Overexpression of Poly(ADP-ribose) Polymerase Promotes Cell Cycle Arrest and Inhibits Neutrophilic Differentiation of NB4 Acute Promyelocytic Leukemia Cells

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Abstract

The t(15;17) translocation causes a disruption of the retinoic acid receptor α (RAR-α) and allows for the expression of the PML-RARα fusion protein considered to be responsible for the differentiation block in acute promyelocytic leukemia (APL). Patients being treated with all-trans retinoic acid (ATRA) undergo remission due to the differentiation of leukemic cells to functional neutrophils but relapse due to subsequent ATRA resistance. Our group has shown recently that NB4 cells, the only in vitro model of APL, are capable of monocytic differentiation in response to 1,25-dihydroxyvitamin D3 and 12-O-tetradecanoylphorbol-13-acetate in addition to the neutrophilic differentiation response that occurs with ATRA treatment. Poly(ADP-ribose) polymerase (PARP) is a ubiquitous protein that plays a role in DNA metabolism and repair. We have shown that, unlike HL-60 cells, NB4 cells completely down-regulate PARP in the neutrophilic lineage and up-regulate PARP 90-fold in the monocytic lineage. To ascertain whether PARP is an active participant in the bipotential differentiation of APL cells, NB4 cells were transiently transfected by lipid-mediated gene transfer with the human PARP gene under the control of the human metallothionein promoter. A 4-fold overexpression of PARP, in response to 8 μM CdCl2, promoted arrest of NB4 cells in the S phase of the cell cycle. Overexpression of PARP alone had no effect on cell viability or induction of phenotypic markers in the monocytic or neutrophilic lineages. However, increased PARP expression did result in an increase in the number of cells in the subdiploïd population likely to include apoptotic cells. Overexpression of PARP, alone with 12-O-tetradecanoylphorbol-13-acetate (200 nm), 1,25-dihydroxyvitamin D3 (200 nm), or a suboptimal dose of the combined agents, did not alter the expected monocytic differentiation marker profile over cells transfected with control plasmid (pSV2Neo). In contrast, PARP overexpression blocked the appearance of phenotypic markers of terminally differentiated neutrophils in 85% of the transfected population in response to 1 μM ATRA. Comparable to wild-type NB4 cells, 90% of cells transfected with pSV2Neo developed neutrophilic differentiation markers (nitroblue tetrazolium-positive and multi-lobed nuclei) in response to 1 μM ATRA. These data suggest that overexpression of PARP arrests APL cells and blocks ATRA-induced terminal neutrophilic differentiation. We propose that normal down-regulation of PARP in NB4 cells is a requirement for neutrophilic maturation.

Introduction

APL is an M3 subtype of myeloid leukemia characterized by a specific t(15;17) translocation and clonal expansion of malignant myeloid cells blocked at a stage of hematopoietic differentiation that results in a high proportion of promyelocytes (1). The efficacy of induction of neutrophilic differentiation by ATRA in vitro and in vivo in APL has heightened interest in the mechanism of ATRA action (2, 3). Although patients receiving ATRA therapy undergo remission, relapse usually ensues due to associated ATRA resistance (4). This has prompted the need to characterize downstream effector molecules during ATRA-induced differentiation as possible targets of alternate differentiation therapy.

Other investigators have entertained the possibility of overcoming this leukemic phenotype with alternative differentiation therapies (5, 6). We have shown recently that NB4 cells, the only in vitro model of APL, are capable of monocytic differentiation in response to VD3 and TPA in combination (7). This provides an alternative pathway to induce the nonmalignant phenotype and potentially bypasses ATRA resistance.

During the process of myeloid differentiation in the monocytic and neutrophilic pathways, biochemical and morphological changes take place that are orchestrated by the genetic program of the leukemic cells. Current research implicates several proto-oncogenes as key players in growth and differentiation processes. These include components of

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4 The abbreviations used are: APL, acute promyelocytic leukemia; ATRA, all-trans-retinoic acid; VD3, 1,25-dihydroxyvitamin D3; TPA, 12-O-tetradecanoylphorbol-13-acetate; PARP, poly(ADP-ribose) polymerase; wt, wild type; NBT, nitroblue tetrazolium.
the AP-1 complex and myc, myb, and max transcription factors (8–11). However, only a few of these have been demonstrated to play instrumental roles in the differentiation process (8, 9).

Poly(ADP-ribose) polymerase (EC2.4.2.30) is a DNA-associated nuclear non-histone protein (12). PARP generates poly(ADP-ribose) by adding multiple ADP-ribose moieties to acceptor proteins, including those found in chromatin, utilizing NAD+ as the ADP-ribose donor (13, 14). PARP activity is stimulated following binding of its zinc fingers to single- or double-stranded breaks in DNA (15). Recently, two DNA-binding activities of PARP have been reported. The zinc finger region binds to DNA strand breaks, while other areas of the enzyme bind to intact double-stranded DNA, especially structures such as cruciform DNA complexes (16, 17).

Involvement of PARP in differentiation has been described in many cell systems, including those of hematopoietic origin (18, 19). Most of this information has been derived from human leukemia models such as myelocytic HL-60 cells, erythrocytic K-562 cells, and myelomonocytic U-937 cells (20–22). In these studies, progression of differentiation was associated with temporal changes in PARP activity, nuclear polymer concentration, and PARP expression. In addition, inhibitors of PARP activity have been shown to induce differentiation alone or synergistically with other differentiation inducers (20, 22). HL-60 cells, induced to differentiate in the neutrophilic lineage, have decreased PARP activity (23). However, PARP is still expressed and appears to behave more like an NAD+ glycohydrolase, degrading NAD+ without forming poly(ADP-ribose) (23).

We showed recently that PARP expression is modulated differentially during monocytic and neutrophilic differentiation in both HL-60 and NB4 cells (24). In NB4 cells, PARP expression was down-regulated to undetectable levels after 36 h of 1 μM ATRA exposure, and this regulation was associated with an increased expression of neutrophil phenotypic markers. In contrast, NB4 cells up-regulated PARP 90-fold in the monocytic lineage in response to a combination of 200 nM VD3 and 200 nM TPA. Acute myelocytic HL-60 leukemia cells, in comparison, did not decrease PARP expression in the neutrophilic pathway in response to ATRA but did increase PARP expression 4-fold in the monocytic pathway in response to either VD3 or TPA (24). In addition, although PARP is considered to be ubiquitously expressed in higher eukaryotic cells, we have shown that human neutrophils are the only nucleated mammalian cells described to this point that are devoid of PARP polypeptide (24).

To define the role of PARP expression during myeloid differentiation, we have transiently transfected a human PARP expression plasmid into acute promyelocytic NB4 leukemia cells that can undergo both neutrophilic and monocytic differentiation (7). We then examined the effects of PARP overexpression on sensitivity to the differentiation agents ATRA, VD3, and TPA. Our results suggest that PARP expression is more than a marker of differentiation and plays a fundamental role in cell division and neutrophil development.

**Results**

Log-phase NB4 cells were transfected using lipid-mediated gene transfer as described in "Materials and Methods." Hundreds of colonies survived when transfected with either the pPARP or pSV2Neo plasmids; then these colonies were transferred to methylcellulose and selected with 1200 μg/ml G418 (data not shown). Similarly treated control cultures generated no colonies in methylcellulose. However, after 7 days in methylcellulose culture, none of the clones survived expansion to liquid culture. Despite repeated attempts to generate stable transfectants using a variety of electroporation and lipid-mediated parameters, including varying time, temperature, DNA/lipid concentrations, voltage, capacitance, cell density, G418 concentration, and liquid versus methylcellulose culture, we have been unable to maintain G418 resistance beyond 7–8 days. Terminal differentiation responses normally occurred within the first 3–4 days of culture, and therefore, effects of transient PARP expression could be seen within this time frame. As a result, we have adopted the transient transfection protocol to assess the effects of PARP expression on NB4 cell differentiation.

Levels of PARP protein were determined by Western blot analysis in NB4 cells transfected with pPARP or pSV2Neo (control) plasmids and cultured in media containing either 8 μM CdCl2 or 32 μM ZnSO4. The concentrations of CdCl2 and ZnSO4 used were chosen in preliminary studies in which they were found to have no effect on proliferation, cell viability, or differentiation features. The quantity of PARP protein was measured following 0, 12, 24, 36, and 48 h of metal exposure (Fig. 1A). NB4 cells transfected with pPARP (NB4-PARP) and exposed to 8 μM CdCl2 increased PARP expression by greater than 2-fold following 12 h of metal exposure, and maximal expression (greater than 4-fold basal) was reached and maintained at 36–48 h. PARP expression in NB4-PARP cells cultured in the presence of 32 μM ZnSO4 was similar; however, the levels achieved were somewhat lower than for CdCl2 (Fig. 1A). NB4 cells transfected with pSV2Neo control plasmid (NB4-SV2) maintained constant PARP expression in the presence of either 8 μM CdCl2 or 32 μM ZnSO4 throughout the 48 h of exposure (Fig. 1A) and served as the control cell population for the remainder of the study. Because CdCl2 was more potent (2-fold) and increased PARP expression earlier than ZnSO4, CdCl2 was used to induce PARP expression in subsequent differentiation experiments.

Proliferation was assessed in NB4-PARP, NB4-SV2, and NB4-wt cells by measuring total cell number (Fig. 1B). Growth of NB4-SV2 cells exposed to either 32 μM ZnSO4 or 8 μM CdCl2 over 48 h were identical to NB4-wt growth patterns. However, NB4-PARP cells showed a marked decrease in proliferation, without changes in cell viability (data not shown) when cultured with either 8 μM CdCl2 or 32 μM ZnSO4 (Fig. 1B). Up-regulation of PARP caused a decrease in growth in NB4-PARP cells, regardless of which metal was used to induce expression (Fig. 1B).

Because growth of PARP-transfected cultures was substantially lower than cultures transfected with the control plasmid, we sought to determine in which stage of the cell cycle NB4-PARP cells were arrested. NB4 cells were transfected with the two plasmids, expression was induced with 8
Fig. 1. Effects of transfection with pPARP expression plasmid (NB4-PARP) and control plasmid SV2Neo (NB4-SV2) in the presence of 8 μM CdCl₂ on the expression of PARP and cell growth. A, quantitation of PARP was performed by scanning of autoradiographs from Western blots as described in “Materials and Methods.” NB4 cells were transfected with plasmid, and expression was induced by treatment with 8 μM CdCl₂ or 32 μM ZnSO₄ and harvested at various intervals after metal addition. a–d columns labeled with differing letters are statistically different from each other (n = 3; P = 0.05). B, cell growth was measured in transfectants without metal or with 8 μM CdCl₂ or 32 μM ZnSO₄. Cultures were harvested at various time points, and the cell number was determined. Results are representative of at least three independent experiments; bars, SD.

μM CdCl₂, and cell cycle analysis was performed 60 h later. NB4-wt and NB4-SV2 cultures displayed nearly identical cell cycle profiles, with 52–53% of the cells in S phase, 40% in G₁, and 7–8% in G₂-M (Fig. 2). In contrast, NB4-PARP profiles showed that 71% of cells were in S phase, 26% in G₁, and 3% in G₂-M. Therefore, the predominant arrest position in NB4-PARP cells appears to be in the S phase of the cell cycle. The rather high proportion of NB4-wt (and NB4-SV2) in S phase may appear unusual, but given that the transfection protocol involves a short period of serum starvation at relatively high cell densities just prior to and during the DNA/lipid incubation steps, one might expect the population to be synchronized. The other major difference was the higher proportion of cells in the subdiploid region of the profile in NB4-PARP cultures (20%) compared to NB4-SV2 or NB4-wt cultures (13%). This subdiploid population is likely to include apoptotic cells, although other markers of apoptosis were not studied.

The marked increase in PARP expression and in vitro polymerase activity during monocytic differentiation (24) led
us to propose that overexpression of PARP may cause changes in monocytic differentiation features in the absence of VD3 and TPA. There were no morphological differences between NB4-PARP, NB4-SV2, and NB4-wt cultures at any time during culture with 8 μM CdCl₂ (Fig. 6A). When the specific monocytic features of adherence and esterase expression were assessed, there were no differences between NB4-PARP and NB4-SV2 cultures after 60 h of CdCl₂ exposure or between transfectants prior to CdCl₂ exposure (Fig. 3A, Pre-Cd). When cells were treated with single agents, 200 nM VD3 or 200 nM TPA, in combination with CdCl₂ treatment, there were no significant differences in monocytic differentiation markers between NB4-PARP and NB4-SV2 cultures (Fig. 3A). This suggests that a 4-fold increase in PARP expression could not replace either differentiation agent in inducing monocyte development. As expected, PARP overexpression was maintained in NB4-PARP cells when treated with VD3 or TPA in the presence of CdCl₂, while NB4-SV2 cultures demonstrated an increase in PARP expression in response to either agent (Ref. 24, Fig. 3B). When NB4-PARP and NB4-SV2 cultures were exposed to a combination of 200 nM VD3 and 200 nM TPA, a treatment shown previously to induce maximal differentiation response in NB4-wt cultures, PARP levels increased in both transfectants (Fig. 3B).
Greater than 80% of each population expressed markers of monocytic differentiation, but no significant difference between NB4-PARP and NB4-SV2 cultures was found (Fig. 3).

We then considered the possibility that although PARP overexpression could not replace VD3 or TPA in monocytic differentiation induction, it could synergize with VD3 and TPA in combination, allowing successful maturation at what would otherwise be suboptimal levels of the agents. To address this question, NB4-PARP and NB4-SV2 cells were treated with VD3 and TPA, at concentrations shown previously to be suboptimal. Both NB4-PARP and NB4-SV2 cultures were treated with CdCl₂ for 18 h (t = 0) and subsequently treated at time 0 (t = 0) with 50 nM VD3 + 100 nM TPA in combination for up to 60 h (Fig. 4A). Neither culture showed any significant difference in adherence or esterase expression prior to CdCl₂ treatment, and no significant increase in these markers was seen after 18 h of CdCl₂ exposure (t = 0, Fig. 4B). Once monocytic differentiation inducers were added (t = 0 h), there was increased esterase and adherence expression in both NB4-PARP and NB4-SV2 cultures (Fig. 4A). However, once again there were no differences in phenotypic markers between NB4-PARP and NB4-SV2 cells at any time point (Fig. 4A). Increased PARP expression was demonstrated by Western blotting through the transient transfection time course in NB4-PARP cells (Fig. 4B).

We have shown previously that down-regulation of PARP occurs coincidently with the increase in ATRA-induced neutrophilic differentiation features in NB4 cells. To investigate the functional consequences of PARP protein regulation in the neutrophilic pathway, we treated cells overexpressing PARP with ATRA as the differentiation inducer. NBT dye reduction, a neutrophilic differentiation marker, was assessed in NB4-PARP and NB4-SV2 cultures prior to or after 18 h of 8 μM CdCl₂ exposure and following treatment with 1 μM ATRA for 0, 20, 40, and 60 h (Fig. 5A). Less than 2–3% of both NB4-PARP and NB4-SV2 cultures expressed NBT reduction features prior to or after CdCl₂ treatment (Fig. 5A). Within 20 h of ATRA treatment, control NB4-SV2 cultures demonstrated a marked increase in the proportion of cells expressing neutrophilic markers, with 80 and 95% of the population being NBT positive at 40 and 60 h, respectively (Fig. 5A). NB4-PARP cultures continued to express high levels of PARP over the entire experimental period of ATRA treatment, and less than 15% went on to reduce NBT dye, an activity characteristic of neutrophilic differentiation. On the other hand, NB4-SV2 cultures, which expressed high levels of NBT...
dye-reducing activity, down-regulated PARP in the same manner as NB4-wt cells (Ref. 24, Fig. 5B). Morphologically, NB4-PARP cultures did not change in response to up-regulated PARP and ATRA treatment for 40 and 60 h, while NB4-SV2 cultures treated with ATRA possessed many band neutrophils at 40 h and cells with bi- and tri-lobed nuclei by 60 h of ATRA exposure (Fig. 6B). This suggests that overexpression of PARP blocks expression of terminal neutrophilic differentiation markers.

**Discussion**

The NB4 cell line offers an excellent model to investigate molecular mechanisms involved in the myelocytic differentiation of human APL cells. APL has been treated successfully with retinoic acid, an observation now explained by the discovery of the retinoic acid receptor \( \alpha \) gene rearrangement in M3 leukemias. Therefore, it is increasingly important to study this model and the mechanism of action of the differentiation inducers, ATRA and VD3 + TPA. Multiple studies have correlated changes in PARP expression and/or activity with differentiation induction in both human nonhematopoietic and hematopoietic cell lines (18–23). The use of inhibitors to differentiate cells implied that higher PARP activity and polymer content were related to immature phenotype, and differentiation could thus be induced by inhibition of PARP (25, 26). It has been shown recently that several of the inhibitors used previously, primarily benzamide derivatives, are nonspecific and modulate several other cellular components (27). The current study provides an unequivocal demonstration of an active role for PARP in the differentiation process.

Our results show that alteration in PARP expression is an important component of neutrophilic differentiation and may also play a role in controlling cell cycle progression. A 2–4-fold increase in PARP expression caused growth inhibition, primarily through arrest in the S phase of the cell cycle, without inducing differentiation. Because PARP is tightly associated with DNA, the S-phase arrest is not surprising. The question now becomes whether it is the physical interactions of PARP with DNA per se or its intact PARP activity that are central to its inhibitory activity. More potent and specific
inhibitors, like isoquinolinediol, may prove useful in this pursuit.

NB4 cells overexpressing PARP and treated with ATRA were substantially inhibited in their expression of mature neutrophilic differentiation markers. Because fewer than 15% of cells from PARP-transfected cultures were NBT positive and displayed neutrophilic nuclei, one might assume that the transfection frequency was about 80%. Although this is conceivable, an alternative explanation is that a smaller proportion of cells are expressing PARP and that these cells then express a factor that is inhibitory to other cells in the culture. To directly answer this question, clonal examination of both PARP expression and neutrophilic differentiation features is required. We are currently developing an immunohistochemical technique to measure PARP expression in individual cells. Both NB4-wt and NB4-SV2 cells responded to ATRA, with 80–95% of cells expressing neutrophilic features, coincidental with the down-regulation of PARP. This shows that down-regulation of PARP is necessary to induce neutrophilic differentiation of APL cells. Whether down-regulation alone is sufficient to induce neutrophilic differentiation could be answered by using antisense nucleic acids to the PARP transcript to artificially down-regulate PARP expression. By inhibiting PARP expression with oligonucleotides, one could also determine whether PARP plays a role later on in the monocytic differentiation pathway. We have thus far tried two different oligonucleotides and have not succeeded in decreasing PARP protein levels.

Despite the fact that monocytes express 90-fold more PARP than control NB4 cells in response to VD3 + TPA, treatment of NB4 cells overexpressing PARP (to 4-fold basal levels) with single differentiation agents (VD3 or TPA) or suboptimal levels of VD3 + TPA failed to demonstrate a potentiating effect. Therefore, either PARP does not play an intimate role in monocytic differentiation or it plays a role downstream of VD3 and TPA action. It is also conceivable that the 4-fold induction achieved here was insufficient to "prime" cells for monocytic differentiation. Regardless of whether PARP controls the acquisition of the phenotype, it nonetheless serves as a valuable marker for monocytic differentiation of APL cells. Studies are ongoing to examine the mechanism by which the 90-fold elevation in PARP in the monocytic pathway occurs. The role of PARP in monocytic differentiation would be examined more effectively by blocking the large increase in PARP protein using antisense, but this could be difficult to accomplish.

Substantial chromatin reorganization is a fundamental process of both cell division and neutrophilic differentiation. PARP is proposed to interact with topoisomerases, polymerases, and DNA, both through physical association and potentially as targets of its ADP-ribosylation activity. PARP could thereby regulate repair, reorganization, and recombination events that accompany cell division and differentiation. In addition, PARP could directly or indirectly modulate the activity of a variety of genes involved in these pathways. At least some of these activities are likely to be mediated through one or both of the zinc fingers found in PARP. Consistent with this hypothesis, Nguyen et al. (28) demonstrated recently that continuous expression of another zinc finger protein, Egr-1, blocks neutrophilic differentiation of
Fig. 6. Morphological features of NB4-PARP, NB4-SV2, and NB4 wt untreated or treated with 1 μM ATRA. Morphology was assessed by microscopic examination of cytospun cultures of Giemsa-Wright-stained preparations (×150), as indicated in "Materials and Methods." A, cells were treated for 60 h with 8 μM CdCl₂. B, cultures were treated with 1 μM ATRA for 40 and 60 h following 18 h of CdCl₂ treatment. Photographs are representative of at least three independent experiments; bars, SD.

HL-60 cells. It would be interesting to know whether egr-1 plays any role in NB4 cell differentiation.

Essential changes in DNA relaxation and genomic separation into polymorphic nuclei in neutrophils may only be accomplished in the absence of PARP protein. Consistent with this premise, cells that are depleted of PARP using antisense constructs express changes in DNA topology (29). Although this is lethal in some cell types (29), it may facilitate nuclear morphological changes during differentiation, or transient changes in PARP expression may facilitate new organization of the chromatin, and in turn, the genome. The maintained overexpression of plasmid-derived PARP during ATRA treatment suggests that ATRA modulates PARP expression at the level of transcription. Studies are currently underway to test this possibility.

Materials and Methods

Cell Culture. NB4 cells were obtained from Dr. M. Minden (Princess Margaret Hospital, Toronto, Ontario, Canada). These cells were originally isolated and characterized from a human patient with APL (2). Transfected and wt NB4 cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) with 10% FCS, supplemented with penicillin and streptomycin (50 U/ml) at 37°C in an atmosphere of 5% CO₂. Light exposure of cultures was minimized to prevent inactivation of light sensitive VD3 and ATRA. Cells were cultured in 22-mm tissue culture dishes (Corning or Falcon) and were routinely passaged in tissue culture flasks (8 ml) as suspension cultures. Cell growth and viability were assessed using a Coulter Counter (model ZM) and trypan blue dye exclusion, respectively. Cultures had a greater than 88% viability in all experimental treatment groups. NB4 cells were used from passages 5 to 13 in the current study. The nonadherent cell fraction was prepared by combining suspended cells with those collected by a gentle PBS wash. Adherent cells were incubated for 10 min with cold PBS-10% fetal bovine serum (FBS) and removed for counting.

Differentiation Inducers. VD3 was purchased from Intercience (Calbiochem) and dissolved in absolute ethanol to produce a stock of VD3 at a concentration of 9.0 x 10⁻⁶ M. ATRA was purchased from Sigma Chemical Co. and dissolved in absolute ethanol to produce a stock of 1.0 x 10⁻⁸ M. Both stocks of VD3 and ATRA were protected from light and were stored at −20°C under nitrogen. TPA was purchased from Sigma and was dissolved first in DMSO and then diluted to a concentration of 170 μM in IMDM with 10% FCS and antibiotics.

All stocks were diluted in culture medium to give final working concentrations that have been shown to maximize the differentiation response. For neutrophilic differentiation, NB4 cells were treated with ATRA to attain a final concentration of 1 μM (2, 20). For monocyte/macrophage differentiation, NB4 cells were treated for monocyte/macrophage differentiation with 2.0 x 10⁻⁷ M VD3, 2.0 x 10⁻⁷ M TPA, and combinations to obtain a maximal response and combined submaximal concentrations as indicated.

Transfection. The human pPARP plasmid is a eukaryotic expression construct in which the human PARP gene cDNA is under the control of the metallothionine promoter, as described previously (30). The vector was constructed by insertion of a 3.2-kb fragment containing the metallothionine promoter and human PARP cDNA into the pSV2Neo expression vector.
plasmid (kindly provided by M. Miwa; Ref. 30). Transfectants were established by introducing linearized plasmid DNA from plasmids pPARP or control plasmid, pSV2neo (kindly provided by M. Baker) into NBE cells (1.0 × 10^5 cells/well) by cationic liposome-mediated transfection (Lipofectamine; BRL) in a ratio of 1:3 (plasmid:lipid). Cells were incubated in serum-free media for 6 h in a mixture of DNA and lipid and allowed to recover overnight. Populations were then pooled and divided into 15-mm wells in a 1-mL volume containing 2.0 × 10^5 cells and treated as indicated.

Cell Cycle Analysis. NBE cells were transfected and incubated with 8 µM CdCl_2 as described above. After 18 h with Cd, cells were diluted to a final cell concentration of 2.0 × 10^5 cells/mL in complete medium containing CdCl_2. Sixty h later, cells were prepared for cell cycle analysis by propidium iodide staining. Briefly, cells were washed three times in PBS and fixed in 75% ethanol for 2 h at 4°C. Cells were pelleted and resuspended in 0.1% Triton X-100 in PBS containing 0.6% NP40. To this was added an equal volume of HBSS containing 2 mg/mL RNase A and incubated at 22°C for 1 h. Cells were filtered through 85 µm Nitex mesh and kept at 4°C until analysis the same day. Cell cycle analysis was provided by a cell cycle analysis using a Beckton-Dickinson FACStarPLUS using the CellFIT Cell Cycle Analysis, Version 2.01.2. The goodness of fit was between 1.17 and 1.63, with at least 9000 events scored.

Markers of Differentiation. Neutrophilic differentiation was assessed by NBT dye reduction as described previously (24). Briefly, using a kit from Sigma (St. Louis), equal volumes of cells in media were combined with a 0.1% solution of NBT in PBS with the addition of stimulant for 20 min at 37°C in 5% CO_2. NBT solutions were prepared, and cells were counted for formazan deposits. A total of 200 cells were counted, and the number of positive cells was expressed as a percentage. Characterization of neutrophilic polymorphic morphology was accomplished by Giemsa-Wright staining of cytosin preparations. Morphology of cultures treated or not treated with plasmids were photographed for cytosin preparations that were Giemsa-Wright stained (150×). Monocyte/macrophage differentiation was assessed by measuring the adherent fraction, α-naphthyl acetate esterase activity using a kit from Sigma (24).

Sample Collection. Cultures containing NBE transfecteds were harvested at specified time intervals after exposure to differentiation-inducing agents and inducers of PARP expression. Cells were washed twice in PBS containing a mixture of protease inhibitors (Boehringer Mannheim) and resuspended in a Tris buffered lysis buffer 6.5 M urea, 6% beta-mercapto- ethanol, 3% SDS, and 0.003% bromophenol blue) to obtain a final cell concentration of 3.2 × 10^6 cells/mL. Lysates were then heated to 60°C for 2 min, sonicated for 45 s, and stored at −20°C until analysis by SDS-PAGE.

Western Blotting. Proteins were separated by SDS-PAGE on 7.5% acrylamide gels and transferred onto a polyvinylidene difluoride membrane on a semidry blotter apparatus 2 h at 100 mA. Molecular weights of proteins were estimated by comparison to migration of standard molecular weight markers (Sigma). Membranes were first blocked with 5% milk powder in Tris-buffered saline (TBS), washed in TBS with 0.05% Tween 20 (TBS-T), and then incubated with primary antibody. A rabbit polyclonal antibody (FII) directed against the human zinc finger domain of PARP was used as the primary antibody (a generous gift from Dr. G. de la Chapelle Institute, Paris, France) at 1:1000 in TBS-Tween powder (TBSTM). Membranes were then washed and incubated with rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:25,000) in TBSTM. P ARP polypeptide was detected using the ECL kit (Amersham) and quantitated by densitometry. Signal intensity and purified P ARP protein from 11 to 124 µg gave a linear response (R = 0.998; P < 0.001), and quantification of all image densities recorded fell within this linear portion. Equal quantities of lysed cells were loaded on each lane in each experiment. Quantitation of P ARP is represented as an average of at least three separate experiments.

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References