Characterization of Human Bone Marrow Fibroblast Colony-Forming Cells (CFU-F) and Their Progeny


The bone marrow stromal cell population is comprised of fibroblasts, endothelial cells, fat cells and "reticular cells." The characteristics and the regulatory role in hematopoiesis of each of these cell types are unknown. 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. Bone marrow CFU-F were adherent and nonphagocytic. The majority (90% ± 2%) were less dense than 1.070 g/cu cm. Velocity sedimentation separation demonstrated a heterogeneous CFU-F sedimentation rate, with a modal sedimentation of 4.95 ± 0.15 mm/hr. Analysis of CFU-F proliferative status by the thymidine suicide technique indicated that this cell was noncycling in individuals with undisturbed bone marrow function. Some of the more distinctive products of fibroblasts, other stromal cells, and hematopoietic colony-forming cells were used as positive and negative markers for CFU-F and the cells derived from them in vitro. Complement-mediated cytotoxicity using anti-la and anti-factor-VIII antigen antisera did not inhibit fibroblast colony formation. In contrast, a striking reduction of granulocyte-macrophage colony formation (CFU-c) was seen when bone marrow cells were treated with anti-la antisera. Immunofluorescence staining was used to characterize the cells derived from CFU-F in vitro. No staining was observed after incubation of subconfluent cultures with anti-la and anti-factor-VIII antigen antisera. A positive immunofluorescent staining was obtained when isolated antibodies against three of the main proteins of bone marrow matrix: type I collagen, type III collagen, and fibronectin were used. Ultrastructural analysis showed that CFU-F progeny, in contrast to endothelial cells, did not contain Weibel-Palade bodies. These data support the conclusion that the colonies described in this study are of fibroblastic nature.

The bone marrow is composed of a heterogeneous mixture of different populations of hematopoietic and stromal cells. Since the introduction of the semisolid agar culture system for cloning murine granulocyte-macrophage precursors, other in vitro techniques have been developed to study the murine and human morphologically unrecognizable hematopoietic progenitor cells. Much information is now available regarding the characterization of these cells and the regulatory interactions occurring between the different subpopulations of hematopoietic cells (for a review see Broxmeyer and Moore2 and Cline and Golde3).

In contrast, little is known about the characteristics of the cells collectively called stromal cells. Morphologic analysis of bone marrow tissue has shown that the stromal population is comprised of fat cells, endothelial cells, fibroblasts, and "reticular cells." There are several lines of evidence, especially in rodents, indicating that marrow stromal elements play a major role in the regulation of hematopoiesis.5-8 Some attempts have been made to dissect the individual role of marrow stromal cells, especially the fibroblast-like cells, although the latter were only characterized morphologically with no definitive evidence to indicate that they were bone marrow fibroblasts.9-11

Myelofibrosis is a human disease state in which there is an abnormal interaction between hematopoietic and stromal cells. It is characterized by an increased deposition of collagen in the bone marrow. Immunofluorescence studies of normal bone marrow and bone marrow experimentally induced by subcutaneous injection of demineralized bone matrix have shown that largely type III collagen occurs in normal bone marrow tissue.12 Current concepts of the pathogenesis of this disorder13,14 indicate that myelofibrosis is a secondary phenomenon associated with a clonal proliferation of hematopoietic cells, but the subpopulation of these cells involved in the regulation of fibroblast function and the factors mediating these interactions are unknown.

The relatively limited amount of collagen-producing cells available in bone marrow aspirates has precluded the direct study of interactions between these cells and the different subpopulations of peripheral blood and bone marrow cells. The ability to grow selectively,

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characterize, and maintain bone marrow fibroblasts in vitro provides a valuable tool to study these interactions. The analysis of these interactions will permit an assessment of the regulatory role of bone marrow fibroblasts on hematopoietic cells and may help to elucidate the mechanisms and/or factors underlying myelofibrosis.

The present report describes a suspension culture technique for cloning human bone marrow fibroblasts, with which we were able to determine the physical characteristics and proliferative status of fibroblast colony-forming cells (CFU-F) and the antigenic characteristics of CFU-F and their fibroblast progeny.

MATERIALS AND METHODS

Bone Marrow and Peripheral Blood Cells

Normal human bone marrow cells were taken from healthy volunteers by aspiration from the posterior superior iliac crest after obtaining informed written consent, in accordance with hospital requirements. Heparin was added as an anticoagulant. Buffy coats were collected after centrifugation of the aspirates at 200 g for 10 min and washed twice in alpha modification of Eagle's medium (Sloan-Kettering Institute, New York, N.Y.) supplemented with 2% fetal calf serum (FCS, Microbiological Associates, Walkersville, Md.). Bone marrow cells obtained from ribs routinely removed from patients undergoing thoracotomy were used in an experiment and were kindly provided by Dr. E. Rabellino (Cornell University Medical Center, New York, N.Y.). Venous blood (450 ml) drawn from healthy volunteers in acid citrate buffer (ACD) or heparin was obtained from the New York Blood Center. A solution of 2% methyl cellulose in alpha medium was added to the blood, and the mixture was allowed to sediment at 37°C for 30 min; then the leukocyte-rich plasma was drawn off with a Pasteur pipette. The leukocytes were centrifuged at 400 g for 10 min and washed twice with culture medium.

Physical Separation of Bone Marrow Cells

Density Cut

Washed buffy coat cells were separated into two fractions according to density by a density "cut" at 1.070 g/cm³ or as indicated in bovine serum albumin (BSA, fraction V, Armour, Reheis Chemical Company, Phoenix, Ariz.), osmolarity 270 mosmole, as described previously.13

Adherence

Washed buffy coat cells or light density cells (<1.070 g/cm³) were diluted to the appropriate cell concentration in alpha medium containing 20% FCS. The cells were allowed to adhere to the bottom of T-75 tissue culture flasks (Corning Glass Works, Corning, N.Y.) or 100 x 20 mm tissue culture dishes (Falcon, Becton, Dickinson and Co., Cockeysville, Md.) at 37°C for different periods of time, after which the nonadherent cells were removed and the adherent cells were washed twice with alpha medium. Both adherent and nonadherent cells were assayed separately for CFU-F.

Because adherence for 60 min resulted in virtually complete separation of CFU-F, subsequent experiments were performed after this time period. Cells were washed 3 times with phosphate-buffered saline (PBS) Ca²⁺, Mg²⁺ free, then trypsin (Grand Island Biological Company, Grand Island, N.Y.) was added and the Petri dishes were incubated at 37°C for 10 min. The cells detached by trypsin were harvested, washed in medium, and replated for CFU-F. CFU-F were also assayed in the population of cells that did not detach following treatment with trypsin.

Carbonyl Iron Phagocytosis

Carbonyl iron particles (Technicon Company, Tarrytown, N.Y.) were mixed with low density bone marrow cells (<1.070 g/cm³) suspended in alpha medium supplemented with 20% FCS. The mixture was incubated at 37°C for 30 min with gentle agitation, then layered over Ficoll-Hypaque (Pharmacia Fine Chemicals Inc., Picataway, N.J.) and centrifuged at 500 g for 20 min. Cells that phagocytosed iron particles appeared in the pellet; nonphagocytic cells appeared at the interface. Both populations were harvested, washed three times, and assayed separately for CFU-F.

Velocity Sedimentation Separation

The general procedure described by Miller and Phillips16,17 was used. The sedimentation apparatus was siliconized in order to minimize the cell loss by adherence. Buffy coat cells 1-2 x 10⁸ suspended in PBS were loaded on a gradient of 0.4%-2% BSA (Fraction V) in PBS. The cells were previously exposed to 0.17 M ammonium chloride for 5 min in an ice bath to lyse residual erythrocytes and then washed 2-3 times with medium. This treatment did not modify CFU-F sedimentation rate. The loaded cells were allowed to sediment at 1 g and 4°C in a 17-cm diameter staut sedimentation chamber. After 4 hr, 35-ml fractions were collected and the cells were counted and assayed for colony-forming cells as described below. Unseparated control samples of bone marrow cells were stored in test tubes at 4°C in 0.2% BSA in PBS during the separation procedure, and assayed for colony-forming cells in parallel with the separated cell fractions.

Colony Assays

Fibroblast Colony-Forming Cells (CFU-F)

Unseparated or fractions of bone marrow cells obtained with the different cell separation procedures were cultured in T-25 or T-75 tissue culture flasks. Cells were plated in alpha medium containing 20% FCS. The flasks were gassed with 5% CO₂ in air and incubated at 37°C. The medium supplemented with FCS was totally renewed every 3-4 days. The cultures were ended at day 8 or as indicated, except when the cells were allowed to grow to confluence. For scoring the fibroblast colonies, the flasks were washed 3 times with PBS, the cells fixed with methanol, and stained with Wright-Giemsa stain. The colonies were scored at 25 x.

Granulocyte-Macrophage Colony-Forming Cells (CFU-c) and G-M Colony-Stimulating Factor(s) Assay

Unseparated or fractions of bone marrow cells were suspended in McCoy's 5A medium (Sloan-Kettering Institute, New York, N.Y.) supplemented with 10% heat-inactivated FCS and 0.3% boactoagar (Difco Laboratories, Detroit, Mich.). The cells were cultured in 35-mm Petri dishes over feeder layers containing 10⁶ peripheral blood leukocytes in 0.5% agar medium as a source of colony-stimulating factor (CSF).18 The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and scored for clusters (3-50 cells/aggregate) and colonies (>50 cells) after 7 and 14 days of incubation. Five plates were scored per point. A similar system was used to test the effect of bone marrow fibroblast conditioned media on G-M colony formation, except that the feeder layers were omitted. The target cells consisted of nonadherent low density
of Iscove et al. Bone marrow cells were plated in alpha medium containing 10% FCS and then incubated in the same medium supplemented with 10% FCS for 4°C for 24 hr and sterilized during the time of exponential growth or when confluence was reached. The fibroblast conditioned medium containing 10% FCS was collected at 37°C for 30 min in alpha medium containing 10% FCS and 100 μCi of 3H-TdR (specific activity 20 Ci/mM, New England Nuclear, Boston, Mass.). The other part was incubated in the same medium conditioned by well characterized endothelial cells derived from human umbilical cords, kindly provided by Dr. E. Jaffe, was used as a positive control. Both conditioned media were left undialyzed or were dialyzed (molecular weight cut-off 6000-8000 daltons) against two changes of PBS at 4°C for 24 hr and sterilized by filtration through a 0.45-μm pore size filter (Millipore, Bedford, Mass.). The volume of conditioned medium added per dish was 0.1 ml.

Erythroid Colony-Forming Cells (BFU-E and CFU-E)

Erythroid colony assays were carried out according to the method of Iscove et al. Bone marrow cells were plated in alpha medium containing a final concentration of 0.8% methylcellulose, 30% fetal calf serum, 1% BSA, and 1 U/ml of human urinary erythropoietin. Colonies, individual colonies were removed, placed on glass slides, washed several times with PBS, fixed with methanol, and stained with benzidine and Wright-Giemsa.

Tritiated Thymidine Suicide

To assess the proportion of colony-forming cells in DNA synthesis, tritiated thymidine (3H-TdR) with high specific activity was used to kill cells in S-phase of the cell cycle. Samples of bone marrow cells were divided in two parts; one part was incubated at 37°C for 30 min in alpha medium containing 10% FCS and 100 μCi of 3H-TdR (specific activity 20 Ci/mM, New England Nuclear, Boston, Mass.). The other part was incubated in the same medium containing an equal amount of cold thymidine (12 μg/ml) as a control. The cells were then washed three times and assayed for colony-forming cells. Cold thymidine at a concentration equal to 100 times the concentration of 3H-TdR was added to the medium used for washing and plating the cells. The reduction in the number of colonies after pulse incubation with 3H-TdR was expressed as a percentage of the number in control cultures.

Latex Ingestion

Latex particles 1.1 μ diameter (Dow Diagnostics, Indianapolis, Ind.) were added to flasks containing nonconfluent primary cultures of bone marrow fibroblasts. They were incubated at 37°C for 1 hr. After that period, the medium was removed, the cell layer was washed several times with PBS, fixed with methanol, and stained with Wright-Giemsa stain.

Immunologic Studies

Antisera

Rabbit antibodies to human fibronectin (FN) were prepared with FN kindly provided by Dr. Peter Harpel. Purity of the antigen was monitored with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which yielded a single band. Human plasma, previously treated on a gelatin-sepharose column and thus rendered free of FN, was used to absorb the harvested rabbit anti-human FN. The absorbed rabbit anti-FN yielded a single precipitation arc against normal human plasma in immunoelectrophoresis, against purified antigen in radial immunodiffusion, and in SDS polyacrylamide gel when analyzed as an immunoprecipitate with fibronectin from another source.

A well characterized rabbit anti-factor-VIII antigen antiserum has been previously described. This was provided courtesy of Dr. Ralph L. Nachman. The IgG fraction of a goat anti-rabbit antiserum (GARlgG) conjugated to fluorescein isothiocyanate was supplied by Dr. Carl Becker.

Indirect immunofluorescence of cell cultures with isolated antibodies against purified interstitial collagen type I and type III was performed as described previously. A specific heteroantiserum was kindly supplied by Dr. R. J. Winchester. Details of preparation, purification, and characterization of antibodies and antigens have been reported previously.

Immunofluorescence Studies

Immunofluorescent staining of membrane- and cytoplasm-asociated components was carried out using the indirect method. Fibroblast-like cells attached to glass cover slips or to the bottom of tissue culture flasks were washed three times with PBS. For membrane staining, cells were incubated with the different specific rabbit antibody preparations at room temperature for 30 min. For cytoplasmic staining, cells were incubated with the antisera after fixation in acetone for 10 min. Following three washes with PBS, the cell preparations were incubated with the fluoresceinated goat anti-rabbit antiserum at room temperature for 30 min, then washed with PBS and mounted in saline for membrane antigens and glycerol phosphate for cytoplasmic antigens. Acetone-fixed confluent cultures of human endothelial cells were used as a control, kindly provided by Dr. E. Jaffe. Cells were examined with a Leitz Ortholux II photo microscope equipped with a Ploem illuminator and phase contrast.

Cytotoxicity Assays

Cytotoxicity experiments were performed using the two-step cytotoxic method and rabbit serum as source of complement. Bone marrow cells resuspended in alpha medium supplemented with 10% FCS were incubated with the antisera at 4°C for 30 min. After the first incubation, cells were washed once by layering over heat-inactivated FCS and then incubated at 37°C for 45 min with rabbit serum. The cells were washed twice in FCS and cultured for CFU-c and CFU-F.

Electron Microscopy

Cultured fibroblasts to be examined by electron microscopy were grown in T-25 flasks or in Petri dishes. The cell monolayers were fixed in situ with 2.5% glutaraldehyde in Dulbecco's medium for 30 min. After 2 washes with medium, cells were detached by scraping with a rubber policeman, collected in tubes, and centrifuged at 200 g for 10 min. Cell pellets were fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 1 hr. Pellets were washed twice with double-distilled water before dehydration in a series of graded ethanol solutions, then treated successively by propylene oxide, epon-propylene oxide mixture, and finally embedded in pure epon. The ultrathin sections were stained with uranyl acetate followed by lead citrate and examined in a Siemens Elmiskop I (Siemens Corporation, Iselin, N.J.).

RESULTS

Growth and Morphology of Colonies and Cells

In preliminary experiments, several media were tested for their ability to support the growth of primary cultures of bone marrow fibroblasts. Alpha medium provided the best conditions for optimal
growth. The other media tested were Dulbecco's modified Eagle medium, McCoy's 5A, RPMI, and Fischer medium.

Fetal calf serum was used as a source of fibroblast growth factor(s). Figure 1 shows the relationship between growth and the concentration of FCS in the flask. While all the FCS we have tested stimulate fibroblast growth, occasionally there were some differences between batches. The figure illustrates a typical dose-response curve for the majority of batches. Plateau growth was obtained with 20% FCS from any batch. Sera from human adults and horses also stimulated the growth of human bone marrow fibroblasts, although the plateau was reached with higher concentration of these sera (25%–35%).

Sequential microscopic observation showed that during the first several hours of culture most of the adherent cells appeared as large mononuclear cells. Later some of these cells became fusiform. Small fibroblast colonies with a few cells were visible on the third or fourth day. The number of colonies increased progressively, and the peak was reached at days 7–10. After that, only the size of the colonies increased. The growth rate was dependent on the number and the composition of the hematopoietic cell population present during the first 3–4 days of cultivation. The peak number of colonies was reached early when unseparated bone marrow was plated, and later when fractions of velocity sedimentation separation were plated.

Figure 2A shows a stained flask after 14 days of incubation. All the colonies contain cells that appear to be fibroblasts, but some isolated macrophages were observed. Figure 2B shows the details of a fibroblast colony. Figures 2C and 2D display the morphologic characteristics of cultured fibroblasts by electron microscopy. They show the typical morphology of fibroblasts in a transverse and longitudinal section. Analysis of several sections of the same cells and of the different cells failed to show the presence of Weibel-Palade bodies, which are typical organelles of endothelial cells.

There was little variation in colony size in a given culture. However, some differences could be observed between sets of cultures established at different times. The CFU-F frequency varied from one donor to another, and in 10 iliac crest aspirations obtained from normal donors, the number of fibroblast colonies for 5 × 10^6 unseparated bone marrow cells plated was 68 ± 10 (x ± 1 SEM) and the range was 45–143 (Table 1). The number of fibroblast colonies when cells obtained directly from costal bone marrow were plated was in the same range, 55 ± 3. CFU-F could not be demonstrated in peripheral blood. Fibroblast colonies were not formed even when plating high concentrations of PBC up to 10^8 cells/flask.

When different concentrations of bone marrow cells were plated, the number of colonies resulting was linearly correlated to the number of cells plated (Fig. 3) (r = 0.98, p < 0.001). If too many cells were plated per unit surface area of the culture flask, the fibroblasts grew into a monolayer, rendering enumeration of colonies impossible. Colony growth was potentiated by the number of colonies growing in the same flask. The more crowded the culture, the larger was the average size of each colony.

**Physical Properties of CFU-F**

**Adherence**

Fibroblast colony-forming cells are extremely adherent cells. As shown in Table 2, they started to adhere within the first minutes of incubation, and the majority of cells were attached after 30 min. The ability to adhere depends also on the number of cells per unit of surface area. When few cells were plated, 100% of CFU-F were attached after 30 min of incubation. CFU-F adherence was sensitive to trypsinization. Of these cells, 80%–90% were detached after treatment of the adherent cells with trypsin (Table 2). This enzyme did not modify the viability of CFU-F.

Most of the low density (<1.070 g/cu cm) adherent bone marrow cells displayed, in Wright-Giemsa stain preparations, the morphologic characteristics of monocytes. This was confirmed by cytochemical staining with the monocyte-specific esterase and phagocytosis of latex particles. The identification of the CFU-F, which is a nonphagocytic cell, was difficult because of the small proportion of this cell in the adherent cells.
Fig. 2. (A) T-75 culture flask containing stained fibroblast colonies 14 days after incubation of the flask with human bone marrow cells. (B) Photomicrograph of a fibroblast colony (200×). All the cells except two are fibroblasts; the two round cells are macrophages. This was demonstrated by adding latex particles to the culture flask and incubating for 1 hr at 37°C. The only cells able to phagocytose these particles were the isolated adherent round cells between or within the fibroblast colonies. (C) and (D) Electron photomicrographs of cultured fibroblasts in a transverse and longitudinal section. Note long irregular mitochondria, bundles of fine filaments, and vacuoles. Weibel-Palade bodies are absent (12,000×).
Table 1. Frequency of CFU-F in Human Bone Marrow

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of Fibroblast Colonies* (per 5 x 10^6)</th>
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<tbody>
<tr>
<td>#1</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>#2</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>#3</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>#4</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>#5</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>#6</td>
<td>143 ± 6</td>
</tr>
<tr>
<td>#7</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>#8</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>#9</td>
<td>78 ± 1</td>
</tr>
<tr>
<td>#10</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>Mean 10 experiments</td>
<td>68 ± 10</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

Unfractionated bone marrow cells were cultivated in T-75 flasks. The colonies were counted on day 8 (colony > 40 cells). Three flasks were used in each experiment.

Table 2. Time-Dependent CFU-F Adherence

<table>
<thead>
<tr>
<th>Time of Incubation</th>
<th>Percent of Fibroblast Colonies*</th>
</tr>
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<tbody>
<tr>
<td>10 min</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>20 min</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>30 min</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>60 min</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>90 min</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>Adherent (60 min) + trypsin</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

Unseparated bone marrow cells (5 x 10^6) were plated in T-75 flasks. The nonadherent cells were removed at the indicated times and both fractions, adherent and nonadherent, were cultured separately for CFU-F and colonies counted at day 10. In one group, the cells remaining attached after 60 min of incubation were trypsinized as described in Materials and Methods and the resulting fractions were assessed for CFU-F. Trypsinization did not modify colony formation. The number of colonies in the groups, untreated adherent cells and trypsin-treated adherent cells (detached cells + nondetached cells), was similar. The results shown are the average of 3 experiments.

Phagocytosis

Since adherence does not allow separation of CFU-F from monocytes, bone marrow cells were exposed to carbonyl iron particles in order to separate the cells on the basis of phagocytic capacity. Fibroblast colony-forming cells were found among the nonphagocytic cells. The cellular yield was relatively low because of the tendency of CFU-F to aggregate and probably to adhere to the iron particles increasing the apparent density of these cells. As a result, some CFU-F appeared in the pellet among the phagocytic cells. Examination of the cells of fibroblast colonies showed that none contained iron particles.

Cell Density

Density cut procedure is a simple and common technique used to enrich the hematopoietic colony-forming cells that are found in the low density fraction when the "cut" is done at 1.070 g/cu cm. The majority of nonphagocytic cells were found among the nonphagocytic cells. Consequently, attempts to differentiate cytochemically bone marrow fibroblasts from monocytes were undertaken. Bone marrow fibroblasts were grown on cover slips and their cytochemical properties were compared to peripheral blood monocytes (Table 3). None of the stains tested gave a positive reaction with fibroblasts and a negative reaction with monocytes. Thus, it was not possible to use this technique for the morphologic identification of CFU-F among the bone marrow adherent cells.

Table 3. Cytochemical Characteristics of Bone Marrow Fibroblasts Grown In Vitro Compared to Peripheral Blood Monocytes

<table>
<thead>
<tr>
<th>Procedure Done</th>
<th>Fibroblasts</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthyl acetate esterase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Naphthol-ASD chloracetate esterase</td>
<td>–</td>
<td>±</td>
</tr>
<tr>
<td>Naphthol-ASD acetate esterase</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Fluoride-resistant acetate esterase</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td>Periodic acid schiff (PAS)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sudan black</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Phagocytosis (latex)</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

(–) Negative; (±) weak or few positive cells; (+) positive; (++) strongly positive (most cells).

Bone marrow fibroblasts grown on cover slips and peripheral blood adherent cells were compared with respect to their cytochemical characteristics using the indicated stains. Note the cytochemical differences between fibroblasts and monocytes.
Table 4. Density Cut Separation of CFU-F

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1.058 g/cm^3</th>
<th>1.062 g/cm^3</th>
<th>1.070 g/cm^3</th>
<th>1.080 g/cm^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buoyant</td>
<td>3 ± 0.2</td>
<td>30 ± 6</td>
<td>90 ± 1.7</td>
<td>95 ± 0.6</td>
</tr>
<tr>
<td>Dense</td>
<td>97 ± 0.2</td>
<td>70 ± 6</td>
<td>10 ± 1.7</td>
<td>5 ± 0.6</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

Bone marrow cells were separated in two fractions by a density cut procedure at different densities. Cells from each fraction were cultured for CFU-F and colonies counted at day 10. The number of colonies per fraction are expressed as percent of the total colonies counted in both fractions. The results shown are the average of four independent experiments. The low and dense fraction of the 1.070 g/cm^3 density cut were also tested for CFU-c. No growth of CFU-c in the dense fraction was observed.

of CFU-F were also found in this fraction. Only 10% ± 1.7% appeared in the high density fraction. In fact, CFU-F had a wide density distribution as demonstrated by density "cut" separation at different densities (Table 4).

Sedimentation Rate in BSA Gradient

The cell profile in a typical experiment is shown in Fig. 4A. The nucleated cells regularly sedimented in two peaks: a small peak at 3–4 mm/hr, where the predominant cell was the small lymphocyte, and another more rapidly sedimenting peak at 5.5–7 mm/hr, consisting almost entirely of peroxidase positive cells. The sedimentation rate distribution of CFU-F was very heterogeneous, with rates varying from 4 to 10 mm/hr. The modal rate was 4.95 ± 0.15, an average of 3 experiments. As separation of cells by velocity sedimentation is primarily on the basis of cell volume and the resolution of this method is 0.5 mm/hr, the heterogeneous distribution of CFU-F may reflect the existence of more than one population in terms of cell size. The same figure illustrates the different sedimentation rate of erythroid and G-M colony-forming cells assayed 7 and 14 days after plating. The colony-forming cells, erythroid or G-M, assayed at the same day have similar sedimentation rates.

Proliferative Status of CFU-F

The number of colony-forming cells (CFU-F and CFU-c) noted after brief in vitro exposure of unseparated normal human bone marrow to 3H-TdR of high specific activity is shown in Table 5. The number of CFU-F was not reduced at all, whereas there was a reduction of 30% ± 10% of the number of CFU-c. This indicates that none of the fibroblast colony-forming cells were in cycle. Velocity sedimentation separation has shown that the distribution of CFU-F cell size is quite heterogenous. This variation is not related to the cell cycle status. Different bone marrow fractions obtained by velocity sedimentation at unit gravity were pooled and assayed for CFU-F in S-phase, treating half of the pooled fractions with 3H-TdR and the other half with equivalent amounts of cold thymidine. There was no reduction of the number of CFU-F in the group treated by 3H-TdR (Table 5).
grown in vitro were tested for its ability to stimulate growth or at the stationary phase. were not stimulated to grow by these conditioned media. The small differences observed are not statistically significant. In Table 6, the growth velocity sedimentation separation were pooled as indicated and each pool was divided in two parts for treatment with 100 µCi of 3H-Thymidine or the equivalent amount of cold thymidine. Cells from each part were plated in triplicate. The small differences observed are not statistically significant. In four independent experiments using unfractionated bone marrow, the percent of reduction was +4.2%, +4.0%, -2.0%, -8.7%. In the experiment depicted in this table, the percent of CFU-c reduction for unseparated bone marrow cells was 42.6%.

Lack of GM Colony-Stimulating Factor(s) in BM Fibroblast Conditioned Media

The media conditioned by bone marrow fibroblasts grown in vitro were tested for its ability to stimulate the growth of G-M colonies. As shown in Table 6, CFU-c present in nonadherent bone marrow cells that were depleted of endogenous CSF-producing cells were not stimulated to grow by these conditioned media collected either at the exponential phase of growth or at the stationary phase. Low molecular weight (6000-8000 daltons) inhibitors were not responsible for the absence of growth; dialyzed or undialyzed samples failed to stimulate CFU-c growth. Media conditioned by endothelial cells contained G-M CSF, as has been described.20

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Table 5. Proliferative Status of CFU-F

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<thead>
<tr>
<th>Bone Marrow Cells</th>
<th>Number of Fibroblast Colonies*</th>
<th>Percent Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>193 ± 6</td>
<td>-1.6</td>
</tr>
<tr>
<td>Velocity sedimentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (2.1-3.6 mm/hr)</td>
<td>18 ± 2</td>
<td>-5.5</td>
</tr>
<tr>
<td>II (3.9-5.9 mm/hr)</td>
<td>65 ± 4</td>
<td>-3.0</td>
</tr>
<tr>
<td>III (6.3-7.9 mm/hr)</td>
<td>30 ± 4</td>
<td>+7.7</td>
</tr>
<tr>
<td>IV (8.3-10.9 mm/hr)</td>
<td>20 ± 3</td>
<td>-5.0</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

Results of a representative thymidine suicide experiment. Fractions of velocity sedimentation separation were pooled as indicated and each pool was divided in two parts for treatment with 100 µCi of 3H-Thymidine or the equivalent amount of cold thymidine. Cells from each part were plated in triplicate. The small differences observed are not statistically significant. In four independent experiments using unfractionated bone marrow, the percent of reduction was +4.2%, +4.0%, -2.0%, -8.7%. In the experiment depicted in this table, the percent of CFU-c reduction for unseparated bone marrow cells was 42.6%.

Table 6. Effect of Bone Marrow Fibroblast Conditioned Media on Granulocyte-Macrophage Colony Formation

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Number of Colonies*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0</td>
</tr>
<tr>
<td>Endothelial cell CM (undialyzed)</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Bone marrow fibroblast conditioned medium</td>
<td></td>
</tr>
<tr>
<td>Exponential phase</td>
<td></td>
</tr>
<tr>
<td>Dialyzed</td>
<td>0</td>
</tr>
<tr>
<td>Undialyzed</td>
<td>0</td>
</tr>
<tr>
<td>Stationary phase</td>
<td></td>
</tr>
<tr>
<td>Dialyzed</td>
<td>0</td>
</tr>
<tr>
<td>Undialyzed</td>
<td>0</td>
</tr>
<tr>
<td>Feeder layers</td>
<td>263 ± 12</td>
</tr>
</tbody>
</table>

*Mean ± SEM. CM, conditioned media.
Nonadherent low density (<1.070 cu/cm) bone marrow cells were plated (10^5/dish) in the presence of different conditioned media. Colony formation was assessed at day 7.

Table 7. CFU-F and CFU-C After Treatment of Bone Marrow Cells With Antisera Against Ia Antigen and Factor-VIII Antigen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Colonies*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>198 ± 7</td>
</tr>
<tr>
<td>Complement (C')</td>
<td>191 ± 6</td>
</tr>
<tr>
<td>Anti-Ia + C'</td>
<td>190 ± 5</td>
</tr>
<tr>
<td>Anti-VIII + C'</td>
<td>212 ± 12</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

Low density (<1.070 g/cm) bone marrow cells were treated with the antisera and then plated in triplicate (3 x 10^5 cells/flask for CFU-F and 0.5 x 10^5/dish for CFU-c). Colony formation was assessed at day 10 and 7, respectively.

Immunologic Characterization of CFU-F and Their Progeny

The antigenic properties of some of the more distinctive components known to be associated with bone marrow fibroblasts, other stromal cells, and hematopoietic cells were used as positive or negative markers of CFU-F and the cells derived from them in vitro. The existence of these markers on the surface of CFU-F was assessed by complement-mediated cytotoxicity of colony formation. The results of the experiments using anti-factor-VIII antigen and anti-Ia antiserum are shown in Table 7. There was no significant reduction of the number of fibroblast colonies induced by either anti-factor-VIII antigen antiserum or anti-Ia antiserum. In contrast, there was a striking reduction of granulocyte-macrophage colonies when the bone marrow cells were treated with anti-Ia antiserum; 90% of CFU-c were killed. The absorption of the antiserum with pooled B-cell lymphoid lines suppressed its ability to inhibit colony formation.

The immunologic characterization of the cells derived from CFU-F was performed by indirect immunofluorescence. The results of these studies are summarized in Table 8. Cells of bone marrow fibroblast colonies labeled with antibodies to type I collagen showed strong staining in the pericellular region, indicating that type I collagen is largely found extracellularly. Label with antibodies to type III

Table 8. Immunofluorescence Studies on Cultured Bone Marrow Fibroblasts

<table>
<thead>
<tr>
<th>Antigen Detected</th>
<th>Exocytoskeleton and Cellular Matrix</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Collagen II</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Collagen III</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Factor VIII Antigen</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ia Antigen</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+ Positive staining, (-) negative staining.)
Fig. 5. Immunofluorescence photomicrographs of (A) confluent cultured fibroblasts treated with rabbit anti-FN (RAFN) and then with fluorescein-conjugated goat anti-rabbit IgG (F-GARigG) after fixation with acetone. Note the extracellular meshwork of fibronectin fibrils (125×). (B) Subconfluent fibroblast cultures stained with RAFN and F-GARigG after acetone fixation. The cells exhibit a bright intracellular granular staining (800×). (C) Confluent cultured endothelial cells derived from human umbilical cord labeled with RAFN and F-GARigG after acetone fixation. Note the polygonal shape of these cells and the bright staining of extracellular fibrils (800×). (D) Confluent endothelial cells stained with rabbit anti-factor-VIII antigen and F-GARigG after acetone fixation. The cells are brightly stained in contrast to the absence of staining of fibroblasts (800×).
collagen also appeared intense, but the staining pattern indicated localization of type III collagen equally in the extracellular and intracellular space. In general, the staining pattern revealed deposition of collagen fibrils similar to that previously demonstrated for skin fibroblasts.

Immunofluorescent staining of acetone-fixed confluent fibroblast cultures after incubation with antifibronectin antiserum produced a characteristic pattern of web-like extracellular fibrils (Fig. 5A). There was some granular cytoplasmic staining. Nonconfluent cultures similarly stained exhibited the extracellular fibrillar pattern in addition to bright intracellular granular staining, particularly in the perinuclear region (Fig. 5B). As the cells become more confluent, the number of intracellular immunofluorescent granules decreased somewhat, and the extent of the extracellular immunofluorescent fibrillar meshwork greatly increased. Cells that were acetone-fixed after double antibody immunofluorescence staining was performed exhibited only the extracellular, peripheral meshwork pattern; no intracellular staining was seen. This represents pure membrane-associated FN. Immunofluorescent staining of fibroblasts was completely inhibited by prior absorption at equivalence of anti-FN serum by purified FN.

No fluorescent staining was seen in fibroblasts treated with rabbit anti-VIII antigen or anti-Ia antisera, regardless of when acetone fixation took place. Similarly, no staining was seen in fibroblast cultures treated with RIgG and F-GARlG or with F-GARlG alone. Fluorescence staining of control cultures of confluent endothelial cells revealed the presence of fibronectin (Fig. 5C) and factor VIII (Fig. 5D), as previously described.

DISCUSSION

Some successful attempts have been made in the past to grow and clone bone marrow stromal cells, in particular fibroblast-like cells. However, no attempts were made to provide definitive evidence that these cells were indeed fibroblasts. The present observations document the fibroblastic nature of these cells. Collagen is the major macromolecule of stromal tissues, and because of its structural, therefore genetic heterogeneity, and its different tissue distribution constitutes an excellent marker for the cells producing the collagen types found in a tissue. We have shown by immunofluorescence studies using collagen-type-specific antibodies that the bone marrow stroma contains predominantly type III collagen with a small proportion of type I. Cells growing from explants of these biopsies also produce the same types of collagen. Bone marrow cells, including the stromal population, can be obtained by aspiration in a single cell suspension; in this manner, the replicating cells can be cloned. The fibroblast is a self-replicating cell able to give rise in vitro to colonies like those we describe in this report, similar in morphology to those obtained by other groups cultivating bone marrow from different species in a suspension culture. We demonstrate here that the cells of these fibroblast-like colonies produce in vitro the same collagens, type III and I, found in situ in bone marrow biopsies. Double labeling immunofluorescence studies on fibroblasts derived from skin have shown that the same fibroblast may synthesize both types of collagen simultaneously; the ratio of type III to type I collagen is much smaller than that produced by cells derived from bone marrow tissue. In addition, we demonstrate that bone marrow fibroblasts produce fibronectin, a high molecular weight glycoprotein of connective tissue matrices present also on the surface of cells and in extracellular fluids. This protein is produced by fibroblasts, as well as by endothelial, epithelial, and astroglial cells.

The present data show that bone marrow fibroblasts described in this report do not have surface characteristics expressed by endothelial cells. The latter are also constituents of the stromal cell population as demonstrated by direct ultrastructural study of bone marrow tissue and by morphologic and functional analysis of the adherent population derived from murine and human bone marrow able to support the proliferation and differentiation of pluripotential hematopoietic stem cells. Studies with bone marrow cells with certain surface characteristics of endothelial cells are in progress. It has been shown that endothelial cells produce a component of antihemophilic factor, the factor VIII antigen, basement membrane collagen, which is antigenically and functionally distinct from the other collagen types, and granulocyte-macrophage colony-stimulating factor(s). None of these products could be demonstrated in the cells of fibroblast colonies, rendering unlikely the possibility of identity of these cells with endothelial cells. An additional finding was the absence of Weibel-Palade bodies, typical organelles of endothelial cells.

The linearity of the regression curve relating the number of cells plated with the number of colonies obtained suggests that the fibroblast colonies arise from a single CFU-F. This is strongly supported by the time lapse cinematography observations and the results of male–female experiments reported by Friedenstein and his group. Individual colonies obtained after culturing a mixture of bone marrow cells from male and female donors contained either male or female chromosomes but not both. The low number of fibroblast colonies obtained after plating a
high number of bone marrow cells reflects more the predominance of hematopoietic cells rather than a low cloning efficiency.

Because of the low number of CFU-F in bone marrow and the inability to enrich them significantly, a direct study of the morphological and surface characteristics has not been performed. Cytotoxicity assays have permitted us to show that this cell, like its progeny, does not express either factor VIII antigen, in contrast to endothelial cells, or Ia antigen, in contrast to hematopoietic colony-forming cells. Ia antigen is a bimolecular glycoprotein encoded by genes mapping within the major histocompatibility complex, similar to the murine I-region-associated antigens.\textsuperscript{43,44} Besides its function in immune response,\textsuperscript{45} studies in our laboratory suggest that Ia antigen is related to the process of differentiation and maturation of hematopoietic cells. Complement-mediated cytotoxicity has failed to reveal the existence of Ia antigen on the surface of the hematopoietic pluripotential stem cell,\textsuperscript{46} but it has demonstrated that Ia is present on the surface of granulocyte-macrophage and erythroid progenitors detectable by colony assays: pre-CFU-c, CFU-c day 14, CFU-c day 7, BFU-E, and CFU-E.\textsuperscript{28,47,48} Ia is then progressively lost during granulocyte and erythroid differentiation. Fibroblasts, in general, in all species studied belong to the Ia-negative cell population.\textsuperscript{79,49}

The lack of this differentiation marker for hematopoietic cells in bone marrow fibroblast colony-forming cells gives additional evidence on the different histogenetic origin of both cell populations. This has been conclusively demonstrated in humans and rodents by analysis of chromosome and enzyme markers of hematopoietic cells and bone marrow fibroblast-like cells obtained from patients with clonal diseases of the hematopoietic pluripotential stem cell,\textsuperscript{13,40,50} and from patients and experimental animals with chimerism in the bone marrow resulting from bone marrow transplantation.\textsuperscript{51}

In spite of the fact that the morphology of CFU-F is unknown, the ability of this cell to originate colonies in vitro has allowed us to obtain information about its physical properties and proliferative status. It has been reported that CFU-F is an extremely adherent and nonphagocytic cell.\textsuperscript{8,10} Our data confirm these observations and bring new information concerning the heterogeneity of biophysical properties of these cells. Velocity sedimentation separation shows that CFU-F has a wide range of sedimentation velocities. This is not related to differences in cycle status, which influences cell size; it is likely to be related to variations in cell density. Although density distribution profile has not been done with these cells, separation by density cut at different densities shows that CFU-F density is distributed in a wide range. This heterogeneity is found in other species; studies in mouse bone marrow fibroblast colony-forming cells have shown that these cells also have a wide density distribution.\textsuperscript{35}

The function served by the heterogeneity of CFU-F and the heterogeneity with respect to sensitivity to growth factors and hormones are unknown.

The assessment of the proportion of cells in S-phase by the thymidine suicide technique indicates that CFU-F is a resting cell in individuals with undisturbed bone marrow function. This is in agreement with

\textsuperscript{3}H-Tdr studies done in rodents. Stromal cells remain labeled for months after administration in utero and during neonatal life, indicating that they are cytokinetically nonproliferating cells.\textsuperscript{52} Flash labeling experiments have shown that stromal cells can modify their proliferative status in response to stimuli leading to reparation.\textsuperscript{53} These findings have widespread implications for the study of human pathologic states, specifically myelofibrosis. Studies on the pathogenesis of this disease have demonstrated indirectly that bone marrow fibrosis is a reactive process associated with a clonal proliferation of hematopoietic cells.\textsuperscript{13,14} We have demonstrated that myelofibrosis is characterized by proliferation of fibroblast-like cells as well as endothelial cells, which leads to a deposition of fibrillar interstitial collagen types I and III and basement membrane collagen.\textsuperscript{54} Collagen deposition in reactive fibrosis represents the last event of a series of phenomena triggered by an insult. Studies in experimental myelofibrosis\textsuperscript{55} and human skin scar formation have shown that the induced fibrotic tissue deposition of type I and III collagen is preceded by an intensive cellular proliferation.\textsuperscript{56} Patients with myeloproliferative diseases like chronic myelogenous leukemia can develop myelofibrosis during the course of their disease.\textsuperscript{57} The monitoring of CFU-F proliferative status in these patients may permit the detection of one of the earliest events occurring in the establishment of myelofibrosis.

In vitro, the entry of CFU-F into cycle and the subsequent development of colonies depend on the growth factors present in the serum. In our experiments, we have observed a typical sigmoid dose–response relationship between the concentration of FCS and the number of fibroblast colonies. A comparable curve was obtained using adult human serum and horse serum. The serum growth factor(s) appear to be derived from platelets,\textsuperscript{58} which are a rich source of fibroblast growth-promoting activity.\textsuperscript{59} Recent studies have demonstrated the intracellular location of these factors in the alpha granules of platelets,\textsuperscript{60} the chemical and biologic characteristics of these factors,\textsuperscript{61,62} and the immunologic identity of the plate-
globin-derived growth factor(s) with the fibroblast growth factor obtained from bovine brain. It is not known if the megakaryocyte produces this growth factor and the platelets merely store it, or if platelets actively synthesize growth factor. The answer to this question will provide new clues in the understanding of the pathogenesis of myelofibrosis, especially the cells, factors, and mechanisms involved in the accumulation of interstitial and basement membrane related tissue in bone marrow.

The present experimental system seems to provide a favorable approach for more quantitative examination of proliferation of bone marrow fibroblasts in primary cultures and the interactions occurring between these cells and hematopoietic cells. Furthermore, it provides a means to amplify in vitro the population of bone marrow fibroblasts. This will permit the study in secondary cultures of the effect of factors derived from the different subpopulations of hematopoietic cells on fibroblast growth and secretory functions.

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Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny


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