Effects of 12-O-Tetradecanoylphorbol-13-acetate on the Proliferation and Differentiation of Normal and Leukemic Myeloid Progenitor Cells

Keiya Ozawa, Yasuo Hashimoto, Yoshiko Kimura, Akio Urabe, and Fumimaro Takaku

ABSTRACT

The effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on myeloid colony formation were investigated by initial and delayed addition of TPA to the cultures. In the human placental conditioned medium-stimulated cultures, TPA inhibited normal myeloid colony formation without any change in colony morphology when added at the beginning of culture. However, macrophage-like transformation of myeloid colonies by TPA was clearly observed after the delayed addition of TPA. Colonies and clusters already formed at the time of TPA addition were exclusively neutrophilic. Two days after TPA addition, many colonies apparently contained macrophage-like cells. Within 4 days after TPA addition, almost all myeloid colonies transformed into the macrophage type. Parallel study of initial and delayed addition of TPA revealed that this macrophage-like transformation of neutrophilic colonies occurred at high concentrations of TPA that would fully inhibit colony formation if added initially. TPA caused similar effects on leukemic colony formation.

INTRODUCTION

The potent tumor promoter TPA is known to modulate the proliferation and differentiation of normal and abnormal hematopoietic cells (4, 6, 12, 20–22, 24, 25). It has been reported by several investigators that human myeloid leukemia cells are transformed to macrophage-like cells by treatment with TPA (5, 7, 13, 16, 18, 23) and that the percentage of monocyte-macrophage colonies and clusters is increased by the addition of TPA to normal bone marrow cultures in the presence of CSF (1, 14). However, it is difficult to determine the effects of TPA on colony morphology in detail, since myeloid colony formation is markedly inhibited by high concentrations of TPA (6, 22).

In the present study, the effects of TPA on myeloid colony formation were investigated by delayed addition as well as initial addition to the cultures. Five days after the initiation of culture, TPA was added to the cultures in which many colonies and clusters had already been formed. This delayed addition of TPA enabled us to examine the effects on colony morphology of high concentrations of TPA that would fully inhibit colony formation if added simultaneously with the initiation of culture.

Moreover, the effects of TPA on the proliferation and differentiation of leukemic progenitor cells were also investigated in the same way. There is relatively little information regarding the effects of TPA on leukemic colony formation (3, 6).

MATERIALS AND METHODS

Normal Myeloid Colony Formation. Bone marrow specimens were obtained from 5 normal human volunteers after written informed consent. Single-layer soft-agar cultures were performed according to the method of Robinson et al. (15) with minor modifications. Briefly, bone marrow buffy coat cells were cultured at a concentration of 2 x 10^6 cells/ml in 0.3% agar medium containing 20% FCS (Flow Laboratories, Inc., Rockville, Md.) and 10% HPCM which was prepared according to the method of Burgess et al. (2). In some experiments, 10% GCT-CM (Grand Island Biological Co., Grand Island, N. Y.) or 10% PHA-LCM was used as a source of CSF instead of HPCM. PHA-LCM was obtained from the supernatant of cultured leukocytes (1 x 10^6 cells/ml) incubated for 7 days in α-medium (Flow) with 10% FCS and 1% phytahemagglutinin (Wellcome HA-15). Since PHA-LCM also stimulates the formation of T-lymphocyte colonies, sheep erythrocyte rosette-negative mononuclear cells were isolated from bone marrow cells by the technique described by Minden et al. (11) with minor modifications and cultured at a concentration of 5 x 10^6 cells/ml. Culture plates were incubated for 9 days at 37° in a fully humidified atmosphere containing 5% CO_2 in air. Colonies containing 40 or more cells were counted under an inverted microscope.

Leukemic Colony Formation. Four cases of acute nonlymphocytic leukemia were studied. The patients' peripheral blood or bone marrow specimens contained a sufficient number of leukemic progenitor cells for analysis. The type of leukemia was classified according to the clinical data, and cytochemical findings were obtained by dual esterase staining (10). Peripheral blood or bone marrow specimens were obtained from the patients before treatment. Leukemic colonies were formed according to the method of Minden et al. (11) with minor modifications. In order to avoid the formation of T-lymphocyte colonies, sheep erythrocyte rosette-forming cells were removed before culture. About 2% or less of sheep erythrocyte rosette-positive cells were left after this rosetting technique. Cells from the sheep erythrocyte rosette-negative fraction were stored in 10% DMSO and 10% FCS at -80°C until use. These cells were thawed, washed, resuspended, and cultured at an appropriate concentration in 0.5% agar medium containing 20% FCS and 10% PHA-LCM. After 9–11 days of culture, colonies containing 20 or more cells were counted under an inverted microscope.

Effects of TPA on Colony Formation. Stock solutions of TPA and 4/2-phorbol (Sigma Chemical Co., St. Louis, Mo.) were prepared in DMSO at 1.8 x 10^{-3} μM and stored at -20°C. These were diluted to the required concentration in α-medium. The effect of TPA on colony formation was tested by initial or delayed addition of TPA to the cultures at concentrations ranging from 1.8 x 10^{-10} to 1.8 x 10^{-8} μM. Initial addition consisted of direct addition of TPA to the culture mixture at the beginning of culture. The colonies were counted, and their morphology was evaluated on Day 9 of culture for normal myeloid colony formation and on Day 9, 10, or 11 for leukemic colony formation. For delayed addition, TPA was added on Day 5 of culture for normal myeloid colony formation and on Day 5, 6, or 7 for leukemic colony formation. Four days after the addition of TPA,
the colonies were counted, and their morphology was evaluated.

Effects of ara-C on Colony Formation. As a control experiment, the effects of ara-C (Nippon Shinyaku Co., Ltd., Kyoto, Japan) were similarly tested by initial and delayed addition to normal bone marrow cultures at concentrations ranging from $1 \times 10^{-6}$ to $1 \times 10^{-5}$ M.

Morphological Analysis of Colonies. Permanent preparations of colonies were made (9) and stained by Wright-Giemsa and dual esterase staining according to the method of Li et al. (10). If most of the cells in a colony were chloroacetate esterase positive or butyrate esterase positive, the colony was considered a neutrophilic or monocyte-macrophage colony, respectively. If a colony consisted of both chloroacetate esterase-positive cells and butyrate esterase-positive cells, the colony was considered a neutrophile-macrophage mixed colony. Esterase reaction of leukemic colonies was often weak and sometimes deficient. The former were classified as similar to normal myeloid colonies, and the latter deficient ones seemed to be mostly blastic colonies on the basis of the results of Wright-Giemsa staining. In some experiments, eosinophils were identified by Biebrich-Scarlet staining (8).

RESULTS

Initial Addition of TPA to Normal Bone Marrow Cultures. Addition of TPA to normal bone marrow cultures at the beginning caused marked inhibition of myeloid colony formation (Table 1). TPA at concentrations of $1.6 \times 10^{-9}$ and $5 \times 10^{-9}$ M fully inhibited colony formation, and $1.6 \times 10^{-8}$ M TPA partially inhibited it. No inhibition was observed at concentrations of $1.6 \times 10^{-10}$ M and lower. In the bone marrow cultures stimulated by HPCM, almost all colonies on Day 9 of culture were neutrophilic as determined by dual esterase staining. No change in colony morphology resulted from the initial addition of TPA. However, the number of scattered macrophage-like cells as background cells increased in the cultures containing TPA at concentrations ranging from $1.6 \times 10^{-6}$ to $1.6 \times 10^{-5}$ M (data not shown).

In the cultures stimulated by PHA-LCM, a significant number of monocyte-macrophage and eosinophilic colonies as well as neutrophilic ones were formed (Table 4). The proportion of monocyte-macrophage colonies increased by the initial addition of $1.6 \times 10^{-9}$ M TPA, although the absolute number of these colonies did not significantly increase because of the reduction in total colony number. In the cultures stimulated by GCT-CM, the results were similar to those of HPCM-stimulated cultures, although a slight increase in the proportion of macro-

<table>
<thead>
<tr>
<th>TPA (M)</th>
<th>No. of colonies/2x10^5 cells</th>
<th>Colony morphology (%)</th>
<th>N^7</th>
<th>NM</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>193 ± 7</td>
<td>100</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1.6 x 10^-12</td>
<td>183 ± 15</td>
<td>100</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1.6 x 10^-11</td>
<td>187 ± 15</td>
<td>100</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1.6 x 10^-10</td>
<td>177 ± 21</td>
<td>100</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1.6 x 10^-9</td>
<td>35 ± 3</td>
<td>100</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>5 x 10^-9</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6 x 10^-8</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Bone marrow buffy coat cells were cultured in the presence of 10% HPCM. Colonies were counted on Day 9 of culture.
* Colony morphology was evaluated on Day 9 of culture by dual esterase staining.
* N, neutrophilic colonies; NM, neutrophil-macrophage mixed colonies; M, monocyte-macrophage colonies.
* Mean ± S.D.

Effects of delayed addition of TPA on normal myeloid colony formation (CSF, HPCM) and relationship between colony morphology and TPA concentration

<table>
<thead>
<tr>
<th>Additive</th>
<th>No. of colonies</th>
<th>Colony morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2x10^5 cells</td>
<td>N^7</td>
</tr>
<tr>
<td>Medium</td>
<td>189 ± 19</td>
<td>100</td>
</tr>
<tr>
<td>Phorbol (1.6 x 10^-8 M)</td>
<td>167 ± 14</td>
<td>100</td>
</tr>
<tr>
<td>TPA (1.6 x 10^-8 M)</td>
<td>155 ± 12</td>
<td>100</td>
</tr>
<tr>
<td>TPA (5 x 10^-9 M)</td>
<td>147 ± 9</td>
<td>11</td>
</tr>
<tr>
<td>TPA (1.6 x 10^-9 M)</td>
<td>170 ± 18</td>
<td>0</td>
</tr>
</tbody>
</table>

* TPA was added to the cultures on Day 5 of culture. Phorbol or α-medium was added instead of TPA as a control.
* Bone marrow buffy coat cells were cultured in the presence of 10% HPCM. Colonies were counted on Day 9 of culture.
* Colony morphology was evaluated on Day 9 of culture by dual esterase staining.
* N, neutrophilic colonies; NM, neutrophil-macrophage mixed colonies; M, monocyte-macrophage colonies.
* Mean ± S.D.

Delay addition of TPA to Normal Bone Marrow Cultures. Since a sufficient number of colonies and clusters had already been formed at the time of TPA addition, we were able to examine the effects of high concentrations of TPA on the differentiation of myeloid cells by the delayed addition method without reducing the number of colonies (Table 2). In the bone marrow cultures stimulated by HPCM, colonies and clusters on Day 5 of culture, when TPA was added, were exclusively neutrophilic (data not shown). Two days after the addition of TPA at a final concentration of $1.6 \times 10^{-9}$ M, many colonies contained macrophage-like cells (Table 3). Within 4 days after TPA addition, almost all of the myeloid colonies became macrophage type as determined by dual esterase staining. Strong α-naphthyl butyrate esterase activity was observed in these colonies. Delayed addition of phorbol or α-medium instead of TPA as a control did not cause any change in colony morphology (Table 2).

Similar experiments were performed using other sources of CSF (Table 4). In the cultures stimulated by PHA-LCM, almost all of the myeloid colonies became macrophage type after the delayed addition of $1.6 \times 10^{-8}$ M TPA. Interestingly, the increase in the proportion of macrophage-like colonies was observed even at a concentration of $1.6 \times 10^{-9}$ M. In the GCT-CM-stimulated cultures, the results were similar to those of HPCM-stimulated cultures, although a slight increase in the proportion of macro-

<table>
<thead>
<tr>
<th>Time after addition (days)</th>
<th>No. of colonies</th>
<th>Colony morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2x10^5 cells</td>
<td>N^7</td>
</tr>
<tr>
<td>Control</td>
<td>152 ± 14^d</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>145 ± 6</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>107 ± 15</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>110 ± 4</td>
<td>99</td>
</tr>
<tr>
<td>TPA^a</td>
<td>160 ± 2</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>171 ± 7</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>150 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>121 ± 17</td>
<td>0</td>
</tr>
</tbody>
</table>

* Bone marrow buffy coat cells were cultured in the presence of 10% HPCM. Colonies were counted on Day 9 of culture.
* N, neutrophilic colonies; NM, neutrophil-macrophage mixed colonies; M, monocyte-macrophage colonies.
* Mean ± S.D.
* TPA at a final concentration of $1.6 \times 10^{-8}$ M was added on Day 5 of culture.

Table 3

Effects of delayed addition of TPA on normal myeloid colony formation (CSF, HPCM) and relationship between colony morphology and the period after TPA addition

- 1.5925
Effects of initial and delayed addition of TPA on the formation of normal myeloid colonies stimulated by other sources of CSF

<table>
<thead>
<tr>
<th>Source of CSF</th>
<th>[TPA] (w)</th>
<th>No. of colonies/3 x 10^4 cells</th>
<th>Colony morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCT-CM</td>
<td>Initial addition</td>
<td>1.6 x 10^-9</td>
<td>N, NM, M, N(-), Est(-)</td>
</tr>
<tr>
<td></td>
<td>1.6 x 10^-8</td>
<td>61 ± 15</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Delayed addition</td>
<td>1.6 x 10^-9</td>
<td>N, NM, M, N(-), Est(-)</td>
</tr>
<tr>
<td></td>
<td>1.6 x 10^-8</td>
<td>117 ± 9</td>
<td>90</td>
</tr>
<tr>
<td>PHA-LCM</td>
<td>Initial addition</td>
<td>1.6 x 10^-9</td>
<td>N, NM, M, N(-), Est(-)</td>
</tr>
<tr>
<td></td>
<td>1.6 x 10^-8</td>
<td>113 ± 15</td>
<td>13</td>
</tr>
</tbody>
</table>

*TPA was added at the beginning of culture or on Day 5 of culture.

Bone marrow sheep erythrocyte rosette-negative mononuclear cells were cultured in the presence of 10% GCT-CM or 10% PHA-LCM. Colonies were counted on Day 9 of culture.

Colony morphology was evaluated on Day 9 of culture by dual esterase staining.

N, neutrophilic colonies; NM, neutrophil-macrophage mixed colonies; M, monocytic-macrophage colonies; N(-), neutrophil-esterase-negative mixed colonies; Est(-), esterase-negative colonies. Most of esterase-negative cells were eosinophils, as determined by Biebrich-Scarlet staining.

Effects of ara-C on Myeloid Colony Formation. Myeloid colony formation was fully inhibited by the initial addition of ara-C at concentrations ranging from 1 x 10^-7 to 1 x 10^-5 M and partially inhibited by 1 x 10^-6 M ara-C. Delayed addition of such concentrations of ara-C did not cause any change in colony type, unlike TPA. Almost all colonies remained neutrophilic (data not shown).

Effects of TPA on Leukemic Colony Formation. Like the effect on normal myeloid colony formation, initial addition of TPA caused marked inhibition of leukemic colony formation (Table 5). As clearly indicated in Case 4, monocytic colonies as well as neutrophilic colonies were also decreased by the initial addition of TPA. Delayed addition of TPA caused an increase in the proportion of macrophage-like colonies. Esterase-negative blasts decreased in number in Case 2.

### DISCUSSION

TPA is known to be a potent inducer of macrophage-like differentiation (5, 7, 13, 18), but, on the other hand, it inhibits

myeloid proliferation in vitro in high concentrations (17, 22). In our culture system, TPA markedly inhibited colony formation at concentrations of 5 x 10^-9 and 1.6 x 10^-8 M. These concentrations are relatively low, compared with other papers (1, 14). This discrepancy may be due to different batches of TPA or the use of DMSO rather than acetone as a solvent. It is reported that autoxidation of TPA is rapid in acetone solution (19). At lower concentrations, TPA did not cause any morphological change in colonies induced with HPCM, which is a potent stimulator of neutrophilic colony growth.

Several investigators reported that, unlike our observation, an increase in macrophage colony formation was caused by even

...
Effects of TPA on Myeloid Cell Differentiation

The initial addition of TPA to normal bone marrow cultures (1, 14). This may be due to different culture conditions, because we also observed a similar phenomenon, an increase in the proportion of macrophage colonies by the initial addition of TPA, using PHA-LCM as a source of CSF instead of HPCM. A low concentration of TPA such as 1.6 x 10-8 M may be enough to induce macrophage colonies, because PHA-LCM itself contains a potent stimulator of macrophage colony growth. However, it is not clear whether TPA added together with CSF selectively stimulated the growth of progenitor cells of monocyte-macrophage lineage and inhibited the growth of progenitors of neutrophilic lineage, or whether TPA unselectively modified the differentiation of progenitor cells stimulated by CSF.

Therefore, in the present study, the effect of TPA on colony morphology was investigated by the delayed addition method. Almost all colonies in the cultures treated with 1.6 x 10-8 M TPA on Day 5 of culture showed dramatic changes in colony morphology and became macrophage type in contrast to unchanged neutrophilic colonies in the control cultures. Colonies and clusters already formed at the time of TPA addition were exclusively neutrophilic. In the HPCM-stimulated cultures, a macrophage-like change in colony type occurred at high concentrations of TPA (5 x 10-8 and 1.6 x 10-8 M) that would fully inhibit colony formation if added simultaneously with culture initiation. A similar change in colony type occurred at a lower concentration of TPA (1.6 x 10-8 M) in the PHA-LCM-stimulated cultures. A time-course study showed that a significant number of macrophage-like cells were present in the colonies even 2 days after TPA addition. This is too late for growth of new colonies. The proportion of macrophage-like cells in a single colony increased thereafter, and almost all colonies became the macrophage type within 4 days after TPA addition. These findings suggest that such effects of TPA are not due to the selective stimulation of a subpopulation of myeloid progenitors already committed to macrophage differentiation, but to the transformation of neutrophilic colonies already formed. The concentrations of TPA necessary for the macrophage-like transformation of neutrophilic colonies were almost the same as those required for the transformation of promyelocytic leukemia cell line (HL-60) cells (19). Thus, delayed addition seems to be a valuable method for investigating the effect of TPA on myeloid cell differentiation.

The delayed addition of ara-C at concentrations that would fully inhibit colony growth upon initial addition did not cause any change in colony type. Therefore, macrophage-like transformation of neutrophilic colonies by TPA is not due merely to the arrest of cell proliferation but seems to be a specific result of the effect of TPA on myeloid differentiation.

The effects of TPA on leukemic colony formation were also investigated by the same method. The results were almost the same as those with normal bone marrow cultures and compatible with the results reported for HL-60 cells (16, 18, 23) and freshly obtained myeloid leukemia cells cultured in suspension (5, 7, 13).

ACKNOWLEDGMENTS

The authors would like to thank Yohko Shiomura, Ikuko Kawamura, Junko Yumoto, and Himiko Suzuki for their excellent technical assistance.

REFERENCES

Fig. 1. Normal myeloid colonies on Day 9 of culture (CSF, HPCM) photographed in situ in agar culture under an inverted microscope (A and B) and stained with Wright-Giemsa (C and D). A and C, a macrophage-like colony observed in the culture to which $1.8 \times 10^{-6}$ M TPA was added on Day 5 of culture; B and D, a neutrophilic colony observed in the culture to which α-medium instead of TPA was added as a control.
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