Internucleosomal DNA Fragmentation during Phorbol Ester–induced Monocytic Differentiation and G_0/G_1 Arrest

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Abstract

The treatment of human myeloid leukemia cell lines with phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is associated with loss of proliferative capacity and induction of monocytic differentiation. The present results demonstrate that treatment of asynchronous human U-937 leukemia cells with 10 nM TPA is also associated with oligonucleosomal DNA cleavage. This pattern of DNA fragmentation, which is observed in programmed cell death, was detectable in populations of TPA-treated cells that had entered a nonproliferative G_0/G_1 phase. Similar findings were obtained after TPA treatment of a synchronous population of G_1 cells. These cells progressed through S and G_2/M phases before undergoing internucleosomal DNA cleavage during G_0/G_1 arrest. These G_0/G_1 cells displayed characteristics of monocytic differentiation, including down-regulation of c-*myc* expression and induction of c-*fms* transcripts. DNA fragmentation was also studied in cells treated with 5 nM TPA for 48 h and then monitored in drug-free long-term culture. Endonucleolytic cleavage was similarly observed in the differentiated G_0/G_1 population. However, longer periods of culture were associated with a decrease in DNA fragmentation to undetectable levels. This effect was followed by retrodifferentiation and reentry of cells into cycle. Taken together, these findings demonstrate that internucleosomal DNA fragmentation occurs during induction of monocytic differentiation, and that both of these events are detectable in G_0/G_1 cells. (*J. Clin. Invest.* 1992. 89:954–960.) Key words: cell cycle • DNA cleavage • retrodifferentiation • TPA

Introduction

Programmed cell death, or apoptosis, is a regulated process during which eukaryotic cells die in response to specific stimuli (1). This process provides a mechanism for deletion of specific cell populations in the developing embryo (2). Apoptosis is also associated with death of lymphocytes treated with glucocorticoids (3) and elimination of autoreactive T-cell clones in the thymus (4, 5). The death of these cells is preceded by chromatin condensation and cleavage at internucleosomal sites (3, 6). Thus, one characteristic of apoptosis is internucleosomal DNA fragmentation. The mechanisms responsible for this endonucleolytic DNA cleavage remain unclear. Nonetheless, similar patterns of DNA fragmentation have been observed in lymphocytes deprived of IL-2 (7) and in myeloid cells deprived of colony-stimulating factors (8, 9). These findings have indicated that certain growth factors may promote survival by suppressing apoptosis (7–9).

Human myeloid leukemia cell lines, such as HL-60 and U-937, proliferate autonomously (10, 11). However, these cells have retained the capacity to respond to inducers of differentiation with cessation of growth and appearance of a more mature phenotype (12). For example, treatment of U-937 or HL-60 cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA)[1] is associated with loss of proliferative capacity, adherence, and increased expression of monocyte surface markers (13, 14). TPA-induced monocytic differentiation of these cell lines is also associated with down-regulation of c-*myc* expression and induction of c-*fos*, c-*jun*, and c-*fms* transcripts (15, 16). The c-*myc*, c-*fos*, and c-*jun* immediate early response genes code for nuclear proteins, whereas the c-*fms* gene codes for the macrophage colony-stimulating factor receptor, a marker of differentiation along the monocytic lineage (17). Although c-*fms* mRNA levels are increased during TPA-induced growth arrest, the relationship, if any, between expression of this gene and cell cycle events is unknown.

Previous studies have demonstrated that differentiation of eukaryotic cells is associated with DNA strand breaks (18–22). However, the mechanisms responsible for this fragmentation of DNA have remained unclear. The present studies were performed to determine whether induction of monocytic differentiation is associated with DNA cleavage at internucleosomal sites. The results demonstrate that DNA of TPA-induced U-937 cells is cleaved into multiples of nucleosome-sized fragments. This pattern of DNA cleavage was found in cells that were arrested in G_0/G_1 and expressed the differentiated phenotype. These findings suggest that induction of differentiation includes at least one characteristic of programmed cell death.

Methods

Cell culture. U-937 and HL-60 myeloid leukemia cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% (U-937) or 15% (HL-60) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were treated with 5 and 10 nM TPA (Sigma Chemical Co., St. Louis, MO) or 1.5% DMSO (Sigma Chemical Co.).

Centrifugal elutriation. Cells in exponential growth phase or after treatment with TPA were subjected to centrifugal elutriation using the JE-5.0 elutriation system (Beckman Instruments, Inc., Palo Alto, CA). Approximately 2×10^8 cells were applied to the standard chamber (1,600 rpm at 27°C) using a digital flow controller (Cole-Parmer Instrument Co., Chicago, IL). The calibrated pump speed was increased

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1. *Abbreviations used in this paper:* MEL, murine erythroleukemia; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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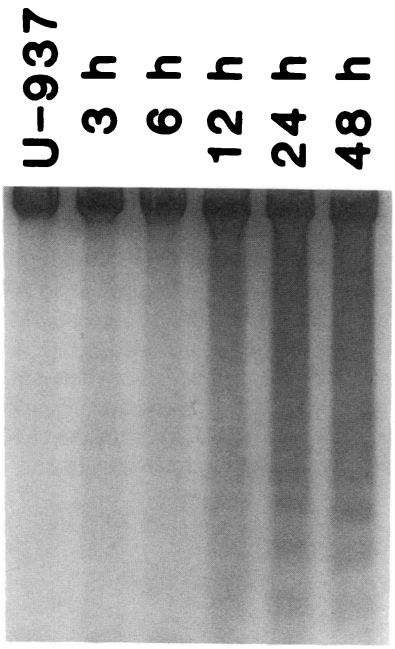


Figure 1. Effects of TPA on DNA fragmentation in asynchronous cells. U-937 cells were treated with 10 nM TPA for the indicated times. The cells were washed and then monitored for DNA fragmentation in 2% agarose gels.

from 10 to 30 ml/min. Enriched cell populations from different phases of the cell cycle were elutriated in 100-ml aliquots of RPMI 1640 medium containing 1% fetal bovine serum. Aliquots (1 ml) were fixed by adding 2 ml of ice-cold methanol for 60 min on ice. After centrifugation, the supernatant was removed and the cell pellet incubated with 500 μl of RNase (200 U/ml) and 500 μl of propidium iodide buffer in the dark at room temperature for 30 min (23). The stained cells were analyzed for DNA content on a FACScan (Becton, Dickinson & Co., Mountain View, CA) using CellFIT cell cycle analysis software.

Analysis of DNA fragmentation. 1×10^6 cells were harvested, washed, and incubated in 20 μl of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, and 0.5 μg/ml proteinase K (Sigma Chemical Co.)

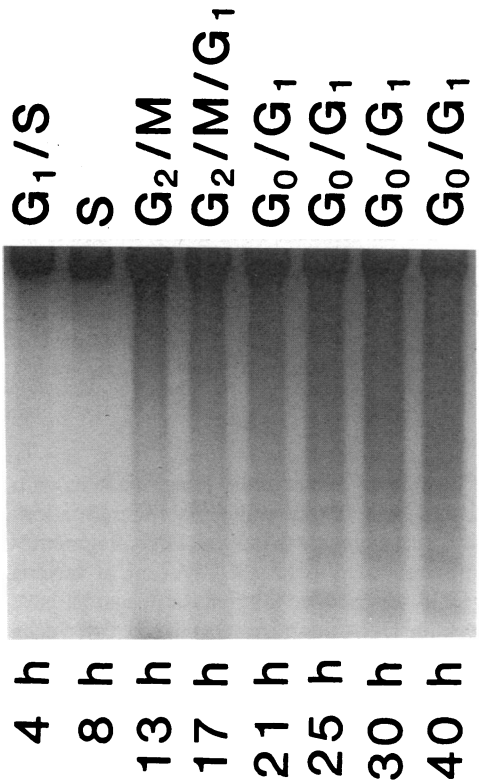
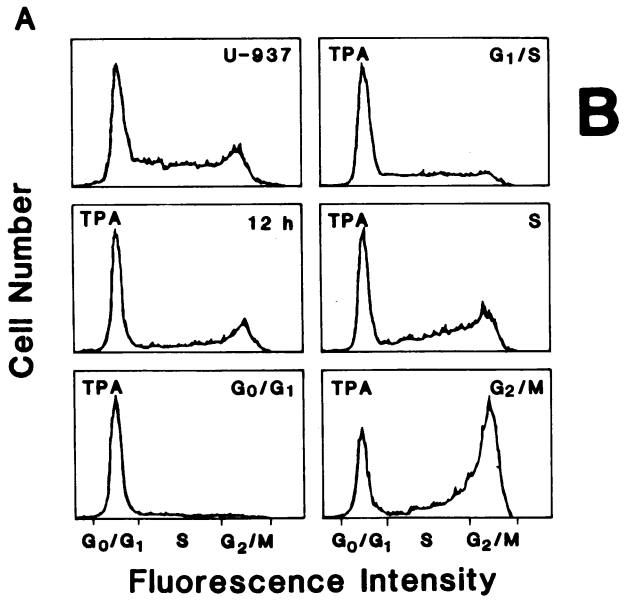


Figure 3. DNA fragmentation after treatment of G_1 cells with TPA. U-937 cells in exponential growth phase were separated by elutriation. The G_1 phase (79% G_1, 18% S, 3% G_2/M) cells were isolated and treated with 10 nM TPA. TPA-treated cells were harvested at the indicated times for analysis of cell cycle distribution and DNA fragmentation. G_1/S: 54% G_1, 34% S, 12% G_2/M. S: 35% G_1, 39% S, 26% G_2/M. G_2/M: 35% G_1, 30% S, 35% G_2M. G_2/M/G_1: 61% G_1, 7% S, 32% G_2/M. G_0/G_1 21 h: 78% G_1, 8% S, 14% G_2/M. G_0/G_1 25 h: 85% G_1, 6% S, 9% G_2/M. G_0/G_1 30 h: 87% G_1, 4% S, 9% G_2/M.



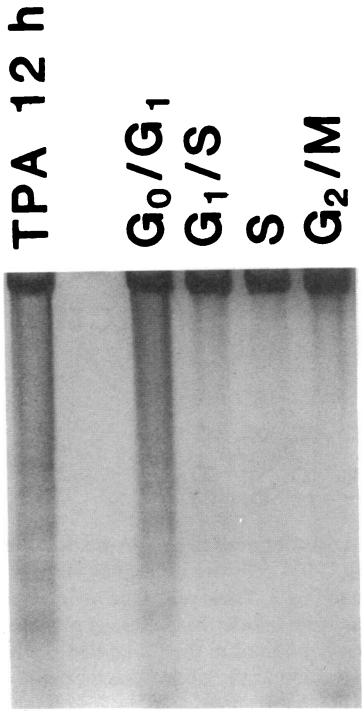


Figure 2. Cell cycle distribution and DNA fragmentation in TPA-treated cells. U-937 cells were treated with 10 nM TPA for 12 h and then separated by centrifugal elutriation. (*A*) Control U-937 cells (*top left panel*) and TPA-treated cells (*middle left panel*) were analyzed by flow cytometry. The elutriated fractions obtained from the TPA-treated cells were also analyzed by flow cytometry for cell cycle distribution. G_0/G_1: 80% G_0/G_1, 19% S, 1% G_2/M. G_1/S: 54% G_1, 35% S, 11% G_2/M. S: 37% G_1, 40% S, 23% G_2/M. G_2/M: 23% G_1, 20% S, 57% G_2/M. (*B*) The same elutriated fractions were monitored for DNA fragmentation in 2% agarose gels.

for 1 h at 50°C. 10 μl of 0.5 μg/ml RNase A was then added and the incubation continued for an additional 1 h. The digested samples were incubated with 10 μl of 10 mM EDTA (pH 8.0) containing 2% (wt/vol) low-melting-point agarose, 0.25% bromophenol blue, and 40% sucrose at 70°C. The DNA was separated in 2% agarose gels and visualized by ultraviolet (UV) illumination after ethidium bromide staining.

Isolation and analysis of RNA. Total cellular RNA was purified as described (15), analyzed by electrophoresis through 1% agarose-formaldehyde gels, transferred to nitrocellulose filters and hybridized to the following ^{32}P-labeled DNA probes: (*a*) the 1.6-kb ClaI/EcoRI fragment of the human c-*myc* 3′ exon purified from the pMC41-3 RC plasmid (24); (*b*) the 4.0-kb EcoRI fragment of the human c-*fms* gene purified from the pc-*fms* 102 plasmid (25); and (*c*) the 2.0-kb PstI fragment of the chicken β-actin gene purified from the pAI plasmid (26).

Results

The treatment of human U-937 myeloid leukemia cells with TPA is associated with induction of monocytic differentiation. To determine whether this agent also induces DNA fragmentation, U-937 cells were exposed to 10 nM TPA and, at various intervals, nuclear DNA was isolated for analysis in agarose gels. There was no detectable DNA fragmentation in untreated cells (Fig. 1). Moreover, there was no evidence of fragmentation after exposure to TPA for 3 or 6 h (Fig. 1). In contrast, DNA fragments at multiples of ~ 200 bp were present at low but detectable levels in cells treated for 12 h; this pattern was more apparent with longer exposures (Fig. 1). This pattern of fragmentation is in concert with internucleosomal DNA cleavage.

To determine whether DNA fragmentation occurs in specific phases of the cell cycle, U-937 cells were treated with 10 nM TPA for 12 h and then subjected to centrifugal elutriation. Cell cycle distribution of the elutriated cells was determined by flow cytometry. Although cells were detectable in S and G_2/M phases, other cells had exited the cycle and entered G_0/G_1 (Fig. 2 *A*). This G_0/G_1 population failed to reenter S phase (data not shown). Moreover, cells remaining in cycle subsequently underwent exit to G_0/G_1 (data not shown). DNA from the elutriated cell fractions was also analyzed in agarose gels. In con-

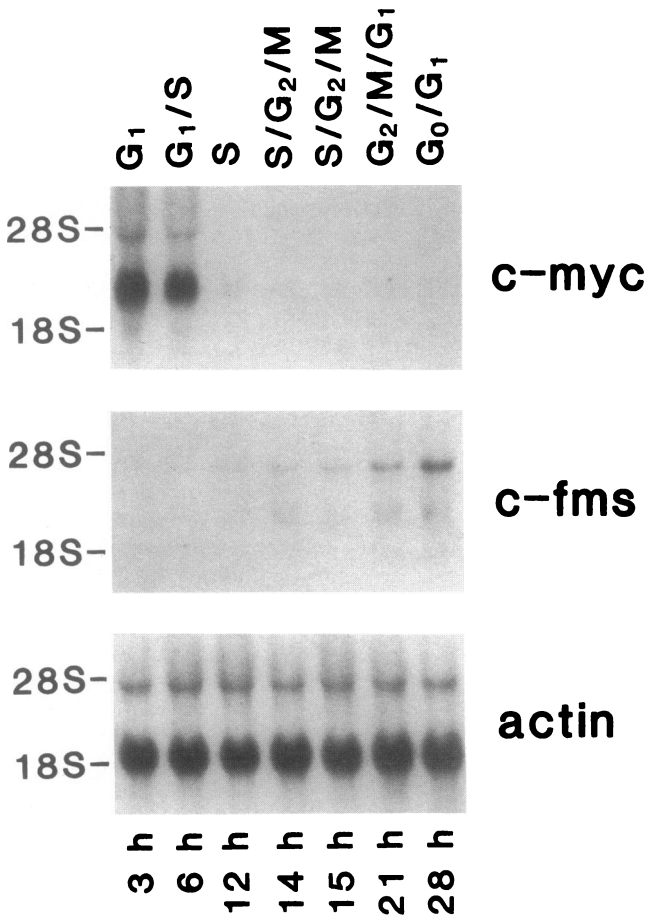


Figure 4. Analysis of c-*myc* and c-*fms* expression in TPA-treated cells. The G_1 fraction of proliferating U-937 cells was isolated by elutriation, as described in the legend to Fig. 3. These cells were maintained in the presence of 10 nM TPA. Total cellular RNA (20 μg) was isolated at the indicated times and hybridized to the ^{32}P-labeled c-*myc* and c-*fms* DNA probes. Hybridization to the labeled β-actin probe demonstrated equal loading of the lanes.

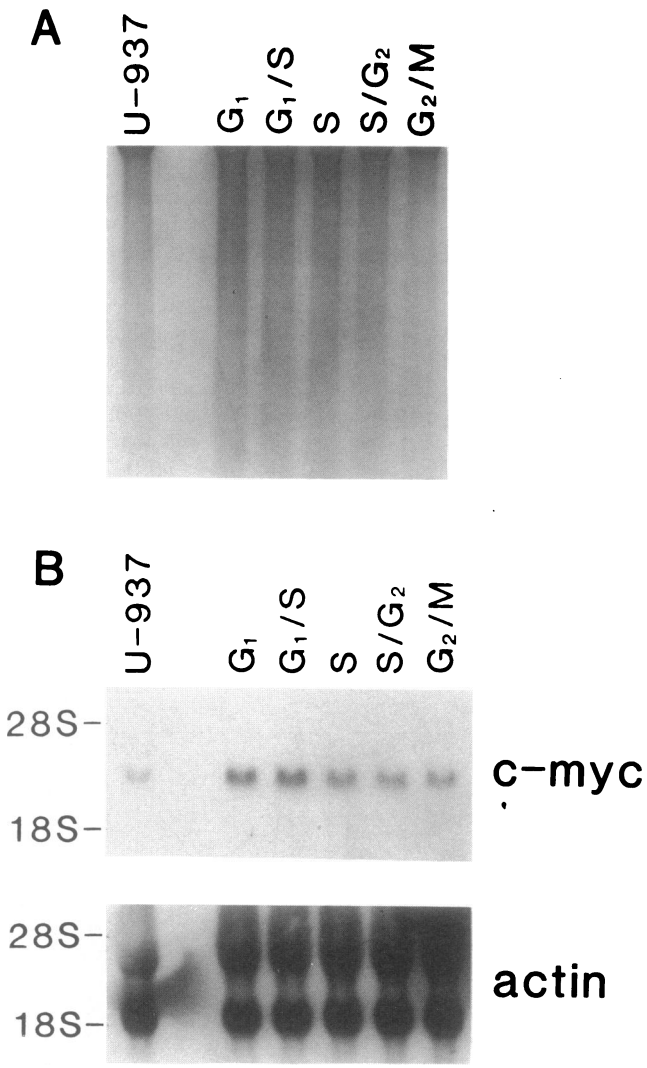


Figure 5. Analysis of uninduced U-937 cells for DNA fragmentation and c-*myc* expression. U-937 cells in exponential growth phase were separated by elutriation. (*A*) Aliquots were analyzed for cell cycle distribution by flow cytometry and for DNA fragmentation in agarose gels. (*B*) Total cellular RNA (20 μg) was also isolated from these cell populations and hybridized to the ^{32}P-labeled c-*myc* probe. Hybridization to the actin probe was used to demonstrate equal loading of the lanes.

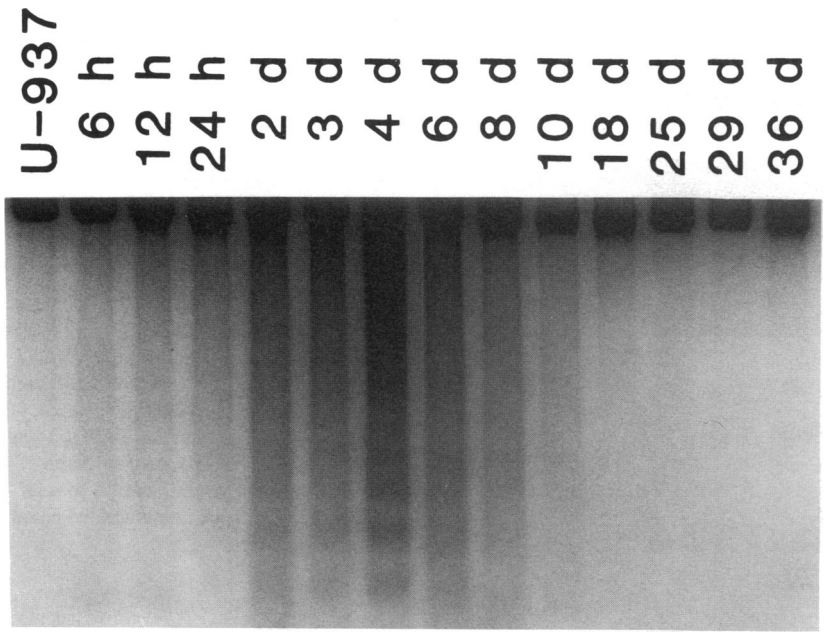


Figure 6. DNA fragmentation during long-term culture after TPA induction. U-937 cells were treated with 5 nM TPA for 48 h and then maintained in the absence of this agent. Cells were harvested at the indicated times and analyzed for DNA fragmentation.

trast to the unfractionated population, there was no evidence for DNA fragmentation in S or G_2/M phase cells (Fig. 2 *B*). However, internucleosomal DNA cleavage was detectable in the G_0/G_1 cell fraction (Fig. 2 *B*). These findings indicated that DNA fragmentation occurs predominantly, if not exclusively, in cells that exit the cycle to G_0/G_1.

Other studies were performed on G_1 cells isolated by elutriation of a population in exponential growth phase. The G_1 cell fraction was treated with TPA and, at various intervals, assayed for cell cycle distribution and DNA fragmentation. These TPA-treated cells progressed through S and G_2/M phases before arrest in G_0/G_1 (Fig. 3). There was no evidence for DNA cleavage in cells that remained in cycle (Fig. 3). However, oligonucleosomal DNA fragmentation was detectable in cells that had undergone exit to G_0/G_1 (Fig. 3). Previous studies have demonstrated that treatment of U-937 cells with TPA is associated with down-regulation of c-*myc* transcripts and induction of c-*fms* gene expression (15). The relationship between these events and internucleosomal DNA fragmentation was studied by performing Northern analyses on RNA from G_1 cells maintained in the presence and absence of TPA. Although c-*myc* expression was detectable in the G_1/S phase fraction of TPA-induced cells, induction of c-*fms* mRNA was found predominantly in the subsequent G_0/G_1 fraction (Fig. 4). In contrast, there was no detectable internucleosomal DNA fragmentation in uninduced U-937 cells subjected to elutriation (Fig. 5 *A*). Moreover, although c-*myc* mRNA levels were highest in G_1/S cells and decreased with cell cycle progression (Fig. 5 *B*), there was no detectable expression of the c-*fms* gene in the absence of TPA treatment (data not shown). These results indicated that the exit of TPA-treated cells to G_0/G_1 is associated with induction of both the monocytic phenotype and internucleosomal DNA cleavage.

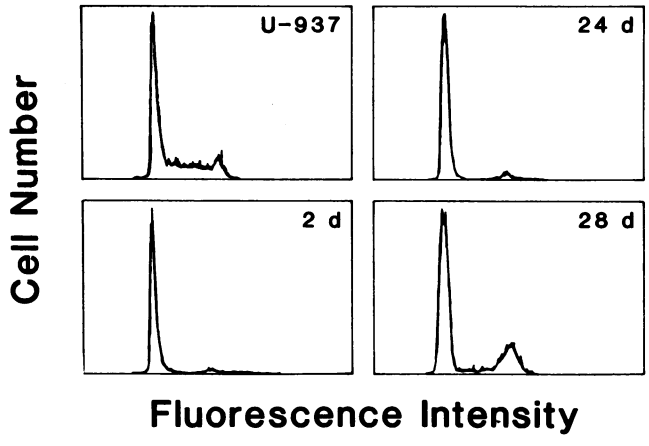


Figure 7. Cell cycle distribution during TPA induction and retrodifferentiation. U-937 cells were treated as described in the legend to Fig. 5. Cells were harvested at the indicated times for analysis of cell cycle distribution by flow cytometry.

The relationship between differentiated G_0/G_1 cells and DNA fragmentation was further studied during long-term culture of TPA-treated U-937 cells. We have previously demonstrated that differentiated U-937 cells grown in the absence of TPA for > 28 d is associated with retrodifferentiation and reentry of growth-arrested cells into the cycle (27). To determine whether internucleosomal cleavage occurs during this process, U-937 cells were treated with 5 nM TPA for 48 h and then maintained in TPA-free medium for up to 36 d. Using these experimental conditions, DNA fragmentation was initially detectable at low levels by 2–3 d (Fig. 6). Maximal DNA cleavage at internucleosomal sites was found at 4 d, whereas longer periods of incubation were associated with a progressive decrease in fragmentation to undetectable levels by 10 d (Fig. 6). These results indicated that the induction of DNA fragmentation is transient during long-term culture.

Flow cytometry to determine cell cycle distribution was performed on TPA-induced cells monitored in the absence of this agent. Before induction with TPA, 50, 40, and 10% of the cells were present in G_1, S, and G_2/M phases, respectively (Fig. 7). An accumulation of G_0/G_1 cells (> 90%) was detectable by 2 d (Fig. 7). Although similar findings were obtained at day 24,

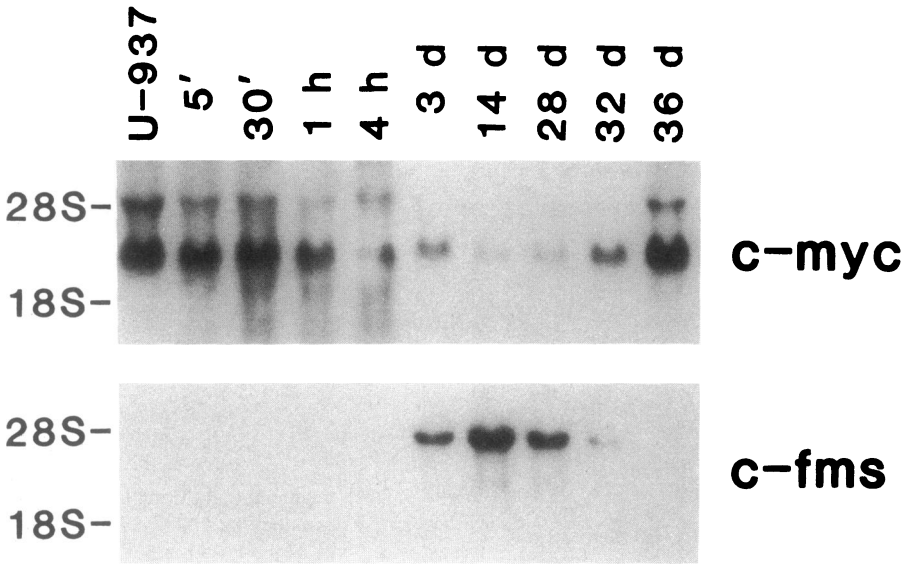


Figure 8. Expression of the c-*myc* and c-*fms* genes during retrodifferentiation. U-937 cells were treated as described in the legend to Fig. 5. Cells were harvested at the indicated times. Total cellular RNA (20 μg) was hybridized to the ^{32}P-labeled c-*myc* and c-*fms* DNA probes. Hybridization to a labeled 28-S DNA probe demonstrated equal loading of the lanes.

these cells were redistributed through S and G_2/M phase by day 28 (Fig. 7). This pattern of reentry into the cell cycle is in concert with the previous demonstration that after day 28 the TPA-induced cells detach and reinitiate proliferation. These changes in cell cycle distribution were also associated with down-regulation of c-*myc* transcripts and induction of c-*fms* expression during periods of G_0/G_1 arrest (Fig. 8). In contrast, reentry of the cells into cycle was preceded by induction of c-*myc* expression and down-regulation of the c-*fms* gene (Fig. 8). Taken together, these results indicated that internucleosomal DNA fragmentation occurs in association with G_0/G_1 arrest and induction of monocytic differentiation.

To determine whether endonucleolytic DNA cleavage is limited to TPA-treated U-937 cells, we performed similar studies with the HL-60 myeloid leukemia cell line. There was no detectable DNA fragmentation in untreated HL-60 cells, while treatment with TPA for 24 h was associated with induction of adherence and internucleosomal DNA fragmentation (Fig. 9 *A*). Recent work has demonstrated that DMSO also induces monocytic differentiation of U-937 cells (28). Treatment of U-937 cells with this agent was similarly associated with internucleosomal fragmentation (Fig. 9 *B*). This effect was predominant at 72 and 96 h when the cells entered G_0/G_1 and exhibited loss of proliferative capacity (Fig. 9 *B*). These findings suggested that induction of differentiation with distinct classes of agents is associated with internucleosomal DNA fragmentation.

Discussion

Previous work has demonstrated that deprivation of growth factors results in programmed cell death in factor-dependent hematopoietic cells (7–9). An early event in this process is the fragmentation of chromatin at internucleosomal sites (7–9). These findings have suggested that apoptosis is suppressed by growth factor stimulation and that programmed cell death may represent a mechanism for selective elimination of cells no longer under autocrine or paracrine control. Indeed, the absence of apoptosis or decreases in the ability of cells to undergo this process may characterize the growth of leukemic populations. However, little is known about the events responsible for the activation of programmed cell death. Furthermore, it is not known whether endonucleoytic DNA cleavage, one characteristic of programmed cell death, is even linked to specific events during cell cycle progression.

The present findings demonstrate that internucleosomal DNA fragmentation occurs during TPA-induced monocytic differentiation of U-937 cells. These findings are not limited to U-937 cells since similar results have been obtained during induction of the HL-60 and THP-1 myeloid leukemia cell lines

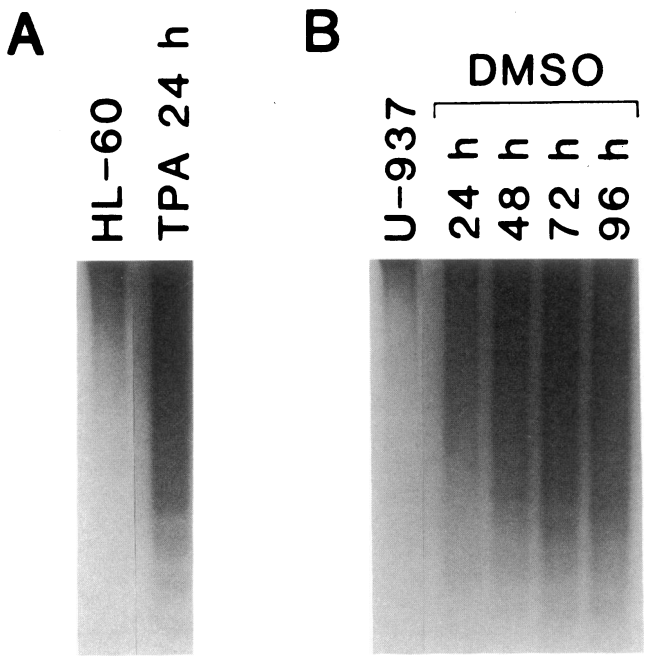


Figure 9. Analysis of internucleosomal DNA fragmentation in TPA-treated HL-60 cells and DMSO-treated U-937 cells. (*A*) HL-60 cells were treated with 10 nM TPA for 24 h. (*B*) U-937 cells were treated with 1.5% DMSO for the indicated times. The cells were washed and monitored for DNA fragmentation.

(data not shown). To our knowledge, there are no prior reports of DNA cleavage during monocytic differentiation. Other studies have demonstrated that induction of murine erythroleukemia (MEL) cell differentiation with various agents is associated with accumulation of DNA strand breaks (18, 19). Moreover, DNA cleavage during MEL differentiation is related to induction of a Ca^{2+},Mg^{2+}-dependent endonuclease activity (22). This activity is similar to that described during internucleosomal DNA cleavage of glucocorticoid-treated thymocytes (29). However, there is no report as yet that oligonucleosomal DNA fragmentation is induced during MEL differentiation. In the present work, the kinetics of DNA cleavage were dependent on the concentration of TPA used to induce differentiation. Although the activation of endonucleolytic cleavage may have been related to the use of TPA rather than to induction of monocytic differentiation, similar patterns of DNA fragmentation were obtained with DMSO, another agent that differentiates U-937 cells along the monocytic lineage (28).

The present results further demonstrate that internucleosomal DNA cleavage is limited to cells that enter a nonproliferative G_0/G_1 phase. In this context, only TPA-induced cells in G_0/G_1, not cells in S or G_2/M phase, had detectable DNA fragmentation. Differentiation of myeloid leukemia cells is associated with loss of proliferation, although it has not been clear whether appearance of the differentiated phenotype is limited to arrest in a certain phase. Expression of the c-*fms* gene represents a specific marker of monocytic differentiation. The finding that c-*fms* transcripts were detectable only in cells that had entered G_0/G_1 suggested that this population was indeed differentiated along the monocytic lineage. Moreover, the finding by in situ hybridization that ~ 99% of TPA-induced cells express the c-*fms* gene (30) indicates that cells that exit the cycle express the mature monocytic phenotype. Taken together, these results would support the activation of endonucleolytic DNA cleavage in differentiated G_0/G_1 cells.

Further support for the induction of endonucleolytic activity during monocytic differentiation was obtained from the study of TPA-induced U-937 cells as they undergo retrodifferentiation. Long-term culture of U-937 cells in the absence of TPA is associated with reentry of a growth-arrested population into the cell cycle (27, 31, 32). Similar findings have been obtained with other myeloid leukemia cell lines (27). Studies with [^{3}H]TPA demonstrate that U-937 cells retrodifferentiate when intracellular levels of this phorbol ester decline to < 1% of initial binding (31). This event is not related to the selection of TPA-resistant cells since reexposure to TPA is associated with reinduction of differentiation (27, 31, 32). In contrast to lymphoid cells, which are known to reenter S from a quiescent G_0/G_1 phase, this capability has not been described for myeloid cells.

The present results confirm that U-937 cells induced with TPA for 48 h subsequently enter a nonproliferative but reversible G_0/G_1 state. Moreover, the finding that this quiescent cell population was adherent and expressed the c-*fms* gene is in concert with induction of the monocytic phenotype. These cells also demonstrated internucleosomal DNA fragmentation, although this effect was transient and disappeared before retrodifferentiation. One possible but unlikely explanation for these findings is that cells can repair oligonucleosomal cleavage. Alternatively, a differentiated cell population with DNA fragmentation may have been eliminated from the culture, while the surviving cells retained the capacity to undergo retrodifferentiation and reenter a proliferative state. Indeed, 25–30% of the cells detach and die during the same period in which we observed the decrease in internucleosomal DNA fragmentation (27). These findings suggest that the balance between differentiated cells committed to programmed cell death or retrodifferentiation could be relevant to expansion of normal or leukemic myeloid cell populations.

Acknowledgments

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