Nucleolin regulating gene expression in CD34 positive hematopoietic cells

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CD34 glycoprotein in human hematopoiesis is expressed on a subset of progenitor cells capable of self-renewal, multilineage differentiation and hematopoietic reconstitution. Nucleolin is an abundant multifunctional phosphoprotein of growing eukaryotic cells, involved in regulation of gene transcription, chromatin remodeling and RNA metabolism, whose transcripts are enriched in murine hematopoietic stem cells, as opposed to differentiated tissue. Here we show that, in human CD34 positive hematopoietic cells, nucleolin activates endogenous CD34 and Bcl-2 gene expression, and cell surface CD34 protein expression is thereby enhanced by nucleolin. Nucleolin-mediated activation of CD34 gene transcription results from direct sequence-specific interactions with the CD34 promoter region. Nucleolin expression prevails in CD34 positive cells mobilized into peripheral blood (PB), as opposed to CD34 negative peripheral blood mononuclear cells (PBMCs). Therefore, in intact CD34 positive mobilized PB cells, a recruitment of nucleolin to the CD34 promoter region takes place, accompanied by nucleosomal determinants of gene activity, which are absent from the CD34 promoter region in CD34 negative PBMCs. Our data show that nucleolin acts as a component of the gene regulation program of CD34 positive hematopoietic cells and provide further insights into processes by which human CD34 positive hematopoietic stem/progenitor cells are maintained.

CD34 glycoprotein in human hematopoiesis is expressed on a subset of progenitor cells capable of self-renewal, multilineage differentiation and hematopoietic reconstitution (1, 2), reviewed in (3). The expression of cell surface CD34 glycoprotein is broadly utilized for enumeration of stem/progenitor cells for clinical bone marrow (BM) transplantation, and the number of 5-year survivors of hematopoietic stem cell transplantation is estimated to be 100000 worldwide (3, 4). Experimental evidence for cellular function of CD34 and its involvement in hematopoietic reconstitution have been presented (5). Regulation of the human CD34 gene has been investigated as a model of hematopoietic stem/progenitor cell-specific gene control (3, 6-15). The promoter region of the CD34 gene as well as the 3′ enhancer have been identified (6-8), and it was shown that multiple regulatory elements, likely acting in the context of chromatin structure, are necessary to provide proper control of expression (8-10). Furthermore, transcription regulators including c-Myb, Ets-2, MZF-1 and nuclear factor Y have been shown to bind to their cognate sites and modulate human CD34 promoter region activity (11-15). It is essential to work out mechanisms controlling expression of the CD34 gene, as well as of other genes involved in the maintenance of CD34 positive human hematopoietic cells to understand the processes underlying homeostasis and reconstitution of the hematopoietic system at a molecular level. For instance, Bcl-2 is the founder member of a family of proteins that play a central role in the regulation of apoptosis, reviewed in (16, 17), and constitutive expression of Bcl-2 increases the frequency and in vivo repopulation potential of hematopoietic stem cells (18).

Nucleolin is a multifunctional DNA and RNA
binding protein, which is abundant in growing and cancerous cells (19, 20), found in the nucleolus, in the nucleus, in the cytoplasm and at the cell surface (19, 21, 22). Interactions of nucleolin with p53 and the retinoblastoma protein have been described (23, 24). A role of nucleolin in regulation of gene transcription, chromatin remodeling and RNA metabolism has been shown (21, 25-29). For instance, nucleolin acts as a subunit of the transcription factor LR1, which activates expression of the c-myc gene in B-cell lymphomas (25, 27). Nucleolin is also directly involved in post-transcriptional inhibition of the p53 gene expression (29). Enrichment of nucleolin gene transcripts in murine hematopoietic stem cells, as opposed to differentiated tissue, has been reported (30, 31).

Here we analyzed a possible involvement of nucleolin in gene regulation in human CD34 positive hematopoietic cells. We show that nucleolin activates endogenous CD34 and Bcl-2 gene expression, and cell surface CD34 protein expression is thereby enhanced by nucleolin. Nucleolin-mediated activation of CD34 gene transcription results from direct sequence-specific interactions with the CD34 promoter region. Nucleolin expression prevails in CD34 positive cells mobilized into PB, as opposed to CD34 negative PBMCs. Therefore, in intact CD34 positive mobilized PB cells, a recruitment of nucleolin to the CD34 promoter region takes place, accompanied by nucleosomal determinants of gene activity, which are absent from the CD34 promoter region in CD34 negative PBMCs. Our data show that nucleolin acts as a component of the gene regulation program of CD34 positive hematopoietic cells and provide further insights into processes by which human CD34 positive hematopoietic stem/progenitor cells are maintained.

Experimental Procedures

Cell culture and creation of stable transfected cells - KG1 cells (32) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), KG1a cells (33) - in RPMI 1640 medium supplemented with 20% FCS; NIH-3T3 cells - in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. Stable KG1-Nuc transfectant cells were obtained using nucleolin expression vector containing the full-length human nucleolin cDNA in frame with N-terminal FLAG-tag, in the pCMV-Tag2B expression vector (Stratagene), and the selection was performed by addition of 200µg/ml G418 to the culture medium. Stable KG1-mock transfectant cells were obtained using the same vector with no insert. Exponentially growing cells were used in all experiments.

Cell separation and fluorescence-activated cell sorter (FACS) analysis - Mobilized PB CD34 positive cells were obtained from apheresis collections from normal adult volunteers and enriched using CD34 MicroBeads (Miltenyi Biotech), after ammonium chloride red cell lysis, according to the supplier’s instructions. For enrichment of natural killer (NK) cells, red cell lysis was followed by selection with the CD56 MultiSort Kit, and depletion of residual T cells using CD3 MicroBeads (Miltenyi Biotech). The purity of all preparations was monitored by FACS analysis with the following antibodies (Abs), directly coupled to phycoerythrin (PE) or fluorescein isothiocyanate: anti-CD34 (8G12), anti-CD56 and anti-CD3 (BD Pharmingen), and isotype-matched control monoclonal antibodies (mAbs). The purity of cell preparations was: CD34 positive cells 96.5 to 97%; CD34-depleted cells 99 to 100%, NK cells >97%.

Transient transfection assays - Reporter construct, containing CD34 promoter region (6, 7) nucleotides -666 to +175, cloned into the pGL3basic vector (Promega), is referred to as CD34WT-pGL3, and derivatives thereof, lacking nucleotide numbers -431 to –427, or -367 to -363 of the CD34 promoter region, are referred to as CD34mutA-pGL3 and CD34mutB-pGL3, respectively. Nucleolin expression vector contained the full-length human nucleolin cDNA downstream of the cytomegalovirus promoter. DNA was purified using a plasmid purification system (Qiagen). KG1 and KG1a cells were transiently transfected using the DMRIE-C transfection reagent (Invitrogen), and NIH-3T3 cells - using the Polyfect transfection reagent (Qiagen), according to the respective manufacturer’s protocols. Measurements of luciferase were performed according to the recommended procedures by the producer of the luciferase kit (Roche), and the results were normalized to protein concentration according to manufacturer’s specifications. Results in Figures represent at least three independent experiments.
Nucleolin downmodulation experiments – Distinct small interfering RNAs (siRNAs) targeted to different regions of the mRNA of human nucleolin were used: a pool of four siRNA molecules targeting human nucleolin (Dharmacon, Lafayette, CO, M-003854-00), referred to as nucleolin siRNA-A, or the siRNA duplex corresponding to nucleolin mRNA nucleotide numbers 1755 to 1774 (nucleolin sequence from Accession NM_005381), referred to as nucleolin siRNA-B. Validation of these siRNAs has been reported previously (34, 35). Non-targeting siRNAs (Santa Cruz, sc-37007, sc-44230) were used as a negative control. SiRNA experiments were performed generally following the procedures recommended by Dharmacon. In brief, siRNA (100 nM) was applied to growing KG1 cells as described above, however, twice at 24h intervals. Cells were harvested 2 days after transfection for analysis of RNA, and 2 to 4 days after transfection for immunoblotting.

For antisense-mediated inhibition of nucleolin expression, phosphorothioate-modified nucleolin antisense oligonucleotide 5’-TCACCATGATGGCGGCGG-3’ was used, that is complementary to the 5’ end of the human nucleolin mRNA encompassing the translation initiation region. The antisense oligonucleotide has been described in detail and validated in our previous study (26). In brief, antisense or sense oligonucleotides (1 µM) were applied to exponentially growing cells as described above, and the selectivity of antisense oligonucleotide was controlled in all experiments by Western blotting.

**Western blotting and electrophoretic mobility shift assay (EMSA)** - Immunoblot analysis followed standard procedures. The following are Abs and their dilutions: Ab specific for a N-terminal peptide of nucleolin, purified on the peptide column (26), Ab specific for CD34 (Santa Cruz, H-140), 1:1500; mAb specific for Bcl-2 (Santa Cruz, C-2), 1:1500; mAb specific for beta-actin (Sigma), 1:2000; mAb specific for proliferating cell nuclear antigen (PCNA) (Santa Cruz, PC10), 1:2000; mAb specific for FLAG-tag (Sigma) 1:1500. Prestained molecular weight marker proteins (BioRad) were used.

The conditions for DNA-binding and EMSA were described (26). In brief, end-labeled double-stranded oligonucleotides from the CD34 gene promoter region (6, 7), corresponding to nucleotide numbers -446 to -412 (CD34A) or -379 to -347 (CD34B) were used, together with 5µg poly(dIdC)(dIdC) and 5µg nuclear extract, in the DNA-binding buffer containing 10 mM tris(hydroxymethyl)aminomethane, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM 1,4-Dithio-DL-threitol and protease inhibitors.

Nucleolin-glutathione S-transferase (GST) fusion protein, comprising amino acid residues 289 to 709 of the human nucleolin cDNA, was generated and employed in EMSA as described (26). In brief, the nucleolin-GST fusion protein was generated by cloning the cDNA fragment corresponding to amino acid residues 289 to 709 of the nucleolin cDNA in frame with GST cDNA into vector pGEX2T. The fusion protein was produced in E.coli and purified by affinity chromatography using glutathione-Sepharose (Pharmacia) according to the manufacturer’s protocol.

**Chromatin immunoprecipitation (ChIP) assay** – Formaldehyde crosslinking procedure (36) was employed, and ChIP kit (Upstate Biotechnology) was used, following the manufacturer's protocol. Average length of sonicated DNA was 400-1000 bp. Abs used were: Ab specific for N-terminal peptide of nucleolin, purified on the peptide column (26), Ab specific for histone H4 acetylated on residue K8 (H4-AcK8) (Upstate Biotechnology), Ab specific for histone H3 trimethylated on residue K4 (H3-tri-methyl K4) (Upstate Biotechnology), Ab specific for histone H3 dimethylated on residue K9 (H3-di-methyl K9) (a kind gift of Prof. T. Jenuwein), together with Ab specific for epidermal growth factor receptor (Santa Cruz, sc-03) or preimmune rabbit serum, as appropriate controls. Immunoprecipitated DNA was quantified by real-time PCR, using a QuantiTect SYBR Green PCR Kit (Qiagen) with an ABI 7700 sequence detector and normalized to input DNA, as described (37). Specificity of PCR products was controlled by melting curve analysis and in Fig. 2C and 4B – additionally on agarose gels. Specific primers used are: CD34 promoter, 5’-GATGGTGATGGGGAACTAAATGG-3’ and 5’-GCCAGTAACAATCTTGCAAAAGG-3’ (size: 338 bp); KIR2DL3 promoter, 5’-TGTATGAGAGGTTGGATCTGAG-3’ and 5’-GCCCTTCCAGGACTCACC-3’ (size: 321 bp).

**Real-time reverse transcription PCR (RT-PCR)** - 2µg total RNA, isolated with RNaseasy kit (Qiagen), was reverse transcribed using oligo(dT) primers, and levels of gene expression were quantified by...
real-time PCR, using a QuantiTect SYBR Green PCR Kit (Qiagen) with an ABI 7700 sequence detector. Sequences of specific primers used are: nucleolin cDNA, 5'-GATCACCCTATGCCAGAAGGACGCCATCCGAC-3' and 5'-CAAAGGCCGCTGCTCCACCAC-3' (size: 297 bp); CD34 cDNA, 5'-CATCACAGAAGCAGCTCAGACA-3' and 5'-ACTCCGCAAGCAGTGAGG-3' (size: 354 bp); Bcl-2 cDNA, 5'-GAATGTCGTCCTCCAGGCC-3' and 5'-TGACATCTCGGCAGAAGTC-3' (size: 209 bp); beta-actin cDNA 5'-GCACCTTCCAGCCTTCC-3' and 5'-CTAGAAGCATTGGCGGTG-3' (size: 351 bp). Specificity of PCR products was controlled by melting curve analysis and on agarose gels. The comparative threshold cycle (CT) method and an internal control (beta-actin) were used to normalize target gene expression.

RESULTS

Activation of endogenous CD34 and Bcl-2 expression by nucleolin in CD34 positive cells. Human BM-derived CD34 positive myeloblast cell line KG1 (32) was stably transfected with a nucleolin expression vector, containing nucleolin cDNA in frame with FLAG-tag under control of cytomegalovirus promoter, referred to as KG1-Nuc cells, or with the empty expression vector, referred to as KG1-mock cells. Nucleolin expression was 3.5-fold increased in KG1-Nuc cells, or with the empty expression vector, compared with control non-targeting siRNA, either nucleolin-targeting siRNA significantly reduced nucleolin protein levels (Fig. 1C, top, lanes 2 and 5). Beta-actin expression was not changed (Fig. 1C, bottom, lanes 1-5) and no significant effect on PCNA protein was detected (data not shown). The level of nucleolin in untransfected cells did not differ detectably from that in cells treated with the control siRNA (Fig. 1C, top, lanes 3 and 1, respectively). The knockdown of nucleolin expression with either nucleolin-targeting siRNA was accompanied by a significant reduction of CD34 and Bcl-2 protein levels, as was demonstrated by immunoblotting of cell extracts with Abs specific for CD34 and Bcl-2 (Fig. 1C, lanes 2 and 5). The levels of CD34 and Bcl-2 transcripts were investigated by quantitative real-time RT-PCR (Fig. 1C, lanes 6-8 and 9-11, respectively). As internal control beta-actin was used, that was not dependent on nucleolin expression. Fig. 1C shows that the treatment with nucleolin-targeting siRNA resulted in a strong reduction of the levels of CD34 and Bcl-2 transcripts, to approximately 20% and 40%, respectively (Fig. 1C, lanes 7 and 10). Very similar effects on the CD34 and Bcl-2 protein expression were also observed after downregulation of nucleolin with a specific antisense oligonucleotide, that has been validated in our previous study (26). After treatment of KG1 cells with nucleolin antisense oligonucleotide, a significant reduction of nucleolin levels was detected (supplemental Fig. S1, top, lane 2). In line with our previous observations (26), antisense downregulation of nucleolin did not inhibit the expression of beta-actin and PCNA (supplemental Fig. S1), and cell viability was not affected under these conditions, as assessed by trypan blue staining (data not shown), consistent with the conclusion that the downregulation of nucleolin is a specific effect. On the other hand, a significant reduction of expression of CD34 and Bcl-2 proteins was reproducibly observed after antisense inhibition of nucleolin in three independent experiments, whereas nucleolin sense
Nucleolin is a CD34 promoter region-binding factor. Two distinct domains of the CD34 promoter region (6, 7) are closely related to high affinity nucleolin binding sites from the human papillomavirus type 18 (HPV18) enhancer (26), and (data not shown). These domains are nucleotide numbers -446 to -412, termed CD34A (promoter, corresponding to the nucleotide numbers 7635 to 7650 and 7721 to 7732 of the HPV18 enhancer (38), related to nucleotide numbers 7635 to 7650 and 7721 to 7732 of the HPV18 enhancer (38), respectively; identical stretches are underlined. Fig. 2 shows EMSA experiments performed with a double stranded oligonucleotide from CD34 promoter, corresponding to the nucleotide numbers -446 to -412, termed CD34A (lanes 1-7), or with an oligonucleotide corresponding to the nucleotide numbers -379 to -347, termed CD34B (lanes 8-11). Retarded protein/DNA complex was detected with nuclear extract from KG1 cells (lane 1), and formation thereof was abolished after addition of a nucleolin peptide Ab (26), resulting in an immunoshift, at moderate levels (Fig. 2A, lane 2). Nucleolin Ab presaturated with the immunizing nucleolin peptide had no effect (lane 3). Correlating with nucleolin expression, retarded nucleolin/DNA complex was present at significantly higher levels in KG1-Nuc cells (Fig. 2A, lanes 4 and 8) as compared with KG1-mock cells (lanes 5 and 9). To demonstrate the sequence-specificity of nucleolin/CD34 promoter interactions, the binding was monitored in the presence of excess of unlabelled wild-type (WT) CD34A oligonucleotide (Fig. 2A, lane 6), mutant CD34A oligonucleotide, lacking nucleotides -431 to -427 (Fig. 2A, lane 7), WT CD34B oligonucleotide (Fig. 2A, lane 10), or mutant CD34B oligonucleotide, lacking nucleotides -367 to -363 (Fig. 2A, lane 11). In the mutant oligonucleotides, fragments of sequences corresponding to nucleolin recognition motifs from HPV18 enhancer were deleted.

To investigate whether nucleolin binds directly to the sequence elements in the CD34 promoter region, affinity-purified recombinant nucleolin-GST fusion protein (26) was employed. This nucleolin-GST fusion protein, comprising amino acid residues 289 to 709, retains the DNA binding activity (26, 39). Fig. 2B shows that the nucleolin-GST protein but not GST protein itself interacted with the CD34A and CD34B oligonucleotide (Fig. 2B, lanes 1-4). Nucleolin-GST protein did not interact with an unrelated binding site of the transcription factor AP1 (Fig. 2B, lane 5). We conclude that nucleolin binds directly to the CD34 promoter region sequence elements.

Nucleolin/CD34 promoter interactions were further analyzed by ChIP experiments, which allow detection of interactions of nuclear factors with regulatory regions of cellular genes within the context of an intact cell (36). Fig. 2C, lane 1, shows that the Ab specific for N-terminal peptide of nucleolin (24, 26) efficiently co-precipitated CD34 promoter region from intact KG1 cells. The identity of the amplified band was reconfirmed by DNA sequence analysis (not shown). CD34 promoter region was neither precipitated with the nucleolin Ab presaturated with the immunizing nucleolin peptide (Fig. 2C, lane 2), nor with a control Ab specific for epidermal growth factor receptor (lanes 3 and 7), or protein A-agarose beads (lane 4). Furthermore, unlike CD34 promoter, intron 1 of the CD34 gene was not detected in the nucleolin Ab immunoprecipitates (Fig. 2C, lane 6). The specificity of nucleolin Ab used for immunoprecipitation (IP) was confirmed by immunoblotting of immunoprecipitates derived from KG1 cell lysates (Fig. 2C, lanes 9-12). We conclude that nucleolin is a CD34 promoter-binding factor.

Nucleolin-mediated activation of CD34 promoter region. Next, co-transfection experiments with a nucleolin cDNA expression vector, and either a WT CD34 promoter-reporter construct, or two mutant derivatives thereof were performed, using KG1 cells (Fig. 3A) or KG1a cells (Fig. 3B), an undifferentiated variant of KG1 (33). The WT CD34 promoter construct comprised nucleotide numbers -666 to +175 of the CD34 gene promoter region (6, 7), referred to as CD34WT-pGL3 throughout. Mutant promoter constructs lacked sequence motifs required for nucleolin binding (Fig. 2A), but not for binding of other molecularly identified CD34 promoter factors: nucleotide numbers -431 to -427 (referred to as CD34mutA-pGL3), or nucleotide numbers -367 to -363.
(referred to as CD34mutB-pGL3). Co-transfection of nucleolin expression vector significantly activated CD34WT-pGL3 reporter construct in all experiments (Fig. 3A and 3B, lane 1). In contrast, neither CD34mutA-pGL3, nor CD34mutB-pGL3 reporter construct could be activated by nucleolin (lanes 3 and 5, respectively), and activity of these mutant reporter constructs was reduced as compared with that of CD34WT-pGL3 (lanes 4, 6 and 2, respectively). Fig. 3B, right, shows that activity of CD34WT-pGL3 reporter construct was dependent on nucleolin concentration, parallelizing the amounts of the nucleolin expression vector added to the transfection reactions (Fig. 3B, lanes 9 to 12). Activation of the CD34 promoter region by nucleolin was also observed in NIH-3T3 cells, a murine CD34 positive embryonic fibroblast cell line (40), suggesting that it is not limited to cells of hematopoietic origins (supplemental Fig. S2). Thus nucleolin-dependent activation of the human CD34 gene transcription results from sequence-specific interactions with the CD34 promoter region.

**Prevalent expression and recruitment of nucleolin to CD34 promoter in CD34 positive mobilized PB cells.** Nucleolin expression in CD34 positive cells mobilized into PB, obtained from normal adult donors, in comparison with CD34-depleted PBMCs, was then analyzed by immunoblotting of cell lysates. Fig. 4A, left, top, shows that nucleolin was significantly enriched in CD34 positive cells, versus CD34-depleted PBMCs derived from the same apheresis collection, as was observed with two independent samples. Beta-actin served as a loading control (Fig. 4A, left, bottom). Fig. 4A, right, shows that nucleolin transcripts were approximately 6-fold enriched in CD34 positive cells versus CD34-depleted PBMCs from the same apheresis collection, or versus purified NK cells, as was determined by real-time RT-PCR, using beta-actin as internal control. Representative amplification plots of real-time RT-PCR for nucleolin and beta-actin, from CD34 positive mobilized PB cells and CD34-depleted PBMCs, belonging to the same run of RNA samples, are shown in Fig. 4A, right, top.

The interaction of nucleolin with CD34 promoter region was then analyzed with CD34 positive cells mobilized into PB, obtained from normal adult donors. Fig. 4B, left, top, shows that nucleolin/DNA complex was detected with nuclear extracts from CD34 positive cells and labeled CD34B oligonucleotide (lane 1), whereas with CD34-depleted PBMCs, derived from the same apheresis collection, the retarded complex was not detected to a significant extent (Fig. 4B, lane 2). Immunoblotting of the extracts confirmed the predominance of nucleolin to CD34 positive cell nuclei, approximately 28-fold in this experiment (Fig. 4B, left, bottom). Formation of the retarded nucleolin/DNA complex was competed by addition of excess of unlabelled WT CD34B oligonucleotide, but not of mutant CD34B oligonucleotide, lacking nucleotides -367 to -363 (data not shown). Fig. 4B, middle and right, shows that the Ab specific for nucleolin efficiently co-precipitated CD34 promoter region from intact CD34 positive mobilized PB cells in ChIP experiments (Fig. 4B, lanes 4 and 16). Co-precipitation was abolished after presaturating the nucleolin Ab with the immunizing nucleolin peptide (lanes 5 and 17). CD34 promoter was neither precipitated with the control Ab (lane 6), nor with protein A-agarose beads (lane 7). Moreover, in identical ChIP experiments, however, performed with CD34-depleted PBMCs derived from the same apheresis collection (Fig. 4B, lanes 8 and 18) or with purified NK cells (lanes 12 and 20), CD34 promoter region was not co-precipitated with the nucleolin Ab to a significant extent. Each experiment shown in Fig. 4B was repeated with two independent samples; altogether four samples were examined.

In view of the ability of nucleolin to facilitate transcription through nucleosomal particles (28), activity of resident CD34 promoter region was then analyzed with intact CD34-depleted PBMCs, in comparison with CD34 positive cells mobilized into PB. Histone H3 tri-methylated on residue K4 and histone H4 acetylated on residue K8 are nucleosomal determinants of gene activity (41-43), and, after IP with Ab specific for H3-tri-methyl K4 or with Ab specific for H4-AcK8, CD34 promoter region was just detected in the immunoprecipitates from CD34-depleted PBMCs (Fig. 4C, lanes 4 and 10), whereas it was efficiently co-precipitated with these Abs from CD34 positive cells, derived from the same apheresis collections (Fig. 4C, lanes 1 and 7). The results were repeated with two independent samples. On the other hand, Ab specific for histone H3 dimethylated on residue K9, a histone modification highly indicative of transcriptionally silenced genes (44), did not co-precipitate CD34...
promoter region from intact mobilized PBMCs (supplemental Fig. S3, lanes 1 and 4). This Ab precipitated, however, NK cell-specific KIR2DL3 gene promoter (45) from PB-derived B-lymphoblastoid RPMI 8866 cells (supplemental Fig. S3, lane 7), and from BM stroma cells L88/5 (46), under the same experimental conditions. CD34 gene transcripts were 75-fold enriched in CD34 positive mobilized PB cells versus CD34-depleted PBMCs, as was determined by quantitative real-time RT-PCR, using beta-actin as internal control (Fig. 4C, lanes 13 and 14).

Thus in intact CD34 positive mobilized PB cells, a recruitment of nucleolin to the CD34 promoter region takes place, accompanied by nucleosomal determinants of gene activity, which are absent from the CD34 promoter region in CD34 negative PBMCs.

**DISCUSSION**

Our study reveals that multifunctional DNA and RNA binding protein nucleolin activates expression of CD34 and Bcl-2 in human CD34 positive hematopoietic cells. Bcl-2 promotes survival of hematopoietic cells, reviewed in (16), and nucleolin is thus involved in the maintenance of CD34 positive cells of hematopoiesis. CD34 is expressed on a subset of hematopoietic stem/progenitor cells, capable of reconstitution, reviewed in (3), and Bcl-2 increases the frequency and in vivo reconstitution potential of hematopoietic stem cells (18, 47). Within human BM, a predominance of Bcl-2 expression to hematopoietic stem/progenitor cells, as compared with their differentiated progeny, has been reported (48). Our data showing the relationship of nucleolin and Bcl-2 expression in CD34 positive cells (Fig. 1) are in agreement with previously undertaken and presented in-depth studies that have identified nucleolin as a Bcl-2 mRNA stabilizing factor (49, 50). It was demonstrated that nucleolin binds to the AU-rich element in the 3′ untranslated region of the Bcl-2 mRNA, that is endowed with a destabilization function, and the binding of nucleolin protects Bcl-2 mRNA from ribonuclease degradation (49-51). On the other hand, it has been reported that expression of p53 is inhibited by nucleolin (29); levels of p53 protein have been demonstrated to increase with maturation in human hematopoietic cells (52), and inhibition of p53 in hematopoietic stem/progenitor cells may facilitate hematopoietic reconstitution after cytoreductive therapy (53).

Regulation of CD34 gene transcription has been investigated as a model of hematopoietic stem/progenitor cell-specific gene control (3, 6-15). The activity of the human CD34 promoter region was shown to be modulated by a number of transcription regulators, including c-Myb, Ets-2, MZF-1 and nuclear factor Y (11-15). Here we show that nucleolin is an additional crucial activator of the CD34 gene transcription. It has been previously reported that a promoter element encompassing one of the here-identified nucleolin binding sites positively regulates the CD34 promoter and acts as an enhancer (54). Nucleolin can facilitate transcription through nucleosomal particles (28), and CD34 promoter region, per se not sufficient for stem/progenitor cell-specific transcription (6, 8-10), thus appears to lack determinants of gene activity in CD34 negative, in contrast to CD34 positive, intact primary hematopoietic cells. In view of the previously proposed role of chromatin remodeling for regulation of the human CD34 gene (3, 8, 9), it should be noted that nucleolin enhances the activity of the SWI/SNF and ACF complexes (28), that contribute to regulation of transcription by altering chromatin structure (55, 56). Furthermore, nucleolin promotes the binding of SWI/SNF to the nucleosome (28). Thus, it is tempting to suggest that the recruitment of nucleolin may contribute to remodeling of the chromatin structure of the CD34 gene regulatory region. Of note, nucleolin is a multidomain protein with amino acid sequence presenting unusual features (19, 57). In a recent study, it was demonstrated that the integrity of the full-length nucleolin protein is needed for the fulfillment of its functions in regulation of gene transcription (28). The structure of nucleolin is largely unknown thus far. Future analysis of the sequences, regions and domains of nucleolin required for activation of CD34 gene transcription, and of the contributions of its specific domains to this process will be important and might aid in understanding how nucleolin functions to regulate CD34 gene expression. This analysis will depend on detailed knowledge of the structure of nucleolin.

Nucleolin is found in the database of non-housekeeping genes, that are preferentially transcribed in murine hematopoietic stem cells derived from fetal liver (database entries SC|C2-57.
and SC(S25-20320), as opposed to stem cell-depleted cell populations (30). Furthermore, it has been shown that nucleolin gene transcripts are selectively enriched in murine BM hematopoietic stem cells, as opposed to differentiated tissue (31), and nucleolin expression is prevalent to murine long-term reconstituting hematopoietic stem cells versus non-long-term reconstituting progenitor cells (58). The reports from the literature, taken together with our data showing the prevalence of nucleolin expression to human CD34 positive mobilized PB cells as compared with CD34 negative PBMCs thus suggest a potential role of nucleolin for basic biological functions of hematopoietic stem/progenitor cells. Taking into account the involvement of nucleolin in the control of gene transcription and RNA metabolism (21, 25-29), as well as its previously suggested role in ribosomal RNA synthesis (19, 59), it will be important to identify the whole spectrum of genes whose expression is regulated by nucleolin in hematopoietic stem/progenitor cells, to establish the impact of nucleolin to the long-term self-renewal and differentiation of these cells and potentially to homeostasis and reconstitution of the hematopoietic system.

It has been shown that human acute myelogenous leukemia (AML) can be viewed as an aberrant hematopoietic tissue initiated by CD34 positive leukemic cells (60, 61). High expression of Bcl-2 promotes survival of leukemic cells and is related to poor clinical prognosis for AML patients (62, 63). Furthermore, Bcl-2 and mitogen-activated protein kinase signaling pathways can act synergistically to promote leukemogenesis (64), and a role for Bcl-2 in the transition from chronic to acute leukemia has been suggested (61). In view of an involvement of nucleolin in the process of cell transformation (20, 24), it is tempting to suggest that, in leukemic cells, deregulation of nucleolin may contribute to the pathogenesis of AML via Bcl-2 overexpression. Future studies will address a potential role of nucleolin in this process.

REFERENCES


FOOTNOTES

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The abbreviations used are: Ab, antibody; AML, acute myelogenous leukemia; BM bone marrow; ChIP, chromatin immunoprecipitation; CT, threshold cycle; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; H4-AcK8, histone H4 acetylated on lysine K8; H3-tri-methyl K4, histone H3 tri-methylated on lysine K4; H3-di-methyl K9, histone H3 di-methylated on lysine K9; HPV18, human papillomavirus type 18; IP, immunoprecipitation; mAb, monoclonal antibody; NK, natural killer; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; PCNA, proliferating cell nuclear antigen; PE, phycoerythrin; RT-PCR, reverse transcription PCR; siRNA, small interfering RNA; WT, wild-type.

FIGURE LEGENDS

Fig. 1. Nucleolin-mediated activation of endogenous CD34 and Bcl-2 expression. A, Left and middle, lysates of KG1-Nuc cells (lanes 1 and 3) and KG1-mock cells (lanes 2 and 4) were analyzed by immunoblotting with Abs specific for nucleolin, FLAG-tag, CD34 (H-140), Bcl-2 and beta-actin. Arrows indicate the positions of proteins detected. Right, cell surface CD34 expression in KG1-mock cells (M1) and KG1-Nuc cells (M2) was monitored by FACS analysis with PE-coupled mAb specific for CD34 (SG12). Dotted line, isotype-matched, PE-coupled control mAb. B, Levels of CD34 mRNA (lanes 1 and 2) and Bcl-2 mRNA (lanes 3 and 4) in KG1-Nuc cells (lanes 1 and 3) and KG1-mock cells (lanes 2 and 4) were analyzed by real-time RT-PCR and normalized to beta-actin. Representative real-time RT-PCR
amplification plots from the same run of RNA samples are shown on the top. Delta CT, average differences of CT values. C, KG1 cells were either not transfected (lanes 3, 8 and 11), or transfected with nucleolin-targeting siRNA-A (lanes 2, 7 and 10), with nucleolin-targeting siRNA-B (lane 5), or with control siRNA (lanes 1, 4, 6 and 9). Left, after application of nucleolin-targeting siRNA-A or siRNA-B, cell lysates were separated identically on two gels (lanes 1-3) and (lanes 4 and 5) and analyzed by immunoblotting with Abs specific for nucleolin, CD34 (H-140), Bcl-2 and beta-actin. Arrows indicate the positions of proteins detected. Band intensity was normalized to beta-actin and is shown at the bottom as a percentage of cells transfected with control siRNA. Right, levels of CD34 mRNA (lanes 6-8) and Bcl-2 mRNA (lanes 9-11) were analyzed by real-time RT-PCR and normalized to beta-actin.

**Fig. 2.** Identification of nucleolin as a CD34 promoter-binding factor. A, EMSA with labeled oligonucleotide CD34A, lanes 1-7, or with oligonucleotide CD34B, lanes 8-11. Lane 1, nuclear extract from KG1 cells; lane 2, nucleolin Ab was added to the binding reaction; lane 3, nucleolin Ab was presaturated with the immunizing nucleolin peptide. Lanes 4 and 8, nuclear extracts from KG1-Nuc cells; lanes 5-7 and 9-11, nuclear extracts from KG1-mock cells. The binding complex was competed by a 100-fold molar excess of oligonucleotides: WT CD34A (lane 6), mutant (Mut) CD34A (lane 7), WT CD34B (lane 10), or mutant CD34B (lane 11). Retarded nucleolin-DNA complex is indicated by an arrow. Lanes 1-3, 4-7 and 8-11 are separate gels. B, Interaction of recombinant nucleolin-GST protein with the CD34 promoter region. EMSA with labeled oligonucleotide CD34A, lanes 1 and 2, with oligonucleotide CD34B, lanes 3 and 4, or with an unrelated AP1 recognition site, lanes 5 and 6. Lanes 1, 3 and 5, EMSA with recombinant nucleolin-GST protein, or with GST backbone protein (lanes 2, 4 and 6). Retarded nucleolin-DNA complex is indicated by an arrow. C, Lanes 1-7, ChIP performed with KG1 cells. ChIP with nucleolin peptide Ab (lanes 1, 5 and 6), with nucleolin Ab presaturated with the immunizing nucleolin peptide (lane 2), with control Ab (lanes 3 and 7), with no Ab (lane 4). Top, lanes 1-5 and 7, semiquantitative PCR with primers specific for CD34 promoter was followed by detection on agarose gel. Lane 6, primers specific for intron 1 of CD34 gene were used. Middle, control showing DNA quantities before IP. Bottom, co-precipitated CD34 promoter was quantified by real-time PCR. The y-axis indicates the ratio between bound and input DNA, as arbitrary units. Lanes 8-12, Western blotting with Ab specific for nucleolin was performed after IP with nucleolin Ab (lane 9), with nucleolin Ab presaturated with the immunizing nucleolin peptide (lane 10), with control Ab (lane 11), or with no Ab (lanes 12). Lane 8, KG1 cell lysate used for IP (10%).

**Fig. 3** Nucleolin-mediated activation of the CD34 promoter region. A, KG1 cells were transiently co-transfected with nucleolin cDNA expression vector and CD34WT-pGL3 reporter construct (lane 1), with empty expression vector and CD34WT-pGL3 reporter construct (lane 2), with nucleolin cDNA expression vector and CD34mutA-pGL3 reporter construct (lane 3), with empty expression vector and CD34mutA-pGL3 reporter construct (lane 4), with nucleolin cDNA expression vector and CD34mutB-pGL3 reporter construct (lane 5), with empty expression vector and CD34mutB-pGL3 reporter construct (lane 6), with nucleolin cDNA expression vector and pGL3basic vector (lane 7), or with empty expression vector and pGL3basic vector (lane 8). The error bars in lanes 5, 7 and 8 are just visible due to identical luciferase activities in the samples. B, Lanes 1-8, KG1a cells were treated as described above for panel A. Lanes 9-12, KG1a cells were co-transfected with CD34WT-pGL3 reporter construct and increasing amounts of nucleolin cDNA expression vector: lane 9, with none; lane 10, with 0.1µg; lane 11, with 0.3µg; lane 12, with 0.5µg (identically to lane 1). Lane 13, co-transfection with empty expression vector and pGL3basic vector. In panels A and B, the y-axis indicates reporter activation relative to empty pGL3basic vector. Luciferase activity was normalized to protein concentration. Figures represent three independent experiments.

**Fig. 4.** Prevalent expression and recruitment of nucleolin to CD34 promoter in CD34 positive mobilized PB cells. A, Left, lysates from CD34 positive cells mobilized into PB (lane 1) and CD34-depleted PBMCs (lane 2) were analyzed by immunoblotting with Abs specific for nucleolin (top) and beta-actin (bottom). Right, levels of nucleolin mRNA in CD34 positive cells mobilized into PB (lane 3), CD34-depleted
PBMCs (lane 4) and purified NK cells (lane 5) were analyzed by real-time RT-PCR and normalized to beta-actin (bottom). Top, representative real-time RT-PCR amplification plots from the same run of RNA samples. Delta CT, average differences of CT values. B, Left, top, EMSA with labeled CD34B oligonucleotide and nuclear extract from CD34 positive cells mobilized into PB (lane 1), CD34-depleted PBMCs (lane 2), or with no extract added (lane 3). Retarded nucleolin/DNA complex is indicated by an arrow. Left, bottom, nuclear extracts were analyzed by immunoblotting with Abs specific for nucleolin and beta-actin. Middle and right, ChIP performed with nucleolin peptide Ab (lanes 4, 8, 12, 16, 18, 20), with nucleolin Ab presaturated with the immunizing nucleolin peptide (lanes 5, 9, 13, 17, 19, 21), with control Ab (lanes 6, 10, 14), or with no Ab (lanes 7, 11, 15). Lanes 4-7, 16 and 17, CD34 positive cells mobilized into PB; lanes 8-11, 18 and 19, CD34-depleted PBMCs; lanes 12-15, 20 and 21, purified NK cells. Lanes 4-15, co-precipitated CD34 promoter was quantified by real-time PCR. The y-axis indicates the ratio between bound and input DNA, as arbitrary units. Lanes 16-21, top, co-precipitated CD34 promoter was detected after semiquantitative PCR on agarose gel; bottom, control showing DNA quantities before IP. Lanes 16-17, 18-19 and 20-21 are adjacent parts of the same exposure of the same gel. C, Left, ChIP performed with Ab specific for H3-tri-methyl K4 (lanes 1 and 4), with Ab specific for H4-AcK8 (lanes 7 and 10), with control Ab (lanes 2, 5, 8 and 11), with no Ab (lanes 3, 6, 9 and 12). Lanes 1-3 and 7-9, CD34 positive cells mobilized into PB; lanes 4-6 and 10-12, CD34-depleted PBMCs. Lanes 1-12, co-precipitated CD34 promoter was quantified by real-time PCR. The y-axis indicates the ratio between bound and input DNA, as arbitrary units. Right, CD34 mRNA levels in CD34 positive cells mobilized into PB (lane 13) and CD34-depleted PBMCs (lane 14) were quantified by real-time RT-PCR and normalized to beta-actin.
Figure 1B

Real-time RT-PCR
Figure 1 C

Western blot analysis showing the expression levels of Nucleolin, CD34, and Bcl-2 under different conditions. The blots are normalized to Beta-actin. The real-time RT-PCR data also indicate the relative mRNA levels of CD34 and Bcl-2.
Figure 2 A

Peptide
Nucleolin
antibody

Nucleolin

Free
CD34A
oligo

Free
CD34A
oligo

Free
CD34B
oligo

Competitor
CD34A oligo
Transfection
Nuc Control

Competitor
CD34B oligo
Transfection
Nuc Control

EMSA
Figure 2 C

CD34 gene region assayed

Bound

Input

IP

ChIP assay
Figure 3 A

Control vector

Nucleolin expression vector

Relative reporter activation, %

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Luciferase reporter assay
Figure 3 B

Luciferase reporter assay
Figure 4 B

**EMSA**

**Western**

**ChIP assay**
Figure 4 C

![Graph showing CHIP assay results for CD34+ and CD34- cells.](image)

**CHIP assay**

**Relative CD34 mRNA levels**

**Real-time RT-PCR**
Nucleolin regulating gene expression in CD34 positive hematopoietic cells
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