Plasminogen activator activity in differentiating leukemia cells

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Plasminogen activator (PA) activity of human promyelocytic leukemia cell line HL-60 was assayed by following the conversion of plasminogen to plasmin and the plasmin-mediated hydrolysis of ^{14}C-labeled globin. When HL-60 cells were induced to differentiate into macrophages by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), cell-associated PA activity and secretion of PA into the conditioned medium increased profoundly. PA activity increased earlier and as a result of lower concentrations of TPA than the ability of the cells to adhere. Exposure to 10^{-6} M dexamethasone did not prevent TPA-induced adherence and produced a slight inhibition of cellular PA activity. These findings imply that TPA-induced differentiation of HL-60 cells to macrophage-like cells is associated with induction of PA activity.

Plasminogen activator *Myeloid leukemic cell* *Differentiation* *Macrophage*

1. INTRODUCTION

Plasminogen activator (PA) has been implicated in malignant transformation of cultured cells, in tumor invasiveness and metastatic potential, in tissue destruction and remodeling, and in cell differentiation [1,2]. Macrophages from inflammatory exudates and macrophages exposed in vitro to inflammatory agents also secrete PA, while cells obtained from noninflamed peritoneal cavities secrete little or no PA [3].

Cultured human myeloid leukemic cells (HL-60) [4] can be induced by TPA to differentiate into macrophage-like cells. The TPA-induced HL-60 cells show several [5], although not all [6], characteristics of normal mature macrophages. One of the early macrophage-specific markers which does appear following treatment with TPA, is the ability of the cells to adhere to and spread on plastic or glass surfaces. We have previously shown that this adherence is inhibited by the protease inhibitor pentamidine isethionate, suggesting that proteolysis is involved in induction of cell adherence [7]

Here we find PA activity in HL-60 cells. Exposure of the cells to TPA results in a 2- to 3-fold enhancement of PA activity, implying that PA is an early marker of adherence in differentiating HL-60 cells. However, since the effect of TPA is seen in the absence of serum, formation of exogenous plasmin by PA does not appear to play a direct role in TPA-induced differentiation.

Abbreviations: PA, plasminogen activator; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate

2. MATERIALS AND METHODS

HL-60 cell line, established from a patient with acute promyelocytic leukemia [4], was maintained in minimal essential alpha medium with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY) by seeding 2.5×10^5 cells/ml every 3–4 days. The cells were incubated at 37°C under humidified atmosphere of 5% CO_2 in air, and 24 h before the experiments the cells were transferred to serum-free medium. Differentiation was induced by the addition of TPA (Consolidated Midland Corp., Brew-

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ster, NY) at 1.0–100.0 ng/ml. TPA stock solution was prepared by dissolving 5 mg/ml in dimethylsulfoxide and further dilution to 1 mg/ml in acetone. Adherence was measured by removing the culture medium and counting the adherent and non-adherent cells [7]. In some experiments adherence was prevented by layering the cells, with or without TPA, on a semi-solid layer of 1% Bacto Agar (Difco, Detroit, MI) in medium. Adherence was also prevented by growing the cells in bacteriological plates or by continuous shaking of the cultures. The cells were harvested at various times as indicated in the text. The adherent cells were obtained by scraping the plastic surface with a rubber policeman, and resuspended in 2 ml of alpha medium. Sonication of the cells was performed for 30 s at 0–4°C in a B-12 sonifier (Branson Sonic Power Company) and the sonicate was centrifuged at 36000 × *g* for 30 min. PA activity was assayed in intact cells and in the supernatant and pellet fractions by a two-step procedure as in [8]. Essentially, the conversion of plasminogen to plasmin was first accomplished by incubating 1 ml of the suspended cells or subcellular fractions with 25 μl plasminogen solution (4 mg/ml) and 50 μl of 0.1 N phosphate buffer (pH 8.0) for 30 min at 37°C. Controls for non-PA globinolytic activity were obtained by a similar incubation without plasminogen and their activity subtracted from the values obtained with plasminogen. The formed plasmin was assayed by measuring its ability to hydrolyze ^{14}C-labeled globin. ^{14}C-Labeled hemoglobin was first prepared as in [9], and the labeled globin separated from heme by denaturation in cold acid acetone. The reaction mixture for the assay of the globinolytic activity of plasmin usually contained 25 μl ^{14}C-labeled globin substrate (15 mg/ml, $4\text{–}7 \times 10^5$ cpm/mg protein) and 400 μl of the plasmin-containing solution. After 90 min of incubation at 37°C, 250 μl of 50% (v/v) trichloroacetic acid was added to each tube to precipitate the undegraded proteins. Following centrifugation (1000 × *g*, 30 min), 0.1 ml aliquots of the supernatant acid-soluble fraction were transferred into scintillation vials and counted in a Packard liquid scintillation counter. The protein content was determined as in [10].

Table 1

PA activity of intact and fractionated HL-60 cells

Source of activity	PA activity (cpm/mg protein)	
	Control	TPA
Intact cells	3669	10812
Supernatant fraction	0	0
Pellet fraction	5133	25924

HL-60 cells were exposed to 10 ng/ml TPA for 24 h. After harvesting the intact cells or the supernatant and pellet fractions obtained by sonication and centrifugation were assayed for PA activity as described in section 2. Similar results were obtained in 4 repeated experiments and the numbers were taken from one representative experiment

3. RESULTS

Table 1 shows that intact HL-60 cells display appreciable PA activity. The activity is located in the 36000 × *g* pellet fraction, while the cytosol is inactive. The specific activity of PA in the pellet is clearly higher than that in intact cells. Table 1 also shows that TPA, added to the serum-free culture for 24 h produced a 3-fold increase in PA activity of intact cells as well as a 5-fold increase in PA activity of the insoluble pellet fraction. TPA under these conditions is known to induce differentiation of HL-60 cells to macrophage-like cells [5,6].

A shorter exposure of the cells to TPA similarly results in a significant increase of PA activity (fig.1). A distinct increase of PA activity can be noted as early as 1 h after addition of TPA, while appearance of cell adherence is only seen about 8 h later. Control HL-60 cells do not adhere and show no change in PA activity throughout the incubation period (fig.1), and up to 48 h (not shown).

The dependence of the PA response on the concentration of TPA added to the culture is shown in table 2. It can be seen that PA activity increases at concentrations of TPA below the minimum levels needed to induce cell adherence. While the lowest concentration of TPA which produces adherence is 5.0 ng/ml, the increase of PA is observed at 1.0 ng/ml and a slight effect was also noticed at 0.1 ng/ml.

The TPA-induced cell adherence can be prevented by shaking of the suspended cells, growth on semi-fluid agar or growth in bacteriological plates.

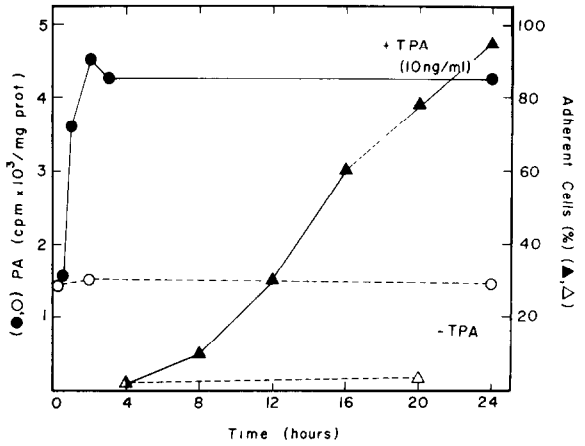

Fig.1. Time course of PA activity and adherence of HL-60 cells following addition of TPA. Cells (2.5×10^5/ml) were grown in the absence or presence of TPA (10 ng/ml). At different times after TPA addition, the percentage of adherent cells and PA activity were determined as described in section 2.

We find that under these conditions TPA does not stimulate PA activity. However, these conditions do not affect the ability of HL-60 cells to express other TPA-induced changes such as phagocytosis and emergence of acid phosphatase and non-specific esterase activities.

Table 3 shows that while untreated HL-60 cells do not secrete PA, exposure of the cells to 10 ng/ml TPA for 24 h stimulates a marked secretion of the enzyme to the medium. The secretion of PA was noted as early as 3 h after addition of TPA (not shown).

Table 2

Effect of TPA adherence and PA activity in HL-60 cells

TPA (ng/ml)	Adherence	PA activity (cpm/mg protein)
0	< 5%	6716 (100)
0.1	< 5%	7057 (110)
1.0	< 5%	8669 (130)
5.0	>90%	15680 (230)
10.0	>90%	17075 (254)

HL-60 cells were exposed to the various concentrations of TPA for 24 h. Percentage of adherent cells and PA activity were determined as described in section 2. Numbers in parentheses are percentages of control activity. Similar results were obtained in 3 repeated experiments and the numbers were taken from one representative experiment.

Table 3

PA activity of HL-60 cells and conditioned medium

Culture	Source of activity	PA activity (cpm/mg protein)
Control	Cells	8829
	Medium	0
TPA-treated	Cells	28620
	Medium	12152

Cells were exposed to TPA, 10 ng/ml, for 24 h. PA activity was determined in the cells and medium as described in section 2. Similar results were obtained in 4 repeated experiments and the numbers were taken from 1 representative experiment

Table 4

Effect of dexamethasone on PA activity in HL-60 cells

Dexamethasone	PA activity (cpm/mg protein)	
	Control	TPA
None	3337	10500
10^{-7} M	3677	9138
10^{-6} M	3700	7801

Exposure to TPA, 10 ng/ml, and to dexamethasone was for 3 h. PA activity was determined as described in section 2. Similar results were obtained in 3 repeated experiments. The numbers were taken from 1 representative experiment

Various macrophage functions including PA activity are suppressed by glucocorticoid hormones [11]. We have therefore examined the effect of the potent synthetic glucocorticoid dexamethasone on PA activity and adhesion of HL-60 cells. Table 4 shows that at the two tested concentrations, dexamethasone failed to affect PA activity in uninduced HL-60 cells. In TPA-treated cells, however, the steroid at 10^{-6} M produced a small reduction in cell-associated PA activity. A 10-fold lower concentration of dexamethasone, as well as a more extended exposure to the hormone (24 h) did not reduce PA activity of TPA-treated cells. Similarly, TPA-induced adherence was not affected by 10^{-7} or 10^{-6} M concentrations of dexamethasone.

4. DISCUSSION

The present findings disclose cellular and secreted PA activity of HL-60 cells to be a sensitive and early marker of TPA-induced adhesion. The direct association between PA and adherence is supported by the finding that prevention of adherence by physical means such as continuous shaking, culture on semi-solid layer or in bacteriological plates also prevents the preceding increase in PA activity. Since adhesion is a marker of differentiation in these cells, the present results corroborate previous reports on the involvement of PA in eukaryotic cell differentiation [12]. The enhancing effect of TPA on PA activity and adherence of HL-60 cells seems to be another manifestation of the pleiotropic effects of this tumor promoter [13]. Interestingly, since TPA-induced changes in membrane properties have also been noted in other cells [13], PA might have a functional role in the membranal changes that are involved in the TPA-induced pleiotropic responses. However, the increase in PA was noted in the absence of plasminogen, since the TPA-induced differentiation was obtained in serum-free medium. Therefore, formation of plasmin through the activity of PA on serum plasminogen is not instrumental for the TPA-induced effect.

The TPA-induced increase in PA activity of HL-60 cells could be a feature of the differentiation process per se. Alternatively, since mature macrophages possess PA activity [14], the appearance of PA might simply reflect emergence of macrophage properties. The first possibility seems more likely since both adherence and the PA activity were almost refractory to dexamethasone, while the hormone markedly inhibits macrophage PA [13]. Further support to this contention comes from the early increase in enzyme activity, much before any other property of macrophages can be observed. The steroid-resistances of PA and adherence are not due to a general lack of steroid responsiveness in HL-60 cells, since these cells respond to the inhibitory effect of glucocorticoids on the expression of Fc receptors [15].

In an earlier study, the differentiation of HL-60 cells to macrophages was observed to be linked to a decrease in intracellular alkaline protease activity and to the emergence of a pentamidine isethionate-inhibitable proteolytic activity [7]. Since this serine protease inhibitor also inhibits plasmin it is not possible to assess its effect on PA in the present system. However, the association between changes in intracellular proteases, PA activity and adherence could suggest that certain proteolytic cleavages lead to changes in cellular characteristics and might produce differentiation. It was already hypothesized that cell surface protease may be important in cell adhesion [16], and changes in PA [12] and surface protease [17] were noted to be associated with differentiation. Furthermore, treatment of erythroleukemic cells in culture with several exogenous proteases initiated their differentiation [18]. In another study we have recently found that exogenous proteases act synergistically with suboptimal concentrations of dimethylsulfoxide to induce differentiation of HL-60 cells to granulocytes (submitted). Taken together, these results support a role for PA, and probably other proteolytic enzymes, in adhesion and differentiation of cells in culture. Interestingly, PA might exert its effect through cleavage of a yet undefined cell-derived substrate, since the presence of exogenous plasminogen is not required for differentiation of HL-60 cells.

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