RAPID COMMUNICATION

Characterization of Murine CD34, a Marker for Hematopoietic Progenitor and Stem Cells

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CD34 is expressed on human hematopoietic stem and progenitor cells, and its clinical usefulness for the purification of stem cells has been well established. However, a similar pattern of expression for murine CD34 (mCD34) has not yet been determined. Two polyclonal anti-mCD34 antibodies that specifically recognize both endogenous and recombinant murine CD34 were developed to characterize the mCD34 protein and to determine its pattern of expression on murine cell lines and hematopoietic progenitor cells. Fluorescence-activated cell sorter analysis showed that mCD34 is expressed on NIH/3T3 embryonic fibroblasts, PA6 stromal cells, embryonic stem cells, M1 leukemia cells, and a subpopulation of normal bone marrow cells. Murine CD34 was found to be a glycoprotein expressed on the cell surface as either a full-length (~100 kD) or truncated (~90 kD) protein in NIH/3T3 and PA6 cells. Recombinant full-length CD34, when expressed in the CHO-K1 cell line, had a molecular weight of approximately 105 kD. Full-length CD34 is expressed on M1 leukemia cells, had a higher apparent molecular weight (110 kD). These results suggest that there are glycosylation differences between CD34 expressed by different cell types. The full-length form, but not the truncated form, is a phosphoprotein that is hyperphosphorylated in response to 12-O-Tetradecanoyl phorbol 13-acetate treatment, suggesting potential functional differences between the two forms. Selection of the 3% highest-expressing CD34+ bone marrow cells enriched for the hematopoietic precursors that form colony-forming unit-spleen (CFU-S), CFU-granulocyte-macrophage, and burst-forming unit-erythroid. Transplantation of lethally irradiated mice with these cells demonstrated both short- and long-term repopulating ability, indicating that this population contains both functional hematopoietic progenitors and the putative stem cell. These antibodies should be useful to select for murine hematopoietic stem cells.

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The human CD34 protein (hCD34) is a strict development stage-specific antigen that marks hematopoietic stem and progenitor cells. During hematopoietic differentiation, expression decreases such that terminally differentiated cells do not express CD34.1,2 This stage-specific expression implicates a potential regulatory function for CD34 in developmental hematopoiesis. The CD34+ bone marrow (BM) population contains colony-forming unit (CFU)-blast and long-term culture initiating cells, as well as multipotential CFU-GEMM (mixed myeloid and erythroid colonies), and committed progenitor cells (ie, CFU-granulocyte-macrophage [CFU-GM], burst-forming unit-erythroid [BFU-E], and CFU-megakaryocyte [CFU-Meg]) as evaluated by in vitro colony-forming assays.3 BM transplantation (BMT) studies in baboons and humans indicate that CD34+ cells may provide long-term reconstitution of the hematopoietic compartment, indicating that the pluripotent stem cell expresses CD34.4,5 The mouse model of BMT affords several advantages for the study of normal hematopoiesis. It is less cumbersome than the baboon model, and inbred animals are available. In the mouse model, progenitor cell growth and stem cell engraftment can be assessed from days to months posttransplantation, serial transplants can be performed to evaluate the self-renewal and differentiation of donor cells, and the donor cells can be manipulated in vitro to optimize transplantation. Results obtained using this model can be directly applicable to the clinical treatment of hematologic malignancies using BMT technology. However, a disadvantage of this model has been the lack of methods to identify the murine stem cell. To date, subpopulations of murine BM that contain putative hematopoietic stem cells have been selected using combinations of various separation procedures including physical characteristics (size and cell density), negative selection to remove lineage-committed cells, and positive selection for expression of Thy-1, Sca-1, or binding to WGA.5,11 Although Thy-1 and Sca-1 are thought to be expressed on stem cells, neither identifies these cells uniquely. If, as anticipated, the pattern of murine CD34 (mCD34) expression on hematopoietic cells is analogous to that for hCD34, it may be useful for positively selecting murine hematopoietic stem cells.

The murine homologue of human CD34 has recently been cloned, but the gene product(s) have not yet been characterized. Transcription studies indicate the presence of two mRNA splice variants that are predicted to code for a full-length and a truncated form of the mCD34 protein.1,2 To characterize the mCD34 protein(s) and study its potential role in murine hematopoiesis, we have developed polyclonal antibodies directed against the predicted extracellular and intracellular domains of mCD34. Using these antibodies, we show that two forms of the mCD34 protein are expressed. Both forms are glycosylated, and there are cell-type specific variations in glycosylation. The full-length, but not the trun-

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cated, protein is phosphorylated. Finally, mCD34 is expressed by a population of normal BM cells that contains both progenitor and stem cells capable of long-term reconstitution (>60 days) of lethally irradiated mice.

MATERIALS AND METHODS

Cell lines and culture conditions. Murine NIH/3T3, CHO-K1, and M1 cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The PA6 stromal cell line14 and 32D C13 (32D) myeloid leukemia cell line15 were generously provided by Dr H. Kodama (Ohu, Japan) and Dr G. Rovera (Philadelphia, PA), respectively. Murine D3 embryonic stem (ES) cells, obtained from Dr T. Doetschman (University of Cincinnati, Cincinnati, OH), were maintained as described.16,17 PA6 and NIH/3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 5% calf serum. The myeloid precursor cell lines DAA1, M1, and the interleukin-3 (IL-3)–dependent 32D were maintained in culture as described.18,19 Synthetic murine IL-3 was kindly provided by Dr I. Clark-Lewis (University of British Columbia, Vancouver, BC, Canada).

Production of an alkaline phosphatase-mCD34 fusion protein. A fusion construct secreted human placental alkaline phosphatase and extracellular murine CD34 was produced using the APtag-1 plasmid, a kind gift of Dr J.G. Flanagan,20 into which mCD34 cDNA encoding the extracellular region of murine CD34 (amino acids 1 through 194) was inserted.21 Reverse transcription-polymerase chain reaction (RT-PCR) of total RNA from NIH/3T3 cells was used to prepare a 618-bp fragment of cDNA coding for the extracellular region of murine CD34 from amino acids 1 through 195. The 5′ PCR primer was a 26-mer (CACCAAGCTTATCTCCGGACGGTAC), similar at 23 nucleotides to sequence 52 to 9 bp upstream of the translation start site. Nucleotide mismatches at three positions (underlined) were introduced to create a HindIII cloning site in the primer. A similar strategy was used to create a BglII cloning site in the 3′ PCR primer (AATCTAGACCTCTAGCTTTAGTCTCCAGG), identical at 24 of 26 nucleotides to bp 560 through 585 of the coding region for murine CD34.13 The final PCR product was sequenced for verification, digested with both HindIII and BglII, and ligated into the HindIII/BglII cloning sites in the APtag vector.20 The resulting pCD34-APtag fusion vector (5 μg) was cotransfected with the pSV2Neo plasmid (0.1 Kg) into Chinese hamster ovary (CHO) cells for verification, digested with both HindIII and BglII, and ligated into the region for murine CD34. The final PCR product was sequenced for verification, digested with both HindIII and BglII, and ligated into the HindIII/BglII cloning sites in the APtag vector. The resulting pCD34-APtag fusion vector (5 μg) was cotransfected with the pSV2Neo plasmid (0.1 Kg) into Chinese hamster ovary (CHO) cells for verification, digested with both HindIII and BglII, and ligated into the region for murine CD34.13 The final PCR product was sequenced for verification, digested with both HindIII and BglII, and ligated into the HindIII/BglII cloning sites in the APtag vector.20 The resulting pCD34-APtag fusion vector (5 μg) was cotransfected with the pSV2Neo plasmid (0.1 Kg) into Chinese hamster ovary (CHO) cells for verification, digested with both HindIII and BglII, and ligated into the region for murine CD34.13 The final PCR product was sequenced for verification, digested with both HindIII and BglII, and ligated into the HindIII/BglII cloning sites in the APtag vector.20 The resulting pCD34-APtag fusion vector (5 μg) was cotransfected with the pSV2Neo plasmid (0.1 Kg) into Chinese hamster ovary (CHO) cells for verification, digested with both HindIII and BglII, and ligated into the region for murine CD34.13

Preparation of full-length recombinant murine CD34 protein. A mammalian expression vector containing the full-length mCD34 cDNA (pMCD34full) was constructed. Total RNA isolated from undifferentiated murine ES cells was used to perform RT-PCR. The 5′ primer spanning the translation start codon was designed to contain an EcoRI site (GGAATTCTGAGGAGGAGATCCGAGTTCCAGGCAGG). The 3′ primer (ACTCTAGATCACAGTTCTGTGTXbaI) was included to cross-link the peptide to the hapten KLH (keyhole limpet hemocyanin). The N-terminal glycine was added to prevent formation of secondary structure through binding of an N-terminal cysteine with histidine. KLH was coupled to the peptide using maleimide-activated KLH according to the manufacturer’s instructions (Pierce, Rockford, IL). The rabbit was immunized with 250 μg of peptide in complete Freund’s adjuvant, and boosted at 3-week intervals with 125 μg of peptide in incomplete Freund’s adjuvant. For transplantation studies, the IgG fraction of Ab 1202, purified as described,22 was used.

Immunofluorescence analysis and fluorescence activated cell sorting (FACS). Cells were stained with anti-CD34 antiserum depleted of anti-alkaline phosphatase antibodies as previously described.23 Briefly, Ab 1202 was used at a 1:100 dilution in immunofluorescence assay (IFA) medium containing 20 mmol/L HEPES, pH 7.4, 4% FCS in isotonic saline. The secondary fluorescent isothiocyanate (FITC)-conjugated goat anti-rabbit Ig antibody (Boehringer Mannheim, Indianapolis, IN) was used at a 1:50 dilution in the IFA medium. Cells were analyzed and sorted on an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL). The laser output was set at 400 mW with emission at 488 nm. Emitted light was collected with a 525/15 nm band-pass filter. Forward angle and perpendicular light scatter were used for elimination of any aggregated cells or debris. Data analysis and sorting were performed with MDADS II software. Negative controls were set at 1% and three populations of cells were sorted using 2-droplet sorting.

Northern analysis. Total RNA was prepared by the single-step guanidinium thiocyanate-phenol-chloroform extraction procedure.24 Fifteen micrograms of total RNA per sample was separated by electrophoresis on a 1.5% formaldehyde agarose gel, transferred to nylon filters, and hybridized with a 32P-labeled 190-bp mCD34 probe containing cDNA from exons 2 and 3.

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tion by immunoprecipitation from the total cell lysate after surface iodination as described below.

**Iodination.** Cells, \(1 \times 10^7\), were disrupted in lysis buffer containing 10 mmol/L Tris-HCl, pH 7.5, 10 mmol/L EDTA, 1% Nonidet P-40, 200 U/mL aprotinin, 0.005% leupeptin, and 1 mmol/L PMSF. Immunoprecipitation and Western analysis were performed with either antibody 1241 or 1202 as described. For competitive inhibition studies, protein A beads bound to Ab 1241 were preincubated for 2 hours at 4°C with 50 \(\mu\)g of the synthetic mCD34 peptide against which Ab 1241 was produced. Immunoprecipitates of mCD34 were desialylated by treatment with 100 nM neuraminidase (Genzyme, Cambridge, MA) in 100 \(\mu\)L 0.1 mol/L sodium acetate buffer (pH 6.5) for 30 minutes at 37°C before addition of sample buffer and electrophoresis.

**Metabolic labeling of cells with \([32P]\)orthophosphoric acid.** NIH/3T3 cells, \(3 \times 10^7\), were washed with phosphate-free medium and equilibrated with carrier-free \([32P]\)orthophosphoric acid for 75 minutes at 37°C as previously described. After treatment with 100 mmol/L TPA for 15 minutes at 37°C, the cells were washed in ice-cold phosphate-buffered saline. Subsequent manipulations were performed at 4°C. Immunoprecipitation and separation of mCD34 by SDS-PAGE were performed as described below.

**Iodination of surface proteins using lactoperoxidase.** Cells were suspended at 0.1 to 1 \(\times 10^7\) per mL in phosphate-buffered saline (PBS) containing 1.5 mmol/L MgCl\(_2\). Surface proteins were labeled at 4°C with \(125\)I using the lactoperoxidase method as previously described, and cell lysates were prepared for immunoprecipitation as described above.

**Two-dimensional tryptic peptide mapping.** SDS-PAGE–purified \(12^p\)- or \(12^p\)-labeled mCD34 was excised from the dried gel, rehydrated, and digested with 50 \(\mu\)g of TPCK-treated trypsin in NH\(_4\)HCO\(_3\) buffer for 16 hours at 37°C. The resulting peptides were separated in two dimensions as described.

**Isolation of murine BM.** Male and female (age-matched) C57B1/J mice served as donors and recipients of BM, respectively. Marrow was obtained by dissecting femoral shafts and plugs were flushed into cold media as described.

**Marrow transplantation and fluorescent in situ hybridization (FISH) analysis.** Female B6D2F1 mice received various numbers of male marrow cells after whole-body irradiation as described. Groups of three to four mice received either whole BM or BM subpopulations intravenously via the tail vein. We assayed spleen colony formation as described by Till and McCulloch on both day 8 and day 12. Survival was determined and long-term donor repopulation of the hematopoietic system was performed using a Y chromosome probe by FISH as described previously.

**In vitro colony-forming assays.** Unseparated and separated populations of BM cells were cultured in semisolid medium consisting of DMEM supplemented with 30% FCS, 10% WEHI-conditioned medium as a source of colony-stimulating factors, 2 U/mL erythropoietin, and 1.5% methylcellulose. The cultures were incubated at 37°C in 5% CO\(_2\) in a humidified incubator and scored for CFU-GM or BFU-E 7 to 9 days later.

**RESULTS**

**Murine CD34 antibody development.** To isolate and characterize mCD34 proteins, two polyclonal antibodies were produced. Rabbit antibody 1202 (Ab 1202) was raised against a mCD34/alkaline phosphatase fusion protein that contains only the extracellular region of mCD34. A second antibody (Ab 1241) was raised against an intracellular peptide at the C-terminal end of the full-length mCD34. Because the C-terminal 57 amino acids are not present in the predicted truncated form of the mCD34, Ab 1241 should react only with the full-length form of the protein. However, Ab 1202 would react with this truncated product as well as full-length mCD34. To confirm the reactivity of both Ab 1241 and Ab 1202 with mCD34, CHO cells were transfected with a full-length mCD34 cDNA, and the expression of recombinant mCD34 protein was assessed by immunoprecipitation. Both antibodies immunoprecipitated a protein migrating as a single diffuse band at approximately 105 kD (Fig 1A). Because untransfected CHO cells do not express mCD34 mRNA (data not shown), no protein could be immunoprecipitated from CHO cells transfected with control vector (Fig 1A). These results confirm that both antibodies react with the full-length recombinant mCD34 protein.

Flow cytometric analysis was used to show that Ab 1202 reacts with mCD34, predicted by Northern analysis to be expressed on several murine cell lines. Cells that express a 2.5-kb mCD34 mRNA include ES, NIH/3T3, PA6, and M1 cells (Fig 1B). Of the cells tested, only these cells were positive for CD34 by flow cytometry (Fig 1C). The NIH/3T3 fibroblast and PA6 stromal cells expressed the highest steady-state levels of mCD34 mRNA, and also had the greatest staining intensity by immunofluorescence. Importantly, cells that did not express the mCD34 transcript (ie, DA1 and 32D) failed to bind Ab 1202. These data show that Ab 1202 specifically binds to mCD34 expressed by a variety of cell types, and suggest that surface expression of mCD34 correlates with steady-state mRNA levels.

**Biochemical properties of murine CD34 protein.** The mCD34 protein was initially characterized using the two cell lines (NIH/3T3 and PA6) that express the highest levels of mCD34. After \(125\)I surface labeling of the cells, immunoprecipitation showed that two proteins of approximately 100 and 90 kD (Fig 2A, 2B, and 2C) were specifically identified by Ab 1202. Western analysis of NIH/3T3 or PA6 cell lysates using Ab 1202 to both immunoprecipitate and probe for mCD34 gave similar results (Fig 2B). The relative mobility of these two bands is approximate, and varies from 90 to 95 kD for the lower band and 97 to 102 kD for the higher band. The bands were present only in the immunoprecipitates using immune, but not preimmune, serum (Fig 2A through C). Human alkaline phosphatase was part of the fusion protein used for immunization. To rule out the possibility that either of the immunoprecipitated bands might be murine alkaline phosphatase, immunoprecipitation was performed using Ab 1202 that had first been exhaustively cleared of anti-alkaline phosphatase activity as described in Materials and Methods. The resulting immunoprecipitation pattern from NIH/3T3 and PA6 cells was unchanged (data not shown). To verify that the 90- and 100-kD bands represent two forms of mCD34, the upper and lower bands from \(125\)I surface-labeled PA6 cells (Fig 2C, left) were isolated, and tryptic peptide mapping was performed. The resulting two-dimensional peptide maps gave identical patterns for both bands (Fig 2C, right), showing that the two proteins have the same extracellular region as predicted by the splice variants.
Fig 1. Ab 1241 and Ab 1202 recognize murine CD34. (A) Autoradiographs of $^{125}$I-labeled mCD34 from $2 \times 10^6$ transfected CHO cells. C refers to cells transfected with control vector (i.e., no mCD34 cDNA insert). T refers to cells transfected with vector pMCD34, containing the entire coding sequence for mCD34. After $^{35}$S-surface labeling, lysates were immunoprecipitated using either Ab 1241 or Ab 1202 as indicated. (B) Northern analysis of CD34. (Left panel) Total RNA extracted from six cell lines was analyzed using a murine CD34 probe, as described in Materials and Methods. Murine CD34 mRNA appears as a 2.5-kb band indicated by the arrowhead. The two mRNA splice variants predicted are not resolved on this blot as their size difference is small (only 156 bp). (Right panel) Ethidium-bromide staining of the loaded RNA. The migration of the 18S and 28S ribosomal RNA is indicated. The cell lines analyzed are indicated above each lane as follows: undifferentiated ES cells (ES), undifferentiated M1 murine leukemia cells (M1), NIH/3T3 (3T3), PA6, DA1, and 32D murine leukemia cells. (C) FACS analysis of surface CD34. Cells from the six murine cell lines in (B) were analyzed for mCD34 surface expression by indirect immunofluorescence using Ab 1202. Fluorescence histograms are shown. The ordinate indicates the number of cells, and the abscissa the relative FITC-fluorescence. The dotted line refers to rabbit preimmune, and the solid line to immune sera. The cell lines analyzed are indicated in the upper right-hand corner of each panel.
Fig 2. Identification and characterization of mCD34 protein from NIH/3T3, M1, and PA6 cells. (A) Autoradiograph of 125I-labeled mCD34 immunoprecipitated by Ab 1241 or Ab 1202 from 1 x 10^6 NIH/3T3 (left) or 1 x 10^6 M1 cells (right) as described in Materials and Methods. The electrophoretic mobility of the mCD34 antigen is indicated by the double arrows for NIH/3T3 cells and by the arrowhead for M1 cells. An iodinated nonspecific band of about 90 kD is detected in M1 immunoprecipitates using both preimmune and immune sera. The antibody used is indicated above each lane. "P" indicates preimmune and "I" indicates immune serum. "Inh" indicates peptide inhibition as described in Materials and Methods. "NA" indicates that the immunoprecipitate was digested with neuraminidase before electrophoresis. (B) Western blot analysis of mCD34 using Ab 1202 was performed on cell lysates from the cell lines indicated. Immunoprecipitation was performed using: lanes 1 and 6, preimmune serum 1241; lanes 2 and 7, Ab 1241; lanes 3 and 8, peptide competition of Ab 1241 as described in Materials and Methods; lanes 4 and 9, preimmune serum 1202; and lanes 5 and 10, Ab 1202. (C) Comparative two-dimensional peptide maps of the upper (100 kD) and lower (90 kD) bands immunoprecipitated by Ab 1202 from 125I-surface labeled PA6 cells (indicated by arrows). The origin is indicated by the open circle.
By contrast, Ab 1241 immunoprecipitated only a single protein of 100 kD from NIH/3T3 and PA6 cells that had been surface-iodinated (Fig 2, A and C). Because Ab 1241 failed to immunoprecipitate a 90-kD protein, this indicated that the 100-kD form is the full-length mCD34, and that the 90-kD band represents the truncated form. This was confirmed by Western blotting because the 100-kD protein immunoprecipitated by Ab 1241 was recognized by Ab 1202 (Fig 2B). The reciprocal experiment was not possible because Ab 1241 is a poor immunoblotting antibody. Immunoprecipitation by Ab 1241 of the 100-kD band was competed with the purified peptide against which the antibody was raised, further demonstrating the specificity of Ab 1241 (Fig 2, A and B). Finally, a mock competition experiment using either purified alkaline phosphatase or albumin as nonspecific polypeptides showed no effect on immunoprecipitation of a single band by Ab 1241, or the two bands by Ab 1202 (data not shown).

To determine whether mCD34 is a sialylated glycoprotein, Ab 1202 immunoprecipitates from NIH/3T3 cells were treated with neuraminidase to cleave sialic acid residues. The resulting proteins had a decreased mobility on SDS-PAGE (Fig 2A). Both the 90- and 100-kD bands were shifted; however, the resultant diffuse 125-kD band was not resolved into two bands. Similar shifts in the migration pattern of mCD34 were obtained after neuraminidase-digestion of mCD34 immunoprecipitated by Ab 1241 (data not shown). The anomalous electrophoretic migration after desialylation has also been observed for hCD34.

Because phosphorylation of hCD34 may regulate its surface expression, we wished to determine whether mCD34 is a phosphoprotein. PA6 cells were metabolically labeled with 32P-orthophosphoric acid, and treated with TPA. In cells that were not treated with TPA, a single band of approximately 100 kD was immunoprecipitated using Ab 1202 (Fig 3). TPA treatment for 15 minutes resulted in hyperphosphorylation of this band, which induced a slight apparent increase in molecular weight. These findings indicate that mCD34, like hCD34, can be hyperphosphorylated after protein kinase C activation by TPA. Because Ab 1202 precipitated only the higher 100-kD form of 32P-mCD34, this suggests that only the full-length, but not the truncated form, is a phosphoprotein. Similar results were obtained with Ab 1241 (data not shown). Finally, the hyperphosphorylated mCD34 had a two-dimensional tryptic phosphopeptide map pattern nearly identical to that published for hCD34 (data not shown), strongly suggesting that the phosphorylation site(s) may be identical for murine and human CD34.

In contrast to the results obtained using NIH/3T3 and PA6 cells, the mCD34 expressed by M1 leukemia cells had a slightly different mobility on SDS-PAGE. Both Ab 1241 and Ab 1202, but not preimmune sera, immunoprecipitated a 110-kD surface-labeled protein (Fig 2A, right). Immunoprecipitation of this band by Ab 1241 was specifically inhibited by addition of excess synthetic peptide against which Ab 1241 was produced (Fig 2A). A characteristic shift-up in molecular weight to 138 kD occurred when the lysate was digested with neuraminidase (Fig 2A). Finally, Ab 1202, directed against extracellular epitopes, was not as efficient in immunoprecipitating mCD34 from M1 cells as was Ab 1241. This is in contrast to NIH/3T3 and PA6 cells, for which Ab 1202 and Ab 1241 had approximately equal efficiency. Collectively, these findings suggest that glycosylation differences may account for the different mobility patterns observed between cell lines.

Isolation of CD34+ BM cells enriches for hematopoietic stem and progenitor cells. To determine whether mCD34 is expressed on hematopoietic stem/progenitor cells, unfractionated murine BM cells were examined by flow cytometric analysis after staining with Ab 1202. There was a broad range in the level of fluorescence intensity with approximately 3% of the cells (population C) expressing at least 100-fold increased levels of mCD34 above background (Fig 4, A and B). For functional studies, three subpopulations were separated by FACS. Population A was comprised of CD34+ cells, and contained approximately 65% of the marrow cells. Population B was comprised of the top 15%, and population C was comprised of the top 3% of the brightest staining cells. Morphologic examination by fluorescence microscopy showed that the CD34+ population contained no fluorescent cells and, by Wright-Giemsa staining, was predominantly composed of mature polymorphonuclear leukocytes, bands, neutrophils, and monocytes (Fig 4C, panel 1). Population B (morphology not shown) contained approximately 10% small round lymphocyte-like cells, 30% blasts, 50% myelomonocytoid progenitor cells, and 10% polymorphonuclear leukocytes. The lower limit of fluorescence intensity for population B was defined to include less than 1%
Fig 4. Expression of mCD34 on murine BM cells. (A) Fluorescence histogram of unseparated murine BM cells stained with Ab 1202. The ordinate represents the relative cell number, and the abscissa relative FITC-fluorescence. The thin solid line refers to staining with preimmune 1202 serum followed by secondary FITC-conjugated antibody, the dotted line represents staining with secondary FITC-conjugated antibody only, and the bold solid line represents staining with Ab 1202 followed by secondary FITC-conjugated antibody. The levels of fluorescence intensity defining three subpopulations (A, B, and C) are indicated by horizontal lines. The threshold for population B was determined to achieve less than 1% positive cells staining with preimmune serum. (B) Fluorescence histogram of the same murine BM cells as shown in (A). The abscissa is a log scale of the relative FITC-fluorescence, the ordinate represents forward angle light scatter (proportional to cell size), and the z axis represents cell number. On the left are cells stained with preimmune serum from rabbit 1202 followed by secondary FITC-conjugated antibody, and on the right are cells stained with Ab 1202 followed by secondary FITC-conjugated antibody. The highly fluorescent cells shown on right are predominantly small cells (for comparison, see [C], lower panels). (C) Fluorescence (left) or Wright-Giemsa staining (right) of BM cells from isolated populations A and C. Pictures shown at left and right are from the same population, but do not represent identical cells. Panel 1, representative polymorphonuclear leukocytes and promyeloblast from population A (CD34- cells); panel 2, representative blasts from population C (top 3% CD34+); panel 3, representative small round lymphocyte-like cells from population C.
of the cells stained with preimmune serum, and therefore this population may contain some cells that do not express CD34. In population C, 60% were small, round, lymphocyte-like cells with relatively dense chromatin surrounded by a thin rim of darkly staining cytoplasm (Fig 4C, panel 3). The remaining 40% appeared to be large blasts and myelomonocytic precursors (Fig 4C, panel 2).

The three populations were analyzed for their ability to (1) form spleen colonies in vivo (CFU-S), (2) form hematopoietic colonies in vitro (CFU-GM and BFU-E), and (3) rescue and reconstitute the BM of lethally irradiated mice. Table 1 shows the results from one of four separate experiments, all of which gave similar results. For CFU-GM and BFU-E, which represent committed hematopoietic progenitors (Table 1), an 8- and 33-fold enrichment, respectively, in population B compared with whole BM. An even greater enrichment of CFU-GM (24-fold) was observed for the brightest 3% of the BM cells (ie, population C). No additional enhancement for BFU-E was found when comparing populations B and C, suggesting that erythroid progenitor cells may have variable levels of CD34 surface expression. Compared with unfraktionated BM, population A, containing only CD34+ cells, had a markedly decreased ability to form hematopoietic colonies in vitro. As a measure of in vivo hematopoietic potential, CFU-S were also assessed. There was a 17- and 38-fold enrichment in populations B and C, respectively, in comparison with unfraccionated BM. Consistent with the results above, the CD34+ population was markedly depleted of CFU-S. As a control, the ability of preimmune serum to select for hematopoietic stem or progenitor cells was also tested. When the top 3% of the cells that stained with preimmune serum were sorted and analyzed, there was no enhancement over population A for their ability to form hematopoietic colonies (Table 1).

To determine whether populations B and C were also enriched for hematopoietic stem cells, the repopulating potential of these cells was tested by BMT. Lethally irradiated female mice were infused with the indicated number of donor male cells from each of the subpopulations (Table 1). Both populations B and C, but not population A, were capable of short-term (30-day) and long-term (60-day) survival and hematopoietic reconstitution of the animals. By contrast, the top 3% of cells stained with preimmune serum were unable to rescue lethally irradiated mice (Table 1). Although population B contained sufficient long-term repopulating cells to rescue a lethally irradiated mouse using 2 × 10^7 cells, population C was able to rescue 2 of 4 mice with as few as 200 cells (Table 1). In additional experiments, not shown, 7 of 12 mice transplanted with 200 male cells from population C survived for greater than 60 days. To document the long-term repopulating potential of the donor transplanted cells, FISH analysis with a Y chromosome probe was performed on the peripheral WBCs from mice that survived greater than 60 days. In 3 of 3 mice given 10^7 cells from population B, 97% to 100% of the WBCs were of donor origin. In 4 of 4 mice given 10^6 cells from population C, all of the animals survived and showed 98% to 100% Y chromosome positive WBCs. Six of six mice transplanted with 200 cells from population C had Y chromosome positive cells (25% to 50% peripheral WBCs) from 60 to 90 days after transplantation. These findings indicate that stem cells capable of long-term hematopoietic reconstitution are present in the CD34+ donor populations. These findings strongly suggest that population C, comprised predominantly of lymphocyte-like, small, round cells, was significantly enriched for the long-term repopulating marrow stem cell.

**DISCUSSION**

We have developed two polyclonal antisera, Ab 1241 and Ab 1202, that specifically bind to murine CD34. Using Ab 1202, which was produced against a fusion protein containing the extracellular region of mCD34, we find that mCD34 is expressed on the surface of a variety of cell types including embryonic stem (ES) cells, myeloid leukemia (M1) cells, embryonic fibroblast-derived NIH/3T3 cells, and PA6 stromal cells.
Analysis of normal murine BM using Ab 1202 shows that it is comprised of cells with a wide range of CD34 surface expression. This is very similar to data published for human BM using the anti-hCD34 antibody MY10. For cells from both human and murine BM, 2% to 3% of the anti-CD34 stained cells were more fluorescent than the 99.9 percentile cell stained with control (eg, preimmune) antibodies. Compared with whole BM, the mCD34+ population (population B) is highly enriched for multipotent progenitor cells including CFU-S, CFU-GM, and BFU-E. However, when the subpopulation of BM cells with the highest 3% surface CD34 expression (population C) is compared with population B that contains cells with less CD34 surface expression, there is further enrichment for CFU-GM (24-fold) and CFU-S (38-fold). This is consistent with findings for human CD34+ cells. That is, CD34+ cells from human BM contain nearly all of the CFU-GM and BFU-E progenitor cells, and selection for the top 2% CD34 staining cells enriches 22-fold for CFU-GM. By contrast, CD34+ murine BM cells are markedly depleted of progenitor cells including CFU-S, CFU-GM, and BFU-E. These data clearly indicate that CD34 marks committed progenitor cells in the murine BM.

BMT results indicate that CD34+ cells are capable of both short-term (30-day) and long-term (60-day) reconstitution of lethally irradiated mice. As few as 200 of the highest expressing cells are capable of rescuing mice from lethal irradiation, and of long-term hematopoietic reconstitution. Because it has been shown previously that 5 x 10^3 whole BM cells can rescue approximately 86% of lethally irradiated mice, this suggests that population C (the highest 3% of CD34+ cells), which rescued 9 of 16 transplanted mice, is enriched approximately 25-fold for the stem cell. Thus, CD34 may be a useful positive selection marker to enrich murine BM stem cells, and should facilitate studies aimed at finally purifying this elusive cell. Because short-term survival relies on the presence of committed progenitor cells in the donor population, the CD34+ cells were unable to rescue lethally irradiated mice. Although it is unlikely that stem cells are present in the CD34+ population, studies are currently underway to definitively rule out this possibility.

For both the murine and human CD34 genes, there are two mRNA splice variants that are predicted to encode a full-length and a truncated form of the protein. The truncated protein would lack all but 16 amino acids in the cytoplasmic domain. Using our polyclonal antibodies, we now show the expression of both forms of the mCD34 protein. Antibody 1202, directed against the extracellular region, immunoprecipitated both the full-length and truncated forms of mCD34 (100- and a 90-kD in NIH/3T3 and PA6 cells). A second antibody (Ab 1241), directed against the intracellular region, reacted with only the full-length protein (100 kD) in NIH/3T3 and PA6 cells. Using a similar strategy, we have also identified two forms of hCD34 (data not shown). In M1 myeloid leukemia cells, which express both splice variants of mCD34 mRNA (data not shown), only one protein was immunoprecipitated by either Ab 1202 or Ab 1241. However, the truncated form could comigrate with the 97-kD nonspecific protein, and therefore not be apparent. We suspect this is the case, and studies are currently underway in our laboratory to investigate this possibility.

Like the human homologue, mCD34 is heavily sialylated, indicating that it is a sialoglycoprotein. Its retarded mobility on SDS-PAGE after removal of sialic acid residues is most likely caused by a loss of acidic groups or to a change in the conformation of the CD34 molecule. The predicted molecular weights for the full-length and truncated mCD34 proteins are 41 kD and 35 kD, respectively. The actual mobilities observed on SDS-PAGE suggest that, like hCD34, mCD34 is highly glycosylated. Furthermore, there may be cell-type variations in the extent of glycosylation of the full-length form. Consistent with this, in NIH/3T3 and PA6 cells CD34 migrated at 100 kD, in CHO cells recombinant full-length mCD34 migrated at approximately 105 kD, whereas in M1 myeloid cells it migrated higher at 110 kD (see Figs 1A, 2A, and 2C). Furthermore, Ab 1202, raised against CHO-derived mCD34, binds less well to mCD34 expressed by myeloid cells than that expressed by other CD34+ cells. At present, whether differential glycosylation can account for these differences is not clear. Murine CD34 expressed by high endothelial venule cells and capable of binding L-selectin was reported to be a 90-kD protein, and it is not yet clear whether this represents full-length or truncated CD34. One possible role for differential glycosylation could be to affect binding of CD34 to its ligand(s). Perhaps mCD34 on endothelial cells is specific for L-selectin, and mCD34 on hematopoietic cells binds to a different, BM-specific ligand.

A functional difference between the full-length and truncated forms of mCD34 has yet to be shown. The absence of the full cytoplasmic domain in the truncated protein may have a profound functional significance, because there is a high degree of amino acid conservation between the cytoplasmic domains of murine and human CD34. Our data show that the full-length, but not the truncated, form of mCD34 is a phosphoprotein that can be hyperphosphorylated by TPA stimulation. It has previously been shown that increased surface expression and hyperphosphorylation of hCD34 are induced by TPA in the absence of protein synthesis. Because increased 32P-labeled mCD34 could be detected after exposure to TPA for just 15 minutes, this phenomenon is also likely to be independent of new protein synthesis. Whether differential phosphorylation can affect expression and/or function of mCD34 remains unclear. Alternatively, the ratio of expression between the full-length and truncated forms of mCD34 could be functionally significant. Differences in the ratio of steady-state levels of mRNA for the two forms have been associated with the leukemic phenotype; several primary human AML samples were shown by RT-PCR to express mRNA for only the full-length form of the hCD34 protein. Whether this is significant with respect to the leukemic phenotype will require further study. Recently published data indicate that binding of anti-CD34 antibodies to hCD34 can induce actin polymerization. It may be that only one form of CD34 can mediate this F-actin formation. We now have the tools to proceed with studies aimed at identifying potential functional differences between the two forms of CD34.
In summary, our findings indicate that the cellular distribution and biochemical properties of murine CD34 are similar to those for human CD34. Moreover, we find that mCD34 can be expressed in both a full-length and a truncated form, and is present on a population of murine BM cells capable of rescuing and reconstituting lethally irradiated mice. These findings indicate that expression of mCD34, like its human homologue, may be functionally important during hematopoiesis. Therefore, CD34 appears to be a useful marker for the identification and isolation of murine hematopoietic stem and progenitor cells.

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