Human Hematopoietic Precursors in Long-Term Culture: Single CD34⁺ Cells that Lack Detectable T Cell, B Cell, and Myeloid Cell Antigens Produce Multiple Colony-Forming Cells When Cultured with Marrow Stromal Cells

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Summary

CD34⁺ human marrow cells not expressing T cell-, B cell-, and myeloid cell–associated antigens (TBM⁻) were cloned by two-color cell sorting into culture wells containing irradiated marrow stromal cells. After 4 wk of culture, $3.7 \pm 2.1\%$ of these cells generated colony-forming cells (CFC), with each of these cells generating 6.3 ± 5.3 CFC. This was not due to the $0.5 \pm 0.5\%$ CFC present in the purified CD34⁺ TBM⁻ cells, as <1% of CFC persist in these cultures. This is the first demonstration that single immature precursor cells in human long-term cultures generate multiple CFC progeny. The immature nature of these clonable CD34⁺ TBM⁻ precursors suggests their candidate status as human hematopoietic stem cells.

Hematopoiesis is maintained in vivo by multipotential stem cells (1–3). In humans, the characterization of these rare cells has been elusive, due to an inability to purify them and the absence of a suitable in vitro clonal assay to study their potential. Precursors of myeloid colony-forming cells (CFC) can be distinguished from their more mature CFC progeny based on surface antigen expression and light scatter properties (4–6). While these cells do not form colonies in soft agar in the presence of most known growth factors, they produce CFC after culture with marrow stromal cells (MSC).

If these precursors of CFC are highly immature cells, then single cells should give rise to multiple CFC in vitro. In previously reported studies, the lack of a clonal assay for precursors of CFC in long-term culture precluded this conclusion. The absolute number of detectable CFC did not exceed the absolute number of cells placed in the cultures (4–6). In the present studies, based on direct cloning of single cells by two-color FACS, we identified a minor subpopulation of CD34⁺ marrow cells (<1% of CD34⁺ cells) in which at least 1–7% of cells were precursors for more than one granulocyte/macrophage (GM)-CFU.

Materials and Methods

Antibody Preparation and Purification. Antibodies 35.1 (anti-CD2) (7), G3-7 (anti-CD7) (7), 24.1 (anti-CD10) (7), 60.3 (anti-CD18) (7), 4119 (anti-CD19) (8), and F13 (anti-CD36) (7) were used as 1:1,000 dilutions of ascites fluids. Isotype controls were 31A (9)

(IgG, anti–mouse Thy-1.1) and H12C12 (5) (IgM, anti–mouse Thy-1.2). IgM antibodies 12-8 (anti-CD34) (10) and H12C12, and IgG antibodies 1F5 (anti-CD20) (7), p67-6 (anti-CD33) (5), and 31A were purified as described (5, 11), and used at 25 μg/ml for IgMs and 5 μg/ml for IgGs (based on titers). 7B9 identifies a novel antigen present on GM-CFU, erythroid (E)-BFU, E-CFU, and MIX-CFU (Bernstein et al., manuscript in preparation).

Separation of Marrow Cells. Marrow samples obtained after informed consent were separated by Ficoll-Hypaque density centrifugation (4, 5). Cells were labeled simultaneously with the IgG antibodies F13, 35.1, G3-7, 24.1, 4119, 1F5, 60.3, p67-6, and 7B9 (ABY-MIX) using previously described methods (5), and were then mixed with goat anti–mouse IgG antibody–conjugated magnetic particles (five particles/cell; Exps. 2–5) (Advanced Magnetics, Cambridge, MA), or with a 1:80 dilution of biotin-conjugated goat anti–mouse IgG antisera (γ chain specific; Southern Biotechnology Associates, Birmingham, AL), washed twice, and then mixed with streptavidin-conjugated magnetic particles (five particles/cell; Exp. 1) (Advanced Magnetics). The cell-particle mixtures were gently rocked for 20 min at 4°C. Cells bound to the particles were removed from suspension using magnets (Biomag Separator; Advanced Magnetics). Unbound cells were stained using two-color indirect immunofluorescent antibody staining techniques with 12-8 or the control antibody H12C12, and to detect residual IgG-bearing cells, as described (5). Unseparated cells labeled with: (*a*) H12C12 and 31A; (*b*) 12-8 and 31A; (*c*) H12C12 and ABY-MIX; and (*d*) 12-8 and ABY-MIX served as controls (5). Cells were separated using a modified FACS-II equipped with a modified single-cell deposition unit (Becton Dickinson & Co., Mountain View, CA), calibrated as described (5). To verify the sorting of single events, we sorted

single fluorescent beads into microtiter wells and directly visualized each well. Of 288 wells cloned in two experiments, 96% contained single beads, 4% contained no beads, and <0.35% contained two beads.

Colony-forming Assays. These were performed as previously described in soft agar with human placental conditioned medium (HPCM) and purified human urinary erythropoietin (EPO) (Terry Fox Cancer Research Center, Vancouver, Canada). In some studies, assays were performed by depositing single cells in microtiter wells containing 50 μl of liquid medium containing HPCM and EPO. 50 μl of agar containing medium was then added to each well and allowed to gel.

Liquid Cultures in Microtiter Wells. MSC layers were generated in 75-cm² tissue culture flasks, transferred to wells of microtiter plates, and were irradiated (1,200 cGy; 80 cGy/min) as previously described (5). Marrow cells were cultured in the microtiter wells containing irradiated MSC (4, 5) for 7 d at 37°C, after which time cultures were maintained at 33°C and fed weekly (4). For CFC assays, the entire contents of each culture well, including the MSC, were removed by vigorous pipetting and scraping.

Results

Single CD34⁺ TBM⁻ Marrow Cells in Long-Term Culture. Marrow cells expressing T cell, B cell, or myeloid cell antigens (TBM⁺ cells) were labeled with mAbs to the CD2, CD7, CD10, CD18, CD19, CD20, CD33, CD36, and 7B9 antigens. By flow cytometry, 2.2 ± 1.1% of all marrow cells, and <1% of all CD34⁺ marrow cells, did not detectably express these antigens. Labeled cells were removed from suspension by two cycles of immunoadsorption to magnetic particles (see Materials and Methods). After adsorption, 3.6 ± 2.9% (range, 9-0.2%) of the marrow cells remained. These unadsorbed cells were labeled with the anti-CD34 antibody 12-8 (IgM) and stained to detect residual TBM⁺ (IgG⁺) cells not depleted by adsorption. Of the unadsorbed cells, CD34⁺ TBM⁻ cells with low right angle light scattering properties identified by two-color FACS represented 0.8 ± 0.9% (range, 2.2-0.06%) of the cells. Based on the frequency of CD34⁺ TBM⁻ cells detectable in these unadsorbed populations, we estimated that the CD34⁺ TBM⁻ cells represented 8.9 ± 6.6 × 10⁻⁵ cells in unseparated marrow.

Single CD34⁺ TBM⁻ cells were deposited by the cell sorter directly into microtiter wells containing irradiated MSC, and were also collected nonclonally for culture in soft agar and in microwells over MSC. In five experiments, a mean of 3.7 ± 2.1% of cloned CD34⁺ TBM⁻ cells generated CFC after 4 wk of culture, whereas after 2 wk of culture, only 0.9 ± 0.9% of these cells generated CFC (Table 1). The finding that most positive wells contained more than three CFC (0.5% of cloned cells at 2 wk, 3.1% of cloned cells at 4 wk) provided evidence for at least limited self-renewal by these individual cells or their immediate progeny. The colonies detected were often associated with stromal cells, but not frequently clustered together, and varied in size from 100 to >1,000 cells, as estimated by counting in the plates. Based on the percent positive wells, the calculated frequency of precursors of CFC in unseparated marrow was 1.4 ± 0.4 × 10⁻⁶.

In two experiments, the agar containing colonies derived from CD34⁺ TBM⁻ cells were fixed, dried on glass slides, and stained with benzidine. One colony was identified that contained benzidine-positive erythroblasts, as well as granulocytes and monocytes, otherwise, only GM-CFU were detected. Both E-BFU and GM-CFU were detected at 4 wk in cultures initiated with ≥100 CD34⁺ TBM⁻ cells in these experiments, though E-BFU were 5-10-fold less frequent than GM-CFU in these cultures.

Single CD34⁺ CD33⁺ 7B9⁺ Cells in Long-Term Culture. Immediately after sorting, 0.5 ± 0.5% of CD34⁺ TBM⁻ cells formed colonies detectable in soft agar. Thus, it was possible that CFC detected after 2 and 4 wk of liquid culture were due to maintenance and expansion of residual CFC. We therefore determined the ability of single CFC to be maintained and self-renew in this culture system. CFC were enriched from marrow by selecting CD34⁺ cells that were also CD33⁺ and 7B9⁺ (Table 2). Single cells were deposited in microtiter wells, to assay for colony formation, and into microtiter wells containing irradiated MSC. On direct culture of single CD34⁺ CD33⁺ 7B9⁺ cells in two experiments, 52 and 47% formed single colonies or clusters (Table 2). None

Table 1. *Cloning of CD34⁺ TBM⁻ Precursors of Myeloid CFC*

| | Clonal liquid cultures | | | | |
| | Wells with CFC (wells assayed) | | CFC/well | | Direct cultures |
Exp.	Week 2	Week 4	Week 2	Week 4	(CFC/No. cells assayed)
1	0/96 (0)	4/88 (4.5)	0	7 ± 3	0/300 (0)
2	1/136 (0.7)	ND	6	ND	15/2,000 (0.8)
3	2/113 (1.8)	2/75 (2.7)	5 ± 2	2 ± 1	14/1,000 (1.4)
4	3/134 (2.3)	9/134 (6.7)	1 ± 0	7 ± 6	1/500 (0.2)
5	0/90 (0)	1/96 (1.0)	0	6	4/1,125 (0.4)
X̄ ± SD	0.9 ± 0.9	3.7 ± 2.1	3.0 ± 2.3	6.3 ± 5.3	0.5 ± 0.5

Numbers in parentheses are percents.

Table 2. *Cloning of CD34⁺ CD33⁺ 7B9⁺ Myeloid Colony-forming Cells*

| | Clonal liquid cultures | | | | |
| | Wells with CFC (wells assayed) | | CFC/Well | | Direct cultures* |
Exp.	Week 2	Week 4	Week 2	Week 4	(CFC/No. cells assayed)
1	0/45 (0)	0/48 (0)	0	0	44/86 (51)‡
2	ND	2/268 (0.7)	ND	1	121/288 (42)§
$\overline{X}$ ± SD	0%	0.4%	ND	1	47%

See Materials and Methods for details. Numbers in parentheses are percents.
* Cells assayed in soft agar immediately after sorting.
‡ Nine GM-CFU, 24 M-CFU, 11 E-BFU; two clusters.
§ 50 GM-CFU, 53 M-CFU, 15 E-BFU, three CFU-MIX; 16 clusters.

of the wells contained more than a single colony, further confirming the single-cell origin of cultures obtained by direct cloning. Of 361 wells containing MSC that were inoculated with single cells and cultured for 2 and 4 wk, only two wells were found that contained single small nonerythroid colonies. These results demonstrate that the multiple CFC detected in the cultures of CD34⁺ TBM⁻ cells most likely arose from immature precursor cells and not from classical growth factor–responsive CFC.

Discussion

Recent data have suggested that the CD34 antigen is expressed by marrow cells that can reconstitute hematopoiesis in vivo in both humans and nonhuman primates (12, 13). In clinical trials of bone marrow transplantation, stem cells that reconstitute in vivo lymphohematopoiesis have not been detectably depleted by using mAbs to T cell– (CD2, CD7), B cell– (CD10, CD19, CD20), or myeloid cell– (CD33) associated antigens to remove malignant and normal lymphohematopoietic cells from marrow (14–20, and unpublished observations). Significantly, depleting marrow of CD33⁺ cells eliminates most unipotent and multipotent CFC (4, 5, 21). We have shown previously that CD34⁺ CD33⁻ marrow cells, representing <1% of marrow-nucleated cells, contain immature precursor cells that, after culture over MSC, gain

the capacity to form CD33⁺ unipotent and multipotent myeloid colonies in the presence of hematopoietic growth factors (4, 5). However, these studies did not determine if single cells were precursors for multiple or single CFC, as the total number of CFC detectable in the cultures was always less than the number of cells used to initiate the cultures.

In the present studies, we have identified a minor population of CD34⁺ TBM⁻ cells in human marrow that are enriched for clonable precursors that generate multiple CFC when cultured with MSC. These isolated precursors have a frequency of ~1.4 ± 0.4 × 10⁻⁶ cells in marrow if the cloning efficiency was 100%, or 1–2 × 10⁻⁵ cells if the cloning efficiency was 10%, similar to that found for stem cells in murine studies by Weissman and colleagues (22). The generation of multiple CFC from single CD34⁺ TBM⁻ cells demonstrating self-renewal and differentiation, and the length of culture required to generate CFC progeny, are compatible with models of hematopoietic stem cells in vivo (23, 24).

The approaches outlined in this report present the possibility of assessing factors that influence self-renewal, proliferation, and maturation of single highly immature hematopoietic precursors, as well as the biologic heterogeneity of this population. Highly enriched stem cell populations may be suitable targets for inserting genes by either retroviral vectors or by microinjection. Formal evidence of the stem cell nature of these isolated cells will require in vivo studies, possibly using immunodeficient mouse models (25, 26).

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