Insensitivity of Chronic Myeloid Leukemia Cells to Inhibition of Growth by Prostaglandin E1

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ABSTRACT

The influence of E prostaglandins on the in vitro growth of chronic myeloid leukemia (CML)-committed granulopoietic precursors [colony-forming unit-culture (CFU-C)] has been investigated in a double-layer agar system in which CFU-C growth was stimulated by adherent monocytes. Addition of the prostaglandin synthesis inhibitor indomethacin to the feeder layer significantly increased the number of normal CFU-C, whereas CML CFU-C were unaffected. Exogenous prostaglandin E1 inhibited CML CFU-C growth at concentrations 1000-fold higher than those necessary to produce a similar effect on normal CFU-C. These data point to a lower than normal sensitivity of CML-committed granulopoietic precursors. It is suggested that derangement of the responsiveness of CML cells to prostaglandin regulation may play a role in the pathogenesis of uncontrolled leukemic proliferation.

INTRODUCTION

Semisolid agar cultures allow the in vitro proliferation and differentiation of committed granulopoietic precursors (CFU-C). In the presence of CSF, CFU-C produce colonies which mature up to the granulocyte-macrophage stage. The stimulatory effect of CSF has been shown to be counterbalanced by PGE. In fact, it has been demonstrated that murine granulopoiesis is inhibited by the exogenous addition of PGE. Moreover, macrophages respond to increasing CSF concentrations by synthesizing PGE. The fact that this synthesis is inhibited by the addition of the cyclooxygenase inhibitor indomethacin suggests that CSF and PGE play a dualistic role in the regulation of murine granulopoiesis. In humans, the number of normal bone marrow colonies increases in cultures stimulated by monocytes in which PGE synthesis has been inhibited by indomethacin.

PGE have also been reported to inhibit the in vitro and in vivo growth of tumor cell lines, suggesting a relevance of PGE in the regulation of both tumor and normal cell proliferation. A main problem, however, is the possibly different sensitivity of tumor cells, as compared to their normal counterparts, to PGE regulation. This possibility has been evaluated with a CML model, because the growth of the committed granulopoietic stem cells can easily be determined in vitro. CML CFU-C differentiate in much the same way as do normal CFU-C from an earlier occurrence of macrophagic colonies. It is therefore possible to study their responsiveness to stimulating and inhibiting factors and to compare such responsiveness to that of normal CFU-C. In the present paper, we have compared the in vitro sensitivity to PGE of normal and CML CFU-C. We present evidence for a less than normal sensitivity of CML CFU-C to PGE inhibition.

MATERIALS AND METHODS

Samples. Normal bone marrow was obtained from patients undergoing chest surgery for nonmalignant diseases. CML peripheral blood or bone marrow cells were obtained from 9 Ph1-positive CML patients in chronic phase who had been off therapy for at least 6 months ( peripheral blood leukocyte range, 21,000 to 280,000).

CFU-C Assay. Bone marrow or peripheral blood cells were collected with preservative-free heparin (50 to 100 units/ml). After 1 to 2 hr sedimentation at room temperature, the WBC buffy coat was collected, washed 3 times with Hank's balanced salt solution, and resuspended in McCoy's 5A modified medium (supplemented with essential and nonessential amino acids, vitamins, sodium pyruvate, and glutamine). One ml of the suspension containing 5 x 10^6 or 1 x 10^7 cells in 0.3% agar plus 15% fetal calf serum (Seromed, Munich, West Germany) was added to the monocyte underlayers prepared 5 to 7 days before. In some cases, the cell suspension was also tested after Lymphoprep separation and removal of adherent cells (see below).

When the effect of PGE1 had to be tested, 0.1 ml of scalar concentrations of highly purified PGE1 (kindly donated by Upjohn, Caponago, Italy) or appropriate ethanol diluent were added to normal or leukemic cells.

After 8 days of incubation in a humidified 5% CO2 atmosphere, 3 dishes/point were scored. All aggregates containing more than 40 cells were counted as colonies.

Morphological examination was performed by transferring single colonies (with the aid of a micropipet) to glass slides. The colonies were stained with May-Grünwald-Giemsa. Seventy-five colonies from 3 normal bone marrow and 75 colonies from 3 CML patients were evaluated.

Feeder Layers. Human monocytes were obtained by density separation of normal peripheral blood cells with Lymphoprep (density, 1.077; Nyegaard, Oslo, Norway). The light-density fraction containing monocytes and lymphocytes was washed 3 times, suspended in McCoy's 5A modified medium plus 15% fetal calf serum. One ml of the suspension (at varying cell concentrations) was allowed to adhere to 35-mm Petri dishes at 37°. After 90 min, the nonadherent cells were removed, and the dishes were washed 3 times with Hanks' balanced salt solution. More than 98% of the adherent cells were monocytes (i.e., α-naphthyl esterase-positive cells). Their number was determined by counting the adherent esterase-positive cells in

1 Supported by a grant from CNR, Progetto finalizzato controllo crescita neoplastica, and a grant from Associazione Italiana per la ricerca sul Cancro.
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3 Fellow of the A. Bosolasco Foundation.
4 The abbreviations used are: CFU-C, colony-forming unit-culture; CSF, colony-stimulating factor; PGE, prostaglandins of the E series; CML, chronic myeloid leukemia; PGE1, prostaglandin E1; PG, prostaglandins.

Received November 2, 1979; accepted March 12, 1980.
Chart 1. Effect of indomethacin on normal and CML CFU-C stimulated by different concentrations of normal monocytes. The adherent monocyte number is a mean from 7 experiments with a variability range of less than 20%. A, data obtained from 7 experiments using normal bone marrow. In each experiment, mean CFU-C colony number of 3 dishes per point was determined and expressed as a percentage of maximum colony number (maximum colony number range, 90 to 322 colonies/10⁵ cells plated). Results are means of these percentages. Bars, S.D. •, ethanol diluent; ▲, plus indomethacin. p values compare in vitro growth with or without indomethacin at the same monocyte concentration. B, data obtained from 7 experiments using 6 peripheral blood samples and 1 bone marrow of 6 CML patients. Procedure as in A. Maximum colony number range, 42 to 414 colonies/10⁵ cells plated. •, ethanol diluent; ▲, plus indomethacin.

Replicate dishes. By varying the number of mononuclear cells initially plated, the number of adherent monocytes could be altered from 2.1 x 10⁴ to 1 x 10⁶ per dish. Adherent cells were overlaid with 1 ml cell-free 0.5% agar prepared in McCoy’s 5A modified medium containing 15% fetal calf serum and were allowed to incubate.

In the first series of experiments, the PG synthesis inhibitor indomethacin (gift of Merck & Co., Rome, Italy) at a concentration of 1.4 x 10⁻⁷ M, or the appropriate ethanol diluent, were added within the monocyte underlayer. After 5 to 7 days of incubation in a humidified atmosphere at 5% CO₂, the feeders were layered with the cell suspension to be tested for CFU-C.
Chart 2. Effect of PGE\textsubscript{i} on normal and CML CFU-C colony formation. The cultures were stimulated by $2.4 \times 10^5$ monocytes plus $1.4 \times 10^{-7}$ M indomethacin. ○, normal bone marrow. Data from 6 experiments. In each experiment, mean CFU-C colony number of 3 dishes per point was determined and expressed as a percentage of control colony number (control range, 98 to 280 colonies/10\textsuperscript{5} cells plated). Results are means of these percentages. Bars, S.D. ■, CML peripheral blood. Data from 6 experiments. The procedure adopted for normal bone marrow was also used here to evaluate CML colony number. Control range, 56 to 175 colonies/10\textsuperscript{5} cells plated. p values compare normal and CML in vitro growth at the same PGE\textsubscript{i} concentration. N.S., not significant.

RESULTS

Chart 1 shows the growth of normal and CML CFU-C stimulated by increasing numbers of monocytes with and without $1.4 \times 10^{-7}$ M indomethacin. The colony number increases from zero to a plateau as a function of the monocyte concentration in the feeder layer. The lack of growth in the absence of an exogenous source of CSF rules out significant endogenous CSF production. The addition of indomethacin to a monocyte concentration above $8.4 \times 10^5$ cells/dish significantly increases the normal CFU-C number, whereas CML CFU-C are unaffected. No difference was observed when $1.4 \times 10^{-8}$ or $1.4 \times 10^{-9}$ M indomethacin was added (data not shown). Moreover, the removal of adherent cells from the sample did not affect the growth pattern.

Chart 2 shows the effect of exogenously added PGE\textsubscript{i} on...
normal and CML CFU-C in cultures where endogenous PG synthesis was inhibited by indomethacin. In normal bone marrow cultures, a significant (p < 0.01) reduction in growth appears at PGE concentrations as low as 10^{-10} M. In CML, a significant (p < 0.01) reduction in growth does not appear before 10^{-7} M PGE; i.e., CML CFU-C are 1000 times less sensitive to PGE inhibition. Comparing normal with CML CFU-C, a significant difference in responsiveness to PGE is present at concentrations from 10^{-10} to 10^{-8} M. Normal bone marrow cultures stimulated by 2.4 \times 10^{5} monocytes plus 1.4 \times 10^{-7} M indomethacin were composed of more than 75% pure granulocytic colonies (the remainder being pure macrophagic or mixed granulocytic-macrophagic colonies); CML CFU-C, with the same stimulus, produced more than 60% pure granulocytic colonies (the remainder being pure macrophagic or mixed granulocytic-macrophage colonies).

**DISCUSSION**

CML is a monoclonal disease (as shown by cytogenetic and enzyme marker studies) (4, 5). It is likely to derive from a defect in a single pluripotent stem cell which expands clonally to produce a leukemia. Although all the myelopoietic lines appear to possess leukemic markers, expanded granulopoiesis due to increased bone marrow and peripheral blood CFU-C, which show a normal in vitro maturation, is the most striking feature of the disease (6, 12, 16, 17). The committed granulopoietic precursors, which possess the leukemic markers (3, 15), have therefore been studied extensively to find a reason for their marked expansion. The response of CML CFU-C to CSF, a putative regulator of granulopoiesis, has been shown to be not significantly different from normal (14). An altered responsiveness to CSF is therefore unlikely to play an important role in the pathogenesis of leukemic proliferation. The pathogenesis of this increased proliferation has been extensively investigated by Broxmeyer et al. Their important data show that leukemic cells have 3 proliferative advantages: (a) CML produce leukemia cell-derived inhibitory activity, an inhibitor of normal but not of leukemic granulopoiesis (1); (b) CML granulocytes produce less colony-inhibiting factor, an inhibitor of monocyte CSF production (lately characterized as lactoferrin) (2, 4); (c) CML monocytes are less sensitive to colony-inhibiting factor inhibition of CSF production (2).

However, all these defects reside in the leukemic progeny. Therefore, they explain the proliferative advantage that leukemic cells have only when many of them are already present in the body.

Our data confirm the normal responsiveness of CML CFU-C to CSF. Both normal and CML CFU-C begin to proliferate in the presence of 8.4 \times 10^{4} monocytes in the feeder layer and reach a plateau at 16.8 \times 10^{4} monocytes/dish. The addition of indomethacin significantly increases the number of normal CFU-C, whereas it does not affect CML CFU-C (Chart 1). It has been reported previously that monocytes, at concentrations similar to ours and under similar experimental conditions, synthesize up to 6 \times 10^{-9} M PGE and that this synthesis is almost completely blocked by indomethacin (9, 10). It has therefore been suggested, by analogy with data in mice, that the indomethacin-dependent increase in normal CFU-C is due to PGE synthesis inhibition. The failure of CML CFU-C to respond to indomethacin seemed to indicate that they are insensitive to PGE concentrations that inhibit normal CFU-C. This finding was confirmed in a second series of experiments wherein exogenous PGE, was added to a culture system in which its endogenous production had been inhibited (Chart 2). It is evident that a significant difference in PGE sensitivity exists between normal and CML CFU-C. The similar morphological composition of normal and CML colonies suggests that the effect of PGE is unlikely to be due only to a different sensitivity of the granulocytic and macrophagic precursors.

Although circulating and tissue PGE levels are difficult to determine in vivo due to the lability of the molecule and its rapid clearance (7), the in vitro evidence points to the probable relevance of PGE in the regulation of granulopoiesis. CML CFU-C are insensitive over a wide range (3 logs) of PGE concentrations inhibitory for normal CFU-C. This fact may well be relevant to the pathogenesis of uncontrolled leukemic proliferation. It would seem that leukemic cells enjoy a proliferative advantage with a mechanism that may be presumed to come into action soon after the leukemogenic event when few leukemic cells are present. Moreover, as prostaglandins are regulatory molecules in many tissues, an altered responsiveness to PG regulation may also play a role in the pathogenesis of other myeloproliferative disorders and of solid tumors.

**ACKNOWLEDGMENTS**

We thank the cardiac surgery team of Professor M. Morea for providing normal bone marrow samples.

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