Pergamon

Biochemical Pharmacology, Vol. 48, No. 9, pp. 1842–1845, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00332-7

Short Communication

TIME COURSE OF PHORBOL-12-MYRISTATE-13-ACETATE (PMA)-INDUCED DOWN-REGULATION OF TOPOISOMERASE II IN HUMAN LEUKEMIA CELLS

AMY L. ELLIS and LEONARD A. ZWELLING*

Department of Clinical Investigation, Division of Medicine, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A.

(*Received* 26 *April* 1994; *accepted* 6 *July* 1994)

Abstract—The time course of the down-regulation of topoisomerase II associated with phorbol-induced differentiation was quantified in two human HL-60 leukemia cell lines. In the line sensitive to phorbol-induced differentiation (S cells), immunoreactive topoisomerase II levels did not begin to fall until 12 hr following exposure of the cells to phorbol ester. Thereafter, levels declined over the next 36 hr. By contrast, phorbol treatment of the phorbol ester tolerant cell line (PET cells) produced a transient decrease in topoisomerase II only to be followed by a recovery to pretreatment levels. The patterns of the alterations in topoisomerase II mRNA levels mirrored those of the alterations in immunoreactive topoisomerase II. Unexpectedly, nuclear run-on studies revealed that transcriptional start sites were not reduced in either cell line following their exposure to phorbol esters. These data suggest that the down-regulation of topoisomerase II is a consequence rather than a cause of phorbol-induced differentiation in HL-60 cells. Furthermore, this down-regulation is not mediated by a decrease in the number of topoisomerase II transcription sites.

Key words: phorbol resistance; differentiation; topoisomerase II; leukemia

Our laboratory has published several papers exploring the potential involvement of the DNA-replicative enzyme topoisomerase II in the phorbol ester-induced monocytoid differentiation of HL-60 cells [1, 2]. We have demonstrated that phorbol treatment causes reductions in drug-induced, topoisomerase II-mediated DNA cleavage [1–3] as well as in the amounts of topoisomerase II enzyme [2] and mRNA coding for this enzyme [1, 2] in HL-60 cells sensitive to the differentiating effects of phorbol esters; these reductions were not observed in HL-60-derived cell lines resistant to phorbol-induced differentiation [1, 2]. In the current study, we further explored the association between topoisomerase II and PMA†-induced monocytoid differentiation of HL-60 by monitoring cell levels of topoisomerase II enzyme and topoisomerase II mRNA in S (phorbol ester sensitive) and PET (phorbol ester tolerant) cells for 2–48 hr following PMA treatment. In addition, we also present data from nuclear run-on experiments indicating that the reduction in topoisomerase II message seen after PMA treatment of S cells is not the result of a PMA-induced decrease in the number of transcriptional initiation complexes.

Materials and Methods

Cells and reagents. The HL-60 S and PET cells used in these experiments were provided by Dr. Donald Macfarlane, University of Iowa [4]. These cells were grown in Iscove's modified Dulbecco's medium (JRH Biochemicals, Lenexa, KS) with 10% fetal bovine serum at 37° in 5% CO_2 and did not adhere to the culture flask in the absence of phorbol esters. The doubling time for both cell lines was approximately 16 hr. All cells were *Mycoplasma* free (American Type Culture Collection, Rockville, MD).

PMA was obtained from the LC Services Corp. (Woburn, MA) and reconstituted as a 10 mM stock solution in 100% DMSO. The concentration of DMSO in the cell medium never exceeded 0.1% in any experiment.

All chemicals utilized were of the highest reagent grade available.

Immunoblotting. S and PET cells treated for 2–48 hr with 10 nM PMA or vehicle were prepared for immunoblotting according to the method of Kaufmann [5, 6]. Cellular protein in each sample was quantified using the BCA Protein Assay from Pierce (Rockford, IL). Seventy-five micrograms of total cellular protein was separated on a 7.5% polyacrylamide gel, electrophoretically transferred to nitrocellulose, and probed with an antibody for topoisomerase II (a gift of Dr. Leroy Liu, Robert Wood Johnson Medical School, UMDNJ) as described previously [2]. Bound antibody was detected with ^{125}I-labeled Protein A (Amersham, Arlington Heights, IL). Radioactivity of the immunoblots was measured using the PhosphorImager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and these readings were used to quantify immunoreactive topoisomerase II in the cells.

Preparation of RNA and dot blotting. S and PET cells were treated with 10 nM PMA or vehicle for 2–48 hr. Cellular RNA was isolated using the acid-guanidine method of Chomczynski and Sacchi [7]. RNA integrity was checked on a 1% agarose gel with ethidium bromide staining. Dot blotting was performed using concentrations of RNA ranging from 1.25 to 10 μg on Hybond (Amersham). The blots were hybridized under standard conditions [8] to the human topoisomerase IIα cDNA probe PCR4 generated in our laboratory [9] (see Fig. 1); then they were stripped and reprobed with β-actin (provided by Dr. Grady Saunders, M.D. Anderson Cancer Center). Probes were

* Corresponding author: Dr. Leonard A. Zwelling, M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 184, Houston, TX 77030. Tel. (713) 792–3695; FAX (713) 794–5531.

† Abbreviations: PMA, phorbol-12-myristate-13-actate; S, phorbol ester sensitive; and PET, phorbol ester tolerant.

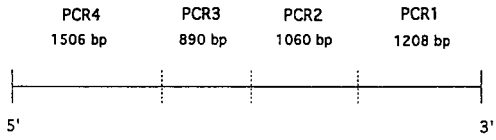

Fig. 1. Map of the cDNA of the human topoisomerase IIα gene. This gene was cloned in our laboratory in 4 pieces (Ref. 9) as indicated in the figure. The 5′ end of the gene (PCR4) was used as a probe to detect topoisomerase II mRNA in dot blots of RNA from S and PET cells treated with PMA or vehicle for various times (Fig. 3). Each of these pieces of the topoisomerase IIα gene was used as a DNA target for the nuclear run-on assays (Fig. 4).

labeled with [^{32}P]CTP using the Amersham Multiprime Labeling System. Radioactivity of the dot blots was measured using the PhosphorImager, and these readings were used to quantify topoisomerase IIα and β-actin RNA in the cells. β-Actin was used to normalize the topoisomerase II mRNA data as well as the data from nuclear run-on experiments (below) because the level of β-actin mRNA remains constant during 48 hr of phorbol treatment of HL-60 S and PET cells.

Nuclei isolation and nuclear run-on. S and PET cells were treated with 10 nM PMA or vehicle for 48 hr before nuclei were harvested according to the method of Groudine *et al.* [10] and stored at −70° (for no more than 1 month) until used. Nuclei were thawed on ice and incubated in a buffer containing 100 μCi[^{32}P]UTP (Amersham), and run-on products were extracted as described by McKnight and Palmiter [11] and as modified by Sagoh and Yamada [12]. Fifteen micrograms of each of the following DNA targets was alkali denatured and fixed to Hybond: topoisomerase IIα, PCR1, PCR2, PCR3, PCR4 (see Fig. 1); topoisomerase IIβ, SP-12 (a gift of Dr. K.B. Tan, SmithKline Beecham); and β-actin. Run-on products were hybridized to targets using Amersham Rapid-Hyb buffer. The PhosphorImager was used to measure radioactivity of the blots, and the readings obtained were used to quantify run-on products bound to the target DNA sequences listed above.

Results and Discussion

The amount of immunoreactive topoisomerase II in HL-60 S cells did not fall during the first 12 hr of PMA treatment (Fig. 2). At 24 hr, the amount declined to approximately 50% of control levels, and after 48 hr of PMA treatment, the amount of immunoreactive topoisomerase II in S cells was approximately 20% of control levels. PET cells treated with PMA for 24 hr contained approximately 40% less immunoreactive topoisomerase II than vehicle-treated PET cells did, but at 48 hr, immunoreactive topoisomerase II had recovered to control levels (Fig. 2) concomitant with the emergence from growth inhibition known to occur at this time in PMA-treated PET cells [4].

After an initial rise (following 8 hr of PMA exposure), the amount of topoisomerase II mRNA in PMA-treated S cells fell to 50% of control levels by 18 hr (Fig. 3). At 24 hr the amount of topoisomerase II mRNA was only 15% of the control level. PMA treatment of PET cells caused topoisomerase II mRNA levels to decline by 40% over the first 24 hr of exposure (Fig. 3). However, over the next 24 hr, PET cell mRNA levels returned to the control level.

Nuclear run-on studies were performed to explore the mechanism by which PMA induced down-regulation of topoisomerase II mRNA in HL-60 S cells. These studies indicated that PMA treatment did not cause a reduction in the number of transcription initiation sites of either α or β topoisomerase II mRNA in S cells or in PET cells (Fig. 4).

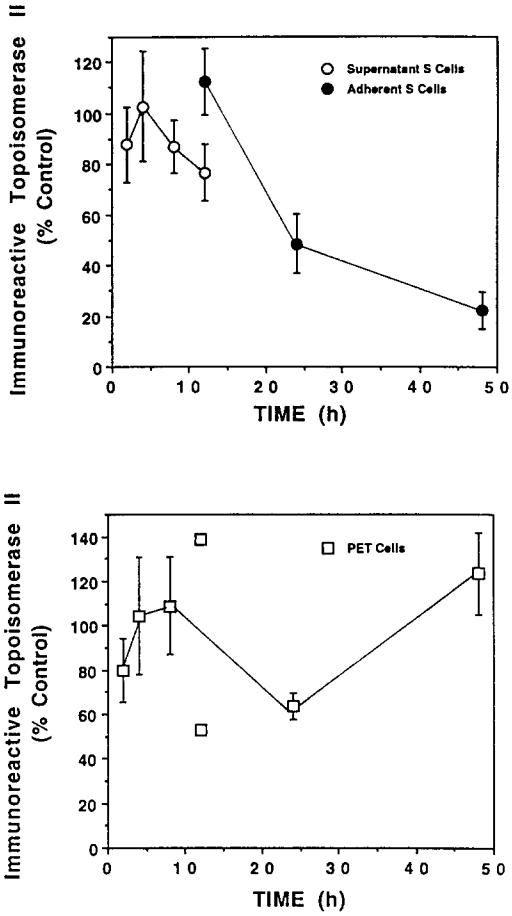

Fig. 2. Levels of immunoreactive topoisomerase II in HL-60 S and PET cells after treatment with PMA. Four PET (squares) and 5 S (circles) individual immunoblots were performed using cells treated with 10 nM PMA or vehicle for 2–48 hr. Radioactivity of the topoisomerase II bands on the blots was measured using a PhosphorImager. For each blot, radioactivity of the topoisomerase II band of PMA-treated samples was expressed as a percentage of the control. Data in the figure are average percent of control ± SEM of immunoreactive topoisomerase II in cells treated with PMA for the indicated times. Average S cell adherence ± SEM: 12 hr, 36.9 ± 3.2; 24 hr, 65.3 ± 6.2; 48 hr, 62.2 ± 5.2. Average PET cell adherence ± SEM: 24 hr, 9.8 ± 2.4; 48 hr, 2.7 ± 0.9.

Phorbol esters induce monocytoid differentiation in HL-60 cells [13, 14], but the mechanism of action is unclear. There is a rapid (within 2 hr) alteration of mRNA species such as c-*myc* (down-regulation) and *fos* (up-regulation) in HL-60 cells during chemically induced differentiation [15–18]. The expression of c-*myc* appears to prevent the differentiation of murine erythroid leukemia cells [19], and c-*myc* down-regulation may be a necessary step in the differentiation process.

The decline in topoisomerase II mRNA was not as rapid as that reported for c-*myc*. We did not observe a drop in topoisomerase II mRNA or immunoreactive topoisomerase II protein in HL-60 S cells for up to 12 hr following PMA addition. At 18 hr, however, there was a 50% reduction of topoisomerase II message, and at 24 hr, a 50% reduction

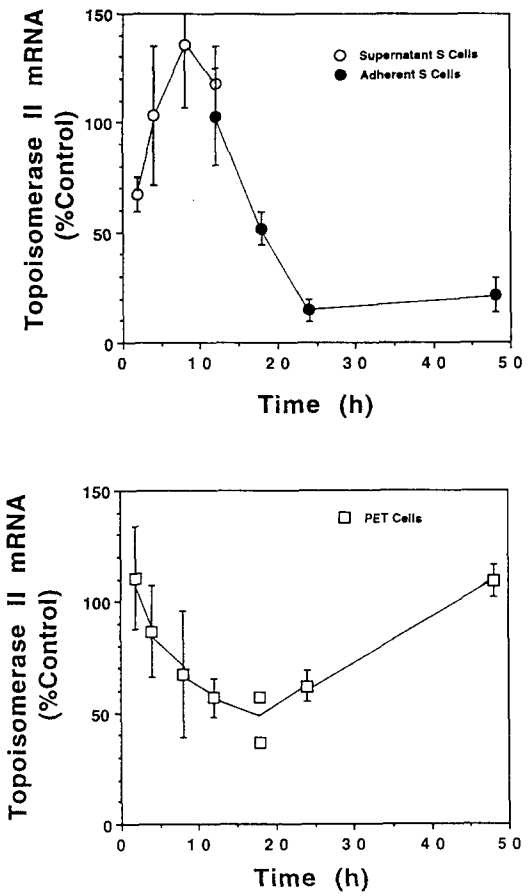

Fig. 3. Levels of topoisomerase II mRNA in HL-60 S and PET cells after treatment with PMA. Three (PET) and 4 (S) individual dot blots were performed with 1.25 to 10 μg of RNA extracted from S and PET cells treated with 10 nM PMA or vehicle for 2–48 hr. Blots were probed with the topoisomerase IIα cDNA probe PCR4, then stripped and reprobed with β-actin. Radioactivity of the dot blots was measured using a PhosphorImager. To minimize variability due to sample loading, PCR4 (topoisomerase II) radioactivity of each dot was divided by β-actin radioactivity of the same dot. For each blot, PCR4/β-actin ratios of vehicle-treated (control) samples were averaged, and PCR4/β-actin ratios of PMA-treated samples were expressed as percentages of the control average. Data in the figure are average percent of control ± SEM of PCR4/β-actin ratios of RNA extracted from cells treated with PMA for the indicated times. Average S cell adherence ± SEM: 12 hr, 35.3 ± 3.0; 18 hr, 64.0 ± 6.9; 24 hr, 64.9 ± 1.6; 48 hr, 73.0 ± 4.9. Average PET cell adherence ± SEM: 24 hr, 5.0 ± 1.2; 48 hr, 2.1 ± 0.3.

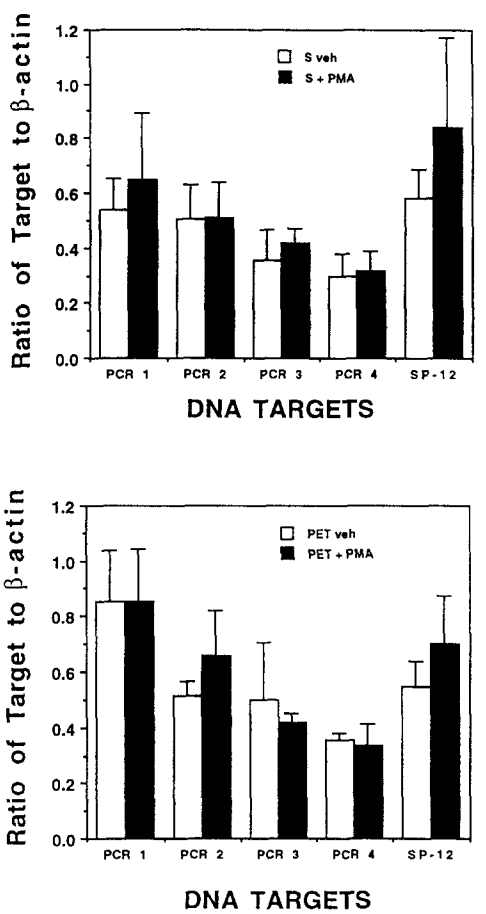

Fig. 4. Nuclear run-on products of HL-60 S and PET cells following 48 hr treatment with PMA or vehicle. Nuclei isolated from cells treated with 10 nM PMA or vehicle for 48 hr were incubated with [^{32}P]UTP for 30 min before extraction. The run-on products extracted from these nuclei were hybridized to target DNA fixed to Hybond. Target DNA (15 μg per spot) was as follows: topoisomerase IIα (PCR1, PCR2, PCR3, PCR4–3′ to 5′, see Fig. 1), topoisomerase IIβ (SP-12), β-actin. Radioactivity of the dot blots was measured using a PhosphorImager. To allow averaging of different experiments and permit comparison between different preparations of nuclei, radioactivity of each topoisomerase II target was divided by radioactivity for the β-actin target on the same blot. Data in the figure are the average topoisomerase II target/β-actin ratios ± SEM. Four determinations using nuclei from three different preparations were performed for each data point.

in immunoreactive topoisomerase II in the phorbol-treated S cells. The decline may occur as the HL-60 S cells commit to differentiation and stop proliferating. The down-regulation of topoisomerase II appears to be more a consequence of cell differentiation than a primary signal in its induction. The results of the run-on experiments suggest that altered RNA processing, decreased RNA stability, or enhanced RNA destruction may explain the apparent decrease in topoisomerase II mRNA and protein in PMA-treated S cells. PMA treatment does not alter the amount of β-actin mRNA in HL-60 S cells or PET cells. Therefore, if an alteration in RNA metabolism explains the topoisomerase II mRNA and protein results, that phenomenon is not generalized. Topoisomerase II may be one of a series of transcriptional products whose level is reduced as cells differentiate.

In summary, DNA topoisomerase II mRNA and enzyme are both down-regulated in HL-60 human leukemia cells undergoing phorbol ester-induced differentiation. We examined the time course of this phenomenon and observed that mRNA down-regulation began after 12 hr but before 18 hr of exposure to PMA. Nuclear run-on assays indicated that the down-regulation of topoisomerase II mRNA did

not appear to be the result of a decrease in transcriptional initiation sites. The length of time before topoisomerase II down-regulation began suggested that it is a consequence of cell differentiation rather than a primary signal in its induction.

Acknowledgements—We are grateful for the expert technical assistance of Ms. Elizabeth Altschuler and Ms. Susan Loughlin. We also wish to thank Ms. Diane Rivera for editing this manuscript. These studies were supported by Grants DHP-39G from the American Cancer Society and RO1CA40090 from the U.S. Public Health Service.

REFERENCES

1. Zwelling LA, Hinds M, Chan D, Altschuler E, Mayes J and Zipf TF, Phorbol ester effects on topoisomerase II activity and gene expression in HL-60 human leukemia cells with different proclivities toward monocytoid differentiation. *Cancer Res* **50**: 7116–7122, 1990.
2. Ellis AL, Altschuler E, Bales E, Hinds M, Mayes J, Soares L, Zipf TF and Zwelling LA, Phorbol regulation of topoisomerases I and II in human leukemia cells: Studies in an additional cell pair sensitive or resistant to phorbol-induced differentiation. *Biochem Pharmacol* **47**: 387–396, 1994.
3. Zwelling LA, Chan D, Hinds M, Mayes J, Silberman LE and Blick M, Effect of phorbol ester treatment on drug-induced, topoisomerase II-mediated DNA cleavage in human leukemia cells. *Cancer Res* **48**: 6625–6633, 1988.
4. Macfarlane DE, Gailani D and Vann K, A phorbol ester tolerant (PET) variant of HL-60 promyelocytes. *Br J Haematol* **68**: 291–302, 1988.
5. Kaufmann SH, Okre S, Wikstrom AC, Gustafsson JA and Shaper JH, Binding of the glucocorticoid receptor to the rat liver nuclear matrix. *J Biol Chem* **261**: 11962–11967, 1986.
6. Kaufmann SH, McLaughlin SJ, Kastan MB, Liu LF, Karp JE and Burke PJ, Topoisomerase II levels during granulocytic maturation *in vitro* and *in vivo*. *Cancer Res* **51**: 3534–3543, 1991.
7. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159, 1987.
8. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*, pp. 9.52–9.55. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989.
9. Hinds M, Deisseroth K, Mayes J, Altschuler E, Jansen R, Ledley FD and Zwelling LA, Identification of a point mutation in the topoisomerase II gene from a human leukemia line containing an amsacrine-resistant form of topoisomerase II. *Cancer Res* **51**: 4729–4731, 1991.
10. Groudine M, Peretz M and Weintraub H, Transcriptional regulation of hemoglobin switching in chicken embryos. *Mol Cell Biol* **1**: 281–288, 1981.
11. McKnight GS and Palmiter RD, Transcriptional regulation of the ovalbumin and conalbumin genes by steroid hormones in chick oviduct. *J Biol Chem* **254**: 9050–9058, 1979.
12. Sagoh T and Yamada M, Transcriptional regulation of myeloperoxidase gene expression in myeloid leukemia HL-60 cells during differentiation into granulocytes and macrophages. *Arch Biochem Biophys* **262**: 599–604, 1988.
13. Rovera G, O'Brien TG and Diamond L, Induction of differentiation in human promyelocytic leukemia cells by tumor promoters. *Science* **204**: 868–870, 1979.
14. Huberman E and Callaham MF, Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. *Proc Natl Acad Sci USA* **76**: 1293–1297, 1979.
15. Eick D and Bornkamm GW, Transcriptional arrest within the first exon is a fast control mechanism in c-myc gene expression. *Nucleic Acids Res* **14**: 8331–8346, 1986.
16. Mitchell RL, Zorkas L, Schreiber RD and Verma IM, Rapid induction of the expression of proto-oncogene *fos* during human monocytic differentiation. *Cell* **40**: 209–217, 1985.
17. Filmus J and Buick RN, Relationship of c-*myc* expression to differentiation and proliferation of HL-60 cells. *Cancer Res* **45**: 822–825, 1985.
18. Grosso LE and Pitot HC, Transcriptional regulation of c-*myc* during chemically induced differentiation of HL-60 cultures. *Cancer Res* **45**: 847–850, 1985.
19. Dmitrovsky E, Kuehl WM, Hollis GF, Kirsch IR, Bender TP and Segal S, Expression of a transfected human c-*myc* oncogene inhibits differentiation of a mouse erythroleukaemia cell line. *Nature* **322**: 748–750, 1986.