Dose-Dependent Effects of a Tumor Promotor on Blast Cell Progenitors in Human Myeloblastic Leukemia

By L. J-A. Chang and E. A. McCulloch

AML blast cell progenitors form colonies in culture when stimulated by a media conditioned by leukocytes in the presence of PHA. Two cellular processes occur during colony formation: self renewal generates new progenitors, while others undergo a change that leads to decreased proliferative potential. We tested the effect of the potent tumor promotor, 12-O-tetradecanoyl phorbol acetate (TPA) on these events. TPA was found to be toxic to blast cell colony formation; doses in excess of 1 ng per ml usually abolished it. At doses lower than this, self renewal, as determined by replating either individual or pooled colonies, was increased. At proliferation inhibiting TPA doses, surviving cells showed a spindle morphology, and had increased ANA esterase activity. We interpret the data to mean that TPA decreases blast cell maturation at low doses and may increase it at high doses.

The BLAST CELL population in acute myeloblastic leukemia (AML) contains cells capable of giving rise to colonies of blast cells in culture. When such colonies are harvested, the cells dispersed and replated, secondary colonies are usually obtained with characteristics similar to those of the primaries. However, the plating efficiency of suspensions obtained from colonies (plating second efficiency or PE2), while variable from sample to sample, is usually low, indicating that only a minority of cells within colonies have colony forming capacity. These findings have been interpreted to indicate that two different growth processes occur during blast colony formation in culture: first, some divisions yield cells that retain colony forming capacity (self renewal). Second, other cells lose proliferative potential during colony formation; this process, although not associated with any change in blast cell morphology, might be considered analogous to terminal differentiation.

The potent tumor promotor, 12-O-tetradecanoyl phorbol-13-acetate (TPA) has been reported to prevent differentiation by Friend erythroleukemic cells in culture and to increase differentiation in a continuous line of human promyelocytes, HL-60. Mouse myeloid leukemias, and primary cultures of acute and chronic human myeloid leukemia cells. These reports suggested to us that TPA might prove a useful probe in the dissection of cellular events during blast colony formation. The present paper contains the data from experiments designed with this objective in mind. We found that low doses of TPA increased the frequency of self renewal a finding compatible with decreased differentiation. In contrast, at higher TPA doses, blast progenitors lost proliferative capacity and acquired a macrophage-like appearance; these changes might be considered as increased differentiation.

MATERIALS AND METHODS

Cell Source

Ten patients with AML were diagnosed using clinical and morphological criteria previously described by Hasselback et al.

One patient (Ar) was diagnosed as acute myelomonocytic leukemia. One patient (Ju) was in the typical stable phase of chronic myeloblastic leukemia (CML).

Peripheral blood was collected in heparin at diagnosis or relapse prior to treatment. Patients presented at the Princess Margaret Hospital, Sunnybrook Hospital or the Toronto Western Hospital. Normal blood was obtained from volunteers.

Cell Separation

Leukocytes were separated as previously described (Minden et al.11). A mononuclear leukocyte fraction was obtained by separation through Ficoll Hypaque (density = 1.077 g/cu cm). These were separated into a T-cell enriched (E+) and T-cell depleted population (E-) by a second Ficoll Hypaque separation after incubation of the mononuclear cells with sheep erythrocytes (SRBC). The fractions were washed twice in alpha medium resuspended in alpha medium plus 10% (v/v) fetal calf serum (FCS) and subsequently used for colony assay.

PHA-LCM

Media conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM) was made according to methods previously described.14 Leukocyte suspensions from the buffy coat of a patient with hemochromatosis were incubated at 37°C, 5% CO2 for 7 days at 10⁶ cells/ml in alpha medium plus 10% FCS and 1% PHA (v/v) (Wellcome HA 15). The culture was then centrifuged and the supernatant filtered prior to use.

Phorbol Esters

TPA (12-O-tetradecanoyl phorbol-13-acetate) and 4β-phorbol were obtained from the Sigma Chemical Company. They were dissolved in acetone at a concentration of 100 mg/ml and stored at 20°C prior to use. In preliminary control experiments, acetone, at the concentrations used to dissolve the chemical, had no effects on blast colony formation.
**Blast Colony Assay**

Blast colony formation was performed as previously described.1 Cells from the E- fraction were cultured in 0.8% methylcellulose with 20% FCS, medium H21 (Gibco H21), plus or minus 10% PHA-LCM, and various esters were present continuously during primary (but not secondary) colony formation. The mixture of cells and media was distributed either as 1-ml volumes into 35 × 10 mm Lux tissue culture dishes (at 2 × 10^5 cells/dish) or as 0.1-ml volumes into 0.6-mm wells of flat bottom Limbro tissue culture trays (2 × 10^5 cells/well). Colonies with more than 20 cells were scored and characterized after 5–7 days incubation at 37°C in a moist atmosphere of 5% CO₂. In the absence of PHA-LCM, blast colony formation was usually not observed and when seen, numerically never exceeded 10% of the colony numbers in cultures with the stimulator. Phorbol or its esters did not stimulate primary blast colony formation in the absence of PHA-LCM.

**Self Renewal Assay**

Self renewal of blast progenitors was measured as described previously.2 Cells from primary cultures were pooled washed twice in alpha medium and replated in 0.8% methylcellulose, 10% PHA-LCM at 2 × 10^5 cells/ml without phorbol esters. The cells were plated at 0.1 ml volumes in Limbro microtitre trays. Colonies were counted and characterized after 5–7 days of incubation.

Single colonies were tested for self renewal by picking them and dispersing them into single cell mixtures in the presence of 0.8% methylcellulose, 20% FCS, 3 × 10^6/ml heterologous normal irradiated T cells (2000 rad) and 10% PHA-LCM. This mixture was plated in microwells and incubated at 5% CO₂, 37°C in a humidified atmosphere for 6 days. Control cultures of irradiated cells only were also plated. Colonies were never seen in these wells.

**Characterization**

Individual colonies were picked and stained using Wright-Giemsa staining, and for presence of myeloperoxidase enzyme using the method of Kaplow.15 Alpha naphthyl acetate esterase (ANA) and naphthol AS-D chloroacetate esterase (NASD) stains were prepared according to the methods of Yam et al.16 E-rosette formation of cells in individual colonies were tested using methods previously described.13

**RESULTS**

AML blast cells were exposed to either TPA or phorbol during primary colony formation; then colonies were harvested, pooled, and replated under standard conditions without further exposure to drug. This design yields data in the following forms: (1) a dose response curve for the effect of TPA or phorbol on blast cell colony formation (PE1 dose response curve); (2) morphological appearance of colonies and cells from colonies obtained during primary colony forma-
EFFECTS OF TPA ON AML BLASTS

Fig. 2. Effect of TPA on cultures of AML blasts. Magnification x 8. All cultures are with 10% PHA-LCM. Figures 2A and 2C illustrate the primary and secondary colonies plated with 10% PHA-LCM alone. Figure 2B illustrates the primary plating in the presence of 10 ng/ml TPA. Figure 2D illustrates the secondary plating with 10% PHA-LCM alone, but after exposure to 0.01 ng/ml for 7 days in primary cultures.

A typical dose response curve for blast colony formation in the presence of TPA or phorbol is shown in Fig. 1. All of the dose response data are presented for PE1 in Table 1 and for PE2 in Table 2. It is apparent that colony formation decreased markedly in concentrations of TPA in excess of 1 ng/ml; phorbol had no effect. The appearance of cultures containing no TPA is shown in Fig. 2A. The morphology of colonies at the low TPA concentrations was no different from that of the control. Secondary colonies of similar morphology were seen in wells seeded with cells from pooled colonies cultured without (Fig. 2C) and in the presence of low concentrations of TPA (Fig. 2D). At high TPA concentrations, the colony forma-
tion was inhibited and the culture dishes contained either scattered single cells or spindle-like cells with one or more processes; this appearance, which we consider macrophage-like, is illustrated in Fig. 2B, a photograph of a primary culture containing 10 ng/ml of TPA.

Cells from controls and cultures containing TPA were harvested and characterized; in Wright-Giemsa stains cells from control cultures and cultures containing low concentrations of TPA had blast-like morphology; colonies from patient Ar, diagnosed clinically as myelomonocytic leukemia, contained cells with a morphology compatible with that diagnosis. At high concentrations of TPA, cells of morphology similar to that in control cultures continued to be seen; others had fragmented nuclei and vacuolated cytoplasm. Such changes might represent either macrophage differentiation or toxic effect of TPA.

Cells were peroxidase negative regardless of whether or not they had been exposed to TPA; a small proportion stained positively for NASD chloracetate and the percentage of positive cells did not increase in cultures exposed to TPA. In three patients studied in detail, cells in control cultures stained positively for ANA esterase; the numbers of positively staining cells increased in cultures treated with TPA. These staining characteristics were also observed in secondary colonies. The data are given in Table 3.

**Plating Single Colonies**

Increased plating efficiency in suspensions from cultures exposed to TPA might represent either a general effect on blast cell progenitors or selection of a specific subclass with high renewal potential. To distinguish between these possibilities, single colonies were replated. The experiment was carried out in cultures of cells from a patient (He) with high self renewal capacity. Cells were harvested from primary cultures and then plated in the presence of varying quantities of TPA; individual secondary colonies were replated in microwells. The distributions of tertiary colonies are shown in Fig. 3. In agreement with results previously reported by Buick et al., an asymmetrical distribution was seen in the absence of TPA, with many tertiary colonies containing few progenitors and a few colonies containing many. Similar distributions were obtained from cultures containing 0.01 or 0.1 ng/ml of TPA; while the number of tertiary colonies increased significantly ($p < 0.05$, rank sum test) the distributions remained broad.

**Effect of TPA on Pooled Colonies**

Cells with macrophage-like morphology (Fig. 1B) might persist in cultures of peripheral blood in the presence of high concentrations of TPA because of selection. To test for this possibility, cells from primary colonies of a patient (Si) with myelomonocytic leukemia were plated for secondary colony formation in the presence of various concentrations of TPA. The data are presented in Fig. 4; the drug dose response curve was similar to that for blast cell progenitors obtained directly from peripheral blood; in addition, cells of morphology similar to that illustrated in Fig. 1B were observed in cultures containing 1

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### Table 3. Alpha Naphthyl Acetate Esterase—TPA Effect

<table>
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<td>&gt;50</td>
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This table represents the results of ANA staining in 3 patients on colonies from PE1 and PE2 cultures grown at varying TPA doses ranging from 0–10 ng/ml. The values are reported as percentages of positive cells present in stained smears obtained by counting 100 cells.
ng/ml of TPA. Since these cultures originated from pooled blast colonies, it is unlikely that the macrophage-like cells were unrelated to blast progenitors.

CML

Experiments of the same design were carried out using peripheral blood from a patient with CML and from normals. From these sources, colonies obtained by plating E-rosette depleted fractions of peripheral leukocytes in the presence of PHA-LCM yield colonies of mature or maturing granulocytes (CFU-C). However, the numbers of CFU-C in blood of CML patients is much greater than normal.\textsuperscript{17} Data from the CML patient and a typical control are shown in Fig. 5; the values were normalized for comparison. It is apparent that leukemic and normal CFU-C were sensitive to TPA over a similar dose range. As reported by others, normal CFU-C were incapable of self renewal as determined by the replating assay; nor was self renewal observed in cultures of normal cells in the presence of TPA. In contrast, CML CFU-C, consisting of granulopoietic cells, were replated successfully; a TPA-induced stimulation of self renewal was observed, with secondary granulocytic colonies, similar to the increase in number seen for the blast progenitors in AML. Since adherent cells were not removed, CFU-C proliferated without added PHA-LCM in primary cultures, although to a lesser extent than when the stimulator was added. This primary growth was not increased by TPA. However, self renewal was increased in cultures with TPA but without PHA-LCM, although values of PE2 were only approximately 50% of those found in the presence of PHA-LCM.

DISCUSSION

To our knowledge, this is the first report of the application of a clonal assay to the study of the effects of TPA on freshly obtained cancer cells. The culture technology permitted observations of the effect of TPA on growth, particularly that component of growth that results in progenitor renewal. Indeed, the major finding of the work was that self renewal increased in cultures containing 1 ng (<1.6 × 10\textsuperscript{-9} M) or less concentrations of TPA. Blast progenitor populations have been shown to be heterogeneous in respect
to their self renewal capacity. Thus, TPA might have acted preferentially to select for progenitors with high renewal capacity. The data obtained by assessing the content of new blast cell progenitors in individual colonies makes such selection unlikely. If a subpopulation of blast progenitors were responding to TPA, self renewal in colonies from such cells would have been apparent in the distribution as a peak. This was not seen; rather, the distributions remained broad and of the same general form. This result is compatible with an effect of TPA on all blast-cell progenitors.

Increased self renewal may be considered as analogous to decreased differentiation; however, renewal of blast progenitors was observed at TPA doses much less than those required to inhibit differentiation by Friend leukemia cells in culture. Rather, the doses of TPA used in the present study were similar to those that had been present to induce differentiation in HL60 cells and in freshly obtained blast cells cultured in suspension. For these cells, morphological changes associated with the development of biochemical markers of differentiation were observed. The appearance of TPA-treated HL60 cells was similar to that of fresh leukemic cells rendered incapable of proliferation by TPA (Fig. 1B), suggesting the possibility that in the latter, as in the former, drug treatment increased differentiation. This suggestion is supported by the increased ANA esterase activity observed in TPA treated blast cells. It should be noted, however, that we have not observed cytochemical markers (peroxidase, NASD, and ANA esterases) in TPA treated cultures where these were not present in the controls. HL-60 cells exposed to TPA showed inhibition of DNA synthesis; this finding may be analogous to the decreased growth observed in our experiments. Regardless of the explanation of the differences and similarities between the present data and those reported by others, our finding underscores the need for dose response curves in assessing the effects of TPA on growth and differentiation, since, in our hands, differentiation was inhibited at low doses and may have been increased at high doses of the drug.

The biologic or clinical significance of the effect of TPA on blast colony formation remains to be determined. Nonetheless, the present observations may be considered in the context of the known effects of TPA on carcinogenesis. TPA is not itself carcinogenic, but is among the most powerful promoters once carcinogenesis has been initiated. AML clones exhibiting low blast cell self renewal have been found to be significantly more likely to respond favourably to treatment than those with high self renewal. This observation is compatible with the view that clones exhibiting low self renewal are not as aggressive, perhaps less progressed, than those with high self renewal. TPA has been reported to accelerate virus-induced generation of leukemic cell lines in long-term cultures of mouse bone marrow. It may be, therefore, that TPA in culture is acting as a promotor when it increases self renewal. From this point of view, it is a noteworthy control that phorbol, which is inactive as a tumor promotor, also failed to affect blast colony formation in culture.

REFERENCES

13. Minden MD, Buick RN, McCulloch EA: Separation of blast


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