Poly(ADP-ribosyl)ation of p53 during Apoptosis in Human Osteosarcoma Cells

Cynthia M. Simbulan-Rosenthal, Dean S. Rosenthal, Ruiba Lu, and Mark E. Smulson

Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, Washington, DC 20007

ABSTRACT

Spontaneous apoptosis in human osteosarcoma cells was observed to be associated with a marked increase in the intracellular abundance of p53. Immunoprecipitation and immunoblot analysis revealed that, together with a variety of other nuclear proteins, p53 undergoes extensive poly(ADP-ribosyl)ation early during the apoptotic program in these cells. Subsequent degradation of poly(ADP-ribose) (PAR), attached to p53 presumably by PAR glycohydrolase, the only reported enzyme to degrade PAR, was apparent concomitantly with the marked induction of expression of the p53-responsive genes bax and fas. These results suggest that poly(ADP-ribosyl)ation may play a role in the regulation of p53 function and implies a regulatory role for PARP and/or PAR early in apoptosis.

INTRODUCTION

p53, a tumor suppressor nuclear phosphoprotein, reduces the occurrence of mutations by mediating cell cycle arrest in G1 or G2-M or inducing apoptosis in cells that have accumulated substantial DNA damage, thus, preventing progression of cells through S phase before DNA repair is complete (1–3). One of the earliest nuclear events that follows DNA strand breakage during DNA repair in response to agents such as γ-irradiation, carcinogens, or alkylating agents is the poly(ADP-ribosyl)ation of various proteins that are localized near DNA strand breaks. PARP catalyzes the poly(ADP-ribosyl)ation of nuclear proteins only when bound to single-or double-stranded DNA ends (4–6) and cycles on and off the DNA ends during DNA repair in vitro (7–10). In addition to undergoing automodification, PARP catalyzes the poly(ADP-ribosyl)ation of various nuclear proteins as histones, topoisomerases I and II (11, 12), SV40 large T antigen (13), DNA polymerase α, proliferating cell nuclear antigen, and ~15 protein components of the DNA synthetase (12). The modification of nuclear proteins also alters the nucleosomal structure of the DNA containing strand breaks and promotes access of various replicative and repair enzymes to these sites (14, 15).

Additionally, depletion of PARP by antisense RNA expression has indicated that poly(ADP-ribosyl)ation plays an auxiliary role in the repair of DNA strand breaks (16, 17), in preferential gene repair (18), in the survival of cells after exposure to various alkylating agents, in gene amplification (19), in differentiation-linked DNA replication (12, 20, 40), and recently, in an early stage of apoptosis (21). Given that PARP is only catalytically active when bound to DNA strand breaks, when PARP undergoes caspase-3-mediated cleavage into M1 89,000 and M2 24,000 fragments during drug-induced (22) or spontaneous (23, 24) apoptosis, separation of its DNA binding domain from its catalytic site essentially inactivates the enzyme. PARP has also been implicated in the induction of p53 expression during apoptosis (25). The specific proteolytic cleavage of PARP by caspase-3 is a key apoptotic event because PARP cleavage and inactivation as well as subsequent apoptotic events are blocked by a peptide inhibitor of this protease (23, 26).

We have shown recently that a transient poly(ADP-ribosyl)ation of nuclear proteins in intact human osteosarcoma cells occurs early in apoptosis, prior to commitment to cell death, and is subsequently followed by cleavage and inactivation of PARP (24). No PAR is synthesized at the later stages of apoptosis, despite the presence of a large number of DNA strand breaks at this time. By depleting 3T3-L1 and Jurkat T cells of PARP by antisense RNA expression, or with the use of immortalized fibroblasts derived from PARP knockout (~−/−) mice, we demonstrated that prevention of this early activation of PARP blocks various biochemical and morphological changes associated with apoptosis (21), thus correlating the early poly(ADP-ribosyl)ation with later events in the cell death cascade.

p53 is induced by a variety of apoptotic stimuli and is required for apoptosis in many cell systems (27); overexpression of p53 is sufficient to induce apoptosis in various cell types (28). Interestingly, p53 can use transcription activation of target genes and/or direct protein-protein interaction to initiate p53-dependent apoptosis. It was shown recently that p53 is poly(ADP-ribosyl)ated in vitro by purified PARP, and that binding of p53 to a specific p53 consensus sequence prevents its covalent modification (29). We now show for the first time that modification of p53 by poly(ADP-ribosyl)ation also occurs in vivo, and that it represents one of the early acceptors of poly(ADP-ribosyl)ation during apoptosis in human osteosarcoma cells. Given that the in vivo half-life of PAR chains on an acceptor has been estimated to be about 1–2 min, we have additionally explored how this posttranslational modification of p53 is altered at the onset of caspase-3-mediated cleavage and inactivation of PARP during the later stages of the death program.

MATERIALS AND METHODS

Cell Culture and Induction of Apoptosis. Human osteosarcoma cells (23, 24) were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cell cultures were maintained as exponentially growing cells in a humidified 5% CO2 incubator.

Immunoprecipitation and Immunoblot Analysis. For immunoblot analysis, SDS-PAGE and transfer of proteins (30) were performed according to standard procedures. The membranes were stained with Ponceau S (0.5%) to confirm equal loading and transfer.

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2 To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, Basic Science Building, Room 351, 3900 Reservoir Road NW, Washington, DC 20007. Phone: (202) 687-1716; Fax: (202) 687-7186; E-mail: smulson@bc.georgetown.edu.
3 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP-ribose); mAb, monoclonal antibody.

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Clonal antibodies to p53 (Calbiochem). After centrifugation, the supernatants were then incubated for 1 h with 0.5 ml of NET-N buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) containing the anti-p53 mAb (2 μg/ml). The beads were washed five times with NET-N buffer, and the proteins bound to the beads were then separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis with the mAb to PAR or sheep polyclonal antibodies to p53 (Calbiochem).

**PARP-Cleavage Assay in Vitro.** PARP-cleavage assays were performed as described previously (23, 24). Full-length PARP cDNA pCD-12 (32) was used to synthesize [35S]methionine-labeled PARP by T7 RNA polymerase-mediated transcription and translation in a reticulocyte lysate system (Promega). Cytosolic extracts were prepared, and PARP-cleavage activity was measured in 25-μl reaction mixtures containing 5 μg of cytosolic protein, [35S]PARP (5 × 10⁴ cpm), 50 mM PIPES-KOH (pH 6.5), 2 mM EDTA, 0.1% 3-(3-cholamidopropyl)dimethy lammonio)-1-propanesulfonate, and 5 mM DTT. After incubation for 1 h at 37°C, reactions were terminated by the addition of SDS sample buffer, and PARP-cleavage products were detected by SDS-PAGE and fluorography.

Detection of Apoptotic Internucleosomal DNA Fragmentation. Total genomic DNA was extracted by lysing cells in 7 M guanidine hydrochloride and purified using a Wizard Miniprep Purification Resin (Promega). Apoptotic internucleosomal DNA fragmentation was then detected by gel electrophoresis (1% agarose) and ethidium bromide staining, as described previously (33).

**RESULTS**

Induction of Biochemical Markers of Apoptosis during Spontaneous Apoptosis in Human Osteosarcoma Cells. A transient burst of poly(ADP-ribosyl)ation of nuclear proteins occurs early during apoptosis in a number of different cell lines (21, 24, 34). Because rapid accumulation of p53 also occurs early during apoptosis (28, 35), we wanted to investguate whether p53 is one of the poly(ADP-ribosyl)ated proteins during the early burst of PARP activity. Human osteosarcoma cells were plated under conditions that result in spontaneous apoptosis over a 10-day period (23, 24). Biochemical markers of apoptosis were initially observed at day 5 and maximized around days 7–9, including caspase-3-mediated in vitro PARP-cleavage activity (Fig. 1A), proteolytic processing of the caspase-3 proenzyme (CPP32) to its active form (p17; Fig. 1B), and internucleosomal DNA fragmentation (Fig. 1C). During apoptosis, PARP is primarily cleaved by caspase-3 (23, 26), a member of the caspase family of aspartate-specific cysteine proteases that play a central role in the execution of the death program (36).

**p53 Accumulation, in Vivo PARP Cleavage, and Poly(ADP-ribosyl)ation of Nuclear Proteins during Spontaneous Apoptosis in Osteosarcoma Cells.** Consistent with previous studies showing p53 accumulation during early apoptosis in different cell lines, immunoblot analysis with anti-p53 mAbs of extracts of osteosarcoma cells at various stages of spontaneous apoptosis revealed that endogenous levels of p53 protein were significantly increased as early as days 2–3, maximized at day 4, and declined thereafter (Figs. 2A and 2B). Immunoblot analysis with antibodies to PARP to monitor in vivo PARP cleavage during the same time frame showed that ~50% of endogenous PARP was cleaved to its Mₗ 89,000 fragment by day 7, and complete cleavage of PARP was noted by day 9 (Fig. 2B).

When the same extracts were subjected to immunoblot analysis with antibodies to PAR, low levels of polymer were observed at day 2 of apoptosis (Fig. 2C), indicating the absence of DNA strand breaks, PARP activity, or both. However, poly(ADP-ribosyl)ation of nuclear proteins was markedly increased at day 3 and was maximal at day 4, a stage at which all of the cells were still viable and could be replated, prior to any evidence of internucleosomal DNA fragmentation. Subsequently, a marked decline in poly(ADP-ribosyl)ation of nuclear proteins was observed at later time points (days 7–9), concomitant with the onset of substantial DNA fragmentation, proteolytic activation of caspase-3, and caspase-3-mediated in vitro and in vivo cleavage of PARP. The specificity of the anti-PAR antibody used in these experiments has previously been confirmed biochemically in experiments showing that removal of PAR from immunoblots by phosphodiesterase treatment eliminates the polymer signal (12). PAR chains are cleaved from proteins by incubation with phosphodiesterase (37). When HeLa cell extracts were incubated in vitro in the presence or absence of NAD, poly(ADP-ribosyl)ated proteins were specifically detected only in the NAD-treated extracts after immunoblot analysis with the antibody to PAR; however, when duplicate lanes of the membrane were incubated with phosphodiesterase, no immunoreactivity was detected when reprobed with anti-PAR, thus verifying the specificity of this antibody (12).

**Poly(ADP-ribosyl)ation of p53 during Spontaneous Apoptosis in Osteosarcoma Cells as Verified by Immunoprecipitation.** Wild-type p53 can be modified by poly(ADP-ribosyl)ation in vitro using purified proteins (29). Poly(ADP-ribosyl)ation of nuclear proteins in response to DNA strand breaks is transient in vivo and is likely restricted mainly to the pool of potential target proteins located adjacent to DNA breaks (6). For example, <1% of the histone H1 pool is poly(ADP-ribosyl)ated both in vivo (38) and in vitro (5). Thus, detection of poly(ADP-ribosyl)ated p53 is likely to be difficult in most cell lines because only a small proportion of the available p53 is expected to be poly(ADP-ribosyl)ated in vivo at any one time, and the abundance of p53 in cells is normally low.

To confirm if p53 undergoes poly(ADP-ribosyl)ation in vivo during apoptosis in human osteosarcoma cells, cell extracts were derived at various times during spontaneous apoptosis and subjected to immunoprecipitation with an anti-p53 mAb. The immunoprecipitated proteins

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**Fig. 1.** Time courses of in vitro caspase-3 (PARP-cleavage) activity (A), proteolytic activation of CPP32 to its active form (p17; B), and internucleosomal DNA fragmentation (C) during spontaneous apoptosis in human osteosarcoma cells. At the indicated times during spontaneous apoptosis, cytosolic extracts were prepared and assayed for in vitro PARP-cleavage activity with [35S]PARP as substrate (A) or subjected to immunoblot analysis with a mAb to the p17 subunit of caspase-3 (B). The positions of full-length PARP and of its Mₗ 89,000 and Mₗ 24,000 cleavage products as well as CPP32 and p17 are indicated. C, total genomic DNA was extracted, and internucleosomal DNA ladders characteristic of apoptosis were detected by agarose gel electrophoresis and ethidium bromide staining. The positions of the DNA size standards (in kilobases) are indicated.

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(mainly p53 and its binding partners) were then subjected to immunoblot analysis with mAb to PAR. This approach revealed marked poly(ADP-ribosyl)ation of p53 at the early stages of apoptosis (days 3–4; Fig. 3A), coincident with the burst of PAR synthesis during this stage (Fig. 2C). The extent of poly(ADP-ribosyl)ation of p53 declined concomitant with the onset of both in vitro and in vivo caspase-3-mediated PARP cleavage (days 5–9; Figs. 1A and 2B). Reprobing of the blot with polyclonal antibodies to p53 confirmed that the modified protein was indeed p53 (Fig. 3B). On the other hand, no nonspecific binding of p53 was apparent when immunoprecipitation was performed with control antibodies (preimmune serum, Fig. 3, Lanes C) or with protein A-Sepharose beads alone (Fig. 3, Lanes B). The observation that p53 is specifically poly(ADP-ribosylated) during the early stages of spontaneous apoptosis in human osteosarcoma cells suggests that this posttranslational modification may play a role in regulating its function during the early phases of the cell death cascade.

Time Course of Induction of Bax and Fas Expression during Spontaneous Apoptosis in Osteosarcoma Cells. PARP can modulate the catalytic activity of a number of DNA-binding nuclear enzymes by catalyzing their poly(ADP-ribosylation), including DNA polymerases α and δ (39, 40) and DNA topoisomerases I and II (11, 41, 42). In most instances, poly(ADP-ribosylation) inhibits the activity of the modified protein, presumably because of a marked decrease in DNA-binding affinity caused by electrostatic repulsion between DNA and PAR. Thus, posttranslational modification of p53 may also alter DNA binding to specific DNA sequences in the promoters of target genes associated with the induction of p53-mediated apoptosis, such as those encoding Bax, IGF-BP3 (43), or Fas (44). The time course of accumulation and poly(ADP-ribosylation) of p53 during the early stages of apoptosis was thus correlated with the induction of expression of the p53-responsive genes bax and Fas. Immunoblot analysis of extracts of cells at various stages of apoptosis in osteosarcoma cells with antibodies to either Bax or Fas revealed that expression of both Bax and Fas (upper and middle panels, respectively) were negligible before and at the peak of p53 accumulation and poly(ADP-ribosylation) (days 3 and 4). Although p53 accumulation was already significantly elevated by day 2 (Fig. 3B), expression of Bax and Fas was markedly induced only at day 5 (Fig. 4), concomitant with a decline in PAR attached to p53 and the onset of caspase-3-mediated PARP cleavage and inactivation. The coincident decrease in PAR covalently bound to p53 and induction of Bax and Fas expression suggest that poly(ADP-ribosylation) may regulate p53 function early in apoptosis; caspase-3-mediated cleavage of PARP may release p53 from poly(ADP-ribosylation)-induced inhibition at the later stages of the apoptotic cascade.

**DISCUSSION**

Both PARP activity and p53 accumulation are induced by DNA damage, and both proteins have been implicated in the normal cellular...
responses to such damage. Whereas PAR synthesis increases within seconds after induction of DNA strand breaks (45), the amount of wild-type p53, which is usually low because of the short half-life (20 min) of the protein, increases 2–5 h after DNA damage as a result of reduced degradation (46, 47). A functional association of PARP and p53 has recently been suggested by coinmunoprecipitation of each protein in vitro by antibodies to the other (48, 49).

Exposure of human cell lines expressing wild-type p53 to various DNA-damaging agents that also stimulate PAR synthesis (including ionizing radiation, bleomycin, and DNA topoisomerase-targeting drugs) results in a rapid increase in the intracellular concentration of p53 (50). Chinese hamster cells that are unable to synthesize PAR because of unavailability of NAD show a marked decrease in baseline p53 concentration and activity, and they fail to exhibit a p53 response and to undergo apoptosis in response to DNA-damaging agents (25). Moreover, compared with wild-type cells, primary fibroblasts from PARP−/− mice express lower constitutive levels of p53 protein and exhibit a defective induction of p53 in response to DNA damage (51), indicating that PARP-dependent signaling may influence the synthesis or degradation of p53 in response to DNA damage.

In human osteosarcoma cells undergoing spontaneous apoptosis, a transient burst of poly(ADP-ribosyl)ation of nuclear proteins occurs early and is followed by caspase-3-mediated cleavage of PARP (24). Such an early burst of poly(ADP-ribosyl)ation was also observed in human HL-60 and Jurkat T cells, mouse 3T3-L1 cells, and immortalized fibroblasts derived from wild-type mice undergoing Fas-mediated or campothecin-induced apoptosis (21). The substantial nuclear poly(ADP-ribosyl)ation early in the death program suggests a potential role for PAR synthesis at this reversible stage in apoptosis, and it is consistent with the presence of large (~1 Mb) chromatin fragments at this time (52), given that the catalytic activation of PARP is absolutely dependent on DNA strand breaks. We recently investigated the effects of preventing this early transient modification of nuclear proteins by depletion of PARP either by antisense RNA expression or by gene disruption on various morphological and biochemical markers of apoptosis (21). Whereas control mouse fibroblasts or Jurkat T cells exhibit proteolytic conversion of the CP32 proenzyme to caspase-3, caspase-3 (PARP-cleavage) activity, internucleosomal DNA fragmentation, and characteristic nuclear morphological changes on induction of apoptosis, cells depleted of PARP by antisense RNA expression or by gene disruption on various morphological and biochemical markers of apoptosis (21). Whereas control mouse fibroblasts or Jurkat T cells exhibit proteolytic conversion of the CP32 proenzyme to caspase-3, caspase-3 (PARP-cleavage) activity, internucleosomal DNA fragmentation, and characteristic nuclear morphological changes on induction of apoptosis, cells depleted of PARP by antisense RNA expression or by gene disruption on various morphological and biochemical markers of apoptosis (21).

Furthermore, whereas immortalized fibroblasts derived from wild-type (PARP+/+) mice show the early burst of poly(ADP-ribosyl)ation and a rapid apoptotic response when induced into Fas-mediated apoptosis, fibroblasts derived from PARP−/− mice exhibit neither the early poly- (ADP-ribosyl)ation nor any of the biochemical or morphological changes characteristic of apoptosis when similarly treated (21). Stable transfection of PARP−/− fibroblasts with wild-type PARP cDNA sensitizes these cells to Fas-mediated apoptosis, suggesting that PARP and poly(ADP-ribosyl)ation may play an essential role in the early stages of apoptosis.

Accordingly, p53 may represent a potentially relevant target for poly- (ADP-ribosyl)ation during the burst of PAR synthesis at the early periods of apoptosis (Fig. 2C). Colocalization of PARP and p53 in the vicinity of large DNA breaks and their physical association (48, 49) suggest that poly(ADP-ribosyl)ation may regulate the DNA binding ability and, consequently, the function of p53. We have now shown that spontaneous apoptosis in osteosarcoma cells is associated with a marked increase in the intracellular p53 concentration early in the death program (Figs. 2A and 3B). This accumulation of p53 may be due to induced expression of the protein by the apoptotic stimuli or stabilization by inhibition of p53 degradation via modification of the protein. Furthermore, immunoprecipitation experiments revealed that p53 undergoes extensive poly(ADP-ribosyl)ation (Fig. 3) during the transient burst of PAR synthesis at the early stages of apoptosis; this occurs at a reversible stage when cells are still viable. This is the first report of poly(ADP-ribosyl)ation of p53 in vivo and suggests a negative regulatory role for PARP and/or PAR early in apoptosis. Subsequent degradation of PAR attached to p53 coincided with the increase in caspase-3 (PARP-cleavage) activity as well as the induction of expression of the p53-responsive genes bax and Fas at a stage when cells are irreversibly committed to death. Although the mechanism(s) of action of the Bax/Bcl2 family of gene products during apoptosis remains to be clarified, induction of Bax expression may influence the decision to commit to apoptosis because homodimerization of Bax promotes cell death, and heterodimerization of Bax with Bcl2 inhibits the antiapoptotic function of Bcl2 (43). Wild-type p53, but not mutant p53, also up-regulates Fas expression during chemotherapy-induced apoptosis, and p53-responsive elements were identified recently within the first intron and the promoter of the Fas gene (44). Binding of Fas to Fas ligand recruits the adapter molecule FADD via shared protein motifs (death domains), resulting in subsequent activation of the caspase cascade leading to apoptosis.

Electrophoretic mobility-shift analysis has shown that PAR attached to p53 in vitro can block its sequence-specific binding to the palindromic p53 consensus sequence, suggesting that poly(ADP-ribosyl)ation of p53 may regulate p53-mediated transcriptional activation of genes important in the cell cycle and apoptosis (53). PARP cycles on and off DNA ends in the presence of NAD, and its automodification during DNA repair presumably allows access to DNA repair enzymes (7–9). Our results with in vivo poly(ADP-ribosyl)ation of p53 suggest that p53 may, similarly, cycle on and off its DNA consensus sequence, depending on its level of negative charge based on its poly(ADP-ribosyl)ation state. This may represent a mechanism for regulating transcriptional activation of bax and Fas by p53 during apoptosis. Alternatively, a polymer binding site in p53 has been localized near a proteolytic cleavage site (53), indicating that PAR binding could protect this sequence from proteolysis; similar protection has been noted after binding of monoclonal antibodies adjacent to this region (54). The significant poly(ADP-ribosyl)ation of p53 early in apoptosis, therefore, suggests that this posttranslational modification could also play a role in p53 up-regulation by protecting the protein from proteolytic degradation.

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