REGULATION OF ACID PHOSPHATASE ACTIVITY IN HUMAN PROMYELOCYTIC LEUKEMIC CELLS INDUCED TO DIFFERENTIATE IN CULTURE

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ABSTRACT

Induction of differentiation of a human promyelocytic leukemic cell line (HL60) in culture is accompanied by changes in acid phosphatase (ACPase) activity. The increase in activity is less than twofold when the leukemic cells are stimulated by dimethylsulfoxide (DMSO) to differentiate into metamyelocytes and granulocytes but is eightfold when the cells are stimulated by the tumor-promoting agent 12-O-tetradecanoylphorbol 13-acetate (TPA) to differentiate into macrophage-like cells. Five different isozymes of ACPase were separated by acrylamide gel electrophoresis. Isozyme 1, the most anodal isozyme, was found to be present in undifferentiated, DMSO-treated and TPA-treated cells; isozyme 2 was a very faint band observed both in DMSO- and TPA-treated cells, the isozymes 3a and 3b were present only in TPA-induced cells; and isozyme 4, the most cathodal isozyme, was present both in TPA- and DMSO-induced cells. A time sequence study on the appearance of the various forms after TPA treatment indicated that the expression of the isozymes is regulated in an uncoordinated fashion. ACPase activity has been shown by ultrastructural cytochemistry to be localized in the entire rough endoplasmic reticulum (RER) and in areas of the smooth endoplasmic reticulum (SER) located near the Golgi complex in differentiating cells but to be extremely weak, if at all detectable, in undifferentiated promyelocytes.

KEY WORDS terminal differentiation, tumor promoter, macrophage-like cells, acid phosphatase isoenzymes, acid phosphatase cytochemistry

Collins et al. (8) established a cell line in tissue culture with cells from a female patient with acute promyelocytic leukemia. This line, HL60, consists of a large (~80%) fraction of promyelocytic cells, of a smaller fraction (~20%) of more mature myeloid elements (myelocytes, metamyelocytes, banded granulocytes), and of a few cells with morphologic characteristics of immature myeloblasts. Collins et al. (9) have reported that treatment of HL60 cells with dimethylsulfoxide (DMSO), dimethylformamide, or butyric acid causes an increase in the fraction of terminally differentiated myeloid cells (metamyelocytes, granulocytes). However, a sizable percentage of the population (20-30%) remains undifferentiated. More recently, Huberman and Callahan (12) have reported that treatment of HL60 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) induces the leukemic population to terminally differentiate.
into cells that were interpreted to be of the myeloid lineage. Instead, we have given evidence that TPA induces terminal differentiation of HL60 cells along the monocytic macrophage lineage rather than the myeloid lineage (23). TPA-treated cells adhere to the substrate and are able to phagocytize particles, bear Fc receptors for IgG on the surface, and secrete increased amounts of lysozyme into the medium; they contain high levels of nonspecific acid esterases, an enzymatic marker of the monocytic macrophage lineage (29). Analogs of the phorbol diester series with tumor-promoting activity in vivo, as well as the antileukemic drug mezerein, have the same effect on HL60 cells; however, phorbol does not (12, 24). In this paper, we report in detail that treatment of HL60 cells with DMSO and TPA results in induction of acid phosphatase (ACPase), an enzyme normally abundant in macrophages.

**MATERIALS AND METHODS**

**Cells**

HL60 human promyelocytic leukemic cells, a gift of Drs. Collins and Gallo, were grown in a 5% CO$_2$ humid incubator in RPMI 1640 medium supplemented with 15% fetal calf serum. HL60 cells and in HL60 cells treated with 1.2% DMSO or 1.6 × 10$^{-4}$ M TPA (Midland Corp. or Polysciences Inc., Warrington, Penn.) diluted from a 10$^{-3}$ M stock dissolved in acetone. Cells were harvested on the day indicated in Results. Adherent TPA-treated cells were collected by trypsinization and scraping with a rubber policeman. Levels of ACPase were determined in untreated HL60 cells and in HL60 cells treated with 1.2% DMSO or 1.6 × 10$^{-4}$ M TPA for different lengths of time. The results, shown in Fig. 1, indicate that detectable ACPase was present in the untreated HL60 cells. After treatment with DMSO, enzymatic activity increased less than twofold within the first 48 h after treatment and remained at that level through the next 2 d. In contrast, after treatment with TPA, enzymatic activity decreased slightly in the first 24 h and then progressively increased, with a peak on the fourth day of treatment, to a value approximately eightfold higher than that of the untreated controls.

We wanted to know whether ACPase activity was present in all the untreated HL60 cells or in only a fraction of the cells. When these cells were...
cytochemically stained for detection of Acpase, only a fraction of them showed intense enzymatic activity. This fraction (~10%) for the most part represented cells with nuclear characteristics of more mature myeloid elements (see electron microscopy results below). After DMSO treatment for 4 d, the percentage of cells demonstrating intense enzymatic activity was 30%. Of the cells treated with TPA for 3 or 4 d, 90% were intensely positive for the enzyme and the remaining 10% were weakly positive. Table I summarizes the percentage of cells positive for Acpase, as determined by light microscopy, at different times after the start of the drug treatment.

**Variation of Acid Phosphatase Isozymes in Differentiating HL60 Cells**

Multiple Acpase isozymes active toward the substrate sodium naphthyl phosphate have been found in bone marrow myeloid precursors and leukocytes (14, 15). We wanted to determine whether specific Acpase isoymes are present in untreated HL60 cells or in HL60 cells treated with DMSO or TPA. Erythrocyte Acpase activity, determined on starch gels with 4-methylumbelliferyl-di-hydrogen phosphate in acetate buffer, pH 6.0 (27), was not detected in either untreated or treated cultures. With the acrylamide gel system described by Axline (1) and a substrate of sodium α-naphthyl phosphate (4), five isozyme bands were detected. These are indicated in Fig. 2 as 1, 2, 3a, 3b, and 4; 1 is the most anodic and 4 the most cathodic. In untreated cells, only isozyme 1 was detected; in cells treated with DMSO, isozymes 1 and 4 were visible; in cells treated with TPA, isozymes 1, 3a, 3b, and 4 were detectable. Isozyme 2 was observed only in trace amounts both in DMSO- and in TPA-treated cells.

The rate of appearance of the different isozymes was determined at different times after treatment of HL60 cells with TPA. The results are presented in Fig. 3. Isozymes 3a and 3b appeared 48 h after TPA treatment. Isozyme 4 was detectable after 72 h.

**Intracellular Distribution of Acid Phosphatase Activity**

The intracellular distribution of Acpase was determined by ultrastructural cytochemistry. Fig. 4 shows comparative electron microscopy pictures of untreated HL60 cells and of HL60 cells treated with DMSO or TPA.

**Table I**

| Treatment | Days after treatment | Percentage of strongly positive cells | Percentage of weakly positive cells | Percentage of negative cells |
|-----------|---------------------|--------------------------------------|------------------------------------|-----------------------------|
| None      | 0                   | 31                                   | 69                                 |                             |
| DMSO, 1.2%| 1                   | 0                                    | 26                                 | 75                          |
|           | 2                   | 38                                   | 38                                 | 24                          |
|           | 3                   | 40                                   | 36                                 | 24                          |
|           | 4                   | 40                                   | 32                                 | 28                          |
| TPA, 1.6 ×10^{-6} M | 1     | 0                                    | 39                                 | 61                          |
|           | 2                   | 20                                   | 56                                 | 24                          |
|           | 3                   | 97                                   | 0                                  | 3                           |
|           | 4                   | 99                                   | 1                                  | 0                           |

Above results were obtained by the counting of 200 cells/slide by two separate operators. Results indicate the average results from duplicate samples in one representative experiment.
Acpase activity was not cytochemically detectable in the majority of untreated cells (Fig. 4a). These cells varied in size and shape but were most frequently round or ellipsoid with large round nuclei and nucleoli. Almost all the cells contained a few osmiophilic lipid droplets and numerous cytoplasmic vacuoles. Some cells were probably undergoing spontaneous differentiation, as seen by changes in their nuclear shape; in these cells, the positive reaction of Acpase was localized in the vicinity of the Golgi apparatus, probably in the limited areas of smooth endoplasmic reticulum (SER) (Fig. 4b). A few cells also contained small vesicles, recognizable as lysosomes, which carried the reaction product (r.p.).

A number of cells treated with DMSO showed a positive reaction for Acpase. In many of these cells, the r.p. appeared in the perinuclear space and in limited areas of the endoplasmic reticulum (Fig. 4c). Under higher magnification (not shown) the r.p. was visible in segments of SER located in the vicinity of the Golgi apparatus and also in a few small lysosomes.

The majority of cells treated with TPA for 4 d showed a positive reaction for Acpase localized in all narrow sacs of the rough endoplasmic reticulum (RER) and in the perinuclear space (Fig. 4d). More data concerning the time sequence of the appearance and distribution of Acpase activity were obtained when HL60 cells were examined after 1, 2, 3, 4, and 5 d of treatment with TPA.

After 24 h of TPA treatment, only a few single cells showed a weak positive reaction for Acpase in the RER; the majority of cells were completely free of the r.p.

After 2 d, numerous cells showed a positive reaction in the perinuclear space and in the narrow...
FIGURE 4  All electron micrographs show unstained sections of untreated or treated HL60 cells incubated for visualization of Acpase activity. (a) Typical appearance of the majority of untreated HL60 cells. No r.p. is visible; arrow points to lipid droplet. (b) Untreated HL60 cell showing the r.p. in the Golgi complex (arrows). (c) HL60 cell treated with DMSO for 4 d. In this cell, the r.p. appears in the perinuclear space (arrow) and in limited areas of the endoplasmic reticulum (arrowhead). (d) Typical appearance of the majority of HL60 cells treated with TPA for 4 d. The r.p. is present in flattened sacs of the RER (arrowheads) and in the perinuclear space (arrow). (N) nucleus; (Nu) nucleolus. Bar, 1 μm. × 9,000.
sacs of the RER. In some cells, the positive reaction was present in the lysosomes and phagolysosomes as well; in these cells, only small segments of the RER contained the r.p. (Fig. 5a). These lysosome-containing cells were frequently in close contact with other, probably degenerating cells. The morphologic pattern suggests that many of the lysosome-containing cells were in the process of engulfing and phagocytizing the degenerating cells. The appearance of the r.p. between the plasma membranes of these cells could be due to the release of lysosomal enzymes (Fig. 5a, arrowheads).

After 3 d of treatment with TPA, the number of Acpase-rich cells increased. The r.p. was present in all flattened sacs of the RER, in the perinuclear spaces and in vacuoles lying in the Golgi area (Fig. 5b).

After 4 d, the intensity and distribution of the r.p. were the same as seen in Fig. 4d. However, in numerous cells, lysosomes were present and the r.p. was seen in all the sacs of the RER. The distribution of the r.p. in these sacs (Fig. 5c, arrowheads) was sparse and the concentration was lower than in areas of the SER (Fig. 5c, arrows). These limited areas of the SER in which the r.p. was highly concentrated were frequently located in the vicinity of the Golgi apparatus. Small vesicles with a high concentration of the r.p., probably lysosomes, were in close proximity to the above-mentioned segments of both the SER and RER (Fig. 5c, L).

After 5 d of TPA treatment, numerous cells showed a distribution of Acpase activity similar to that observed after 4 d. However, the number of dead and degenerating cells, as well as of cell remnants, was greatly increased. Numerous transformed, macrophage-like cells contained phagocytized and partially digested cells in their cytoplasms.

No reaction was observed in either untreated or treated cells after incubation in a control medium containing NaF, an inhibitor of lysosomal Acpase activity.

DISCUSSION
In this paper, we report that treatment of human promyelocytic leukemic HL60 cells with TPA, a phorbol diester with in vivo tumor-promoting activity, causes, in addition to various morphologic and functional changes (23), a marked increase of Acpase activity. DMSO, a drug which induced differentiation of a large fraction of leukemic HL60 promyelocytes into more mature myeloid elements (9), causes a smaller increase in activity.

Using the acrylamide gel procedure described by Axline (1) and employed for leukocytes by Li et al. (14, 15), we were able to detect five Acpase isozymes in TPA-treated HL60 cells (Fig. 2e). Two bands (1 and 4) were detectable after DMSO treatment of HL60 cells (Fig. 2b). These results are similar to those reported for normal granulocytes by the above mentioned authors. The activity observed in TPA-treated cells is similar to that seen in monocytes (14, 15); here, isozymes 3a and 3b have been detected.

The fast moving band usually seen on starch gels when lysates of granulocytes are electrophoresed (this band represents sialated forms of slower isozymes and can be destroyed by neuraminidase treatment) have not been observed in DMSO-treated cells. This indicates that the granulocytic differentiation induced by the drug may be defective in some posttranslational stage. Whether the increase and change in enzymatic activity reported here reflects (a) a pattern of gene activation induced by TPA or DMSO, (b) activation of the enzyme previously present in undifferentiated promyelocytes or (c) posttranslational modifications in the enzyme remains to be determined.

Acpase activity starts to increase on the second day of TPA treatment and reaches its maximum level on the fourth day (Fig. 1). These quantitative data are in good agreement with the morphologic studies performed with light and electron microscope cytochemistry (Figs. 4 and 5). It is possible, however, that the quantitative results are less relevant to the cytochemical findings. It is known in fact that when β-glycerophosphate is used as substrate the acid phosphatase (as we have done for the cytochemical studies) activity is limited to the lysosomal sites, and that when p-nitrophenyl-phosphate is used as a substrate (as we have done for the quantitative studies) the enzyme can be detected in extralysosomal sites (16, 17).

Ultrastructural examination reveals an interesting and rather unusual phenomenon: high Acpase activity in the entire RER. The association of the ER with formation of lysosomes and related particles has been described and discussed in detail by Novikoff (19) and by Novikoff and Novikoff (21). A positive reaction for Acpase in the ER has been observed in various cells in physiological (3) or pathological conditions (11, 13) but not to the extent found in the present studies. Thus, TPA-treated HL60 cells might constitute a unique bio-
All electron micrographs show HL60 cells treated with TPA and incubated in cytochemical medium for detection of Acpase activity. Bar, 1 µm; (M) mitochondria; (N) nucleus. (a) Cells treated for 2 d with TPA. Lower part of the photograph is occupied by the cell showing a positive reaction for Acpase in the small segments of the ER (arrows), in the lysosomes (L) and in the phagolysosomes (P). This cell is engulfing an apparently degenerating cell (D). The r.p. also appears in the narrow space between the plasma membranes of both cells (arrowheads); section stained with uranyl acetate and lead citrate. × 6,300.

(b) Cell treated with TPA for 3 d. The r.p. is present in all narrow sacs of the RER (arrowheads) and in the perinuclear space (PS). In the vicinity of the Golgi area (G), the r.p. is highly concentrated in flattened sacs and vacuoles (arrows); unstained section. × 25,000.

(c) Cell treated with TPA for 4 d. Sparse deposits
logical model in which unusual stimulation of the production of Acpase occurs and the enzymatic activity is spread over the entire endomembrane system involved in protein synthesis and processing.

The concentration of reaction product appears to be greater in the limited segments of the SER lying in the Golgi areas than it is in the RER, recalling in this respect the structures called GERL observed by Novikoff in several cell types (18-20) and by Benfield and Bainton in megakaryocytes (6).

The sequential changes in isozyme pattern observed at different times after treatment of HL60 cells with TPA may represent either sequential activation of the expression of different genes or enzymatic modifications related to the physical location of the enzyme within the cellular structure. This subject is presently being investigated in our laboratory.

The technical assistance of Elsa Aglow in light microscopy, cytochemistry, and of Danuta Dobrogowska in enzymatic modifications related to the physical concentration of the r.p. is located in tubular parts of the SER (arrows) and in lysosomes (L). Section of the r.p. can be seen in the perinuclear space and in all flattened sacs of the RER (arrowheads); a high concentration of reaction product appears in tubular parts of the SER (arrows) and in lysosomes (L).

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