Mechanism of human Hb switching: a possible role of the kit receptor/miR 221-222 complex

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Design and Methods

Blood samples

Cord blood (CB) was obtained after informed consent from healthy mothers according to institutional guidelines. The gestational age was determined essentially according to standard gynecological criteria: specifically, pre-term CB samples 33-37 weeks, mid-full term samples 38-41 weeks of gestational age. Adult peripheral blood (APB) was obtained from healthy donors after informed consent and blood (450 ml) was collected in preservative-free citrate/phosphate/dextrose/adenine (CPDA-1) anticoagulant.

Evaluation of KL plasma levels

Plasma was obtained from anticoagulated CB and APB samples and stored at -80°C. The level of KL was evaluated by immunoenzymatic assay using a commercial kit (R&D Systems, Minneapolis, USA) whose threshold detection limit is approximately 20 pg/mL. Its specificity was confirmed against a large panel of recombinant cytokines.

Reticulocyte purification

Reticulocytes were purified from CB and APB samples by Percoll density gradient. Two ml of packed red blood cells diluted 1:2 in RPMI 1640 medium were centrifuged on a discontinuous three-step Percoll gradient (55%, 60% and 65% Percoll concentration) for 30 min at 1600 rpm at room temperature. The fractions floating on the 60% and 65% Percoll cushions were recovered, washed in PBS and analyzed for reticulocytes by supravital staining with Brillant Cresyl Blue. The purification of reticulocytes ranged from 75 to 96%.

HPC purification

Low-density (less than 1.077 g/ml) mononuclear cells (MNCs) were isolated from CB and APB by Ficoll-Hypaque density-gradient centrifugation and $CD34^+$ cells were then purified by MACS columns (Miltenyi, Bergisch, Gladbach, Germany). Purified cells were greater than 90-95% $CD34^+$, as evaluated by FACS analysis.

Flow cytometry analysis

Kit expression on the membrane of purified $CD34^+$ samples was analyzed by FACS (FACScan, Becton Dickinson, San Jose, CA) using a monoclonal anti-CD117 Ab (5 µg/mL, final concentration) conjugated with Cy-Chrome (Pharmingen, San Jose, CA, USA). Negative controls were incubated with irrelevant mouse IgGs conjugated with Cy-Chrome (5 µg/mL). The same batches of Abs (control IgG and anti-CD117) was used for all analyses performed in this study. Briefly, $5x10^4$ $CD34^+$ cells (for each labeling) were washed twice at 4°C with PBS containing 1 mg/ml BSA and then resuspended in 0.1 ml of the same solution containing either 5 µg/mL of control Cy-Chrome labeled Ig or 5 µg/ml Cy-Chrome labelled anti-human CD117 Ab. After 60 min of incubation at 4°C the cells were washed twice with PBS/BSA, resuspended in 0.2 mL of this solution and analyzed for fluorescence emission using a FACScan (Becton-Dickinson, USA). In all assays the fluorescence of the negative control was set at a similar level (in 47 assays the control stained cells showed a fluorescence level of 5.46±0.24, with a range of between 4.34 and 6.66). The intensity of fluorescence was evaluated on the basis of arbitrary units, i.e. the ratio between mean fluorescence intensity level of cells incubated with anti-kit and that of cells treated with control IgGs.

HGFs and chemical inducers

Recombinant human interleukin-3 (IL-3), granulocyte macrophage-colony stimulating factor (GM-CSF), kit ligand (KL) and erythropoietin (Epo) were purchased from Behringwerke AG (Marburg, Germany), Sandoz (Basel, Switzerland), Peprotech (London, UK) and Amgen (Thousand, Oaks, PA), respectively. STI571 (Imatinib or Gleevec), kindly provided by Novartis Pharma, Basel, Switzerland) was dissolved in DMSO and stored frozen in aliquots. Each aliquot was diluted in culture medium and then added into the culture.

HPC unilineage erythropoietic culture

(a) *Mini-bulk culture*. Purified HPCs were grown in fetal calf serum (FCS)-free liquid suspension culture ($5x10^4$ cells/mL) in a fully humidified 5% CO_2/5% O_2/90% N_2 atmosphere and were induced to unilineage erythropoietic differentiation by an erythroid-specific HGF cocktail (saturating dosage of Epo and low-dose IL-3/GM-CSF) as previously reported.[1,2] The HGF cocktail was supplemented or not with KL (1-100 ng/mL) ± Gleevec in a range of between 10^{-5} and 10^{-6}M. (b) *Unicellular sibling BFU-E culture*. Unicellular cultures (0.5 cell/well) were performed in flat-bottomed 96-microwell plates in 0.1 ml of the above

erythroid medium supplemented with 5% FCS.[3] At day 3-4, the 4 cell clones were identified. The sibling cells were picked up by a micromanipulator and seeded in 4 different wells containing 0.1 ml of the erythroid medium supplemented with graded amounts of KL (1,10 and 100 ng/mL) or KL+Gleevec (100 ng/mL and 2 μM, respectively) and incubated as indicated above. Clones were analyzed from day 14 through day 28.

Morphology analysis

Cells were harvested from day 14 to day 28-30 of culture, smeared on glass slides by cytospin centrifugation, and stained with standard May-Grunwald-Giemsa.

Assay of γ-chain content

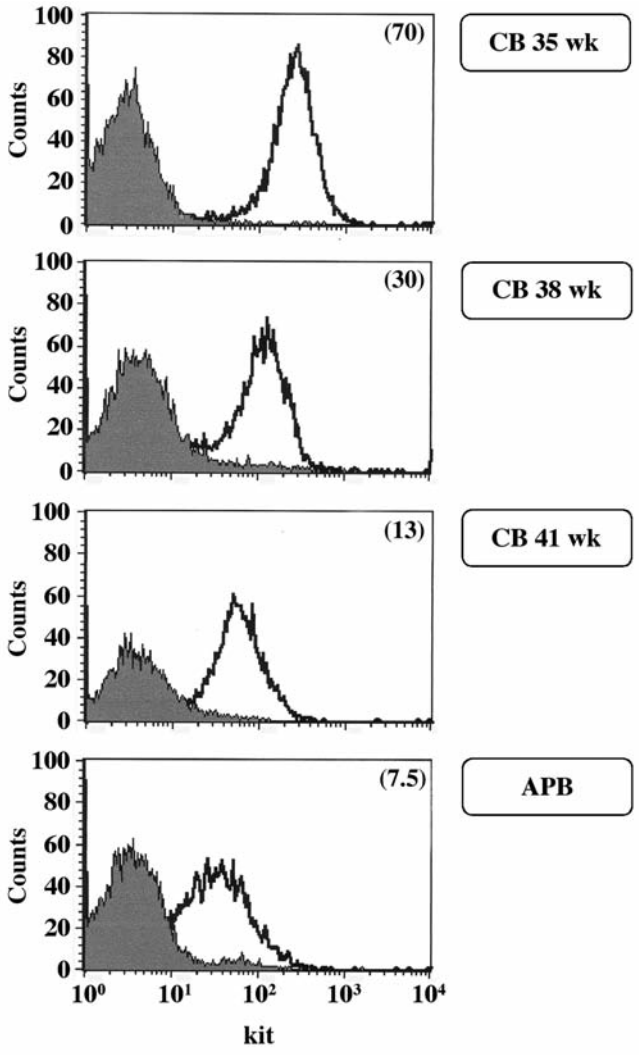
High-performance liquid chromatography (HPLC) separation of globin chains was performed according to previously published methods.[4] Briefly, cell lysates from enriched reticulocytes or bulk culture were separated on chromatographic columns (Merck LiChrospher 100 CH8/2.5 μm; E. Merck, Darmstadt, Germany) using as eluents a linear gradient of acetonitrile/methanol/0.155M sodium chloride (eluent A; pH2.7, 68:4:28 vol/vol/vol) and acetonitrile/methanol/0.155M sodium chloride (eluent B; pH2.7, 26:33:41 vol/vol/vol). Gradient was from 20% to 60% eluent A in 60 min at a flow rate of 0.8 mL/min. The absorbance of globin chains was evaluated at 214 nm.

Cell cycle analysis

Cell-cycle analysis was carried out on nuclei stained with propidium iodide (PI), as previously described,[5] using the Cycle Test Plus Kit (Becton Dickinson, USA). Cells were then analyzed by flow cytometry using a FACS equipped with software for cell-cycle analysis.

MiR-221/222 expression in CB and APB CD34+cells

(a) *Real-time RT–PCR*. Total RNA was purified with TRIZOL Reagent (Invitrogen) from pre- and full-term CB or APB CD34$^+$ HPCs. Reverse transcription and real-time PCR were performed as described.[6] Expression of mature miRNAs was determined using miRNA-specific quantitative real-time PCR (qRT–PCR; Applied Biosystems, Foster City, CA, USA). U6 snRNA was used for normalization. (b) *Transfection experiments*. In day 3 erythroid cultures, cells were seeded in antibiotic-free media and cotransfected with the stability enhanced mature miRNA 221, 222 (160 nM) or a control scrambled double-stranded RNA oligonucleotide (160 nM) plus a FITC-conjugated double-stranded RNA (16 nM; Dharmacon, Lafayette, CO, USA) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection with antagomir-221 or -222 (160 nM) was performed on day 0 CD34$^+$ HPCs. Antagomirs were synthesized as described.[7] Sequences were 5'-g$_s$a$_s$aacccagcagacaauguasg$_s$c$_s$u$_s$–Chol 3' (antagomir-221), 5'g$_s$a$_s$gacccaguagccagaugua$_s$g$_s$c$_s$u$_s$ - Chol 3' (antagomir-222), 5'- a$_s$c$_s$agcugguug aagggga$_s$c$_s$a$_s$a$_s$–Chol 3' (control antagomir-133). After transfection, FITC-positive cells were sorted (FACS Aria, Becton Dickinson, Franklin Lakes, NJ, USA) and grown in unilineage erytroid liquid suspension culture.



Online Supplementary Figure S1. Flow cytometry analysis of kit expression on CD34$^+$ cells purified from CB samples of 35, 38 and 41 gestational wks and APB. The cells were labeled with either an irrelevant mouse IgG (negative control, gray area) or anti-CD117 Ab (white area). The number in brackets indicates the MFI ratio between the mean fluorescence intensity of cells incubated with anti-CD117 Ab and that of cells treated with control IgGs. Representative results are presented.

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