Induction of Maturation in Cultured Human Monocytic Leukemia Cells by a Phorbol Diester

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ABSTRACT

Suspension cultures of a human monocytic leukemia cell line, THP-1, were treated with 0.16 to 160 nM 12-O-tetradecanoylphorbol-13-acetate (TPA). In an original cell line, THP-1-O, cultured again from -80°C cryopreservation, more than 80% of the cells adhered to the glass substrate with marked morphological change within 3 hr of TPA treatment. Adherent cells became flat and amoeboid in shape, and many microvilli and flaps of the cell surface disappeared. Well-developed Golgi apparatus, rough endoplasmic reticula, and a large amount of free ribosomes were seen in the cytoplasm. On the other hand, in THP-1-R cells cultured continuously without cryopreservation for 26 months, approximately 80% of the cells adhered to the substrate 48 hr after TPA treatment. Round and ovoid shapes were kept in THP-1-R cells treated with TPA. Surface Fc receptors for immunoglobulin G were present on more than 90% of THP-1-O and THP-1-R cells and were little affected by treatment with TPA. Sixty to 70% of the TPA-treated THP-1-O and THP-1-R cells were able to phagocytize yeasts and immunoglobulin G-coated sheep erythrocytes. Less than 20% of the untreated THP-1 cells were able to phagocytize yeasts and immunoglobulin G-coated sheep erythrocytes. In histochemical staining, α-naphthyl butyrate esterase was enhanced after treatment with TPA. Lysozyme activity in culture supernatants was not affected by TPA treatment. When exposed to latex beads and TPA, increased 14CO₂ production from [1-14C]glucose in THP-1-O cells was observed. These results indicate that, after treatment with TPA, human monocytic leukemia cells may be converted into mature cells with functions of macrophages.

INTRODUCTION

It is well known that some murine and human leukemic cells can be induced to differentiate into mature cells by many chemicals, such as DMSO,3 TPA, sodium butyrate, hexamethylene bisacetamide, glucocorticoid, and so on (1, 8, 9, 13, 15–18, 21). Of these chemicals, TPA, a phorbol diester, has pleiotropic effects on the differentiation of normal and malignant cells. It has been reported that TPA has induced differentiation of Friend erythroleukemic and protein-induced differentiation of M1 cells (11, 24).

We have established recently a human leukemic cell line (THP-1) with characteristics of monocytes (22). In view of the biological properties of TPA, we examined the effects of TPA on the differentiation of THP-1 cells and found that TPA treatment changes THP-1 cells into cells with the functional characteristics of mature macrophages.

MATERIALS AND METHODS

Cells. The characteristics of THP-1, a human monocytic leukemia cell line, have been described previously in detail (22). THP-1-O was an original type of THP-1 cell which had been cryopreserved at -80°C for 24 months and cultured again for this study. THP-1-R was a type of cell continuously cultured after establishment. These cell lines were maintained in suspension cultures in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 5% fetal calf serum and kanamycin (60 µg/ml). Both THP-1-O and THP-1-R cells retained the same characteristics as described previously, but THP-1 cells adapted, showing rapid growth in vitro culture. When one-half of the medium was changed once a week, maximum cell density was 5 × 10⁶/ml in THP-1-O and 1 × 10⁶/ml in THP-1-R. Five LCLs established in our laboratory by infection with Epstein-Barr virus, one B-lymphoma cell line (THP-2-B) without Epstein-Barr virus genome also established in our laboratory,4 and the HL-60 cell line were cultured as control cells under the same conditions as those of THP-1. The cell lines used were not checked for microbial infections.

TPA (Consolidated Midland Corporation, Brewster, N. Y.) was solubilized in DMSO at a concentration of 1.8 × 10⁻³ M and added to suspension cultures of THP-1 cells at the various concentrations indicated.

For light-microscopic observation, the cells were smeared on coverslips and stained with Wright-Giemsa.

TEM. Suspension cultures of untreated THP-1-O cells were centrifuged, and the pellets were fixed for the TEM study. Specimens of TPA (160 nM)-treated THP-1-O and THP-1-R cells were obtained directly from monolayer cultures. Cell specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, and postfixed in 1% osmium tetroxide. They were dehydrated in graded concentrations of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-100C electron microscope.

Scanning Electron Microscopy. Specimens were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated in graded concentrations of acetone, and dried with the critical point method. They were coated with platinum and observed with a Hitachi S-700 scanning electron microscope.

1 This work was supported in part by a research grant from the Intractable Disease Division, Public Health Bureau, Ministry of Health and Welfare, Japan.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: DMSO, dimethyl sulfoxide; TPA, 12-O-tetradecanoylphorbol-13-acetate; LCL, lymphoblastoid cell line; TEM, transmission electron microscopy; SRBC, sheep erythrocytes; ER, endoplasmic reticulum.
Surface Markers of THP-1 Cells. Rosette-forming assay was performed according to the method of Tachibana and Ishikawa (20). Cells with IgG Fc receptors were assayed by their capacity to form rosettes with IgG antibody-coated SRBC (3). Cells with C3b and C3d receptors were examined by rosette formation with IgG antibody (human complement)-coated SRBC and IgM antibody (C5-deficient mouse complement)-coated SRBC, respectively (5). Spontaneous rosette formation with SRBC was carried out using neuraminidase-treated SRBC (23).

Phagocytic Activity. Immediately after treatment of the cells (1 × 10⁶/ml) with indicated concentrations of TPA, 0.1 ml of heat-killed yeasts (2.5 × 10⁶/ml), 2% SRBC, or 2% IgG-coated SRBC in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 20% fetal calf serum were added to each tube and incubated for 4 hr at 37°C. After washing in phosphate-buffered saline, pH 7.2, the cells were smeared on coverslips. On the occasion of SRBC, coverslip smears were made after treatment with Tris-ammonium chloride buffer, pH 7.65, to remove unphagocytized SRBC.

Oxidation of [1-¹⁴C] and [6-¹⁴C]Glucose in THP-1 Cells. Oxidation of glucose was measured according to the method of Stossel et al. (19). Glucose (1 × 10⁻³ M) and 0.25 μCi of [1-¹⁴C]glucose (8.2 mCi/mmol; New England Nuclear) or [6-¹⁴C]glucose (9.0 mCi/mmol; New England Nuclear) were added to cell suspension (1 × 10⁶/vial) with or without 160 nM TPA and 0.15 μl latex beads (size, 0.81 μm; Difco Laboratories, Inc., Detroit, Mich.). These suspensions were incubated in vials stopped by caps fitted with cups in which filter paper wicks impregnated with 100 μl of 2 N H₂SO₄ were held. After a 30-min incubation, the wicks were removed and assayed for radioactivity.

Mononuclear control cells obtained from healthy volunteers were prepared by Ficoll-isopaque gravity sedimentation. After staining with peroxidase, the percentage of monocytes was counted and were adjusted to 1 × 10⁶ monocytes in each vial. The assay was performed as described above in the presence of lymphocytes.

Cytotoxic Staining and Lysozyme Assay. a-Naphthyl butyrate esterase, naphthol ASD chloroacetate esterase, periodic acid-Schiff, Sudan Black B, and peroxidase stainings were performed as reported previously (22). Lysozyme activity of the culture supernatants was assayed by the turbidimetric technique using Micrococcus lysodeikticus (Sigma Chemical Co., St. Louis, Mo.) (14).

RESULTS

Adherence of THP-1 Cells after Treatment with TPA. THP-1-O cells grew as a single-cell suspension, and only a few cells spread onto the glass substrate. TPA-treated THP-1-O cells adhered to the glass substrate. Since the adherent cells could not be detached from the substrate with EDTA and trypsin treatment, the number of adherent cells was measured by subtracting the number of suspended cells from that of seeded cells (Table 1). Approximately 80% of the cells were attached to the substrate as early as 3 hr after the addition of TPA (16 to 160 nM). No more adherent cells grew in culture, and after 48 hr, dead cells gradually became detached from the substrate. Treatment of low concentrations of TPA (0.16 to 1.6 nM) induced adherence of less than 50% of the cells to the substrate. On the other hand, THP-1-R cells were found to adhere to the substrate 48 hr after treatment with 160 nM TPA, although at lower concentrations of TPA (0.16 to 16 nM), adherence of the cells was not observed. Adherent THP-1-R cells were able to grow in culture with many aggregated clumps. Five LCLs treated in the same manner were loosely attached to the substrate, but the attached cells were easily detached from the substrate by treatment with EDTA. Addition of DMSO only at the same dilutions required for obtaining the given concentrations of TPA in the culture system did not induce adherence of the cells to the substrate.

Morphological Changes of THP-1 Cells after Treatment with TPA. The untreated THP-1-O cells stained with Wright-Giemsa showed a moderate amount of basophilic cytoplasm containing small azurophilic granules, a few vacuoles, and nuclei indented and irregular in shape with prominent nucleoli. After a 24-hr treatment with TPA, cytoplasmic vacuoles of THP-1-O cells were increased, and the nuclei became reniform in shape with distinct nucleoli. Scanning electron microscopy showed TPA-treated THP-1-O cells spreading onto the substrate to be irregular and flattened in shape with almost complete loss of microvilli and flaps (Figs. 1A and 3A). A slightly raised nuclear pole was noted. TEM showed well-developed structures of rough ER, Golgi apparatus, and abundant free ribosomes in the cytoplasm. Many vesicles including the cored type disappeared (Figs. 1B and 3B). TPA-treated THP-1-R cells retained their round shape, but microvilli and flaps on their surface decreased (Fig. 2A). Changes in cytoplasmic structures were similar to those in TPA-treated THP-1-O cells, except that the Golgi apparatus were poorly developed (Fig. 2B). Morphological characteristics of untreated and TPA-treated THP-1 cells are summarized in Table 2.

In histochemical stainings, both THP-1-O and THP-1-R cells were positive for a-naphthyl butyrate esterase and periodic acid-Schiff, increasing in intensity after treatment with TPA, but negative for naphthol ASD chloroacetate esterase, Sudan Black B, and peroxidase. a-Naphthyl butyrate esterase was inhibited by NaF.

Surface Markers and Lysozymes of THP-1 Cells after Treatment with TPA. The effect of TPA treatment on the expression of surface receptors of THP-1 cells was examined by rosette-forming assay. Ninety-seven % of THP-1-O cells and 92% of the THP-1-R cells were IgG Fc receptor positive. With TPA treatment, little change in the expression of IgG Fc, C3b, and C3d receptors on THP-1-O cells was observed (Table 3).

Supernatants obtained from the 3-day culture that had been initiated with THP-1 cells (5 × 10⁶/ml) were subjected to lysozyme assay. The levels of lysozyme were 4.7 ± 0.3 μg/ml in THP-1-O and 1.9 ± 0.3 μg/ml in THP-1-R with or without addition of TPA.

Table 1

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>TPA (nM)</th>
<th>THP-1-O</th>
<th>THP-1-R</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>160</td>
<td>6.8 × 10⁴</td>
<td>1.0 × 10⁴</td>
</tr>
<tr>
<td>3</td>
<td>160</td>
<td>8.0 × 10⁴</td>
<td>1.4 × 10⁴</td>
</tr>
<tr>
<td>24</td>
<td>160</td>
<td>ND</td>
<td>6.5 × 10⁴</td>
</tr>
<tr>
<td>48</td>
<td>160</td>
<td>ND</td>
<td>2.0 × 10⁴</td>
</tr>
</tbody>
</table>

a ND, not done.
Phagocytosis of THP-1 Cells after Treatment with TPA.

Table 3
Surface markers of THP-1 cells after treatment with TPA

<table>
<thead>
<tr>
<th>% of rosette forming</th>
<th>THP-1-O cells with</th>
<th>THP-1-R cells with</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA (nM)</td>
<td>EA, EAC, EAC, EN</td>
<td>EA, EAC, EAC, EN</td>
</tr>
<tr>
<td>160</td>
<td>98.3</td>
<td>78.5</td>
</tr>
<tr>
<td>16</td>
<td>95.6</td>
<td>82.9</td>
</tr>
<tr>
<td>1.6</td>
<td>97.0</td>
<td>90.6</td>
</tr>
<tr>
<td>97.2</td>
<td>35.1</td>
<td>92.6</td>
</tr>
</tbody>
</table>

* EA, IgG antibody-coated SRBC; EAC, IgM antibody (human complement)-coated SRBC; EAC, IgM antibody (CS-deficient mouse complement)-coated SRBC; EN, neuraminidase-treated SRBC.

Table 4
Phagocytosis of THP-1 cells after treatment with TPA

<table>
<thead>
<tr>
<th>% of phagocytic THP-1-O cellswith</th>
<th>% of phagocytic THP-1-R cells with</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA (nM) Yeast SRBC IgG-coated SRBC</td>
<td>Yeast SRBC IgG-coated SRBC</td>
</tr>
<tr>
<td>160</td>
<td>74.1 34.5 64.2</td>
</tr>
<tr>
<td>16</td>
<td>68.7 22.3 38.4</td>
</tr>
<tr>
<td>1.6</td>
<td>37.2 6.9 14.1</td>
</tr>
<tr>
<td>0.16</td>
<td>16.2 0.6 1.4</td>
</tr>
<tr>
<td>0</td>
<td>9.8 0.5 1.2</td>
</tr>
</tbody>
</table>

Phagocytosis of yeasts, SRBC, and IgG-coated SRBC by untreated THP-1-O cells was seen in 9.8, 0.6, and 1.2% of the cells, respectively. When exposed to 160 nM TPA, phagocytosis increased to 74.1% for yeasts, 64.2% for IgG-coated SRBC, and 34.5% for SRBC (Table 4; Fig. 4, A and B). Phagocytosis of yeasts, SRBC, and IgG-coated SRBC by untreated THP-1-R cells was 21.2, 2.6, and 6.3%, respectively. Treatment of THP-1-R cells with 160 nM TPA induced maximum phagocytosis of the cells up to approximately the same level as that of THP-1-O cells. There was no noticeable effect of DMSO added to the suspension culture on the phagocytic activity of the cells. Five LCLs were treated with TPA and examined for phagocytic activity. IgG-coated SRBC were phagocytized by less than 1% of the cells and yeasts, and SRBC were phagocytized by none of the cells.

Carbon Dioxide Production of THP-1 Cells from [1-14C]- and [6-14C]-Glucose. One of the well-known metabolic changes of polymorphonuclear leukocytes and monocytes exposed to phagocytizable matter is glucose oxidation through the pentose monophosphate pathway (2, 7, 19). We examined whether the pathway could be activated when THP-1 cells were exposed to latex beads and TPA (Table 5). When treated with latex beads or TPA, THP-1-O cells showed an increase in 14CO2 production from [1-14C]glucose of 14 times and 34 times, respectively, as compared with untreated cells. On the other hand, in THP-1-R cells treated with latex beads or TPA, 14CO2 production from [1-14C]glucose was only about twice that of untreated cells. There were almost no effects of latex beads and TPA on 14CO2 production from [6-14C]glucose in the cells tested. Increased 14CO2 production from [1-14C]glucose in peripheral mononuclear cells was considered to be due to the presence of monocytes. Among 4 leukemic cell lines, HL-60 and THP-2-B
showed little increase in $^{14}\text{CO}_2$ production from [1-$^{14}$C]- and [6-$^{14}$C]glucose.

**DISCUSSION**

Human monocytic leukemia cells (THP-1-O) had spread onto the glass substrate as early as 1 hr after exposure to TPA. These adherent cells were still considered to belong to the mononuclear phagocyte series according to the following criteria: (a) positivity in staining with α-naphthyl butyrate esterase inhibitable by NaF; (b) the presence of lysozyme activity; (c) the presence of IgG Fc receptors on the cell surface; and (d) increased phagocytosis of yeasts and IgG-coated SRBC. The SRBC and yeasts had actually been ingested by the TPA-treated THP-1 cells because (a) SRBC simply adhering to the external membrane of the THP-1 had been lysed by treatment with Tris-NH₄Cl buffer and then made into coverslip smears, (b) SRBC and yeasts phagocytized were present in the phagosomes, and (c) THP-1 cells and ingested cells were capable of being focused simultaneously in a light microscope. The increase in the phagocytosis of yeasts and IgG-coated SRBC by TPA-treated cells may indicate that THP-1-O cells develop the characteristics of activated macrophages.

Differences in growth patterns and adhesiveness to the substrate between THP-1-O and THP-1-R cells were observed after exposure of the cells to TPA. It is generally accepted that long-term culturing gives rise to a gradual loss of the original nature of those cells which adapt to in vitro culture. HL-60 cells cultured in our laboratory remained positive for peroxidase and naphthol ASD chloroacetate esterase (16-18), but they lost the ability to differentiate and were able to grow in culture after treatment with TPA. Recently, it has been shown that there is a specific target in the cells for the phorbol ester tumor promoters (4). Changes of the binding capacity of the target and TPA during long-term culture may result in the loss of the capacity of THP-1-R cells to adhere to the substrate.

TEM findings for TPA-treated THP-1-O cells revealed the development of rough ER and Golgi apparatus, corresponding to the findings for TPA-treated HL-60 cells in which the differentiation of the cells into a more mature form was indicated (18). Abundant free ribosomes were also seen in the cytoplasm of TPA-treated THP-1 cells. In general, immature cells or tumor cells with rapid growth have abundant free ribosomes and poorly developed rough ER (6). In the case of TPA-treated HL-60 cells, the presence of many free ribosomes in the cytoplasm has not been reported. Whatever the meaning of the coexistence of the well-developed rough ER and abundant free ribosomes in TPA-treated THP-1 cells, the acquisition of vigorous phagocytosis by TPA-treated THP-1 cells may indicate that the cells have been changed to macrophage, a more mature form.

While both THP-1-O and THP-1-R cells bore IgG Fc receptors, phagocytosis of IgG-coated SRBC by these cells was seen in less than 6% of the cells. TPA treatment induced a prominent increase in phagocytosis, especially via IgG Fc receptors. This evidence may indicate that simple binding of IgG-coated SRBC via IgG Fc receptors on the cells is not a sufficient state for phagocytosis to occur in the case of THP-1 cells.

TPA-treated THP-1-O cells spread to the substrate and had an amoeboid shape, but in the same conditions, THP-1-R cells did not spread to the substrate and retained their round shape. Therefore, adhesion of the THP-1 cells on the substrate seems not to be necessary for the expression of phagocytic activities. After exposure to TPA, phagocytosis of uncoated SRBC increased 57 times in THP-1-O and 14 times in THP-1-R cells. The phagocytosis of uncoated SRBC and yeasts was independent of the presence of IgG Fc receptors. These results also suggest that Fc receptor-positive THP-1 cells may be converted to Fc receptor-positive macrophages with phagocytic capacity by treatment with TPA.

Not only polymorphonuclear leukocytes but also monocytes, when exposed to phagocytizable matter and chemicals which are thought to induce alteration of the plasma membranes, exhibit increased glucose oxidation through the pentose monophosphate pathway and the resultant production of CO₂ (2, 10). Latex beads belong to the former, and TPA is considered to belong to the latter substance (12). THP-1-O cells were shown to produce CO₂ through the pentose monophosphate shunt immediately after stimulation with latex beads and TPA, indicating that the cells retained some metabolic characteristics of the mononuclear phagocyte series. TPA-induced increase in glucose oxidation and CO₂ production was seen to precede these morphological changes in THP-1-O cells and may have some relation to the acquisition of the capacity for vigorous phagocytosis.

The present study has shown that THP-1-O cells can be converted with TPA treatment into activated macrophages which spread to the substrate and phagocytize yeasts and IgG-coated SRBC, as well as being morphologically changed. Metabolic changes associated with phagocytosis were also observed in THP-1-O cells. The cell line may offer a useful model for the study of the mechanism of maturation or differentiation from monocytes to macrophages and of the biochemical mechanism of phagocytosis.

**Table 5**

Comparison of the effect of latex ingestion and TPA treatment of leukemia cell lines and leukocytes from control subjects on $^{14}\text{CO}_2$ production from [1-$^{14}$C]glucose and [6-$^{14}$C]glucose

<table>
<thead>
<tr>
<th>Cells</th>
<th>Nothing</th>
<th>Latex</th>
<th>TPA</th>
<th>Nothing</th>
<th>Latex</th>
<th>TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1-O</td>
<td>0.34</td>
<td>4.77</td>
<td>11.5</td>
<td>0.07</td>
<td>0.19</td>
<td>0.21</td>
</tr>
<tr>
<td>THP-1-R</td>
<td>1.72</td>
<td>3.15</td>
<td>3.27</td>
<td>0.17</td>
<td>0.20</td>
<td>0.23</td>
</tr>
<tr>
<td>HL-60</td>
<td>1.01</td>
<td>1.07</td>
<td>1.75</td>
<td>0.24</td>
<td>0.26</td>
<td>0.35</td>
</tr>
<tr>
<td>THP-2-B</td>
<td>0.18</td>
<td>0.29</td>
<td>0.16</td>
<td>0.14</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>Control subjects</td>
<td>5.2 ± 0.7</td>
<td>23.1 ± 2.6</td>
<td>93.4 ± 8.5</td>
<td>0.32 ± 0.08</td>
<td>0.72 ± 0.17</td>
<td>2.3 ± 0.4</td>
</tr>
</tbody>
</table>

*These data were calculated as $^{14}\text{CO}_2$ production from $1 \times 10^6$ monocytes.*
REFERENCES

Fig. 1. A, untreated THP-1-O cell. Many microvilli and flaps were seen. × 11,000. B, untreated THP-1-O cell. Lobated nucleus and many vesicles in the cytoplasm were seen. × 9,500.

Fig. 2. A, TPA-treated THP-1-R cell (160 nM, 24 hr). Microvilli and flaps were decreased from the surface membrane, but the shape was still round. Bar, 1.0 μm. × 18,000. B, TPA-treated THP-1-R cell (160 nM, 24 hr). Well-developed rough ER and abundant free ribosomes were seen. Bar, 1.0 μm. × 13,500.
Fig. 3. A, TPA-treated THP-1-O cell (160 nM, 24 hr). The cell spread onto the substrate, becoming irregular and flattened in shape with almost complete loss of microvilli and flaps. Bar, 1.0 μm. × 9,200. B, TPA-treated THP-1-O cell (160 nM, 24 hr). Well-developed structures of rough ER, Golgi apparatus, and abundant free ribosomes in the cytoplasm were seen. Bar, 1.0 μm. × 12,500.

Fig. 4. A, phagocytosis of sensitized SRBC by THP-1-O cells treated with 160 nM TPA for 4 hr. Wright-Giemsa, × 4,500. B, phagocytosis of yeasts by THP-1-O cell treated with 160 nM TPA for 24 hr. Wright-Giemsa, × 4,500.
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