

## **Purification of fetal hematopoietic progenitors and demonstration of recombinant multipotential colony-stimulating activity.**

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### **Research Article**

To facilitate the direct study of progenitor cell biology, we have developed a simple and efficient procedure based upon negative selection by panning to purify large numbers of committed erythroid and myeloid progenitors from human fetal liver. The nonadherent, panned cells constitute a highly enriched population of progenitor cells, containing 30.4 +/- 13.1% erythrocyte burst forming units (BFU-E), 5.5 +/- 1.9% granulocyte-macrophage colony forming units (CFU-GM), and 1.4 +/- 0.7% granulocyte-erythroid-macrophage-megakaryocyte colony forming units (CFU-GEMM) as assayed in methylcellulose cultures. These cells are morphologically immature blasts with prominent Golgi. This preparative method recovers 60-100% of the committed progenitors detectable in unfractionated fetal liver and yields 2-30 X 10<sup>6</sup> progenitors from each fetal liver sample, and thus provides sufficient numbers of enriched progenitors to allow direct biochemical and immunologic manipulation. Using this technique, a purified recombinant protein previously thought to have only granulocyte-macrophage colony stimulating activity (GM-CSA) is shown to have both burst promoting activity and multipotential colony stimulating activity. Progenitor purification by panning thus appears to be a simple, efficient method that should facilitate the direct study of committed hematopoietic progenitors and their differentiation.

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## Purification of Fetal Hematopoietic Progenitors and Demonstration of Recombinant Multipotential Colony-stimulating Activity

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### Abstract

To facilitate the direct study of progenitor cell biology, we have developed a simple and efficient procedure based upon negative selection by panning to purify large numbers of committed erythroid and myeloid progenitors from human fetal liver. The non-adherent, panned cells constitute a highly enriched population of progenitor cells, containing  $30.4 \pm 13.1\%$  erythrocyte burst forming units (BFU-E),  $5.5 \pm 1.9\%$  granulocyte-macrophage colony forming units (CFU-GM), and  $1.4 \pm 0.7\%$  granulocyte-erythroid-macrophage-megakaryocyte colony forming units (CFU-GEMM) as assayed in methylcellulose cultures. These cells are morphologically immature blasts with prominent Golgi. This preparative method recovers 60–100% of the committed progenitors detectable in unfractionated fetal liver and yields  $2\text{--}30 \times 10^6$  progenitors from each fetal liver sample, and thus provides sufficient numbers of enriched progenitors to allow direct biochemical and immunologic manipulation. Using this technique, a purified recombinant protein previously thought to have only granulocyte-macrophage colony stimulating activity (GM-CSA) is shown to have both burst promoting activity and multipotential colony stimulating activity. Progenitor purification by panning thus appears to be a simple, efficient method that should facilitate the direct study of committed hematopoietic progenitors and their differentiation.

### Introduction

The cellular and molecular analysis of hematopoietic regulation has been hindered by the extreme rarity of the critical hematopoietic progenitor cells in the bone marrow and peripheral blood (1, 2, 3). Identification and accurate characterization of colony stimulating activities have been particularly difficult due to the endogenous production of these activities by accessory cells that contaminate the progenitor cells isolated by current techniques. Thus, a source of purified progenitors and purified factors would be invaluable for the accurate analysis of hematopoietic regulation. Approaches to the purification of progenitor cells have included differential density centrifugation (4) and density gradient electrophoresis (5), immune selection using rosetting (6) or fluorescence-activated cell sorting (7, 8), and various combinations of these physical and immunologic techniques (9).

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While these techniques have led to a significant progenitor enrichment, cell yields have been notably low, particularly when the prefractionation sample consists of normal, nonneoplastic human hematopoietic cells. This limitation has restricted biochemical analysis of the enriched progenitors, and has prevented detailed studies of growth factor and accessory cell interactions with human progenitor cells.

We have therefore developed a simple and efficient procedure to purify large numbers of human erythroid and myeloid progenitors. Beginning with fetal liver cells (10, 11, 12) and utilizing negative selection by panning (13, 14, 15), the technique greatly enriches the progenitor cells and provides, for the first time, sufficient numbers of progenitors to allow direct morphological and biochemical studies of these cells. In this report, we describe this progenitor purification technique, we present preliminary morphologic analysis of the progenitors, and we show that a purified recombinant protein initially characterized as granulocyte-macrophage colony stimulating activity (GM-CSA) (16) in fact possesses both burst promoting activity (BPA)<sup>1</sup> and multipotential colony stimulating activity (multi-CSA).

### Methods

*Isolation of mononuclear cells from fetal liver.* Abortuses induced by prostaglandin infusion were obtained within 30 min of delivery from patients who had previously signed consent forms for research studies, under a protocol approved by the Brigham and Women's Hospital Human Investigation Committee. Livers from 17- to 23-wk fetuses were sterilely dissected and teased into Iscove's modified Dulbecco's medium (IMDM) containing 50 mg% collagenase Type IV (Sigma Chemical Co., St. Louis, MO) using forceps and scissors. Hepatic stroma was digested by incubating the collagenase-rich cell suspension at 37°C under 5% CO<sub>2</sub> for 15–30 min (17) and large clumps were allowed to settle out by standing the cell suspension in vertical 50-ml centrifuge tubes. A mononuclear cell preparation was prepared by centrifugation over Ficoll Hypaque SG 1.077, through which sedimented mature blood cells and hepatocytes. The cells at the interface were washed three times, and then subjected to a second buoyant density separation over Ficoll Hypaque SG 1.077. The cells at the interface were washed three times and then depleted of adherent cells by overnight adherence to 100 × 15 mm plastic tissue culture dishes (Lux, Miles Laboratories, Naperville, IL) in IMDM with 20% fetal calf serum (FCS) at 37°C under 5% CO<sub>2</sub>.

*Immune depletion by adherence to anti-Ig coated plates (panning).*

1. *Abbreviations used in this paper:* BPA, burst promoting activity; BFU-E, erythrocyte burst forming units; CFU-GEMM, granulocyte-erythroid-macrophage-megakaryocyte colony forming units; CFU-GM, granulocyte-macrophage colony forming units; CSA, colony stimulating activity; FCS, fetal calf serum; GM-CSA, granulocyte-macrophage colony stimulating activity; GM-CSF, granulocyte-macrophage colony stimulating factor; IMDM, Iscove's modified Dulbecco's medium; multi-CSA, multipotential colony stimulating activity.

Nonadherent cells were incubated with saturating quantities of a panel of eight murine monoclonal antibodies for 1 h at 4°C. The antibodies, their sources, specificities, and the quantities per 10<sup>6</sup> cells which were employed were as follows: anti-Leu-1 (Becton-Dickinson & Co., Oxnard, CA), T cells, 100 ng; anti-leu 5b (Becton-Dickinson & Co.), T cells and NK cells, 40 ng; anti-leu-10 (Becton-Dickinson & Co.), HLA-DC, 400 ng; anti-leu-M1 (Becton-Dickinson & Co.), mature and precursor myeloid and monocytic cells, 200 ng; anti-BA-1 (Hybritech, Inc., La Jolla, CA), B cells, 200 ng; anti-CALLA (Hybritech), pre-B cells, 300 ng; TG-1 (Peter Beverley [7]), granulocytes, myeloid precursors, some monocytes, 5 μl hybridoma supernatant; R.18.84 (Paul Edwards [18]) glycophorin A, 1 μl hybridoma supernatant. Labeled cells were then washed three times to remove excess antibody before panning. Preparative separation of antibody-negative cells (panning) was performed by a modification of the method of Biddison et al. (14). Anti-Ig plates were prepared by incubating 100 × 15 mm plastic culture plates (Lux) with 5 ml affinity purified rabbit anti-mouse Ig (100 μg/ml in phosphate-buffered saline [PBS]) overnight at 4°C and washing with cold PBS before use. Coating the plates with <250 μg antibody resulted in suboptimal cell binding. Murine antibody-labeled, washed cells were suspended in 5 ml PBS at 1–5 × 10<sup>7</sup> cells/ml with 5% heat-inactivated FCS and incubated over the rabbit anti-mouse Ig coated plates for 1 h at 4°C, after which time the nonadherent antibody negative cells were recovered by gentle pipetting without disrupting the antibody-coated cells bound to the plates. Incubation times of <40 min were ineffective at allowing maximal cell binding to the plates, while cells could be allowed to adhere to the plates for at least 2 h without decreased binding or subsequent plating efficiency.

**Immunofluorescence staining.** After immune depletion by panning, the presence of residual hematopoietic precursor cells was assessed morphologically and by indirect fluorescent labeling under three conditions: (a) Cells were immediately relabeled with fluoresceinated goat anti-mouse Ig to detect residual mouse antibody coated cells; (b) other aliquots of cells were immediately relabeled with the individual murine monoclonal antibodies, followed by fluoresceinated goat anti-mouse Ig to assay cells that had been ineffectively labeled; and (c) a final aliquot was cultured at 37°C for 4 h before relabeling, to identify cells that might have modulated surface antigens on exposure to the murine antibodies and so escaped the secondary antibody-coated plates. Fluorescent antibody-stained cells were quantitated by flow cytometric analysis (fluorescence analyzer; Becton-Dickinson & Co.)

**In vitro short-term cultures.** Cells were cultured in 0.9% methylcellulose in IMDM plus 30% FCS, 0.9% deionized bovine serum albumin (Fraction V; Sigma Chemical Co.), and 10 M<sup>-4</sup> β-mercaptoethanol. To measure erythrocyte burst forming unit (BFU-E) derived colonies the cells were cultured in the presence of 1 u/ml erythropoietin (Terry Fox Laboratories, Vancouver, British Columbia) (19). Granulocyte-macrophage colony forming units (CFU-GM) and granulocyte-erythroid-macrophage-megakaryocyte colony forming units (CFU-GEMM) were assayed by culturing the cells in the presence of 10% vol/vol Mo conditioned medium as well. Red colonies (usually containing over 1,000 cells and always at least 100 cells) present on day 14 were scored as BFU-E; colonies containing nonhemoglobinized cells (primarily granulocytic precursors and macrophages) were scored as CFU-GM, while colonies containing both erythroid elements and one or more classes of phagocytic on megakaryocytic cells were scored as CFU-GEMM. Cultures were plated either in quadruplicate 250-μl volumes in 24-well tissue culture plates or in 10 replicates in 100-μl volumes in 96-well plates (Falcon Labware, Becton-Dickinson & Co.). The cultures were established at sufficiently low density to clearly distinguish individual colonies, usually 5 × 10<sup>4</sup> cells/ml for cells after one Ficoll gradient, 10<sup>4</sup> cells/ml for cells obtained after the second gradient step and adherence depletion, and 10<sup>3</sup> cells/ml obtained after depletion by panning. No filler cells or irradiated feeder cells were added to the cultures. Progenitor recovery was measured by normalizing the progenitor frequency assayed in each fraction by the percentage of cells removed during the purification, taking the number of progenitors present in the original ficoll interface mononuclear cell fraction as 100%: % recovery = colonies per 10<sup>5</sup> cells × proportion of cells recovered ÷ colonies per 10<sup>5</sup> cells in the unfractionated (Ficoll × 1) sample × 100.

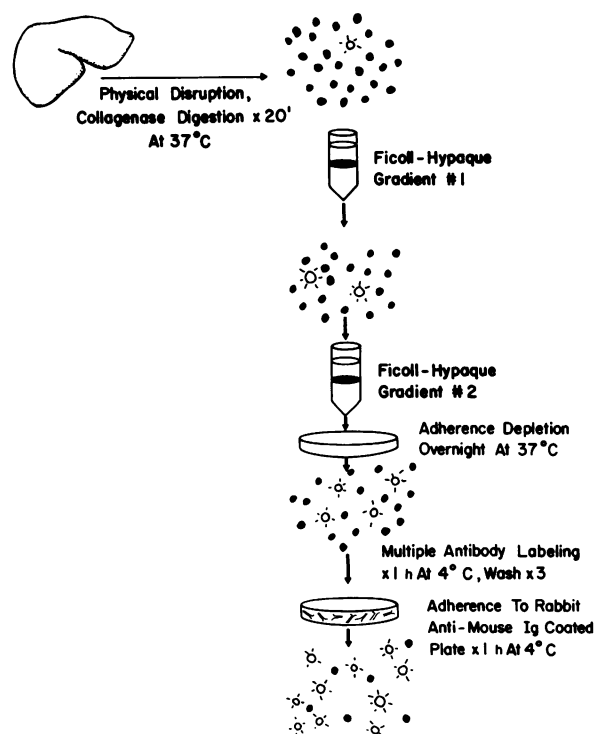
**Demonstration of GM-CSA, BPA, and multi-CSA.** Recombinant

colony stimulating activity cloned and purified from the complementary DNA (cDNA) of the Mo cell line was prepared as previously described (16). Briefly, a cDNA library was derived from messenger RNA (mRNA) of lectin-stimulated Mo cells, and active clones coding for colony stimulating activity (CSA) were detected by transfection of monkey COS-1 cells. The recombinant CSA was then purified from serum-free COS-1 supernatants by gel filtration and high performance liquid chromatography. GM-CSA, BPA, and multi-CSA were assayed on the final purified progenitor fraction by initiating methylcellulose cultures in the presence of graded concentrations of the HPLC purified recombinant protein, and after 3 d 1 U/ml erythropoietin was added to each replicate (20, 21). No additional source of BPA or CSA was included in the cultures. BFU-E, CFU-C, and CFU-GEMM were enumerated on day 14, as before.

## Results

**Cell recovery and analysis through the fractionation.** The fetal progenitor purification schema, with two ficoll gradients, one adherence depletion, and a final immune depletion by panning, is shown in Fig. 1. The first ficoll step removed the mature erythrocytes, rare mature myeloid cells, and hepatocytes. The cells at the interface were >95% mature erythroid precursors with <5% myeloid and mononuclear precursors. The second ficoll step further removed 70–90% of the cells, including >80% of metamyelocytes, myelocytes, and orthochromatophilic and polychromatophilic normoblasts. The cells at the interface consisted of 10–15% blasts, 20–30% promyelocytes, and 50–70% basophilic and polychromatophilic normoblasts.

After adherence depletion, the remaining cells were labeled with a panel of eight murine monoclonal antibodies including at least two antibodies that detect each major class of leukocyte, and antiglycophorin A to detect the predominant antigen on



**Figure 1.** Purification of fetal progenitors by panning. Single cell suspensions of fetal liver mononuclear cells were subjected to two sequential Ficoll gradients and adherence depletion, followed by antibody labeling and depletion of antibody-coated cells by panning. Progenitor cells are depicted as open stars, nonprogenitor cells as closed circles.

Table I. Progressive Purification of Fetal Progenitors

Fractionation step	Cells recovered	BFU-E colonies/10 <sup>5</sup> cells	Percent yield	CFU-GM colonies/10 <sup>5</sup> cells	Percent yield	CFU-GEMM colonies/10 <sup>5</sup> cells	Percent yield
Ficoll × 1	300–3,000 × 10 <sup>6</sup>	218 ± 18*	100	87 ± 8	100	11 ± 2	100
Ficoll × 2, Ad <sup>-</sup>	30–600 × 10 <sup>6</sup>	2,200 ± 340	95 ± 3	950 ± 82	97 + 2	120 ± 2	97 + 2
Ficoll × 2, Ad <sup>-</sup> , Ab <sup>-</sup>	2–60 × 10 <sup>6</sup>	30,400 ± 13,000 (Range, 18,000–55,000)	88 + 10	5,500 ± 1,900 (Range, 3,200–15,000)	62 + 20	1,400 ± 700 (Range, 500–2,800)	98 + 10

\* Mean values ± standard deviations from 12 experiments.

erythroid precursors. In preliminary experiments each antibody was shown to bind to precursor cells and mature cells, without detecting progenitor cells. The combined use of several overlapping anti-leukocyte antibodies and a very potent anti-erythroid antibody allowed for excellent cell depletion after a single 1-h pan at 4°C. Under a variety of relabeling conditions, no more than 2% of the remaining cells were stained with antibodies detecting known hemato-lymphoid subsets. The single pan removed 90–95% of the cells, and the remaining antibody-negative cells were at least 97% viable by trypan blue exclusion.

*Progressive purification of hematopoietic progenitors.* This sequential fractionation procedure resulted in marked enrichment of the progenitor cells (Table I). The mononuclear cells obtained after one Ficoll step contained ~0.3% progenitors. After the second gradient step and adherence depletion, over 3% of the remaining cells scored as progenitors in vitro. After the panning step, the progenitors were greatly enriched with 22–75% of the cells scoring as progenitors in short-term culture. No progenitors could be detected by culturing cells obtained from either the Ficoll gradient pellets or the antibody-coated plates.

The majority of the progenitors present in the original fetal liver mononuclear cell suspension were recovered throughout the fractionation procedure. In the final fraction, after double ficoll separation, adherence depletion and panning, 88 ± 10% of BFU-E, 62 ± 20% of CFU-GM, and 98 ± 10% of CFU-GEMM were recovered. Thus, the 2–60 × 10<sup>6</sup> cells obtained from each fractionation contained most of the progenitors present in the original fetal liver.

*Linear plating efficiency to low cell concentration.* This high degree of progenitor purity permitted short-term in vitro culture at low cell density (Fig. 2). Cells obtained after the first ficoll step were routinely cultured at 10<sup>5</sup> cells/ml, and could only be diluted to 10<sup>4</sup> cells/ml while still retaining normal growth characteristics. Below this concentration the colonies which began to develop disintegrated a few days into the culture. Cells obtained after a second gradient separation and adherence depletion could be cultured over a range of from 10<sup>3</sup>–10<sup>5</sup> cells/ml with linear plating characteristics.

The highly enriched progenitors isolated after panning could be cultured at 10<sup>2</sup>–10<sup>4</sup> cells/ml while maintaining qualitatively normal colony growth and linear plating characteristics. When cultured at higher density, the colonies overlapped in the plates and could not be accurately enumerated. When the cells were plated at <10<sup>2</sup>/ml (10–25 cells per well), colony growth was poor, with only small poorly differentiated colonies that disintegrated prematurely.

*Initial characterization of the purified progenitors.* Sufficient quantities of purified progenitors were thus obtained to permit direct morphological and histochemical characterization of these cells. When stained with routine Wright-Giemsa, the cells appeared to be undifferentiated blasts of variable size with basophilic cytoplasm, prominent Golgi zones, and often containing

multiple nucleoli. In addition, the cell membranes showed many small areas of extrusion suggesting uropod formation (Fig. 3). There were no detectable cells resembling small lymphocytes, and as expected, only very rare contaminating recognizable erythroid or myeloid precursors were found. The panned progenitor cells did not stain with standard histochemical dyes, including myeloperoxidase, periodic acid-Schiff, nonspecific esterase, alkaline phosphatase, and acid phosphatase.

*Detection of BPA and multi-CSA in recombinant protein.* These highly enriched progenitors were then used to detect the range of myeloid stimulating activities present in a purified recombinant protein cloned from the Mo cell line (16), which was initially thought to have only a restricted GM-CSA. Short-term cultures were initiated in methylcellulose without erythropoietin, which was added on day three. Under these conditions no colonies developed, as all of the accessory cells that contribute to the survival and proliferation of the progenitor cells had been removed and no exogenous source of BPA or CSA (e.g. Mo CM) had been added (Fig. 4). However, when the recombinant protein was added at the initiation of the cultures, erythroid bursts and mixed GEMM colonies as well as purely myeloid colonies were detected. Maximal BPA was detectable with as little as 1 ng/ml added recombinant protein, and both BPA and multi-CSA were detectable with as little as 10 pg/ml. The use of purified progenitors depleted of known accessory cells thus results in extremely low background that permits the detection of BPA and multi-CSA in this protein.

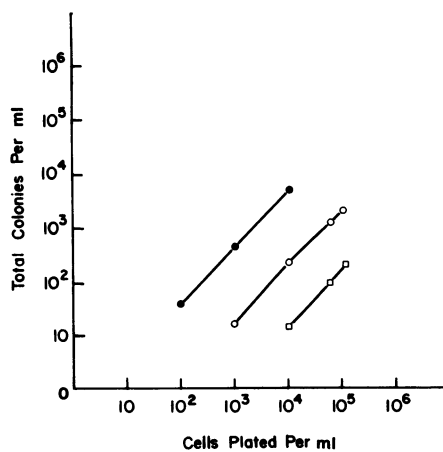


Figure 2. Linear plating efficiency of fetal liver progenitors. Cells obtained from three stages of the purification (after the first Ficoll gradient, after the second Ficoll gradient, and after the adherence panning depletion step) were plated in methyl cellulose at 10<sup>2</sup>–10<sup>5</sup> cells per ml. The data are shown as total colonies (BFU-E + CFU-GM + CFU-GEMM) per ml, and represent mean values from one of four similar experiments. ●, two Ficolls, nonadherent cells, antibody negative cells after panning; ○, two Ficolls, nonadherent cells; □, one Ficoll.



Figure 3. Wright-Giemsa stain of the panned progenitor cells. Note the prominent Golgi, nucleoli, and membrane exfoliation. Colony frequencies from this particular preparation were: BFU-E, 39%; CFU-GM, 9.5%; CFU-GEMM, 1.5%.

## Discussion

This technique provides a superior method for the isolation of large numbers of highly enriched, relatively unmanipulated progenitors. Of note, the calculated progenitor frequency of 22–75% may be an underestimate of the actual number of progenitors present in the final fraction, due to the limitations of in vitro culture per se. Visser et al. (22) have found that murine progenitor cells which they calculate to be 100% in vivo primary CFU-S have only a 25–30% plating efficiency in vitro. Ogawa and his colleagues (23) have found that the replating efficiency in methylcellulose of observable, dividing colony progenitors is only 50–75%. Thus the actual frequency of progenitors in the post-panning fraction may be closer to 50–100%, with the lower observed frequency due to the imperfect plating efficiency of the methyl cellulose system itself.

Panning for negative selection has several advantages over other techniques for progenitor purification. Unlike rosetting (6, 24, 25) or complement mediated lysis, panning involves no potential nonspecific toxins, such as  $\text{CrCl}_3$  or complement, which can damage the progenitors. This is extremely important for normal progenitors, which we have found are quite sensitive to nonspecific damage by  $\text{CrCl}_3$ -coupled erythrocytes used for rosetting, unlike leukemic progenitors, which may be somewhat more resistant. Moreover, the panning procedure is based on the labeling of only undesired cells, without direct manipulation of the progenitors themselves. Thus, the in vitro behavior of the recovered progenitors is unlikely to be artifactually influenced by the antibody treatment per se. Finally, panning is very simple

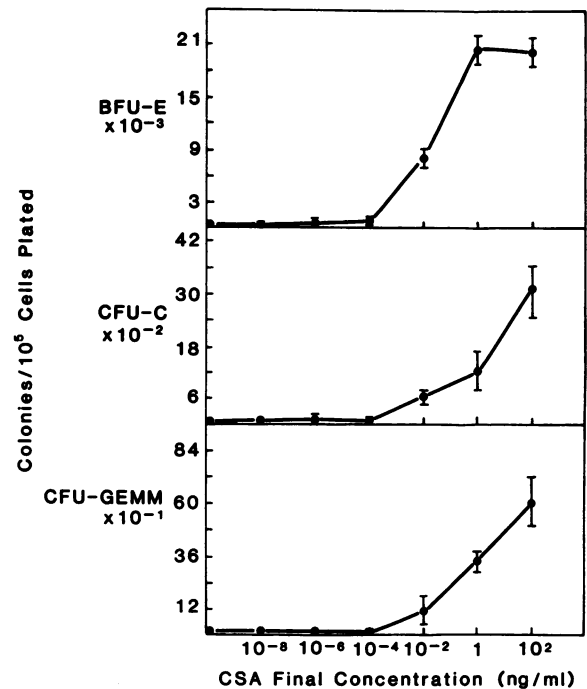


Figure 4. Multipotential colony stimulating activity demonstrated using purified progenitor target cells. Purified progenitors were cultured in methylcellulose in the presence of graded concentrations of purified recombinant colony stimulating activity, with erythropoietin added on day three. The data shown are mean  $\pm$  standard deviations from quadruplicate samples and are from one of three similar experiments.

and easy to perform, and results in an extremely high yield of recovered progenitors. In these respects it is superior to multiparameter fluorescence-activated cell sorting, for example, which requires sophisticated equipment and still results in typical cell yields of only 20–50%.

The isolated progenitors appear to be basophilic, undifferentiated blasts with prominent Golgi zones and frequent membranous extrusions. The active Golgi suggests that these cells are actively synthesizing membrane glycoproteins, likely including membrane receptors for hematopoietic growth factors. Thus these cells might be an excellent cell population for identification and study of such receptors. The unusual folded membrane may reflect submembranous cytoskeletal structures responsible for the immature progenitors' motility, which are responsible for the multicentric nature of erythroid bursts and multilineage colonies.

Ideally, one would like to be able to culture these progenitors at limiting dilution to address directly questions such as the role of soluble factors, and auxiliary cells in altering the stochastic program of differentiating progenitors. So far, however, we have only succeeded in routinely culturing these cells at 100 cells/ml (10–25 cells/well), despite trials of irradiated feeder layers, added progenitor conditioned culture medium, and a variety of added soluble factors. Whether fetal progenitors require a continuous supply of autocrine factors, have stringent buffering requirements which we have not yet solved, or require an as yet unidentified accessory cell to respond to the trophic effects of added soluble CSAs, remains to be determined. If fetal progenitors do require intermediary accessory cells to respond to added soluble factors, however, their behavior would be quite different from that of circulating adult BFU-E, which respond directly to BPA (25).

The high progenitor concentration and the lack of T cells,

monocytes, and other cells known to produce hematopoietic growth factors allow the purified progenitor pool to be an extremely sensitive and accurate test population for assaying the activity of soluble factors and defined cell populations. In this study these purified progenitors were used to demonstrate that a purified recombinant protein previously thought to have only limited GM-CSA in fact processes both BPA and multi-CSA. Removing the active accessory cells was in fact essential to demonstrating the full spectrum of activity of the recombinant CSA molecule.

Although this protein has ~60% DNA sequence homology with murine granulocyte-macrophage colony stimulating factor (GM-CSF) and none with murine interleukin-3 (IL-3) (16), its activity resembles that of murine IL-3. Inasmuch as no human DNA sequences homologous to murine IL-3 have yet been detected, it is possible that during evolution the human GM-CSA gene has taken over the multipoietin role characteristic of IL-3 in the mouse. It will be of interest to determine, once their DNA and protein sequences are known, whether molecules with putative multipotential CSA purified from other sources (26) are identical to the protein analyzed in this study, and to measure the full target cell specificities and phylogenetic ranges of activities of these hormones.

This method for progenitor purification should prove to be extremely valuable for the further study of the regulation of hematopoiesis. The preparations provide, for the first time, sufficient normal cells for the direct biochemical isolation of human progenitor membrane proteins, including membrane receptors for such proteins as erythropoietin and multipotential CSA. Similarly, the purified progenitors are an ideal immunogen and screening cell source for the generation and detection of antibodies truly specific for hematopoietic progenitors. Purification by panning is thus a simple, highly efficient technique for the isolation of fetal liver progenitors, and is a valuable tool for improving our understanding of the molecular and cellular regulation of human hematopoiesis.

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