[CANCER RESEARCH 41, 5049-5051, December 1981]
0008-5472/81/0041-0000$02.00

Different Responsiveness of Colony-forming Cells from Normal Subjects and Chronic Myeloid Leukemia Patients to 12-*O*-Tetradecanoylphorbol-13-acetate[1]

Luigi Pegoraro,[2] Gianpaolo Bagnara, Laura Bonsi, Graziella Biagini, Giovanni Garbarino, and Giovanni Luca Pagliardi

Istituto di Medicina Interna, Università di Torino, 10126 Torino, Italy [L. P., G. G., G. L. P.], and Istituto di Istologia ed Embriologia Generale [G. Ba., L. B.] and Istituto di Microscopia Elettronica Clinica [G. Bi], Università di Bologna, Bologna, Italy

ABSTRACT

The effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent inducer of differentiation, upon the *in vitro* proliferation of normal bone marrow and chronic myeloid leukemia granulocyte-macrophage progenitor cells were investigated in the presence or absence of exogenous colony-stimulating activity. In the absence of colony-stimulating activity, no colony formation occurred, whereas its presence was followed by the appearance of colonies almost exclusively composed of macrophagic cells. However, both normal and leukemic granulocyte-macrophage progenitor cells showed a different response to TPA in terms of colony number and size. The number of colonies obtained from normal bone marrow was reduced to about 45% at three TPA concentrations (1×10^{-8}, 1×10^{-10}, and 1×10^{-12} M). By contrast, the plating efficiency of leukemic granulocyte-macrophage progenitor cells was not significantly affected by 1×10^{-10} and 1×10^{-12} M TPA, but was strongly reduced by 1×10^{-8} M. Moreover, TPA treatment did not significantly influence the size of colonies from normal bone marrow, while at 1×10^{-10} and 1×10^{-12} M, it remarkably increased that of the leukemic colonies. These different responses to TPA can be related to the abnormal sensitivity of chronic myeloid leukemia stem cells to factors regulating proliferation.

INTRODUCTION

Some tumor promoters of the phorbol diester series reversibly inhibit the differentiation of Friend erythroleukemia cells (16, 24) and mouse myeloid M1 leukemia cells (6) and induce differentiation in other mouse erythroleukemia cells (10) and human promyelocytic HL60 leukemia cells (5, 9, 17). TPA[3], the most potent of these promoters, rapidly arrests proliferation and irreversibly transforms this human line into cells with the morphological, cytochemical, and functional properties of macrophages (18, 19).

In a previous study, qe demonstrated that blast cells of acute nonlymphoid leukemia patients can also be transformed into macrophage-like cells by TPA (13). Similar results have been obtained by Svet-Moldavskaya *et al.* (21) on cells of the granulocytopoietic lineage from normal subjects and patients with CML.

Among human leukemias, CML is a unique model because its stem cells can still differentiate along the myeloid lineage and form colonies in response to normal stimulating factors (11, 12).

In the present study, we investigated the effects of TPA on the *in vitro* proliferation and differentiation of human normal and CML early myeloid progenitor cells, *i.e.*, the CFU-C.

MATERIALS AND METHODS

Patients. Normal human bone marrow was obtained from nonhematological patients. Leukemic cells were obtained from bone marrow or peripheral blood of 11 Ph[1]-positive untreated patients in chronic phase with peripheral blood WBC ranging from 50,000 to 150,000/µl (less than 5% myeloblasts).

Cell Separation. Sodium citrate solution (3.4%; 1:5 for bone marrow and 1:10 for peripheral blood) was used as anticoagulant agent. Cell suspensions were centrifuged on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) at 1.077 density to remove the majority of the granulocytes and erythroblasts. We assumed that this fraction contained the total CFU-C population. In addition, a fraction containing light-density CFU-C was obtained by centrifugation on bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo.) gradient at 1.063 density (290 mOsmol; Ref. 2).

Colony Forming Assay. Cells were washed twice in McCoy's medium 5A supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Adherent bone marrow and peripheral blood cells were eliminated by incubation in plastic culture flasks for 1 hr at 37° in the same medium. Possible synergism between TPA and mononuclear adherent cells as a source of endogenous CSF was tested by using gradient separated cells *in toto*. The colony-forming capacity of normal and leukemic cells was assayed in soft agar cultures as described by Pike and Robinson (15). Cells (0.5 to 1×10^5) were seeded in 1-ml layers containing 0.3% agar (Difco Laboratories, Inc., Detroit, Mich.) in McCoy's modified Medium 5A plus 10% fetal calf serum. The feeder layers were prepared from normal peripheral blood according to Pike and Robinson (15). Colonies (*i.e.*, the aggregates with more than 50 cells) were scored after 8 days of incubation following staining with 2% orcein. Normal bone marrow cells obtained by centrifugation on Ficoll and bovine serum albumin produced 84 ± 29 and 210 ± 44 (S.E.) colonies per 1×10^5 cells, respectively. For cytological and functional studies, colony cells were harvested from agar with a capillary pipet and resuspended in the liquid medium.

Phagocytosis. Phagocytic activity was assayed by incubating the harvested cells for 24 hr in the presence of 0.8-µm latex particles as described by Levine and Cox (8).

Morphology and Cytochemistry. Morphology and cytochemistry of the colony cells were studied on cytospin preparations. Chloroacetate esterase and α-naphthyl acetate esterase were performed by the method of Yam *et al.* (23). Electron microscopy examination was performed following *in situ* fixation with 1.7% glutaraldehyde.

Chemicals. TPA (Peter Borchert, Eden Prairie, Minn.) was dissolved

[1] Supported by Grant CT80.00645.04 from Consiglio Nazionale delle Ricerche.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CML, chronic nmyeloid leukemia; CFU-C, colony-forming unit-culture; CSF, colony-stimulating factor.

Received February 12, 1981; accepted July 31, 1981.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/41/12_Part_1/5049/2408725/cr04112p15049.pdf by guest on 27 February 2024

at 1 × 10^{-3} M in acetone, further diluted with McCoy's modified Medium 5A, and added to the overlayer cell suspensions before plating. The final acetone concentration (less than 0.01% of the cell suspension) did not affect colony growth in control cultures.

RESULTS

Both in normal bone marrow and in CML, no colony formation was observed in the cultures in the absence of the feeder layer of Pike and Robinson as source of CSF. The addition of TPA at final concentrations of 1 × 10^{-8}, 1 × 10^{-10}, and 1 × 10^{-12} M to these unstimulated cultures always failed to induce CFU-C proliferation in both the presence and the absence of endogenous mononuclear adherent cells.

Colony Number. The effects of TPA on colony formation in stimulated cultures are shown in Chart 1. The growth of both light-density and Ficoll-separated CFU-C from normal bone marrow was inhibited. The addition of 1 × 10^{-12} M TPA resulted in an approximately 55% decrease in colony formation ($p < 0.001$), but no further significant decrease was observed increasing the concentration up to 1 × 10^{-8} M. TPA at 1 × 10^{-12} M in CML cultures did not produce a significant change in the plating efficiency, while at 1 × 10^{-10} M, it slightly increased the number of colonies. By contrast, at 1 × 10^{-8} M, less than 20% of the CFU-C retained their ability to proliferate ($p < 0.01$). As in normal bone marrow, light-density and Ficoll-separated CFU-C from CML did not significantly differ in their response to TPA.

Colony Size. The effects of TPA on colony size are illustrated in Chart 2. No difference in the number of cells per colony was observed in control and TPA-treated normal bone marrow cultures. By contrast, the size of the colonies obtained from CML was strongly increased in cultures treated with TPA at 1 × 10^{-10} and 1 × 10^{-12} M.

Colony Morphology. Colonies from normal subjects and CML patients in the absence of TPA were predominantly of the granulocytic and the granulocytic-monocytic type. Macrophagic colonies, as defined by intense phagocytic activity of latex beads, positivity to α-naphthyl acetate esterase, and negativity to chloroacetate esterase cytochemical stainings and typical ultrastructural features, did not exceed 15% of the total colonies in normal and leukemic cultures. Conversely, all colonies grown with TPA were almost entirely composed of macrophage-like cells.

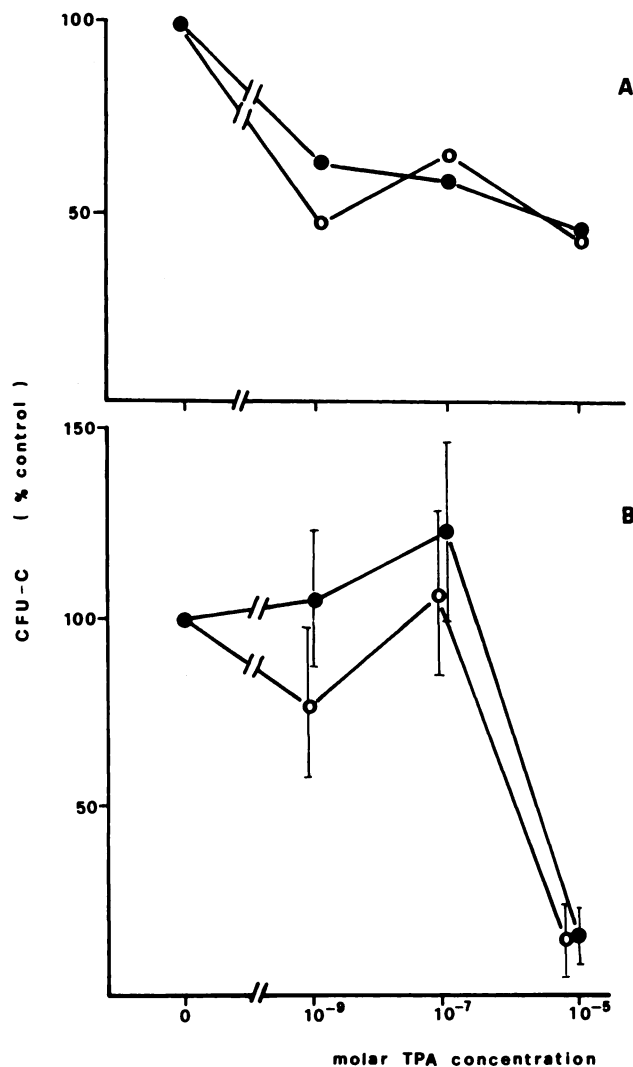

Chart 1. Effects of different concentrations of TPA on the number of CFU-C from (*A*) normal bone marrow (mean of 4 cases) and (*B*) peripheral blood or bone marrow of CML patients (mean of 11 cases). Plating efficiency of CFU-C obtained by Ficoll centrifugation at 1.077 specific density (○) and by albumin density cut at 1.063 specific density (●). *Ordinate*, number of colonies expressed as a percentage of the controls. Each *point* represents the mean of quadruplicate cultures; *bars*, S.E.

DISCUSSION

Previous studies have shown that TPA is able to transform immature myeloid cells from normal human bone marrow and CML blood and bone marrow cultured in liquid media into macrophage-like cells (13). The present data demonstrate that normal and leukemic CFU-C proliferating in the presence of TPA produce colonies formed almost solely of cells showing the morphological, ultrastructural, and functional features of macrophages. It is not clear whether such CFU-C belong to a subpopulation already committed to macrophagic differentiation, or whether the committed stem cells, after the given number of generations required for colony formation, become sensitive to the differentiative effect of TPA.

Moreover our data, unlike those obtained by others in mouse bone marrow cultures (4, 20), indicate that, in humans, both normal and CML CFU-C cannot proliferate in the presence of

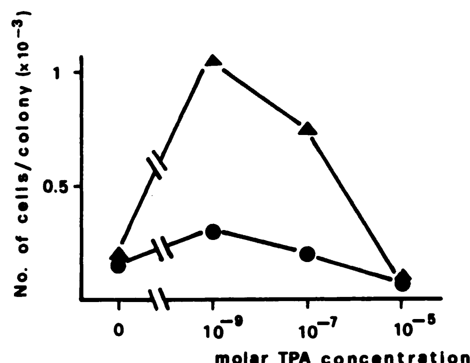

Chart 2. Effects of different concentrations of TPA on the size of colonies obtained from normal bone marrow (mean of 4 cases; ●) and from CML patients (mean of 11 cases; ▲). The results refer only to CFU-C recovered after Ficoll centrifugation.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/41/12_Part_1/5049/2408725/cr04112p15049.pdf by guest on 27 February 2024

TPA alone, and that exogenous CSF is always required. However, we also found that normal and leukemic CFU-C display a markedly different response to increasing concentrations of TPA. In normal bone marrow cultures, in fact, colony numbers are reduced in a virtually non-dose-dependent manner by 1×10^{-12} to 1×10^{-8} M TPA. This finding can tentatively be explained by assuming that one committed stem cell population is directly pushed into terminal differentiation without proliferation, while another population representing about 45% of the total CFU-C is insensitive to TPA, and can still proliferate even at 1×10^{-8} M. It is a matter of speculation whether this difference in behavior is dependent upon the presence in the first CFU-C population and the absence in the second of specific membrane receptors for TPA, as proposed by Stuart and Hamilton to explain the response of mouse bone marrow CFU-C to this compound (20). Conversely, in CML cultures, 1×10^{-10} and 1×10^{-12} M TPA does not significantly decrease the number of colonies, while it greatly increases the number of cells per single colony. This agrees with the finding of Kasukabe *et al.* (7) that TPA acts synergistically with CSF in stimulating the colony growth of the mouse myeloid Mm-1 cell line, and with the observation of Chang and McCulloch (3) that the proliferation of blast progenitor cells from human acute myelogenous leukemia is stimulated by very low TPA concentrations.

Our finding that leukemic as opposed to normal myeloid stem cells are stimulated by low and strongly inhibited by high TPA concentrations can be related to the abnormal sensitivity of CML CFU-C to regulatory factors (1, 14, 22). The present results lend support to the view that leukemic precursor cells can be selectively eliminated *in vitro* by treatment with agents inducing terminal differentiation.

REFERENCES

1. Aglietta, M., Piacibello, W., and Gavosto, F. Insensitivity of chronic myeloid leukemia cells to inhibition of growth by prostaglandin E. Cancer Res., *40:* 2507–2511, 1980.
2. Broxmayer, H. E., Baker, F. L., and Galbraith, P. R. *In vitro* regulation of granulopoiesis in human leukemia: application of an assay for colony inhibiting cells. Blood, *47:* 389–402, 1976.
3. Chang, L. J. A., and McCulloch, E. A. Dose dependent effects of a tumor promoter on blast cell progenitors in human myeloblastic leukemia. Blood, *57:* 361–367, 1981.
4. Greenberger, J. S., Newburger, P. E., and Sakakeeny, M. Phorbol myristate acetate stimulates macrophage differentiation and replication and alters granulopoiesis and leukemogenesis in long-term bone marrow cultures. Blood, *56:* 368–379, 1980.
5. Huberman, E., and Callaham, M. F. Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. Proc. Natl. Acad. Sci. U. S. A., *76:* 1293–1297, 1979.
6. Kasukabe, T., Honma, Y., and Hozumi, M. Inhibition of functional and morphological differentiation of cultured mouse myeloid leukemia cells by tumor promoters. Gann, *70:* 119–123, 1979.
7. Kasukabe, T., Honma, Y., and Hozumi, M. Enhancement of a mouse macrophage cell line by a tumor promoter and granulocyte-macrophage colony-stimulating factor. Cancer Res., *40:* 472–476, 1980.
8. Levine, M. R., and Cox, R. P. Use of latex particles for analysis of heterokaryon formation and cell fusion. Somatic Cell Genet., *4:* 507–512, 1978.
9. Lotem, J., and Sachs, L. Regulation of normal differentiation in mouse and human myeloid leukemic cells by phorbol esters and the mechanism of tumor promotion. Proc. Natl. Acad. Sci. U. S. A., *76:* 5158–5162, 1979.
10. Miao, R. M., Fieldsteel, A. H., and Fodge, D. W. Opposing effects of tumor promoters on erythroid differentiation. Nature (Lond.), *264:* 271–272, 1978.
11. Moore, M. A. S. Agar culture in CML and blastic transformation. Semin. Hematol., *8:* 11–27, 1976.
12. Moore, M. A. S., Williams, N., and Metcalf, D. *In vitro* colony formation by normal and leukemic haematopoietic cells. Characterization of the colony forming cells. J. Natl. Cancer Inst., *50:* 603–629, 1973.
13. Pegoraro, L., Abrahm, J., Cooper, R. A., Levis, A., Lange, B., Meo, P., and Rovera, G. Differentiation of human leukemias in response to 12-*O*-tetradecanoyl-phorbol-13-acetate in vitro. Blood, *55:* 859–862, 1980.
14. Pelus, L. M., Broxmeyer, H. E., Clarkson, B. D., and Moore, M. A. S. Abnormal responsiveness of granulocyte-macrophage-committed colony-forming cells from patients with chronic myeloid leukemia to inhibition by prostaglandin E. Cancer Res., *40:* 2512–2515, 1980.
15. Pike, B. L., and Robinson, W. A. Human bone marrow colony growth in agar gel. J. Cell. Physiol., *76:* 77–84, 1970.
16. Rovera, G., O'Brien, T. G., and Diamond, L. Tumor promoters inhibit spontaneous differentiation of Friend erythroleukemia cells in culture. Proc. Natl. Acad. Sci. U. S. A., *74:* 2894–2898, 1977.
17. Rovera, G., O'Brien, T. G., and Diamond, L. Induction of differentiation in human promyelocytic leukemia cells by tumor promoters. Science (Wash. D. C.), *204:* 868–870, 1979.
18. Rovera, G., Olashaw, N., and Meo, P. Terminal differentiation in human promyelocytic leukemic cells in the absence of DNA synthesis. Nature (Lond.), *284:* 69–70, 1980.
19. Rovera, G., Santoli, D., and Damsky, C. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with phorbol diesters. Proc. Natl. Acad. Sci. U. S. A., *76:* 2779–2783, 1979.
20. Stuart, R. K., and Hamilton, J. A. Tumor-promoting phorbol esters stimulate hematopoietic colony formation *in vitro*. Science (Wash. D. C.), *208:* 402–404, 1980.
21. Svet-Moldavskaya, I. A., Zinzar, S. N., Svet-Moldavsky, G. J., Mann, P. E., Bekesi, J. G., Holland, J. F., Clarkson, B. D., Arlin, Z., and Koziner, B. Macrophage-like cell transformation and $CFU_{(c)}$ fluctuations in normal and leukemic human marrow cultures treated by phorbol diester. Biomed. (Paris), *31:* 252–257, 1979.
22. Taetle, R., and Koessler, A. Effects of cyclic nucleotides and prostaglandins on normal and abnormal human myeloid progenitor proliferation. Cancer Res., *40:* 1223–1229, 1980.
23. Yam, L. T., Li, C. Y., and Crosby, W. M. Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol., *55:* 283–290, 1971.
24. Yamasaki, H., Fibach, E., Nudel, U., Weinstein, I. B., Rifkind, R. A., and Marks, P. A. Tumor promoters inhibit spontaneous and induced differentiation of murine erythroleukemia cells in culture. Proc. Natl. Acad. Sci. U. S. A., *74:* 3451–3455, 1977.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/41/12_Part_1/5049/2408725/cr04112p15049.pdf by guest on 27 February 2024