Selective Expression of Two Homeobox Genes in CD34-Positive Cells From Human Bone Marrow

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Proteins coded by homeobox-containing genes are sequence-specific DNA-binding proteins that have been implicated in the control of gene expression both in developing as well as in adult tissues. Two recently characterized human homeobox genes, HB24 and HB9, were found to be highly expressed in bone marrow cells enriched for CD34-positive cells, present at low levels in unfraccionated bone marrow cells, and essentially undetectable in bone marrow cells depleted of CD34 cells. Treatment of CD34-enriched cells with recombinant interleukin-3 (IL-3) and granulocyte macrophage-colony-stimulating factor for 24 hours increased expression of HB24 threefold and HB9 fourfold. Based on studies with actinomycin D, the HB24 and HB9 transcripts in human CD34-positive cells have short half-lives, estimated to be 30 to 45 minutes. Downregulation of HB24 and HB9 expression was found following the treatment of cultures of CD34-positive cells with IL-3. Thus, the differentiation of CD34-positive cells along a specific cell lineage likely requires downregulation of both HB24 and HB9.

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MATERIALS AND METHODS

Preparation of BM suspensions and purified CD34-positive cells. BM aspirates were obtained from the posterior iliac crest of normal volunteers after informed consent. The samples were immediately diluted 1:1 with Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY) containing 20 U/mL of sodium heparin. This mixture was passed through a 150-μm screen and layered over an equal volume of Ficoll-Paque (specific gravity, 1.077 g/cm³; Pharmacia Fine Chemicals, Piscataway, NJ). Density centrifugation was performed at 500g for 25 minutes at 4°C and the interface layer of low density mononuclear cells was collected, washed, and resuspended in phosphate-buffered saline (PBS):EDTA (PBS, pH 7.4 containing 5% fetal bovine serum [FBS], vol/vol; 0.01% EDTA, wt/vol; and 1.0 g/L D-glucose). The low density marrow cells were treated with anti-HPCA-1 (My10) murine monoclonal antibody (MoAb; Becton Dickinson, Mountain View, CA) for 30 minutes at 4°C, washed three times, and mixed...

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with sheep antimonious Ig-conjugated magnetic beads (Dynabeads; Dynal, Oslo, Norway) at a ratio of 1:20 (cell to bead). The CD34-positive cells were separated by a cobalt-samarium magnet-equipped device (Dynabeads). After separation, the cells were washed twice with PBS and used for preparation of cytoplasmic RNA. The purity of the magnetic bead sorted cells was determined by re-analyzing aliquots of the separated subsets on a Coulter Epics 753 (Coulter, Hialeah, FL). The CD34-enriched fraction contained greater than 92% My10-positive cells and the CD34-depleted fraction contained less than 0.8% My10-positive cells, respectively. The low-density BM cell fraction before the separation contained approximately 2.8% My10-positive cells.

RNase protection assay. Cytoplasmic RNA was isolated from BM cells using a miniprep method. Briefly, cells were washed once with ice-cold tris-saline (25 mM/L Tris pH 7.4, 130 mM/L NaCl, 5 mM/L KCl), spun at low speed in an Eppendorf centrifuge (5,000 rpm) at 4°C for 1 minute, resuspended in 400 μL of tris-saline, and 100 μL of NDD buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.02% dextran sulfate in tris saline) was added to the suspended cells. The tubes were inverted 10 times and spun at low speed as above. The supernatant was transferred to a fresh tube and 500 μL of phenol/chloroform, 20 μL of 20% sodium dodecyl sulfate (SDS), and 15 μL of 5 mol/L NaCl were added. After centrifugation at high speed (12,000 rpm), the upper phase was collected, the phenol/chloroform extraction repeated until the interface was clear, and a last extraction with chloroform alone was performed to remove any residual phenol. The cytoplasmic RNA was ethanol precipitated and resuspended in diethylpyrocarbonate-treated water and frozen until use. The RNase protection assays were performed using standard methodology. An HB24 EcoRI-Sma I fragment (351 bp) and HB9 EcoRI-Xho I fragment (295 bp) were each subcloned into pBluescript (Stratagene, La Jolla, CA). An actin BamHI-Hind III fragment (145 bp) from the 3’ noncoding region of a γ-actin cDNA was also subcloned into pBluescript. 32P-labeled HB24, HB9, and control actin RNA transcripts were made in the sense and anti-sense direction and hybridized to 10 μg of total cytoplasmic RNA. Cytoplasmic RNA transcripts protected from RNase digestion were size-fractionated on polyacrylamide gels and visualized by autoradiography. No fragments were protected with the sense probes.

In vitro culture of CD34 cells. The CD34-positive cells were cultured in IMDM (GIBCO) supplemented with 5% bovine calf serum (Flow Laboratories, Inc, McLean, VA) and recombinant interleukin-3 (IL-3) (200 U/mL) in 24-well plates (Costar, Cambridge, MA) for 3 weeks in a CO2-incubator at 37°C. Fresh media was added twice a week. Cytospin slides of both untreated CD34-positive cells and differentiated cells were prepared for in situ hybridization.

In situ hybridization to analyze HB24 and HB9 gene expression. The HB24 and HB9 antisense RNA probe (“S-labeled transcripts of HB24 EcoRI-Sma I 462 bp with T3 promoter and HB9 EcoRI-Xho I 406 bp with T7 promoter) detecting the sense HB24 and HB9 RNA or the HB24 and HB9 RNA sense probe (same fragment with T7 for HB24 and T3 promoter for HB9 were made by standard methods). Slides were prepared, prehybridized, hybridized, and washed as previously described. After 4 days of exposure to a 1:1 dilution of Kodak NTN-2 emulsion (Eastman Kodak, Rochester, NY), the slides were developed in Kodak D-19 and Rapid Fix, and stained with Hematoxylin-Eosin and Giemsa solution.

RESULTS

Analysis of HB24 and HB9 mRNA expression in CD34-positive BM cells. Examination of unfractionated BM cells for the expression of either HB24 or HB9 mRNA by in situ hybridization with anti-sense RNA probes previously showed hybridization in approximately 5% of the cells (Y. Deguchi, manuscripts submitted for publication). Because homeobox gene mRNAs are often present at relatively low copy numbers and may be difficult to detect by standard Northern blots, a more sensitive RNase protection assay was used to study the expression of HB9 and HB24. Cytoplasmic RNA was extracted from unfractionated BM cells, highly enriched CD34-positive cells purified from BM, and unfractionated BM cells depleted of CD34-positive cells and analyzed by RNase protection assay using either HB24- or HB9-specific RNA probes. High levels of both the HB24 and the HB9 mRNA transcripts were detected in the highly enriched CD34-positive fraction, whereas low levels of each gene were evident in total BM cells, and very low levels of the transcripts were present in the fraction depleted of CD34-positive cells (Fig 1). A human actin

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**Fig 1.** Expression of human homeobox genes HB24 and HB9 in CD34-positive human cells. RNase protection assays were performed to assess HB24 (top, left panel) and HB9 (top, right panel) expression (18 hours of exposure) and actin (lower panels) expression was used as a control (6 hours of exposure). Lane 1, CD34-depleted cells; lane 2, total unfractionated BM cells; and lane 3, CD34-positive-enriched mononuclear cell fraction. Lanes 1 and 3 are reversed for HB9. Ten micrograms of cytoplasmic RNA was analyzed per lane. The sizes of the protected fragments are indicated.
RNA probe was used as a control to ascertain that equal amounts of intact RNA were analyzed. The actin mRNA level in each RNA sample was similar (Fig 1). Quantification of expression levels by densitometric analysis and measurement of 32P incorporation in excised bands with a beta counter showed that the levels of HB9 and HB24 transcripts in the CD34-positive–enriched fractions were approximately 40 times and 50 times higher than in the CD34-depleted fraction, respectively.

To determine the half-lives of HB24 and HB9 mRNA transcripts in CD34-positive cells, gene transcription was blocked by the addition of actinomycin D, and the levels of HB24 and HB9 mRNA transcripts were analyzed at various time intervals. The estimated half-lives of the HB24 and HB9 mRNAs in CD34-positive cells were approximately 45 minutes and 30 minutes, respectively (Fig 2). Inhibition of protein synthesis with cycloheximide for 1 hour did not significantly alter the HB24 and HB9 mRNA transcript levels in CD34-positive cells (Fig 2).

Further induction of HB24 and HB9 mRNA transcripts in IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF)-treated CD34-positive cells. Because the HB24 and HB9 mRNA transcripts are induced in lymphocytes activated with the appropriate stimuli such as phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) for T lymphocytes or Staphylococcus aureus cowan I (SAC) and PMA for B lymphocytes, the levels of HB24 and HB9 expression were examined following treatment of CD34-positive cells with recombinant IL-3 and GM-CSF. IL-3 and GM-CSF provide a potent proliferative and differentiative stimulus for hematopoietic progenitor cells. Exposure of the CD34-enriched fraction to IL-3 and GM-CSF for 24 hours increased the levels of HB24 and HB9 mRNA transcripts approximately threefold (3.2-fold) and fourfold (4.3-fold), respectively, as determined by densitometric analysis and excision of the appropriate band for analysis on a beta counter (Fig 3). No morphologic changes suggestive of differentiation of CD34-positive–enriched BM cells occurred during the first 48 hours of these cultures as assessed by an inverted light microscope using standard criteria.

Downregulation of HB24 and HB9 expression following the differentiation of CD34-positive cells in vitro. While the HB24 and HB9 mRNA transcripts are expressed highly in CD34-positive BM cells and can be further increased following a brief exposure to IL-3 and GM-CSF, the absence of HB24 and HB9 mRNA in CD34-depleted BM cells suggested that both of these genes are downregulated following differentiation. To examine whether the levels of HB24 and HB9 mRNA expression were altered in CD34 cells induced to differentiate in vitro, we examined the levels of HB24 and HB9 mRNA by in situ hybridization and RNase protection assay following in vitro treatment with recombinant IL-3 for 3 weeks. IL-3 is known to differentiate CD34-positive cells along the myeloid lineages. CD34-positive cells were strongly HB24 and HB9 mRNA positive by in situ hybridization (Fig 4). No hybridization signal was found when either the HB24 or HB9 sense control probes were used (data not shown). When treated with IL-3 for 3 weeks, the CD34-positive cells differentiated as assessed by an inverted light microscope using standard criteria and no longer had detectable levels of HB24 and HB9 mRNA by in situ hybridization (Fig 4). Downregulation of HB24 and HB9 mRNA expression was also confirmed following the differentiation of CD34-positive cells in vitro by RNase protection assay with RNAs from untreated and cultured cells (Fig 5).

**DISCUSSION**

Differentiation of hematopoietic progenitor cells along specific cell lineages must be accompanied by altered gene transcription of a variety of regulatory and tissue-specific genes to account for the appearance of various mature phenotypes. We have identified two putative transcript-
I for 24 hours with cytokines. Ten micrograms of cytoplasmic RNA was analyzed in each lane. Recombinant GM-CSF- and IL-3- (300 U/mL with a specific activity of 10^6 U/mg protein) treated CD34-positive cells. The cells were treated with IL-3 and GM-CSF. RNase protection assays were performed to assess HE24 (left panel) and HE9 (right panel) expression. Lane 1, untreated CD34-positive cells; lane 2, recombinant GM-CSF- and IL-3- treated CD34-positive cells. The cells were treated with IL-3 and GM-CSF.

Fig 3. Increased HB24 and HB9 expression following treatment of CD34-positive cells with IL-3 and GM-CSF. RNase protection assays were performed to assess HB24 (left panel) and HB9 (right panel) expression. Lane 1, untreated CD34-positive cells; lane 2, recombinant IL-3- (300 U/mL with a specific activity of 10^6 U/mg protein) and recombinant GM-CSF- (100 U/mL with a specific activity of 5 x 10^7 CFU/mg protein) treated CD34-positive cells. The cells were treated for 24 hours with cytokines. Ten micrograms of cytoplasmic RNA was analyzed in each lane.

HN has been shown to be inducible in lymphocytes and present in most lymphoid cell lines examined. In addition, the HB9 gene is present in stromal cells derived from human tonsils. Examination of a limited number of fetal tissues showed HB9 expression in 18 week brain and testis while HB24 was also present in brain, but in addition to developing inferior vena cava. The two genes are likely to be conserved evolutionarily because Southern blots show cross-hybridization with DNA from a variety of other species. In addition, both genes cross-hybridize with mRNAs derived from a murine teratocarcinoma cell line F9

Other homeobox genes have been found to be expressed in BM-derived cells as well as in hematopoietic cell lines. For example, HOX 1.1 and HOX 6.1 were isolated from a mouse BM cDNA and found to be expressed in a variety of hematopoietic cell lines. HOX 1.1 was expressed in all cell lines tested including cell lines of erythroid, myeloid, and lymphoid lineage while HOX 6.1 was present in a limited number of the tested cell lines although it was present in at least one cell line from each lineage. The expression of these genes in normal BM cells was not examined. Based on low stringency hybridization studies, it has been estimated that as many as 20 homeobox-containing genes are expressed within the hematopoietic compartment, suggesting that homeodomain-containing proteins are likely to play an important role in the hematopoietic cells. There is also some indirect evidence that homeobox genes may be involved in leukemogenesis. In the mouse myeloid leukemia cell line, WEHI-3B, HOX 2.4 is transcribed constitutively as a result of the insertion of an intracisternal A particle. In addition, 55 of 59 mouse myeloid leukemias tested contained a deletion in the HOX 4.1 locus and, recently, a translocation found in human acute pre-B-cell lymphoblastic leukemia was found to involve a homeobox gene, PRL, at the chromosomal break point. Because the differentiation of hematopoietic progenitor cells along lineage-specific lines likely requires the downregulation of HB24 and HB9, the dysregulation of either HB24 or HB9 expression may impair normal differentiation and contribute to oncogenesis.
Fig 4. Downregulation of HB24 and HB9 expression following the differentiation of CD34-positive cells cultured in vitro with recombinant IL-3. In situ hybridization assays were performed to assess HB24 (A) and HB9 (B) expression. (A, a) Bright field photomicrograph of CD34-positive cells hybridized with an anti-sense HB24 probe. (A, b) Dark field photomicrograph of the same cells as shown in (A, a). (A, c) Bright field photomicrograph of purified CD34-positive cells that have been cultured with IL-3 for 3 weeks and hybridized with the anti-sense HB24 probe. (B, a) Bright field photomicrograph of CD34-positive cells hybridized with the anti-sense HB9 probe. (B, b) Bright field photomicrograph of purified CD34-positive cells treated with IL-3 for 3 weeks and hybridized with the anti-sense HB9 probe.

Fig 5. RNase protection assay of HB24 and HB9 expression following the differentiation of CD34-positive cells cultured in vitro with recombinant IL-3. The left panel is from the analysis of HB24 mRNA transcripts and the right panel is from HB9 mRNA transcript. Lane 1, untreated CD34-positive human cells; lane 2, CD34-positive cells treated with recombinant IL-3 (300 U/mL) for 3 weeks. Ten micrograms of total RNA was used in each lane.

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