Changes in Isoenzyme Profiles during Induction of Differentiation in Human Myelomonocytic Leukemia Cell Lines

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ABSTRACT

The human leukemia cell lines HL-60, KG-1, KLM-2, ML-3, THP-1, and U-937 were treated with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA). TPA partially or completely inhibited the proliferative activity of the cell cultures. The number of cells with the ability to reduce nitroblue tetrazolium increased in the TPA-treated cell lines HL-60, ML-3, THP-1, and U-937, whereas the cell lines KG-1 and KLM-2 remained nitroblue tetrazolium negative. Except for KG-1 and KLM-2, all TPA-treated cell lines showed varying degrees of strong adherence to plastic surface. The carboxylic esterase, acid phosphatase, hexosaminidase, and lactate dehydrogenase isoenzyme profiles from these cell lines were analyzed by isoelectric focusing on horizontal polyacrylamide gels. The new or stronger expression of an esterase isoenzyme which is specific for monocytes-macrophages was induced in HL-60, ML-3, THP-1, and U-937 but not in KG-1 or KLM-2. The new expression of the tartrate-resistant acid phosphatase isoenzyme was induced in ML-3, THP-1, and U-937. The number of esterase and acid phosphatase isoenzymes and the staining intensity of isoenzymes characteristic for myeloid cells were increased by TPA in all cell lines. The loss of the hexosaminidase I isoenzyme which is a marker of immature hematopoietic cells was noted in KG-1, ML-3, THP-1, and U-937. TPA triggered an increase in number and staining intensity of the lactate dehydrogenase isoenzymes in all cell lines. Some isoenzyme changes (e.g., monocyte-specific esterase, tartrate-resistant acid phosphatase, hexosaminidase I) appear to correlate with TPA-induced differentiation while other alterations in the isoenzyme patterns do not (e.g., lactate dehydrogenase, other esterases and acid phosphatase isoenzymes). Differentiation of nonmonocytoid cells appears, at the isoenzyme level, to be quite different from that of the monocytoid cell lines.

INTRODUCTION

Human leukemia cell lines are characterized by three common features: (a) monoclonality of the cell population; (b) arrest of the cells at a given stage of differentiation; and (c) stability of the phenotypic marker profiles in long-term culture (1). Due to virtually unlimited growth, cell lines have become valuable tools for leukemia research. The analysis of acute myeloid leukemia differentiation is aided substantially by the establishment of human acute myeloid leukemia cell lines. The cell lines HL-60 and KG-1 show in their original, untreated state predominantly features of early myeloid cells, but HL-60 can also be triggered to differentiate to macrophage-like cells (2-5). The cell lines ML-3, U-937, and THP-1 are characterized by monocye-associated phenotypes (4-7). A variety of distinct morphological, immunological, cytochemical, biochemical, and functional markers of maturation are expressed as the cell lines undergo differentiation along one or several cell lineages induced by a number of agents (5). The cell line KLM-2 established from a case of acute monocytic leukemia (8) displays an immunological marker profile of pre-B-cells (9) but could be induced to differentiate to macrophage-like cells (10). Recently we described the isoenzyme profiles of a panel of leukemia-lymphoma cell lines (9, 11-13) and detected lineage- and/or stage-related expression of isoenzymes or isoenzyme patterns: the increase in number and staining intensity of esterase, acid phosphatase, and lactate dehydrogenase isoenzymes paralleled the sequences of differentiation along the lymphoid and myeloid pathways; one particular esterase isoenzyme was seen only in myelomonocytic cell lines; one hexosaminidase isoenzyme (isoenzyme I) was a marker of immature cell lines. Here, we wish to report the enzymatic changes found in the myelomonocytic cell lines HL-60, KG-1, ML-3, THP-1, and U-937 and in the cell line KLM-2 during induction of differentiation. The isoenzyme profiles of carboxylic esterase (EC 3.1.1.1), acid phosphatase (EC 3.1.3.2), hexosaminidase (= alpha-N-acetylgalactosaminidase, EC 3.2.1.30), and LDH (EC 1.1.1.27) were used as main parameters for monitoring the effects of the phorbol ester TPA on these cell lines.

MATERIALS AND METHODS

Cell Lines

All cell lines were grown as single cell suspension cultures in RPMI 1640 containing 5% heat-inactivated fetal calf serum at 37°C in a 5% CO2-humidified atmosphere. Cultures free of infection with Mycoplasma and with sufficiently high viability (95% or more) were used for this study. The cell lines used are HL-60 (established from a case of acute promyelocytic leukemia) (2), KG-1 (acute myeloblastic leukemia) (3), KLM-2 (acute monocytic leukemia) (8), ML-3 (acute myeloblastic leukemia) (4), THP-1 (acute monocytic leukemia) (7), and U-937 (histiocytic lymphoma) (6).

Induction Experiments

Cells were harvested in the exponential growth phase and resuspended in 50 ml RPMI 1640 at a concentration of 0.5 x 10^6 cells/ml. TPA (10^-9 or 10^-10 M) was added to the cultures. Controls without TPA and TPA-treated cells were maintained under the above described conditions without further addition or exchange of culture medium. Cells were harvested and examined for the described parameters at 24, 48, 72, and 96 h.

Cell Counts and NBT Reduction Test

Cell viability was examined by the trypan blue dye exclusion test. Cells were counted in a hemocytometer. NBT reduction assay was performed by incubating the cells in a solution containing 0.1% (w/v) NBT dissolved in 5% (v/v) dimethyl sulfoxide and 95% (v/v) RPMI 1640 plus 100 ng TPA for 30 min at 37°C. NBT reduction was assessed in a hemocytometer by examining at least 200 cells for black-bluish deposits.

Adherence of Cells

Adherence to plastic surface was examined visually in the culture flasks. Cells were considered to be adherent if they resisted vigorous shaking of the culture flask and washing by pipetting with culture medium. Most of the adherent cells could be removed by treatment with 0.25% (w/v) trypsin solution for 5-10 min.

Isoelectric Focusing

After harvest and washing of cells with RPMI 1640, cells were resuspended in Tris-sucrose buffer, pH 7.4 (9). Cells were lysed by 3
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Fig. 1. Number of viable cells in control and TPA-treated cultures of HL-60, KG-1, KLM-2, ML-3, THP-1, and U-937. ———, controls; ———, TPA-treated cells; Δ, ○, C, •, □, ▲, 10^{-9} M TPA; △, ○, □, 10^{-8} M TPA.

cycles of freezing/thawing; enzymes were solubilized by addition of 1% triton X-100. After centrifugation, aliquots of the supernatant from equal numbers of viable cells were applied for separation of isoenzymes by analytical isoelectric focusing.

Isoelectric focusing was performed on horizontal polyacrylamide thin-layer gels containing 4.8% (w/v) acrylamide/bisacrylamide, 12.5% (w/v) sucrose, 0.015% (w/v) ammonium persulfate/riboflavin, and 0.1% (v/v) tetramethylethylenediamine. A 2% (w/v) ampholyte of pH 2-11 (Serva, Heidelberg, Federal Republic of Germany; for separation of esterase, acid phoshatase, and LDH isoenzymes) or of pH 3-10 (Sigma Chemical Co., St. Louis, MO; for separation of hexosaminidase isoenzymes) was added to the gel matrix. Runs were performed for 1 h at 5°C cooling temperature and 30 W constant power with limitation of voltage to 1400 V using a LKB-Multiphor/Power Supply unit (9).

Visualization of Isoenzymes

Immediately after isoelectric focusing, isoenzymes were visualized on the gels using modified histocytochemical staining methods (9, 11-13).

Carboxylic Esterase. Phosphate buffer (pH 7.2) and α-naphthyl acetate dissolved in acetone were used as a substrate and Fast Blue RR was the coupling dye. Staining was for 1 h at room temperature. For inhibition of a monocyte-specific isoenzyme 40 mM sodium fluoride was added to the staining bath.

Acid Phosphatase. Barbituric acid-sodium acetate buffer (pH 5.0) and naphthol-AS-Bi-phosphate dissolved in dimethylformamide were used as substrate and Fast Garnet GBC was the dye. Staining was for 3-3.5 h at room temperature. For detection of the tartrate-resistant acid phosphatase isoenzyme, 75 mM sodium tartrate was added to the staining solution.

Hexosaminidase. Citrate buffer (pH 4.5) and naphthol-AS-Bi-N-acetyl-β-D-glucosaminide dissolved in ethyleneglycol monomethyl ether were used as substrate and Fast Garnet GBC was the dye. Staining was for 1.5 h at 37°C. The isoenzymes A, I, and B could be demonstrated by isoelectric focusing.

LDH. Sodium lactate, NAD, NaCl solution, MgCl2 solution, phosphate buffer (pH 7.4), NBT, and phenazine methosulfate were used as substrate. Staining was for 20-25 min at room temperature. By isoelectric focusing the isoenzymes 1, 2, 3, 4a, 4b, 5a, 5b, and 5c could be separated (13).

RESULTS

Cell Proliferation

No cell proliferation was noted in cell cultures treated with 10^{-9} M TPA (Fig. 1). The number of viable cells decreased at day 1 and then (except for ML-3) remained stable at this level. TPA (10^{-10} M) partially inhibited cell growth in HL-60, KG-1, THP-1, and U-937; KLM-2 and ML-3 growth was fully inhibited. The percentage of viable cells remained between 60 and 80% in the experiments using 10^{-9} M TPA whereas the viability was above 80% in the cultures treated with 10^{-10} M TPA.

NBT Reduction Test

An increase in the number of NBT-reducing cells was detected for the following TPA-treated cell lines: HL-60, ML-3, THP-1, and U-937 (Table 1).

Adherence of Cells

Adherence to plastic surface was seen in HL-60 cultures treated with TPA (35 and 42% of the total viable cell population in two experiments), ML-3 (44%, 53%), THP-1 (73%, 79%), and U-937 (38%, 44%). The majority of these adherent cells could be removed by treatment with trypsin solution; however, about 10% of the adherent populations could not be detached. On day 2 of treatment the maximum number of adherent cells

Fig. 2. Schematic diagrams of esterase isoenzyme patterns of 10^{-9} M TPA-treated leukemia cell lines at day 0, 2, and 4 (top cathode, bottom anode); arrows, NaF-sensitive, monocyte-specific isoenzyme in HL-60, ML-3, THP-1, and U-937. Numbers in boxes, number of days in culture.
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Fig. 3. Schematic diagrams of acid phosphatase isoenzyme patterns of 10^{-9} M TPA-treated leukemia cell lines; arrows, tartrate-resistant acid phosphatase isoenzyme in ML-3, THP-1, and U-937.

Table 1 Results of NBT reduction test in 10^{-9} M TPA-treated cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% of positive cells (per viable cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>HL-60</td>
<td>0</td>
</tr>
<tr>
<td>KG-1</td>
<td>0</td>
</tr>
<tr>
<td>KLM-2</td>
<td>0</td>
</tr>
<tr>
<td>ML-3</td>
<td>19</td>
</tr>
<tr>
<td>THP-1</td>
<td>19</td>
</tr>
<tr>
<td>U-937</td>
<td>58</td>
</tr>
</tbody>
</table>

Values given are results from two separate experiments.

was reached and did not change until day 4. Only a few cells (<1%) were attached to the flasks in the control cultures. KG-1 and KLM-2 cells were loosely attached to the plastic surface but were easily removed by vigorous shaking and pipeting.

Isoenzyme Analysis

A description of all detectable isoenzymes referring to the biochemical characteristics, isoelectric points, and occurrence in various types of leukemia cell lines has been given earlier (9, 11–13). In accompanying experiments, it was found that 10^{-11} M and 10^{-12} M TPA did not cause any changes in the isoenzyme profiles whereas 10^{-7} and 10^{-8} M TPA concentrations were too cytotoxic and only a small number of cells "survived" the treatment. The differences in the isoenzyme profiles induced by either 10^{-9} or 10^{-10} M TPA were of quantitative nature reflected in different staining intensities of the newly induced isoenzyme bands.

Carboxylic Esterase. An increase in the number of isoenzymes and in the staining intensity of several bands was found in all treated cell lines (Fig. 2). This led to a pattern which appears to be characteristic for normal and leukemic myeloid cells.

KG-1 and HL-60 lost an isoenzyme that migrates to a pH of approximately 6.0. The expression of the NaF-sensitive, monocyte-specific isoenzyme (9) could be induced in HL-60 (Fig. 2). The staining intensity of this particular isoenzyme increased strongly in the monocytoid cell lines ML-3, THP-1, and U-937 which also showed this isoenzyme in the untreated cells.

Acid Phosphatase. TPA triggered an increase in number and staining intensity of isoenzymes in all cell lines studied, especially in the first group (counting from the cathode) composed of 3 isoenzymes (Fig. 3). A strong staining of one or all of these 3 isoenzymes as seen in the treated cells appears to be characteristic for myelomonocytic cells. The tartrate-resistant acid phosphatase band (11) could be induced in the monocytoid cell lines ML-3, THP-1, and U-937.

Hexosaminidase. The uninduced cell lines KG-1, ML-3, THP-1, and U-937 expressed the isoenzyme hexosaminidase I (Fig. 4). TPA-treated cells no longer displayed this isoenzyme which is a marker of immature hematopoietic cells (14). An increase in the staining intensity of the hexosaminidase A isoenzyme was seen for HL-60 and KLM-2 leading to an isoenzyme profile consisting only of the isoenzyme A in all 3 cell lines. This picture has been found in the majority of fresh AML cells. On the other hand, the 3 monocytoid cell lines ML-3, THP-1, and U-937 showed the following changes: decrease and increase of staining intensity of isoenzyme A and isoenzyme B, respectively; loss of isoenzyme I.

LDH. No significant differences between the nonmonocytic and monocytoid cell lines were found for the LDH isoenzyme profiles (Fig. 5). TPA led to an increase in the staining intensity of the isoenzymes 1 and 2 (in KG-1, KLM-2, ML-3, THP-1, and U-937), isoenzyme 3 (in HL-60 and ML-3), and isoenzyme 5b (in HL-60, KLM-2, ML-3, and U-937). TPA induced in all cell lines the new or stronger expression of isoenzyme 5c.

No changes were seen in the isoenzyme profiles of the control cultures.

DISCUSSION

While a large number of papers are dealing with the effects of TPA on the cell lines HL-60, KG-1, and U-937 (reviewed in Ref. 5), very few reports describe phenotypical changes induced...
Fig. 4. Schematic diagrams of hexosaminidase isoenzyme patterns of $10^{-7}$ M TPA-treated leukemia cell lines; A, B, C, hexosaminidase isoenzymes.

Fig. 5. Schematic diagrams of LDH isoenzyme patterns (separated by isoelectric focusing) of $10^{-7}$ M TPA-treated leukemia cell lines; 1, 2, 3, 4a-b, 5a-c, LDH isoenzymes.

in ML cell lines (15–18), in KLM-2 (10, 19), and in THP-1 (20). Qualitative (iso-) enzyme alterations induced by treatment with TPA have been shown for esterase in HL-60 (19, 21), U-937 (22), and KLM-2 (19), for acid phosphatase in HL-60 (23, 24), and for LDH in HL-60 (25).

During treatment with TPA HL-60 cells converted to cells of the monocyte-macrophage complex (26). Our results confirm these data because TPA-treated HL-60 cells ceased to proliferate, became adherent and NBT positive, and expressed newly the monocyte-specific esterase isoenzyme. TPA increased the number of isoenzymes and staining intensity of several bands of acid phosphatase, hexosaminidase, and LDH in HL-60. The isoenzyme patterns of TPA-treated HL-60 cells differed from those of ML-3, THP-1, and U-937, even though by conventional assays (NBT reduction, adherence) the treated cell populations were similar.

Because TPA-exposed KG-1 cells did not adhere to plastic surface and did not show the monocyte-specific isoenzyme, it can be assumed that these cells did not differentiate along the monocyte-macrophage cell lineage. However, KG-1 cells displayed changes in their isoenzyme patterns indicating the expression of a more mature isoenzyme picture. The loss of hexosaminidase I which is a marker of immature hematopoietic cells (14) demonstrates that (a) our untreated KG-1 cells were arrested at a relatively immature stage of differentiation and (b) the arrest of differentiation could be overcome by TPA. Koeffler et al. (27) reported that a subclone of KG-1, termed "KG-1a," which appears to be blocked at a less differentiated myeloid blast cell stage than the original KG-1 culture, was completely resistant to TPA-induced macrophage differentiation.

In our study KLM-2 cell proliferation was inhibited by TPA and the cells remained NBT negative. Regarding isoenzyme profiles, TPA caused changes in all 4 isoenzyme patterns of KLM-2 leading to an overall picture which was different from that of HL-60 and the monocytic cell lines and which did not contain features of monocytes-macrophages.

The TPA-treated monocytoid cell lines ML-3, THP-1, and U-937 showed strong adherence to plastic surface; were all...
NBT positive; and expressed strongly the monocyte-specific esterase isoenzyme. These results confirm earlier reports which found that these 3 cell lines can be converted by TPA treatment into activated macrophages according to morphological, immunological, and functional characteristics (15, 20, 28).

ML-3, THP-1, and U-937 originally expressed hexosaminidase I which disappeared during exposure to TPA. This underlies the fact that this particular isoenzyme can serve as an indicator of immature cells (14). In contrast to the nonmonocytic myeloid cell lines where TPA triggered the stronger expression of hexosaminidase A, the monocytic cell lines showed stronger staining intensities of hexosaminidase B and a gradual loss of hexosaminidase A. This might be a characteristic feature discerning monocytoid from myeloid cells. ML-3, THP-1, and U-937 could be induced to express the tartrate-resistant acid phosphatase isoenzyme. Tartrate-resistant acid phosphatase activity was found not only in hairy cell leukemia but also in activated normal macrophages (29); thus, it might be a correlate of an activated status of normal and malignant cells.

The differentiation of all cell lines was accompanied by an increase in the number of several isoenzymes, a loss of some isoenzymes, and a stronger staining intensity of several isoenzyme bands in all 4 enzymes studied. Some isoenzyme changes (e.g., the monocyte-specific esterase, the tartrate-resistant acid phosphatase, and hexosaminidase I) appear to correlate with TPA-induced differentiation as assessed by NBT reduction or adherence while other isoenzymatic alterations do not (e.g., LDH isoenzymes, other esterase and acid phosphatase isoenzymes). Differential of nonmonocytic cells appears to be, at the isoenzyme level, quite different from that of the monocytoid cell lines.

The new expression of isoenzymes might be the qualitative result of de novo induction of gene expression or simply the quantitative amplification of already expressed enzymatic activity; prior to treatment the isoenzymes might be present at a lower level not detectable by the methods used. It remains to be determined whether the isoenzymatic alterations are quantitative or qualitative in nature.

In conclusion, isoenzyme marker analysis provides sensitive parameters for monitoring subtle changes occurring during differentiation of hematopoietic cells. Isoenzyme studies should give more insight into the processes associated with the arrest of differentiation of malignant cells.

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