CONCISE REPORT

Stimulation of Proliferation of Human Myeloid Leukemia Cells in Culture: Applications for Cytogenetic Analysis

By Joseph Michaeli, Israela Lerner, Eliezer A. Rachmilewitz, and Eitan Fibach

The use of chromosome banding techniques has provided a valuable diagnostic tool in various malignancies. The application of these methods, however, is often restricted by a low yield of mitotic cells and the patient’s unwillingness to comply with repeated bone marrow aspiration. In an attempt to promote mitotic activity of leukemic cells from the bone marrow and peripheral blood, we employed a new method based on culturing the cells in the presence of a conditioned medium derived from a human bladder carcinoma cell line (5637). In addition to colony stimulating factor, this conditioned medium contains a factor that is capable of stimulating leukemic myeloblast proliferation. Bone marrow and peripheral blood mononuclear cells from 58 patients with a variety of myeloid leukemias were cultured for 24 to 120 hours in the presence or absence of conditioned medium. These bone marrow cells showed a pronounced increase in the mitotic index (5- to 50-fold) as compared to unstimulated cultures, and a greater than 100-fold increase as compared to fresh, uncultured bone marrow cells. Analyzable metaphases could be obtained even in marrow samples in which direct or 24-hour G-banding techniques had failed to reveal metaphases. The effect observed on peripheral blood cells was even more dramatic because prior to culture no mitotic cells were detected, whereas up to 2% mitotic cells were found in conditioned medium–stimulated peripheral leukemic cells. Karyotype analysis of 36 out of the 58 leukemic patients has shown that the chromosome changes discovered in conditioned medium stimulated cells were identical to those found in unstimulated cells. New chromosome aberrations, attributable to the stimulation of growth by conditioned medium, were not found. The quality of the metaphases analyzed following conditioned medium stimulation was considerably better than that of unstimulated samples. Frozen cells, when cultured with conditioned medium, were also suitable for cytogenetic analysis. Thus, the use of this conditioned medium permits adequate cytogenetic analysis even in cases where such analysis was previously impossible.

A LTHOUGH CYTOGENETIC STUDIES have become a valuable diagnostic tool for leukemic patients, their use is often restricted by two major obstacles: the paucity or complete absence of dividing cells, rendering the bone marrow (BM) samples inadequate for analysis, and patient unwillingness to comply with repeated BM aspirations. Recently, we have demonstrated that a conditioned medium (CM) derived from cultures of a human urinary bladder carcinoma cell line (5637) is capable of stimulating the proliferation and growth of human myeloid leukemic cells (in preparation).

In an attempt to promote mitotic activity of leukemic cells and to permit cytogenetic analysis of peripheral mononuclear cells, we have employed a new method based on karyotype analysis of BM and peripheral blood (PB) leukemic cells cultured in the presence of this CM.

MATERIALS AND METHODS

Patients. The effect of the CM on the mitotic activity was determined in cultures of cells obtained from 58 patients with acute and chronic myeloid leukemia and the myelodysplastic syndrome. Informed consent was obtained from all patients. Detailed karyotype analysis was performed on 36 of these patients [15 with myelodysplastic syndrome, 11 acute myelogenous leukemia (AML) and 10 chronic myelogenous leukemia (CML)]. Cells from patients with myelodysplastic syndrome or acute leukemia were studied prior to initiation of chemotherapy. Three patients with acute lymphatic leukemia (ALL) and six normal controls were also studied. The type of leukemia or myelodysplastic syndrome was classified according to the French-American-British criteria, following a review of Wright’s stained PB smears, BM aspirates, and bone core biopsies. Cytocchemical studies were also performed in all cases.

Cultures. Blood or marrow samples were collected in preservative-free heparin. In most cases they were processed immediately, and in other cases they were left overnight at room temperature before further processing. The mononuclear cell fraction was isolated using Ficoll-Hypaque density gradient centrifugation. Cells from the interphase band were washed twice in phosphate buffered saline and cultured at 2.5 to 5 × 10⁶ cells/mL in alpha minimal essential medium supplemented with 10% fetal calf serum (FCS) (GIBCO, Grand Island, NY) either in the presence or absence of 10% (vol/vol) CM. The cultures were incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C.

In some experiments human myeloid leukemic cells which had been stored were also studied. The cells were frozen as follows: PB and BM cells were isolated by Ficoll-Hypaque density gradient centrifugation, washed twice and resuspended at 2 to 4 × 10⁷/mL in alpha medium supplemented with 10% FCS and 10% dimethylsulfoxide. One-mL aliquots were divided into 48 × 12.5-mm polystyrene tubes (Nunc, Roskilde, Denmark) and frozen at 1 °C/min in a Linde CRFC-1 + CRC freezing units (Union Carbide, Indianapolis, Ind). Tubes were stored in liquid nitrogen. Before use, cells were thawed rapidly in a 37 °C water bath and washed twice in medium. Viable cells were assessed using trypan blue exclusion and cultured as described for fresh cells.

Determination of mitotic index. Since the percentage of mitotic cells in both fresh and cultured cells was usually <1%, we have utilized the following method for more accurate scoring. Approximately one million cells were treated with a hypotonic solution (0.075 mol/L KCl) for ten minutes and then fixed for ten minutes in a mixture of methanol:acetic acid (3:1). Following centrifugation,

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the number of cells (mitotic index) was calculated by dividing a light microscope (x 250 magnification). The percentage of mitotic counting on the hemocytometer. Using this procedure it was possible to score the mitotic cells in a population of 10^4 to 10^5 cells and to determine the number of mitotic cells even when at concentrations as low as 0.01%.

Cytogenetic analysis. Samples were analyzed following 24 to 120 hours in culture in the presence or absence of CM. Cultured for 24 hours were synchronized with methotrexate. Chromosome preparations were tryptic G-banded.

Preparation of conditioned medium. The human bladder carcinoma cell line (5637)^8 was provided by Dr. J. Fogh, Human Tumor Cell Line Bank, Sloan Kettering Institute for Cancer Research, Walker Laboratory, Rye, NY and was subcloned in our laboratory. One subclone was passaged routinely every 2 weeks in alpha medium supplemented with 20% FCS. For production of CM, cells were seeded at 5 x 10^5/75 cm^2 culture flask in 50 mL medium and serum.

Results

The mitotic indices of PB and BM leukemic cells of representative cases prior to culture and after four days of culture in the presence or absence of CM of representative cases are shown in Table 1. Direct examination of PB cells from patients with either chronic or acute myeloid leukemia failed to reveal mitotic cells, even when patients had over 100 x 10^9/L circulating blasts. The mitotic index of normal or leukemic fresh BM cells was <0.01%. Although mitotic cells were present in most unstimulated marrow leukemic samples, the mitotic yield was considerably higher (5- to 50-fold) in CM-stimulated BM cultures. Moreover, ample analyzable metaphases could be obtained in several marrow samples in which direct or 24-hour G-band methodology had failed to reveal metaphases. Mitotic cells of CM-stimulated BM and PB samples were evident on day 1 and their number progressively increased during the following days (Fig 1). The rate of this increase, however, varied. In some patients it was evident as early as 24 hours following stimulation, whereas in others a longer period of culturing (48 to 120 hours) was required (Fig 2). When added to samples obtained from normal controls and patients with ALL, CM had no stimulatory effect during this culture period.

The karyotype analysis of 36 CM-stimulated samples whether originated from PB or BM was virtually identical to the karyotype of the unstimulated samples. Nineteen patients exhibited one or more chromosomal abnormalities before stimulation. The same abnormalities were rediscoversed in the CM-stimulated samples. One patient with myelodysplastic syndrome exhibited the following complex abnormalities: 44XX;IMS;13q-;18q-;4p- and two marker chromosomes. These abnormalities were also detected following stimulation. Fourteen patients had a normal karyotype both in CM-stimulated and unstimulated cells. In two patients with CML, the typical Philadelphia chromosome could be detected in PB cells only after CM stimulation, since no mitotic cells were found prior to culture. In one patient with AML, analysis of unstimulated samples revealed 18 mitotic cells with normal karyotype, whereas analysis following 24-hour stimulation with CM showed that 20% of the dividing cells exhibited an abnormal karyotype (40xy;12p-).

CM effectively stimulated the growth of cells from freshly isolated samples, as well as cells derived from samples which were left overnight at room temperature. In addition, myeloid cells stored for 2 to 5 months in liquid nitrogen and thawed and cultured with CM were also suitable for cytogenetic analysis. Ten frozen samples were examined, three

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<tr>
<th>Mitotic Index of Marrow and Peripheral Blood Cells</th>
<th>Before and After Culture</th>
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<td>Diagnosis</td>
<td>Source</td>
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<tr>
<td>CML PB</td>
<td>&lt;0.01</td>
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<tr>
<td>BM</td>
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<td>CML-BC PB</td>
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<tr>
<td>AMML PB</td>
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<td>BM</td>
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Cells were cultured for 4 days with or without 10% 5637-CM.
Fig 2. The mitotic index of peripheral blood myeloid leukemic cells in culture. Peripheral blood cells from three patients with AML were isolated on Ficoll-Hypaque and cultured either in the presence (full symbols) or absence (empty symbols) of 10% CM.

acute myelogenous leukemia (AML) (BM and PB cells), one acute promyelocytic leukemia (APL) (PB), one chronic myelogenous leukemia (CML) (BM and PB) and one CML in blast crisis (PB). The mitotic index of these cells was 0.1% to 1% within five days of culture, whereas the mitotic index of unstimulated cells was <0.01%. In patients with APL and CML, the characteristic translocations t(15:17) and t(9:22) were found even after the samples were frozen for an extended period (up to 5 months). The quality of metaphases found in both PB and BM samples was considerably better in CM-stimulated cells (Fig 3).

DISCUSSION

The recognition and identification of subtle chromosomal changes in leukemic cells has greatly been facilitated since the advent of high-resolution banding techniques. However, efficient utilization of these methods is often hampered by the inadequate mitotic activity of the leukemic cells, and by the inconvenience of repeating marrow aspirations. An additional drawback stems from the uncertainty as to whether these methods detect chromosomal changes which are exclusively representative of the affected malignant clone. In an attempt to circumvent some of these difficulties and to increase mitotic activity, we have performed cytogenetic analysis of human myeloid leukemic cells cultured in the presence of a CM which is derived from a human bladder carcinoma cell line (5637). A pronounced increase in the mitotic index of both PB and BM myeloid cells was found. Moreover, this culture technique substantially improved the ability to obtain analyzable metaphases even in samples in which direct or 24-hour G-banding methods had failed to reveal metaphases. This was relevant for BM, and particularly for PB cells, since their mitotic index prior to culture was consistently very low. The quality of metaphases in CM-stimulated samples, whether derived from BM or PB, was also improved (Fig 3). In addition, the ability to perform studies on frozen myeloblasts stimulated by CM was comparable to that of freshly isolated BM or PB cells. This method, therefore, may provide a useful aid for experiments where retrospective analysis of chromosomal changes might be needed.

Although we found that the CM was highly effective in enhancing cell proliferation, the rate of increase in the mitotic activity was not uniform. In some patients, maximal stimulation was noted shortly after incubation, whereas in others exposure for a longer period was necessary. It is advisable, therefore, that samples of CM-stimulated cells be evaluated repeatedly within 24 to 120 hours following incubation. The procedure described in this study for quantitative assessment of the mitotic index may therefore be valuable in determining the exact timing of analysis.

Although 5637-CM contains colony stimulating factors supporting the development of normal myeloid colonies in
semisolid culture, it has only a negligible effect on the mitotic index of normal bone marrow cells in short term (1- to 5-day) liquid culture (Table 1). Rather it is likely that this CM contains a growth factor which is distinct from colony stimulating factor, and which specifically stimulates human myeloid leukemic cells (manuscript in preparation).

In addition to the unresponsiveness of normal cells to stimulation by CM in short term culture, we have shown that when samples which contained a chromosomal abnormality in all dividing cells were subsequently stimulated by CM, no enrichment of cytogenetically normal cells was found. It is therefore likely that the chromosomal analysis of CM-stimulated cells was confined to the malignant cell population.

Our study indicates that the chromosomal pattern of leukemic cells stimulated by CM for up to five days was consistent with that of the unstimulated cells. This was confirmed both when leukemic cells without detectable chromosomal abnormality and when cells exhibiting abnormal karyotypes were stimulated.

In one AML patient, however, a chromosomal aberration (46 xy; 12P-) could be detected only following stimulation with CM. In view of the fact that this deletion is known to occur nonrandomly in myeloid leukemias,9 it seems that it represented an existing malignant clone that was exposed following stimulation by CM, rather than a new culture-induced alteration.

Although prolonged growth in culture may induce chromosomal changes, it would be of interest to culture for longer periods and to determine whether this may ultimately lead to the selection of new malignant clones. It would also be intriguing to explore the origin of such additional clones, whether they are merely culture artifacts or are truly representative of the patient karyotype. If the latter proves true, then the use of this CM for an extended duration may have prognostic value, as it may presage the clinical emergence of new malignant clones.

The determination of the percentage of cytogenetically normal cells in samples exhibiting mosaicism is also of prognostic importance, although this notion has recently been disputed in CML.10 We are currently attempting to address this question by determining the proportions of cytogenetically normal and abnormal cells in CM-stimulated and unstimulated cells. Although in some patients we were able to detect more abnormal metaphases following stimulation, our data are still insufficient for final conclusions. We also intend to study whether the use of this CM or growth factor, by virtue of its specificity, will help detect minimal residual leukemic cells in patients attaining clinical remission.

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