ABSTRACT

From a spontaneous AKR/Ms thymic leukemia symbiotically cultured with thymic epithelial reticular cells, a tumor promoter-dependent cell line A65T was established by passaging the cells in medium containing 12-O-tetradecanoylphorbol-13-acetate (10 ng/ml). The in vitro growth of A65T was strictly dependent on the presence of active tumor promoters. Their action was reversible, since withdrawal of 12-O-tetradecanoylphorbol-13-acetate resulted in rapid decrease in viability of the cells. Three classes of chemically unrelated compounds sharing tumor-promoting activity in mouse skin could support the in vitro growth of A65T: plant diterpene esters; indole alkaloids; and polyacetales. Their growth effect on A65T cells quantitatively correlated well with the tumor-promoting activity in mouse skin. However, other growth stimulators of epithelial cells such as cholera toxin and epidermal growth factor failed to support the growth of A65T. It is suggested that lymphokines such as interleukin-2 and interleukin-3 were not responsible for 12-O-tetradecanoylphorbol-13-acetate-stimulated growth of A65T because concanavalin A-stimulated spleen cell-conditioned medium containing both interleukin-2 and interleukin-3 activities as well as WEHI-3 cell culture supernatant containing potent interleukin-3 activity did not stimulate the proliferation of A65T cells. Furthermore, 12-O-tetradecanoylphorbol-13-acetate did not induce production of any significant amount of either activity in A65T cells. This cell line is useful for the screening of tumor promoters in environments although, so far, all the compounds capable of stimulating A65T growth have been limited to those competing with phorbol esters for the cellular receptor. Also, the cell line provides a potential model for analyzing growth requirements of developing mouse thymic leukemias.

INTRODUCTION

Thymic leukemogenesis in the mouse has been assumed to be a sequential process corresponding to the steps of normal T-cell lineage development. Haran-Ghera et al. (17–19) showed the presence of dormant preleukemia cells among bone marrow prothymocytes long before development of overt leukemias. We have postulated that the thymus provides permissive microenvironments for migrating prothymocyte transformants to progress to an autonomously growing T-cell neoplasia (22, 23). This hypothesis is based on consistent isolation of symbiotic complexes of leukemia cells and TERs3 from primary leukemia thymuses (22). In vitro growth and survival of the leukemia cells from such complexes are dependent on close contact with TERs (22) in a unique form of cell interaction known as pseudoeuphorb, which has been defined by us previously (22, 24, 40). Furthermore, tumor-promoting agents including TPA (23) and teleocidin (30) are found to stimulate markedly the in vitro growth of such microenvironment-dependent leukemias without growth supporting TERs and also to inhibit complex formation (23, 30, 40). Thus, the in vitro effects of tumor promoters on microenvironment-dependent leukemias may well simulate the progression of leukemias supposedly occurring in late preleukemic thymuses. In order to analyze the action of tumor promoters, it is desirable to establish a continuous cell line, the growth of which is stably dependent on tumor promoters.

In this paper, we will report the establishment and properties of a TPA-dependent leukemia cell line derived from one of the microenvironment-dependent leukemias. The growth of the A65T cells was a sensitive measure for quantitative screening of tumor-promoting agents. The growth-stimulating activity of tumor promoters on A65T cells was not due to lymphokines IL-2 or IL-3.

MATERIALS AND METHODS

Chemicals. TPA, 4α-phorbol, phorbol-12,13-diacetate, phorbol-12,20-diacetate, phorbol-12,13-dibenzzoate, phorbol-12,13-dibutyrate, and 4α-phorbol-12,13-didecanoate were purchased from CMC Cancer Research Chemicals, Inc., Brewster, N. Y., and mezerein was from LC Service Corp., Wobum, Mass. Telodocin (51), lyngbyatoxin A (3, 10), and aplysia toxin and debronia lysosiecin (11, 31) were prepared as described in each reference. They were dissolved in ethanol and stored in small aliquots at −20°. Lyophilized cholera toxin was obtained from Sigma Chemical Co., St. Louis, Mo. Con A was a product of Miles-Yeda, Rehovot, Israel. Culture grade EGF purified from mouse submaxillary gland was obtained from Collaborative Research Inc., Waltham, Mass. (Lot 83-111).

Tissue Culture. The medium was the high-glucose version of Dulbecco’s modified Eagle’s minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.), fortified by amino acids and vitamins as described previously (22) and supplemented with 10% unheated FCS. The method of symbiotic culture of mouse leukemias was described in detail previously (22). The A65T cell line was isolated from a syngeneic AKR-L65 leukemia (30) by passage 10³ leukemia cells every 4 to 5 days in medium containing TPA (10 ng/ml). Dependence on TPA was examined every week and, when the stimulation index was less than 5, the cells were discarded and replaced by a frozen stock. The A65TND was a variant cell line of A65T which spontaneously lost dependence on TPA during long-term passage.

The C/T was a cytotoxic T-cell line established by E. Nakayama from a spleen of a BALB/c x C57BL/6 F1 mouse immunized against a BALB/c radiation leukemia virus-induced leukemia. It was maintained in medium containing 20% Con A-stimulated spleen cell CM, prepared by culturing rat spleen cells for 20 hr in 40 ml Dulbecco’s modified Eagle’s minimum essential medium with Con A (2 μg/ml), 1% FCS, and 50 μM 2-mercaptopropionylglycine (22). In order to analyze the action of tumor promoters, it is desirable to establish a continuous cell line, the growth of which is stably dependent on tumor promoters.

In this paper, we will report the establishment and properties of a TPA-dependent leukemia cell line derived from one of the microenvironment-dependent leukemias. The growth of the A65T cells was a sensitive measure for quantitative screening of tumor-promoting agents. The growth-stimulating activity of tumor promoters on A65T cells was not due to lymphokines IL-2 or IL-3.

1 Supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture, Japan.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: TER, thymic epithelial reticular cell; TPA, 12-O-tetradecanoylphorbol-13-acetate; IL-2, interleukin-2; IL-3, interleukin-3; Con A, concanavalin A; EGF, epidermal growth factor; FCS, fetal calf serum; CM, conditioned medium; dThd, thymidine; CSA, colony-stimulating activity.

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The action of TPA was reversible since the viability of the cells as low as 0.1 ng/ml, and 50% effective dose was 0.068 ng/ml. Conversely (Chart 1). Significant growth stimulation was observed at the presence of active tumor promoters. In the presence of they were replaced by the frozen stock. Otherwise noted, whenever the cells lost dependence on TPA, they were frozen in liquid nitrogen. Passage of A65T cells for an additional 2 to 6 months resulted in occasional appearance of independence (23, 30). The leukemia cells from TPA responders could be increasing concentrations of TPA, A65T cells proliferated vigorously (30) in medium with TPA (10 ng/ml). It was maintained stably as supporting TERs. The A65T cell line was obtained by serially passaging the leukemia cells of the symbiotic AKR-65 cell line (30) in medium with TPA (10 ng/ml). It was maintained stably as a TPA-dependent cell line for over 6 months and thereafter stored frozen in liquid nitrogen. Passage of A65T cells for an additional 2 to 6 months resulted in occasional appearance of independently growing variants, one of which was A65T cell line. Unless otherwise noted, whenever the cells lost dependence on TPA, they were replaced by the frozen stock.

The survival and growth of A65T cells were strictly dependent on the presence of active tumor promoters. In the presence of increasing concentrations of TPA, A65T cells proliferated vigorously (Chart 1). Significant growth stimulation was observed at as low as 0.1 ng/ml, and 50% effective dose was 0.068 ng/ml. The action of TPA was reversible since the viability of the cells promptly decreased after removal of TPA (Chart 2).

Similar TPA-dependent cell lines could be obtained from various lymphokines and culture supernatants on A65T, C/T, and FDC-P2 cells were evaluated similarly. Final concentrations of growth stimulators were given in tables.

**RESULTS**

**Establishment of TPA-dependent Cell Line.** Approximately 70% of cell lines obtained from symbiotically cultured AKR/Ms-thymic leukemias were responders to growth stimulation by TPA (23, 30). The leukemia cells from TPA responders could be maintained indefinitely in TPA-containing medium without growth supporting TERs. The A65T cell line was obtained by serially passaging the leukemia cells of the symbiotic AKR-65 cell line (30) in medium with TPA (10 ng/ml). It was maintained stably as a TPA-dependent cell line for over 6 months and thereafter stored frozen in liquid nitrogen. Passage of A65T cells for an additional 2 to 6 months resulted in occasional appearance of independently growing variants, one of which was A65T cell line. Unless otherwise noted, whenever the cells lost dependence on TPA, they were replaced by the frozen stock.

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Tumor Promoter-dependent Leukemia Cell Line

**Effect of Lymphokines.** In order to determine whether the growth stimulation by TPA and other tumor promoters was mediated by lymphokines, the effect of exogenous IL-2 and IL-3 typically all TPA-responder symbiotic leukemias. However, out of 12 cell lines passaged in TPA-containing medium, 7 lost dependence on TPA spontaneously within 4 weeks, and 4 more lost dependence after an additional 4 weeks. Therefore, stability of dependence of A65T seemed rather exceptional.

**Effect of Various Tumor Promoters.** A65T cells were stimulated to grow not only by TPA but more widely by various chemicals with tumor-promoting activity in mouse skin (Table 1). We examined 3 classes of structurally different chemicals with tumor-promoting activity: plant diterpene esters, indole alkaloids, and polyacetates. The diterpene esters included tigliane, daphnane, and ingenane derivatives (21). Among tigliane derivatives, active tumor promoters such as TPA, phorbol-12,13-dibutyrate, and phorbol-12,13-dibenozoate (46) stimulated the growth of A65T, whereas inactive derivatives such as 4α-phorbol, phorbol-13,20-diacetate, and 4α-phorbol-12,13-didecanoate did not. A marginally active tumor promoter phorbol-12,13-diacetate could stimulate the A65T growth, but it was approximately 400 times less efficient than TPA. Mezerein (32), a daphnane derivative from Daphne mezereum L., had moderate activity to stimulate the proliferation of A65T cells. Among ingenane derivatives, we examined ingenol only (1), which was inactive both in tumor promotion and A65T growth stimulation. Indole alkaloid tumor promoters, teleocidin from Streptomyces mediocidicus (51) and lyngbyatoxin A from the blue-green alga Lyngbya majuscula (3), stimulated A65T cells to grow. The activity of teleocidin was comparable to that of TPA but one of its stereoisomers, lyngbyatoxin A, was much weaker. Polyacetate tumor promoters aplysiasatoxin and debromopolysiasatoxin, which also were isolated from Lyngbya majuscula, could induce proliferation of A65T cells, but aplysiasatoxin was much stronger in this activity. Aplysiasatoxin is reported to have stronger tumor-promoting activity in mouse skin than debromopolysiasatoxin (11). These results indicated general quantitative correlation between tumor-promoting activity in mouse skin and growth-stimulatory activity in A65T. However, cholera toxin, which had been demonstrated to induce proliferation of mouse epidermal cells both in vivo (33) and in vitro (15), failed to stimulate the growth of A65T cells at 10^6 to 10^8 ng/ml. Also, EGF was totally without effect on A65T at 10^8 to 10^11 ng/ml.

**Effect of Lymphokines.** In order to determine whether the growth stimulation by TPA and other tumor promoters was mediated by lymphokines, the effect of exogenous IL-2 and IL-3...
Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Diterpene esters</th>
<th>Tigliane derivatives</th>
<th>Phorbol-12,13-dibutyrate</th>
<th>Phorbol-12,13-dibenzoate</th>
<th>Phorbol-12,13-diacetate</th>
<th>4α-Phorbol-12,13-didecanoate</th>
<th>Daphnane derivative</th>
<th>Ingenane derivative</th>
<th>Mezerein</th>
<th>Indole alkaloids</th>
<th>Polypeptide toxins</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt; (ng/ml)</td>
<td>0.068</td>
<td>1.03</td>
<td>1.08</td>
<td>28.0</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0.27</td>
<td>&gt;1000</td>
<td>0.048</td>
<td>2.6</td>
<td>0.23</td>
<td>3.8</td>
</tr>
<tr>
<td>Growth-stimulatory</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>(21)</td>
<td>(21)</td>
<td>+</td>
<td>(21)</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>(1)</td>
</tr>
<tr>
<td>Promoter activity</td>
<td>0.016</td>
<td>0.7</td>
<td>0.24</td>
<td>17.6</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0.03</td>
<td>&gt;1000</td>
<td>0.008</td>
<td>0.011</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>Irritation of mouse ear skin ID&lt;sub&gt;50&lt;/sub&gt; (nmol/ear)</td>
<td>0.068</td>
<td>0.7</td>
<td>0.24</td>
<td>17.6</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0.03</td>
<td>&gt;1000</td>
<td>0.008</td>
<td>0.011</td>
<td>0.005</td>
<td>0.005</td>
</tr>
</tbody>
</table>

"Numbers in parentheses, references.

Table 2

Failure of lymphokines to stimulate the growth of TPA-dependent A65T leukemia cells

<table>
<thead>
<tr>
<th>Growth stimulator</th>
<th>A65T</th>
<th>C/T</th>
<th>FDC-P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium control</td>
<td>1,420 ± 115</td>
<td>808 ± 35</td>
<td>241 ± 193</td>
</tr>
<tr>
<td>TPA (10 ng/ml)</td>
<td>8,720 ± 653</td>
<td>460 ± 125</td>
<td>400 ± 330</td>
</tr>
<tr>
<td>Con A CM (20%, v/v)</td>
<td>456 ± 79</td>
<td>356 ± 79</td>
<td>236 ± 32</td>
</tr>
<tr>
<td>WEHI-3 CM (20%, v/v)</td>
<td>1,350 ± 460</td>
<td>139 ± 43</td>
<td>397 ± 162</td>
</tr>
</tbody>
</table>

Table 3

Absence of lymphokine activities in the supernatant of A65T and its independently growing variant A65IND cultured with or without TPA

<table>
<thead>
<tr>
<th>Growth stimulator</th>
<th>C/T</th>
<th>FDC-P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium control</td>
<td>53 ± 12</td>
<td>99 ± 64</td>
</tr>
<tr>
<td>A65T CM</td>
<td>144 ± 107</td>
<td>258 ± 51</td>
</tr>
<tr>
<td>A65IND CM</td>
<td>132 ± 42</td>
<td>746 ± 147</td>
</tr>
<tr>
<td>Con A CM (20%, v/v)</td>
<td>62 ± 9</td>
<td>156 ± 54</td>
</tr>
<tr>
<td>WEHI-3 CM</td>
<td>50,772 ± 679</td>
<td>3,715 ± 1074</td>
</tr>
<tr>
<td>TPA (10 ng/ml)</td>
<td>156 ± 136</td>
<td>128 ± 44</td>
</tr>
</tbody>
</table>

DISCUSSION

The A65T cells were stimulated to proliferate by wide varieties of chemicals with tumor-promoting activity in mouse skin. Chemically, they were classified into 3 groups: diterpene esters (tigliane, daphnane, and ingenane derivatives); indole alkaloids; and polypeptide toxins. Their activity to stimulate the growth of A65T, shown as the 50% effective dose, was determined from a graph of dose-response curve like Chart 1.

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Debromoaplysiatoxin, which is chemically identical with aplysiatoxin except for the lack of one bromide atom (31), is a much weaker promoter than aplysiatoxin (11). The 2 compounds, however, had comparable skin-irritating and ornithine decarboxylase induction activities (11). In contrast, induction of adhesion of HL-60 cells to substrate (9, 28, 44) and aggregation of NL-3 cells (27) quantitatively correlate better with tumor-promoting activity (11). In the growth stimulation of A65T cells, aplysiatoxin was approximately 16 times stronger than debromoaplysiatoxin. Therefore, the growth stimulation of A65T may provide another sensitive measure to search for tumor-promoting agents in environments. However, it must be noted here that the compounds capable of stimulating A65T cell line growth may be limited to those sharing the same or a closely related cellular receptor. Actually, teleocidin (54) and aplysiatoxin (26, 45) compete with phorbol esters for the binding to cellular receptor. In other words, this system may not be effective in detecting possible tumor promoters working by other mechanisms.

Subsequently, we studied the effect of 2 biologically active substances sharing growth-stimulatory activity in mouse epidermal cells. Cholera toxin strongly stimulates proliferation of epidermal cells by increasing intracellular cyclic adenosine 3′:5′-monophosphate level (15, 33). However, in our system, it was totally without effect. Since normal thymocytes have a receptor for cholera toxin (25), the failure of A65T cells to respond to the toxin is not likely to be due to the lack of a cellular receptor but probably to the differential growth control depending on cell types. Actually, cholera toxin is reported to inhibit mitogen-induced DNA synthesis of lymphoid cells (25). Another growth factor, EGF, shares a number of biological effects with tumor promoters (2, 4, 36, 39, 49, 53). EGF binding on the cellular receptor is inhibited by active tumor promoters (37, 38), possibly by decreasing the affinity of receptor (38, 47). Furthermore, EGF itself has been suggested to enhance methylcholanthrene-initiated mouse skin carcinogenesis (42). However, A65T cells did not multiply in response to EGF. Thus far, this is the only case in which A65T cells failed to respond to substances with any tumor-enhancing activity in mouse skin. These observations also indicate that growth stimulation of epidermal cells is not directly linked to that of A65T leukemia cells. The growth of lymphoid cells is controlled by a number of factors produced by antigen- or mitogen-activated lymphocytes (lymphokine) or macrophages (monokine). IL-2 stimulates the growth of predominantly Thy-1+, Lyt-1−,23+ cytotoxic T cells. The production of IL-2 by antigen-activated T-cells requires an interleukin-1 signal released by macrophages (34, 48). Tumor promoters have been found to replace the interleukin-1 activity to induce the production of IL-2 in both normal (7, 12, 43) and leukemic T-cells (6, 55). In the growth of A65T cells, however, IL-2 was not likely to be responsible since exogenous IL-2 did not support their growth (Table 2) and A65T cells incubated with TPA did not release IL-2 activity (Table 3).

Tumor Promoter-dependent Leukemia Cell Line

WEHI-3 CM contains a unique lymphokine IL-3 (29, 35) that induces proliferation of Thy-1+, Lyt-1−,23+ T-cells (16) and hematopoietic progenitor cells (5, 13) and induces 20a-hydroxy-steroid dehydrogenase characteristic of mature T-cells in splenic lymphocytes of nu/nu mice (29). FDC-P2 is one of the WEHI-3 growth factor-dependent hematopoietic cell lines (5) whose growth is supported by purified IL-3 (13). Our preparation of WEHI-3 CM intensely stimulated the growth of FDC-P2; Con A CM also contained a low level of this activity. However, the A65T cells were not supported by WEHI-3 CM. A65T CM prepared without TPA slightly increased [3H]TdR uptake by FDC-P2. Whether this increase is a result of IL-3 production by A65T cells or due to another reason is unclear. TPA enhances production of IL-2 but not of IL-3 in normal cells (35), but FDC-P2 are refractory to IL-2. TPA also substitutes CSA in soft agar culture of committed hematopoietic precursor cells (8, 50). However, CSA seems unlikely to be responsible for the growth of A65T, since WEHI-3 CM, a rich source of CSA (5) as well as IL-3, failed to support A65T. Finally, an intriguing possibility to be considered is the production of autostimulatory factors by transformed cells which may be lacking in normal or not yet autonomously growing cells. Todaro and De Lareco (52) described the sarcoma growth factor elaborated by virustransformed cells. In our preliminary experiment, the culture supernatant of A65TND, an independently growing variant of A65T, did not stimulate A65T cells to grow. Therefore, the nature of TPA dependence is probably not due to failure of autostimulatory factor production.

One of the important steps of thymic leukemogenesis is the formation of symbiotic complexes with TER (22). Under the support of TER, growth behavior of leukemias is supposed to progress from dependence on to independence of thymic microenvironments. However, the nature of cell interaction between developing leukemia cells and TER has remained obscure. Transmission of the growth signal from TERs seems to require closed-cell contact or mediation by a labile, short-range factor (22). The present work explores several possible mechanisms of growth stimulation of TPA-dependent and thus microenvironment-dependent leukemia cells. Although the basic questions remain unanswered, this cell line will provide a useful model to analyze growth requirements of developing leukemias.

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Tumor Promoter-dependent Mouse Leukemia Cell Line

Hideto Kaneshima, Hiroshi Hiai, Hirota Fujiki, et al.


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