# Generation of Procoagulant Activity (PCA) by Phorbol-Esters-Induced Macrophages Derived From a Leukemic Promyelocytic Cell Line (HL-60)

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**Normal human monocytes and macrophages, as well as leukemic promyelocytes, generate potent procoagulant activity (PCA) resembling thromboplastin. In the present study only mild PCA was detected in a leukemic promyelocytic cell line (HL-60) and in promyelocytic cells induced to differentiate into neutrophils by dimethylsulfoxide (DMSO). After exposure to 12-0-tetradecanoylphorbol-13-acetate (TPA), the leukemic promyelocytic cells assume characteristics specific to monocytes and macrophages. This differentiation was associated with potent PCA generation. No PCA was found in lymphoblasts and several lymphoid cell lines after exposure to TPA. The PCA in TPA-induced HL-60 cells resembles tissue thromboplastin and normal monocyte PCA in several aspects: (A) All three accelerate clotting through the extrinsic coagulation pathway; (B) all three are inhibited by concanavalin-A and protected by methyl-α-D-mannopyranoside, suggesting that the PCA contains carbohydrate moieties; (C) the PCA is located in the cell membrane. However, the mechanism of PCA generation in normal monocytes and in TPA-induced HL-60 cells differs in several aspects: (A) Endotoxin and phytohemagglutinin, which are potent stimuli of PCA generation in normal monocytes, have no effect on TPA-induced HL-60 cells; (B) HL-60 cells treated with TPA develop PCA before acquiring any other specific characteristic of monocyte or macrophages; (C) the development of PCA in TPA-induced macrophages is not dependent on cell adhesion or the presence of T cells. In spite of these differences, the production of PCA is additional evidence of the similarity between TPA-induced macrophages and normal human monocytes and macrophages. The HL-60 cells may provide a unique model for studying PCA generation in mononuclear cells.**

HUMAN PERIPHERAL BLOOD leukocytes can generate potent procoagulant activity (PCA) in response to a variety of stimuli, including endotoxin,[1-3] phytohemagglutinin (PHA), purified protein derivative,[4,5] aggregated immunoglobulins, antigen–antibody complexes,[6] the mixed lymphocyte reaction,[7] and adherence.[8] The procoagulant substance activates an extrinsic cascade of blood clotting and is neutralized by antibodies to human purified tissue factor.[1,3] It has been identified, therefore, as tissue factor or thromboplastin. It was demonstrated that the monocytes and macrophages are responsible for the bulk of PCA generation,[2,9,10] and optimal production requires the cooperation of T lymphocytes.[10] Increased PCA was also found in human leukemic promyelocytes, and it is responsible for the high incidence of disseminated intravascular coagulation in patients with acute promyelocytic leukemia.[11,12]

The HL-60 line is derived from human leukemic promyelocytes.[13] In the presence of dimethylsulfoxide (DMSO), the HL-60 cells mature into granulocyte-like cells.[14] When exposed to phorbol esters, such as 12-0-tetradecanoylphorbol-13-acetate (TPA), the cells express characteristics specific to monocytes and macrophages, such as adherence, phagocytosis, and elaboration of acid phosphatase, nonspecific esterase, lysozyme, and NADase.[15,16] Recently it has been shown that following incubation with TPA, leukemic cells from patients with acute and chronic myelogenous leukemia also differentiate into macrophage-like cells, whereas lymphoblasts are unaffected.[17,18]

In the present study we questioned the ability of HL-60 cells in various stages of differentiation, and particularly macrophage-like cells obtained in the presence of TPA, to generate PCA. Our results demonstrate that the macrophage-like cells develop a potent PCA that resembles thromboplastin and mononuclear cell tissue factor.

## MATERIALS AND METHODS

### *Cells and Culture Conditions*

HL-60 cell line, originally established by Collins et al.[13] from the peripheral blood of a patient with acute promyelocytic leukemia, was maintained in our laboratory for 10 mo. The cells were subcultured every 3–4 days at $2.5 \times 10^5$/ml in alpha Minimal Essential Medium supplemented with 15% FCS (Gibco, Grand Island, N.Y.) and incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Determination of cellular differentiation was based on morphology, cell adherence, phagocytic capability, and cytochemical staining for acid phosphatase and fluoride-inhibited non-specific esterase as previously described.[18]

Peripheral mononuclear cells were obtained from normal volunteers. Heparinized blood was centrifuged for 10 min at 800 *g* and the platelet-rich plasma removed. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation.[19]

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### *Assay of Procoagulant Activity*

Cells were incubated at $2.5 \times 10^5$ cells/ml in the above-mentioned medium in the presence of various compounds. At the end of the incubation, cells were collected into conical tubes, washed twice with Dulbecco's phosphate-buffered saline, and resuspended in isotonic veronal-acetate buffer, pH 7.35, to a final concentration of $3 \times 10^6$ cells/ml.[20] Adherent cells were removed by scraping the plastic flasks with a rubber policeman. Cell populations were used only if their viability, as tested by the trypan blue exclusion test, exceeded 90%. Procoagulant activity was determined using either intact cells or cells disrupted by sonification with ultrasonic vibration for 60 sec in an ice bath (Branson Sonic Power Co., Danbury, Conn.) or by freeze–thawing. To obtain the membrane-rich fraction, nuclei were sedimented at 400 *g* and then the supernatant centrifugated at 40,000 *g*, 20°C, for 20 min (Sorval, Rc-5, Du Pont). The activity was measured by the recalcification test:[11,20] 0.1 ml of pooled oxalated normal human plasma (General Diagnostics, Morris Plains, N.J.) was incubated with 0.1 ml cell suspension at 37°C for 1 min, and then 0.1 ml 0.025 *M* $CaCl_2$ added and the clotting time recorded. In some experiments, PCA was assayed using plasma from patients congenitally deficient in one of the following factors: VII, VIII, IX, X (Dade Co., Miami, Fla.).

### *Reagents*

Dimethylsulfoxide (DMSO) (Sigma Co., St. Louis, Mo.), concanavalin-A (Con-A) (Miles-Yeda, Rehovot, Israel), endotoxin (*Salmonella typhimurium*) (Sigma Chemical Co., St. Louis, Mo.) and phytohemagglutinin (PHA) (Wellcome, Beckenham, England) were all dissolved in medium. Phorbol and 12-0-tetradecanoyl-phorbol-13-acetate (TPA) (Consolidated Midland Co., Brewster, N.Y.) were dissolved in DMSO to a concentration of 5 mg/ml and further diluted in acetone to 1 mg/ml. Final concentrations of DMSO and acetone in culture medium were 0.002% and 0.01%, respectively. At these concentrations, neither solvent had any effect on cell culture. Commercial thromboplastin (Simplastin) was purchased from General Diagnostics, Morris Plains, N.J., and methyl-α-D mannopyranoside (αMM) from Phanstiehl Lab., Ill.

## RESULTS

### *Generation of Procoagulant Activity During Differentiation of HL-60 Cells*

The temporal changes in PCA occurring upon differentiation of the HL-60 cells into either granulocyte or macrophage-like cells are presented in Fig. 1. Uninduced HL-60 cells generate a mild PCA (clotting time 120–150 sec). When 1.5% DMSO was added to the culture, the cells differentiated into neutrophil-like cells. Only a slight increase in PCA was observed after 96 hr in the presence of DMSO, indicating that in vitro differentiation into granulocytes was not associated with any significant increase in the generation of PCA. In agreement with previous reports,[16] addition of 10 ng/ml TPA to cultures of HL-60 cells induced the appearance of many characteristics similar to those of normal human macrophages. More than 90% of the cells adhered to the surface of the culture dish, acquired phagocytic activity, and generated fluoride-inhibited nonspecific esterase and acid phosphatase. These properties first appeared about 20 hr after addition of TPA and were maximal at 48 hr. In TPA-treated cells, a significant increase in PCA was observed as early as 8 hr after addition of the chemical and progressively increased to a maximum level 48 hr after addition (Fig. 1.). The rise in PCA preceded the appearance of any other macrophage-specific properties.

In preliminary studies, potent PCA was also demonstrated in TPA-induced macrophages derived from myeloblasts of five patients with acute myelogenous leukemia and from cells of four patients with chronic granulocytic leukemia. No PCA was detected in lymphoblasts from four ALL patients and in various human cell lines derived from malignant B cells (Burkitt's lymphoma—Raji line), normal B cells, and erythroleukemia cells (K-562) after treatment with TPA (clotting time always exceeded 150 sec).

A previous report emphasized that in vitro PCA generation by normal mononuclear cells was dependent on adherence of the cells to glass or plastic surfaces.[8] In order to investigate the possible relationship between cell adhesion and procoagulant generation, HL-60 cells were cultured for 48 hr on an agar underlayer in the presence of 10 ng/ml TPA in order to prevent contact with the plastic flasks. Although cell adhesion was totally prevented, the cells developed all

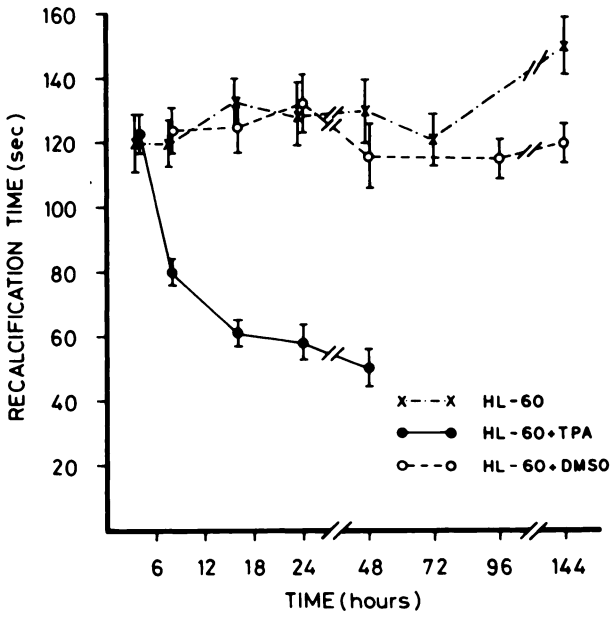


**Fig. 1. Time-course of generation of procoagulant activity in HL-60 cells in various stages of differentiation. Cells were cultured with either 10 ng/ml TPA or 1.5% DMSO. After various intervals, the cells were assayed for PCA by the recalcification test. The results are means of 6–8 experiments.**

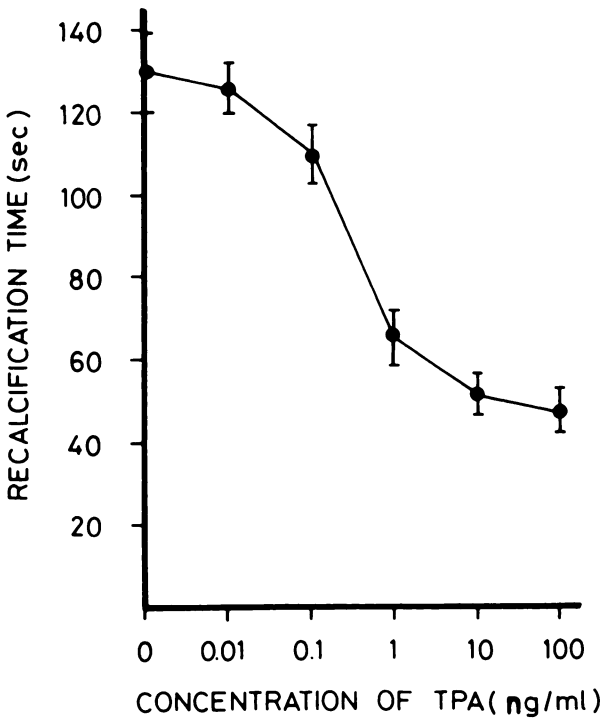


**Fig. 2. The effect of various concentrations of TPA on generation of procoagulant activity by HL-60 cells. Cells were assayed for PCA after 48-hr incubation with different concentrations of TPA. The results are the mean of 6–8 experiments.**

the other above-mentioned properties characteristic of macrophages. In addition, the quantity of PCA generated was similar to that produced when cells were allowed to adhere and spread over the plastic surfaces of the flasks (clotting time 60 ± 6 and 51 ± 5 sec, respectively).

The dose-response relationship between TPA concentration and generation of PCA is presented in Fig. 2. Concentrations of 1–100 ng/ml TPA were found by us and others[16] to induce HL-60 cells to differentiate into macrophage-like cells. The same concentrations were also found to be optimal for induction of PCA. These results suggest that induction of differentiation into macrophage-like cells and generation of PCA are closely related phenomena.

**Table 1. The Effect of Various Compounds on Procoagulant Activity of HL-60 Cells and Human Peripheral Blood Mononuclear Cells**

| Compound Added | Concentration | Recalcification Time (sec) HL-60 Cells | Recalcification Time (sec) Normal Mononuclear Cells |
|---|---|---|---|
| None | | 130 ± 10 | 98 ± 8 |
| TPA | 10 ng/ml | 51 ± 5 | 62 ± 10 |
| Phorbol | 10 ng/ml | 155 ± 6 | 108 ± 10 |
| PHA | 1 μg/ml | 135 ± 5 | NA |
| | 10 μg/ml | 142 ± 7 | 58 ± 5 |
| Endotoxin | 1 μg/ml | 146 ± 9 | 77 ± 6 |
| | 10 μg/ml | 134 ± 6 | 60 ± 8 |
| | 100 μg/ml | 139 ± 9 | 52 ± 6 |

HL-60 cells and normal human peripheral mononuclear cells were incubated with various compounds and PCA determined. The results in the table represent incubation period of maximal PCA generation, which is 12 hr for mononuclear cells and 48 hr for HL-60 cells. Recalcification time of veronal acetate buffer, alpha medium, or TPA + alpha medium in the absence of cells always exceeded 150 sec. The results are the mean ± SD of 6–8 experiments.

NA, not assayed.

A variety of compounds may stimulate the generation of PCA by normal human monocytes.[1-6] Two of them, endotoxin and PHA, were tested for their effect on HL-60 cells as compared to human monocytes. The results (Table 1) indicate that under conditions in which these compounds strongly stimulate normal peripheral blood monocytes, they had no effect on PCA generation by HL-60 cells, neither could they induce macrophage differentiation in these cells. No PCA was generated in the presence of phorbol, a plant diterpene similar in structure to TPA but lacking tumor-promoting activity.[21]

### *Characterization of the Procoagulant Activity Generated by HL-60 Cells*

Procoagulant activity generated by HL-60 cells 2 days after exposure to 10 ng/ml TPA was compared to commercial thromboplastin (Simplastin) (Table 2). Using the recalcification test, both activities accelerated the clotting time of normal plasma and plasma

**Table 2. The Effect of Simplastin and TPA-Induced Macrophages on Recalcification Time of Normal and Various Factor-Deficient Plasmas**

| | Recalcification Time of Plasma (sec) Normal | Factor VII Deficient | Factor VIII Deficient | Factor IX Deficient | Factor X Deficient |
|---|---|---|---|---|---|
| Simplastin (dilution 1:16) | 53 ± 4 | 148 ± 7 | 51 ± 5 | 55 ± 5 | 162 ± 8 |
| TPA-induced macrophages | 51 ± 5 | 160 ± 8 | 48 ± 6 | 50 ± 4 | 183 ± 10 |

TPA-induced macrophages were obtained after incubation of HL-60 cells with 10 ng/ml TPA for 48 hr. The results are the mean ± SD of 6–8 experiments.

deficient in factors VIII or IX, and had no effect on the clotting time of plasmas deficient in factors X or VII. These results indicate that like Simplastin, PCA produced by TPA-induced macrophages affects clotting through the extrinsic pathway of coagulation.

It has been previously demonstrated that thromboplastin is a glyco-protein and that its activity is inhibited by the lectin Con-A, which preferentially binds mannosyl or glycosyl residues.[22,23] Inhibition can be prevented by the addition of αMM prior to incubation of Con-A with thromboplastin.[24] In our studies, incubation of Con-A with TPA-induced macrophages resulted in dose-related inhibition of PCA that could be prevented by the addition of αMM (Table 3). The results suggest that the procoagulant material in TPA-induced macrophages contains carbohydrate residues and may be a glycoprotein. The composite results suggest a similarity in several aspects between HL-60 PCA and Simplastin.

In some of the experiments in which HL-60 cells were cultured in the absence of serum and in the presence of TPA, the same potent PCA was observed. The results exclude the possibility that the activity originated in the serum.

### *Distribution of Procoagulant Activity Within HL-60 Cells*

In order to localize PCA within the cells, uninduced HL-60 cells and macrophage-like cells were disrupted by sonification, freeze–thawing or freeze–thawing and sonification. Cell nuclei were sedimented at 400 *g*, and the membrane-rich fraction was obtained by centrifugation of the supernatant at 40,000 *g*. The findings (Table 4) demonstrate that the PCA of the membrane-rich pellet was the same as that of the intact cells. No activity was found in the supernatant, containing mainly the cytosol or in the culture media (clotting time >150 sec). The results suggest that PCA is associated with the cell membrane. There was no activity generated upon incubation of the disrupted HL-60 cells with TPA. Thus, the induction of PCA by TPA requires intact metabolically active cells.

**Table 3. Con-A Inhibition of Procoagulant Activity Generated by TPA-Induced Macrophages**

| | Recalcification Time | | | |
|---|---|---|---|---|
| | Cells + Con-A | | Cells + Con-A + αMM | |
| Con A (μg/ml) | Seconds | Percent Inhibition | Seconds | Percent Protection |
| 0 | 65 | 0 | 63 | — |
| 4 | 82 | 26 | 66 | 94 |
| 16 | 100 | 53 | 68 | 92 |
| 64 | 122 | 87 | 74 | 84 |
| 256 | 128 | 97 | 72 | 58 |

The effect of Con-A was determined after 1 hr of incubation with HL-60 cells treated with 10 ng/ml TPA for 48 hr. When used, 20 mg/ml methyl-α-D-mannopyranoside (αMM) was added 5 min before the addition of Con-A.

**Table 4. The Generation of Procoagulant Activity by Various Cell Fractions**

| | | Recalcification Time (sec) | |
|---|---|---|---|
| Cell | Cell Fraction | Cells | Cells + TPA |
| Intact | Whole | 130 ± 11 | 51 ± 5 |
| Sonicated | Supernatant | 160 ± 7 | 152 ± 6 |
| | pellet | 134 ± 99 | 57 ± 6 |
| Freeze-thawed | Supernatant | 148 ± 7 | 144 ± 10 |
| | pellet | 128 ± 8 | 55 ± 6 |
| Freeze-thawed* + TPA | — | — | 148 ± 9 |

HL-60 cells were incubated with or without 10 ng/ml TPA for 48 hrs. The cells were lysed and the supernatant and pellet were obtained after centifugation at 40,000 *g* as described in Materials and Methods.

*Cells cultured for 48 hr without TPA were collected, washed, and freeze-thawed. TPA was then added (10 ng/ml) to the cell lysate, the mixture incubated for 48 hr, and PCA determined. The results are the mean of 6-8 experiments.

## DISCUSSION

Normal human monocytes and macrophages generate potent PCA identical to tissue thromboplastin in response to various stimuli.[5,8-10,25] In the present studies we show that a similar PCA is generated by an in vitro cell line of human leukemic promyelocytes (HL-60) when induced to differentiate into macrophage-like cells by the phorbol ester TPA. In our preliminary studies, potent PCA was also demonstrated in TPA-induced macrophages derived from myeloid cells of patients with acute and chronic myelogenous leukemia. The untreated HL-60 promyelocytes and HL-60 cells induced to differentiate into neutrophils by DMSO generated only mild PCA. Comparable levels of PCA were found in isolated acute promyelocytic leukemia cells.[11] No PCA was detected in acute lymphoblastic leukemic cells and various human lymphoid cell lines.

PCA generated by macrophage-like cells resembles thromboplastin and mononuclear cell tissue factor in the following characteristics: (A) All three accelerate clotting through the extrinsic coagulation pathway; (B) TPA-induced macrophage PCA and thromboplastin are inhibited by Con-A and protected by αMM, suggesting that both substances contain carbohydrate residues; (C) it has been shown that mononuclear cell tissue factor and thromboplastin are located in the plasma membrane of monocytes and other cell types.[2,4,9,20,26] The finding of PCA on intact HL-60 cells and in the membrane-rich fraction, but not in the cytosol, suggests that it is also located in the cell membranes.

However, there are some differences in the mechanism of PCA generation by the macrophage-like cells and normal human monocytes and macrophages: (A) HL-60 cells treated with TPA developed PCA before acquiring any other specific monocyte or macrophage characteristics; (B) T cells play a significant role in the generation of PCA by normal monocytes,[10,27] but are not required for the development of PCA in TPA-treated HL-60 cells; (C) the adherence of normal monocytes, as demonstrated by Van Ginkel et al.[8] and Hiller et al.,[28] stimulates the production of PCA; in HL-60 cells, the development of PCA is not dependent on cell adhesion; (D) endotoxin and PHA, which stimulate the production of PCA in normal monocytes,[4,5,9,10] had no effect on HL-60 cells.

It is possible that endotoxin may be present as a contaminant in the culture system.[5] PCA generation may result from stimulation of HL-60 cells by the endotoxin after the cells were induced to differentiate into macrophages-like cells in the presence of TPA. However, it is unlikely, since our studies demonstrated that addition of either endotoxin or PHA, at various concentrations, together with TPA did not accelerate or enhance PCA generation. Moreover, TPA was found to stimulate PCA generation directly also in normal human peripheral blood mononuclear cells (Table 1). A similar observation was recently reported by Lyberg and Prydz.[29]

Although the generation of PCA by HL-60 cells after treatment with TPA differs in several aspects from the production of PCA in normal monocytes and macrophages, additional to morphological, immunologic, and histochemical characteristics, the ability of TPA-induced macrophages to generate potent PCA resembling mononuclear cell tissue factor is yet further support of the similarity between these cells and normal monocytes and macrophages. Therefore, HL-60 cells can provide a unique model for the study of the mechanism of PCA generation in human monocytes and macrophages. The significance of PCA in TPA-induced macrophages derived from acute and chronic myelogenous leukemic cells merits further investigation.

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