Antisense knockout of HOXB4 blocks 1,25-dihydroxyvitamin D₃ inhibition of *c-myc* expression

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Abstract

The expression of *c-myc* is decreased by 1,25-(OH)₂D₃ during HL-60 cell differentiation. Concomitantly, 1,25-(OH)₂D₃ increases the expression and DNA binding activity of HOXB4, a homeobox gene. HOXB4 binds to the *c-myc* gene at sites involved in blocking *c-myc* transcription elongation. In this study, a phosphorothioate antisense oligonucleotide targeted against HOXB4 was examined for its effect on 1,25-(OH)₂D₃ inhibition of *c-myc* expression. Alone, 1,25-(OH)₂D₃ (20 nM) increased HOXB4 levels by 103 ± 7% (mean ± s.e., *n* = 3) and decreased *c-myc* levels by 89 ± 5% (mean ± s.e.m., *n* = 3) at 48 h of treatment. HOXB4 antisense treatment

completely blocked the induction of HOXB4 by 1,25-(OH)₂D₃. In addition, HOXB4 antisense partially blocked 1,25-(OH)₂D₃-mediated decrease in *c-myc* levels (46 ± 6% inhibition) and promotion of HL-60 cell differentiation (20 ± 2% and 25 ± 3% inhibition as assessed by nitroblue tetrazolium and non-specific esterase assays respectively). The data further establish that HOXB4 levels are regulated by 1,25-(OH)₂D₃ and reveal that HOXB4 participates in the down-regulation of *c-myc* expression.

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Introduction

The seco-steroidal hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) induces terminal differentiation of HL-60 promyelocytic leukemia cells into monocytes/macrophages (McCarthy *et al.* 1983, Grosso & Pitot 1985, Studzinski *et al.* 1985, Simpson & Weishaar 1986). Early in the induction of differentiation, the expression of *c-myc* is down-regulated (Bentley & Groudine 1986, Simpson *et al.* 1987, Spencer & Groudine 1991). This reduction in *c-myc* expression is at least in part a result of a transcriptional elongation block at the first intron/exon of the gene (Simpson *et al.* 1989). Recently, our laboratory reported that three protein binding sites located at intron 1 of the *c-myc* gene, termed MIE1, MIE2 and MIE3, have an important role in 1,25-(OH)₂D₃ regulation of *c-myc* transcription during HL-60 cell differentiation (Pan & Simpson 1999). A homeobox gene-product, HOXB4, was identified as an MIE1 binding protein by MIE1-affinity purification and HOXB4 levels were enhanced by 1,25-(OH)₂D₃ (Pan & Simpson 1999). Interestingly, MIE1–3 each have one or two core HOX-like response elements, TTAT. Members of the HOX gene family participate in the regulation of hematopoiesis (Kongsuwan *et al.* 1988, Levine & Hoey 1988, Shen *et al.* 1989). Investigators have shown that blood cells at different stages of hematopoietic differentiation express different sets of HOX genes (Shen

et al. 1989, Magli *et al.* 1991). In addition, overexpression of certain HOX genes has been demonstrated to affect hematopoiesis and leukemogenesis (Shen *et al.* 1989, Magli *et al.* 1991, Celetti *et al.* 1993). In this study, a HOXB4 antisense oligonucleotide was found to block 1,25-(OH)₂D₃ increases in HOXB4 levels. Moreover, HOXB4 antisense inhibited 1,25-(OH)₂D₃-mediated decreases in *c-myc* levels and partially blocked 1,25-(OH)₂D₃ promotion of cell differentiation. These results indicate that HOXB4, a 1,25-(OH)₂D₃-regulated gene product, is involved in *c-myc* transcriptional elongation blockage and promotion of HL-60 cell differentiation.

Materials and Methods

Chemicals

1,25-(OH)₂D₃ was purchased from Tetriconics Inc. (Madison, WI, USA). Vitamin D₃ metabolite purity and structural integrity were confirmed by high performance liquid chromatography and UV spectroscopy. All other reagents were reagent grade or better.

Oligonucleotides

Phosphorothioate oligonucleotides were synthesized by the DNA Synthesis Center at the University of Michigan.

HOXB4 antisense had the nucleotide sequence: 5'-AAGAACTCATAGCCATTA-3'. The HOXB4 sense had a sequence complementary to that of the antisense oligonucleotide. Oligonucleotides were purified by HPLC and quantified by UV spectroscopy at 260 nm/280 nm. Cells were treated with HOXB4 sense and antisense (diethyl pyrocarbonate H₂O) for periods and concentrations as described.

Cell culture

HL-60 promyelocytic leukemia cells were obtained from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% horse serum, 1000 U/ml penicillin G and 0.5 mg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Cells used in this study were from passages 21 to 45. Experiments were performed with cells in log phase growth at 2×10^5 cells/ml. Cells were allowed to equilibrate in growth medium for 24 h before any treatment. Cellular differentiation was assessed by nitroblue tetrazolium (NBT) dye reduction and non-specific esterase (NSE) activity as previously described (Martell *et al.* 1987, 1988, Pan *et al.* 1997).

Western blot analysis of HOXB4 and *c-myc* protein concentrations

After treatments, cells were washed twice with PBS then resuspended in lysis buffer (0.2 M Tris, 0.5 mM EGTA, 0.5 mM EDTA, 0.5% Triton-X100, 100 mM leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, pH 7.5) and homogenized using a dounce homogenizer. Protein content of total cell homogenates was determined by the Bradford protein assay (Bradford 1976). Equal amounts of protein from each condition were run on a 10% polyacrylamide gel and proteins were subsequently transferred to Immobilon paper (Millipore). The blot was blocked with buffer containing 1% bovine serum albumin (10 mM Tris, 0.1% Tween-20 and 1% bovine serum albumin, pH 7.4). It was then probed for 2 h with primary antibodies (HOXB4, Berkeley Antibodies, Richmond, CA, USA; *c-myc*, Oncogene Science, Cambridge, MA, USA), then washed three times with blocking buffer and incubated for 1.5 h with a secondary antibody conjugated with horseradish peroxidase (Sigma Chemicals, St. Louis, MO, USA). The blot was then washed five times with 10 mM Tris and 0.2% Tween-20, pH 7.4. Finally, it was developed using enhanced chemiluminescence (Amersham, Boston, MA, USA) and exposed to X-ray film.

Statistical analysis of data

Differences between 1,25(OH)₂D₃-treated cells and untreated cells for all assays were evaluated by unpaired Student's *t*-test.

Results

Effect of HOXB4 antisense on 1,25-(OH)₂D₃ induction of HOXB4

HL-60 cells were treated with vehicle (0.1% ethanol), 1,25-(OH)₂D₃ (20 nM), HOXB4 sense (30 μM), HOXB4 antisense (30 μM), 1,25-(OH)₂D₃ and HOXB4 sense, or 1,25-(OH)₂D₃ and HOXB4 antisense for 48 h. HOXB4 protein concentrations were determined by western blot analysis (Fig. 1). Cells treated with 1,25-(OH)₂D₃ in the presence of HOXB4 sense oligonucleotide showed a similar increase in HOXB4 concentrations as did cells exposed to 1,25-(OH)₂D₃ alone ($92 \pm 5\%$ compared with $103 \pm 7\%$, mean $\pm$ S.E.M., $n=3$). In contrast, cells treated with HOXB4 antisense exhibited a marked inhibition in 1,25-(OH)₂D₃ induction of HOXB4. HOXB4 concentrations were decreased by $97 \pm 4\%$ (mean $\pm$ S.E.M., $n=3$) relative to sense- or oligonucleotide-free cultures. These results demonstrate that the HOXB4 antisense oligonucleotide is able to block 1,25-(OH)₂D₃-mediated increases in HOXB4 concentrations.

Effect of HOXB4 antisense on 1,25-(OH)₂D₃ inhibition of *c-myc*

Recently, investigators in our laboratory identified HOXB4 as the predominant MIE1 binding protein in HL-60 cells (Pan & Simpson 1999). Cell transfection experiments with *c-myc* promoter deletion constructs revealed that 1,25-(OH)₂D₃ inhibition of *c-myc* expression during HL-60 cell differentiation required *c-myc* intron element (MIE) binding proteins (Pan & Simpson 1999). Here, we examined the capacity of a HOXB4 antisense construct to block 1,25-(OH)₂D₃-enhanced binding of nuclear proteins to MIE1. HL-60 cells were treated with vehicle (0.1% ethanol), 1,25-(OH)₂D₃ (20 nM), HOXB4 sense (30 μM), HOXB4 antisense (30 μM), 1,25-(OH)₂D₃ and HOXB4 sense, or 20 nM 1,25-(OH)₂D₃ and HOXB4 antisense for 48 h. As shown in Fig. 2, 1,25-(OH)₂D₃ significantly enhanced the binding of nuclear proteins to MIE1. A HOXB4 antisense was able to reduce MIE1 binding by $56 \pm 4\%$ (mean $\pm$ S.E.M., $n=3$). Next, we determined whether this antisense construct blocks 1,25-(OH)₂D₃ inhibition of *c-myc*. Amounts of *c-myc* were determined by western blot analysis (Fig. 3). 1,25-(OH)₂D₃ decreased *c-myc* levels by $89 \pm 5\%$ (mean $\pm$ S.E.M., $n=3$). HOXB4 antisense blocked 1,25-(OH)₂D₃ reduction of *c-myc* levels by $46 \pm 6\%$, but the sense construct had no effect. Taken together, these results further support our contention that HOXB4 has a role in the regulation of *c-myc* expression.

Effect of HOXB4 antisense on HL-60 cell differentiation

Cell differentiation promoted by 1,25-(OH)₂D₃ was determined by NBT dye reduction and NSE activity

Figure 1 Effect of HOXB4 antisense on 1,25-(OH)₂D₃ induction of HOXB4 protein. HL-60 cells were treated with vehicle (0.1% ethanol), HOXB4 sense (s) (30 μ M), HOXB4 antisense (as), 20 nM 1,25-(OH)₂D₃ and HOXB4 sense, or 20 nM 1,25-(OH)₂D₃ and HOXB4 antisense for 48 h. HOXB4 proteins were visualized by western blot analysis and quantified by densitometry. (A) This HOXB4 western blot is representative of three independent experiments. (B) Blots from three independent experiments were densitometrically scanned and the relative intensity of the HOXB4 band was calculated and averaged (mean and s.e.).

Figure 2 Effect of HOXB4 antisense on the MIE1 binding proteins. HL-60 cells were treated with vehicle (0.1% ethanol), HOXB4 sense (s) (30 μ M), HOXB4 antisense (as), 20 nM 1,25-(OH)₂D₃ and HOXB4 sense, or 20 nM 1,25-(OH)₂D₃ and HOXB4 antisense for 48 h. MIE1 binding was assessed by electrophoretic mobility shift assay. This figure is representative of three independent experiments.

assays (Fig. 4). A submaximal concentration of 1,25-(OH)₂D₃ (20 nM for 48 h) promoted $36 \pm 2\%$ (mean $\pm$ s.e.m., $n=5$) of cells to differentiate as determined by NBT assay and $33 \pm 1\%$ (mean $\pm$ s.e.m., $n=5$)

of cells to differentiate as determined by NSE assay. HOXB4 sense did not affect 1,25-(OH)₂D₃ promotion of cell differentiation. HOXB4 antisense (30 μ M) significantly blocked the ability of 1,25-(OH)₂D₃ to promote

Figure 3 Effect of HOXB4 antisense on 1,25-(OH)₂D₃ inhibition of c-myc protein concentrations. HL-60 cells were treated with vehicle (0.1% ethanol), HOXB4 sense (s) (30 μM), HOXB4 antisense (as), 20 nM 1,25-(OH)₂D₃ and HOXB4 sense, or 20 nM 1,25-(OH)₂D₃ and HOXB4 antisense for 48 h. c-Myc proteins were visualized by western blot analysis and quantified by densitometry. (A) This c-myc western blot is representative of three independent experiments. (B) Blots from three experiments were scanned and the relative intensity of the c-myc band calculated and averaged (mean and S.E.).

cell differentiation (NBT: 20 ± 2% inhibition, *n* = 5, *P* < 0.02; NSE: 25 ± 3% inhibition, *n* = 5, *P* < 0.002). Greater concentrations of HOXB4 antisense (60 and

Figure 4 Effect of HOXB4 sense (s) and antisense (as) (30 μM) on 1,25-(OH)₂D₃ (20 μM) promotion of HL-60 cell differentiation. Cell differentiation was determined by NBT dye reduction and NSE activity. Data are presented as percent of differentiated cells with the vehicle value subtracted and represent the mean ± S.E.M. of five independent experiments. **P* < 0.02; ***P* < 0.002.

100 μM) did not increase the effectiveness of the antisense to block cell differentiation.

Effect of HOXB4 antisense and a protein kinase C activity inhibitor on MIE1 binding and HL-60 cell differentiation

Previously, we showed that HOXB4 binding to MIE-1, 2 and 3 was dependent on protein kinase Cβ (PKC) activity (Pan & Simpson 1999). Therefore we determined whether HOXB4 antisense and a PKC activity inhibitor would have an additive effect on blocking MIE1 binding and HL-60 cell differentiation. Consistent with the data of Fig. 2, HOXB4 antisense significantly blocked MIE1 binding (Fig. 5). Chelerythrine chloride, a PKC inhibitor, decreased MIE1 binding in a dose-dependent manner, ranging from 22 ± 5% (mean ± S.E.M., *n* = 3) inhibition with 0.3 μM to complete inhibition with 3 μM. Treatment with HOXB4 antisense and chelerythrine chloride together did not have an additive effect on blocking MIE1 binding. Similar results were obtained when cell differentiation was assessed: chelerythrine chloride inhibited 1,25-(OH)₂D₃ promotion of cell differentiation in a

Figure 5 Effect of HOXB4 sense (s) and antisense (as) and chelerythrine chloride (CR), a PKC activity inhibitor, on the MIE1 binding proteins. HL-60 cells were treated with: 20 nM 1,25-(OH)₂D₃ alone; 20 nM 1,25-(OH)₂D₃ and HOXB4 sense; 20 nM 1,25-(OH)₂D₃ and HOXB4 antisense; 20 nM 1,25-(OH)₂D₃ and 0.3 μM chelerythrine chloride; 20 nM 1,25-(OH)₂D₃ and 1.0 μM chelerythrine chloride; 20 nM 1,25-(OH)₂D₃ and 3.0 μM chelerythrine chloride; 20 nM 1,25-(OH)₂D₃, HOXB4 antisense and 0.3 μM chelerythrine chloride; 20 nM 1,25-(OH)₂D₃, HOXB4 antisense and 1.0 μM chelerythrine chloride; or 20 nM 1,25-(OH)₂D₃, HOXB4 antisense and 3.0 μM chelerythrine chloride for 48 h. MIE1 binding was assessed by electrophoretic mobility shift assay. This figure is representative of three independent experiments.

Figure 6 Effect of HOXB4 antisense (as) and a PKC activity inhibitor, chelerythrine chloride (CR), on HL-60 cell differentiation. Cell differentiation was determined by NBT dye reduction and NSE activity. Data are presented as percent of differentiated cells with the vehicle value subtracted, and represent the mean $\pm$ S.E.M. of three independent experiments.

dose-dependent manner (Fig. 6). Chelerythrine chloride and HOXB4 antisense did not have an additive effect on blocking MIE1 binding or cell differentiation promoted by 1,25-(OH)₂D₃. These data suggest that blocking of HOXB4 activity is one of several processes inhibited by chelerythrine chloride.

Discussion

HOXB4 expression is increased by 1,25(OH)₂D₃ and is involved in the regulation of *c-myc* oncogene expression during 1,25-(OH)₂D₃ promotion of HL-60 cell differentiation (Pan & Simpson 1999). In addition, HOXB4 is the predominant protein that binds to MIE1, a DNA binding site located at intron 1 of the *c-myc* gene (Pan & Simpson 1999). This action was correlated to 1,25-(OH)₂D₃-mediated blockage of *c-myc* transcription elongation (Pan *et al.* 1996). In this study, a HOXB4 antisense construct inhibited 1,25-(OH)₂D₃-mediated increases in HOXB4 concentrations and blocked, by 46%, hormone-induced decreases in *c-myc* protein concentrations. This result is consistent with our *c-myc* promoter deletion studies that showed the MIE1 binding site to be partially responsible and cooperates with the MIE2 and MIE3 binding sites in down-regulating *c-myc* during cell differentiation (Pan & Simpson 1999). A reduction in basal concentrations of HOXB4 – that is, cell HOXB4 concentrations in untreated cultures – was not required for the HOXB4 antisense block of the actions of 1,25-(OH)₂D₃ to reduce *c-myc* concentrations and MIE1 binding (Figs 2 and 3). This observation suggests that it is 1,25-(OH)₂D₃ enhancement of *de novo* synthesis of HOXB4 that increases MIE1 binding and down-regulates *c-myc* during HL-60 cell differentiation. The data suggest that two processes, increased *de novo* synthesis and PKC phosphorylation, are required for HOXB4 block of *c-myc* transcription elongation. HOXB4 antisense inhibited 1,25-(OH)₂D₃ promotion of HL-60 cell differentiation by 20–25%. The action of the antisense to inhibit 1,25-(OH)₂D₃ promotion of cell differentiation was not as great as its action to block 1,25-(OH)₂D₃ down-regulation of *c-myc*. Thus promotion of cell differentiation and regulation of *c-myc* are seemingly two distinct processes regulated by 1,25-(OH)₂D₃. This suggestion is of interest in light of our studies showing that PKC inhibitors and a PKCβ antisense construct blocked 1,25-(OH)₂D₃ promotion of cell differentiation but had no effect on 1,25-(OH)₂D₃ inhibition of cell proliferation (Simpson *et al.* 1998). The data suggest that HOXB4 is directly involved in 1,25-(OH)₂D₃ down-regulation of *c-myc* and the antisense effect observed on cell differentiation may be a result of reduced *c-myc* concentrations. This is consistent with studies demonstrating that a *c-myc* antisense construct partially promoted HL-60 cell differentiation (Yokoyama & Imamoto 1987, Holt *et al.* 1988).

The HOX gene family encodes transcription factors that contain a common homeodomain DNA-binding motif (Scott *et al.* 1989). HOX genes are involved in the regulation of cellular development in all organs of every species. A knockout experimental approach has provided important data on the functions of HOXB4 (Ramirez-Solls *et al.* 1993). Interestingly, a major defect

noted by the investigators was to bone, a primary target organ for 1,25-(OH)₂D₃. HOXB4 knockout newborn homozygous mice develop with a profoundly mutated sternum. These authors also reported defective sternal fusion in heterozygous knockout mice (Ramirez-Solls *et al.* 1993). In addition and relevant to our study, several reports have shown that HOX genes play a part in hematopoiesis (Kongsuwan *et al.* 1988, Levine & Hoey 1988, Shen *et al.* 1989). Numerous investigators have reported that HOX genes are differentially expressed in blood cells (Shen *et al.* 1989, Magli *et al.* 1991, Celetti *et al.* 1993). Modulation of HOX gene expression induces alterations in the proliferation, differentiation and phenotypic characteristics of hematopoietic cells (Kongsuwan *et al.* 1988, Shen *et al.* 1989, Magli *et al.* 1991, Celetti *et al.* 1993). In addition, studies have suggested that mutations of HOX genes may be leukemogenic (Shen *et al.* 1992). HOX genes have been shown to be involved in translocation events that occur in certain leukemias and lymphomas (Blatt *et al.* 1988, Ben-David *et al.* 1991, Sato *et al.* 1987). For example, a fused gene composed of HOXA9 and a nucleoporin gene NUP89 was identified in cases of acute myeloid leukemia with an unusual t(7;11)(9p15;p15) translocation (Sato *et al.* 1987, Borrow *et al.* 1996). This chimeric protein may interfere with the normal functions of HOXA9. Other HOX genes are altered during cell differentiation. 1,25-(OH)₂D₃ has been shown to increase HOXB7 expression in HL-60 cells during terminal differentiation (Lill *et al.* 1995). In U937 human myelomonocytic leukemia cells, 1,25-(OH)₂D₃ increases the expression of HOXA10 during monocytic differentiation (Rots *et al.* 1998). HOXA10 protein concentrations induced by 1,25-(OH)₂D₃ were maximal at 36 h and gradually returned to basal values after 96–120 h of hormone exposure – a time of phenotype conversion. Moreover, overexpression of HOXA10 in U937 cells promoted monocytic differentiation (Rots *et al.* 1998). Interestingly, HOXA10 expression is also increased by 1,25-(OH)₂D₃ in MCF-7 human breast cancer cells (Rots *et al.* 1998). The overexpression of HOXA10 in MCF-7 cells results in G₁ arrest and growth inhibition (Rots *et al.* 1998). These studies suggest that HOX genes have a role in the growth and differentiation of various cell types.

In summary, this study supports a regulatory role of the HOXB4 gene product in the control of *c-myc* expression during 1,25-(OH)₂D₃ promotion of HL-60 cell terminal differentiation.

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