Transient Poly(ADP-ribosyl)ation of Nuclear Proteins and Role of Poly(ADP-ribose) Polymerase in the Early Stages of Apoptosis*

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A transient burst of poly(ADP-ribosyl)ation of nuclear proteins occurs early, prior to commitment to death, in human osteosarcoma cells undergoing apoptosis, followed by caspase-3-mediated cleavage of poly(ADP-ribose) polymerase (PARP). The generality of this early burst of poly(ADP-ribosyl)ation has now been investigated with human IL-60 cells, mouse 3T3-L1, and immortalized fibroblasts derived from wild-type mice. The effects of eliminating this early transient modification of nuclear proteins by depletion of PARP protein either by antisense RNA expression or by gene disruption on various morphological and biochemical markers of apoptosis were then examined. Marked caspase-3-like PARP cleavage activity, proteolytic processing of CPP32 to its active form, internucleosomal DNA fragmentation, and nuclear morphological changes associated with apoptosis were induced in control 3T3-L1 cells treated for 24 h with anti-Fas and cycloheximide but not in PARP-depleted 3T3-L1 antisense cells exposed to these inducers. Similar results were obtained with control and PARP-depleted human Jurkat T cells. Whereas immortalized PARP +/+ fibroblasts showed the early burst of poly(ADP-ribosyl)ation and a rapid apoptotic response when exposed to anti-Fas and cycloheximide, PARP −/− fibroblasts exhibited neither the early poly (ADP-ribosyl)ation nor any of the biochemical or morphological changes characteristic of apoptosis when similarly treated. Stable transfection of PARP −/− fibroblasts with wild-type PARP rendered the cells sensitive to Fas-mediated apoptosis. These results suggest that PARP and poly(ADP-ribosyl)ation may trigger key steps in the apoptotic program. Subsequent degradation of PARP by caspase-3-like proteases may prevent depletion of NAD and ATP or release certain nuclear proteins from poly(ADP-ribosyl)ation-induced inhibition, both of which might be required for late stages of apoptosis.

It is characterized by marked morphological changes such as membrane blebbing, chromatin condensation, nuclear breakdown, and the appearance of membrane-associated apoptotic bodies, as well as by internucleosomal DNA fragmentation. The enzyme poly(ADP-ribose) polymerase (PARP) catalyzes the poly(ADP-ribosyl)ation of various nuclear proteins with NAD as substrate, and, because it is activated by binding to DNA ends or strand breaks, PARP has been suggested to contribute to cell death by depleting the cell of NAD and ATP (1). PARP undergoes proteolytic cleavage into 89- and 24-kDa fragments that contain the active site and the DNA-binding domain of the enzyme, respectively, during drug-induced apoptosis in a variety of cells (2). More recently, PARP has been implicated in the induction of both p53 expression and apoptosis (3), with the specific proteolysis of the enzyme thought to be a key apoptotic event (4–6).

Caspase-3, a member of the caspase family of 10 or more aspartate-specific cysteine proteases that play a central role in the execution of the apoptotic program (7), is primarily responsible for the cleavage of PARP during cell death (4, 5). Other caspases, such as caspase-7, also cleave PARP in vivo but at lower efficiencies. Composed of two subunits of 17 and 12 kDa that are derived from a common proenzyme (CPP32), caspase-3 is related to interleukin-1β-converting enzyme and CED-3, which is required for apoptosis in Caenorhabditis elegans (8).

In human osteosarcoma cells that undergo confluence-associated apoptosis over a 10-day period, caspase-3-like activity, measured with a specific [35S]PARP-cleavage assay in vitro, peaks at 6–7 days after initiation of apoptosis, concomitant with the onset of internucleosomal DNA fragmentation (4).

We recently examined the time course of PARP activation and cleavage during apoptosis in intact osteosarcoma cells by immunofluorescence microscopy with antibodies to PARP, to the 24-kDa cleavage product of PARP, and to poly(ADP-ribose) (9). We observed a transient burst of synthesis of PAR from NAD that increased early and was maximal 3 days after initiation of apoptosis, prior to the appearance of internucleosomal DNA cleavage (at day 7) and before the cells became irreversibly committed to apoptosis. During this early period, expression of full-length PARP was detected by both immunofluorescence and immunoblot analysis. The amounts of both PAR and PARP decreased thereafter, and at 6 days, the 24-kDa cleavage product of PARP was detected both immunocytochemically and by immunoblot analysis. PAR was not observed during days 8 to 10, despite the presence of abundant DNA strand breaks, potential activators of PARP, during this time.

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These observations suggested that short-lived PARP-catalyzed poly(ADP-ribose)ylation may be important at an early stage of apoptosis and is followed by the cleavage of PARP by mid-apoptosis. We have now investigated whether this transient poly(ADP-ribose)ylation occurs in other cell lines and with other inducers of apoptosis. We examined both cell lines stably transfected with inducible PARP antisense constructs (9–11) and immortalized fibroblasts derived from PARP knockout mice (12) to determine the effect of preventing the early burst in PARP activity on specific markers of apoptosis.

Several PARP knockout mice have been established by disrupting the PARP gene in embryonic stem cells; these animals neither express PARP nor exhibit any poly(ADP-ribose)ylation (12, 13). In contrast, >80% of primary embryonic fibroblasts derived from the earlier PARP knockout mice lost viability when exposed to anti-Fas (1000 ng/ml) for 8 h; thus, the absence of PARP does not seem to interfere with programmed cell death in these primary cells (14).

Our previous studies with clonal cells depleted of PARP by expression of PARP antisense RNA have supported accessory roles for PARP and/or poly(ADP-ribose)ylation in adipocyte differentiation (11), DNA replication associated with this differentiation (15, 63), genomic stability (10), and DNA repair (33, 16). In DNA repair, for example, although the absence of PARP did not totally prevent the repair of single-strand breaks, it resulted in a significant delay in this process (16). Primary cell cultures presumably consist of a mosaic of different stages of development, many of which perhaps possess compensatory routes to overcome gene disruption. It is possible that biochemical roles, not easily observable in the context of the whole animal or in primary cultures of cells, can be identified in clonal cells because of more profound effects observed in these cells.

The present study demonstrates the occurrence of a transient poly(ADP-ribose)ylation of nuclear proteins at an early stage of apoptosis induced by serum deprivation, camptothecin, or antibodies to Fas in different cell lines. When this early poly(ADP-ribose)ylation was prevented as a result of depletion of endogenous PARP, either by gene disruption or by antisense RNA expression, several morphological and biochemical markers of apoptosis were no longer observed in response to such inducers.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Vectors, and Transfection**—A 1.1-kilobase fragment of murine PARP cDNA encoding the DNA-binding domain and the NH₂-terminal automodification domain (for the mouse 3T3-L1 cell transfections) or a 3.7-kilobase XhoI full-length human PARP cDNA (for human Jurkat T cell transfection) was subcloned in an antisense orientation in the expression vector pMAM-neo (CLONTECH) under the control of the dexamethasone-inducible mouse mammary tumor virus promoter. The resulting pMAM-A (antisense) or pMAM-neo (control) plasmids were transfected into cells by calcium phosphate precipitation (3T3-L1 cells) or by electroporation (Jurkat cells). Transfectants were selected in appropriate medium with G-418 (400 μg/ml) (Life Technologies, Inc.). Stably transfected 3T3-L1 cells and fibroblasts derived from both wild-type and PARP knockout mice (12) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The PARP+/+ and −/− fibroblasts, immortalized by a standard 3T3 protocol, were kindly provided by Z. Q. Wang (International Agency for Research on Cancer, Lyon, France). Jurkat T cells and HL-60 cells were maintained in RPMI 1640 supplemented with 10% and 20% fetal bovine serum, respectively. PARP −/− fibroblasts were either cotransfected with a plasmid expressing wild-type PARP (pCD-12; Ref. 17) along with the plasmid pTracerCMV (Invitrogen), a zeocin-based vector system, or with the rescue construct pCMVΔ-CMV alone using lipofectamine (Life Technologies, Inc.). This vector system was utilized as the PARP −/− fibroblasts expressed an endogenous neo gene, which was used to establish the original PARP knockout mice. Stable transfectants were colony-selected in growth medium containing 500 μg/ml Zeocin.

**PARP-cleavage Assay**—In vitro PARP-cleavage assays were performed essentially as described previously (9, 42). In brief, full-length human PARP cDNA was excised from pCD-12 (17), ligated into the XhoI site of pBluescript II SK⁺ (Stratagene), and used to synthesize [32P]methionine-labeled PARP by coupled T7 RNA polymerase-mediated transcription and translation in a reticulocyte lysate system (Promega). Cytosolic extracts of various cells were prepared by rapid freezing and thawing of cells in a solution containing 10 mM Hepes-KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS detergent, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, peptatin A (10 μg/ml), leupeptin (10 μg/ml), and aprotinin (10 μg/ml), followed by centrifugation of the cell lysate at 100,000 × g for 30 min and recovery of the supernatant. In vitro PARP-cleavage activity was measured in 25-μl reaction mixtures containing 5 μg of cytosolic protein, [32P]PARP (5 × 10⁴ cpm), 50 mM Pipes-KOH (pH 6.8), 100 mM NaCl, 5 mM dithiothreitol. After incubation for 1 h at 37°C reactions were terminated by the addition of SDS sample buffer (4% SDS, 4% 2-mercaptoethanol, 10% glycerol, 125 mM Tris-HCl (pH 6.8), and 0.02% bromophenol blue). Proteins were resolved by SDS-polyacrylamide gel electrophoresis, and PARP-cleavage products were visualized by fluorography.

**Indirect Immunofluorescence Microscopy and Immunoblot Analysis**—The procedures for fixation and staining with monoclonal antibodies to PAR (10HA) (18) have been described previously (9). Cells were transferred to a slide in a Cytospin (IEC Centra), fixed with 10% (v/v) ice-cold trichloroacetic acid for 10 min, and dehydrated in 70%, 90%, and absolute ethanol for 3 min each at −20°C. The slides were then incubated overnight in a humid chamber at room temperature with antibodies to PAR (1:250 dilution) in phosphate-buffered saline (PBS) containing 12% bovine serum albumin. After washing with PBS, cells were incubated for 1 h with biotinylated anti-mouse IgG (1:400 dilution) in PBS containing 12% bovine serum albumin. After washing with PBS, cells were incubated with streptavidin-conjugated Texas red (1:800 dilution in PBS containing 12% bovine serum albumin). Cells were finally mounted with PBS containing 80% glycerol and observed with a Zeiss fluorescence microscope. All exposure times were identical to allow comparisons of relative staining intensities at various times during apoptosis.

For immunoblot analysis, SDS-polyacrylamide gel electrophoresis and transfer of proteins (30 μg per lane) to nitrocellulose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. After blocking of non-specific sites, membranes were incubated with antibodies to PARP (1:2000 dilution) (19) or to caspase-3 (1:10,000 dilution). After blocking of nonspecific sites, membranes were probed with appropriate peroxidase-labeled antibodies to mouse or rabbit IgG (1:3000 dilution), and immune complexes were detected by enhanced chemiluminescence (Pierce). For detection of PAR bound to the separated proteins in the same blots, the membranes were stripped of antibodies by incubation at 50°C for 30 min in a solution containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7); after blocking of non-specific sites, they were reprobed with monoclonal antibodies to PAR (1:250 dilution).

**PARP Activity Assays**—At indicated time intervals, cells were harvested by scraping, washed with ice-cold PBS, and assayed for PARP activity as described previously (20). Briefly, cells were sonicated for 20 s (three times) to lyse cells and introduce DNA strand breaks required for PARP activity, followed by measurement of [32P]NAD incorporation into acid-insoluble acceptors at 25°C for 1 min.

**Detection of Apoptotic Internucleosomal DNA Fragmentation**—Cells were washed in PBS and lysed in 7% guanidine hydrochloride, and total genomic DNA was extracted and purified using a Wizard MiniPrep DNA Purification Resin (Promega). After RNase A treatment (1 μg/50 μl) of the DNA samples for 30 min, apoptosis-associated DNA fragmentation was detected by gel electrophoresis on a 1% agarose gel and ethidium bromide staining (0.5 μg/ml) as described previously (64).

** Hoechst Staining for Apoptotic Morphology**—Cells were centrifuged at 1000 rpm for 5 min, fixed for 10 min in PBS containing 4% formalin, washed with PBS, and stained with Hoechst 33258 (24 mg/ml) in PBS containing 80% glycerol. An aliquot (25 μl) of the cell suspension was
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RESULTS

A Transient Burst of Poly(ADP-ribosyl)ation of Nuclear Proteins Occurs during Early Stages of Apoptosis in HL-60 Cells—To investigate whether the brief activation of PARP during the early stages of apoptosis, detected initially by immunofluorescence in osteosarcoma cells (9), is a general phenomenon, we examined several different cell types. The transient PARP activation occurs prior to the induction of caspase-3-like activity, as measured by the in vitro cleavage of [35S]methionine-labeled PARP into 89- and 24-kDa fragments by cell extracts derived at various stages of apoptosis. This burst of PAR synthesis also occurs before the onset of internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis, which has been shown to begin on day 7 and peak at day 10, when almost 100% of the cells have undergone apoptosis, as evidenced by agarose gel electrophoresis and ethidium bromide staining. C, caspase-3-like PARP-cleavage activity in cytosolic extracts was assayed with [35S]PARP as substrate.

then dropped onto a slide, and nuclear morphology was observed with an Olympus BH2 fluorescence microscope.

FIG. 1. Time course of poly(ADP-ribosyl)ation of nuclear proteins (A), DNA fragmentation (B), and PARP-cleavage activity (C) during camptothecin-induced apoptosis in human HL-60 cells. A, apoptosis was induced in human HL-60 cells by incubation with camptothecin (10 μM); at the indicated times, cell extracts were prepared and subjected to immunoblot analysis with antibodies to PAR. B, apoptosis was monitored by extraction of total genomic DNA and detection of characteristic internucleosomal DNA ladders by agarose gel electrophoresis and ethidium bromide staining. C, caspase-3-like PARP-cleavage activity was assayed by immunoblotting of total cytosolic extracts with anti-PARP antisera. Densitometric scanning showed that caspase-3-like activity (6–8 h), even in the presence of massive DNA fragments (Fig. 1B) at this time.

Transient Poly(ADP-ribosyl)ation of Nuclear Proteins Occurs Early during Fas-mediated Apoptosis in Murine 3T3-L1 Cells but Not in PARP-depleted 3T3-L1 Antisense Cells—We next examined murine 3T3-L1 cells that can be depleted of endogenous PARP by antisense RNA expression. We have previously used these cells, which are stably transfected with a dexamethasone-inducible PARP antisense construct, to investigate the role of PARP in differentiation (11, 15, 63). PARP was shown to be required for a round of DNA replication that precedes the onset of differentiation in these cells.

Because apoptosis has previously been induced in 3T3-L1 cells only by deregulated expression of c-Myc under conditions of serum deprivation (21), it was first necessary to establish conditions under which apoptosis could be triggered by exogenous inducers. 3T3-L1 cells transfected with the control vector (mock-transfected) were preincubated in the presence or absence of 1 mM dexamethasone for 72 h and then treated with various inducers of apoptosis. Tumor necrosis factor-α (TNF-α), camptothecin, or antibodies to Fas (anti-Fas) (even at a dose of 500 ng/ml, which is 10 times the concentration required to induce apoptosis in other cell lines) induced only a slight increase in caspase-3-like activity in mock-transfected cells (Fig. 2). However, incubation of cells with a combination of anti-Fas and cycloheximide resulted in a marked induction of caspase-3-like activity, as indicated by the generation of the 89- and 24-kDa cleavage fragments of PARP. Densitometric scanning showed that ∼60% of [35S]PARP substrate was converted to the 89-kDa cleavage product under these conditions. Cycloheximide alone did not induce apoptosis in these cells (data not shown). Cycloheximide has been previously shown to potentiate TNF-α-induced apoptosis (22–24) as well as overcome resistance to Fas-mediated apoptosis in various cells (24–26); this latter effect of cycloheximide does not seem to be mediated by inhibition of translation because resistant cells can be sensitized to anti-Fas by subinhibitory concentrations of the drug. The presence of dexamethasone during the 72-h preincubation of mock-transfected 3T3-L1 cells had no effect on induction of caspase-3-like activity (Fig. 2B).

It was important to determine the kinetics of PARP depletion induced by antisense RNA expression in 3T3-L1 cells under the present conditions, given that our previous studies with this cell line had focused on the role of PARP in differentiation.
were incubated in the absence or presence of 1 μM dexamethasone (Dex) for the indicated times, after which equal amounts of total cellular protein (30 μg) were resolved by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with rabbit antibodies to full-length PARP (A). The immunoblot was also stained with Ponceau S to confirm equal protein loading and transfer among the lanes (B). At indicated time intervals after exposure to dexamethasone, control and PARP antisense cells were harvested by scraping, washed with ice-cold PBS, and assayed for PARP activity as described under “Experimental Procedures” (C).

Immunoblot analysis revealed that the amount of PARP in mock-transfected 3T3-L1 cells was not affected by incubation with dexamethasone (Fig. 3A). However, in the PARP-antisense cells, dexamethasone induced a time-dependent depletion of PARP, with only ~5% of the protein remaining after 72 h. Ponceau S staining for total protein on the same immunoblot confirmed essentially equal protein loading and transfer among lanes (Fig. 3B). Whereas the in vitro PARP activity of control cells was not affected by incubation with dexamethasone, exposure of PARP-antisense cells to dexamethasone for 72 h resulted in an ~80% decrease in PARP activity (Fig. 3C).

3T3-L1 control cells that had been preincubated with dexamethasone for 72 h were exposed to anti-Fas and cycloheximide for various times and then subjected to immunoblot analysis with antibodies to PAR. As in HL-60 and osteosarcoma cells, the extent of poly(ADP-ribosyl)ation of nuclear proteins peaked early, 4 h after the induction of apoptosis in control 3T3-L1 cells, a stage at which all the cells were still viable and could be replaced, and subsequently decreased (Fig. 4A). The array of poly(ADP-ribosyl)ated nuclear proteins was consistent with automodification of PARP as well as the poly(ADP-ribosyl)ation of histones and other nuclear acceptor proteins (27). As anticipated, poly(ADP-ribosyl)ation of nuclear proteins was not detected in PARP-depleted 3T3-L1 antisense cells exposed to anti-Fas and cycloheximide for 24 h (Fig. 4B).

**Effects of the Absence of Early Transient Poly(ADP-ribosyl)ation on Morphological and Biochemical Markers of Apoptosis in 3T3-L1 Cells**—We next tried to determine whether prevention of the early burst of PAR synthesis by PARP antisense RNA expression could affect the development of other biochemical or morphological markers of apoptosis when these cells are exposed to apoptosis inducers. The combination of anti-Fas and cycloheximide induced a marked increase in caspase-3-like activity in mock-transfected 3T3-L1 cells that had been preincubated in the absence or presence of dexamethasone (Fig. 5A); this effect was maximal 24 h after induction of apoptosis. Whereas PARP-antisense 3T3-L1 cells that were not exposed to dexamethasone showed a similar increase in caspase-3-like activity in response to anti-Fas and cycloheximide, no such increase was apparent in PARP-antisense cells that had been depleted of PARP by preincubation with dexamethasone before exposure to anti-Fas and cycloheximide (Fig. 5B).

Caspase-3, similar to other members of the caspase family, is expressed in cells as an inactive 32-kDa proenzyme (CPP32). During apoptosis, CPP32 is activated by cleavage at specific Asp residues, with the active enzyme (caspase-3) consisting of a heterodimer of a 17-kDa subunit (p17), containing the catalytic domain, and a 12-kDa subunit (p12) (4). To confirm that CPP32 is proteolytically processed to p17 during apoptosis in control 3T3-L1 cells and to determine whether the transient early poly(ADP-ribosyl)ation is necessary for this activation, control and antisense cells were preincubated with dexamethasone and exposed to anti-Fas and cycloheximide for the indicated times; cell extracts were then subjected to immunoblot analysis with antibodies to the p17 subunit of caspase-3 (Fig. 5C). The amount of CPP32 increased in both control and antisense cells after exposure to anti-Fas and cycloheximide. However, whereas CPP32 was proteolytically processed to p17 by 24 h, coinciding with the peak of in vitro caspase-3-like PARP-cleavage activity, in control cells, proteolytic processing of CPP32 was not apparent in the PARP-depleted antisense cells. Furthermore, using DNA fragmentation analysis as another assay for apoptosis, control 3T3-L1 cells exposed to anti-Fas and cycloheximide for 24 h exhibited marked internucleosomal DNA fragmentation (DNA ladders), but not the PARP-depleted
Consistently, whereas antisense cells preincubated in the absence of dexamethasone showed changes in nuclear morphology typical of apoptosis when exposed to anti-Fas and cycloheximide, those depleted of PARP by preincubation with dexamethasone did not (Fig. 6A). Whereas 75% of the antisense cells, which were not preincubated with dexamethasone, exhibited chromatin condensation (arrowheads) and nuclear fragmentation (arrows) after 48 h of exposure to anti-Fas and cycloheximide for the indicated times as in (A) and (B). Cell extracts were subjected to immunoblot analysis with a monoclonal antibody to the p17 subunit of caspase-3. The positions of CPP32 and p17 are indicated. (D) 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 DNA size standards (in kilobases) are indicated (M).

Effects of PARP Depletion by Antisense RNA Expression on Induction of Apoptosis in Human Jurkat Cells—To confirm our results with 3T3-L1 control and antisense cells, we examined human Jurkat T cells stably transfected with either a PARP antisense RNA construct or the empty vector. Immunoblot analysis showed that preincubation of two different Jurkat antisense cell clones for 72 h with dexamethasone resulted in depletion of endogenous PARP by 99% (Fig. 7A). Because Jurkat cells express high levels of the Fas antigen (24, 29), anti-Fas alone was used to induce apoptosis in these cells. Similar to the other cell lines used in this study, Jurkat cells also exhibited an early transient peak of PAR synthesis 3 h after induction with anti-Fas (data not shown). Mock-transfected Jurkat cells preincubated in the absence of dexamethasone showed increases in PARP protein levels when exposed to anti-Fas and cycloheximide (10 μg/ml) for the indicated times. Cells were fixed, stained with Hoechst stain, and observed with a fluorescence microscope for morphological changes associated with apoptosis (pyknotic nuclei, chromatin condensation, nuclear fragmentation). Original magnification, 40×. Arrows indicate nuclear fragmentation; arrowheads indicate chromatin condensation.
depleted antisense cells treated with anti-Fas for the same time (Fig. 7B).

Anti-Fas also induced changes in nuclear morphological consistent with apoptosis in control Jurkat cells (Fig. 7C). More than 80% of mock-transfected cells that had been preincubated in the absence or presence of dexamethasone, as well as antisense cells preincubated without dexamethasone, showed either chromatin condensation or nuclear fragmentation after 24-h treatment with anti-Fas. However, consistent with the results obtained with the 3T3-L1 antisense cells, no such
PARP knockout and wild-type mice. PARP +/+ (left panels) and PARP −/− (right panels) fibroblasts were exposed to anti-Fas (100 ng/ml) and cycloheximide (10 μg/ml) for 0 h (upper panels) or 24 h (lower panels). Cells were fixed, stained with Hoechst stain, and observed under a fluorescence microscope for nuclear morphological changes associated with apoptosis. Original magnification, 40×.

**Fig. 10.** Effects of anti-Fas and cycloheximide on nuclear morphology of immortalized fibroblasts from PARP knockout and wild-type mice. PARP +/+ (left panels) and PARP −/− (right panels) fibroblasts were exposed to anti-Fas (100 ng/ml) and cycloheximide (10 μg/ml) for 0 h (upper panels) or 24 h (lower panels). Cells were fixed, stained with Hoechst stain, and observed under a fluorescence microscope for nuclear morphological changes associated with apoptosis. Original magnification, 40×.

Changes were evident in PARP-depleted Jurkat antisense cells exposed to anti-Fas (Fig. 7C).

**Transient Poly(ADP-ribose)ylation of Nuclear Proteins Also Occurs Early during Fas-mediated Apoptosis in PARP +/+ Fibroblasts but Not in PARP −/− Cells**—To investigate whether prevention of the early burst of PAR synthesis by gene disruption could likewise affect the induction of biochemical or morphological markers of apoptosis when these cells are exposed to apoptosis inducers, fibroblasts derived from wild-type (PARP +/+ ) and PARP knockout mice (PARP −/− ) (12), immortalized by the standard 3T3 protocol (28), were utilized. PARP −/− cells were confirmed devoid of PARP and PAR by immunoblot analysis with the corresponding antibodies (Fig. 8A). As with the other cell lines, these cells also exhibited a transient burst of poly(ADP-ribose)ylation of nuclear proteins as early as 1 h after exposure to anti-Fas and cycloheximide (Fig. 8B), and PAR synthesis markedly declined thereafter, presumably by a combination of caspase-3-like mediated PARP cleavage and PAR-glycohydrolase activity. As anticipated, no burst of poly(ADP-ribose)ylation was observed in PARP −/− fibroblasts after exposure to inducers of apoptosis for up to 6 h (Fig. 8B).

**Immortalized Fibroblasts Derived from PARP Knockout Mice Do Not Exhibit Morphological and Biochemical Markers Characteristic of Apoptosis**—Anti-Fas and cycloheximide induced a marked increase in caspase-3-like activity in PARP +/+ cells; this effect was maximal 24 h after induction of apoptosis, as indicated by the complete cleavage of PARP into 89- and 24-kDa fragments (Fig. 9A). In contrast, no such increase in caspase-3-like activity was evident in PARP −/− cells after exposure to anti-Fas and cycloheximide for up to 24 h.

To verify that CPP32 is proteolytically activated to caspase-3 during induction of apoptosis in these cells, extracts of cells that had been exposed to anti-Fas and cycloheximide for various times were subjected to immunoblot analysis with antibodies to CPP32. Whereas CPP32 was proteolytically processed to p17 in PARP +/+ fibroblasts exposed to anti-Fas and cycloheximide, no such effect was evident in the PARP −/− cells (Fig. 9B). The band migrating slightly faster than CPP32 corresponds to a protein that reacts nonspecifically with the antibodies to CPP32.

PARP +/+ cells showed substantial nuclear fragmentation and chromatin condensation 24 h after induction of Fas-mediated apoptosis (Fig. 10); ~97% of nuclei exhibited apoptotic morphology by this time. In contrast, no substantial changes in nuclear morphology were apparent in the PARP −/− fibroblasts even after exposure to anti-Fas and cycloheximide for 24 h (Fig. 10) or 48 h (data not shown).

**Transfection of PARP −/− Fibroblasts with Wild-type PARP Sensitizes These Cells to Fas-mediated Apoptosis**—PARP −/− fibroblasts were stably transfected with pcD-12, a plasmid expressing wild-type PARP (17). Immunoblot analysis showed that three different cell clones (1, 2, and 3) and pooled clones (P) expressed PARP protein similar to the PARP +/+ cells, whereas PARP −/− cells and the clone transfected with the vector alone (“vec”) did not (Fig. 11A). The ability of these clones to express PARP was also confirmed by in vitro PARP activity assays (data not shown).

These cells were induced to undergo apoptosis by exposure to anti-Fas and cycloheximide for up to 48 h. In vivo caspase-3-like PARP-cleavage activity was monitored by immunoblot analysis with antisera to PARP that recognizes both the 116-kDa PARP and its 24-kDa cleavage fragment (P1–23, Biomol) (Fig. 11B). PARP +/+ cells as well as PARP −/− clones stably transfected with PARP (clones 2 and 3) exhibited significant caspase-3-like activity after 48 h; ~95% of the PARP protein was cleaved in vivo to the 24-kDa cleavage fragment by 48 h (Fig. 11B). As expected, PARP was not expressed in the PARP −/− fibroblasts nor in −/− cells transfected with vector alone. Consistently, whereas exposure to anti-Fas and cycloheximide induced marked internucleosomal DNA fragmentation in PARP +/+ fibroblasts and PARP −/− cells stably transfected with PARP (clones 2 and 3), no apoptotic DNA ladders were evident in the PARP −/− cells when similarly treated (Fig. 11C).

Furthermore, exposure to anti-Fas plus cycloheximide for 48 h induced apoptotic nuclear morphology in PARP −/− cells transfected with PARP (clone 2) (Fig. 11D), almost to the same extent as the PARP +/+ cells (Fig. 10). Whereas ~85% of PARP-transfected cells showed either chromatin condensation or nuclear fragmentation after treatment with anti-Fas for 48 h (Fig. 11D), no such changes were apparent in mock-transfected PARP −/− cells when exposed to these inducers for the same time. These results confirm that the inability of the PARP −/− cells to undergo Fas-mediated apoptosis is attributed to the lack of PARP due to disruption of the gene and not due to other genetic alterations resulting from immortalization or clonal differences between the PARP +/+ and −/− cells.

**Table**

| Time after induction (h) | PARP +/+ | PARP −/− |
|-------------------------|----------|----------|
| 0                       |          |          |
| 24                      |          |          |
Transient Activation of PARP Early in Apoptosis

To investigate, without the use of possibly nonspecific chemical inhibitors (30–32), the potential roles of PARP and poly(ADP-ribosyl)ation in nuclear processes that require cleavage and rejoicing of DNA strands, we have previously established and characterized several mammalian cell lines, including HeLa (16), keratinocytes (33), and 3T3-L1 preadipocytes (11), that are stably transfected with PARP antisense cDNA under the control of an inducible promoter. Establishment of conditions at which endogenous PARP protein and activity can be substantially depleted at specific times by expression of PARP antisense transcripts has enabled us to investigate the roles of PARP in DNA repair, recovery of cells from exposure to mutagenic agents (10, 33), gene amplification (10), and differentiation-linked DNA replication (11, 15, 63).

A role for PARP in apoptosis has been suggested by studies showing that the enzyme undergoes proteolytic cleavage into 89- and 24-kDa fragments during chemotherapy-induced (2) or spontaneous (4) apoptosis. PARP cleavage by caspase-3 has been shown to be necessary for apoptosis (4, 5); the cleavage and inactivation of PARP as well as subsequent apoptotic events are blocked by a peptide inhibitor of this protease. We recently showed by immunofluorescence microscopy that poly-(ADP-ribosyl)ation of nuclear proteins occurs early in apoptosis, prior to commitment to cell death, and is followed by cleavage and inactivation of PARP; only small amounts of PAR remained during the later stages of apoptosis, despite the presence of a large number of DNA strand breaks (9). We have now shown that the transient burst of poly(ADP-ribosyl)ation of nuclear proteins during the early stages of apoptosis occurs in several other cell systems as well. Furthermore, by depleting the normally abundant PARP from 3T3-L1 and Jurkat cells by antisense RNA expression prior to the induction of apoptosis, or with the use of immortalized fibroblasts derived from PARP knockout mice, we have demonstrated that prevention of this early activation of PARP blocks various biochemical and morphological changes associated with apoptosis, thus correlating the early poly(ADP-ribosyl)ation with later events in the Fas-mediated cell death cascade.

In contrast, it was recently shown that primary, nonimmortalized PARP −/− splenocytes and fibroblasts, from the same strain of PARP knockout mice from which the immortalized fibroblasts used in the present study were derived, undergo apparently normal apoptosis in response to anti-Fas, TNF-α, γ-irradiation, or dexamethasone (14). Although the concentration of anti-Fas used to induce apoptosis in these primary PARP −/− cells was substantially higher than that used in our study with immortalized fibroblasts, the apparent discrepancy between the responses of the primary and immortalized cells remains to be clarified. This difference between the two types of cells may be related to the process of immortalization. The immortalized fibroblasts used in the present study are essentially clonal in comparison, whereas the primary cultures used in previous studies (13, 14) contain cells at various stages of development. Although the physiological relevance of using immortalized cell lines requires further study, our results with PARP-antisense cell lines (both murine and human) are consistent with our data obtained with the immortalized PARP −/− fibroblasts.

In both 3T3-L1 and Jurkat PARP-antisense cells, endoge-
nous PARP protein was substantially depleted after incubation with 1 μM dexamethasone for 72 h, whereas PARP abundance was unaffected by dexamethasone treatment in control cells. With the use of an in vitro PARP-cleavage assay, marked induction of caspase-3-like activity was observed after exposure to anti-Fas and cycloheximide in control 3T3-L1 cells and immortalized PARP+/+ fibroblasts, as well as in control Jurkat cells treated with anti-Fas, and HL-60 cells exposed to camptothecin. These various treatments also induced a transient burst of poly(ADP-ribosyl)ation before the onset of biochemical events associated with apoptosis in these cells. In contrast, PARP-depleted 3T3-L1 or Jurkat antisense cells as well as immortalized PARP−/− fibroblasts did not undergo this early transient poly(ADP-ribosyl)ation nor did they show any increase in caspase-3-like activity. These results show that induction of spontaneous apoptosis in osteosarcoma cells is associated with an increase in intracellular abundance of p53. Immunoprecipitation and immunoblot analysis further indicate that extensive poly-(ADP-ribosyl)ation of p53 occurs concomitantly with the burst of poly(ADP-ribosyl)ation and that subsequent degradation of PAR attached to p53 occurs concomitant with the increase in caspase-3-like activity. Thus, this posttranslational modification may play a role in the regulation of p53 function, or, alternatively, in its degradation during p53-dependent apoptosis. These results are consistent with recent studies showing substantial poly(ADP-ribosyl)ation of p53, with polymer chain lengths from 4 to 30 residues, in cells undergoing apoptosis in response to DNA damage (57, 58). Electrophoretic mobility shift analysis further showed that ADP-ribose polymers attached to p53 blocked its sequence-specific binding to a 26-base pair oligonucleotide containing the palindromic p53 consensus binding sequence, suggesting that poly(ADP-ribosyl)ation of p53 may negatively regulate p53-mediated transcriptional activation of genes important in the cell cycle and apoptosis (59). Recently, primary fibroblasts from PARP−/− mice were further shown to have a 2-fold lower basal level of p53 and are defective in the induction of p53 in response to DNA damage (60).

Finally, the activity of Ca2+, Mg2+-dependent endonuclease is inhibited by poly(ADP-ribosyl)ation in vitro (61), and the enzyme is implicated in internucleosomal DNA cleavage during apoptosis; it is identical in size and kinetic properties to DNase γ, which is thought to be responsible for DNA fragmentation during thymic apoptosis (62). This enzyme also seems to be a target of the early burst of poly(ADP-ribosyl)ation during spontaneous apoptosis in osteosarcoma and HL-60 cells (data not shown), suggesting a possible negative regulatory role for PARP in apoptosis, whereby inactivation by caspase-3-catalyzed cleavage may release specific nuclear proteins from poly-(ADP-ribosyl)ation-induced inhibition. These ongoing studies aim to clarify the apparently essential requirement, at least in the clonal immortalized mouse and human cells studied here, for the early and brief poly(ADP-ribosyl)ation that occurs during the initial stages of apoptosis.

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