The Role of the Homeobox Gene, HOX B7, in Human Myelomonocytic Differentiation

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Homeobox genes encode transcription factors known to be important morphogenic regulators during embryogenesis. An increasing body of work implies a role for homeobox genes in both hematopoiesis and oncogenesis. We have analyzed the role of the homeobox gene, HOX B7, in the program of differentiation of the biphenotypic myeloid cell line, HL60. Induction of monocytic differentiation in HL60 cells by vitamin D₃ resulted in rapid expression of HOX B7 mRNA, but stimulation with phorbol ester or dimethyl sulfoxide (DMSO) did not. Constitutive overexpression of HOX B7 in the HL60 cell line inhibited the granulocytic differentiation associated with stimulation with DMSO or retinoic acid, but had no effect on the monocytic differentiation induced by vitamin D₃. Normal human monocytes do not constitutively express HOX B7, nor are they able to be induced to do so by stimulation with colony-stimulating factor 1 (CSF-1) and gamma interferon (IFNγ), or with vitamin D₃ and lipopolysaccharide. Human bone marrow (BM) cells were found to express HOX B7 in response to granulocyte-macrophage CSF (GM-CSF) and antisense oligonucleotides directed against HOX B7 inhibited the formation of colonies derived from GM-CSF-stimulated BM. These data suggest a critical role for HOX B7 in myelomonocytic differentiation.

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HOMEOBOX GENES encode DNA-binding proteins that contain a characteristic 60-amino acid DNA-binding motif, the homeodomain. The sequences encoding the homeodomain are highly conserved throughout evolution and are found in organisms as diverse as Drosophila, yeast, and man. A large body of work shows a critical role for these genes in embryogenesis. Homeobox genes may also have a regulatory function in differentiation processes ongoing in the adult animal, including hematopoiesis. Kongswan and colleagues surveyed 36 mammalian cell lines of lymphoid, myeloid, or erythroid lineages using cDNA clones derived from murine spleen and marrow and a human leukemic cell line, K562. All cell lines tested expressed at least one homeobox gene transcript, and the pattern of expression differed, depending on the lineage of the cell line tested. Interestingly, HOX B7 (formerly known as HOX 2.3) expression was essentially confined to the four murine macrophage lines studied, and was absent in all other myeloid lines, including premacrophages. Wu et al. used antisense oligonucleotides directed against HOX B7 to inhibit granulocyte-macrophage colony-forming unit’s (CFU-GM’s) development in a murine system. Other laboratories have subsequently shown expression of HOX B7 in human cell lines from many other lineages.

These results suggest that homeobox genes play a role in normal hematopoiesis, and that HOX B7 may be involved in myelomonocytic differentiation. Therefore, we designed experiments using human cell lines and primary human cells to analyze the effect of increased or inhibited expression of HOX B7 on myeloid differentiation.

MATERIALS AND METHODS

Cloning of HOX B7. Polymerase chain reaction (PCR) primers (5’ primer (nucleotide [nt] –18 to –1): AAATCATCGGC-CAAATT; 3’ primer (nt +650 to +667): TTCTCTCACTCT-CACCT) were designed from the published sequence of HOX B7, synthesized on a PCR Mate oligonucleotide synthesizer (Applied Biosystems; Foster City, CA) and purified by affinity chromatography using a Nensorb column according to the manufacturer’s instructions (DuPont, Wilmington, DE). Total RNA was extracted from K562 cells using a urea lysis/NP40 method. Reverse transcription was performed using avian myeloblastosis virus (AMV) reverse transcriptase (RT) (Boehringer Mannheim, Indianapolis, IN) and an oligo dT primer (Boehringer Mannheim) according to the manufacturer’s instructions.

Five microliters of cDNA was added to the PCR reaction mix consisting of 1.25 U Taq polymerase (Boehringer Mannheim) in the manufacturer’s buffer with 0.2 mmol/L of each dNTP (dNTP) (Pharmacia, Piscataway, NJ) and 1 μmol/L each of 5’ and 3’ primers. PCR was performed in a thermocycler (Perkin-Elmer Cetus, Piscataway, NJ) and ligated into the TA vector (Invitrogen; San Diego, CA) and the Division of Research Immunology, Children’s Hospital of Los Angeles, Los Angeles, CA.

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orientiation to produce LHB7SN (Fig 1). Expression of the HOX B7 insert is driven by the Moloney murine leukemia virus long terminal repeat, whereas expression of the selectable marker gene, neomycin phosphotransferase (neo), is driven by the SV40 promoter. The eukaryotic murine fibroblast line, PE501, was transfected and the supernatant was used to infect the amphotropic fibroblast packaging cell line, PA317. High-titer, stably transfected PA317 clones were selected for transduction of cell lines. 

Transduction and characterization of HL60 cell lines. HL60 cells were transduced by cocultivation with irradiated PA317 packaging cells expressing LHB7SN, or with a control packaging line expressing LXSN to produce the cell line, HL60-neo. Mock transductions were performed by cocultivation with the parent packaging cell line, PA317. HL60 cells at a density of 10⁶/mL in Iscove's modified Dulbecco's medium (IMDM; Irvine Scientific, Santa Ana, CA) and 10% fetal bovine serum (FBS; Gemini Bioproducts, Calabasas, CA) were added to the packaging cells together with 20 μg/mL protamine sulfate (Elkins Sln Inc, Cherry Hill, NJ), and cocultivated for 6 hours. After cocultivation, the nonadherent cells were removed, washed twice, and cultured in IMDM and 10% FBS for 48 hours. The HL60 cells were then selected in 1.0 mg/mL active G418 (GIBCO, Grand Island, NY) until all mock-infected cells were dead (≈3 weeks). The HL60 cells were then maintained in IMDM and 10% FBS, with intermittent passage through G418 to ensure persistent expression of neo. Expression of HOX B7 was confirmed using RT-PCR on several occasions.

PCR primers and methodology. Total RNA was extracted from 10⁶ cells using the urea lysis/NP40 method, except in the experiments involving human monocytes in which mRNA was extracted using FastTrack kits (Invitrogen) according to the manufacturer's instructions. HOX B7-specific primers were as follows. 5' (I'A), nt 65 to 84, (TGGCAATCTTATTTTCTA) and 3' (I'B), nt 513 to 532, (AAGTACGGATTGATGAAAA), resulting in a 477-bp product. Oligonucleotide primers were synthesized and purified as above.

Reverse transcription was performed on 5 μg of total RNA using either AMV RT (Boehringer-Mannheim) or Superscript (GIBCO-BRL) according to the manufacturer’s instructions. Negative controls consisting of the total reaction mix without the RT were performed in every instance. PCR conditions were 94°C for 90 seconds, 48°C for 90 seconds and 72°C for 90 seconds for 40 cycles. The reaction mix was fractionated on a 1.2% agarose gel in TBE. PCR gels were stained with ethidium bromide to visualize PCR products.

HL60 cells were stimulated with tetradeoxyl phorbol acetate (TPA; Sigma, St Louis, MO) at a concentration of 50 ng/mL added once at time 0. Controls consisting of addition of an equivalent amount of ethanol diluent were included in the differentiation experiments. Cell lines were stimulated with DMSO (Sigma) at a concentration of 1.25% added once at time 0. Xl-trans retinoic acid (ATRA; Sigma) was dissolved as a 1,000× solution in alcohol and stored in the dark at −20°C. The solution was freshly made every 2 weeks. ATRA was added to cell lines once at time 0 at a final concentration of 10⁻⁶ mol/L.

FACS analysis of cell lines. Cells were washed twice in FACS buffer and resuspended at 10⁶ cells/mL in 50 μL of FACS buffer. Human AB serum was added at room temperature for 1 minute to block nonspecific binding of antibody. Monoclonal antibody directed against the CD11b antigen (Leu-15 conjugated with phycoerythrin) was added to cells on ice according to the manufacturer’s recommendations (Becton Dickinson, San Jose, CA). Cells were vortexed and incubated at 4°C for 30 minutes in the dark, fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS), and analyzed on a FACSscan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Purification and induction of differentiation of human monocytes. Peripheral blood was obtained from healthy volunteers. Monocytes were isolated by Ficoll-Hypaque separation and adherence to culture dishes in the presence of 5% (vol/vol) human AB serum. After successive washing procedures, cultures contained greater than 97% pure monocytes. Adherent monocytes were cultured in IMDM supplemented with 10% (vol/vol) heat-inactivated FBS, 1% glutamine, and antibiotics at 37°C in a 5% humidified CO₂ incubator.

Monocytes were cultured with and without 10⁻⁸ mol/L colony-stimulating factor 1 (CSF-1) (generously provided by National Institutes of Health, Rockville, MD) and 500 U/mL IFNγ (Boehringer Mannheim) for 7 days, or with and without 10⁻⁸ mol/L vitamin D₃ (Calbiochem) for 7 days. Lipopolysaccharide (LPS) (100 ng/mL) (Fisher,ustin, CA) or diluent was added to the cultured monocytes 6 hours before harvesting. mRNA was extracted at 0, 24, and 72 hours poststimulation. Induction of differentiation was confirmed by cytoflorometry at day 7.

RT-PCR screen for HOX gene expression in GM-CSF–stimulated human monocytes. Total RNA was extracted from 10⁶ mononuclear cells using the QuickPrep Micro mRNA purification kit (Pharmacia) and resuspended in 16 μL of H₂O. Four microliters of this mRNA was reverse transcribed into cDNA using AMV RT in a 20-μL reaction volume per manufacturer’s instructions.

This cDNA was used in a PCR reaction using degenerate primers complementary to conserved sequences belonging to the mammalian Antennapedia class of HOX genes: 5'-AT(A/C/T)TGA(C/T)CC(A/C/G/T)TGATG(TG)3' (5' primer) and 5'-TTTCTAT(A/G/C/T)G(A/G)/CG(T/T)TG(A/G)AAACCA (3' primer). Oligonucleotides were prepared and purified as above. To control against the presence of false-positive PCR products resulting from genomic DNA contamination in the mRNA, this primer set spans an intron, and parallel cDNA reactions lacking reverse transcriptase were also performed.

The PCR reaction mix contained 5 μL of cDNA. 0.5 mmol/L
each of 3′ and 5′ primers, 0.25 mmol/L of each dNTP (Boehringer Mannheim), 16.6 mmol/L (NH4)2 SO4, 67 mmol/L TRIS-HCl (pH 8.8) (Sigma), 6.7 mmol/L MgCl2 (Sigma), 6.7 mmol/L EDTA (Sigma), 0.17 mg/mL bovine serum albumin (Sigma), 10% DMSO (Sigma), and 1.25 U of Taq polymerase (Boehringer Mannheim). 

PCR cycling was performed in a Perkin-Elmer Cetus thermocycler as follows: 94°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes for 35 cycles, followed by a 72°C extension for 10 minutes. Five microliters of this reaction was then reamplified in a fresh reaction mix under the same conditions for 25 cycles. This reamplified PCR product was then directly ligated into the TA vector (Invitrogen). 

Clones were sequenced to identify the number and types of homeobox genes present. Complete sequences were identified with the GENBANK database (Bethesda, MD).

Antisense oligonucleotide colony assay. Mononuclear cells were obtained from normal human BM, as above. Colony assays were performed in duplicate by plating 103 viable cells in serum-free medium (HSCM, GIBCO-BRL) containing 0.3% agar (Difco, Detroit, MI), 1 mmol/L GM-CSF (generously provided by Larry Souza) and antisense, sense, or nonsense oligonucleotides added at a concentration of 0.5 mmol/L, 5.0 mmol/L, or 15.0 mmol/L in a total volume of 0.5 mL into the wells of a 24-well plate. Oligonucleotide sequences were as follows: B7-Antisense, 5′-ATGAGTTCTCCCTTCT-3′; B7-Sense, 5′-ATGAGTTCCGTTATAT-3′; Nonsense, 5′-AACAATATCGTT-3′. Oligonucleotides were synthesized as above, purified by butanol extraction and ethanol precipitation, and resuspended in culture medium. The specificity of the antisense and sense oligonucleotides for HOX B7 and the nonspecificity of the nonsense oligonucleotide for any known sequences was determined by screening the GENBANK database. Clones were sequenced to identify the number and types of homeobox genes present. Complete sequences were identified with the GENBANK database (Bethesda, MD).

RESULTS

Expression of HOX B7 in HL60 cells. HL60 cells were originally isolated from a patient with acute myeloblastic leukemia, and have the phenotype of promyelocytes.9,10 They can be induced to differentiate to either granulocytes or monocyte/macrophages with the appropriate stimuli.9 We examined the effect of various inducers of monocytic or granulocytic differentiation on expression of HOX B7 mRNA in the HL60 cell line. RNA was extracted from HL60 cells stimulated with the monocyte-inducing agent, vitamin D3, over a time course of 12 hours. Induction of monocytic differentiation was confirmed by examining the morphology of the cells on cytopreps obtained from the same tissue culture after 5 days of stimulation. HOX B7 mRNA was assayed by RT-PCR. Constitutive expression was not detected in the HL60 cell line, but expression of HOX B7 was induced within 2 hours of stimulation with vitamin D3 (Fig 2). The specificity of the band obtained was confirmed by Southern blot. All PCR reactions also included multiple negative controls consisting of all the components of the cDNA reaction except the RT. 

Stimulation of HL60 cells with the macrophage differentiation inducing agent, TPA, at 50 ng/mL, or with the granulocyte inducing agent, DMSO, at 1.25%, did not induce HOX B7 expression (data not shown).

Inhibition of granulocytic differentiation by HOX B7. To assess the effect of overexpression of HOX B7 on differentiation, HL60 cells were transduced with a retroviral vector containing HOX B7 and the selectable marker, neo (HL60-HOX B7). Control cells were transduced with the vector containing only the neo gene (HL60-neo). A population of cells was selected in G418 and the expression of HOX B7 was confirmed by PCR. Subsequently, cells were intermittently assayed for HOX B7 expression by RT-PCR. No phenotypic changes were observed in the cell population constitutively expressing HOX B7.

The HL60-HOX B7 and HL60-neo cell lines were stimulated for 1 week with DMSO, an inducer of granulocytic differentiation. Differentiation was assessed using FACS analysis of CD11b expression (Fig 3) over 7 days. The cell line which constitutively expressed HOX B7 (HL60-HOX B7) had dramatically reduced expression of CD11b (~5% of cells) relative to the control cell line, HL60-neo (~50%), even after 7 days of stimulation with DMSO. The same result was observed in two other independent experiments. Similar results were obtained after stimulation with the granulocytic inducer, ATRA (Fig 4); the induction of CD11b expression by ATRA in the HL60-neo cells was over sixfold greater than that seen in the HL60-HOX B7 cells at 168 hours.

Experiments performed using vitamin D3 or TPA to induce monocyte/macrophage differentiation showed no effect of constitutive expression of HOX B7. CD11b was induced to a similar level in all cells. A range of concentrations of vitamin D3 was used, but there was no evidence of an increased sensitivity to the monocyte-inducing activity of this agent as a result of overexpression of HOX B7 (data not shown).

HOX B7 is not expressed by human monocytes. These results suggested a role for HOX B7 in the early lineage commitment steps of monocyte differentiation. Therefore, we examined HOX B7 expression in primary human monocytes. mRNA was extracted over a time course of 72 hours from human monocytes stimulated with either CSF-1 and IFNγ or with vitamin D3 ± LPS. Induction of macrophage differentiation was confirmed by cytopreps obtained after 7 days of stimulation. RT-PCR was performed using either GM-CSF receptor alpha subunit primers (positive controls) or HOX B7 primers A and B. HOX B7 was not detected in primary human monocytes, nor could expression be induced by stimulation with several agents known to induce differentiation or functional activation of monocytes (data not shown).

These data suggest that HOX B7 is not critical for the differentiation or activation of mature committed monocytes.

Inhibition of HOX B7 expression results in decreased colony formation by human BM cells stimulated with GM-CSF. HOX B7 was not present in mature human monocytes, but could be detected in HL60 cells undergoing monocytic differentiation. Therefore, we studied the role of HOX B7 in earlier events of myelomonocytic differentiation. GM-CSF is a potent growth factor for myeloid progenitor cells, resulting in formation of colonies containing both monocyctic and granulocytic cells. BM cells in semisolid culture were stimulated with GM-CSF and mRNA isolated from four to eight cell clusters on day 4. RT-PCR using degenerate oligonucleotide primers followed by subcloning and sequencing showed the presence of HOX B7 in seven of the nine clones.
containing homeobox genes obtained from clusters of cells differentiating toward a myeloid lineage.

Having shown expression of HOX B7 in early myelomonocytic cells, we then examined the function of HOX B7 by suppressing its expression. Addition of antisense oligonucleotides directed against HOX B7 dramatically inhibited colony formation by human BM mononuclear cells stimulated with GM-CSF (Fig 5). This inhibition showed a dose-response effect and was specific for HOX B7, as neither the sense or nonsense controls showed any inhibition at these concentrations. These results show that HOX B7 is expressed during CFU-GM development, and that this expression is necessary for proliferation and maturation of the CFU-GM.

DISCUSSION

Many previous studies of homeobox genes in hematopoiesis have surveyed cell lines of various lineages or primary human hematopoietic cells to delineate the pattern of expression of homeobox genes. These studies have shown that homeobox genes are expressed in all hematopoietic cells studied. There is a degree of lineage restriction of expression, but there is also considerable redundancy of expression.\(^1\)

Kongsuwan et al\(^1\) studied murine cell lines and noted that HOX B7 expression was limited to cells of monocyte/macrophage origin. Additional studies by Shen et al\(^2\) have also shown complex patterns of expression of homeobox mRNA in various human leukemic cell lines, although the particular pattern of lineage restriction observed was somewhat different to that reported by Kongsuwan et al. Similarly, Deguchi et al\(^3\) showed expression of HOX B7 in human lymphoid cells, leading to the idea that the pattern of homeobox gene expression may be different in mice and men. More recently, Vieille-Grosjean et al\(^4\) showed lineage-specific expression of homeobox genes in leukemic cell lines, and also showed expression of nine normal homeobox genes in a CD34-enriched population of chronic myeloid leukemia blast cells.\(^5\)

None of these studies addressed the question of a specific function for a particular homeobox gene or combination thereof.

Several authors have used strategies of overexpression or inhibition of expression of homeobox genes to further investigate the impact of the pattern of homeobox gene expression on hematopoietic differentiation. Wu et al used an antisense oligonucleotide directed against Hox B7 in a mu-
The inhibition of ATRA-induced granulocytic differentiation by HOX B7 alone is not sufficient for monocytic differentiation, and at a later stage, HOX B7 may function by restricting the lineage commitment choices that an individual cell makes rather than by specifically driving the cell down a particular pathway. The data from Wu et al showing that Hox B7 must be inhibited early to inhibit CFU-GM development are consistent with this hypothesis. This observation is analogous to the phenomenon of posterior dominance seen in embryologic studies of Drosophila, in which the effect of expression of a posteriorly expressed homeobox gene predominates over the effect of an anteriorly expressed homeobox gene. Therefore, inhibition of the posteriorly expressed homeobox gene is required for the anteriorly expressed gene to exert a phenotypic effect.16

The homeobox-containing family of transcription factors is assuming an increasing importance in the studies of regulation of hematopoiesis. Functional studies looking at the effect of overexpressing or underexpressing the gene of interest have the ability to greatly add to our understanding of mechanisms of regulation of hematopoiesis. We have shown a role for HOX B7 in human myelomonopoiesis, but have also shown that this role may well be considerably more complex than that suggested by the original observations of lineage restriction.

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Fig. 5. HOXB7 antisense oligonucleotides inhibit human bone marrow colony formation in a dose-dependent manner. Colony assays from human BM incubated with HOX B7 sense or antisense oligonucleotides at differing concentrations. BM mononuclear cells were analyzed for colony formation in response to 1 nmol/L human GM-CSF, as described in the Materials and Methods. Colonies were scored on day 14 in a blinded fashion. Data are expressed as a percentage of the colonies formed by incubation with nonsense oligonucleotides at the same concentration. ([ ]) Sense; (■), antisense.

We have extended these observations by identifying a role for HOX B7 in myelomonocytic differentiation in a human cell line and in primary human cells, and suggest that this gene may have differing roles, depending on the stage of maturation of the cell expressing the gene. An exact role for HOX B7 in human myelomonocytosis remains difficult to assign. We have shown, using the human myeloblastic leukemic cell line, HL60, that HOX B7 is expressed only after stimulation with vitamin D₃, which results in monocytic differentiation. Vitamin D₃ and TPA are known to induce differentiation through differing pathways and result in a monocytic versus a macrophage phenotype, respectively.9 These results suggest that the monocytic and macrophage differentiation phenotypes of HL60 cells differ in their pattern of expression of homeobox genes. Overexpression of HOX B7 in this system is able to inhibit granulocytic differentiation in response to DMSO and to ATRA, but did not increase monocytic differentiation in response to either TPA or vitamin D₃. The inhibition of ATRA-induced granulocytic differentiation by HOX B7 is particularly interesting, as retinoic acid is known to regulate expression of homeobox genes, including HOX B7, in other systems.15 This suggests that overexpression of a single homeobox gene may be able to override the effects of multiple homeobox genes whose expression is correctly regulated. We could not detect HOX B7 expression in primary human monocytes, nor could it be induced. However, primary human marrow cells stimulated with GM-CSF did express HOX B7, and inhibition of HOX B7 expression abrogated colony formation in response to GM-CSF.

One hypothesis which ties these observations together is that expression of HOX B7 is required at an early, yet committed stage of myelomonocytic development for further proliferation and differentiation. However, expression of HOX B7 alone is not sufficient for monocytic differentiation, and at a later stage, HOX B7 may function by restricting the lineage commitment choices that an individual cell makes rather than by specifically driving the cell down a particular pathway. The data from Wu et al showing that Hox B7 must be inhibited early to inhibit CFU-GM development are consistent with this hypothesis. This observation is analogous to the phenomenon of posterior dominance seen in embryologic studies of Drosophila, in which the effect of expression of a posteriorly expressed homeobox gene predominates over the effect of an anteriorly expressed homeobox gene. Therefore, inhibition of the posteriorly expressed homeobox gene is required for the anteriorly expressed gene to exert a phenotypic effect.16

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