Enhancement of Growth of a Mouse Macrophage Cell Line by a Tumor Promoter and Granulocyte-Macrophage Colony-stimulating Factor

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ABSTRACT

The macrophage-like cell line Mm-1 consists of spontaneously differentiated cells of a clonal line of mouse myeloid leukemia cells (M1). Mm-1 cells were seeded in soft agar on a harder agar base in the presence or absence of ascitic fluid from rats bearing hepatoma AH-130. Although a few colonies developed in the absence of ascitic fluid, the number of colonies was significantly increased by addition of the ascitic fluid. Further addition of 12-O-tetradecanoylphorbol-13-acetate (TPA) synergistically enhanced formation of colonies of Mm-1 cells in the presence of the ascitic fluid, although TPA alone had no effect. In the presence of 2.5 or 5% ascitic fluid, TPA (3.2 x 10^{-7} M) caused a 1.5- to 3-fold increase in the number of colonies over the number without TPA.

A positive correlation was found between the tumor-promoting activities of several plant diterpenes and their enhancing effects on growth of Mm-1 cells in semisolid agar in the presence of ascitic fluid from rats bearing hepatoma AH-130. Ascitic fluid contains granulocyte-macrophage colony-stimulating factor (GM-CSF). Conditioned media containing GM-CSF from embryo cells, peritoneal macrophages, and Yoshida sarcoma cells, or highly purified GM-CSF's from human urine and mouse L-cells also acted synergistically with TPA in increasing the number of colonies of Mm-1 cells. On the other hand, neither epidermal growth factor nor fibroblast growth factor with or without TPA stimulated colony formation of Mm-1 cells. These results indicate that colony formation of Mm-1 cells is stimulated as a result of the specific interaction of the tumor promoter with growth factor(s), possibly GM-CSF.

INTRODUCTION

The phorbol ester TPA and related plant diterpenes are potent tumor-promoting agents in a 2-stage mouse skin carcinogenesis system (2, 10, 40). Recent studies have shown that TPA and related phorbol esters have a variety of effects in cell culture, including stimulation of DNA synthesis (7, 45) and induction of ornithine decarboxylase (45), induction of plasmid activation (42), changes in membrane properties (1, 35), phospholipid synthesis (1, 29), and in vitro transformation of fibroblast cultures (25). Furthermore, it has been shown that these tumor-promoting agents either inhibit (5, 6, 18, 19, 28, 30, 44) or induce (14, 15, 24, 31, 32) various types of cell differentiation of cultured cells. These findings suggest that these tumor-promoting agents affect the regulation of cell growth and differentiation.

The mouse myeloid leukemia cell line M1 was established in culture from a spontaneous myeloid leukemia of an SL mouse (16). M1 cells differentiate into forms that resemble mature macrophages and granulocytes in the presence of proteinaceous inducers in conditioned media from various cells, various body fluids (11-13, 33), or chemicals, such as glucocorticoid hormones, lipopolysaccharides, poly (adenosine diphosphoribose), polyinosine, prostaglandins, and chloroquine (11-13, 33). Spontaneously differentiated macrophage-like cells (Mm-1) were established from a clonal line of M1 cells (23). These macrophage-like cells resemble normal macrophages in morphology, adherence to the dish, high phagocytic activity, and synthesis of lysozyme (20, 23). Mice inoculated with 10^5 M1 cells all died of leukemia, whereas those inoculated with 10^6 Mm-1 cells did not (23). On the other hand, the plating efficiency of M1 cells in soft agar medium was much higher than that of Mm-1 cells, although the plating efficiency of the latter was enhanced by the addition of certain conditioned media of cells (23).

We are now attempting to analyze the mechanisms of action of tumor promoters on regulation of growth and differentiation of myeloid leukemia M1 cell lines. We previously found that the tumor promoter TPA and its analogs inhibited induction of differentiation of M1 cells by various inducers (20). In the present experiments, we report that these tumor promoters markedly enhanced formation of colonies of Mm-1 cells.

MATERIALS AND METHODS

Chemicals. TPA, phorbol-12, 13-didecanoate, 4a-phorbol-12, 13-didecanoate, and phorbol were obtained from Consolidated Midland Corp., Brewster, N. Y. These compounds were dissolved in acetone. The final concentration of acetone in semisolid agar, 0.04%, alone had no detectable effect on cell growth in semisolid agar.

Preparations of Various Growth Factors. Ascitic fluid was obtained from rats (Donryu) that had been given i.p. injections of ascites hepatoma AH-130 (12). Conditioned media were prepared from primary cultures of Syrian hamster embryo cells and peritoneal macrophages of mice by the method of Ichikawa (16). Conditioned medium of Yoshida sarcoma cells (YSSF-212T) was obtained by culturing the cells as a suspension in the serum-free medium described by Ohno et al. (27). Highly purified preparations of GM-CSF from human urine and conditioned medium of mouse L-cells (39) were gifts from Drs. Mikio Shikita and Kazuko Tsuneoka (National Institute of Radiological Sciences, Chiba, Japan). Purified preparations of epidermal growth factor (mouse submaxillary glands) and fibroblast growth factor (bovine pituitary glands) were obtained from Collaborative Research, Inc., Waltham, Mass.

Cell Culture. Mm-1 cells grew as monolayers in liquid culture (23). Mm-1 cells were maintained by subculture in 6-cm Falcon
Tumor Promoter and Growth of Macrophage-like Cells

**Table 1** Effect of TPA on growth of Mm-i cells in liquid culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (µM)</th>
<th>No. of viable cells (x 10^-6)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>1.45</td>
<td>100</td>
</tr>
<tr>
<td>TPA</td>
<td>3.2 x 10^-10</td>
<td>1.42</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>3.2 x 10^-9</td>
<td>1.24</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>3.2 x 10^-8</td>
<td>1.17</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>3.2 x 10^-7</td>
<td>0.88</td>
<td>46</td>
</tr>
</tbody>
</table>

Data are means for duplicate dishes.

plastic dishes in Eagle's minimum essential medium with twice the normal concentrations of amino acids and vitamins, and supplemented with 10% heat-inactivated calf serum and 160 µg of kanamycin sulfate per ml. They were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air.

**Colony Formation of Mm-i Cells.** Eagle's minimum essential medium with double concentrations of amino acids and vitamins was supplemented with 20% heat-inactivated calf serum. Mm-i cells were seeded at 1000 cells/0.5-cm plastic dish in soft agar (0.33%) in the presence or absence of tumor promoter on a harder agar base (0.5%), which contained ascitic fluid, conditioned media, or GM-CSF. Colonies of more than about 1000 cells were scored microscopically on Days 12 to 15 of culture.

**Calculation of the Number of Cells per Colony.** After addition of 5 ml of phosphate-buffered saline (138 mM sodium chloride, 2.7 mM potassium chloride, 8 mM dibasic sodium phosphate, and 1.5 mM monobasic potassium phosphate, pH 7.4), each dish was shaken for a few min so that the 0.33% agar layer became freed from the 0.5% agar layer, which remained attached to the bottom of the dish. The upper layer was then transferred to a glass tube and adjusted to 8 ml with phosphate-buffered saline. After vigorous pipetting, the number of cells in the homogenate solution was counted in a hemocytometer, and the number of cells per colony was calculated. The efficiency of recovery of intact cells from the agar was more than 99%.

**RESULTS**

**Effect of TPA on Growth of Mm-i Cells in Liquid Culture.** Mm-i cells grew as monolayers in liquid culture as described previously (23). We first examined the effect of TPA on growth of Mm-i cells in liquid culture, since TPA is known to be a potent mitogen in several culture systems (7, 8). Under the conditions used, TPA did not stimulate growth of Mm-i cells but in fact inhibited it significantly at concentrations of 3.2 x 10^-10 to 3.2 x 10^-7 M (Table 1). A similar inhibitory effect of TPA was observed when Mm-i cells were grown with suboptimal concentrations of calf serum (0.25 to 2%).

**Enhancement of Growth of Mm-i Cells by TPA in Semisolid Agar in the Presence of Ascitic Fluid.** We next examined the effect of TPA on the colony formation of Mm-i cells in semisolid agar (Fig. 1; Chart 1). Mm-i cells were seeded at 1000 cells/dish in soft agar on a harder agar base in the presence or absence of ascitic fluid of tumor-bearing rat, which is known to contain GM-CSF (38). Few colonies developed in the absence of ascitic fluid, but in the presence of ascitic fluid the colony number increased and the increase was dose dependent. TPA had no effect on colony formation of Mm-i cells in the absence of ascitic fluid. However, in the presence of ascitic fluid, the number of colonies increased progressively with increase in TPA concentration over the range of 3.2 x 10^-10 to 3.2 x 10^-7 M. In the presence of 2.5 or 5% ascitic fluid, TPA caused a 1.5- to 3-fold increase in the number of colonies over the number without TPA. Thus, there is a clear synergistic interaction between ascitic fluid and TPA in stimulating colony formation of Mm-i cells. We observed enhancement of colony formation of Mm-i cells by TPA on seeding 200 to 1000 cells/dish. The final concentration of the solvent, acetone, in the soft agar medium had no effect on colony formation of Mm-i cells in the presence or absence of the ascitic fluid.

In addition to examining the effect of TPA on the plating efficiency of Mm-i cells, we also examined whether TPA af-
Effects of TPA on colony formation of Mm-1 cells. As shown in Chart 2, the number of colonies of Mm-1 cells and the cell number per colony were enhanced 3.2- and 2-fold, respectively, by $3.2 \times 10^{-7}$ M TPA with 5% ascitic fluid.

**Effect of Various Plant Diterpenes on Colony Formation of Mm-1 Cells.** Next we examined whether the ability of plant diterpenes to increase the number of colonies of Mm-1 cells in the presence of ascitic fluid was correlated with their tumor-promoting activity in the mouse skin 2-stage carcinogenesis system (2, 10, 40) (Table 2). Phorbol-12,13-didecanoate, which has the same phorbol nucleus as does TPA and is a potent tumor-promoting agent, also enhanced colony formation by Mm-1 cells. However, the isomer $4\alpha$-phorbol-12,13-didecanoate and phorbol itself, which are not promoters in the mouse skin system (2, 10, 40), did not enhance colony formation. This stereospecificity suggests that tumor-promoting plant diterpenes may enhance colony formation by interacting with a receptor macromolecule in the cells.

**Effects of Various Growth Factors on Colony Formation of Mm-1 Cells in the Presence or Absence of TPA.** As mentioned above, tumor-promoting plant diterpenes acted synergistically with ascitic fluid in increasing colony formation of Mm-1 cells. We next examined whether TPA had a similar effect with GM-CSF from other sources than ascitic fluid (Table 3). It is known that conditioned medium of mouse embryo cells stimulates colony formation of Mm-1 cells (23), and we showed that conditioned medium from hamster embryo cells also enhanced growth of Mm-1 cells in soft agar. We also found that conditioned medium of hamster embryo cells acted synergistically with TPA in enhancing colony formation of Mm-1 cells. Furthermore, conditioned medium of macrophages or Yoshida sarcoma cells (27) stimulated colony formation of Mm-1 cells, and TPA also enhanced the action of these conditioned media. Since these conditioned media and ascitic fluid were known to contain GM-CSF (4, 27, 37, 38), we examined the effect of highly purified cerebrospinal fluid on colony formation of Mm-1 cells. GM-CSF from human urine or L-cells stimulated colony formation of Mm-1 cells, and TPA enhanced growth of Mm-1 cells in soft agar in the presence of either human GM-CSF or mouse GM-CSF. On the other hand, growth of colonies of Mm-1 cells was not affected by epidermal growth factor or fibroblast growth factor, and the latter in fact slightly inhibited colony formation. Furthermore, TPA did not enhance formation of colonies of Mm-1 cells in the presence of epidermal growth factor or fibroblast growth factor. A high concentration (100 ng/ml) of epidermal growth factor or fibroblast growth factor also did not enhance the growth of Mm-1 cells in soft agar.

**DISCUSSION**

The present experiments indicate that TPA and related plant diterpenes that are known to be tumor promoters on mouse skin (2, 40) acted synergistically with GM-CSF from various sources, including ascitic fluid in stimulating colony formation of Mm-1 cells and growth of these colonies. Conversely, derivatives of TPA that were not tumor promoters (2, 10, 40) did not enhance formation of colonies of Mm-1 cells in the presence of ascitic fluid. In liquid culture, growth of Mm-1 cells was not stimulated by 1 to 10% ascitic fluid and TPA (data not shown). Conceivably, this difference may reflect a difference in growth of the cells; in liquid medium containing calf serum, Mm-1 cells grow as monolayers adhering to the substratum, whereas in semisolid agar medium in the presence of calf...
serum and other growth factors, such as ascitic fluid, conditioned media from embryo cells, macrophages, or Yoshida sarcoma cells, growth is not dependent on anchorage of the cells.

The ability of cells to grow in semisolid agar culture is known to be closely correlated with their tumorigenicity in vivo (34), and this correlation is apparent with differentiated Mm-1 cells and the original leukemic Mi cells. M1 cells are highly tumorigenic in syngeneic SL mice and form colonies with high efficiency in soft agar medium in the presence of 20% calf serum alone, whereas Mm-1 cells have very low tumorigenicity and do not readily form colonies in soft agar with 20% calf serum. Formation of colonies of Mm-1 cells was markedly enhanced by ascitic fluid and TPA. Therefore, TPA might act synergistically with growth factor(s) in ascitic fluid in enhancing colony formation of Mm-1 cells. Recently, phorbol esters were found to enhance growth of transformed rat embryo cells in semisolid agar (25). This growth-enhancing interaction of tumor-promoting agents with growth factors observed in semisolid agar may be involved in the mechanism of tumor promotion in carcinogenesis.

Recent works showed that TPA acts synergistically with growth factors, such as epidermal growth factor, insulin, and fibroblast-derived growth factor, in stimulating DNA synthesis in quiescent cultures of mouse and human fibroblasts (3, 7) and that TPA enhances the cell proliferation of BALB/c-3T3 cells in the presence of hormonal growth factors (9). Other synergistic responses reported between TPA and other materials have been in stimulating plasminogen activator (21, 41, 43) and enhancing survival of ganglion cells (17). Furthermore, TPA specifically alters the affinity of membrane receptors to epidermal growth factor of mouse and human cells (3, 22, 36). Our results indicate that TPA acts synergistically with GM-CSF from various sources in enhancing colony formation of Mm-1 cells. Other growth factors, such as epidermal growth factor and fibroblast growth factor, did not significantly increase colony formation of Mm-1 cells even in the presence of TPA. Thus, the specific action of TPA with a certain growth factor(s), possibly GM-CSF, stimulates colony formation of the cells. Interaction of TPA with the growth factor may depend on the cell type.

We are now examining whether TPA and related plant diterpenes enhance colony formation of the original leukemic M1 cells and normal bone marrow stem cells in the presence of the growth factors that stimulate colony formation of Mm-1 cells. We found previously that various factors induced M1 cells to differentiate into mature macrophages and granulocytes and that this induced differentiation was reversibly inhibited by tumor-promoting plant diterpenes in liquid culture (18). The present work showed that tumor promoters and GM-CSF from various sources acted synergistically in enhancing colony formation of Mm-1 cells, established from spontaneously differentiated macrophage-like M1 cells. Therefore, our experimental system should be useful for studying the mechanisms by which TPA and related plant diterpenes initiate the chain of events causing alteration in growth and differentiation of myeloid leukemic cells.

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