

# Serial In Vitro Bone Marrow Fibroblast Culture in Human Leukemia

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Human fibroblast colony formation from bone marrow was performed in liquid culture. Fetal calf serum was used as a stimulator of the fibroblast colony formation. The colony formation took place not only in normal donors, but also in patients with acute leukemia and chronic myelocytic leukemia. At the diagnosis of the disease, significant colony suppression was observed in most cases of acute leukemia, while the number of colonies increased in half of the cases of chronic myelocytic leukemia. However, there was no correlation between the colony-forming efficiency and the initial

number of peripheral platelets or bone marrow megakaryocytes that contained growth-promoting factor. The number of colonies increased after chemotherapy, recovered at the stage of complete remission, and then decreased to low levels at relapse in the patients with acute leukemia; it decreased after treatment with busulfan in the patients with chronic myelocytic leukemia. This fibroblast culture method is useful for counting fibroblast colony-forming cells in the bone marrow of human leukemia.

CLONING of fibroblasts from bone marrow has been described of late in rodents<sup>1-5</sup> and somewhat more recently in humans.<sup>6-9</sup> A liquid culture system has been used to clone and to characterize human bone marrow fibroblast colony-forming cells (CFU-F). The linear relationship between the number of cells plated and the number of colonies formed suggests that fibroblast colonies originate in a single cell.<sup>6,7</sup>

There is some evidence indicating that marrow fibroblasts play a role in the regulation of hematopoiesis.<sup>5,8</sup> However, little is known about the quantitative observation of CFU-F in patients with hematologic diseases. Bone marrow fibrosis may be primary or secondary. Myelofibrosis associated with acute leukemia or chronic myelocytic leukemia is considered to be a secondary phenomenon, with a clonal proliferation of hematopoietic cells.<sup>10-12</sup> The in vitro assay system for the quantitative and qualitative changes of CFU-F will permit detection of one of the earliest events occurring in the establishment of myelofibrosis and study of the microenvironmental influences on the hematopoietic components in patients with hematologic malignancies.

Although several reports have described the cloning efficiency of CFU-F obtained from normal subjects, attempts to study the cloning efficiency of those from leukemic patients are rare. We devised a simplified method of fibroblast colony formation from human bone marrow<sup>6</sup> and demonstrated that bone marrow from most patients with acute leukemia grew only a small number of colonies or failed to produce any growth at all.<sup>6</sup> This report is concerned with serial studies of the quantitative changes of bone marrow CFU-F in patients with acute leukemia and chronic myelocytic leukemia in liquid culture as well as the relationships between initial hematologic findings and their CFU-F.

## MATERIALS AND METHODS

### Fibroblast Colony Assay

The method of collection of human bone marrow and the liquid culture method are as previously described.<sup>6</sup> Briefly, 35 × 10 mm

plastic dishes (Falcon Plastic, New York) were prepared by adding 2 ml of RPMI 1640 containing 20% fetal calf serum and  $2 \times 10^5$  bone marrow nucleated cells. Three dishes were plated per sample. The cultures were incubated for 7 days in humidified 5% CO<sub>2</sub> at 37°C. The culture medium was not changed during the incubation period. The serial marrow samples were plated with the same lots of fetal calf serum. Aggregates containing 6 or more fibroblasts were scored as colonies.

### Normal Controls

Bone marrow was obtained from 10 normal healthy individuals. These controls, who were 20–40 yr old, had peripheral leukocyte counts within the normal range (5000–10,000/cu mm) with normal cell differential counts. The marrow was normally cellular on smear, with normal M:E ratios.

### Patient Populations and Chemotherapy

All patients were under care at Tokai University Hospital. Acute leukemia was confirmed by the findings of the peripheral blood and bone marrow. Each type of acute leukemia was diagnosed by May-Giemsa stain and the cytochemical method. Chronic myelocytic leukemia was confirmed by the findings of the peripheral blood, bone marrow, alkaline phosphatase of neutrophils, and Ph<sup>1</sup> chromosomes of a bone marrow myeloid series. The patients' blood counts and bone marrow findings are shown in Tables 1 and 2. Marrow from patients with acute leukemia and chronic myelocytic leukemia were serially cultured.

Patients with acute leukemia were treated with two major intensive combination chemotherapy protocols. The protocol for acute nonlymphocytic leukemia consisted of a combination of daunorubicin (30 mg/sq m intravenously), neocarzinostatin (1500 U/sq m intravenously), cytosine arabinoside (60 mg/sq m intravenously) and prednisolone (50 mg/sq m intravenously) given for 4 days. The schedule was repeated when normal bone marrow elements recovered. The protocol for acute lymphocytic leukemia consisted of a combination of vincristine (1.5 mg/sq m on day 1), daunorubicin (30 mg/sq m × 4), and prednisolone (50 mg/sq m × 4). This course was repeated upon recovery of normal bone marrow cellularity. Patients

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**Table 1. Hematologic Characteristics, Age, and In Vitro Fibroblast Growth in Acute Leukemia**

Type	No.	Age	WBC/cu mm $\times 10^3$	Platelets/cu mm $\times 10^4$	Hb (g/dl)	Percent Blasts		Fibroblast Colonies/ $2 \times 10^5$
						Blood	Marrow	
Normal control	10	32 $\pm$ 5*						46 $\pm$ 7
AML	16	58 $\pm$ 11	31.6 $\pm$ 57.0	4.3 $\pm$ 3.2	7.7 $\pm$ 3.0	56 $\pm$ 34	74 $\pm$ 24	16 $\pm$ 26
APL	4	46 $\pm$ 8	16.2 $\pm$ 9.2	2.3 $\pm$ 0.6	7.4 $\pm$ 0.8	66 $\pm$ 36	95 $\pm$ 4	15 $\pm$ 6
AMML	2	48 $\pm$ 8	18.1 $\pm$ 2.7	6.0 $\pm$ 0.2	7.5 $\pm$ 0.9	53 $\pm$ 35	72 $\pm$ 26	28 $\pm$ 7
AMoL	3	50 $\pm$ 16	39.1 $\pm$ 34.7	5.7 $\pm$ 4.8	8.8 $\pm$ 0.5	39 $\pm$ 20	77 $\pm$ 16	7 $\pm$ 5
Erythro leukemia	2	40 $\pm$ 15	33.4 $\pm$ 19.6	18.2 $\pm$ 15.4	8.5 $\pm$ 1.3	24 $\pm$ 4	42 $\pm$ 7	10 $\pm$ 8
ALL	8	38 $\pm$ 15	27.7 $\pm$ 35.3	5.9 $\pm$ 7.8	9.3 $\pm$ 2.9	64 $\pm$ 27	89 $\pm$ 16	16 $\pm$ 15

\*Mean  $\pm$  SD.

with chronic myelocytic leukemia were treated with busulfan (2 mg/sq m orally). The drug was given until the peripheral blood leukocyte count reached 20,000/cu mm. In the patients with acute leukemia, bone marrow samples for in vitro culture were obtained before chemotherapy and just prior to the next sequential course of chemotherapy, usually from 10 to 28 days after the preceding chemotherapy course. In the patients with chronic myelocytic leukemia, marrow samples were obtained before chemotherapy and serially thereafter.

Complete remission in the patients with acute leukemia was achieved when the neutrophil counts had risen to 1500/cu mm or more, the platelet counts to at least 100,000/cu mm, and bone marrow aspiration showed a normocellular marrow with less than 5% blasts.

### Statistical Analysis

Data were compared by the analysis of variance and the Student's *t* test. Correlation was tested by the nonparametric Spearman's test.

## RESULTS

### In Vitro Growth Characteristics in Acute Leukemia

The data from serial studies of patients with acute leukemia and from normal controls are shown in Fig. 1. The incidence of CFU-F in normal marrow was 46  $\pm$  7 (SD) colonies per  $2 \times 10^5$  cells. Although the incidence of CFU-F among 35 patients with acute leukemia before chemotherapy varied over a very wide range, it was significantly decreased compared to

normal marrow ( $p < 0.005$ ). Nine patients failed to produce fibroblast colonies, and 21 had a greatly reduced incidence of CFU-F. Three patients showed a normal colony number, and two showed a larger colony number than normal bone marrow. In vitro colony culture was studied among 20 patients during chemotherapy, among 18 patients at complete remission, and among 8 patients at relapse. In most cases, the colony number was significantly increased after chemotherapy as compared with that at diagnosis ( $p < 0.05$ ), recovered to within almost normal range during complete remission, and significantly decreased at the stage of relapse as compared with that during remission ( $p < 0.01$ ). There was no correlation between the number of CFU-F and survival time (data not shown).

The number of colonies in the different morphological variants of acute leukemia is shown in Table 1. No correlation was found between the hematologic findings and the efficiency of colony formation. Figure 2 shows in vitro growth characteristics and incidence of different morphological types of acute leukemia. The incidence of nongrowing colony formation was the highest in AML. A high incidence of less than normal colony formation was observed in patients with APL, AMoL, erythroleukemia, and ALL.

**Table 2. Hematologic Characteristics and In Vitro Fibroblast Growth in Chronic Myelocytic Leukemia**

Case	Age	Sex	WBC/cu mm $\times 10^4$	Platelet/ cu mm $\times 10^4$	Hb (g/dl)	Marrow Megakaryocyte/cu mm	Fibroblast Colonies/ $2 \times 10^5$	
							At Diagnosis	After Chemotherapy
1	59	M	8.2	68.8	12.1	300	62 $\pm$ 5*	28 $\pm$ 3
2	70	F	2.2	37.3	11.7	80	66 $\pm$ 4	4 $\pm$ 0.5
3	55	M	3.8	34.8	13.3	75	126 $\pm$ 8	47 $\pm$ 6
4	41	M	7.2	18.8	12.3	210	41 $\pm$ 3	15 $\pm$ 2
5	55	F	4.4	34.4	11.6	135	6 $\pm$ 1	4 $\pm$ 0.8
6	55	F	10.0	122.4	9.9	495	77 $\pm$ 5	13 $\pm$ 3
7	61	M	3.2	84.6	12.2	990	84 $\pm$ 6	12 $\pm$ 2
8	39	M	30.2	61.8	11.6	915	10 $\pm$ 2	3 $\pm$ 0.5
9	23	M	27.3	51.4	12.2	150	31 $\pm$ 4	5 $\pm$ 0.8
10	66	M	2.8	91.0	11.9	1935	13 $\pm$ 2	2 $\pm$ 0.3

\*Mean  $\pm$  SD.

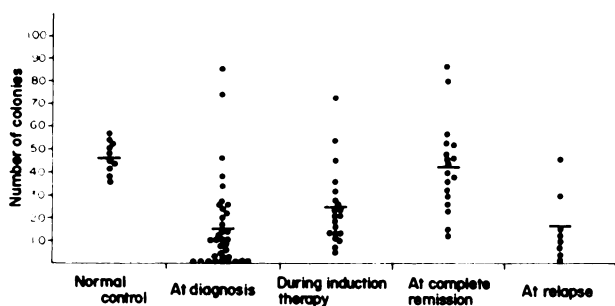


Fig. 1. Serial studies of CFU-F in patients with acute leukemia. Each culture contains  $2 \times 10^5$  bone marrow cells. Horizontal bars are mean values. Each point is a mean value from 3 culture dishes.

*In Vitro Growth Characteristics in Chronic Myelocytic Leukemia*

The hematologic characteristics and the incidence of CFU-F in the patients with chronic myelocytic leukemia are shown in Table 2. The patients were studied at diagnosis and 1-3 mo after busulfan therapy. In 5 of 10 patients, the incidence of CFU-F was increased to more than normal. In two, the culture was within normal range, and in the other three, decreased to less than normal. No correlation was found between the WBC count, platelet count, or megakaryocyte count and the efficiency of colony formation. The number of CFU-F was decreased after busulfan therapy as compared with that at diagnosis ( $p < 0.05$ ).

DISCUSSION

The occurrence of secondary myelofibrosis is common in patients with chronic myelocytic leukemia, and it is not uncommon in those with acute leukemia. Hann et al.<sup>12</sup> reported that secondary myelofibrosis was present at diagnosis in 57% of 40 children with acute lymphoblastic leukemia. Kundel et al.<sup>13</sup> showed that reticulin fibrosis was found in 53% of 40 patients with

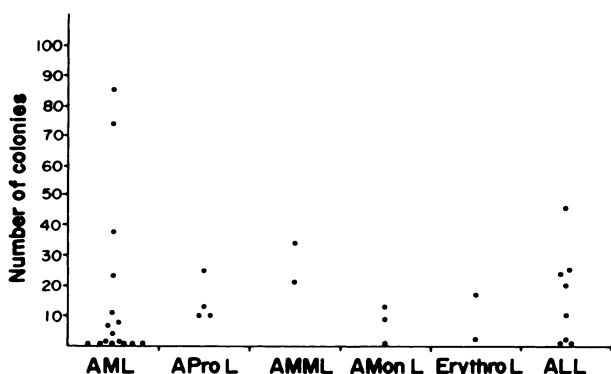


Fig. 2. Number of fibroblast colonies in different morphological types of acute leukemia. Each culture contains  $2 \times 10^5$  bone marrow cells. Each point is a mean value from 3 culture dishes.

acute lymphoblastic leukemia. Clough et al.<sup>14</sup> observed that a significant number of patients with chronic myelocytic leukemia showed myelofibrosis, which was indistinguishable histologically from that found in agnogenic myeloid metaplasia at diagnosis, and that many cases that showed a progressive myelofibrosis were often supervened by accelerated disease or blast crisis. However, the pathogenesis of bone marrow fibrosis in these diseases is unknown. The bone marrow fibrosis in such diseases probably arises by way of different mechanisms. Fibroblasts produce the silver-staining collagen that replaces normal marrow stroma in disorders with myelofibrosis. We were interested in the bone marrow fibroblasts and aimed to clarify the genesis of marrow fibroblast proliferation in acute leukemia and chronic myelocytic leukemia with myelofibrosis. In the current study, fibroblast colonies were decreased in 30 of 35 patients with acute leukemia and increased in 5 of 10 patients with chronic myelocytic leukemia at the time of initial diagnosis. From these results, it is considered that the bone marrow fibrosis is due to the increase of the CFU-F number in some patients with chronic myelocytic leukemia. However, the genesis of bone marrow fibrosis in acute leukemia cannot be explained by the CFU-F number.

Observations of bone marrow fibroblast colony-forming cells in leukemia are very few.<sup>6,8</sup> Greenberg et al.<sup>8</sup> reported that the mean values of CFU-F for the normal and leukemic groups were similar, though a much greater range in cloning efficiency was obtained for the leukemic group. Our results concerning acute leukemia do not agree with the observations of Greenberg et al.<sup>8</sup> This discrepancy may be related to the different culture conditions, counting stages, and colony criteria.

The pathogenesis of myelofibrosis in some cases involves the intramedullary release of a newly described hormone, the platelet-derived growth factor, contained in the alpha granules of platelets and mitogenic for a variety of mesenchymal cells, including fibroblasts. Recently, Castro-Malaspina et al.<sup>15</sup> have demonstrated that human megakaryocytes are a source of the growth factor derived from platelets and that they may play a role in the pathogenesis of the marrow fibrosis observed in myeloproliferative disorders by stimulating fibroblast and collagen secretion. In our study, a comparison of the colony-forming efficiency and hematologic findings such as RBC, WBC, platelets, leukemic content, or type of acute leukemia failed to show a correlation in the patients with acute leukemia. Nearly every patient with chronic myelocytic leukemia had high platelet counts in the peripheral blood and high megakaryocyte counts in the

bone marrow. However, no correlation was found between the colony-forming efficiency and the platelet or megakaryocyte counts. It is considered that, except for platelets or megakaryocytes, there may be several factors that influence the efficiency of in vitro fibroblast colony formation. By comparing the number of CFU-F with the findings of bone marrow biopsy, the significance of fibroblast colony assay will be clarified.

When clinical remission was attained in the patients with acute leukemia and leukemic blasts were reduced to 5% or less, the colony-forming capacity returned to normal. It decreased again at the relapse stage. These results provide evidence that the leukemic blasts interfere with the colony-forming activity of CFU-F or that

CFU-F failed to be formed from an abnormal marrow precursor and further that the fibroblast colony-forming efficiency of leukemic bone marrow is related to disease status. A study concerned with fibroblast colony suppression by leukemic blasts is now in progress.

The finding that the number of colonies was decreased after therapy with busulfan in the patients with chronic myelocytic leukemia is of some interest. Moore et al.<sup>16</sup> reported that the number of CFU-F in the peripheral blood was decreased after busulfan therapy. Our data concerned with the influence of busulfan therapy on fibroblast colony formation are similar to their results. These findings suggest the suppressive effect of busulfan on fibroblast colony-forming cells in the bone marrow.

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