Expression of HOXC4, HOXC5, and HOXC6 in Human Lymphoid Cell Lines, Leukemias, and Benign and Malignant Lymphoid Tissue


Besides their regulatory role in embryogenesis, homeobox (HOX) genes are expressed in a specific manner in hematopoietic cell lineages, implying a role in the molecular regulation of hematopoiesis. Some HOX C cluster genes are found to be expressed in lymphoid cells of mice and humans. Their function and expression in normal hematopoiesis are still largely unknown. We have studied the mRNA expression of HOXC4, HOXC5, and HOXC6 in several stages of lymphocyte maturation by reverse transcriptase-polymerase chain reaction (RT-PCR) and RNA in situ hybridization (RISH). We examined CD34+/CD38low and CD34+/CD38high cells obtained from normal donor bone marrow (BM), a panel of 19 lymphoid cell lines, several types of leukemias and non-Hodgkin’s lymphomas (NHL), and lymphocytes isolated from tonsillar tissue and peripheral blood (PB). HOXC4 and HOXC6 were found to be expressed during maturation in B- and T-lymphoid cells. The expression of each gene was found to be initiated at different cell maturation stages. HOXC4 transcripts were present in CD34+/CD38low cells, which are thought to comprise stem cells and noncommitted progenitor cells, and in subsequent stages to terminally matured lymphoid cells. HOXC6 expression is initiated in equivalents of prothymocyte and pre-pre-B cell stage and remains present in mature cells. However, HOXC5 is only expressed in neoplastic cell lines and in neoplastic cells of NHL, but not in CD34+ BM cells, nor in resting or activated lymphoid cells isolated from tonsil, PB, or in leukemia cells. In cell lines, weak expression of HOXC5 is initiated in equivalents of pre-B cell and common thymocyte stage and is continuously expressed in mature cell lines. Semi-quantitative RT-PCR showed that expression levels of HOXC5 were much lower than those of HOXC4 and HOXC6; furthermore an increase of expression of HOXC4, HOXC5, and HOXC6 during lymphoid cell differentiation was demonstrated. Thus, mainly mature lymphoid cell lines and neoplastic cells of NHL do express HOXC5, in contrast to the lack of expression in normal lymphoid cells and leukemias. These findings suggest involvement of HOXC5 in lymphomagenesis.

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normal in vivo situation, were examined on the expression of HOXC4, HOXC5, and HOXC6.

MATERIALS AND METHODS

Cell lines and cell culture. Lymphocytic cell lines studied are 697, Nalm-6, SMS-SB (pre-B cell lines); JJB, Ramos, Jijoye, Raji, JVM-3, JY, (mature B-cell lines), RPMI 8226 (plasma cell line); Cem, Molt 4, HPB-ALL, HS6, Peer (early T-cell lines); Jurkat, H9, Hut 78, and Karpas 299 (mature T-cell lines). Cells were classified into stages of maturation according to their marker profile. Cells were cultured at 37°C in RPMI-1640 medium with 25 mmol/L HEPES buffer (GIBCO BRL, Life Technologies, Gaithersburg, MD) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Hyclone Laboratories Inc, Logan, UT), 1 mmol/L glutamine, 100 U/mL natrium Penicillin G (Gist Brocades NV, Delft, The Netherlands) and 100 U/mL Streptomycin Sulphate (Pharmacie BV, Haarlem, The Netherlands). Cultured cell lines were frequently tested for the presence of mycoplasma infection by PCR and were found to be clean continuously. Adherent cells (PBMC) were isolated from a buffy coat by centrifugation over a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient, according to a standard procedure. T cells were isolated by adhering monocytes and B lymphocytes to a type 200L Nylon Wool column (Robbins Scientific Corp, Sunnyvale, CA). Adherent cells were then washed from the column with RPMI-1640 medium supplemented with 10% FCS. From this cell fraction monocytes were depleted by adherence to the bottom of a plastic cell culture bottle (Greiner GmbH, Frickenhausen, Germany) for 2 hours at 37°C. To induce or enhance HOX gene expression, T- and B-cell fractions were cultured in the presence of 20 μg/mL phytohemagglutinin (PHA; GIBCO BRL) or 300 U/mL interferon-γ (IFN-γ; Boehringer Ingelheim, Ingelheim am Rhein, Germany) and 5% human ABnormal (Calbiochem-Novabiochem Corp, La Jolla, CA) in combination with 50 U/mL interleukin-2 (rIL-2; Cetus, Amsterdam, The Netherlands), respectively.

Three fresh tonsils were obtained immediately after surgery and kept on ice. Lymphocytes were extracted from the tissue matrix by eluting them with cold phosphate-buffered saline (PBS) over a small wire netting. B cells were negatively selected by a twice repeated column (Robbins Scientific Corp, Sunnyvale, CA). Adherent cells were then washed from the column with RPMI-1640 medium supplemented with 10% FCS. From this cell fraction monocytes were depleted by adherence to the bottom of a plastic cell culture bottle (Greiner GmbH, Frickenhausen, Germany) for 2 hours at 37°C. To induce or enhance HOX gene expression, T- and B-cell fractions were cultured in the presence of 20 μg/mL phytohemagglutinin (PHA; GIBCO BRL) or 300 U/mL interferon-γ (IFN-γ; Boehringer Ingelheim, Ingelheim am Rhein, Germany) and 5% human ABnormal (Calbiochem-Novabiochem Corp, La Jolla, CA) in combination with 50 U/mL interleukin-2 (rIL-2; Cetus, Amsterdam, The Netherlands), respectively.

Leukemias and lymphomas. PBMCs from 13 children with B-acute lymphoblastic leukemia and 4 children with T-ALLs were provided by the Department of Pediatrics. Fresh samples, containing 3 to 5 × 10⁶ cells, were immediately frozen and stored until RNA was isolated. In general, ALL samples contained 95% or more blast cells, blast cell count was never lower than 90%. Frozen material of three B-cell lymphomas (immunoblastic lymphoma, immunocyto- toma, and a maltona) were collected from the tissue bank. RNA was isolated from 10 tissue sections of 5 μm.

RNA isolation. Total RNA was isolated in a single step by liquid-phase separation, using RNAzol (Campro Scientific, Elst, The Netherlands), based on a protocol of Chomczynski and Sacchi. After lysis with RNAzol, containing guanidine thiocyanate and phenol, RNA was directly extracted after adding 1/10 (vol/vol) chloroform.

RNA was precipitated with isopropanol, centrifuged, and washed with 70% ethanol. RNA pellets were vacuum dried and dissolved in sterile double-distilled water. RNA concentration was measured spectrophotometrically at 260/280 nm.

Primer selection. Primer pairs for HOXC4, P1BHOXC4 (forward) 5′-COCGTCATTATACTCTGACTTCTC-3′, P2BHOXC4 (reverse) 5′-CGATCCTTAGATCTTCTCCTC-3′, HOXC5 PSHOX5 (forward) 5′-CAACTGTGGGAACCATTGATCGG-3′, PHOX5 (reverse) 5′-GAGGCTTCTGAGCCGCTGAGTGC-3′ and HOXC6 PSHOX6 (forward) 5′-TAGTACCTGAGCAGGGCCAGGACTGG-3′, P6HOXC6 (reverse) 5′-CCGCTCTCGTATCCGCCCCACTGGT-3′, and their internal oligomer probes O1HOXC4 (internal oligomer) for HOXC4 5′-CTCCCTCAACCCGGCAAGCA-3′, O2HOXC5 (internal oligomer) for HOXC5 5′-CATGCTATGTCTACCCGGTAGGAG-3′ and O3HOXC6 (internal oligomer) for HOXC6 5′-CCGCCTCACCTCCAGGCCACTGTG-3′ were designed with the PCRPLAN program of PCGene software (IntelliGenetics, Mountain View, CA) and give products of 375 bp, 438 bp, and 223 bp, respectively. Each primer pair was selected to span an intron to distinguish amplified cDNA products from amplified genomic DNA. RNA quality control was performed using primers for the mRNA encoding the U1 small nuclear ribonucleoprotein specific A protein (snRNP U1A mRNA). U1A1 (forward) 5′-CAGTATGCAACAGGCGACC-3′, U1A2 (reverse) 5′-GGCCCGCGATGGTGCGATAA-3′, and internal oligomer probe 5′-GAAGAGGAAAGCCCAAAGGCCCA-3′ (primer and probe sequences are kindly provided by Dr P. Sillekens, Organon Technika, Boxtel, The Netherlands). The reproducible expression of this gene in all types of cells and cell lines tested was also used for an internal standard.

RT-PCR. One to five micrograms of total RNA was reverse transcribed according to an adapted protocol of Van den Brule et al. as described by Rieger et al. Briefly, 40 pmol of each of three antisense primers was annealed separately to RNA in 5 μL of water for 5 minutes at 65°C and cooled at ice. Subsequently, 15 μL of a mixture of 10 mmol/L dithiothreitol (DTT), 1 mmol/L dNTPs, RNAsin 8 U and avian myeloblastosis virus (AMV) reverse transcriptase 1 U (Pharmacia), 50 mmol/L Tris pH 8.3, 60 mmol/L KCl, and 3 mmol/L MgCl₂ was added and incubated for 60 minutes at 42°C. For PCR, 5 μL of synthesized cDNA was used in a PCR mix containing 25 pmol of each primer, 0.1 mmol/L dNTPs, 1 U Taq DNA polymerase, 5 mmol/L Tris pH 8.3, 44 mmol/L KCl, and 1.2 mmol/L MgCl₂ in a total volume of 50 μL. After 5 minutes denaturation at 94°C, amplification was done for 40 cycles (GeneAmp PCR system 9600; Perkin Elmer, Branchburg, NJ). Each cycle included 45 seconds of denaturation at 94°C, 45 seconds of annealing at 60°C, and 45 seconds of extension at 72°C. Samples containing distilled water and samples without RT enzyme were included as negative controls. As a control for the quality of isolated RNA, amplification of U1A mRNA was performed on specific synthesized cDNA. Samples with very low or undetectable levels of U1A mRNA were excluded from the study.

For semi-quantitative RT-PCR, 1 μg of total RNA was transcribed to cDNA using an antisense primer for one particular HOX gene together with the antisense primer for the control gene encoding U1A. Each HOX gene was then amplified together with the internal standard in one tube for 25 cycles, which showed linearity at this number of PCR cycles in a PCR assay performed for 17, 25, 33, and 40 cycles in the tested cell lines. After exposure of the hybridized products to a phosphor imager (Molecular Dynamics, Sunnyvale, CA) signal intensities of the HOX genes were compared with those of the U1A gene.

Gel electrophoresis and blotting. Ten microliters of each PCR product and a PBR 322 Hind I marker was loaded on a 1.5% agarose gel (Ultra Pure; BRL Life Technologies, Gaithersburg, MD) and stained with ethidium bromide. Electrophoresis was performed in a
Fig 1. Expression of 3' end genes of the HOX C cluster, HOXC4, HOXC5, and HOXC6 in B- (A) and T-cell (B) lines and B- and T-cell fractions isolated of the HOX C cluster, HOXC4, HOXC5, and HOXC6. Exposure time for HOXC4 is 2 days for B-cell lines 697 and Nalm 6, and 1 day for other cell lines. For HOXC5 hybridization signals are obtained after 2 days of exposure. Cell lines 697, CEM, and Molt 4 showed hybridization signals for HOXC5 only after 3 days or longer. RNA quality is controlled using primers for the snRNP U1A gene, indicated by snRNP. Results are based on at least three independent experiments.

RESULTS

HOXC4, HOXC5, and HOXC6 expression in cell lines. To establish the stages of maturation, CD marker profiles of the cell lines were analyzed by FACS. RT-PCR analysis shows expression of HOXC4, HOXC5, and HOXC6 RNA during the maturation of both B- and T-cell lines (Fig 1). All B-cell lines reflecting maturation stages ranging from pre-B cell to plasma cell, express HOXC4, HOXC5, and HOXC6 RNA (Fig 1A). Also, all T-cell lines, representing maturation stages from the common T-lymphocyte stage to mature T cells, express HOXC4, HOXC5, and HOXC6 RNA (Fig 1B). Although these B- and T-cell lines express HOXC4, HOXC5, and HOXC6 using the same amount of RNA, a large quantitative difference in expression was observed. In particular, RT-PCR analysis shows that products of HOXC5 could only be detected in some cell lines by using higher amounts of RNA (2.5 and 5 μg) and a longer exposure time (2 days), whereas products of HOXC4 and HOXC6 usually could be detected using 1 μg RNA and 1 night exposure. Repeated RT-PCR experiments (n = 3 to 10) for HOXC4 on mRNA of pre-B cell lines 697 and Nalm 6 and immature T-cell line CEM showed very faint signals or no signal at all after 1 night of exposure, indicating a low expression level for HOXC4. Using a semi-quantitative RT-PCR assay pre-B cell line 697 and Nalm 6 indeed showed no signals of HOXC4 and HOXC6 amplimer products after 25 cycles. In contrast, the more mature B-cell lines showed clear HOXC4 and HOXC6 expression, whereas signals of amplimer products from control gene U1A were identical for all used cell lines (Fig 2). Likewise, HOXC5 expression levels were shown to be lower than HOXC4 and HOXC6. Signals for HOXC4, HOXC5, and HOXC6 were always stronger in mature cell lines than in immature cell lines (Fig 2).

HOXC4, HOXC5, and HOXC6 expression in leukemias and lymphomas. RT-PCR analysis, performed on B- and T-ALLs, reflecting more immature maturation stages than our lymphoid cell line panel showed expression of HOXC4 and HOXC6, whereas HOXC5 was not expressed under optimal conditions (Table 1). Twelve and 11 of 13 B-ALLs and T-ALLs (n = 4) investigated were positive for both HOXC4 and HOXC6. Signal intensities of the samples varied from weak to strong for HOXC4 as well as for HOXC6, whereas identical signal intensities using control gene U1A were obtained in all samples. These heterogeneous expression levels of both HOXC4 and HOXC6 suggest low expression levels in the early stages of lymphoid cell differentiation. Remarkably, three B-NHL, reflecting more mature maturation stages, showed strong signals of HOXC5 amplimer products in addition to the expression of HOXC4 and HOXC6. The obtained signals were even stronger than those obtained with
our cell line panel for HOXC4 and HOXC6. Results are summarized in Table 1.

Expression of HOXC4, HOXC5, and HOXC6 in normal lymphoid cells. To test the most immature lymphoid cells, CD34+ cells were isolated from healthy donor BM using affinity columns and purified up to 100% by FACS. The total population of CD34+ cells does express HOXC4 RNA, but does not express HOXC5 and HOXC6 (Fig 1C). Further fractionation was performed by FACS using the differential expression of CD38 resulting in a CD34+/CD38low and a CD34+/CD38high subpopulation, comprising noncommitted and committed hematopoietic progenitor cells, respectively. HOXC4 transcripts were detected in both fractions (data not shown). Normal mature B cells derived from PB and tonsillar tissue, prepared to 99% purity, showed expression of HOXC4 and HOXC6 like in cultured lymphoid cell lines. But HOXCS expression could not be detected. T cells, isolated from PB with a purity of 99%, express HOXC4 and C6, and also do not express HOXC5 (Fig 1C) at a detectable level. Stimulation of isolated B-cell fractions with Sac-1 combined with IL-2 and T-cell fractions with PHA or IFN-γ did not result in detection of HOXC5 expression.

Table 1. Expression of HOXC4, HOXC5, and HOXC6 in B- and T-ALLs and NHL, Detected by RT-PCR

<table>
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<th>Patient</th>
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<th>HOXC5</th>
<th>HOXC6</th>
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Based on FACS analysis of their differentiation markers ALLs were grouped according to their maturation stage. Primers for the U1A gene are used to control RNA quality.

Abbreviations: ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin’s lymphoma; ND, not determined; negative results indicate that after 3 days exposure still no signal was detected.

DISCUSSION

Several studies indicate that HOX genes are involved in the proliferation and differentiation of stem cells.17,18,22,23,41
Fig 3. (A) RISH on Raji cells with a digoxigenin-labeled antisense probe for HOX4. Expression is demonstrated by the presence of black silver granules. (B) RISH with a sense control probe was negative. (C) RISH on lymph node tissue with a digoxigenin labeled antisense probe for HOX6. Expression can be seen as black silver granules. Cells of the germinal center (G), mantle cells (M), and T cells (T) in the paracortex show expression (original magnification $\times 520$).
Studies on the expression of the clustered HOX genes in cell lines derived from the four hematopoietic cell lineages clearly show different patterns of expression.25 Genes belonging to the C cluster are predominantly found to be expressed in lymphoid cell lines.25 HOX4 especially showed a striking lymphoid-specific expression pattern.25 How- ever, HOX C gene expression during lymphoid cell differenti-
ation has not been studied systematically thus far. This prompted us to study the order of expression of 3' end lo-
cated genes of the HOX C cluster, HOX4, HOX5, and
HOX6, in relation to the maturation stage of lymphoid cells, using cell lines and leukemias, arrested in various stages of the B- and T-cell development, and nonneoplastic lymphoid cells including early and late fractions of CD34+ progenitor cells, determined by CD38.28,11

We used RT-PCR analysis and showed a large quantita-tive difference in HOX4, HOX5, and HOX6 expression. These results were further substantiated by the observa-
tions that: (1) In reconstruction experiments using serial genomic DNA dilutions, all three primer sets showed a comparable efficiency when amplifying the three genes (data not shown), excluding the possibility that the HOX C gene-specific primer sets differed in their annealing charac-
teristics. (2) A limited number (n = 25) of amplification cycles was proven to be within a linear range of expression of HOX genes, thereby avoiding to reach the plateau level for amplification efficiency. (3) The expression levels of HOX5 genes were compared with the housekeeping gene showing a constant level of expression independent of the cell type used (see Fig 2).
data could be confirmed by morphologic technique such as RISH.

Previously, Lawrence et al.25 investigated the expression of HOX C cluster genes in a few lymphoid cell lines by Northern blotting and RNase protection assays. With these techniques no HOXCS expression could be detected in the immature cell lines Molt 4 and CEM. In this study these cell lines do express very low levels of HOXCS, as detected by the more sensitive RT-PCR technique, indicating that this discrepancy is the result of the use of less-sensitive techniques.25 For the same reason, HOXCS also has not been detected before in the T-cell lines Molt 4 and CEM.25 Our data show that these cell lines express lower levels of HOXCS than more mature T-cell lines (Fig 2).

The present study shows that the initial HOX gene expression for each gene occurs in different stages of maturation. The HOXCS gene is actively transcribed in the most earliest undifferentiated hematopoietic cells, since CD34+CD38− positive cells derived from BM are positive (Fig 4). This cell fraction includes noncommitted progenitor cells and likely also stem cells.28−31 Subsequently, HOXCS is expressed in cell lines and leukemias representing the neoplastic equivalents of prothymocytes and pre-pre-B cells. At last HOXCS expression is initiated in more mature cell lines representing the neoplastic equivalents of the common thymocyte and the pre-B cell stage (Fig 1 and summarized in Fig 4A and B). Thus, the order of expression of these genes in lymphoid differentiation is not colinear with the order on the chromosome, like in normal vertebrate embryonic development.42−44 Neither are HOX4C, HOX5C, and HOX6C transcribed as one primary transcription unit and additionally alternatively processed as found in human embryonal tissue by Simeone et al.34 Our observations indicate that another mechanism is involved in expression patterning in different stages of lymphocyte maturation.

In agreement with the cell line model, HOX4C and HOX6C were both expressed in normal B and T cells (Fig 1 and summarized in Fig 4A). Additionally, data obtained for HOX4C and HOX6C by RNA in situ hybridization on several cell lines and lymph node and tonsillar tissue confirmed RT-PCR data for both neoplastic and normal lymphoid cells. We noted that by RISH detection weaker signals were found in small T and B lymphocytes than in T or B blast cells (Fig 3C). This was especially clear in centroblast cells of the germinal center.

In contrast to HOX4C and HOX6C, HOX5C is expressed predominantly in cell lines, representing mature stages of lymphoid cell differentiation. In accordance, lymphomas corresponding with more mature lymphoid cells show a strong HOX5C signal, in contrast to leukemias, representing immature stages of lymphoid differentiation, which show a lack of HOX5C expression. Interestingly, no expression of HOX5C was found in normal lymphocytes derived from tonsillar tissue or PB. Activation of B- and T-cell fractions with SAC, interleukin-2, and PHA or IFN-γ, respectively, did not result either in an induction or enhancement of HOX5C expression (data not shown). Therefore, based on the absence of HOX5C transcripts in normal lymphoid tissue and the contrasting presence in mature lymphoid cell lines and in lymphomas, we put forward the hypothesis that HOX5C plays an important role in lymphogenesis.

Because HOX5C is expressed in other hematopoietic cell lineages,23,25 lack of HOX5C expression in normal lymphoid cell differentiation might be crucial for lymphoid cell lineage commitment. Because HOX4C and HOX6C are also expressed in myeloid and erythroid cell lineages as well these genes are not likely to play a dominant role in lineage commitment5,23 (and our own unpublished results, February 1995). Their function might be more important in the differentiation of lymphoid cells and other hematopoietic cells in general. Whether the presence of alternative transcripts or modulation of the expression levels of HOX4C and HOX6C are important in lineage commitment has to be further established.45

Finally, the question arises how this expression pattern of regulatory genes is converted into morphologic characteristics. Morphoregulatory proteins like cell adhesion and substrate adhesion molecules (CAMs and SAMS) as well as growth factors are suggested to be candidates for modulating cell surface events and cellular mechanisms through adhesion.46,47 Because N-CAM expression in mice is regulated by Xenopus-derived HOX6C,48 and L-CAM expression in chicken liver cells is controlled by human HOX9D proteins,49 adhesion molecules are a direct target for homeoproteins and especially for HOX6C.46 Furthermore, there is increasing evidence that the expression of HOX genes is involved in cell adhesion of hematopoietic cells.50,51 This is important because adhesion molecules are involved in the spreading of lymphomas and leukemias.52,53 Additionally, sequence database homology searches showed that the target motifs for HOX6C can also be found in sequences of adhesion molecules such as VLA-4 (CD49d), I-CAM-1 (CD54), L-selectin (CD62L), and CD44. The sole expression of HOX5C in neoplastic cells of NHL, in contrast to the lack of expression in lymphatic leukemias and normal lymphocytes, suggests a similar role for HOX5C. However, further studies are needed to clarify the relationship of HOX5C expression and the expression of adhesion molecules.

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