Granulopoietic Effects of Human Bone Marrow Fibroblastic Cells and Abnormalities in the “Granulopoietic Microenvironment”

By Bernard R. Greenberg, Floyd D. Wilson, and Linda Woo

The in vitro granulopoietic effects of adherent bone marrow fibroblastic cells (FC) were studied in normal humans and in patients with acute myelogenous leukemia (AML) and myeloproliferative disorders (MPD). To determine their influence on granulopoiesis, we established FC in liquid-phase cultures, overlaid the adherent FC with normal bone marrow cells in agar, and subsequently measured the growth of CFU-C. When using target marrows containing few spontaneous colonies, increased numbers of CFU-C were found above the FC obtained from normals. No growth greater than controls was observed in those areas lacking FC. If target marrows contained large numbers of spontaneous CFU-C, actual inhibition of colony formation was produced by FC co-incubation. In contrast to normals, FC obtained from untreated AML and MPD patients typically failed to enhance granulopoiesis. Regardless of source, FC were not synergistic with the effects of placenta-conditioned media (typically being inhibitory) for colony number, but were synergistic for colony size. Conditioned media obtained from FC cultures did not enhance colony formation and actually inhibited spontaneous colony formation. Thus, microenvironmental abnormalities in interactions between “stromal cells” and hematopoietic progenitors may be important in the pathogenesis and clinical expression of hematopoietic malignancies in humans.

The demonstration in mice that the long-term in vitro persistence of hematopoietic stem cells is absolutely dependent on the presence of bone marrow adherent cells has stimulated a renewed interest in defining the role of “stromal elements” in the regulation of hematopoiesis.1-4 Recent reports have also described the long-term maintenance of human CFU-C and presumptive stem cells (pre-CFU-C) in cultures containing appropriate populations of adherent cells.5-6

Although a major function of hematopoietic stroma has been proposed in rodents,7-10 knowledge of a similar function of stroma has been lacking in humans. Myelofibrosis, however, which occurs relatively frequently in humans in association with various myeloproliferative disorders, most certainly represents a pathologic alteration in hematopoietic stromal elements. A recent example of the involvement of abnormalities in hematopoietic microenvironment in human disease was provided by the demonstration of a functional defect in the erythroid microenvironment in a patient with aplastic anemia.11

While most studies in stromal cell microenvironmental influences have concentrated on the hematopoietic components in long-term culture systems, relatively few studies have focused on the adherent cell components of such cultures. The majority of studies published to date indicate that bone marrow fibroblasts are not related to hematopoietic stem cells,12-14 nor will they engraft during usual conditions of transplantation.15-17

Employing relatively “short-term” semisolid18 and liquid culture systems,19 we have been investigating the origin,12,13,15 structure,13,20,21 cyogenetic composition,12,13,15,18 and functional22 capacities of the human bone marrow fibroblastic cells (FC), which represent one distinct component of the adherent cell complex (ACC). Our studies on human FC have been supported by numerous studies in experimental animals.23-26

In our previous studies, we demonstrated that the Philadelphia (Ph') chromosome is absent in the fibroblastic colonies (FC) obtained from Ph'-positive CML patients12,13 and that FC are not transplanted along with hematopoietic stem cells.15 This is in agreement with the work of Castro-Malaspina et al.,14 who demonstrated the absence of the la antigen in bone marrow fibroblast colony-forming cells. We have also recently shown that bone marrow FC are cytogenetically distinct from skin fibroblasts in patients with Fanconi's anemia.27

We have previously reported on the ability of FC to enhance the growth of CFU-C in rodents25 and canines.26 In the studies reported herein, we have investigated the effects of FC obtained from normals and from patients with various granulopoietic disorders on the growth of CFU-C. In addition, we studied whether these effects were similar to or synergistic with the effects of colony-stimulating factor (CSF).
MATERIALS AND METHODS

Liquid Culture for FC

The liquid culture system we use represents a modification of the technique described by Friedenstein et al. Bone marrow cells were obtained by aspiration from the posterior iliac crest of normal volunteers and patients with acute myelogenous leukemia (AML) and myeloproliferative disorders (MPD) (myelofibrosis, essential thrombocytopenia, and splenomegaly vera). Five million viable nucleated cells were placed into 75 cm² tissue culture flasks containing 10 ml supplemental MEM (GIBCO, Grand Island, N.Y.), 1% L-glutamine (GIBCO), and 1% antibiotic-antimycotic (GIBCO). The cells were incubated in a 37°C, 7% CO₂, humidified environment. Following 24-28 hr of incubation, the supernatant and nonadherent cells were decanted, fresh medium added, and the cultures incubated for an additional 5-7 days. The cells were then examined microscopically for the presence of surface-related fibroblastic colonies. Upon reaching approximately 75% confluency, cultures were passed with a 0.1% protease solution until a sufficient number of cells of a morphologically "pure" population of fibroblastic elements (FC) was obtained. FC were maintained in culture from 1 to 4 mo and were only studied after the fourth or fifth passage of the influences of the presence or absence of FC on CFU-C in normal cultures incubated for growth of FC for 2-10 wk as described above. Previous studies using nonspecific esterase staining have demonstrated that it is possible to obtain 95% or greater purity of FC using this culture procedure.

In patients who were inapplicable, a biopsy was obtained with a Jamshidi needle. A portion of the biopsy specimen was minced using sterile scissors and a 3-cc syringe containing complete MEM. The specimen was teased and washed in complete MEM until only fragments remained. These fragments were placed on the bottom of a flask that was incubated on its side for 1-3 hr to allow the fragments to become adherent. The flask was subsequently set upright and incubated following the further addition of complete MEM.

In additional experiments, 2 × 10⁵ viable nucleated cells were placed into 75 cm² tissue culture flasks containing 10 ml alpha medium (GIBCO) supplemented with 20% fetal calf serum. These flasks were incubated for 30 min, the supernatant and nonadherent cells discarded, fresh medium added, and the remaining adherent cells incubated for growth of FC for 2-10 wk as described above.

Stimulation of Granulopoiesis

The ability of the FC to stimulate granulopoiesis was determined using a refill technique. The surface-adherent FC were removed from the flasks with protease, resuspended in supplemented MEM, and reseeded into 35-mm culture dishes at a concentration of 5 × 10⁴ FC/dish. In some experiments, additional cultures were prepared in which 5 × 10⁴ FC were carefully pipetted onto only one side of each dish. The entire tray containing these dishes was then tilted at an angle in the incubator, permitting the FC to adhere to only one-half of each dish. Thus, when such preparations were overlaid with agar containing fresh marrow targets, only half the surface contained adherent fibroblasts, allowing for direct observations of the influences of the presence or absence of FC on CFU-C in the same culture plate. Following overnight incubation, the medium above the FC was removed and the dishes refilled with 1 ml of agar medium containing 10³ fresh normal bone marrow cells. In additional experiments, adherent cells were removed from the bone marrow by the method of Messner et al. Prior to suspension in the agar medium. Only nonadherent marrow cells were employed in these additional refill experiments. The remaining adherent cells received complete MEM and were observed for FC growth. Following 14 days of incubation, colonies (composed of 40 or more cells) and clusters (composed of 20-40 cells) were counted with an inverted microscope. Control cultures consisted of bone marrow cells in agar medium in plates without FC.

To determine whether the granulopoietic effects of FC were synergistic with traditional sources of colony-stimulating factor (CSF), placenta-conditioned media (PCM) was added, at optimal strength, to the above cultures on days 0, 4, 8, and 11 of incubation.

To determine the granulopoietic effects of the supernatant from the FC cultures, only flasks with greater than 75% confluency were used. At this time, the medium in the flasks was discarded and replaced with fresh medium. Following 4 days, the FC-conditioned media was collected and frozen until just prior to use.

CFU-C

The in vitro growth of granulocyte-macrophage colonies (CFU-C) was assayed as previously described. Cultures consisted of 10⁴ nucleated bone marrow cells suspended in agar culture medium containing MEM alpha medium supplemented with 20% fetal calf serum and incubated in a humidified incubator at 37°C and 7% CO₂. The cells were either plated alone (control or spontaneous CFU-C) or with PCM added at optimal strength as a source of CSF. FC-conditioned media was also tested with this system. Colonies of 40 or more cells and clusters of 20-40 cells were counted with a inverted microscope on day 14.

RESULTS

Adherent Cells Only

We have observed no formation of FC in cultures containing solely adherent cells, in spite of extending the period of incubation to 10 wk. Furthermore, removal of the initially adherent cells from the marrow did not significantly affect the subsequent growth of FC. Removal of adherent cells from the target marrow, however, did result in reduction of the effect of FC on CFU-C formation (Table 1). Human FC have been maintained for up to 12 mo with subculturing, after which time they usually become senescent. A detailed description of the ultrastructural appearance of human FC has been previously reported. We have observed that the FC are lipase negative and nonphagocytic, in contrast to macrophages, which are lipase positive and actively phagocytize latex particles.

Cloning Efficiency of FC

Our preliminary results for cloning efficiency of FC obtained from normals and patients with AML are

<table>
<thead>
<tr>
<th>Table 1. Effect of Removal of Adherent Cells on CFU-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-C/10⁴ Cells</td>
</tr>
<tr>
<td>Source of FC</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>AML (complete remission)</td>
</tr>
<tr>
<td>AML (complete remission)</td>
</tr>
</tbody>
</table>

From www.bloodjournal.org by guest on November 9, 2017. For personal use only.
Fig. 1. Summary of stimulatory and inhibitory granulopoietic effects of FC from various sources upon normal marrows with minimal (0–2 colonies/10^5 cells) or considerable (>9 colonies/10^5 cells) spontaneous (unstimulated) granulopoiesis. Each point represents the mean of usually four plates counted.

**Table 2. Cloning Efficiencies for FC**

<table>
<thead>
<tr>
<th>Subject</th>
<th>No. of FC/10^5 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals 1</td>
<td>27.2</td>
</tr>
<tr>
<td>2</td>
<td>26.5</td>
</tr>
<tr>
<td>3</td>
<td>14.5</td>
</tr>
<tr>
<td>4</td>
<td>20.0</td>
</tr>
<tr>
<td>5</td>
<td>20.3</td>
</tr>
<tr>
<td>6</td>
<td>14.5</td>
</tr>
<tr>
<td>Mean ± range</td>
<td>20.5 (14.5–27.2)</td>
</tr>
<tr>
<td>AML 1</td>
<td>11.0</td>
</tr>
<tr>
<td>2</td>
<td>12.0</td>
</tr>
<tr>
<td>3</td>
<td>25.5</td>
</tr>
<tr>
<td>4</td>
<td>28.0</td>
</tr>
<tr>
<td>5</td>
<td>12.0</td>
</tr>
<tr>
<td>6</td>
<td>38.0</td>
</tr>
<tr>
<td>Mean ± range</td>
<td>23.1 (11.0–38.0)</td>
</tr>
</tbody>
</table>

*At diagnosis or relapse.

presented in Table 2. Although the limited data do not allow for statistical analysis, the mean values for the normal and leukemic groups are quite similar, though a much greater range in cloning efficiency was obtained for the AML group.

**Granulopoietic Effects of Normal FC**

The results of the effects of FC from normal subjects on the growth of CFU-C are presented in Fig. 1 and Table 1. If the bone marrow target cells employed in the refill experiments contained few or no spontaneous (unstimulated) CFU-C, FC from normal subjects stimulated the growth of CFU-C to a variable extent. In the experiments with fibroblasts adhered to only one-half of the culture dish, we observed that the stimulation was local in nature, i.e., the vast majority of CFU-C were observed directly above the FC. In those areas lacking FC, few CFU-C were observed, suggesting an in vitro granulopoietic microenvironmental effect. Compared to the effects of PCM, FC stimulated fewer CFU-C and the range of colony number was greater. For a total of 10 normal marrows studied, a mean value of 18 (range 0–52.5) CFU-C was obtained with FC stimulation compared to a mean of 48 (range of 38–70) CFU-C when the marrows were stimulated by PCM. In contrast, when using target marrows containing relatively large numbers of spontaneous CFU-C (>9 colonies/10^5 cells), the FC usually inhibited CFU-C formation, i.e., fewer colonies were observed in cultures containing FC than in unstimulated control plates containing neither FC nor PCM.

**FC From Patients With AML and MPD**

FC from patients with AML (at diagnosis or relapse) and MPD usually did not stimulate the growth of CFU-C from marrow target populations containing few or no spontaneous CFU-C with one striking exception (Fig. 1). The FC from an unusual patient with AML who presented with overt extramedullary leukemia, including spinal cord compression, actively stimulated CFU-C to a degree exceeded by only one normal. Due to the atypical nature of both the clinical manifestations and the FC effects, this patient was analyzed separately. Of the more typical AML patients studied prior to therapy or in relapse, FC in 5 of 6 produced essentially no stimulation, while in one patient low normal activity was seen when employing target marrows containing few or no spontaneous CFU-C. The FC obtained from three patients with AML in complete remission, however, did produce substantial stimulation of CFU-C. We had the opportunity to sequentially study two of these patients and found that the FC obtained when they were in an early or overt relapse produced little or no stimulation of CFU-C (Table 3). The FC obtained from the three patients with MPD did not stimulate CFU-C (Fig. 1).
It is of interest that when considered as a group, FC from patients with AML and MPD stimulated CFU-C in target marrows containing few spontaneous colonies to a far lesser degree than did normals. Indeed, 10 of the 15 patients in the former group produced essentially no stimulation. Of the five patients producing relatively high stimulation, three were in complete remission of AML when studied and another presented as an atypical form of AML with prominent extramedullary involvement. Analysis of all typical AML and MPD patients yielded significantly lower values (p < 0.02 and < 0.05, respectively) than normals (Table 4). Although only a small number of patients with AML in complete remission were studied, their mean value of stimulated CFU-C was essentially the same as controls.

Similar to the FC effects in normals, FC from patients with AML and MPD inhibited granulopoiesis in marrow target populations containing large numbers of spontaneous CFU-C. The ability of FC obtained from three different normals and two patients with AML in complete remission to stimulate granulopoiesis was greatly diminished when adherent cells were removed from the target marrow (Table 1).

Studies on FC Plus PCM

The addition of PCM to FC on day 0 produced no synergistic effects. The number of CFU-C observed in cultures containing both FC and PCM was usually much less than found with PCM alone (Fig. 2). The results were similar with FC obtained from two normal subjects, two with AML in relapse, one with preleukemia, and another with MPD. When PCM was serially added to cultures containing FC and normal marrow cells on days 4, 8, and 11, the stimulation produced by PCM was greatest when added on day 4. At that time, the colonies observed were approximately three times larger than those without PCM, i.e., with FC alone. The increase in colony number was compatible with the expected effects of PCM and added effects, i.e., greater than PCM alone, were observed on only one occasion (Fig. 3). The FC from patients with AML markedly diminished the expected stimulation with PCM. The addition of PCM on day 4 had no significant influence on the number of clusters produced by FC stimulation alone (Fig. 4).

Effects of FC-Conditioned Media on CFU-C

Supernatants obtained from FC cultures were in all instances inhibitory to the growth of CFU-C. The addition of the supernatant from two normal FC cultures decreased unstimulated granulopoiesis to one-third of control, while the FC-conditioned media from cultures of two patients with AML and one with preleukemia had an even greater inhibitory effect (Fig. 5).

DISCUSSION

Several reports have described the proliferation of in vitro surface-adherent colonies or cells with mesen-
Fig. 3. Effect of colony number due to the addition of PCM on day 4 to cultures containing normal marrow cells and FC from normal subjects and patients with AML. NG, no growth; PCM, placenta-conditioned medium.

This present study was concerned with the functional capacities of human in vitro fibroblastic cells (FC) as they relate to granulopoiesis. A somewhat unexpected finding was that although fibroblastic colonies and FC are extremely adherent, their progenitors are initially nonadherent. Cultures of cells allowed to adhere for 30 min on no occasion gave rise to fibroblastic colonies. Thus, the property of surface adherence is not initially present but appears to develop early in the maturation sequence of the FC, perhaps with the first division in vitro. These results also suggest that the fibroblastic colonies are derived from progenitor cells within the bone marrow or other sites rather than from “fixed” or mature stromal cells, which is in agreement with the morphological studies of Daniels. Our results do not agree with the observations of Castro-Malaspina et al., who found that the fibroblast colony-forming cells were extremely adherent, beginning to adhere within the first minutes of incubation. This discrepancy may be related to the different culture conditions.

In all of our experiments, the granulopoietic effects of the FC were observed to be local in nature, suggesting an in vitro granulopoietic microenvironmental effect. Indeed, in cultures containing adherent FC only on half the surface of the plate, the vast majority of the CFU-C stimulated were found directly above the FC areas. Those regions of culture plates lacking FC contained CFU-C only equal to controls. Such in vitro microenvironmental effects of FC on granulocyte-macrophage colonies were previously reported for mice.

The effects of the FC on the target marrow depended on the amount of endogenous colony formation. While FC stimulated growth of CFU-C in target marrows containing few or no spontaneous CFU-C, actual inhibitory effects of FC were observed if large numbers of endogenous CFU-C were present.
Whether this observation is due to competition between CSF and FC for receptors on CFU-C, release of inhibitors by CFU-C, a reflection of CSF and FC operating on different subpopulations of CFU-C, or other mechanisms is not presently known.

The stimulatory effects of the FC were greatly diminished if adherent cells were removed from the target marrow prior to culturing with FC. This finding suggests the possibility that macrophages and/or other adherent cells augment the granulopoietic effects of FC. In this context, adherent mononuclear phagocytic elements, as well as large “fat cells,” are components of the adherent complex reported to be required for the long-term maintenance of hematopoietic stem cells in vitro. It is also possible that adherence of target marrows depletes some FC potential, although our results indicate that short-term adherence had no effect on the formation and stimulating effects of FC.

Unlike FC from normal subjects, those derived from patients with typical AML at diagnosis or in relapse did not stimulate target marrows containing few spontaneous CFU-C; however, similar inhibitory effects were observed with marrows containing large numbers of spontaneous CFU-C. Reports by several groups have indicated that cells or cellular factors from leukemic patients inhibit growth of normal CFU-C.

An observation of potentially major importance in the study of patients with AML was provided by serial cultures performed in two patients who we studied both in complete remission and in subsequent relapse. During remission, their FC stimulated CFU-C similar to normals; however, upon relapse, the FC stimulatory effect was entirely lost. Though FC are not derived from the hematopoietic stem cell from which leukemic clones originate, the functional properties of FC may depend on the number of leukemic cells present in the marrow. Conversely, defective interactions between stromal elements and leukemic progenitors could be involved in the occurrence of relapse and the maturation arrest observed. Similar to our observations on FC, the ability of marrow-adherent cells of the monocyte-macrophage series to produce CSA has been reported to be impaired in AML patients at diagnosis or in relapse in contrast to normal or increased CSA found in AML patients in complete remission. Furthermore, those AML patients with low marrow CSA tended to have poorer responses to chemotherapy than those who had normal CSA at diagnosis.

Blackburn and Patt reported that when mouse bone marrow or spleen cells were incubated in medium conditioned by marrow adherent (stromal) cells, the survival of pluripotent hematopoietic stem cells (CFU-S) was considerably greater than that of stem cells incubated in fresh or spleen-conditioned medium. This factor, which increased CFU-S survival, was produced by marrow stromal cells independent of proliferation. In more recent studies by Blackburn and Goldman, it was reported that human FC enhanced not only growth of cocultured CFU-C and BFU-E, but also conditioned media obtained from human FC increased survival of murine CFU-S. In agreement with our results, FC-conditioned media did not enhance CFU-C or BFU-E when added directly to the assay cultures but did stimulate their growth after one week.

The results of our own studies demonstrated that FC-conditioned media inhibited or totally suppressed the growth of spontaneous CFU-C in marrows containing a large number of endogenous CFU-C (≥9 colonies/10⁵ cells). Thus, some of the inhibitory effects of FC on marrow populations containing large numbers of CFU-C may be related to the release of inhibitory substances by FC, such as prostaglandins, into the culture medium. Similar inhibitory effects were seen in our studies of the relationship of the stimulatory effects of FC to CSF. Employing PCM as a source of CSF, we found that the presence of the FC actually diminished the stimulatory effects of PCM. The inhibitory effects of the FC on PCM-stimulated CFU-C were observed with FC derived from normals and patients with AML and other granulopoietic disorders. Thus, inhibitory effects of FC on spontaneous CFU-C were observed using both conditioned media and refilled methods, while stimulatory effects by normal FC were seen only when using target marrows containing few spontaneous CFU-C. Similar results were obtained by Castro-Malaspina et al., who reported that media conditioned by bone marrow fibroblasts did not stimulate the growth of granulocyte-macrophage colonies in marrows depleted of CSF-producing cells.

In contrast to our results for mice, dogs, and humans demonstrating enhancement of CFU-C growth by FC, two recent reports indicate either no activity or actual inhibitory effects of FC on CFU-C. The differences observed by various investigators on adherent cells could reflect such variables as the number of macrophages or other adherent cells intermixed with the FC (mice) or, as our results would suggest, the number of spontaneous CFU-C present in the target marrow (human). Furthermore, intentionally depleting the target marrow of adherent cells, as many investigators do, markedly diminishes or eradicates the stimulatory effects of FC, as we have demonstrated.

The cellular and humoral events influencing granulopoiesis are complex. While the number of factors reported to either stimulate or inhibit hematopoiesis
appear to be increasing exponentially, the influence of direct cellular interactions has not been fully appreciated.

Our studies demonstrating in vitro granulopoietic effects of bone marrow FC do not, in themselves, establish a physiologic role for these cells for the regulation of in vivo granulopoiesis. It is of interest, however, that recent in vivo studies have described fibroblastic stromal cells with morphology similar to the in vitro FC, which are intimately associated with granulopoietic regions in bone marrow and spleen. Further studies comparing the in vivo and in vitro stromal populations are clearly indicated. We have previously reported that human bone marrow FC give rise to "mesenchymal" tumors composed of fibroblasts when injected subcutaneously into nude mice. Our studies indicate that bone marrow FC should be included as candidates for the granulopoietic microenvironment. In agreement with previous results, our studies also demonstrate that the nature of physiologic and pathologic interactions between stromal (adherent) cells and hematopoietic elements can be studied in vitro using relatively "short-term" bone marrow culture systems. Our studies suggest that these effects vary, depending on whether the stromal cells are obtained from normal individuals or patients with acute myelogenous leukemia or other granulopoietic disorders.

ACKNOWLEDGMENT

This investigation was performed after review and approval by the Chancellor’s Advisory Committee on Research Involving Clinical or Physiological Studies of Human Subjects, University of California, Davis, in accordance with an assurance filed with and approved by the Department of Health, Education and Welfare.

REFERENCES


42. La Pushin RW, Trentin JJ: Identification of distinctive stromal elements in erythroid and neutrophil granuloid spleen colonies: Light and electron microscopic study. Exp Hematol 5:505, 1977
Granulopoietic effects of human bone marrow fibroblastic cells and abnormalities in the "granulopoietic microenvironment"

BR Greenberg, FZD Wilson and L Woo