CD34 Expression by Stromal Precursors in Normal Human Adult Bone Marrow

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Normal bone marrow cells were isolated by fluorescence-activated cell sorting (FACS) on the basis of CD34 antigen expression and then assayed in vitro for colonies of fibroblastic cells (fibroblast colony-forming units [CFU-F]). Greater than 95% of detectable CFU-F were recovered in the CD34+ population, while their numbers were markedly depleted in the CD34- population. Additional experiments showed that the majority of CFU-F exhibited high forward and perpendicular light scatter and low-density CD34 antigen. Growth of sorted cells in medium optimized for long-term marrow culture (LTMC) produced a complex mixture of adherent stromal elements including fibroblasts, adipocytes, smooth muscle cells, and macrophages. Monoclonal antibody STRO-1, which identifies bone marrow stromal cells, reacted with approximately 5% of CD34+ cells, which included all CFU-F and stromal precursors in LTMC. Experiments using soybean agglutinin (SBA) further showed that these stromal elements were restricted to a population of bone marrow cells with the phenotype CD34+/SBA+. These properties of stromal precursors are quite distinct from those of primitive hematopoietic progenitors, showing that although the precursors of the hematopoietic and stromal systems share expression of CD34, they are otherwise phenotypically distinct cell types.

MATERIALS AND METHODS

BM cells. BM cells were obtained from normal donors after informed consent as defined by the Internal Review Board at the Fred Hutchinson Cancer Research Center (FHCRC). Cells were separated over Ficoll (Lymphoprep, Nygaard, Oslo; density 1.077 g/mL) to obtain a mononuclear cell fraction, filtered through Nitex (85 μm pore size), and passed repeatedly through a 21-gauge needle to ensure preparation of a single-cell suspension.

Labeling of cells for fluorescence-activated cell sorting (FACS). BM mononuclear cells were resuspended in calcium- and magnesium-free Hanks Balanced Salt Solution (HBSS) supplemented with 10 mmol/L HEPES buffer (pH 7.3) and 2% fetal calf serum (FCS) (HHF) and 2% human AB serum. Cells were incubated for 45 minutes on ice with saturating concentrations of any one of four anti-CD34 antibodies: MY-10 (HPCAl [Becton Dickinson, Sunnyvale, CA], IgG1); BI-3C5 (IgGI)*; ICH3 (generously provided by Warren Myers, Applied ImmunoSciences, Palo Alto, CA; IgG2a); 12.8 (Dr R.G. Andrews, FHCRC; IgM)*; or with equivalent concentrations of isotype-matched antibodies of irrelevant specificity. After washing, the cells were incubated with optimally diluted goat antimouse IgG-fluorescein isothiocyanate (FITC) (γ- or μ-specific as appropriate) (Southern Biotechnology Associates, Birmingham, AL) for a further 45 minutes at 4°C, washed again, and resuspended in HHF containing propidium iodide (2 μg/mL) for sorting.

Some experiments were performed using BM samples enriched for stromal elements by means of negative selection. BM cells were incubated with a cocktail of five murine IgG MoAbs including: 35.1 (CD2), 64.1 (CD3), 60.3 (CD18IS; generously provided by Paul Martin, FHCRC), Leu-M3 (CD14; Becton Dickinson), and R10 (glycophorin A). After incubation for 45 minutes at 4°C, cells were washed twice in HHF and then incubated with immunomagnetic beads covalently coupled with sheep antiozym IgG (Dynal, Oslo, Norway) according to the manufacturer's recommendations. Cells binding the antibody-coated beads were then removed using a magnet. Alternatively, the procedure was performed using the sheep-antimouse–coated beads “prearmed” by overnight incubation at 4°C with saturating concentrations of the antibodies listed above. Identification of CD34+ cells was performed by means of indirect immunofluorescence using antibody 12.8 followed by goat antiozym IgM coupled to either FITC or phycoerythrin (PE; Southern Biotechnology Associates).

In some experiments, BM cells were double-labeled with HPCAl and STRO-1, a mouse IgM MoAb that binds to a determinant on 100% of fibroblast CFU (CFU-F). HPCAl antibody was shown by sequential incubation with biotinylated IgG MoAbs including: 35.1 (CD2), 64.1 (CD3), 60.3 (CD18IS; generously provided by Paul Martin, FHCRC), Leu-M3 (CD14; Becton Dickinson), and R10 (glycophorin A). After incubation for 45 minutes at 4°C, cells were washed twice in HHF and then incubated with immunomagnetic beads covalently coupled with sheep antiozym IgG (Dynal, Oslo, Norway) according to the manufacturer's recommendations. Cells binding the antibody-coated beads were then removed using a magnet. Alternatively, the procedure was performed using the sheep-antimouse–coated beads “prearmed” by overnight incubation at 4°C with saturating concentrations of the antibodies listed above. Identification of CD34+ cells was performed by means of indirect immunofluorescence using antibody 12.8 followed by goat antiozym IgM coupled to either FITC or phycoerythrin (PE; Southern Biotechnology Associates).

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Additional experiments were performed using the BM samples enriched for CFU-F as described above, double-labeled with FITC-conjugated soybean agglutinin (SBA; 20 μg/mL; Vector Laboratories, Burlingame, CA) and antibody 12.8 followed by PE-coupled antiozym IgM. Specificity of staining with SBA-FITC was shown by perform-
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...ing parallel incubation in the presence of 0.2 mol/L D-galactose (Sigma, St Louis, MO).

Cells were analyzed or sorted using either an EPICS 753 (Coulter, Hialeah, FL) or FACS II (Becton Dickinson). Sort gates were set according to the staining obtained using isotype-matched control IgM (H12C12) and IgG (1A14) MoAbs of irrelevant specificity. All cells sorted as CD34+ showed a fluorescence intensity greater than that detected on 98% of the cells labeled with the isotype-matched control antibody. The population of CD34+ cells had a mean fluorescence intensity between fivefold and 15-fold greater than the control population. Likewise, cells sorted as SBA+ showed a level of SBA fluorescence greater than that detected on 98% of cells stained with SBA-FITC in the presence of 0.2 mol galactose.

Indirect immunofluorescence assay. Aliquots of 10⁶ mononuclear BM cells were incubated for 1 hour at 4°C with the anti-CD34 antibody ICH3 (20 μg/mL) or with 1:1,000 dilution of ascites fluid containing the isotype-matched antibodies 60.5 (anti-HLA class I) or the nonbinding antibody 9E8 (antimouse thy 1), used as positive and negative controls, respectively. After washing twice with HHF at 4°C, the samples were further incubated with 20 μg/mL of sheep antimouse IgG coupled to pseudomonas exotoxin (SAM-pE; generously provided by Dr Oystein Fodstad, Institute for Cancer Research, Oslo, Norway) for 4 hours at 4°C with constant agitation. After three additional washes in HHF, aliquots of the cells were assayed for CFU-GM and BFU-E (as previously described) while the remainder were assayed for CFU-F as described below.

BM CFU-F assay. This procedure has been described in detail elsewhere. Depending on the number of cells obtained and the anticipated degree of enrichment or depletion of CFU-F, all sorted samples were plated in triplicate over a range of concentrations from 10⁴ to 10⁶ cells/plate in a-MEM supplemented with 20% FCS (Hyclone, Logan, UT), penicillin (100 U/mL), and streptomycin (100 μg/mL). Colonies of fibroblastic cells were scored on day 10. In all experiments, CFU-F exhibited a linear growth response consistent with clonal growth of the stromal colonies.

Establishment of heterogeneous adherent cell layers. Sorted cells were plated in long-term BM culture (LTBMC) growth medium, comprising Iscove’s medium, 365 mOsm, supplemented with 20% FCS (Hyclone, Logan, UT), penicillin (100 U/mL), streptomycin (100 μg/mL), 1% sodium pyruvate, 1% L-glutamine, folic acid (0.01 mg/mL), myo-inositol (0.4 mg/mL), and 10⁻⁴ mol/L hydrocortisone sodium succinate (Sigma). Cultures were fed weekly by replacement of half of the growth medium. Confuent cultures were examined at weeks 4 to 6 for phenotypic markers of stromal cells.

Phenotypic analysis of stromal cells. CFU-F colonies and LTBMC adherent layers were stained in situ, as previously described, using a panel of MoAbs and polyclonal antibodies that included: STRO-1 (anti-CFU-F/marrow fibroblastic cells); 6.19 (antineuroblastoma; Dr C. Frantz, Rochester, NY); 1B10, TE723 (antifibroblast/macrophage, antifibroblast; Dr K. Singer, Duke University, Durham, NC); HHF35, 1A4 (antismooth muscle actin; Dr A. Gown, University of Washington, Sigma); 24.15 (CD10, endopeptidase; Dr Paul Martin, FHCRC); My-7 (CD13, aminopeptidase-N; Dr J. Griffin, Dana-Farber Cancer Center, Boston, MA); Leu-M3 (CD14, macrophages; Becton Dickinson); and polyclonal rabbit or goat antisera to factor VIII-related antigen (Dako, Santa Barbara, CA); collagen types I, III, IV, and V (Southern Biotechnology Associates); fibronectin and laminin (Dr W.G. Carter, FHCRC). Bound antibody was detected by indirect immunofluorescence or immunoperoxidase staining, as previously described. Background staining was minimized by first incubating the fixed cells with a solution of 0.2 mol glycine in phosphate-buffered saline (PBS) to block free aldehyde groups, followed by the inclusion of Tween 20 (0.05%) during the incubation in primary and secondary antibodies and during all wash steps. Staining for alkaline phosphatase activity and lipid, using Oil Red-O, was performed according to established techniques.

RESULTS

CFU-F express CD34 and exhibit a broad range of perpendicular light scatter (PLS). A series of experiments were performed in which we assayed for CFU-F in different fractions of BM, gated according to 90° and forward light scatter (FLS) (Fig 1), and then sorted on the basis of CD34 expression. These experiments were performed using both unfractionated BM samples and marrow samples enriched for stromal cells by means of negative selection using a panel of MoAbs previously shown to be nonreactive with CFU-F. This procedure resulted in the depletion of 79% to 93% of BM mononuclear cells with a concomitant 2.4- to 8.4-fold enrichment of CFU-F in the negatively selected population. Mean recovery of CFU-F was 86% (range, 72% to 129%; n = 5). The incidence of CD34+ cells in un gated samples after negative selection was increased by a mean of 8.8-fold (range, 3.3- to 12.4-fold). Sorting of this preenriched fraction on the basis of CD34 expression consistently showed greater than 95% of the CFU-F in the CD34+ fraction and yielded an overall mean recovery of CFU-F of 78.8% (range, 65.1% to 84.5%; n = 4).

A representative experiment is shown in Table 1. CFU-F were recovered from CD34+ BM cell populations exhibiting a broad range of 90° and forward scatter. In accordance with the previous data, CFU-F were present in the fraction...
of cells with low 90° scatter (Fig 1, window 2). However, CD34+ cells exhibiting high 90° scatter (Fig 1, window 1) generated approximately sevenfold higher numbers of CFU-F than cells with low 90° scatter. Allowing for the proportion of cells with high 90° scatter (mean 78%) and the incidence of CD34+ cells in this population (mean 2.2%), a mean of 42% (range, 30% to 60%) of the total CFU-F assayed exhibited high PLS. The recovery of CFU-F in the sorted fractions ranged from 52% to 90.4% of the number assayed from the unsorted BM samples. Gating by means of FLS further showed that cells with high FLS demonstrate the highest frequency of CFU-F, while lymphoid-sized cells with low FLS and 90° scatter (Fig 1, windows 3 and 5) are largely devoid of CFU-F. Accordingly, CFU-F were present at high frequency in the population gated simultaneously for CD34+/high PLS/high FLS (Fig 1, window 6).

To confirm these cell-sorting data, we attempted to inhibit CFU-F growth by means of immunotoxin-mediated cytotoxicity using an indirect method using antibody ICH3 and a sheep antimouse Ig antiserum coupled to SAM-pE. Under the conditions of the assay, there was in all experiments a ≥95% reduction in colony formation by hematopoietic progenitor cells (CFU-GM, BFU-E) after treatment of the cells with ICH3 and SAM-pE (data not shown), showing the efficacy of this procedure in eliminating CD34+ cells. Assay of CFU-F in the immunotoxin-treated samples showed no effect on CFU-F growth in the presence of SAM-pE alone or in combination with the isotype-matched control antibody 9E8, but a marked inhibition in CFU-F number when the cells were labeled with ICH3, the positive control antibody 60.5 (anti-HLA class I), or with antibody 6.19, previously shown to be lytic for CFU-F in the presence of rabbit complement23 (Table 2).

**CFU-F are CD34+/STRO-1**. Previous studies have shown that MoAb STRO-1 binds to 100% of CFU-F and can be used to isolate CFU-F by FACS.16 As shown in Fig 2, analysis of BM stained by dual-color immunofluorescence with antibodies HPCA-1 (CD34) and STRO-1 shows that approximately 4.4% of CD34+ cells bind STRO-1. To determine whether CFU-F were present within this population, BM was separated into CD34+/STRO-1+ and CD34+/STRO-1- subpopulations according to the gates shown in window 6.

![Image](https://example.com/image.png)

**Fig 2.** Dual immunofluorescence analysis of the expression of the CD34 (HPCA-1) and STRO-1 antigen on BM mononuclear cells.
and CD34+ marrow cell populations showed no consistent differences between the two groups. The predominant cellular component in each case showed a fibroblastic or polygonal morphology with heterogenous alkaline phosphatase positivity. In both groups there were cells that stained for interstitial collagen types I and III, cell surface labeling with antibodies STRO-1 and 6.19, and cytoplasmic staining with TE7. FVIII-rAg+ endothelial cells were not detected in either group.

In contrast, the phenotype of stromal cells generated from CD34+ cells grown under LTBMC assay conditions differed in one respect from those generated by unseparated marrow. LTBMC initiated with CD34+ cells failed to generate FVIII-rAg+ cells, whereas such cells are easily detected in all cultures initiated with unseparated BM. However, stromal cells grown from either unseparated and CD34+ marrow cells showed synthesis of basal lamina extracellular matrix protein such as collagen type IV and laminin. In addition, Oil Red O+ adipocytes and CD14+ macrophages were present in both groups.

DISCUSSION

The CD34 glycoprotein is generally recognized as a differentiation antigen that is expressed on a minor population of mature hematopoietic cells that include progenitors committed to myeloid, erythroid, and lymphoid development, as well as primitive stem cells with the capacity to reconstitute the entire hematopoietic system. Previous studies have shown the selective reactivity of CD34 antibodies within hematopoietic cell populations and the absence of binding to a wide variety of cell types in nonhematopoietic tissues. An exception to this is the reactivity of capillary endothelial cells and stromal matrix components of skin, breast, and other tissues. In this report, we have shown that an additional nonhematopoietic cell type, namely, the cells that give rise to fibroblast-like stromal elements of the BM, is recovered in the CD34+ fraction of BM cells after cell sorting, suggesting that this cell type also expresses the CD34 antigen.

That these results are not simply due to contamination of the CD34+ population with stromal elements during sorting is shown both by experiments in which the CD34+ cells are resorted to confirm purity and by data showing inhibition of stromal cell growth after treatment with CD34 antibody and immunotoxin. Furthermore, in the cell sorting experiments, essentially equivalent results were obtained using four anti-CD34 antibodies that identify distinct or partially overlapping epitopes on the 115-Kd glycoprotein, including HPCA-1, 12.8, ICH3, and BI-3CS (data not shown). These data suggest that the recovery of CFU-F in the CD34+ population is not due to the expression of a cross-reactive epitope by marrow stromal cells, but represents binding of the antibodies to the CD34 gene product. Definitive proof of this will, of course, require biochemical analysis of the cell surface molecule(s) identified by CD34 antibodies on stromal elements and evidence of CD34 gene expression by these cells. In the present study, this has not been feasible due to the extremely low frequency of stromal precursors in the BM and the fact that, after culture in vitro, stromal cells were found to be nonreactive with CD34 antibodies. Similar observations were made by Watt et al., who also showed that primary cultures of umbilical cord vein endothelial cells were nonreactive with CD34 antibody, despite their reactivity in tissue sections. A recent study has extended this observation by showing the presence of CD34 messenger RNA in cultured endothelial cells despite their lack of binding of CD34 antibodies, suggesting that, in vitro, the CD34 protein is either downregulated or modified to a form that is nonreactive with CD34 antibodies.

Further experiments showed that CFU-F are characterized by low-density expression of CD34 antigens and heterogeneous light scatter properties, with the majority showing high FLS and a broad range of PLS. It is of interest that these properties of CFU-F are quite distinct from those of primitive hematopoietic progenitors that are reported to be small lymphoid-sized blast cells with high CD34 antigen density. Other experiments provided additional evidence of differences between these two populations. First, CFU-F were shown to be restricted to a minor subpopulation of CD34+ cells that also express the stromal cell-associated antigen STRO-1. In a previous report, we have shown that STRO-1 is not expressed on committed hematopoietic progenitors or their more primitive precursors. Secondly, CFU-F were shown to be CD34+/SBA+. This finding is in accordance with other published data showing the presence of binding sites for SBA on CFU-F and the lack of SBA binding to cells with the ability to reconstitute the hematopoietic system. Consistent with these observations, we have recently reported that cells able to initiate hematopoiesis on irradiated stromal layers in LTBMC are restricted to a minor population of BM cells with the phenotype CD34+/SBA+. Collectively, these data provide direct evidence that in the adult, although both cell types express CD34, hematopoietic stem cells and stromal precursors are otherwise phenotypically distinct cell types, confirming data obtained in the transplant setting.

The significance of CD34 expression by stromal cells remains to be determined. By analogy with the developmentally regulated expression of CD34 by primitive hematopoietic cells, CD34 may also be a marker of marrow stromal cell precursors that is lost as the cells differentiate into phenotypically distinct stromal elements. Analysis of the structural characteristics of the CD34 glycoprotein based on biochemical and cDNA sequence data has so far not provided insight into the possible function of the molecule. The CD34 gene has recently been mapped to 1q12, a region that contains a cluster of genes encoding...
leukocyte/endothelial adhesion molecules, leading to speculation that CD34 may also function as a cell adhesion molecule. Thus far, however, CD34 antibodies have not been shown to block binding of hematopoietic progenitors to stromal cells in vitro. Therefore, the relevance of our observation that CD34 is expressed by stromal cell precursors awaits the functional definition ascribed to the CD34 molecule.

REFERENCES


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