Modulation of homeobox gene expression alters the phenotype of human hematopoietic cell lines

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We have previously reported that certain genes of the HOX 2 cluster of homeobox genes on human chromosome 17 are specifically expressed in human leukemic cell lines with erythroid potential, suggesting that these genes are involved in hematopoietic differentiation. We now show that the expression of the HOX 2.2 gene decreases during erythropoietin-induced differentiation of the erythroid cell line MB02. In order to study the role of the HOX 2.2 homeobox gene in hematopoiesis, vectors producing sense or antisense transcripts were introduced into K562 and HEL cells, pluripotent lines with erythroid and myeloid features. Overexpression of HOX 2.2 is associated with loss of erythroid features in both lines and an increase in certain myelomonocytic markers in K562 cells. Expression of antisense HOX 2.2 is associated with an increase in erythroid features in HEL cells and a mild decrease in myeloid characteristics in K562 cells. Overexpression of the adjacent HOX 2.1 gene in K562 cells does not produce similar phenotype changes. These data demonstrate that modulation of a specific HOX 2 homeobox gene can change the phenotype of somatic cells and suggest that certain HOX 2 genes play a role in blood cell differentiation.

Key words: differentiation/homeotic genes/leukemic cells/transcription factors

Introduction

Homeobox-containing genes encode transcription factors involved in the establishment of body plan and tissue identity during embryogenesis (for reviews, see Gehring, 1987; Levine and Hoey, 1988; Akam, 1989). Many of the identified mammalian homeobox genes are organized in four highly conserved clusters which are expressed in precise spatial patterns in the embryo (Duboule and Dolle, 1989; Graham *et al.*, 1989). Aberrant expression of *Hox* genes in transgenic mice results in a variety of morphologic derangements, indicating an important role for these genes in development (Balling *et al.*, 1989; Wolgemuth *et al.*,

1989; Chisaka and Capecchi, 1991). We hypothesized that homeobox gene products also play a role in hematopoiesis, a life-long process of differentiation of highly specialized blood cells from pluripotent stem cells (Metcalf, 1989).

Previous studies have shown that many homeobox genes of the human HOX 1, HOX 2 and HOX 3 loci are expressed in hematopoietic cells in lineage-restricted patterns, suggesting a role in blood cell differentiation (Kongsuwan *et al.*, 1988; Shen *et al.*, 1989; Lowney *et al.*, 1991; Mathews *et al.*, 1991a). Expression of the HOX 2.1 and HOX 2.2 homeobox genes is limited to cell lines with primitive erythroid features (Shen *et al.*, 1989, 1990; Mathews *et al.*, 1991a). We now report that the expression of HOX 2.2 but not HOX 2.1 is decreased during erythropoietin-induced differentiation in the MB02 cell line. Furthermore overexpression of HOX 2.2 in the K562 and HEL cell lines is associated with inhibition of erythroid differentiation. These data demonstrate that modulation of the expression of a specific HOX 2 homeobox gene can change the phenotype of a human hematopoietic cell, suggesting a role in the regulation of blood cell differentiation.

Results

Decreased HOX 2.2 expression during erythroid differentiation

The GM-CSF dependent cell line MB02 shows erythroid differentiation in response to erythropoietin (Morgan and Brodsky, 1989). When MB02 cells are treated with erythropoietin (Epo) for 6–7 days, the level of α -globin mRNA increases ~2-fold, and β -globin mRNA, which is not present before induction, becomes readily detectable (Figure 1a). In addition, the fraction of heme-containing cells increases from 10 to 80%, as assessed by benzidine positivity. After Epo-induced differentiation, HOX 2.2 homeobox mRNA levels are markedly reduced (Figure 1b); by contrast, the mRNA levels of a neighboring homeobox gene in the HOX 2 locus, HOX 2.1, do not change. The expression of several other HOX 2 genes, i.e. HOX 2.3, 2.4 and 2.7, also does not change significantly with Epo treatment (data not shown).

Transfection of K562 cells with HOX 2.2 sense and antisense constructs

To investigate the role of HOX 2.2 in the determination of the erythroid phenotype, sense and antisense HOX 2.2 expression vectors were transfected into erythroid cell lines. The replicon vector system, which has been used successfully by others to transfect hematopoietic cell lines (Hauer *et al.*, 1989), was employed in these studies. This vector contains an Epstein–Barr virus origin of replication (Yates *et al.*, 1985) and replicates episomally in eukaryotic cells without requiring integration into the host genome (Figure 2).

Attempts to transfect MB02 cells were not successful,

Fig. 1. Expression of globin and HOX 2.2 transcripts in MB02 cells. (A) Northern gel analysis of β -globin and α -globin mRNA levels in MB02 cells treated with GM-CSF alone (lane 1), GM-CSF with Epo (lane 2) and with Epo alone (lane 3). (B) RNase protection analysis of HOX 2.2 and HOX 2.1 transcripts from MB02 cells before and after treatment with Epo. The HOX 2.2 probe protects a 300 nt fragment, while the HOX 2.1 probe protects a 240 nt band. A G3PD probe was used as a control for loading. Control lanes with tRNA and probe alone are also shown.

Fig. 2. The Rep-3 vector system and the HOX 2.2 sense and antisense inserts (see Materials and methods). The letter 'A' identifies the probe used for RNase protection assays of MB02 cell mRNA (shown in Figure 1).

apparently due to their inability to sustain growth under conditions of low cell density during antibiotic selection. However, two pluripotent cell lines, K562 and HEL, which express primitive erythroid features (Lozzio *et al.*, 1981; Martin and Papayannopoulou, 1982), were successfully transfected. In a parallel experiment the HOX 2.1 gene was

placed into the replicon vector in the sense orientation and transfected into the K562 cell line. As an additional control, K562 cells were also transfected with the replicon vector alone.

Of note is the fact that endogenous HOX 2.2 and HOX 2.1 mRNA levels in parent K562 cells are very low and detected

Fig. 3. Expression of HOX 2 mRNAs in transfected K562 and HEL cells. RNase protection was used to measure the transcript levels in transfected cell lines. (A) K562 cells transfected with HOX 2.2 vectors: lanes are vector control, sense HOX 2.2 vector or antisense HOX 2.2 vector, respectively. The first two lanes were hybridized with an antisense probe, while the third lane was hybridized with a sense strand probe. The unfilled arrow marks the band protected by antisense HOX 2.2 transcripts at 325 nt, while the filled arrow marks the band protected by sense HOX 2.2 transcripts at 304 nt. Each hybridization contained 5 μ g of mRNA. The autoradiograph shown is 24 h exposure; a 3 day autoradiograph shows the weak endogenous HOX 2.2 message in K562 cells (results not shown). (B) K562 cells transfected with HOX 2.1 vector: lanes are replicon control or sense HOX 2.1, respectively. The open arrow marks the band protected by both endogenous and sense HOX 2.1 at 240 nt. The replicon control lane represents 10 μ g of mRNA, while the HOX 2.1 transfected lane represents 100 ng of mRNA added to the hybridization reaction, respectively. (C) HEL cells transfected with HOX 2.2 vectors: lanes represent cells transfected with vector alone, sense HOX 2.2 vector or antisense HOX 2.2 vector, respectively. The replicon and sense transfectants were hybridized with an antisense HOX 2.2 probe, while the antisense HOX 2.2 transfectant probed with a sense HOX 2.2 probe. The unfilled, filled and open arrows represent the expected bands produced by protection with antisense HOX 2.2 mRNA (325 nt), endogenous HOX 2.2 mRNA (304 nt) and the exogenous sense HOX 2.2 mRNA (175 nt), respectively. Note that the breakdown product in the antisense HOX 2.2 transfected cells does not represent endogenous sense HOX 2.2 message, since the probe is a sense HOX 2.2 construct. Each lane represents 5 μ g of mRNA in the hybridization reaction. Molecular weight markers were end-filled, *Msp* digested Bluescript minus DNA (Stratagene).

only by RNase protection assays while expression in HEL cells is ~ 10 -fold higher (Shen *et al.*, 1989; Mathews *et al.*, 1991a). The expected expression of sense and antisense constructs in transfected cells was confirmed by RNase protection assays (Figure 3). Compared with endogenous mRNA amounts, exogenous HOX 2.2 and HOX 2.1 transcripts are both elevated ~ 100 -fold in K562 sense transfectants. Antisense HOX 2.2 mRNA in antisense transfected K562 cells is 10-fold higher than parent cell HOX 2.2 mRNA. The sense HOX 2.2 transcripts in HEL cells are ~ 5 -fold increased over endogenous levels, while the amount of antisense HOX 2.2 mRNA is similar to the endogenous HOX 2.2 mRNA level in HEL cells.

Loss of erythroid features in K562 cells overexpressing HOX 2.2

K562 cells transfected with sense HOX 2.2 show a dramatic decrease of several erythroid features. Alpha and gamma globin mRNA levels are markedly reduced as compared with the parent cell line, vector transfected cells or cells transfected with antisense HOX 2.2 (Figure 4A). Heme content, as gauged by benzidine staining, is reduced in

sense HOX 2.2 transfected cells grown in liquid culture (Table I), even after treatment with the erythroid inducer, δ -aminolevulinic acid (ALA). K562 sense HOX 2.2 transfectants grown in semi-solid media shows a virtual absence of benzidine-positive colonies (Figure 5 and Table I). Cells overexpressing HOX 2.2 also show decreased expression of the erythroid-specific surface marker glycophorin A (Table I and Figure 6). It should be noted that K562 cells transfected with replicon alone showed small but consistent increases in erythroid features, i.e. benzidine positivity and glycophorin, as compared with untransfected parental K562 cells (Table I).

To control for the possibility that phenotypic changes seen in transfected cells represented random clonal variation in the parent cells and not an effect of the HOX 2.2 insert, three different HOX 2.2 transfectants, derived from separate transfection experiments were studied and showed identical phenotypic changes, including globin mRNA levels (Figure 4), glycophorin expression and benzidine positivity (data not shown). Furthermore, following growth of the first batch of K562 sense transfectants (K562-sense HOX 2.2 #1) in semi-solid media, two separate pools (K562-sense HOX 2.2

Fig. 4. Expression of globin mRNA in HOX 2.2 transfected K562 and HEL cells assayed by Northern gel analysis. (A) α - and γ -globin mRNA levels in K562 cells transfected with sense HOX 2.2 vectors: lanes are K562 parent cells; cells transfected with vector alone; two separate sense HOX 2.2 transfectants, #1 and #2; and two sets of sense HOX 2.2 pooled colonies, 1A and 1B (see text); and an antisense HOX 2.2 transfectant. A representative set of actin signals shows equal RNA loading. K562 cells do not synthesize β -globin chains. (B) K562 cells transfected with sense HOX 2.1 vector: lanes are K562 parent cells, cells transfected with vector alone or cells transfected with sense HOX 2.1. (C) Globin mRNA levels in HEL cells transfected with sense or antisense HOX 2.2 vector: lanes are HEL parent cells, cells transfected with vector alone, sense HOX 2.2, or antisense HOX 2.2, respectively. A representative actin lane is shown.

1A) and K562-sense HOX 2.2 1B) of ~50 colonies were picked and expanded for phenotypic study. Both pools show high levels of HOX 2.2 expression indistinguishable from the original sense HOX 2.2 transfectants, as well as low globin mRNA levels (Figure 4A) and low benzidine positivity (results not shown).

Concomitant with a decrease in erythroid features, K562 cells overexpressing HOX 2.2 display increases in markers of myelomonocytic differentiation, including increased phagocytic activity, chloroacetate esterase levels and My4 surface antigen expression (Table I).

Phenotype changes in K562 cells transfected with antisense HOX 2.2

K562 cells expressing antisense HOX 2.2 transcripts show no change in erythroid features, but do show a mild but consistent decrease in certain myelomonocytic surface antigens, including My7 and Mo2 (Table I). Furthermore, they no longer produce non-specific esterase in response to phorbol ester (Table I).

Overexpression of HOX 2.1 in K562 cells does not change globin or heme content

K562 cells transfected with a sense HOX 2.1 replicon vector do not show decreased α - or γ -globin expression compared with parent K562 cells (Figure 4B), despite the expression

of approximately equal amounts of HOX 2.1 message to that observed for the HOX 2.2 transfected cells (Figure 3A and B). In addition, the HOX 2.1 transfectants do not show a significant decrease in heme content as compared with replicon control (Table I). The sense HOX 2.1 transfectants do, however, exhibit a decreased content of glycophorin A which is of a similar or even greater magnitude to that observed for the sense HOX 2.2 transfectants (Table I). The only change in myeloid phenotype observed for the HOX 2.1 transfectants is an increase in basal expression of non-specific esterase (Table I).

Overexpression of sense and antisense HOX 2.2 alter HEL cell erythroid phenotype

HEL cells expressing sense HOX 2.2 (Figure 3C) show a reproducible 2- to 3-fold reduction in α -, β - and γ -globin levels (Figure 4C). Expression of antisense HOX 2.2 mRNA in HEL cells, produces a reproducible 2-fold increase in α - and β -globin mRNA levels. In addition, ALA-treated HEL cells producing antisense HOX 2.2 show a reproducible increase (mean 76%) in heme content measured by spectrophotometric assessment of benzidine positivity, as compared with ALA-treated cells transfected with vector alone ($P < 0.005$). No significant changes in the erythroid surface marker glycophorin A or in myeloid surface antigens were noted for either the sense or antisense HOX 2.2 transfected HEL cells.

Table I. Changes in K562 cell phenotype induced by sense and antisense HOX 2.2 transcripts

Marker	Parent control	Replicon control	HOX 2.2 sense	HOX 2.2 antisense	HOX 2.1 sense
Benzidine positivity^a					
Liquid -ALA	10.7 ± 2.1	17.8 ± 2.9	3.8 ± 3.8*	16.5 ± 6.4	21
Liquid +ALA	26.3 ± 6.7	39.0 ± 12.7	14.5 ± 0.7**	29.5 ± 0.7	ND
Colonies	17.3 ± 3.5	42.0 ± 18	4.2 ± 6.1***	30.0 ± 5.7	40.2 ± 2.5
Cell surface antigens^b					
Glycophorin A					
Microscope	43.5 ± 10.3	68.4 ± 20.6	29.6 ± 11.1*	66.7 ± 11.6	ND
FACS	55	47	26	79	11
My4	5.8	1.5	13.9	0	3.5
My7	14.2	15.0	16.0	0	6.7
Mo2	1.5	5.6	7	0	ND
Phenotypic markers^c					
Latex particle ingestion					
	11.3 ± 5.9	10.7 ± 4.0	36.0 ± 16.1***	12.0 ± 4.0	13
Non-specific esterase					
	0	0	5	0	21
Non-specific esterase/PMA ^d					
	50	30	60	0	ND
Chloroacetate esterase					
	ND	0	22	6	3

^aPercent positive cells or colonies. Cells were grown in liquid suspension or semi-solid media with or without ALA and stained with benzidine.

^bMicroscopic analysis of glycophorin expression by direct visualization with a fluorescence microscope. Results represent the means of five individual experiments. FACS analysis scored as specific fluorescence intensity, subtracting background fluorescence. Results are taken from representative FACS runs. All experiments were repeated two to three times.

^cScored as percentage of positive cells by two independent reviewers. The latex particle ingestion results are the means of three separate experiments.

^dCells were treated with 10⁻⁹ M PMA for 72 h prior to assay.

* $P < 0.005$ as compared with replicon control.

** $P < 0.025$ compared with replicon control.

*** $P < 0.05$ compared with replicon control.

Discussion

The MB02, K562 and HEL cell lines are human leukemic lines with primitive erythroid features (Lozzio *et al.*, 1981; Martin and Papayannopoulou, 1982; Morgan and Brodsky, 1989) which express differing levels of HOX 2.2 transcripts as well as other genes of the HOX 2 homeobox complex (Shen *et al.*, 1989, 1990; Mathews *et al.*, 1991a). MB02 cells, which express moderate amounts of HOX 2.2, show a marked diminution of HOX 2.2 transcripts during more 'terminal' erythroid differentiation. K562 cells, pluripotent cells with the capacity for both myeloid and erythroid differentiation, express low amounts of HOX 2.2, and overexpression of this gene in K562 cells leads to loss of several erythroid features and a partial shift towards a myelomonocytic phenotype. Inhibition of the already low level of HOX 2.2 expression in K562 cells leads to a mild decrease in myeloid features, most notably the loss of responsiveness to phorbol esters. The phenotypic changes seen in transfected K562 cells do not appear to represent random clonal variation in the parent cell line since (i) very similar phenotypic changes were seen with repeated transfections, and (ii) transfectants with vector alone do not show phenotypic changes. That these effects are specific for HOX 2.2 (and not simply due to random modulation of a homeobox gene) is suggested by the observation that K562 cells transfected with a sense HOX 2.1 construct do not show the changes in globin and heme expression seen in HOX 2.2 sense transfectants.

HEL cells contain ~10-fold higher amounts of HOX 2.2 mRNA than K562 cells. Expression of the replicon vector

system in this line did not lead to the marked overexpression observed in K562 cells. This may reflect different transcriptional milieus in the two lines. The moderate increase in HOX 2.2 message achieved with the replicon vector in HEL cells resulted in a reproducible but small diminution in all three globin chain transcripts expressed by this line. Heme content is sufficiently low in HEL cells that it is difficult to detect a decrease of this erythroid marker in the sense HOX 2.2 transfectants; however, the antisense HOX 2.2 transfected HEL cells did show a modest, reproducible increase both in globin chain expression and in heme content.

The three erythroid lines studied show parallel changes in HOX 2.2 mRNA levels and erythroid character, with a shift toward a more mature erythroid phenotype being associated with a decrease in HOX 2.2 message content. These observations, taken together with our previous finding that HOX 2 gene expression is largely limited to pluripotent cell lines with erythroid potential, suggest that HOX 2.2 gene expression is required for pluripotent stem cells or for early stages of erythroid development, but progression to a more mature erythroid phenotype requires diminution of HOX 2.2 gene expression. The molecular basis for the phenotypic changes induced in transfected cell lines overexpressing HOX 2.2 is unclear but may well involve direct binding of HOX 2 proteins to regulatory regions of erythroid-specific genes. We have demonstrated that the HOX 2.2 protein binds to the second intron and the 3' enhancer of the β -globin gene, important regulatory regions for globin expression (Vallerga *et al.*, 1991). In addition, Lavelle *et al.* (1991) have recently shown that the HOX 2.8 protein binds to the 3' regulatory region of the human γ -globin gene.

Fig. 5. Changes in heme content in K562 colonies transfected with HOX 2.2 sense and antisense constructs. Benzidine staining of colonies of transfected K562 cells grown for 5–6 days in semi-solid media reveals no staining of the HOX 2.2 sense transfectants (panel A) as compared with cells transfected with an antisense HOX 2.2 construct (panel B) or with Rep-3 vector alone (panel C).

It seems very probable that many transcription factors, such as the erythroid-specific GATA-1 (Tsai *et al.*, 1989) and other homeobox genes of the HOX 2 complex, participate in the determination of cell lineage of hematopoietic stem cells. Supporting this hypothesis are recent experiments in our laboratory showing that HOX 2.6 expression is upregulated during induction of erythroid differentiation in both K562 and MB02 cells (K. Detmer, unpublished observations). Taken together, these results suggest that the eight HOX 2 homeobox genes which are

GLYCOPHORIN A

Fig. 6. Changes in surface glycophorin expression of transfected K562 cells. FACS analysis of K562 cells transfected with HOX 2.2 vectors. The y axis represents cell number and the x axis represents the log of fluorescence intensity. The solid dark line represents sense HOX 2.2 transfectants, the dashed line antisense HOX 2.2 transfectants and the dotted line K562 cells transfected with vector alone.

expressed in human erythroid cell lines play a complex regulatory role in control of erythroid phenotype.

Materials and methods

Human leukemic cell lines and differentiation experiments

The erythroid line MB02 (Morgan and Brodsky, 1989) was maintained in RPMI with 10% heat-inactivated human serum supplemented with 50 U/ml of recombinant human GM-CSF (Amgen, Thousand Oaks, CA). Erythroid differentiation was induced with 2 U/ml of recombinant human erythropoietin (Amgen, Thousand Oaks, CA). K562 and HEL cells were maintained in RPMI with 10% fetal calf serum (Papayannopoulou *et al.*, 1987). Monocytic differentiation of K562 cells was induced by the addition of 10^{-9} M phorbol 12-myristate 13-acetate (PMA) for 72 h prior to assay (Papayannopoulou *et al.*, 1987). Erythroid differentiation of K562 cells was induced by the addition of 2×10^{-5} M δ -aminolevulinic acid (ALA) for 5–6 days prior to assay (Lozzio *et al.*, 1981).

Northern gel analysis of globin expression

RNA was isolated by the guanidinium thiocyanate method (Chirgwin *et al.*, 1979) and poly(A)⁺ RNA was prepared using standard techniques (Ausubel, 1990). mRNA was electrophoresed in 1.5% agarose–formaldehyde gels, transferred to GeneScreen, and cross-linked to the filters by UV irradiation. Probes for α -globin, β -globin, γ -globin and actin were labeled by the random primer method (Feinberg and Vogelstein, 1983). Separate gels were run for each globin transcript since they are of similar size.

RNAse protection assays

Cytoplasmic poly(A)⁺ RNA was isolated, and RNA probes synthesized from Bluescript vectors were used in RNase protection assays as previously described (Ausubel, 1990). The HOX 2.2 probe used for the MB02 RNase protection experiment (labeled 'A' in Figure 2) protects a 300 nucleotide (nt) fragment; the HOX 2.1 probe protects a 240 nt fragment. A glyceraldehyde-3-phosphate dehydrogenase (G3PD) probe protecting an 83 nt band was used as a control. The RNase protection probe used to assess sense HOX 2.2 expression in K562 cells protects a 304 nt fragment which represents both the endogenous and exogenous transcripts, while the probe used to protect sense HOX 2.2 in the HEL cells was constructed to protect a 302 nt fragment for the endogenous mRNA and a 175 nt fragment for the exogenous message. The probe used to detect antisense HOX 2.2 expression from the replicon plasmid in either K562 or HEL cells protects a 325 nt fragment. The probe used to detect sense HOX 2.1 expression in the K562 cells is the same probe as used for the MB02 experiments and protects a 240 nt fragment.

Transfection of K562 cells with sense and antisense HOX 2 constructs

In the Rep-3 vector, the sequence to be expressed is inserted into cloning sites between the Rous sarcoma virus 3' long terminal repeat and an SV40 polyadenylation signal (Hauer *et al.*, 1989). The vector also contains a hygromycin resistance gene to permit selection. The rep-3 HOX 2.2 sense construct contains a 747 nt *NotI*-*SmaI* sequence encoding a 224 amino acid homeobox-containing protein (Figure 2). To make the corresponding antisense construct a *NotI*-*XhoI* HOX 2.2 fragment (Figure 2) extending from 5' to the translation start site to ~193 nt downstream from the protein termination codon was inserted in the antisense orientation. The sense HOX 2.1 replicon expression vector contains a full-length 2.0 kb cDNA (Hauser *et al.*, unpublished results). Cells (2×10^6) were transfected by lipofection and selected in medium containing 200 μ g/ml hygromycin, using published methods (Hauer *et al.*, 1989). Previous studies using K562 cells suggested that numerous cells were transfected in each experiment (McNally *et al.*, 1988; Hauer *et al.*, 1989). Since the Rep-3 vector does not integrate into the host genome, true 'clones' of transfected cells were not expected; therefore subcloning of transfected cells was not attempted. Thus the transfected cells in each experiment represent a mixture derived from the original transfected cells, containing varying numbers of the replicon plasmid.

Assessment of phenotypic changes

Surface antigen expression was analyzed with a Becton-Dickinson FACS analyzer and a panel of lineage-specific monoclonal antibodies, using manufacturers' suggested dilutions. Results are expressed as specific fluorescence intensity, after subtracting non-specific fluorescence, and represent the mean of two to four separate experiments. All cytochemical stains were performed using commercial staining kits following manufacturer's suggestions (Sigma, St Louis, MO). Heme content in K562 cells was assessed by direct benzidine staining of individual cells in liquid suspension or of individual colonies in semi-solid medium (Rowley *et al.*, 1985). Since the endogenous heme content of HEL cells is too low for visual scoring, heme content in HEL cells was measured spectrophotometrically following reaction of cell lysates with benzidine; all samples were run in triplicate and the experiment was repeated twice (Clarke *et al.*, 1982). Latex particle ingestion were assessed by published methods (Takeda *et al.*, 1982); results are expressed as the percentage of cells containing latex particles and represent the means of three separate experiments. Statistical analysis was performed using a one-sided Student's *t* test.

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