Etoposide-induced Differentiation of U937 Promonocytic Cells: AP-1-dependent Gene Expression and Protein Kinase C Activation[1]

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Abstract

The administration of 150 nM etoposide, an inhibitor of DNA topoisomerase II activity, decreased the proliferation and induced the differentiation of U937 human promonocytic cells, as determined by nitroblue tetrazolium reduction, surface accumulation of CD11b/CD18 and CD11c/CD18 integrins, and c-*fms* protooncogene expression. The expression of these differentiation markers started to be detected at 24 h of treatment. Etoposide caused little cell damage, as determined by trypan blue exclusion and by apoptotic-like DNA degradation, which was slightly initiated at 48 h. The treatment induced a transient increase in c-*fos*, c-*jun*, and *jun B* mRNA levels, with maximum values at 12 h, a transient increase in collagenase mRNA level, with maximum value at 48 h, and a progressive increase in vimentin and lamin A and C mRNAs. These changes were qualitatively similar to those produced by 12-*O*-tetradecanoylphorbol-13-acetate. Etoposide also caused a transient increase of total AP-1 binding activity, with maximum value at 12 h of treatment, as determined by gel retardation assays. The drug produced an early transient activation (3–6 h) of membrane-bound protein kinase C, followed by the later activation (48 h) of both the membrane and cytosolic enzyme. The protein kinase C inhibitors, sphinganine and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), attenuated the induction of differentiation markers by etoposide. These results suggest that protein kinase C and AP-1-dependent gene expression could be involved in myeloid cell differentiation by DNA topoisomerase II inhibitors.

Introduction

DNA topoisomerases are enzymes capable of generating local changes in DNA topology which are required for DNA replication, transcription, recombination, and chromatid segregation (1). In addition, topoisomerases are the targets for an important number of antitumor drugs. This is the case of DNA intercalating agents, such as doxorubicin or amsacrine, and of nonintercalators, such as the epipodophyllotoxins etoposide and teniposide. These compounds cause the stabilization of the cleavable DNA topoisomerase II covalent complexes, preventing subsequent DNA religation and producing, as a consequence, DNA strand breaks (2). Although these breaks are rapidly repaired upon drug removal, the toxic action may be persistent, leading to cell death (2). In fact, the primary topoisomerase-linked DNA strand breakage generated by high concentrations of topoisomerase inhibitors may be rapidly followed by irreversible, secondary oligonucleosome-sized DNA fragmentation, which is characteristic of apoptosis (3, 4). Nevertheless, we and others have demonstrated that topoisomerase II inhibitors may also induce the *in vitro* differentiation of myeloid cells (5–14). This observation is of great importance since it opens the possibility for the clinical use of antitopoisomerase drugs in differentiation therapies. However, the mechanisms responsible for such a differentiation induction are unknown. Possible explanations are that: (*a*) the reduction in topoisomerase II activity is *per se* a signal or a condition for differentiation (5, 11, 15); (*b*) differentiation is facilitated by the perturbations of the cell cycle caused by cytostatic agents such as antitopoisomerase drugs (14); and (*c*) these drugs elicit intracellular signals and activate the expression of genes that are important for differentiation.

The latter hypothesis is examined in the present work by analyzing the action of subcytotoxic concentrations of etoposide on the expression of differentiation markers, the expression of early protooncogenes and AP-1-regulated genes, and the activity of PKC[3] in U937 human promonocytic cells (16). The results are compared with those produced by TPA, a powerful inducer of differentiation and PKC activity in this cell line (17).

Results

Cell Growth and Differentiation. We measured the effect of continuous treatments with etoposide on the growth of U937 cells. At the dose of 150 nM the drug progressively decreased the growth activity, as revealed by cell counting (Fig. 1). The treatment preferentially affected cell transit throughout G_2, as indicated by the accumulation of cells in this phase of the cycle (Fig. 1).

Alkaline elution assays indicated that a 3-h treatment with 150 nM etoposide sufficed to produce a slight amount of primary, topoisomerase-linked DNA strand breaks (results not shown). Nevertheless, secondary DNA degradation characteristic of apoptosis was only slightly initiated at the second day of treatment, as observed by electrophoresis (Fig. 2). In addition, the proportion of nonviable cells,

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[3] The abbreviations used are: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NBT, nitroblue tetrazolium; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; PBS, phosphate-buffered saline; kb, kilobase(s).

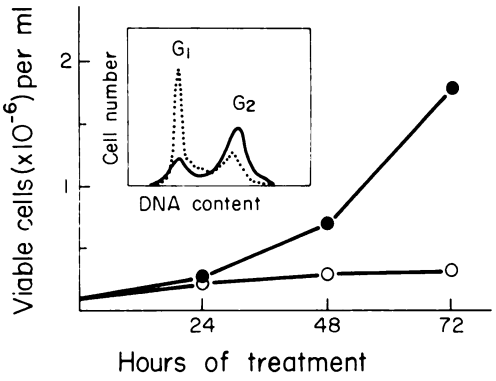

Fig. 1. Effect of 150 nM etoposide on U937 cell growth and cycle distribution. The main graph shows the increase in cell number in untreated (●) and drug-treated (○) cultures. Data are the average of two determinations with similar results. The *inset* shows the cell cycle distribution in untreated (*dotted line*) and 72-h treated (*continuous line*) cultures, as determined by flow cytometry analysis of propidium iodide-stained cells.

which represented the 4% in untreated cultures, remained unchanged until the second day and underwent only a slight increase (18%) at the third day of treatment, as indicated by trypan blue exclusion. Hence, the concentration of 150 nM etoposide was considered subcytotoxic and was adopted for further experiments.

The capacity of etoposide to induce the expression of myeloid differentiation markers, *i.e.*, the surface accumulation of the CD11b/CD18 and CD11c/CD18 leukocyte integrins (18, 19) and the reduction of NBT (20), was afterwards determined. We also measured the expression at the RNA level of the c-*fms* protooncogene, which is a specific characteristic of the mature monocyte-macrophage phenotype (21). The phorbol ester TPA was used as a positive control. The results in Fig. 3 indicate that etoposide increased the expression of all these differentiation markers, the increase being already observed at 24 h of treatment.

Gene Expression and AP-1 Binding. Northern blot assays were carried out to analyze the effect of etoposide on the expression of other genes which might be involved in differentiation. This included the c-*fos*, c-*jun*, and *jun B* protooncogenes, which encode protein constituents of the AP-1 transcription factor (22); the collagenase and vimentin genes, the expression of which is regulated by AP-1 (23, 24); and the A lamin gene, which encodes the nuclear A and C intermediate filament proteins (25). The results are represented in Fig. 4. Etoposide caused a transient increase in c-*fos*, c-*jun*, and *jun B* mRNA levels, which reached the maximum values at 12 h of treatment, to decrease thereafter. The drug also caused a transient increase in collagenase mRNA levels, with maximum value at 48 h. Finally, the levels of vimentin and of lamin A and C mRNAs underwent a progressive increase (24 h and thereafter). TPA (15 nM), which was included as a positive control, also activated the expression of those genes, although at earlier times than etoposide (Fig. 4).

Fig. 5 shows the effect of etoposide on total AP-1 binding activity, as determined by gel retardation assays. The drug caused an increase in binding activity, which reached the maximum value at 12 h, to decrease at 48 h to control levels. An increase in AP-1 binding was already observed at 3 h of treatment with 15 nM TPA, which was included as a positive control.

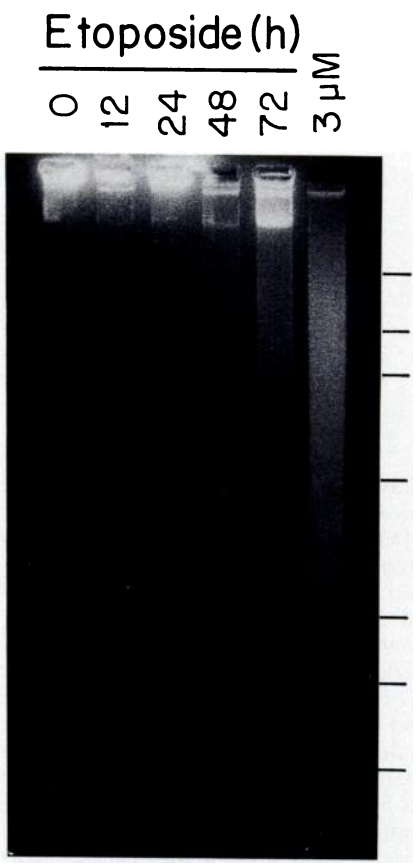

Fig. 2. DNA degradation following etoposide treatment. DNA extracted from cells treated with 150 nm etoposide for the indicated time periods was fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV illumination. DNA extracted from cells treated for 24 h with 3 μM etoposide was also analyzed as a control of apoptotic DNA degradation. The *horizontal lines* at the *margins* indicate the position of simultaneously run markers (from *top* to *bottom*: 4.4, 2.5, 1.9, 1.1, 0.61, 0.45, and 0.27 kb).

PKC Activity. Since etoposide and TPA had similar effects on gene expression and TPA is a potent activator of PKC, we were interested to measure the possible action of etoposide on PKC activity. The results are represented in Fig. 6. An increase in membrane-associated PKC activity could be detected at 3 and 6 h of treatment, to decrease again at 12 h. By contrast, no significant changes in cytosolic PKC activity were observed at these times. At 48 h of treatment, PKC activity was again increased, affecting both the membrane-bound and cytosolic fractions.

Effect of PKC Inhibitors. To determine whether PKC activation could be effectively involved in differentiation induction by etoposide, experiments using the PKC inhibitors sphinganine, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), and staurosporine were carried out. Three different experimental designs were used (Fig. 7). In the first design, cells were treated for 48 h with etoposide, either alone or in the presence of the PKC inhibitors (Fig. 7*A*). This was aimed at examining the total (early and late) PKC activation. In the second design, cells were first incubated for 24 h with etoposide, either alone or in the presence of the PKC inhibitors, then washed and let to recover for 24 h in the absence of all drugs (Fig. 7*B*). This was aimed to

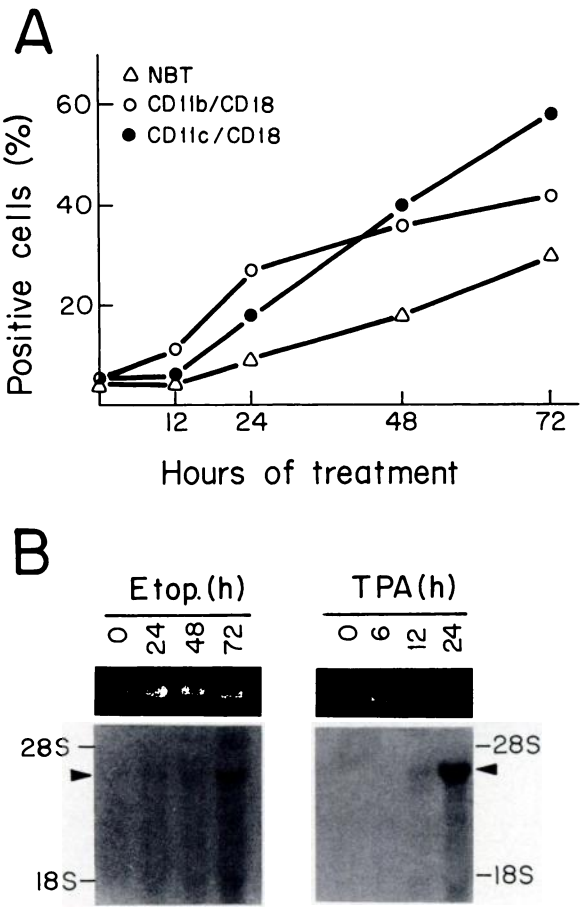

Fig. 3. Time-course expression of differentiation markers. *A*, percentage of cells which reduce NBT and express the CD11b/CD18 and CD11c/CD18 leukocyte integrins at different times of treatment with 150 nM etoposide. Data are the average of two determinations with similar results. Values in untreated cells were 4% for NBT and 5% for both CD11b/CD18 for CD11c/CD18. Values in TPA-treated cells (24 h at 15 nM) were 65% for NBT, 71% for CD11b/CD18, and 82% for CD11c/CD18. *B*, changes in the level of c-*fms* mRNA (*arrowheads*) in cells treated with 150 nM etoposide or 15 nM TPA. Total cytoplasmic RNA samples (15 μg/lane) were separated by electrophoresis, transferred to nylon membranes, and hybridized with a c-*fms*-specific cDNA probe. The position of the 28 S and 18 S ribosomal RNAs is indicated. Ethidium bromide staining of 28 S ribosomal RNA in the gel is shown as a control of sample loading.

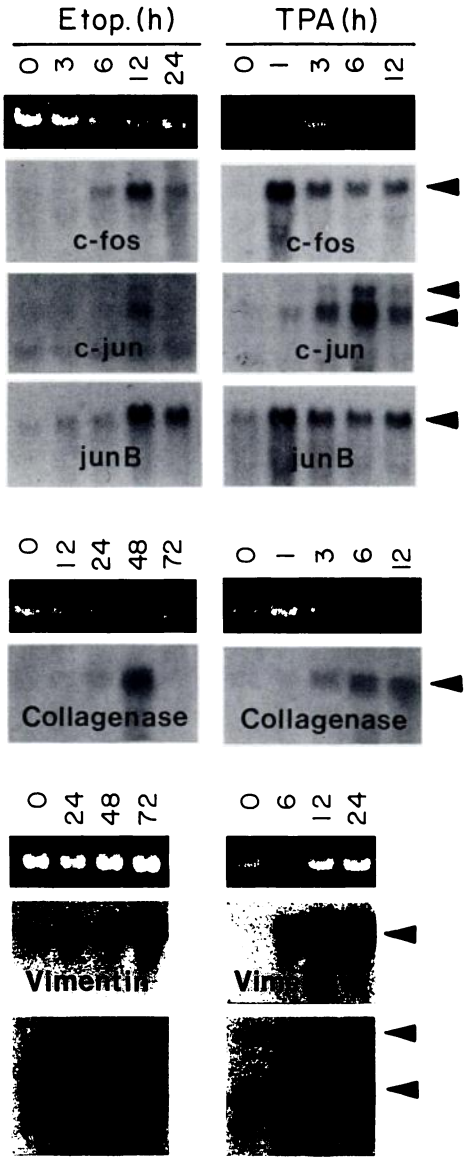

Fig. 4. Effect of etoposide and TPA on specific mRNA levels. Total cytoplasmic RNA was analyzed by Northern blot. A filter was successively hybridized with c-*fos*, c-*jun*, and *jun B*-specific probes, another one with vimentin and lamin probes, and a third one with a collagenase probe. All other conditions were as in Fig. 3*B*. The c-*jun*-specific probe recognized two mRNA species of 3.4 and 2.7 kb. The lamin-specific probe recognized two mRNA species of 3.0 and 2.0 kb corresponding to lamins A and C, respectively.

specifically examine the early activation of membrane-bound PKC activity. In the third design, cells were incubated for 24 h with etoposide alone and then for 24 h more with etoposide either alone or in the presence of the PKC inhibitors (Fig. 7*C*). This was aimed at specifically examining the late activation of PKC activity. As a control, cells were incubated for 24 h with TPA, either in the absence or in the presence of the PKC inhibitors (Fig. 7*D*). The obtained results are expressed in Table 1. It was found that sphinganine and H7 attenuated the etoposide-mediated induction of the CD11b/CD18 integrin. By contrast, staurosporine did not attenuate and even potentiated the etoposide-mediated induction of this differentiation marker. Allowing for some quantitative differences, the same results were obtained when the PKC inhibitors were applied for the total 48 h or the partial 24-h treatment periods. The three PKC inhibitors attenuated the TPA-induced expression of the differentiation marker, which is in agreement with earlier published observations (26, 27). At the concentrations used, none of the PKC inhibitors caused significant cell mortality (more than 90% of the cells excluded trypan blue at 48 h of treatment). Sphinganine and H7 did not alter the basal level of CD11b/CD18 expression, but staurosporine slightly activated the expression of this marker at 48 h of treatment (Table 1).

Discussion

The results in this work indicated that subcytotoxic concentrations of the topoisomerase II inhibitor etoposide, which

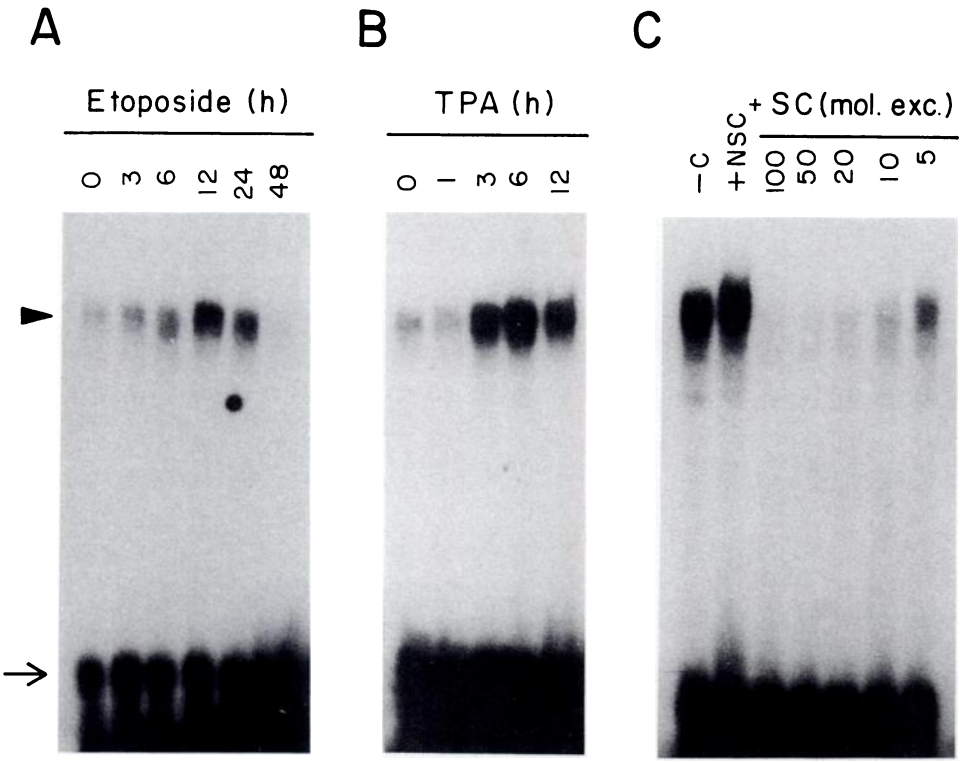

Fig. 5. Effect of etoposide on AP-1 binding activity. Nuclear extracts from cells treated for the indicated time periods with either 150 nM etoposide (*A*) or 15 nM TPA (*B*) were analyzed by gel retardation assay for AP-1 binding. The AP-1-specific signal in these assays was totally suppressed by the presence of 100-fold molar excess of specific competitor (not shown). *C*, nuclear extracts from cells treated for 6 h with TPA were assayed for AP-1 binding in the absence of competitor (*−C*), in the presence of 100-fold molar excess of nonspecific competitor (*+NSC*), and in the presence of different amounts (from 100- to 5-fold molar excess) of specific competitor (*+SC*), as indicated in "Materials and Methods." The position of free oligoprobe (*arrow*) and oligoprobe-AP-1 complexes (*arrowhead*) are indicated.

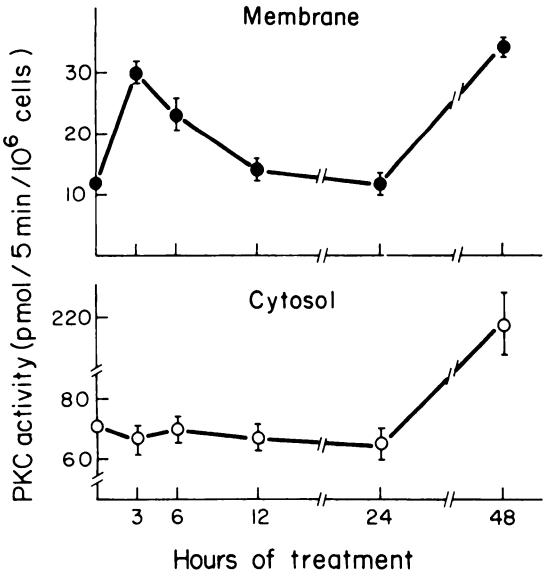

Fig. 6. Effect of etoposide on PKC activity. The graphs represent the membrane-bound and cytosolic PKC activities at different times of treatment with 150 nM etoposide. *Points*, mean of two determinations; *bars*, SD.

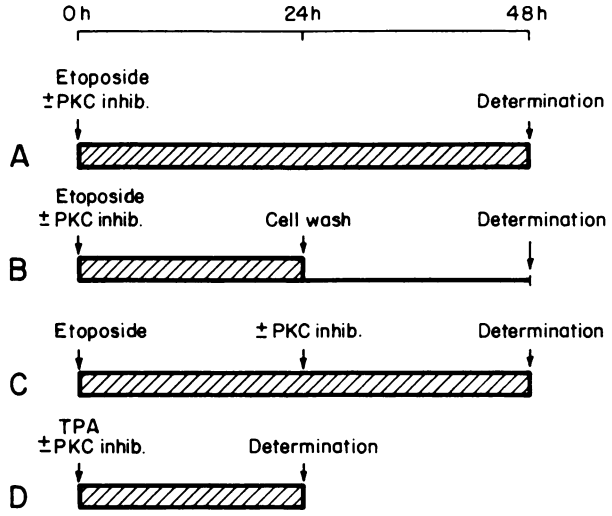

Fig. 7. Experimental designs used to determine the effect of PKC inhibitors (H7, sphinganine, and staurosporine) on the etoposide- and TPA-induced expression of CD11b/CD18. *Design A* was aimed at examining the total (early plus late) PKC activation (see Fig. 6), while the early and the late enzyme activations were separately examined in *Designs B* and *C*, respectively. As a control, the effect of the PKC inhibitors was also examined in TPA-treated cells (*Design D*). The results are indicated in Table 1.

induced the differentiation of U937 promonocytic cells, also caused the activation of AP-1-regulated gene expression. This was demonstrated by measuring total AP-1 binding activity as well as the expression of individual genes. Some of these genes could be important for the acquisition of the mature phenotype, as we had earlier demonstrated in the case of vimentin (28). The expression of c-*fos*, c-*jun*, and *jun B* (12 h) preceded the expression of differentiation markers (24 h) and then could be involved in the trigger of differentiation. In fact, it is known that these early protooncogenes may be implicated in the activation of diverse cellular processes such as proliferation, differentiation (22), and apoptosis (29). In an earlier work, Rubin *et al.* (30)

Table 1 Effect of PKC inhibitors, etoposide, and TPA on CD11b/CD18 surface integrin expression

A, B, and C correspond to the experimental designs in Fig. 7. Values represent the mean ± SD of at least three determinations. Sphinganine (SPH) was used at 5 μM, H7 at 100 μM, staurosporine (ST) at 4 nm, etoposide at 150 nm, and TPA at 3 nm. Exp., Experimental.

Exp. design	Treatment	Positive cells (%)
	None	4 ± 0.7
	SPH, 48 h	3 ± 1.1
	H7, 48 h	6 ± 1.7
	ST, 48 h	19 ± 3.2
A	Etoposide	40 ± 2.3
	Etoposide + SPH	17 ± 2.8
	Etoposide + H7	21 ± 2.4
	Etoposide + ST	50 ± 3.3
B	Etoposide	32 ± 1.9
	Etoposide + SPH	11 ± 0.9
	Etoposide + H7	15 ± 2.1
C	Etoposide	42 ± 4.1
	Etoposide + SPH	24 ± 1.8
	Etoposide + H7	27 ± 2.2
D	TPA	53 ± 6.0
	TPA + SPH	31 ± 3.6
	TPA + H7	23 ± 3.2
	TPA + ST	9 ± 2.2

showed that etoposide activated c-*fos* and c-*jun* expression in HL-60 human promyelocytic cells. However, the drug was used at highly toxic concentrations, which rapidly provoked oligonucleosome-sized DNA degradation. Hence, it is possible that c-*fos* and c-*jun* could be mainly regulating the trigger of apoptotic cell death. Our experiments do not clearly allow the separation of events leading to differentiation from those regulating apoptosis. Nevertheless, the fact that at the subcytotoxic concentrations of etoposide used apoptotic-like DNA fragmentation was only slightly initiated at 48 h of treatment might indicate that c-*fos* and c-*jun* were preferentially signaling cell differentiation instead of cell death.

The possibility that PKC could be involved in differentiation by antitopoisomerase drugs was earlier suggested by the observations of Constantinou *et al.* (5), which indicated that the nonspecific topoisomerase II inhibitor Novobiocin caused differentiation of TPA-sensitive but not of TPA-resistant HL-60 cells and that the PKC inhibitor H7 prevented the Novobiocin-induced differentiation. Our results directly demonstrated that differentiation of U937 cells by etoposide is accompanied by the activation of PKC. The treatment caused and early transient activation (3–6 h) of membrane-bound PKC, which was followed by a late activation (48 h) of total, membrane-bound plus cytosolic enzyme. The early PKC activation preceded and then could be involved in the activation of early protooncogene expression (12 h) and the trigger of differentiation (24 h). The late activation of total enzyme, which has currently been observed with other differentiation inducers (31, 32), might be required for the achievement of the differentiated phenotype (32). The fact that the etoposide-induced expression of differentiation markers was reduced when the PKC inhibitors H7 and sphinganine were applied for discrete time periods confirmed that both the early and late activation of PKC were important for differentiation. It could be surprising that the PKC inhibitor staurosporine potentiated the action of etoposide, although it inhibited differentiation by TPA. The action of staurosporine seems to depend on the inducer used, since it was also found to potentiate differentiation by 1α,25-dihydroxyvitamin D_3 and retinoic acid, which activate PKC, while decreasing differentiation by TPA in HL-60 cells (27). Such a discrepancy may be explained by differences in the signaling pathways elicited by TPA and the other inducers as well as by the poor specificity of action of the protein kinase inhibitors (33).

Our results give rise to several questions. One of them concerns the mechanism by which etoposide, the primary target of which are the topoisomerase-DNA complexes, rapidly activated membrane-bound PKC. A possible explanation is that the limited amount of primary DNA strand breakage, resulting from topoisomerase-DNA complex stabilization, could itself elicit PKC activation throughout yet unknown pathways. In this regard, Radler-Pohl *et al.* (34) suggested that DNA damage could be the primary step signaling other cellular events, such as activation of cytoplasmic protein kinases and stimulation of AP-1 activity, in UV-irradiated cells. Another explanation is that PKC could be activated as a consequence of nonspecific membrane perturbations, since epipodophyllotoxins (etoposide and teniposide) are known to interact with membrane lipids and alter membrane fluidity (35). Experiments using topoisomerase II inhibitors which do not cause topoisomerase-DNA cleavable complex stabilization nor primary DNA strand breakage are in progress to investigate this problem.

Another question is the reason for the temporal dissociation between the early activation of membrane-bound PKC, observed at 3 h, and the maximum AP-1 binding activity, detected at 12 h. It is believed that PKC may induce AP-1 activation by both pre- and posttranslational mechanisms. For instance, Boyle *et al.* (36) proposed that the rapid AP-1 activation by TPA in some epithelial and fibroblastic cells could be due to decreased phosphorylation at specific sites of pre-existing c-*jun*, resulting in an enhanced DNA binding activity. By contrast, AP-1 activation at prolonged times of treatment could be due to new synthesis of c-*fos* and c-*jun*. As indicated by the same authors, the importance of the former, posttranslational mechanism would be negligible in cell types in which the basal levels of c-*jun* are very low. If this is the case in nonstimulated U937 cells, AP-1 activation could only occur at the expense of new production of c-*fos* and c-*jun*, which under our conditions was detected at the RNA level at 12 h of etoposide treatment.

Materials and Methods

Cell Culture and Drug Treatment. U937 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 0.2% sodium bicarbonate in a humidified 5% CO_2 atmosphere at 37°C. TPA, etoposide, DL-sphinganine, staurosporine, and H7 were obtained from Sigma Química, Madrid, Spain. TPA and etoposide were dissolved in dimethyl sulfoxide at 1.5 and 20 mM, respectively, sphinganine at 10 mM in ethanol, and H7 and staurosporine at 1 mM in distilled water. The solutions were stored at −20°C. At the final concentrations used, the solvents were without significant effects on cell growth and differentiation. Cells were seeded for experiments in a mixture of old and fresh media (1:3, v/v) at 10^5 cells/ml. Cells from control cultures were harvested at the exponential

growth phase. Cells from treated cultures were harvested at the times indicated in each experiment. To prevent long-term cultures from reaching plateau densities or nutrient exhaustion, at the second day of treatment, they were supplemented with an equal volume of fresh medium containing the corresponding differentiation inducer. Cell growth and viability were checked using an hemocytometer and trypan blue exclusion, respectively. Cell cycle distribution was determined by flow cytometry determinations after propidium iodide staining, as described earlier (10).

Measurement of Differentiation Markers. The ability of the cells to reduce NBT was determined by incubating them for 20 min at 37°C in PBS solution containing 0.2% NBT and 0.15 μM TPA; then the cells were examined with a microscope for the presence of formazan precipitates. The surface expression of CR3 (CD11b/CD18) and p150,95 (CD11c/CD18) leukocyte integrins was determined by indirect immunofluorescence combined with flow cytometry, as it was described in a previous work (10).

Determination of Apoptotic DNA Degradation. Cells (10^6) were washed with PBS, resuspended in 100 μl of 45 mM Tris-borate buffer (pH 8.0) containing 0.25% Nonidet P-40 and 0.1% RNase A, and incubated for 1 h at 37°C. Proteinase K was then added to a final concentration of 1 mg/ml, and the incubation was followed for 1 h more. The samples were mixed at 70°C with 20 μl of 10 mM EDTA (pH 8.0) containing 0.25% (v/v) bromophenol blue, 40% sucrose, and 1% low-melting point agarose; then 25 μl of the mixture were electrophoresed in 2% agarose gels containing 0.5 μg/ml of ethidium bromide. *Hind*III-digested ϕ29 DNA was used as a marker.

RNA Blot Assays. Total cytoplasmic RNA was prepared as described in a previous work (37). RNA samples (15 μg/lane) were denatured, electrophoresed in 1.1% agarose-formaldehyde gels, and blotted to nylon membranes (Hybond-N; Amersham). RNA blots were prehybridized, hybridized with excess ^{32}P-labeled probes, washed under highly stringent conditions, and finally autoradiographed. The probes used were: the 2.7-kb *fos*-specific *Xho*I-*Nco*I fragment of pc-fos^{human} clone (38); the 1.8 plus 0.8-kb human *jun*-specific *Eco*RI-*Pst*I fragments of AH119 clone (39); a pGEM-2 plasmid containing the human *jun B* cDNA (40); the 1.1-kb human vimentin-specific *Xho*I fragment of p4F1 clone (41); the 2.0-kb *Eco*RI fragment of a human C lamin clone (25); the 2.0-kb human collagenase-specific *Hind*III-*Sma*I fragment of pCIIase 1 clone (Ref. 42; American Type Culture Collection); and the 1.2-kb human *fms*-specific *Eco*RI fragment of pcfms 104 clone (Ref. 43; American Type Culture Collection). The fragments were labeled to approximately 10^9 cpm/μg of DNA with [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA) by random hexanucleotide priming (44).

AP-1 Binding Assays. Nuclear extracts from 2×10^7 cells were prepared according to the method of Schreibert *et al.* (45) and stored at −70°C. The partially complementary oligonucleotides 5′-GGCTAGTGATGAGTCAAGCCG-GATC-3′ and 5′-GGGATCCGGCTTGACTCATCACTAG-3′, synthesized in a Gene Assembler Plus (Pharmacia LKB, Uppsala, Sweden), were used to prepare a double-strand oligonucleotide containing an AP-1-recognizing sequence (AP-1 probe). This oligonucleotide was labeled with [α-^{32}P]dCTP following the method of Sambrook *et al.* (46). The oligonucleotides 5′-GCGAAACCCCTGGAATATTC-CCGACCTGGC-3′ and 5′-GGGCCAGGTCGGGAATAT-TCCAGGGGTTTCG-3′ were used to prepare a double-strand oligonucleotide which contained a heat-shock element, recognized by the heat-shock transcription factor (HSE probe). Binding reactions were carried out for 15 min at room temperature in 20 μl of binding buffer [25 mM NaCl, 60 mM KCl, 2 mM dithiothreitol, 2% Ficoll, 0.01% (v/v) Nonidet P-40, 0.1 mg/ml bovine serum albumin, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4)] containing 5 ng of the labeled AP-1 probe, 8 μg of total nuclear proteins, 2 μg of poly(dI:dC), and 2 μg salmon sperm DNA. When required, unlabeled AP-1 or HSE probes were added as specific and nonspecific competitors, respectively. The samples were electrophoresed in 4% polyacrylamide gels, and the gels were dried and autoradiographed.

Protein Kinase C Assays. Cells (5×10^6) were collected, washed with PBS, and suspended in 0.4 ml ice-cold extraction buffer [20 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml aprotinin, and 25 μg/ml leupeptin). The cells were disrupted with a Dounce homogenizer, after which the homogenates were first centrifuged for 2 min in a microfuge to remove the cellular debris and then in an ultracentrifuge for 30 min at 100,000 × *g*. The supernatants (cytosolic fractions) were kept at 4°C until used. The pellets (membrane fractions) were resuspended in extraction buffer containing 0.5% Triton X-100 and kept for 30 min on ice with occasional vortexing, after which they were centrifuged for 10 min in a microfuge to collect the supernatants. The cytosolic fractions and the detergent-extracted membrane fractions were eluted at 4°C with 2 ml elution buffer [20 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA, 0.2 M NaCl, and 10 mM β-mercaptoethanol] in Sepharose 2B columns. PKC activities were determined with the Protein Kinase C Assay System (GIBCO/BRL, Grand Island, NY), according to the instructions described by the manufacturer. This assay system follows the procedure described by Yasuda *et al.* (47), which uses as phosphate acceptor the myelin basic protein sequence Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu (MBP_{4-14}), and is considered to be highly specific for the PKC-α, PKC-β, and PKC-γ subspecies.

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