Reversal of Enzymic Phenotype of Thymidine Metabolism in Induced Differentiation of U-937 Cells

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ABSTRACT

Exposure of U-937 cells to 12-O-tetradecanoylphorbol-13-acetate (TPA) resulted in specific alterations in thymidine metabolism. Within 24 h after treatment with 1.62 x 10^{-8} M TPA, the reciprocal alteration in the activities of opposing enzymes of thymidine metabolism observed during normal cell culture growth was reversed. In TPA-treated cells, the activities of anabolic enzymes thymidine kinase (EC 2.7.1.75) and thymidylic acid synthase (EC 2.1.1.45) declined with time linearly to 20 and 16% of those of untreated cells by 72 h. Incorporation of [H]thymidine and [H]deoxyuridine into acid-insoluble fractions also decreased in parallel with the decline in enzyme activities. In contrast, the activities of catabolic enzymes thymidine phosphorylase (EC 2.4.2.4) and dihydrothymine dehydrogenase (EC 1.3.1.2) increased. The rise in thymidine phosphorylase activity peaked at 48 h with a 406% elevation over the control. The activity of dihydrothymine dehydrogenase was not altered for the first 24 h, but it increased up to 338% by 96 h. Immunotitration of dihydrothymine dehydrogenase with monoclonal antibody against this enzyme showed that the rise in activity in the differentiated cells was due to the increase in the amount of enzyme protein. No significant difference was observed in the K values for the substrate of each enzyme between untreated and TPA-treated cells. These metabolic alterations during induced differentiation were in line with the changes in cell morphology and accompanied by an accumulation of the cells in G phase at the expense of S phase. These observations indicate that induced differentiation of U-937 cells results in a reversal of the enzymic phenotype of thymidine metabolism and suggest that emergence of thymidine metabolic imbalance may serve as an early marker of differentiation of these cells.

INTRODUCTION

TPA has been shown to induce morphological and functional differentiation in various cultured leukemic cell lines (1-4). The human histiocytic lymphoma cell line, U-937, can be differentiated to macrophage/monocyte-like cells (5-8). Induced differentiation in leukemic cells was accompanied by an arrest of the proliferation of the cells (1-3) and changes in the cell cycle kinetics (9-12). Although these cellular responses to TPA are thought to be triggered by specific receptors on the cell membrane of the leukemic cells (1-4), induced differentiation may be also controlled to a critical degree by the metabolic involvement with the cell. Several nucleosides of purine and pyrimidine and antimetabolites have been shown to induce differentiation of leukemic cells (2, 3, 12-15), suggesting a possible role of DNA metabolism in induced differentiation. Alterations in purine metabolism in HL-60 cells during maturation have been demonstrated (15). However, little information is available on the pyrimidine metabolism. Thymidine is salvaged by dThd kinase, providing an alternate route for dTMP production for DNA biosynthesis. Thymidine also may be degraded by dThd phosphorlyase and through the rate-limiting enzyme of dThd catabolism, DHT DH, for eventual catabolism to CO2 and ammonia (16, 17). Since dThd phosphorlyase is an equilibrium enzyme, the balance of dThd kinase and DHT DH may determine the metabolic routing of dThd (16-19). Thymidylate, an important precursor of DNA synthesis, also may be produced by the de novo pathway through dTMP synthase. Recently, we have demonstrated a reciprocal regulation in the behavior of the activities of these opposing enzymes of dThd metabolism in human leukemia-lymphoma cell lines (16). The activities of the anabolic enzymes, dThd kinase and dTMP synthase, increased, and those of catabolic enzymes, dThd phosphorlyase and DHT DH, decreased compared with those of normal lymphocytes. We purified DHT DH to homogeneity (20), prepared monoclonal antibody against DHT DH, and showed that the decreased activity of DHT DH in leukemic cells was due to the decline in the amount of enzyme protein (16). These studies indicate that the enzymic balance of anabolism versus catabolism, especially the activity of DHT DH, is a primary factor determining dThd utilization. Therefore, the elucidation of the behavior of the enzymic capacities in the opposing pathways of dThd metabolism during induced differentiation should lead to a deeper insight into the regulation of dThd utilization.

In this paper, we present the reversal of the reciprocal alterations in the behavior of the enzymic capacities for synthesis of DNA (dThd kinase, dTMP synthase, and incorporation of [H]dThd and [H]deoxyuridine) and in that of catabolic enzymes (dThd phosphorlyase and DHT DH) in U-937 cells which were induced to differentiate by TPA. There was a close relation with the changes in morphology and cell cycle kinetics. Evidence is provided that the rise in DHT DH activity in differentiated cells was due to the increase in the amount of enzyme protein.

MATERIALS AND METHODS

Chemicals. [methyl-3H]dThd (20.0 Ci/mmol), [methyl-3H]thymine (12.6 Ci/mmol), [6-3H]dThd (15.5 Ci/mmol), [2-14C]dThd (58.0 mCi/mmol), [2-3H]thymine (55.2 mCi/mmol), and [6-3H]deoxyuridine (15.5 Ci/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA). [5-3H]UMP (8.9 Ci/mmol) and ACS-II were obtained from Amersham Corp. (Arlington Heights, IL). RPMI 1640 culture medium was from GIBCO (Grand Island, NY). TPA was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were also of the highest available analytical grade.

Cells and Cell Culture. The human histiocytic lymphoma cell line, U-937 (21), was provided by Dr. Reinhard Kurth (Paul Ehrlich Institute, West Germany). Cells were maintained in the logarithmic phase of growth in RPMI 1640 medium supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO2 in air. Cells were seeded in a plastic dish (100 x 20 mm; Becton Dickinson, Lincoln Park, NJ) at a density of 5 x 10^5/ml in 10 ml of medium. TPA, dissolved in absolute ethanol, was added to the dishes in a final concentration from 0.162 x 10^{-8} to 162 x 10^{-8} M and cultured for 24, 48, 72, and 96 h. Ten ml of additional culture medium containing the same concentration was added 48 h after plating.
of TPA were added at the third day in order to support cell growth. Control cultures contained an equivalent amount of ethanol (0.1%) which did not affect cell growth, enzyme activities, or incorporation of [3H]deoxynucleosides. Cell numbers and viability were determined by trypan blue exclusion. Adherent cells, recovered by mechanical pipetting, and nonadherent cells were harvested by centrifugation and washed with PBS. Duplicate cultures were carried out for each experiment. TPA-treated cells were examined for morphological changes under a scanning electron microscope (Model S-550; Hitachi, Tokyo, Japan) (22). For histochemical studies, the cells were stained for α-naphthylbutyrate esterase, naphthol AS-D chloroacetate esterase, myeloperoxidase, and Wright/Giemsa (22, 23). Functional differentiation was analyzed by phagocytosis of Enterococcus faecalis using acridine orange staining, as described previously (22).

Flow Cytometry. The cell cycle distribution was determined by flow cytometry with ethidium bromide-stained cells (22). At the indicated time, untreated and TPA-treated cells (1 to 2 × 10⁶ cells) were collected by centrifugation and washed with PBS twice. The cells were fixed with 50% methanol in PBS while vortexing and then stored at −20°C. Fixed cells were pelleted by centrifugation and washed once with PBS. The cells were then incubated with RNase (final concentration, 1 mg/ml) for 30 min at 37°C. After centrifugation, the cells were washed once with PBS and then resuspended in the staining solution (50 μg/ml of ethidium bromide, 0.1 M sodium citrate, and 0.1% Triton X-100). The mixture was incubated for 45 min at 4°C. DNA histograms were obtained by analyzing 10⁵ cells in Ortho Cytofluorograf 50H (Ortho Diagnostic System Inc., Westwood, MA). The proportions of cells in G₁, S, and G₂ plus M of the cell cycle were determined by computer analysis of DNA histograms by the method of Dean and Jett (24).

Enzyme Assays. Cells were resuspended at 1 to 2 × 10⁷ cells/ml in 50 mM Tris-HCl, pH 7.4, and disrupted by rapid freezing and thawing in liquid nitrogen 5 times. The extracts were centrifuged at 100,000 × g at 4°C for 60 min, and the resulting supernatants were immediately assayed for the dThd-metabolizing enzyme activities as described previously (16, 25). Protein was determined by a routine method using bovine serum albumin as standard (26). Enzyme activity is expressed as nmol of product formed/h/mg of protein. All assays were performed in duplicate and were measured at two enzyme concentrations to ensure the linearity of the reaction.

Incorporation of [3H]deoxynucleoside into Acid-insoluble Fraction. [6-3H]dThd or [6-3H]deoxyuridine was added to untreated and TPA-treated cells in a final concentration of 1.0 μCi/ml, 64.5 μM, at various times during culture and was incubated for 30 min. The labeling was terminated by the addition of 10 ml of cold PBS, and the dishes were stored on ice immediately. Aliquots were taken for cell number and protein determination. Cells were harvested by centrifugation at 3000 rpm at 4°C for 5 min, washed with PBS 3 times, and finally suspended in 10 ml of cold 10% TCA. The acid-insoluble materials were collected on glass fiber disc papers (Whatman; 2.2 cm in diameter) that were washed twice with 10 ml of cold 10% TCA and once with 10 ml of distilled water. Finally, the discs were washed with 70% ethanol once, put into counting vials, and dried in the oven at 60°C for 30 min. Ten ml of ACS II were added to the vials and counted for radioactivity by a liquid scintillation counter.

Immunotitration of DHT DH by Monoclonal Antibody against DHT DH. Preparation of monoclonal antibody against DHT DH and the method of immunotitration are described elsewhere (16). Monoclonal antibody, clone 2A2, was used for the estimation of the amount of DHT DH enzyme protein. In brief, immunotitration of DHT DH was carried out by incubation of the cytosol extracts from untreated and TPA-treated cells with increasing amounts of hybridoma culture medium immunoglobulin G of clone 2A2 in a final volume of 300 μl with 0.9% NaCl solution for 4 h at 4°C, and then the anti-mouse immunoglobulin G was added in excess. The incubation was continued for another 4 h at 4°C. After centrifugation at 10,000 rpm for 10 min, DHT DH activity remaining in the supernatants was assayed.

RESULTS

Effect of TPA on U-937 Cell Growth. The effect of TPA at different concentrations on the growth of U-937 cells was examined (Fig. 1). The growth of the cells was not affected significantly by 0.162 × 10⁻⁹ M TPA, and most of the cells grew in suspension. However, above 1.62 × 10⁻⁹ M, cell growth was retarded in a dose- and time-dependent manner. After 24-h exposure to 1.62 × 10⁻⁹ M TPA, cell growth was inhibited by 36% compared with that of untreated cells cultured for the same period. The cell number at 96 h decreased to 6% of control. The percentage of viable cells remained between 60 and 80% in 1.62 × 10⁻⁹ M TPA, whereas viability was above 80% in the cultures treated with 0.162 × 10⁻⁹ M TPA (data not shown). The TPA concentration of 1.62 × 10⁻⁹ M was used for the following experiments.

Morphological and Histochemical Evidence for Induced Differentiation. The inhibition of proliferation of cells treated with 1.62 × 10⁻⁹ M TPA was accompanied by striking morphological changes consistent with the induction of macrophage/monocyte-like cells. Greater than 80% of the cells adhered to the plastic substrate within 24 h. Wright/Giemsa staining of cytoplasmic cell preparations revealed an increase in the cytoplasm and vacuolization. The percentage of cells positive for α-naphthylbutyrate esterase activity and phagocytosis of E. faecalis increased after treatment with TPA (data not shown). In addition, TPA-treated cells developed ruffle-like protrusions, as shown by scanning electron microscope (Fig. 2), became adherent, and spread with pseudopods on plastic surfaces. These results indicate that, with TPA treatment, U-937 cells terminally differentiated into macrophage/monocyte-like cells.

Effect of TPA on Cell Cycle. Changes in the distribution of the cell cycle of U-937 cells by TPA treatment were analyzed by flow cytometry (Table 1). In untreated cells, the percentages of cell population in G₁, S, and G₂ plus M phases at seeding were 67, 20, and 13%, respectively. At 24 h after plating, the population of cells in the S fraction increased to 38%, and that in G₁ decreased to 50% and then returned to the range of

![Fig. 1. Effect of TPA on U-937 cell growth. U-937 cells were seeded at a density of 5 × 10⁵ cells/ml in 10 ml of medium and cultured for 96 h in the absence (○) or presence of 0.162 (△), 1.62 (●), or 162 (□) × 10⁻⁹ M TPA. Total numbers of viable cells were counted at the indicated times by trypan blue exclusion. Points, mean of 3 determinations; bars, SE.](attachment:fig1.png)
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Fig. 2. Scanning electron micrographs of U-937 cells before (upper) and after (lower) induced differentiation with TPA. U-937 cells were cultured in the absence or presence of 1.62 x 10^-8 M TPA for 48 h.

Table 1 Effect of TPA on U-937 cell cycle distribution

<table>
<thead>
<tr>
<th>No. of h of culture</th>
<th>% of cell population</th>
<th>Control</th>
<th>TPA (1.62 x 10^-8 mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G_1</td>
<td>S</td>
<td>G_2 + M</td>
</tr>
<tr>
<td>0</td>
<td>67 ± 7^a</td>
<td>20 ± 2</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>24</td>
<td>50 ± 4^a</td>
<td>38 ± 3^a</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>48</td>
<td>62 ± 5</td>
<td>27 ± 2^a</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>72</td>
<td>68 ± 6</td>
<td>21 ± 2</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>96</td>
<td>69 ± 8</td>
<td>19 ± 3</td>
<td>12 ± 2</td>
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^a Mean ± SE for 3 determinations.
^b Significantly different from the value of 0 time (P < 0.05).

Following treatment with TPA, there was a progressive increase in the fraction of G_1 and a decrease in S phase. The total population in G_1 phase at 96 h increased to 86%. In contrast, the percentage in S phase decreased from 20 to 12% within 24 h and to 2% by 96 h. The percentage of G_2 plus M phases was not altered significantly during TPA treatment. These results suggest that TPA-treated U-937 cells accumulated in G_1 at the expense of S phase.

Behavior of the Activities of dThd-metabolizing Enzymes during Untreated Culture Growth of U-937 Cells. Cultures were grown from low population density (5 x 10^5 cells/ml) through the logarithmic and into the plateau phases for 4 days. At seeding, the absolute activities (mean ± SE) of dThd kinase, dTMP synthase, dThd phosphorylase, and DHT DH were 8.51 ± 3.26, 67.20 ± 19.60, 88.0 ± 18.0, and 1.38 ± 0.60 nmol/h/mg of protein, respectively. The activities of the anabolic enzymes, dThd kinase and dTMP synthase, increased after seeding and showed a peak level at 24 h with a maximal elevation of 3.1- and 4.0-fold over the value of 0 time. The activities declined to that observed in the cells at seeding (Fig. 3). A similar pattern was observed in the behavior of [3H]-labeled deoxynucleoside incorporation (data not shown), which might be a reflection of that of the synthetic enzymes. The activities of the catabolic enzymes, dThd phosphorylase and DHT DH, declined in activity, with a nadir at 24 h (to 60 and 67% decrease of the values at seeding). Subsequently, activities returned to that of the cells at seeding. Thus, a reciprocal regulation was observed in the activities of opposing enzymes of dThd metabolism during the normal culture growth of U-937 cells.

Inhibition of [3H]-labeled Deoxynucleoside Incorporation into the Acid-insoluble Fraction by TPA. Incorporation of [3H]dThd and [3H]deoxyuridine into acid-insoluble fractions reflects the overall synthetic capacity of DNA through the salvage and the de novo pathways. The rate of incorporation of [3H]dThd and [3H]deoxyuridine in TPA-treated cells declined with time linearly by 72 h to 11 and 10% of the value of the cells seeded at 0 time and then remained constant (Fig. 4). There was no significant difference in the extent of the decline of incorporation in both [3H]-labeled deoxynucleosides.

Behavior of dThd-metabolizing Enzyme Activities during Induced Differentiation by TPA. The effect of TPA on the behavior of the activities of dThd-metabolizing enzymes in U-937 cells is shown in Fig. 5. Activities of dThd kinase and dTMP synthase declined with time linearly by 72 h to 20 and 16% of values of untreated cells, remaining at this low level for the rest of the incubation period. In contrast, the activity of dThd phosphorylase increased until 48 h to 406% of the control value and then remained at this level. DHT DH activity was not altered for the first 24 h, but it progressively increased over a 3-day incubation period (338% increase over the control value). The specific activities of dThd kinase, dTMP synthase, dThd phosphorylase, and DHT DH in the cells treated with TPA for 96 h were 1.99 ± 0.49, 17.20 ± 3.78, 303.7 ± 60.7, and 4.39 ± 0.81 nmol/h/mg of protein, respectively. As a result, the met-
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Fig. 4. Effect of 1.62 x 10^{-9} M TPA on the incorporation of ^H-labeled deoxynucleoside into an acid-insoluble fraction. The incorporation rate is expressed as the percentage of that of cells cultured without TPA for the same periods. A, ^H]dThd; •, [3H]deoxyuridine. Points, mean of 3 determinations; bars, SE.

Fig. 5. Behavior of the activities of dThd-metabolizing enzymes during induced differentiation by 1.62 x 10^{-9} M TPA. Enzyme activity is expressed as the percentage of that of cells cultured without TPA for the same periods. A, dThd kinase; •, dTMP synthase; A, dThd phosphorylase; O, DHT DH. Points, mean of 3 determinations; bars, SE.

abolistic imbalance observed during normal culture growth of U-937 cells was reversed by the treatment with TPA.

Comparison of the Kinetic Parameters of the Enzymes between Untreated and TPA-treated Cells. Kinetic parameters of dThd-metabolizing enzymes were determined in the cells untreated or treated with TPA for 96 h (data not shown). \( K_m \) values for dThd of dThd kinase and for dUMP of dTMP synthase before and after induced differentiation were 5.1 ± 0.3 and 4.9 ± 0.5 \( \mu M \) for dThd kinase and 8.2 ± 0.6 and 9.0 ± 0.6 \( \mu M \) for dTMP synthase. \( K_m \) values for dThd of dThd phosphorylase and for thymine of DHT DH were 0.42 ± 0.03 and 0.39 ± 0.05 \( \mu M \) for dThd phosphorylase and 4.0 ± 0.1 and 4.2 ± 0.2 \( \mu M \) for DHT DH. \( K_m \) values for thymine, which is also the substrate for dThd phosphorylase, were 0.50 ± 0.05 and 0.52 ± 0.04 \( \mu M \), respectively. These results suggest that alterations in enzyme activities are not due to changes in kinetic properties of these enzymes.

Evidence for the Increased Amount of DHT DH Enzyme Protein in TPA-treated U-937 Cells. The protein amount in DHT DH before and after induced differentiation by TPA (1.62 x 10^{-9} M) was compared in cells cultured for 96 h (Table 2). DHT DH from untreated cells was completely neutralized by 0.8 mg of monoclonal antibody. Complete neutralization of DHT DH from TPA-treated cells was obtained by addition of 2.8 mg of monoclonal antibody. As a result, the amount of antibody required for 100% neutralization of TPA-treated cells was 350% of that of untreated cells, which is in good agreement with the increase in the activity of the enzymes of crude extracts to 338% (4.39 versus 1.30 nmol/h/mg of protein). These results indicate that the increased activity of DHT DH in TPA-treated cells was due to an increase in concentration of enzyme protein.

DISCUSSION

Although it has been demonstrated that TPA-induced differentiation of leukemic cells did not require DNA synthesis, which was estimated only by measuring the rate of ^[3]H]dThd incorporation (9, 27-29), DNA is synthesized through not only the salvage but also the de novo pathway in pyrimidine metabolism (16-19). Therefore, the elucidation of the behavior of the capacities in both pathways of dThd metabolism would be most relevant to understand the role of DNA synthesis during induced differentiation.

The present study shows that metabolic imbalance revealed by the increase in DNA synthetic capacity and the decrease in the activity of dThd catabolism during normal culture growth of U-937 cells is reversed by the treatment with TPA. The marked decrease in the capacities of DNA synthesis in TPA-treated cells indicates that synthetic steps of DNA are down-regulated in induced differentiation. Although the predominant contribution of the salvage over the de novo pathway in purine metabolism was described in HL-60 cells during maturation (15), no significant difference between these pathways in pyrimidine metabolism was observed in our study, because the synthetic capacities through both pathways are inhibited to the same extent by TPA. The increase in the levels of dThd degradative enzymes is as important as the decrease in those of DNA synthetic enzymes, since dThd is channeled not only to DNA synthesis...
synthesis but also to catabolism in a competitive manner (16–19). Therefore, the increased enzymic capacities in dThd catabolism during induced differentiation should limit dThd utilization for DNA synthesis, resulting in the decline in DNA synthetic capacities. Thus, a rise in dThd catabolic activities might not be a consequence of cellular differentiation, but rather it might trigger differentiation or permit differentiation to occur. The delayed onset in the increase in DHT DH activity as compared with that in dThd phosphorylase might suggest a different function for these enzymes in dThd degradation. The \( K_m \) value for thymine, the substrate for both enzymes (16), and the specific activity of DHT DH in the cells treated with TPA for 96 h were orders of magnitude lower than those of dThd phosphorylase (4.2 \( \mu \)M versus 0.52 \( \mu \)M and 4.39 versus 303.7 nmol/h/mg of protein). These results indicate that DHT DH in U-937 cells is the rate-limiting enzyme and the candidate for determining the overall capacity for dThd catabolism. Therefore, the rate of dThd degradation should depend on the activity of DHT DH.

Regarding the mechanism of alterations in the activities of dThd-metabolizing enzymes, several possibilities might be considered: (a) an artifact due to choice of an inappropriate denominator, such as cell size; (b) appearance of inhibitors or activators for the enzymes; (c) changes in enzyme properties represented by an alteration of \( K_m \) value for the substrate; (d) altered population of cell cycle; and (e) an alteration in the amount of enzyme protein. Although a relative increase in cell size during induced differentiation (1.2- to 1.4-fold of control) was observed (data not shown), as demonstrated by others (10, 11), the magnitude of changes in enzyme activities was greater than what would be expected if cell size was the sole determinant of enzyme activities. Changes in the distribution of cell cycle would be one important factor. An association of the inhibition of DNA synthesis with the changes in the population of the cell cycle during TPA-induced differentiation has been demonstrated, i.e., decrease in S phase and increase in G1 cells during differentiation of HL-60 (9, 11), HeLa cells (30), and breast carcinoma cells MCF-7 (10). We identified such changes in U-937 cells in the present study. As dThd catabolic enzyme might be active in G1, as demonstrated here and by others (31), elevated activities of dThd catabolism might be due at least in part to the rise in the population of G1 cells. Moreover, immunotitration of DHT DH with monoclonal antibody indicates that increased DHT DH activity in TPA-treated cells was due to the increased amount of enzyme protein. Therefore, the increased level of DHT DH might be attributed to the increase in G1 fraction and to the elevated enzyme protein amount.

We have demonstrated previously a positive or negative relation between the levels of the catabolic or anabolic enzymes and the degree of differentiation of the cell in normal adult and fetal liver tissues (18, 19, 32). It is likely that the elevated level of dThd catabolic capacity and decline in that of anabolism might be required for the maintenance of the differentiated state, since functionally mature lymphocytes isolated from the peripheral blood of normal human donors appear to possess a similar level of these enzyme activities (16). As cellular effects caused by TPA may be mediated in part by modulation of dThd metabolic capacity and as rapid alteration in dThd utilization is in line with the onset of morphological differentiation, the emergence of dThd metabolic imbalance should be an early marker of differentiation.

In conclusion, the enzymic phenotype of dThd metabolism was reversed in TPA-induced differentiation of U-937 cells in parallel with the changes in morphology and cell cycle kinetics.

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