Regulation of Erythroid Colony Formation by Bone Marrow Macrophages

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We present evidence that macrophages stimulate red cell production by human bone marrow cells in vitro and that this effect is mediated by soluble substances. Bone marrow cells from donors were cultured in liquid media; after 7–14 days, cells highly enriched for macrophages and supernatants from these cultures were collected and cocultured in plasma clots with fresh human bone marrow cells (CFU-e) for 7 days or peripheral blood mononuclear cells (BFU-e) for 15 days. When added in numbers generally noted in marrow aspirates, bone marrow cells enriched for macrophages enhanced erythroid colony (EC) formation. Thus, at macrophage concentrations of 0% versus 1% and 2%, 4% and 10%, the numbers of EC/6×10⁶ BM cells were: 677 ± 46 (mean ± SEM) versus 846 ± 98, 938 ± 85, 1039 ± 93, and 956 ± 68, respectively. Stimulation was noted until unphysiologically high ratios of macrophages (>15%) were added, when EC formation was progressively inhibited. The same erythroid growth curve was obtained whether macrophages and red cell precursors were from the same or different donors. Nonadherent cells from liquid culture did not modulate in vitro erythroid growth, whereas adherent cells at low concentrations enhanced and at higher concentrations inhibited EC formation. Supernatants from these macrophage cultures contained a substance, not erythropoietin, with similar erythropoietic effects.

MORPHOLOGICAL OBSERVATIONS by light and electron microscopy suggest that erythropoietic cells are not scattered randomly through the bone marrow matrix, but in fact are organized anatomically into erythroblastic islands. These islands consist of one or two central reticular cells or macrophages surrounded by a ring of developing erythroblasts. High resolution electron microscopy has shown that ferritin molecules essential to normal erythroblast development adhere to the surface of the erythroblast membrane. From this observation has come the hypothesis that the central macrophage, as the cell of origin of these molecules, assumes a “nursing” function in erythropoiesis.

The development of simple cloning techniques in semisolid medium has allowed further characterization of the physiology of hemopoiesis and has further clarified the function of peripheral blood and bone marrow mononuclear cells. Thus, it has been shown that colony formation in culture depends on certain exogenous and endogenous stimulators and is subject to a variety of interacting regulatory systems. One such system appears to involve the peripheral blood monocyte, which has been shown to be a source of regulatory factors that influence hemopoiesis; that is, monocytes produce soluble substances including prostaglandins and colony-stimulating factor, which interact at a cellular level with developing stem cells to alter proliferation.

Because of the morphological “nursing” function of macrophages, we have investigated the interaction of bone marrow macrophages with developing erythroblasts, using clonal culture techniques. Our results indicate that normal human bone marrow macrophages influence the growth of both early (BFU-e) and late (CFU-e) committed erythroid precursors. Furthermore, our investigations demonstrate that bone-marrow-derived mononuclear phagocytes modulate erythropoiesis both by synthesis of soluble factors and through cell–cell interaction. The results suggest a central regulatory function of marrow macrophages that is different from that seen with peripheral blood monocytes.

MATERIALS AND METHODS

Liquid Culture of Bone Marrow

Human bone marrow was grown in liquid culture as previously described. Briefly, marrow aspirates were obtained by iliac puncture either in normal donors or in patients undergoing bone marrow aspiration for staging purposes for evaluation of Hodgkin’s disease or for staging of solid tumors. Three to five milliliters of marrow mixed with 500 U of heparin was passed through successively smaller gauge needles (nos. 18–25). After addition of an equal volume of CMRL 1066 (Grand Island Biological Co., Grand Island, N.Y.) pH 7.8, containing 1% glutamine, 1% fetal calf serum (Reheis Chemical Co., Kankakee, Ill.), and 1% penicillin-streptomycin (Grand Island Biological Co.), the mixture was layered onto a Ficoll-Hypaque gradient and centrifuged at 400 g for 30 min at 4°C to remove mature RBC and polymorphonuclear leukocytes (PMNs). Cells at the interface were collected, washed 3 times with media, counted, and tested for viability by trypan blue exclusion. The cell concentration was adjusted to 2–3×10⁶ viable cells/ml of media; 1 ml of this suspension was placed into the chamber of a modified Marbrook flask with 75 cc of media in the outer portion of the flask. The flasks were incubated at 37°C with humidity and 5% CO₂. The cells were harvested at 7–14 days, as previous experiments have shown that 68% ± 26% of viable cells remaining in culture at that time morphologically resemble macrophages. Further experi-
ments have shown that these cells also manifest other characteristics of macrophages, such as phagocytosis, membrane Fc receptor activity, surface adherence, and chemotaxis.

**Coculture Studies**

The macrophages obtained from the liquid cultures and their cell-free supernatants were cocultured with freshly prepared human bone marrow buffy coat cells or peripheral blood mononuclear cells (PBMN), with or without erythropoietin, in plasma clots. PBMN were prepared by diluting the blood 1:1 with Seligman's balanced salt solution, layered over Ficoll-Hypaque (1.077 specific gravity), and centrifuged at 400 g for 25 min at 15°C. Interface cells were washed, and resuspended in RPMI-25% autologous sera at a concentration of 4 x 10⁶ cells/ml. Eight-milliliter aliquots were placed in plastic tissue culture flasks (75 sq cm, Falcon) and incubated at 37°C for 45 min. The flask was then carefully turned so the contents were now in contact with the other surface and incubated for an additional 45 min. The nonadherent cells were removed, washed, and used in coculture studies. This procedure resulted in reducing the monocyte concentration of these cell preparations from 24% ± 3% to 2.3% ± 0.4%. The plasma clot system used was as previously described, except that varying amounts of macrophages and/or macrophage culture conditioned media were added to the final plasma clot culture. In these cocultures, varying concentrations of macrophages were added to either 6 x 10⁶ bone marrow cells or 10⁵ monocyte-depleted mononuclear cells in the presence of erythropoietin (marrow, 1.5 IU/ml; blood, 3 IU/ml). Thus, the number of erythroid progenitors/ml of culture remained constant, while the total number of cells/ml culture varied depending on the number of macrophages added. We have previously demonstrated that this variation in total cell concentration did not play a significant role in these coculture studies. After 7 days (for CFU-e) or 15 days (BFU-e) in culture, erythroid colonies in each plasma clot were counted.

**Adherence Studies**

In some experiments the macrophage population was depleted by adherence to baby hamster kidney cell microexudate-coated flasks as previously described. Alternatively, adherent cells were collected using a modification of the method of Rinehart et al.

Bone marrow cells were cultured for 10-14 days in liquid media and then were adhered to plastic culture dishes for 2 hr in the presence of 20% human AB serum. The dishes were rinsed 3 times with culture media and the nonadherent population collected. Adherent cells were subsequently removed by exposure to 30 mM lidocaine in media at room temperature for 30 min and washed 3 times.

**RESULTS**

When low concentrations of human bone marrow macrophages from 15 patients were cocultured with fresh bone marrow cells in plasma clots, erythroid colony (CFU-e) formation was stimulated (Fig. 1). Thus, if macrophages comprised between 1% and 10% of the total cells plated, the resulting erythroid colony numbers (per 6 x 10⁵ bone marrow cells plated) were 125%-150% of control samples devoid of added macrophages. Conversely, larger numbers of added macrophages caused progressive inhibition of erythroid formation until, at macrophage concentrations greater than 40%, no erythroid colonies were seen.

Fractionation of the modulator cells by adherence demonstrated that, in two experiments, cells not adherent to baby hamster kidney microexudate flasks showed no effect, whereas, in two separate experiments, adherent cell populations produced stimulatory effects (120%-157% of control CFU-e) at low concentrations and inhibitory effects (44%-54% of control CFU-e) at higher concentrations. The modulations by adherent cells are identical to those observed with unfractionated liquid culture bone marrow cells.

Supernatants from macrophage cultures also modulate erythroid colony formation in vitro. When 0.05-0.15-ml aliquots of the macrophage-conditioned media

![Fig. 1. Effect of human bone marrow macrophages on erythroid colony formation by normal human bone marrow CFU-e. When added to plasma clots with freshly obtained bone marrow mononuclear cells, bone-marrow-derived macrophages in small numbers (1%-10%) caused stimulation of erythroid colony formation, while at higher concentrations there is a progressive inhibition of erythroid colony formation.](image1)

![Fig. 2. Effect of macrophage-conditioned media on erythroid colony formation by normal human bone marrow CFU-e. Macrophage-conditioned media (MCM), when added to plasma clots in low concentrations, causes stimulation of erythroid colony formation, while at high concentrations it causes inhibition.](image2)
(1 ml/10^6 cultured cells) from 10 patients were added to the plasma clot system, erythroid colony formation was enhanced (Fig. 2); at higher concentrations conditioned media was inhibitory.

Since cocultures frequently were done using red cell precursors and macrophages from different donors, we excluded the role of heterologous cell interactions in our results by performing experiments using the same donor as a source of both red cell precursors and macrophages. Erythroid proliferation characteristics were the same as with the heterologous systems (mean of 185% of control at 6% macrophages, and 52% of control BFU-e at 20% autologous macrophages), indicating that neither macrophage-induced stimulation nor inhibition can be attributed to heterologous cell-cell interactions.

As with CFU-e, added macrophages also modulated BFU-e proliferation. Thus, addition of bone-marrow-derived macrophages of 8 patients in concentrations of 4%-10% enhanced erythroid colony formation from peripheral blood cells whereas at higher concentrations erythroid colony formation was progressively inhibited (Fig. 3). These modulatory effects on BFU-e colony formation by macrophages were duplicated by supernatants derived from macrophage cultures (Fig. 4).

Since in these cultures no red cell colonies are generated in the absence of erythropoietin, the possibility that at least the stimulatory effect might result from erythropoietin production by macrophages in culture was also considered. Exhypoxic polycythemic mice were used to assay macrophage-conditioned supernatant for erythropoietin, and neither the conditioned media nor a concentrate of it showed measurable erythropoietin activity.

DISCUSSION

Our experiments indicate that bone-marrow-derived macrophages may modulate erythroid colony formation by human CFU-e and BFU-e, thus, in low concentrations (1%-10%) bone marrow cells enriched for macrophages enhance erythroid colony formation, while at higher concentrations, erythroid colony formation is progressively inhibited. Although approximately 20%-40% of cells in the liquid culture are not macrophages, the adherent cells in our cultures altered in vitro erythropoiesis whereas nonadherent cells had no effect, indicating that the macrophage is the active cell. Our data support and extend a previous report that adherent bone marrow cells modulate in vitro erythroid growth characteristics. These results, using a system that allows isolation of a population of bone-marrow-derived macrophages, are different from those using peripheral blood monocytes reported earlier from our laboratory. Thus, monocytes, although supposedly derived from precursors in the bone marrow, exert only inhibitory effects on the peripheral blood BFU-e at all added concentrations. Whether this difference in function between bone-marrow-derived and peripheral blood mononuclear phagocytic cells results from changes in surface membrane antigens, secretory capacities, or other unknown factors remains for further study.

Previous evidence suggests that competitive demands on erythroid and granulocytic lines of differen-
cell-free macrophage-conditioned media expressed the stimulating factor (CSF) causes the suppression of colony-nulopoiesis, is suggested by the observation that colony-stimulating factor (CSF) causes the suppression of erythropoietin-stimulated hemoglobin synthesis, while erythropoietin can suppress CFU-c proliferation. Monocyte-macrophage involvement in such competitive regulation is suggested by their ability to produce colony-inhibiting prostaglandins as well as colony-stimulating factor (CSF). Such capabilities make these cells potential negative (PGE) and positive (CSF) feedback modulators of myeloid stem cell differentiation. The possibility that erythropoietin production by macrophages might underlie the observed stimulation of in vitro erythropoiesis was explored but found unlikely, since no erythropoietin activity could be detected in the macrophage-conditioned media. Although cell-cell interaction with direct transfer of erythropoietin from macrophage to erythroid cell is possible, this seems unlikely since cell-free macrophage-conditioned media expressed the same stimulatory effect as adherent cells and since

cells enriched for macrophages (or conditioned media) failed to support erythroid colony formation in the absence of erythropoietin. The identity of other soluble substances secreted by the mononuclear cells that may be operative in regulating stem cell proliferation is unknown at present. However, data to be presented elsewhere indicate that lysozyme, a 15,000 molecular weight protein synthesized continuously by cells of the monocyte-macrophage system and found to alter certain membrane-dependent cell functions, is one such factor.

To summarize, cultured bone marrow cells enriched for macrophages or conditioned media therefrom can regulate both bone marrow and peripheral blood stem cell differentiation into erythroid cells and do so evidently without involvement of erythropoietin. This modulating function of these cells, we believe, may have implications for understanding the abnormal hemopoiesis in states of chronic infection, neoplastic disease, and aplastic anemia, where derangements in bone marrow macrophage number or function may exist. A preliminary report from our laboratory of macrophages inhibiting excessive erythroid colony-inhibitory activity in chronic histoplasmosis may serve as an internal validation of this prediction.

REFERENCES

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