage event on the catalytic activity of pADPRp was unknown. In the present study, the nature of this proteolytic cleavage and its effect on pADPRp activity have been studied in relation to changes in NAD levels in etoposide-treated HL-60 cells. In addition, a number of different experimental systems that undergo apoptosis were examined for pADPRp cleavage. Finally, to clarify the importance of this early proteolysis, the effect of protease inhibitors on the fragmentation of pADPRp and DNA was examined.

MATERIALS AND METHODS

Materials. Etoposide was kindly provided by Bristol-Myers (Syracuse, NY). Phosphoramidone and E-64 were from Boehringer-Mannheim (Indianapolis, IN). Puromycin, cytosine arabinoside, methotrexate, cis-platinum, 3,AB, trifluorothymidine, 5-fluoro-2-deoxycytidine, bisbenzimidazole, and the remaining protease inhibitors were obtained from Sigma (St. Louis, MO). 32P-labeled NAD and nitrocellulose for activity blots were from New England Nuclear (Boston, MA) and Amersham (Oakville, Ontario), respectively. Sources of all other reagents have been previously described (58).

Buffers. Medium A consisted of RPMI 1640, 10% (v/v) heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 μg/ml penicillin G, and 2 mM glutamine. Buffer B consisted of 6 M urea, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 0.15 mM β-mercaptoethanol, and 0.1% (w/v) bromphenol blue. Buffer C consisted of 6 M guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 21°C), 10 mM EDTA, and 1% (v/v) β-mercaptoethanol. Immediately before use of each aliquot of buffer C, α-phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM from a 100 mM stock in anhydrous isopropanol. Buffer D consisted of 0.7 M β-mercaptoethanol, 190 mM glycine, 25 mM Tris-HCl (pH 8.0), and 0.1% (w/v) SDS. Buffers E and F consisted of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM dithiothreitol, 20 μM Zn(II) acetate, 2 mM MgCl2, and 0.3% (v/v) Tween-20. Buffer F consisted of buffer E without Zn(II) acetate and MgCl2. PBS contained 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8.0 mM Na2HPO4.

Drug Treatment and Irradiation. Logarithmically growing HL-60 human progranulocytic leukemia cells or K562 chronic myelogenous leukemia cells (propagated at densities of <1 × 106/ml in medium A) were freed of senescent cells by sedimentation over a Ficoll-Hypaque step gradient (density = 1.119 g/cm3) and resuspended at a concentration of 1–1.5 × 106/ml in fresh medium A. Etoposide was added from a 1000-fold concentrated stock in dimethyl sulfoxide. After various periods of incubation at 37°C in an atmosphere containing 5% (v/v) CO2:95% air, cells were sedimented at 3200 × g for 1 min and lysed in SDS-proteinase K in preparation for agarose gel electrophoresis (59). For Western blots, cells were sedimented at 200 × g for 10 min, washed once in serum-free RPMI 1640 containing 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (pH 7.4 at 21°C), and lysed by sonication in buffer C (60). For activity blots, drug-treated cells were washed as described for Western blotting and solubilized by vigorous vortexing for 1 min in buffer B at a concentration of 4 × 107 cells/ml. Cells for NAD levels were sedimented at 400 × g for 10 min, washed twice at 4°C with PBS, and extracted for 15 min on ice with 0.5 M perchloric acid. After the extracts were neutralized with 0.5 volume of 1 M KOH containing 0.2 M potassium phosphate (pH 7.5 after neutralization), NAD was determined using an enzymatic cycling assay exactly as described by Bernofsky and Swan (61). Cells for morphological examination were washed twice with PBS, fixed in 3:1 (v/v) methanol:acetic acid, pipetted dropwise onto glass slides, rehydrated with PBS, stained with 1 μg/ml bisbenzimide in 50% glycerol, and examined by fluorescence microscopy using a Zeiss universal microscope equipped with an epi-illuminator and appropriate filters.

In some experiments, etoposide was replaced with cytosine arabinoside, methotrexate, or cis-platinum (prepared as 1000-fold concentrated stocks in dimethyl sulfoxide). Alternatively, HL-60 cells were treated with puromycin or cycloheximide (added from 1 mM stocks in tissue culture medium) or were irradiated at 1.0 Gy/min to a final dose of 10 Gy using a 137Co source.

MDA-MB-468 cells (kindly provided by Dr. Marc Lippman, Georgetown University, Washington, DC) were exposed to 100 μM trifluorothymidine or 5-fluoro-2'-deoxyuridine (prepared as filter-sterilized 10 μM aqueous stocks that were frozen at −20°C until use). Adherent and nonadherent cells were either harvested separately as previously described (14) or combined, washed with serum-free medium, and solubilized in buffer C as described above.

Rat thymocytes (isolated as described in Ref. 62) were incubated at 37°C in serum-free RPMI 1640 medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (pH 7.4) in the presence or absence of 1 μM dexamethasone (added from a 1000-fold concentrated ethanolic stock). Alternatively, thymocytes were irradiated to 6.0 Gy from a 137Co source at a dose of 1 Gy/min prior to incubation in the absence of dexamethasone. Analyses of protein and DNA were performed as described above except that cells were sedimented at 12,000 × g for 2 min prior to lysis.

Western Blotting. After reduction, alkylation with iodoacetamide, and subsequent preparation for electrophoresis (60), samples containing 50 μg of protein (MDA-MB-468 cells, thymocytes) or protein from 3 × 107 leukemia cells were applied to SDS gels prepared with a 5–15% (w/v) polyacrylamide gradient. After electrophoretic transfer of the separated polypeptides to nitrocellulose, Western blotting was performed as previously described (58). The epitopes recognized by the various antibodies are indicated in Fig. 2C. C2–10 is a mouse monoclonal antibody that recognizes an epitope located at the carboxyl end of the DNA-binding domain (63). Anti-F2 (generously provided by Dr. G. deMurcia, Centre National de la Recherche Scientifique, Strasbourg Cedex, France) is a rabbit polyclonal antiserum raised against a synthetic polypeptide corresponding to the second zinc finger of human pADPRp (64).

Activity Blotting. Electrophoretically separated polypeptides were assayed for pADPRp activity as described by Simonin et al. (65). Briefly, samples containing protein from 4 × 105 cells were applied to adjacent wells of SDS-containing minigels prepared with 8% (w/v) polyacrylamide. After electrophoresis at 200 V, gels were soaked for 60 min at 37°C in buffer D and electrophoretically transferred to nitrocellulose. Immunobilized polypeptides were renatured for 1 h in buffer E, incubated with 2 μCi/ml 32P-labeled NAD in buffer E (with or without 1 μg/ml of DNase-activated DNA), and washed with isopeptide-free buffer F.

RESULTS

Etoposide-induced Apoptosis in HL-60 Cells. Treatment of HL-60 human progranulocytic leukemia cells with the topoisomerase II-directed chemotherapeutic agent etoposide resulted in morphological changes consistent with the process of apoptosis (Fig. 1). Initially a rim of heterochromatin appeared at the nuclear periphery (Fig. 1F). The nucleus simultaneously disappeared. The nuclei subsequently fragmented, and each cell formed multiple membrane-enclosed vesicles (Fig. 1C; see also Fig. 9C). As is the case in other cell systems undergoing apoptosis, mitochondria and the plasma membrane remained intact throughout the course of these morphological changes. Consequently, ~95% of the cells continued to exclude trypan blue after 6 h.

These morphological changes were accompanied by progressive internucleosomal degradation of DNA to yield a ladder of DNA fragments (Fig. 2A) as previously reported by this laboratory and others (15, 58, 66–68). When the proteins in these etoposide-treated cells...
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Cleavage of pADPRp was observed at etoposide concentrations as low as 4 µM (data not shown).

Location of the Cleavage Site in pADPRp. To assess the site of cleavage of pADPRp, Western blotting was repeated with anti-F2, a polyclonal antibody raised against the second zinc finger of pADPRp (Fig. 2F). During the course of etoposide treatment, loss of the Mr 116,000 polymerase (Fig 2D, lane 1) was accompanied by the appearance of an Mr ~25,000 polypeptide recognized by this antibody.

were separated by SDS-PAGE and stained with Coomassie blue, the bulk of the cellular polypeptides remained intact (Fig. 2B). However, Western blotting with monoclonal antibody C-2-10, an antibody that recognizes an epitope at the carboxyl end of the DNA-binding domain of pADPRp (Fig. 2F), revealed that the Mr 116,000 pADPRp molecule was degraded to a relatively stable Mr ~85,000 fragment with a time course that paralleled the DNA fragmentation (Fig. 2C). Similar proteolytic cleavage of pADPRp accompanies endonucleolytic DNA degradation in etoposide-treated HL-60 cells. HL-60 cells were treated with 68 µM etoposide for 0, 1, 1.5, 2, 3, or 4 h (lanes 1-6, respectively). Aliquots were prepared for agarose gel electrophoresis (A) or SDS-PAGE (B-E). One gel was stained with Coomassie blue (B). Companion gels were transferred to nitrocellulose and reacted with monoclonal antibody C-2-10 (C), polyclonal anti-F2 (D), or antibody against the nucleolar protein B23 (E), followed by their respective radiolabeled secondary antibodies. F, schematic of pADPRp indicating known cleavage sites for trypsin (t), papain (P), and chymotrypsin (C) as well as the two zinc fingers (F1 and F2) and the location of epitopes recognized by C-2-10 and anti-F2. Lower schematic indicates functional domains of pADPRp (98). Numbers at left and right, sizes of standard DNA fragments in kilobases or polypeptides in thousands.

Fig. 2. Proteolytic cleavage of pADPRp accompanies endonucleolytic DNA degradation in etoposide-treated HL-60 cells. HL-60 cells were treated with 68 µM etoposide for 0, 1, 1.5, 2, 3, or 4 h (lanes 1–6, respectively). Aliquots were prepared for agarose gel electrophoresis (A) or SDS-PAGE (B–E). One gel was stained with Coomassie blue (B). Companion gels were transferred to nitrocellulose and reacted with monoclonal antibody C-2-10 (C), polyclonal anti-F2 (D), or antibody against the nucleolar protein B23 (E), followed by their respective radiolabeled secondary antibodies. F, schematic of pADPRp indicating known cleavage sites for trypsin (t), papain (P), and chymotrypsin (C) as well as the two zinc fingers (F1 and F2) and the location of epitopes recognized by C-2-10 and anti-F2. Lower schematic indicates functional domains of pADPRp (98). Numbers at left and right, sizes of standard DNA fragments in kilobases or polypeptides in thousands.
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Fig. 3. Activity blot of pADPRp performed in the absence (A and C) or presence (B and D) of exogenous nicked DNA. HL-60 cells treated with 68 μM etoposide for 0, 1, 1.5, 2, 3, or 4 h (lanes 1–6, respectively) were subjected to SDS-PAGE. After polypeptides were transferred to nitrocellulose and renatured in situ as described in the "Materials and Methods," blots were incubated with 32P-labeled NAD in the absence (A and C) or presence (B and D) of exogenous nicked DNA. In A and B, simultaneously prepared autoradiographs demonstrate that the M₄ ~85,000 fragment is labeled by 32P-NAD in the presence (B and D) of exogenous DNA. In A and B, simultaneously prepared autoradiographs demonstrate that the M₄ ~85,000 fragment is labeled by 32P-NAD in the absence of exogenous DNA (e.g., A, lane 6) but is less strongly labeled in the presence of exogenous DNA (e.g., B, lane 6). C and D, simultaneously prepared autoradiographs were used to demonstrate the activation of the M₄ 116,000 polymerase by exogenous nicked DNA (D) compared to baseline activity (C). E, glycohydrolase digestion to confirm that bound radiolabel was incorporated into poly(ADP-ribose) polymer. Lanes 1–3, samples from HL-60 cells treated with 68 μM etoposide for 0, 1, or 2 h, respectively. Lanes 4–6, after autoradiography, the samples in lanes 1 and 2 were incubated with buffer containing (lane 4) or lacking (lane 5) glycohydrolase. Autoradiography revealed that glycohydrolase completely removed the bound radiolabel (lane 4). The piece of nitrocellulose corresponding to lane 5 was inadvertently reversed during remounting (cf. lanes 2 and 5). Ordinate, M₄ in thousands.

Fig. 2D, lanes 4–6). These results suggested that pADPRp was cleaved to fragments of M₄ ~85,000 (lacking the zinc finger domain) and M₄ ~25,000 (containing the zinc finger domain). In contrast, the nuclease polypeptide B23/nucleohosmin, which has a nuclear localization similar to pADPRp (69), remained intact during etoposide-induced apoptosis (Fig. 2E).

M₄ 85,000 Fragment Retains DNA-insensitive Catalytic Activity. If pADPRp were fragmented without significant proteolysis at its carboxyl terminus, the M₄ ~85,000 fragment described above would be expected to retain enzyme activity (64). Moreover, removal of the zinc fingers would be expected to render the M₄ ~85,000 fragment insensitive to activation by the addition of exogenous nicked DNA (64). To confirm these predictions, polypeptides from control and etoposide-treated HL-60 cells were separated by SDS-PAGE, transferred to nitrocellulose, renatured, and assayed for activity in situ by incubating the blots with 32P-labeled NAD as previously described by Simonin et al. (65).

When control samples were analyzed in this fashion, an M₄ 116,000 polypeptide (the automodified pADPRp) became 32P-labeled (Fig. 3A, lane 1). The bound radiolabel was not removed by subsequent incubation of the blots with 3% SDS (data not shown), a treatment that removes noncovalently bound radiolabel. In contrast, the radiolabel was removed by incubation with purified glycohydrolase, an enzyme that selectively degrades poly(ADP-ribose) polymers (Fig. 3E). Additional experiments (not shown) revealed that the radiolabel could also be released from the immobilized M₄ 116,000 polypeptide by NaOH treatment. Electrophoresis of this NaOH-released product in 20% (w/v) polyacrylamide gels (44) revealed a ladder of polymer sizes characteristic of poly(ADP-ribose). Collectively, these results indicate that the radiolabel bound to the M₄ 116,000 polypeptide represents poly(ADP-ribose) polymers that have become covalently attached to the enzyme as a consequence of automodification.

When extracts from etoposide-treated cells were analyzed, a polypeptide of M₄ ~85,000 also became radiolabeled (Fig. 3A, lanes 4–6). Although it was not possible to isolate enough radiolabeled material for size determination, control experiments revealed that the radiolabel remained bound to the M₄ ~85,000 band after treatment with 3% SDS but was removed by incubation with purified glycohydrolase. Thus, the M₄ ~85,000 polypeptide, like the intact M₄ 116,000 polymerase, appears to be covalently modified by an NAD derivative that is degraded by an enzyme that selectively degrades poly(ADP-ribose). These observations are consistent with the view that the M₄ ~85,000 pADPRp fragment contains functional catalytic and automodification domains (Fig. 2F). Furthermore, since removal of as few as 45 amino acids from the carboxyl terminus of pADPRp abolishes pADPRp activity (64), these results suggest that the carboxyl terminus of pADPRp is largely or completely intact in the M₄ ~85,000 fragment.

When the activity blotting was repeated in the presence of exogenous nicked DNA, labeling of the M₄ 116,000 polypeptide was markedly increased (cf. C and D, Fig. 3). In contrast, labeling of the M₄ ~85,000 fragment was not enhanced and was probably diminished (cf. A and B, Fig. 3). Thus, the M₄ ~85,000 fragment exhibits basal catalytic activity, but its activity under conditions of maximal stimulation appears to be much lower than that of the intact polymerase.

Relationship of pADPRp Cleavage to Diminished NAD Levels. The decline of NAD levels observed in cells undergoing apoptosis has been attributed to increased NAD consumption as a consequence of pADPRp activation by internucleosomal DNA strand breaks (see "Introduction"). On the other hand, the cleavage of pADPRp described above might be expected to diminish the cellular activity of pADPRp. In order to reconcile the consumption of NAD and the cleavage of pADPRp, we examined NAD levels and pADPRp fragmentation in HL-60 cells treated with etoposide in the absence and presence of the NADp inhibitor 3-AB. In the absence of 3-AB, NAD levels diminished to 70% of control levels 60–90 min after addition of etoposide, to 30% of control levels after 2 h, and to 20% of control levels by 3 h (Fig. 4C). Thus, the bulk of the cellular NAD was consumed prior to the cleavage of pADPRp (cf. B and C, Fig. 4, lanes 1–6).

When the same experiment was performed in the presence of 200 μM 3-AB, a concentration that selectively inhibits pADPRp (56), NAD levels remained at control levels for the first 2 h (Fig. 4C). Nonetheless, fragmentation of the cellular DNA and proteolytic cleavage of pADPRp became evident by 2 h (Fig. 4, lane 10) and proceeded at the same rate as in the absence of 3-AB. Although these results implicate pADPRp in the consumption of NAD that occurs early in the course of etoposide-induced apoptosis (cf. lanes 4 and 10, Fig. 4C), they also suggest that pADPRp activity and consumption of NAD do not play an obligate role in DNA fragmentation or pADPRp cleavage.

Cleavage of pADPRp after Treatment with Other Cytotoxic Agents. To ascertain whether the cleavage of pADPRp was unique to etoposide-induced apoptosis, HL-60 cells were treated with a variety of cytotoxic agents including cytosine arabinoside, methotrexate, cis-diaminedichloroplatinum(II), and colcemid. Results obtained with cytosine arabinoside and colcemid are shown in Fig. 5. Despite the

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Fig. 4. Temporal relationship among DNA fragmentation (A), pADPRp cleavage (B), and cellular NAD levels (bar graph) in the absence (lanes 1-6) or presence (lanes 7-12) of 3-AB. HL-60 cells were treated with 68 μM etoposide for the indicated time in the absence of 3-AB and analyzed as described in “Materials and Methods.” NAD levels were expressed as % of control (0 h, -3-AB) levels.

Appearance of the pADPRp Fragment in the Presence of Protein Synthesis Inhibitors. In certain cell types, generation of endonucleolytic DNA damage is prevented by inhibitors of RNA and protein synthesis (70-74). Even though the time course for the appearance of DNA fragments varied (cf. Figs. 2A, 5A, and 5C), in each case the initial appearance of oligonucleosomal DNA fragments was accompanied by cleavage of pADPRp (data not shown). Thus, the cleavage of pADPRp was not unique to treatment with etoposide.

Fig. 5. Cytosine arabinoside (Ara-C; A and B) or colcemid (C and D) induce DNA fragmentation (A and C) and cleavage of pADPRp (B and D). In A and B, HL-60 cells were incubated with 1 μM cytosine arabinoside for the indicated time (lanes 2-6) and then analyzed as described in “Materials and Methods.” As controls, cells were incubated for 4 h in the presence (lane 1) or absence (lane 7) of 68 μM etoposide (VP). In C and D, HL-60 cells were incubated for the indicated time with 1 μg/ml colcemid.

A nucleosomal DNA ladder (Fig. 6A) and degradation of pADPRp (Fig. 6B) within 2 h. Similar results were obtained with cycloheximide (data not shown). Therefore, it appears that the cleavage of pADPRp, like the endonucleolytic degradation of DNA, can occur despite extensive inhibition of protein synthesis. These results raise the possibility that the endonuclease and protease detected in these assays are preexisting cellular enzymes, although the selective synthesis of the endonuclease and protease in the presence of puromycin or cycloheximide also cannot be ruled out.

Cleavage of pADPRp in Other Cells Undergoing Apoptosis. To exclude the possibility that the cleavage of pADPRp occurred only in HL-60 cells, the fragmentation of DNA and pADPRp were examined in a variety of cell types undergoing chemotherapy-induced apoptosis. In human KG1a acute myelogenous leukemia cells as well as Molt 3 acute lymphocytic leukemia cells treated with etoposide, internucleosomal DNA degradation was again accompanied by cleavage of pADPRp (data not shown). In contrast, human K562 chronic myelogenous leukemia cells, a cell type previously reported to resist diphteria toxin-induced apoptosis (76), failed to show evidence of endonucleolytic DNA degradation (Fig. 7A) and pADPRp cleavage (Fig. 7B) after a 24- to 48-h treatment with lethal doses of etoposide. These observations strengthen the association between endonucleolytic DNA degradation and pADPRp fragmentation.

To determine whether proteolytic cleavage of pADPRp occurred only in leukemia cell lines, MDA-MB-468 human breast cancer cells were treated for up to 48 h with trifluorothymidine or 5-fluoro-2'-
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deoxyuridine, two pyrimidine analogues that result in thymidineless cell death. Cleavage of pADPRp was evident in these cells at 24 h (Fig. 8A). Previous results (14, 80) have revealed that apoptosis-associated internucleosomal DNA cleavage in adherent cell lines is most readily demonstrated in the cells that lift off the tissue culture plates. Western blotting revealed that cleavage of pADPRp also occurred exclusively in the nonadherent cell population (Fig. 8B). Furthermore, this analysis revealed that fragmentation of pADPRp occurred in the small population of cells that spontaneously underwent apoptosis in this cell culture (Fig. 8B, 0h). Thus, cleavage of pADPRp is not a consequence of the drug treatment per se. Instead, this cleavage is a component of the apoptotic process.

Finally, to rule out the possibility that proteolytic cleavage of pADPRp only occurred in neoplastically transformed cells, rat thymocytes were treated with glucocorticoids or γ-irradiation, treatments that have previously been shown to result in apoptosis (32–34, 70, 71). Cleavage of pADPRp accompanied the apoptosis induced by these treatments (Fig. 9).

Effect of Protease Inhibitors on pADPRp Cleavage, DNA Fragmentation, and Cell Survival. In an attempt to characterize the protease(s) involved in cleavage of pADPRp, HL-60 cells were incubated with etoposide for 1 h, washed, and incubated in drug-free medium in the absence or presence of various protease inhibitors. A variety of agents including phenylmethylsulfonyl fluoride, sodium bisulfite, E-64, benzamidine, leupeptin, antipain, and phosphoramidon had no effect on the cleavage of pADPRp (see Fig. 10 for examples). In contrast, the chloromethyl ketones TLCK and tosyl-L-phenylalanine chloromethyl ketone as well as the sulfhydryl blocking reagents iodoacetamide, N-ethylmaleimide, and p-chloromercuribenzenesulfonate all prevented cleavage of pADPRp to the Mr ~85,000.

Fig. 6. Puromycin induces endonucleolytic DNA degradation (A) and cleavage of pADPRp (B). HL-60 cells were incubated with 100 μM puromycin for the indicated time. In separate experiments, this concentration of puromycin was found to inhibit [35S]-methionine incorporation into trichloroacetic acid-precipitable material by 95%.

Fig. 7. Resistance of K562 chronic myelogenous leukemia cells to etoposide-induced DNA fragmentation and pADPRp cleavage. HL-60 cells (lanes 1–5) or K562 cells (lanes 6–10) were simultaneously incubated with 68 μM etoposide for 0 h (lanes 1 and 6), 4 h (lanes 2 and 7), 8 h (lanes 3 and 8), 12 h (lanes 4 and 9), or 24 h (lanes 5 and 10). Duplicate samples were subjected to agarose gel electrophoresis (A) or SDS-PAGE followed by Western blotting with antibody C-2-10 (B). Similar results were obtained when K562 cells were harvested after 48 h. Additional experiments indicated that the formation of K562 colonies in soft agar was reduced to 10% of control values by a 1-h incubation in 10 μM etoposide.

Fig. 8. Cleavage of pADPRp in human breast cancer cells undergoing apoptosis. In A, MDA-MB-468 cells were treated with 100 μM trifluorothymidine (TFT) or 5-fluoro-2’-deoxyuridine (5-FdUrd) for 0–48 h. Adherent and nonadherent cells were combined. Samples containing 50 μg of protein from each sample were blotted with antibody C-2-10. In B, nonadherent (N) and adherent (A) MDA-MB-468 cells treated with 100 μM 5-FdUrd for 0–48 h were processed separately. Western blotting was performed as in A. Although 50 μg of protein was loaded for each sample, the actual proportion of protein in the nonadherent cells increased from 4.2% of the total (adherent + nonadherent) protein at 0 h to 12.5% of the total at 48 h.

Fig. 9. Etoposide-induced apoptosis in K562 cells. Cells were incubated with 100 μM etoposide for 0 h (lanes 1–5) or 10 μM etoposide for 12 h (lanes 6–10). Duplicate samples were subjected to agarose gel electrophoresis (A) or SDS-PAGE followed by Western blotting with antibody C-2-10 (B). Similar results were obtained when K562 cells were harvested after 48 h. Additional experiments indicated that the formation of K562 colonies in soft agar was reduced to 10% of control values by a 1-h incubation in 10 μM etoposide.
fragment (Fig. 10B, lanes 3–5, 8, and 9). Interestingly, each of these agents prevented etoposide-induced internucleosomal DNA fragmentation (Fig. 10A, lanes 3–5, 8, and 9) and the morphological changes of apoptosis as well (Fig. 10C). Similar results were obtained in HL-60 cells and rat thymocytes after γ-irradiation (data not shown). Despite these effects, these compounds did not prevent etoposide- or irradiation-induced cell death. On the contrary, cells treated with iodoacetamide or TLCK (in the presence or absence of etoposide) uniformly excluded trypan blue for 2–3 h and then became permeable to this dye. After 6 h of treatment, all of the cells treated with iodoacetamide and up to 80% of the cells treated with TLCK were permeable to trypan blue. Thus, agents such as TLCK and iodoacetamide prevented the biochemical and morphological changes of apoptosis but did not prevent cell death.

DISCUSSION

In this present study, we have shown that proteolysis of pADPRp to an Mr ~85,000 fragment displaying DNA-independent enzyme activity is an early event that accompanies apoptosis induced by a variety of chemotherapeutic agents. The location of the proteolytic site on the pADPRp molecule and the biological significance of this observation are discussed below.

Cleavage of pADPRp. When HL-60 cells were treated with etoposide, the polypeptide recognized by monoclonal antibody C-2-10 (Fig. 2C) showed a shift in mobility from Mr, 116,000 to ~85,000. Although the activation of transglutaminase has been observed in other cells undergoing apoptosis (reviewed in Ref. 81), the increased mobility of pADPRp was not due to the formation of intramolecular isopeptide bonds. Blotting with an antiserum directed against the second zinc finger of pADPRp revealed that the epitopes recognized by this serum were recovered in a second fragment of Mr, ~25,000 (Fig. 2D). This result strongly suggests that proteolytic cleavage of pADPRp rather than the formation of intramolecular isopeptide bonds occurs during apoptosis.

This apoptosis-associated proteolytic cleavage of pADPRp is readily distinguished from the fragmentation of pADPRp that occurs during the course of normal protein turnover. During normal turnover, pADPRp is cleaved toward its carboxyl terminus to yield an enzymatically inactive Mr, 60,000 fragment containing the DNA-binding and automodification domains (82). In contrast, apoptosis-associated cleavage of pADPRp removes the amino-terminal domain to yield an Mr, ~85,000 fragment that retains basal catalytic activity but lacks the DNA-binding domain and cannot be stimulated by nicked DNA (Fig. 3).

The identity of the protease that is responsible for the cleavage of pADPRp is currently unknown. Bruno et al. (83) reported that chloromethyl ketones inhibited DNA fragmentation in a number of experimental systems in which apoptosis was induced. Our experiments confirm and extend those observations. The studies presented in Fig. 10 suggest that pADPRp cleavage in HL-60 cells can be inhibited by irreversible sulfhydryl-blocking reagents as well as chloromethyl ketones but not by serine esterase inhibitors. The sulfhydryl-dependent cathepsins are inhibited by this spectrum of inhibitors (84–86). Interestingly, this class of proteases is also inhibited by Zn²⁺ (86, 87), a cation that has been observed to prevent apoptosis in a variety of settings (88–90). Although these observations are all consistent with the view that the protease involved in pADPRp cleavage might be a sulfhydryl-dependent cathepsin, a number of additional considerations

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Fig. 9. Cleavage of pADPRp in rat thymocytes. Nonirradiated (lanes 1–12) or irradiated (lanes 13–18) thymocytes were incubated in the absence (lanes 1–7 and 13–18) or presence (lanes 8–12) of 1 μM dexamethasone for the indicated time and then prepared for Western blotting with antibody C-2-10. Under the conditions of this assay, >95% of the untreated and treated cells excluded trypan blue during the first 8 h.

Fig. 10. Effect of protease inhibitors on DNA degradation (A), cleavage of pADPRp (B), and cell morphology (C). In A and B, after a 1-h treatment with 68 μM etoposide, HL-60 cells were incubated for 3 h in the absence of protease inhibitors (lane 2) or in the presence of 10 mM iodoacetamide (lane 3), 10 mM N-ethylmaleimide (NEM, lane 4), 1 mM p-chloromercuribenzenesulfonate (pCMBS, lane 5), 1 mM phenylmethylsulfonyl fluoride (PMSF, lane 6), 1 mM TLCK (lane 8), 0.2 mM tosyl-L-phenylalanine chloromethyl ketone (TPCK, lane 9), 2 μg/ml phosphoramidone (phosphor, lane 10), or 5 μg/ml antipain (lane 11). Untreated cells were examined in lanes 1 and 7. C, Cell morphology as determined by phase contrast (left) and fluorescence microscopy (right) after staining with bisbenzimide. 1,1', control HL-60 cells. 2,2', cells incubated for 1 h with 68 μM etoposide followed by 5 h in fresh medium lacking protease inhibitors. 3,3', cells incubated for 1 h with 68 μM etoposide followed by 5 h in fresh medium containing 0.5 mM TLCK.

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* Subsequent experiments have revealed that cleavage of pADPRp in etoposide-treated HL-60 cells is also prevented by treatment of the cells with 1 mM ZnCl₂ (S. H. Kaufmann, unpublished observations).
argue against this conclusion. Intracytoplasmic release of a sulfhydryl-dependent cathepsin would be expected to cause degradation of many cellular polypeptides (85, 91). The observation that most cellular polypeptides remain intact during apoptosis (Fig. 2, B and E) appears to argue against the widespread accessibility of cathepsins B, H, or L to intracellular polypeptides. Likewise, the inability of leupeptin or E-64, two other inhibitors of sulfhydryl-dependent cathepsins (84–86, 91), to inhibit pADPRp cleavage also argues against the possibility that the enzyme involved is a sulfhydryl-dependent cathepsin, although the inability of these inhibitors to penetrate into cells in a timely fashion might also explain these results. Further studies are therefore required to identify the protease(s) involved in pADPRp cleavage.

**Biological Consequences of pADPRp Cleavage.** Previous results from a number of laboratories have shown that the internucleosomal degradation of DNA in cells undergoing apoptosis is accompanied by depletion of cellular NAD stores (see “Introduction”). This NAD depletion has been attributed to the consumption of NAD by pADPRp, which is in turn thought to be activated by internucleosomal DNA breaks that appear during apoptosis. On the other hand, direct measurement of enzyme activity suggested a decrease in pADPRp activity in thymocytes undergoing irradiation-induced apoptosis (52). These results have been difficult to reconcile with each other.

The simultaneous monitoring of NAD levels and pADPRp cleavage described in Fig. 4 suggests that both of these previous interpretations are correct. When 3-AB was applied to HL-60 cells at a concentration that selectively inhibits pADPRp (56), the etoposide-induced decline in NAD levels was delayed (Fig. 4C, lanes 7–12). This result (see also Refs. 30, 31, and 51) directly implicates pADPRp in the consumption of NAD that ordinarily occurs early during the course of apoptosis (Fig. 4C, lanes 1–4). Furthermore, when cells were incubated in the absence of 3-AB, NAD levels began to decline prior to the earliest detectable cleavage of pADPRp (Fig. 4, B and C, lanes 1–4). This observation suggests that the intact pADPRp molecule rather than the Mr, ~85,000 fragment is responsible for the initial consumption of NAD. By the time nucleosomal DNA fragments appear, however, levels of the Mr, 116,000 polymerase are diminishing and the Mr, ~85,000 fragment is being formed (Fig. 4, lanes 4–6). Although this Mr, ~85,000 fragment might play a role in the continuing consumption of NAD late in the course of apoptosis (Fig. 4C, lanes 5 and 6), the fragment appears to have much lower catalytic activity in the presence of nicked DNA than does the intact polymerase (Fig. 3B). The formation of this less active fragment (see also Fig. 9, lanes 15–18) appears to provide an explanation for the decreasing pADPRp catalytic activity that has been previously reported during radiation-induced apoptosis (46, 52).

Although it is tempting to speculate that the proteolytic cleavage of pADPRp serves a feedback function by decreasing the activity of pADPRp after it has been stimulated by DNA strand breaks, the data do not appear to support this model. In particular, treatment with 3-AB delays NAD consumption (presumably by inhibiting pADPRp) but does not delay DNA fragmentation and pADPRp cleavage in the etoposide-treated HL-60 cells (Fig. 4, lanes 10–12). This result suggests that activation of pADPRp activity is not required for proteolytic cleavage of pADPRp. If the downregulation can occur without the preceding upregulation, it is difficult to view this as a feedback mechanism. Instead, the cleavage of pADPRp appears to represent one of several proteolytic events that commonly occur during apoptosis (see below).

**Implications for Programmed Cell Death.** Studies in a number of laboratories are directed at elucidating the common features of physiological cell death (see “Introduction”). Because of its almost ubiquitous activation during apoptosis (reviewed in Refs. 5, 26, and 27), much current effort is focused on the identification and characterization of the “apoptotic endonuclease.” Other frequently observed features of the apoptotic process such as the induction of the TRPM-2 gene (92) or the requirement for new protein synthesis (70–74) have been shown to be absent from certain cell types [absence of TRPM-2 induction (14, 93); apparent absence of requirement for protein synthesis (58, 75–79)]. On the other hand, the proteolytic process described in the present work appears to be widespread.

There is ample precedent for the suggestion that proteases might play a role in apoptosis. Experimental results in the 1960s and 1970s revealed that cathepsin activity increased in tissues undergoing apoptosis (94, 95). Increased protein turnover rates were subsequently demonstrated in isolated thymocytes undergoing apoptosis (96). More recent results suggested that quantitatively abundant nuclear proteins such as pADPRp (58, 59) and lamin B (58, 59, 97) were proteolytically cleaved during apoptosis in the limited number of experimental systems examined. The present results extend these earlier studies by demonstrating that specific cleavage of the Mr, 116,000 pADPRp polypeptide to an Mr, ~85,000 fragment accompanies internucleosomal DNA degradation in a variety of model systems and appears to be a hallmark of chemotherapy-induced apoptosis.

Since treatments that inhibit this proteolytic cleavage also inhibit the internucleosomal DNA degradation (Fig. 10A; see also Ref. 83) and the morphological changes associated with physiological cell death (Fig. 10C), the possibility that limited proteolysis is an important controlling factor in initiating apoptosis needs to be considered. In this context, the recent suggestion that the apoptotic endonuclease is a preexisting cellular enzyme which decreases in molecular mass as cells undergo apoptosis (34) raises the interesting possibility that a proteolytic process similar to the one described in the present paper plays a role in activating the apoptotic endonuclease. Further studies are required to assess this possibility.

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CLEAVAGE OF POLY(ADP-RIBOSE) POLYMERASE


Specific Proteolytic Cleavage of Poly(ADP-ribose) Polymerase: An Early Marker of Chemotherapy-induced Apoptosis

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