Differential Induction of Apoptosis in Undifferentiated and Differentiated HL-60 Cells by DNA Topoisomerase I and II Inhibitors

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The effects of monocytic/macrophage and granulocytic differentiation induced by phorbol myristate acetate (TPA) and all-trans retinoic acid, respectively, were tested on the induction of apoptosis in human promyelocytic leukemia HL-60 cells treated with topoisomerase I and II inhibitors. Using a filter-binding assay, we observed a strong inhibition of DNA fragmentation induced by 3- and 24-hour continuous exposure to camptothecin, VP-16, VM-26, and m-AMSA in TPA-differentiated cells. The inhibition of the typical internucleosomal DNA fragmentation was confirmed by agarose gel electrophoresis. By contrast, drug-induced DNA fragmentation was not inhibited in retinoic acid-differentiated cells, and apoptosis occurred in these cells after 4 to 5 days in the absence of drug treatment. The TPA inhibitory effect was maximal after 24 hours of treatment and was correlated with differentiation, because phorbol dibutyrate ester was active, whereas 4-O-TPA, a nontumor promoter that does not induce differentiation, was not active. Using alkaline elution, we observed that TPA and retinoic acid differentiation were associated with changes in topoisomerase-mediated DNA breaks that were not correlated with their differential effects on drug-induced DNA fragmentation. Moreover, TPA also inhibited DNA fragmentation induced by vinblastine, cycloheximide, calphostin C, and x-rays. Using a cell-free system, we observed that DNA fragmentation was not inhibited in nuclei from TPA-differentiated cells. Rather, inhibition of apoptosis seemed to take place in the cytoplasm. We conclude that phenotypic changes associated with TPA-induced differentiation include inactivation of a cytoplasmic activity that can induce DNA fragmentation associated with apoptosis. This is a US government work. There are no restrictions on its use.

APOPTOSIS is an active mode of cell death that is essential for normal regulation of tissue cell number. It can be defined by characteristic morphologic changes associated with the digestion of chromatin into DNA fragments in multiples of approximately 180 bp after activation of an endonuclease. Additional genetic and biochemical markers have been described, but the relationship between these markers and the process of cell death remains to be determined. Apoptosis is implicated in processes as diverse as embryogenesis, carcinogenesis, hormone-dependent atrophy of tissues and tumors, immune system regulation, cytolytic cell killing of virally infected cells, and cell survival promoted by hematopoietic colony-stimulating factors. Apoptosis can be induced by various physical and chemical stimuli in the human promyelocytic cell line HL-60.

In contrast to what has been observed in murine thymocytes, no requirement for RNA or protein synthesis seems necessary to the apoptotic process in HL-60 cells as well as in most other human cell lines. Not only does cycloheximide prevent apoptosis in acute leukemia blast cells but it also causes apoptosis in HL-60 cells and in a human T-cell leukemia cell line. It seems that protein activation/modification, such as phosphorylation/dephosphorylation, rather than de novo protein synthesis, may play an important role in the mechanism of apoptosis in human cells. Phorbol esters can prevent DNA fragmentation in thymocytes and isolated nuclei. The tumor-promoting 12-O-tetra-decanoyl-phorbol-13-acetate (TPA) can rescue differentiating myeloid cells from apoptosis induced by deprivation in growth factor. Okadaic acid, a protein phosphatase inhibitor, prevents radiation-induced apoptosis in human lymphoid tumor lines but, at the same concentration, induces apoptosis by itself in various other human or rodent cell lines. Staurosporine, a relatively nonspecific protein kinase C inhibitor, induces apoptosis very rapidly in numerous cell lines. Taken together, these data suggest that activation of a phosphatase(s) or loss of activity of a kinase(s) could be of central importance in triggering the apoptotic process. The expression of various oncopogenes, such as c-myc, c-ras, bcl-2, or p53, also modulates the cell sensitivity to the induction of apoptosis by various stimuli.

HL-60 cells have been used extensively as a model to study the control of proliferation and differentiation. These cells can be induced to differentiate into functional monocytes/macrophages by agents such as phorbol myristate acetate (TPA), sodium butyrate, or 1a,25-dihydroxyvitamin D3(1,25-(OH)2D3) whereas treatment with retinoic acid (RA) or dimethyl sulfoxide (DMSO) induces them to differentiate into granulocytes.

DNA topoisomerases I and II (top 1 and 2), which are ubiquitous nuclear enzymes involved in essential nuclear functions such as DNA replication, transcription, recombination, and chromatid segregation, have been involved in cell differentiation. Top 2 activation was suggested to play a role in phorbol ester–induced HL-60 cells differentiation. Conversely, a decrease of top 2 level was reported to be associated with the granulocytic differentiation of these cells. Camptothecin (CPT), an antineoplastic agent that inhibits top 1 by stabilizing the cleavable enzyme-DNA complex, and genistein, a top 2 and tyrosine kinase inhibitor, were reported to interfere with the differentiation of HL-60 as well as other cell lines.

A variety of clinically useful antineoplastic agents, including epipodophyllotoxins, anthracyclines, anthracenediones, ellipticines, and aminoacridines, kill cells by inhibiting DNA
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MATERIALS AND METHODS

Chemicals. CPT, m-AMSA, doxorubicin, and nitrogen mustard were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (NCI; Bethesda, MD) and VP-16 and VM-26 were a gift from Bristol-Myers-Squibb Laboratories (Wallingford, CT). All agents were dissolved in DMSO at 10 mmol/L, aliquoted, and stored at −70°C. Further dilutions were made in culture medium just before use. The final concentration of DMSO in culture medium did not exceed 1% (vol/vol), which was nontoxic to the cells. [methyl-3H]Thymidine (78.5 Ci/mmol) and [2-14C]Thymidine (59 mCi/mmole) were purchased from New England Nuclear (Boston, MA). All other chemicals were of reagent grade and purchased from either Sigma Chemical Co (St Louis, MO) or other local sources.

Cell culture, labeling, and drug treatment. HL-60 cells were a gift from Dr T. Breitman (NCI). Cells were grown in suspension in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and 2 mmol/L glutamine. They were passed twice a week and studied between passages 20 and 40. Chinese hamster lung fibroblasts DC3F, used as internal standard cells in alkaline elution, were grown in Eagle's Modified Minimum Essential Medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 0.1 mmol/L nonessential amino acids. Cells were grown at 37°C in an atmosphere of 95% air and 5% CO2. All cell culture products were purchased from Advanced Biotechnologies Incorporated (Columbia, MD) and routinely monitored for the absence of Mycoplasma contamination. Labeling was performed by adding 0.1 μCi/mL of [methyl-3H]Thymidine or 0.02 μCi/mL of [2-14C]Thymidine for two
Fig 2. Agarose gel electrophoresis of DNA extracted from undifferentiated HL-60 cells (lanes 1, 3, and 5) and adherent TPA-differentiated HL-60 cells (lanes 2, 4, and 6) either untreated (lanes 1 and 2) or treated for 3 hours with CPT at 1 pmol/L (lanes 3 and 4) or 10 pmol/L (lanes 5 and 6).

doubling-times to exponentially growing cells seeded at an initial cell concentration of $5 \times 10^6$/mL. Cells were then chased in isotope-free medium overnight and treated with differentiating agents for the indicated times. Cell viability was determined using the trypan blue exclusion test. Cell number was determined from triplicate samples using an electronic cell counter (Coulter Electronics Inc, Hialeah, FL).

Quantification of DNA fragmentation by filter-binding assay. DNA fragmentation was measured as reported previously. Briefly, at specified times, approximately $0.5 \times 10^6$ cells were suspended in 10 mL ice-cold Hank’s Balanced Salt Solution (HBSS; Quality Biological Incorporated, Gaithersburg, MD) and then loaded onto protein absorbing filters (vinyl/acrylic copolymers filters, 0.8-μm pore size, 25-mm diameter; Metricel membrane; Gelman Sciences Inc, Ann Arbor, MI). Adherent TPA-differentiated cells were washed once with 5 mL HBSS before being harvested using a cell scraper. Cells loaded on the filters were washed with an additional 10 mL ice-cold HBSS. Lysis was subsequently performed with 5 mL LS10 buffer (0.2% sodium sarkosyl, 2 mol/L NaCl, 0.04 mol/L EDTA, pH 10.0). Filters were washed with 10 mL 0.02 mol/L EDTA, pH 10.0, and then processed as in the case of alkaline elution. DNA fragmentation was determined as the percentage of disintegrations per minute (dpm) in the lysis + wash fractions (fragmented DNA) divided by total intracellular dpm (total DNA).

Analysis of DNA fragmentation by agarose gel electrophoresis. Cellular DNA was extracted by a salting-out procedure as described previously. Electrophoresis was performed in 1.25% agarose gel in Tris-borate buffer (pH 8) containing 0.1% sodium dodecyl sulfate (SDS) (wt/vol) at 30 V for 14 hours. After electrophoresis, DNA was visualized by ethidium bromide staining.

Alkaline elution—Measurement of DNA SSB. DNA damage was quantitated by alkaline elution filter methods, as described in detail by Kohn et al. Briefly, HL-60 cells were labeled with 0.02 μCi/mL of [2-¹⁴C]-thymidine (59 mCi/mmol; New England Nuclear, Boston, MA) for 1.5 to 2 cell doublings at 37°C. All cells were then chased by resuspension in nonradioactive fresh medium before drug treatment. To measure the production of DNA lesions produced by CPT, VP-16, or m-AMSA, cells were washed in ice-cold HBSS (10 mL) containing drug before loading onto the filters. The [¹⁴C]-labeled, drug-treated cells were loaded onto polycarbonate filters (2-μm pore size, 25-mm diameter; Nucleopore Corp, Pleasanton, CA) that had been prewashed with ice-cold HBSS. Cells were lysed in 5 mL of 2% (wt/vol) SDS, 0.5 mg/mL of proteinase K (Boehringer Mannheim, Indianapolis, IN) at pH 10. The [³H]-labeled, DC3F-internal standard

Fig 3. Spontaneous and CPT-induced DNA fragmentation determined by filter-binding assay in adherent (△, ●) and nonadherent (○, ■) cells as a function of time of TPA pretreatment. HL-60 cells were induced to differentiate by treatment with 10 nmol/L TPA for the indicated times, before treatment with 1 μmol/L CPT or solvent (control). After 8 additional hours, DNA fragmentation was measured in adherent and in floating cells, either untreated (control: ○ — ○, △ — △) or treated with CPT (● — ●, ■ — ■).
cells, which had been irradiated on ice with 2,000 rad of a $^{137}$Cs source, were then added to the filters in a minimum volume (<0.3 mL) and immediately lysed with 5 mL of 2% (wt/vol) SDS, 0.5 mg/mL of proteinase K. The lysis solution was washed from the filters with 0.02 mol/L EDTA, pH 10 (5 mL), and the DNA was eluted with tetrapropylammonium hydroxide (RSA Corporation, Ardsley, NY)/EDTA, pH 12.1, containing 0.1% (wt/vol) SDS, at a flow rate of 0.08 to 0.12 mL/min. Fractions were collected at 5-minute intervals for 30 minutes. Elution curves were normalized with respect to internal standard cells to compensate for differences in flow rates between individual elution lines. SSB frequency expressed in rad-equivalents was calculated from the formula:

$$SSB = \left[ \log \left( \frac{r_i}{r_0} \right) / \log \left( \frac{R_0}{r_0} \right) \right] \times 2,000$$

where $R_0$, $r_i$, and $r_0$ are the fractions of $[^{14}C]$-DNA retained on the filter for 2,000-rad irradiated control cells, unirradiated control cells, and drug-treated cells, respectively.

**Measurement of DNA fragmentation in a cell-free system.** Cytoplasmic and nuclear fractions were prepared by washing HL-60 cells four times in ice-cold phosphate-buffered saline (PBS) (without Ca$^{2+}$ and Mg$^{2+}$) and then incubating the cells for 10 minutes on ice at a density of $10^7$ cells/mL in lysis buffer (150 mmol/L NaCl, 1 mmol/L K$_2$HPO$_4$, 5 mmol/L MgCl$_2$, 1 mmol/L EGTA, 10% glycerol, 0.1 mmol/L phenylmethylsulfonil fluoride, 0.15 U/mL aprotinin, 1.0 mmol/L Na$_2$VO$_4$, 5 mmol/L HEPES, pH 7.4) containing 0.3% Triton X-100. Lysates were centrifuged (2,000g for 10 minutes at 4°C) and supernatants collected as cytoplasmic fractions. Pellets (nuclei fractions) were then washed twice by centrifugation/resuspension in the lysis buffer without Triton. Cytoplasmic fractions from undifferentiated or TPA-differentiated (10 nmol/L, 24 hours) HL-60 cells either untreated or treated with CPT (1 pmol/L, 3 hours) or m-AMSA (5 pmol/L, 3 hours) were incubated with isolated nuclei from labeled undifferentiated or differentiated HL-60 cells at 37ºC for 15, 30, or 60 minutes. DNA fragmentation was then measured by the filter-binding assay. Controls were performed by incubating labeled nuclei with lysis buffer without triton, in the absence or presence of CPT (1 pmol/L) or m-AMSA (5 pmol/L).

**RESULTS**

Inhibition of DNA fragmentation induced by top inhibitors in TPA-differentiated HL-60 cells. HL-60 cells were induced to differentiate along a monocytic/macrophagic pathway by 24 hours of treatment with 10 nmol/L TPA. Control and TPA-differentiated HL-60 cells were then continuously treated for 3 or 24 hours with CPT (top 1 inhibitor) or VP-16, VM-26, or m-AMSA (top 2 inhibitors). DNA fragmentation was measured by filter-binding assay in control and TPA-treated adherent cells (Fig 1). The four drugs tested induced DNA-fragmentation in undifferentiated HL-60 cells, which increased with drug concentration and duration of exposure. After 3 hours of continuous exposure, the lowest drug...
concentration that induced significant DNA fragmentation in control cells was 0.1 \mu M for CPT, 5 \mu M for m-AMSA and VM-26, and 50 \mu M for VP-16. In contrast, no DNA fragmentation was observed in adherent TPA-treated cells after 3 hours of continuous treatment with CPT, m-AMSA, VM-26 (until 10 \mu M), or VP-16 (until 100 \mu M). This inhibition of drug-induced DNA fragmentation was even observed after 24 hours of continuous drug exposure. At this time, the lowest concentration at which significant DNA fragmentation was measured in control cells was 0.1 \mu M for CPT, 1 \mu M for m-AMSA, 0.5 \mu M for VM-26, and 1 \mu M for VP-16. In adherent TPA-treated cells, a slight increase of DNA fragmentation was observed after 24 hours continuous exposure to the highest tested concentrations of CPT (5 and 10 \mu M) and m-AMSA (10 \mu M) (Fig 1).

Inhibition of DNA fragmentation in adherent TPA-treated cells was confirmed by agarose gel electrophoresis (Fig 2). Whereas a characteristic ladder of nucleosome-size DNA fragments was observed in camptothecin-treated undifferentiated HL-60 cells, no DNA ladder was detectable in control cells or in adherent TPA-differentiated cells, whether untreated (Fig 2, lane 2) or treated with CPT. Hence, 24-hour TPA treatment did not induce apoptosis in HL-60 cells but rather inhibited drug-induced DNA fragmentation associated with apoptosis.

Time course of TPA-induced inhibition of DNA fragmentation. TPA treatment of HL-60 cells induces the rapid adherence of the differentiated cells to the culture flask because adherent cells can be observed as early as 1 hour after the beginning of the treatment (15%) and represent 70% of the total cells after 24 hours. Figure 3 shows the effect of duration of TPA pretreatment on DNA fragmentation induced by CPT. DNA fragmentation was measured in adherent and nonadherent cells, either untreated or treated with CPT at various times after TPA exposure.

In flasks that were not treated with CPT, the 10% background DNA fragmentation measured in adherent TPA-differentiated cells did not increase with time. By contrast, a time-dependent increase of DNA fragmentation was measured in floating cells. These data indicate that HL-60 cells that are not undergoing differentiation and do not attach to the flask are dying via apoptosis.

CPT-induced DNA fragmentation was detectable in the early attached cells and decreased slowly as a function of time of TPA pretreatment, indicating that the adherent phenotype is not sufficient to inhibit CPT-induced DNA fragmentation. By contrast, floating cells exhibited increasing CPT-induced DNA fragmentation, indicating that floating cells remain sensitive to the induction of DNA fragmentation by CPT.

TPA-induced inhibition of drug-induced DNA fragmentation is linked to differentiation of HL-60 cells into adherent macrophage-like cells. Pretreatment of HL-60 cells with a nontumor-promoting phorbol ester, 4\alpha\textit{TPA} (10 \textit{nmol}/L, 24 hours), which does not induce HL-60 cell differentiation, did not inhibit CPT-induced DNA fragmentation. On the other hand, treatment of HL-60 cells with phorbol dibutyrate ester (0.2 \mu M/L, 24 hours) that induces monocytic/macrophage-like differentiation similar to TPA, induced a complete suppression of CPT-induced DNA fragmentation in adherent cells (data not shown).

RA-induced HL-60 differentiation does not inhibit DNA fragmentation induced by top 1 and 2 inhibitors. DNA-fragmentation induced by 3- or 24-hour exposure to CPT, VP-16, VM-26, and m-AMSA was determined in HL-60 cells that had been treated for 4 days with all-trans RA (1 \mu M/L) to induce granulocytic differentiation. Figure 4 demon-
strates the lack of inhibition of drug-induced DNA fragmentation in RA-differentiated compared with control undifferentiated HL-60 cells. Increased DNA fragmentation was observed at day 5 in RA-differentiated cells in the absence of top inhibitors, indicating that RA-induced differentiation is associated with DNA fragmentation and apoptosis.\textsuperscript{53} Therefore, granulocytic differentiation of HL-60 cells does not inhibit drug-induced apoptosis.

Relationship between differentiation and topoisomerase inhibition. Because TPA-induced differentiation but not RA-induced differentiation of HL-60 cells was associated with an inhibition of DNA fragmentation induced by top 1 and 2 inhibitors, we looked at the effect of differentiating agents on drug-induced DNA SSB. These breaks correspond to the top-DNA cleavage complexes and are a direct measure of enzyme inhibition in drug-treated cells.

Figure 5 shows that TPA-induced differentiation did not modify the level of SSB induced by CPT. Conversely, monocytic/macrophagelike differentiation was associated with a slight decrease of the level of SSB induced by m-AMSA and a stronger decrease of SSB induced by VP-16. Therefore, monocytic/macrophage differentiation did not affect top 1 inhibition by CPT and reduced top 2 inhibition differently for VP-16 and m-AMSA. By contrast, Fig 6 demonstrates that RA-induced differentiation of HL-60 cells is associated with a slight and progressive decrease of SSB induced by either CPT, VP-16, or m-AMSA. These data indicate that the decrease of DNA fragmentation in TPA-differentiated HL-60 cells is not correlated with a decrease of SSB induced by top inhibitors.

The inhibition of DNA fragmentation in TPA-differentiated cells is not restricted to top 1 and 2 inhibitors. As shown in Fig 7, the DNA fragmentation induced by various inhibitors, vinblastine sulfate (tubulin), calphostin C (protein kinase C), cycloheximide (protein synthesis), or \textgamma-irradiation was also inhibited in TPA-treated adherent cells. Additional experiments also demonstrated a similar suppression for UV light, hyperthermia, nitrogen mustard, or EGTA (data not shown). Therefore, TPA-induced differentiation is associated with a global inhibition of apoptosis induced by a broad range of chemical and physical agents.

The inhibition of apoptosis in TPA-differentiated HL-60 cells takes place in the cytoplasm. To study the mechanism of inhibition of apoptosis in TPA-differentiated HL-60 cells, we designed a cell-free system in which labeled nuclei from control or TPA-differentiated cells were incubated with the cytoplasms from drug-treated control or TPA-differentiated cells.
measured by filter-binding assay, in isolated nuclei from HL-60 cells. Isolated nuclei were incubated in the presence of drug alone (5 [μmol/L]) or in the presence of the cytoplasm from undifferentiated HL-60 cells previously treated for 3 hours with either CPT (1 [μmol/L]) or m-AMSA (5 [μmol/L]).

![Graph showing DNA fragmentation time course](image)

**Fig 9.** Time course of the induction of DNA fragmentation, as measured by filter-binding assay, in isolated nuclei from HL-60 cells. Isolated nuclei were incubated in the presence of drug alone (△) or in the presence of the cytoplasm from undifferentiated HL-60 cells previously treated for 3 hours with either CPT (1 [μmol/L]) or m-AMSA (5 [μmol/L]) (●).

As shown in Fig 8, the level of spontaneous DNA fragmentation was slightly lower in TPA-differentiated than in control HL-60 cells. Direct treatment of the nuclei with either CPT (1 [μmol/L]) or m-AMSA (10 [μmol/L]) did not induce DNA fragmentation. By contrast, cytoplasms from CPT- or m-AMSA-treated undifferentiated HL-60 cells were able to induce DNA fragmentation in the nuclei from either undifferentiated or TPA-differentiated HL-60 cells. This first part of the experiment indicates that the chromatin of TPA-differentiated cells remains sensitive to the induction DNA fragmentation.

Secondly, no DNA fragmentation was observed in nuclei from either control or TPA-treated cells when incubated with the cytoplasms from TPA-differentiated cells treated by either CPT or m-AMSA. Therefore, inhibition of DNA fragmentation in TPA-differentiated HL-60 cells takes place in the cytoplasm and is the consequence of the inactivation of either an endonuclease or a pathway of activation of an endonuclease.

Figure 9 shows that the induction of DNA fragmentation in isolated nuclei incubated with cytoplasms from cells treated with top inhibitors is very rapid, being detectable after 15 minutes of incubation, and confirms the absence of induction of nuclear DNA fragmentation by the drugs in the absence of cytoplasmic extract.

**DISCUSSION**

Apoptosis has been reported to be the mode of cell death of some differentiated cells. In the absence of treatment by top inhibitors, we observed that HL-60 granulocytic differentiation is associated with DNA fragmentation starting 4 to 5 days after the initiation of RA treatment. This observation is in agreement with a previous study. Peripheral blood neutrophils also die by apoptosis while being recognized and engulfed by macrophages. We found also that TPA induces DNA fragmentation associated with morphologic changes typical of apoptosis in the population of cells that fail to attach early to the culture dish. By contrast, no apoptotic cells were observed among adherent TPA-differentiated HL-60 cells up to 5 days after the beginning of TPA treatment.

DNA top inhibitors inhibit cell growth by different mechanisms. CPT, a top 1 inhibitor, and novobiocin, a top 2 inhibitor, were reported to induce terminal differentiation in several cell lines. Top inhibitors also induce cell death by either G2-arrest or apoptosis, depending on the cell types. G2 arrest can be observed in various cell lines, including human colon carcinomas (HT-29 and KM20L2) and Chinese hamster ovary (CHO) and lung (DC3F) cell lines, whereas apoptosis can be induced in HL-60 cells by a broad range of chemical and physical agents. The cytotoxicity of top inhibitors is thought to result primarily from the induction of enzyme-mediated protein-linked DNA breaks.

In accordance with previous reports, we observed that macrophagelike differentiation by TPA was associated with a significant reduction of VP-16–induced and, to a lesser extent, m-AMSA–induced top 2-mediated DNA breaks. Conversely, phorbol ester–induced differentiation of HL-60 cells did not modify the level of DNA SSBs induced by the top 1 inhibitor CPT, which is consistent with a previous study. Top 2 levels were reported to be decreased in HL-60 cells induced to differentiate toward the granulocytic pathway. However, the level of DNA SSB induced by m-AMSA and VP-16 as well as the level of CPT-induced top 1-mediated DNA breaks were only slightly decreased in granulocytodifferentiated HL-60 cells. Therefore, drug-induced apoptosis in differentiated HL-60 cells is not correlated with modulation of drug-induced and top-mediated DNA cleavage.

Because the drug-induced DNA breaks reverse in cells that are programmed to die either by G2-arrest or apoptosis, additional mechanisms are necessary to trigger the cytotoxic effect of top inhibitors. It is known that inhibition of DNA synthesis by aphidicolin or hydroxyurea abolishes the cytotoxicity of camptothecin without changing the level of protein-linked DNA breaks. These results suggested that an interference of moving DNA replication forks with drug-stabilized top-DNA complexes is critical for cell death. Because TPA-induced differentiation is associated with a progressive inhibition of DNA synthesis, as determined by incorporation of tritiated thymidine (Rovera et al and data not shown), this effect could explain the suppression of CPT-induced DNA fragmentation. However, inhibition of DNA synthesis cannot explain the complete inhibition of VP-16 or m-AMSA–induced DNA fragmentation in TPA-differentiated cells because aphidicolin does not block apoptosis in HL-60 cells. Lastly, the appearance of endonucleolytic DNA damage in HL-60 cells does not require new protein synthesis, is not specific for inhibitors of top and is inhibited in TPA-differentiated cells treated with a variety of agents that are not S-phase specific (present report).
Phorbol esters have been demonstrated to inhibit apoptosis in other systems. In thymocytes, sustained increase in the cytosolic level of calcium or cAMP induced by various agents can trigger apoptosis, an effect inhibited by phorbol esters as well as other signals activating protein kinase C. This inhibitory effect of phorbol esters can be prevented by the protein kinase C inhibitor H-7. Phorbol esters were also reported to prevent radiation-induced apoptosis in mouse fibroblasts and to mimic the effects of growth factors in preventing apoptosis in various cell types. As fibroblast growth factor, phorbol esters prevent apoptosis of human vascular endothelial cells observed under serum-free culture conditions. Similarly, they were shown to inactivate the mechanism of cell death triggered by interleukin-2 (IL-2) deprivation in IL-2-dependent T lymphocytes and were demonstrated to rescue IL-3–dependent differentiating leukemic cells when deprived in IL-3. Lastly, phorbol esters were reported to inhibit apoptosis induced by VP-16 in chronic lymphocytic leukemia cells. Thus, phorbol esters were proposed to act as tumor promoters by inhibiting programmed cell death. TPA-induced inhibition in HL-60 cells seems to be different from effects reported in other cell systems. Indeed, short-term treatment with TPA had no effect on the induction of DNA fragmentation by top inhibitors. As progressive inhibition was observed only in adherent cells, genotypic and phenotypic changes associated with the phorbol ester-induced, macrophagelike differentiation might have modified the susceptibility of the cells to the induction of apoptosis.

The expression of the early response genes c-jun and c-fos, whose products act as key regulatory transcription factors when associated to form the AP-1 transcription factor, is rapidly induced in TPA-treated HL-60 cells, whereas RA was shown to be a negative regulator of AP-1–responsive genes by inducing the α retinoid receptor to form a nonproductive complex with c-jun. The induction of c-jun was associated with CPT-induced internucleosomal DNA fragmentation in human U-937 leukemia cells and proposed to be part of the signaling cascade inducing apoptosis in these cells. Whether the changes in c-jun expression associated with differentiation modify the susceptibility to apoptosis remains speculative. The deregulation of c-myc expression was recently demonstrated to be a potent inducer of apoptosis, to potentiate apoptosis in IL-3–dependent myeloid cell lines in response to IL-3 deprivation, and to be a component of activation-induced apoptosis in T lymphocytes. Because various cell lines, including HL-60, that exhibit an amplification of c-myc were shown to be very sensitive to the induction of apoptosis by cytotoxic agents, c-myc expression has been suggested to be also involved in this apoptotic pathway. According to this hypothesis, the marked decrease in c-myc RNA levels observed in mononcytic and macrophagelike differentiated HL-60 cells could account for the inhibition of apoptosis observed in these cells. Similarly, the decrease in c-myc RNA reported in normal bone marrow progenitors cells undergoing terminal differentiation in culture could explain the absence of DNA fragmentation in mature granulocytes treated by VP-16. However, c-myc RNA levels are also decreased in granulocytic differentiated cells in which we did not observe a strong inhibition of apoptosis, suggesting that a pathway independent of c-myc remains effective in these cells.

The bcl-2 gene, one of the genes currently known to be involved in apoptosis regulation, was shown to be operative in myeloid cells, but a marked decrease in bcl-2 mRNA and protein levels was observed in mononuclear and granulocytic differentiated HL-60 cells. Because we observed a decrease of apoptosis induced by various stimuli in the TPA-differentiated HL-60 cells, we conclude that the death pathway operating in HL-60 cells treated by cytotoxic or metabolic agents is not correlated with bcl-2 expression. The existence of multiple apoptosis pathways that can be distinguished by their dependence on bcl-2 has also been demonstrated in the thymus, using a transgenic model.

We observed recently that DNA fragmentation induced in HL-60 cells by top inhibitors was inhibited by spermine and 3 aminobenzamide, suggesting that chromatin structure and poly(ADP)ribosylation play a role in the susceptibility of HL-60 cells to apoptosis. To determine whether TPA is inducing changes at the chromatin or the cytoplasm level, we used a cell-free system in which cytoplasm from top inhibitor-treated cells was incubated with nuclei from either undifferentiated or TPA-differentiated HL-60 cells. We observed that nuclei from adherent TPA-treated HL-60 cells remained as sensitive to the endonuclease as nuclei from un-differentiated cells. By contrast, cytoplasm from TPA-differentiated cells treated in the same conditions with top inhibitors was unable to induce fragmentation. These results are consistent with a previous report indicating that untreated HL-60 cells contain an endonuclease capable of generating the nucleosomal DNA degradation pattern but that this endonuclease is normally limited to an extranuclear compartment of the cell. The differentiation-associated modifications could be either an inhibition of the endonuclease or a modification of some other step in the activation pathway of this endonuclease.

Inhibition of apoptosis induced by various stimuli in adherent TPA-differentiated HL-60 cells could be used as a model for modulation of apoptosis in human cells. Understanding of the mechanisms of modulation of apoptosis, either its potentiation in tumor cells or its inhibition in normal cells, may be useful to provide new therapeutic strategies and improve the tolerance to cancer treatments.

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