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BAALC, a novel marker of human hematopoietic progenitor cells

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Objective. **The gene *BAALC* (*Brain And Acute Leukemia, Cytoplasmic*), a novel molecular marker involved in leukemia, is highly expressed in a subset of patients with acute leukemia and predictive of clinical outcome in patients with acute myeloid leukemia and normal karyotype. The role of *BAALC* in hematopoiesis and leukemogenesis is unknown.**

Material and Methods. **We used real-time RT-PCR to show that *BAALC* is strongly expressed in $CD34^+$ cells from the bone marrow and blood and only weakly expressed in total normal bone marrow and blood cells.**

Results. **Expression analyses of FACSorted cells revealed high *BAALC* transcript levels in $CD34^+$ bone marrow cells including $CD34^+/CD38^-$, $CD34^+/CD33^+$, as well as $CD34^+/CD19^+/CD10^+$, $CD34^+/CD7^+$, and $CD34^+/CD71^+/CD45^-$ cell fractions. Expression was significantly lower in all $CD34^-$ fractions. In vitro differentiation of $CD34^+$ bone marrow cells showed downregulation of *BAALC* and *CD34* transcripts as early as day 4 in suspension cultures supplemented with lineage-specific cytokines (G-CSF, M-CSF, or EPO). In cultures with only lineage-unspecific cytokines (IL-3, SCF, GM-CSF), *BAALC* transcripts persisted up to day 20, while *CD34* transcripts disappeared earlier. These observations suggest that expression of *BAALC* is stage specific.**

Conclusion. ***BAALC*** **expression is restricted to progenitor cells, and downregulation of *BAALC* occurs with cell differentiation. We postulate that *BAALC* represents a novel marker of an early progenitor cell common to the myeloid, lymphoid, and erythroid pathways.** **© 2003 International Society for Experimental Hematology. Published by Elsevier Inc.**

During hematopoiesis a complex network of differentially expressed genes controls the steady state of dormant multipotent hematopoietic stem cells, highly proliferating progenitor cells, and differentiated mature blood cells [1,2]. Alterations in the expression of genes belonging to signal transduction pathways as well as transcription factors are known to be implicated in the process of leukemogenesis [3–5]. Many of these genes have been identified based on their involvement in chromosomal rearrangements, and aberrant expression of these genes has been linked to leukemic transformation [6–8]. Insight into the regulation of genes active in hematopoiesis may elucidate yet unknown mechanisms affecting differentiation. The characterization of these genes and their underlying pathways could potentially aid the design of specific targeted therapies [9].

In a search for novel genes involved in leukemia, the human gene *BAALC* (*Brain And Acute Leukemia, Cytoplasmic*) was identified when the expression levels of blasts from acute myeloid leukemia (AML) with trisomy 8 were compared with cytogenetically normal AML [10]. *BAALC*, located on chromosome 8q22.3, was implicated in neuroectodermal and hematopoietic development. The DNA sequence and expression pattern of *BAALC* are highly conserved among mammals, whereas no orthologs were found in lower organisms. The protein sequence shows no homology to any known proteins and suggests no known functional domains. Expression of *BAALC* was mainly found in neuroectoderm-derived tissues and immunohistochemistry showed cytoplasmic localization of the protein in neuronal cells. It was previously shown that blood and unselected bone marrow cells from healthy donors showed no or very low *BAALC* expression, whereas higher levels of

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BAALC transcripts were detected in hematopoietic progenitor cells [10]. In addition, high levels of *BAALC* expression were present in leukemic blasts in subsets of acute lymphoblastic leukemia (ALL) and AML patients and turned out to be predictive of adverse clinical outcome in AML patients with a normal karyotype [11]. No expression was found in the chronic phase of chronic myelogenous leukemia (CML) or in chronic lymphocytic leukemia (CLL) [10].

To further explore the role of *BAALC* in hematopoiesis, we studied *BAALC* expression in subpopulations of human bone marrow and blood cells, as well as during in vitro differentiation of bone marrow $CD34^+$ cells.

Materials and methods

Cells

Human blood and bone marrow samples were obtained from 29 healthy donors after informed consent. Density gradient centrifugation (Ficoll-Hypaque; Amersham Pharmacia Biotech, Uppsala, Sweden) was used to isolate mononuclear cells from blood and bone marrow. $CD34^+$ cells were immunomagnetically enriched from blood (nonmobilized) and bone marrow using MACS CD34 isolation kit (Miltenyi Biotec, Auburn, CA, USA) following the manufacturer's recommendations.

Approval was obtained from the Ohio State University Institutional Review Board for these studies.

Immunocytochemical staining of BAALC

Cytospin preparations of immunomagnetically enriched $CD34^+$ cells as well as $CD34^-$ bone marrow cells were fixed in 10% formalin and stained using the polyclonal rabbit anti-human BAALC antibody GN2214 (1:200 dilution) [10]. Species-specific biotinylated goat anti-rabbit antibody was used to visualize protein-antibody complexes (Vectastain Elite ABC, Vector Laboratories, Inc., Burlingame, CA, USA) and cells were counterstained with hematoxylin.

Immunofluorescence staining and fluorescence-activated cell sorting (FACSorting)

Three-color flow cytometry was performed on a FACSort flow cytometer using the CellQuest (Becton-Dickinson, San Jose, CA, USA) software for data acquisition and analysis. Fluorochrome-conjugated control immunoglobulin (IgG) or antigen-specific antibodies were used for immunofluorescence staining of isolated cells from blood and bone marrow. Nonspecific staining was blocked by preincubation with 30µg mouse Ig (Sigma-Aldrich Corp., St. Louis, MO, USA) on ice for 10 minutes. Cells were stained for 30 minutes on ice using fluorescein isothiocyanate (FITC)-conjugated mouse anti-human antibodies against CD34 as well as phycoerythrin (PE)-conjugated mouse anti-human antibodies against CD33, CD38, CD71, CD19, CD7, and Cy-Chrome (Cy)-conjugated mouse anti-human CD10 and CD45RA. Control samples were incubated with isotype-matched IgG-FITC, IgG-PE, or IgG-Cy (all Becton-Dickinson, San Jose, CA, USA). Cells were washed and resuspended in cold FACS staining buffer (phosphate-buffered saline [PBS], 1% bovine serum albumin, 0.1% NaN_3). For each sample 10,000 events were analyzed. Fluorescence was considered positive when it was greater than 99% of the matched isotype control. $CD34^+$ and $CD34^-$ bone marrow cells were used for FACSorting. Samples were processed in cold Iscove's modified Dulbecco's medium (IMDM; GIBCO BRL, Grand Island, NY, USA) with 20% fetal calf serum (FCS) and stained for various surface markers as described above. Different cell populations were analyzed and sorted based on their immunophenotype using a Coulter Elite cell sorter. Collected cells were kept on ice and immediately processed for RNA isolation. Equal numbers of cells (20,000 cells) were sorted for all subpopulations.

In vitro differentiation of $CD34^+$ cells

Immunomagnetically purified $CD34^+$ bone marrow cells (90–95% pure) were cultured in IMDM medium, 20% FCS, and various cytokines including stem cell factor (SCF; 50 ng/mL), interleukin-3 (IL-3; 20 ng/mL), Flt3-ligand (100 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/mL), granulocyte colony-stimulating factor (G-CSF; 50 ng/mL), macrophage colony-stimulating factor (M-CSF; 50 ng/mL), and erythropoietin (EPO; 3 U/mL; all cytokines were purchased from R & D Systems, Minneapolis, MN, USA). Cells were cultured in 6-well tissue culture plates seeded at an initial density of 0.5×10^5/mL in 2 mL per well. Cultures were semi-depleted every 4 days (day 4, 8, 12, 16, 20). At these time points medium and cytokines were replaced, and harvested cells were processed for RNA isolation. In addition, harvested cells were used for slide preparations, which were subsequently stained with Giemsa for morphological analysis.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's directions. Cells were pelleted and resuspended in 1 mL Trizol; for samples with less than 10^5 cells 100 µL Trizol was used. Precipitation of RNA was facilitated by the addition of 60 µg glycogen (Roche, Indianapolis, IN, USA). Precipitated and air-dried RNA from sorted subpopulations was resuspended in 15 µL RNase-free water and 5 µL RNA used for the synthesis of complementary DNA (cDNA); for samples containing more than 10^5 cells RNA was quantified by spectrophotometry and 0.5 µg RNA was reverse transcribed. cDNA synthesis was performed using avian myeloblastosis virus reverse transcriptase (Roche, Indianapolis, IN, USA) with oligo$(dT)_{24}$ or gene-specific primers at 50°C for 60 minutes in the presence of RNase inhibitor (RNasin; Roche, Indianapolis, IN, USA).

Semi-quantitative multiplex RT-PCR

Multiplex RT-PCR was carried out in a final volume of 50 µL containing 1 µL of cDNA, 2.5 U AmpliTaq Gold, 100 nM of each dNTP (all Roche, Indianapolis, IN, USA), and 20 pmol of each primer. Conditions were 95°C for 10 minutes, 32 cycles of 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 1 minute with a final step of 72°C for 5 minutes. Levels of *BAALC* were compared to the mRNA expression levels of *CD34* and the housekeeping gene *Glucose-phosphate isomerase* (*GPI*) by nondenaturing polyacrylamide gel electrophoresis after staining with silver nitrate [12].

Quantitative real-time RT-PCR

For real-time RT-PCR, cDNA was synthesized using gene-specific primers for *GPI* and *BAALC* at 50°C for 60 minutes. 5′-nuclease comparative real-time RT-PCR assays were performed as previously described [11]. Briefly, *GPI* and *BAALC* were coamplified in the same tube using 2 µL cDNA, 1 × universal master mix (Applied Biosystems, Foster City, CA, USA), 250 nM human

GPI (VIC-labeled) with 600 nM each of the forward and reverse primers, and 250 nM of *BAALC* TaqMan oligo (6-FAM-labeled) with 900 nM each of the *BAALC* forward and reverse primers. Probes were labeled with quencher TAMRA at the 3′-end. Amplification was carried out at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 PCR cycles at 95°C for 15 seconds, and 60°C for 1 minute. All reactions were done in MicroAmp optical 96-well plates using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). The comparative cycle threshold (C_T) method was used to determine the relative expression levels of *BAALC* (Applied Biosystems, Foster City, CA, USA). The threshold cycles for *BAALC* and *GPI* were determined, and the cycle number difference ($\Delta C_T = GPI - BAALC$) was calculated for each replicate. Relative *BAALC* expression values were calculated using the mean of ΔC_T from the three replicates, that is $\mu(\Delta C_T) = (\Sigma \Delta C_T)/3$.

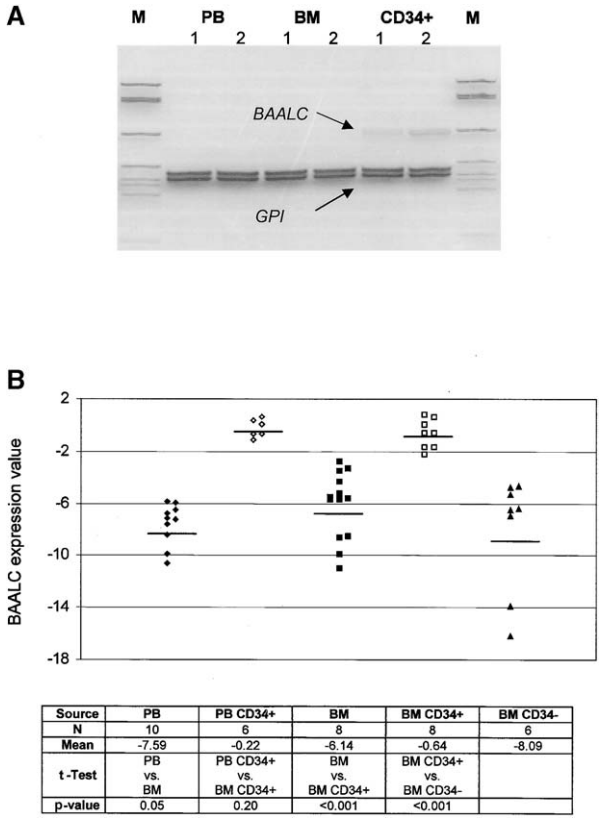
Results

BAALC expression in normal blood, bone marrow, and CD34$^+$ cells

BAALC expression levels in bone marrow and blood cells were evaluated by semi-quantitative and real-time RT-PCR. As shown in Figure 1A, *BAALC* transcripts were detectable in CD34$^+$ bone marrow cells, whereas no bands were present in unselected blood or bone marrow cells. This observation was confirmed by real-time RT-PCR, showing high *BAALC* expression values only for CD34$^+$ cells including CD34$^+$ bone marrow cells (mean expression value: −0.64) and CD34$^+$ blood cells (mean: −0.22). Virtually no *BAALC* was present in total blood (mean: −7.59) and very low levels of *BAALC* transcripts were detected in total bone marrow samples (mean: −6.14), and in CD34$^-$ bone marrow samples (mean: −8.09). *BAALC* expression was higher in bone marrow compared to blood (mean: −6.14 vs −7.59; $p = 0.05$), and *BAALC* expression of CD34$^+$ bone marrow cells was significantly higher than that of total bone marrow cells (mean: −0.64 vs −6.14; $p < 0.001$). The levels of *BAALC* expression detected in CD34$^+$ cells isolated from bone marrow and blood were similar ($p = 0.20$; Fig. 1B).

Immunocytochemical staining of BAALC

Immunocytochemical demonstration of CD34$^+$ bone marrow cells using a polyclonal anti-*BAALC* antibody revealed the staining to be cytoplasmic with a granular pattern. As shown in Figure 2, typical CD34$^+$ cells had roundish, relatively large granules localized in the cytoplasm. Within the fraction of CD34$^+$ cells only those with morphological features of maturation did not stain for *BAALC*. These few cells negative for *BAALC* most likely reflect residual, mature CD34$^-$ cells within the CD34$^+$ fraction (purity 90–95% CD34$^+$ cells after immunomagnetic selection). No *BAALC* protein expression was detected in the fraction of CD34$^-$ cells (Fig. 2). To exclude the possibility of false-positive staining due to the presence of microspheres on the CD34$^+$ cells, CD34$^+$ cells were also isolated using Dynal CD34 progenitor cell selection system (Dynal Biotech, Lake Success, NY, USA), in which the beads can be released after the enrichment producing CD34$^+$, phenotypically unaltered cells. Immunochemical staining of such selected cells resulted in the same granular staining pattern of CD34$^+$, but not CD34$^-$, cells.



Source	PB	PB CD34+	BM	BM CD34+	BM CD34-
N	10	6	8	8	6
Mean	-7.59	-0.22	-6.14	-0.64	-8.09
t-Test	PB vs. BM	PB CD34+ vs. BM CD34+	BM vs. BM CD34+	BM CD34+ vs. BM CD34-	
p-value	0.05	0.20	<0.001	<0.001	

Figure 1. *BAALC* mRNA expression in peripheral blood (PB), bone marrow (BM), and CD34$^+$ and CD34$^-$ cells. **(A)**: Semi-quantitative RT-PCR comparing *BAALC* expression to the housekeeping gene *GPI*. Two samples from different donors (1 and 2) are shown for PB, total BM, and CD34$^+$ BM cells; M, size marker. **(B)**: *BAALC* expression values determined by real-time RT-PCR. Number of different donors analyzed (N), mean expression levels (horizontal bars), and *p*-values comparing differences in expression levels are shown below. The mean *BAALC* expression level measured in BM CD34$^+$ cells was significantly higher than the mean *BAALC* expression for BM cells and for BM CD34$^-$ cells (*p*-values both <0.001). Mean *BAALC* expression level was higher in unsorted BM cells compared to unsorted PB cells ($p = 0.05$). No difference in mean *BAALC* expression of PB CD34$^+$ cells and BM CD34$^+$ cells was observed ($p = 0.2$). All differences in mean *BAALC* expression values were evaluated using a one-sided *t*-test with unequal variances except for the test between PB CD34$^+$ and BM CD34$^+$ cells, which was a two-sided test.

FACSorting of subpopulations and evaluation of BAALC expression

FACSorting using a variety of appropriate antibodies followed by real-time RT-PCR analyses revealed high levels of *BAALC* expression in all subsets of CD34$^+$ bone marrow progenitor populations examined, including lineage-committed CD34$^+$/CD38$^+$ cells (mean expression value: −1.32)

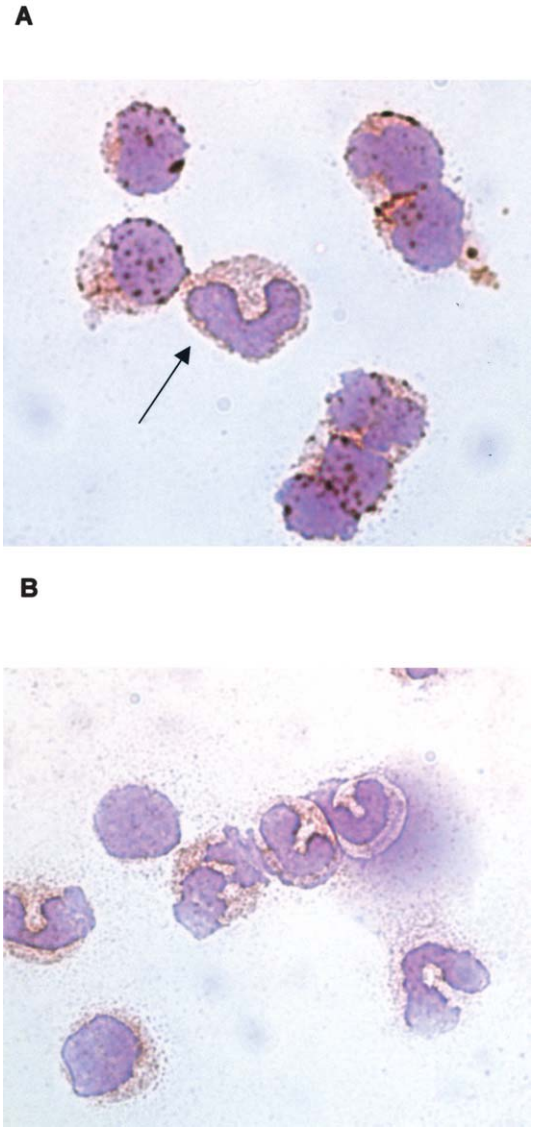


Figure 2. Immunocytochemical staining of BAALC in $CD34^+$ and $CD34^-$ fractions of bone marrow cells. Cytospin preparations of enriched $CD34^+$ and $CD34^-$ cells were stained using a polyclonal rabbit anti-human BAALC antibody. **(A)**: A granular cytoplasmic staining is seen for most cells in the $CD34^+$ fraction. A mature myelocytic cell (arrow) is unstained. **(B)**: Cells in the $CD34^-$ fraction do not express BAALC protein.

and myeloid $CD34^+/CD33^+$ cells (mean: −2.23), as well as $CD34^+/CD7^+$ T-cell progenitor cells (mean: −1.92), $CD34^+/CD19^+/CD10^+$ B-cell progenitor cells (mean: −1.56), and $CD34^+/CD71^+/CD45^-$ erythroid progenitor cells (mean: −2.51). No significant difference in *BAALC* expression levels was observed in comparison to the non-committed population of $CD34^+/CD38^-$ cells (mean: −1.59). In contrast, all sorted subpopulations from the $CD34^-$ bone marrow cells showed significantly lower levels of *BAALC* expression (Fig. 3).

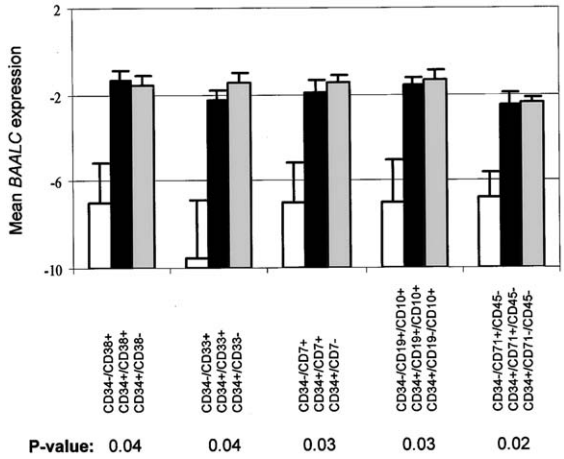


Figure 3. Mean *BAALC* expression values of sorted bone marrow subsets determined by real-time RT-PCR. The mean expression level for each subset from at least three different donors was analyzed. Bars indicating the standard error of the mean are shown. Differences between $CD34^-$ and $CD34^+$ populations of each subset were statistically significant (*p*-values are shown for each subset), whereas no significant differences were observed within the $CD34^+$ populations of each subset.

BAALC expression during in vitro differentiation of $CD34^+$ bone marrow cells

To evaluate the expression of *BAALC* during hematopoietic differentiation, transcripts were analyzed in $CD34^+$ marrow cells cultured in the presence of various cytokines to promote differentiation. Duplicate experiments on cells from three different donors gave almost identical results. Purified $CD34^+$ cells were maintained in suspension culture supplemented with lineage-unspecific cytokines supporting proliferation of early-stage progenitors (including SCF, IL-3, Flt3-ligand, GM-CSF). Differentiation was induced by the addition of lineage-specific cytokines controlling proliferation and maturation of committed progenitors (including G-CSF, M-CSF, or EPO) [1]. Morphologic analyses on day 16 confirmed lineage-specific maturation with the emergence of granulocytic, macrophage, or erythroid features, respectively (Fig. 4-II). In vitro differentiation of $CD34^+$ cells revealed downregulation of *BAALC* transcripts by day 4 in cultures supplemented with lineage-unspecific cytokines (SCF and IL-3 plus GM-CSF) in combination with G-CSF, M-CSF, or EPO (Fig. 4-IB–D). In contrast, maintenance culture using different combinations of lineage-unspecific cytokines (IL-3 and SCF; IL-3 and SCF plus GM-CSF; IL-3 and SCF plus Flt3-ligand) allowed detection of *BAALC* transcripts up to day 20 (Fig. 4-IA, and data not shown). Multiplex RT-PCR showed that *BAALC* and *CD34* expression were independently regulated. Whereas low levels of *BAALC* expression could be detected until day 20 of maintenance cultures, *CD34* transcripts were only maintained until day 12. Downregulation of *BAALC* and *CD34* is promptly (day 4) induced by lineage-specific cytokines (Fig. 4-IB–D).

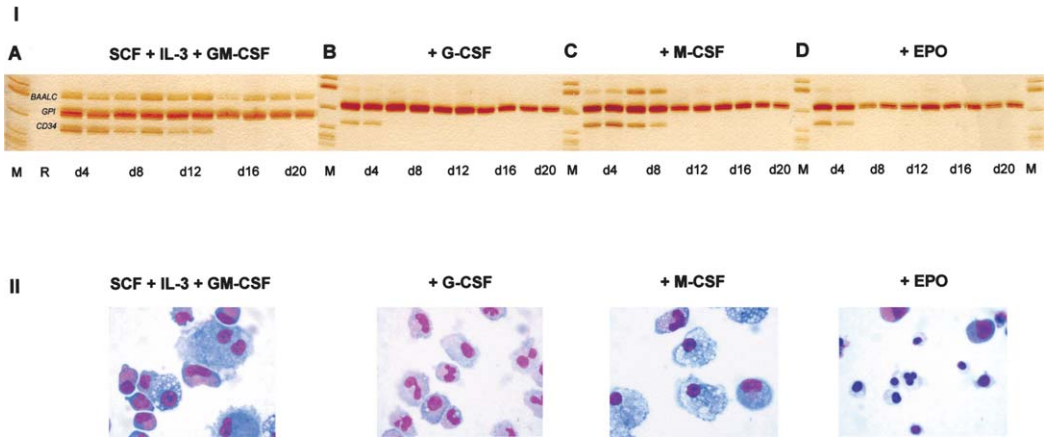


Figure 4. *BAALC* expression during in vitro differentiation of CD34$^+$ progenitor cells. **(I)**: Multiplex RT-PCR was performed on days 4, 8, 12, 16, and 20 of in vitro differentiation (each time point in duplicate). M, marker; R, reagent control. **(A)**: *BAALC* expression can be detected until day 20 in the presence of SCF, IL-3, and GM-CSF, whereas mRNA of *CD34* can only be detected until day 12. In contrast **(B,C,D)**, *BAALC* and *CD34* are downregulated by day 4/8 in the presence of additional lineage-specific cytokines (G-CSF, M-CSF, or EPO). A representative result from three independent experiments is shown. **(II)**: Giemsa staining of cytospin preparations on day 16 confirmed differentiation with typical morphological features of granulocytic (+G-CSF), macrophage (+M-CSF), and erythroid (+EPO) maturation.

Discussion

We assessed the expression of *BAALC* in different hematopoietic cells as well as its regulation during in vitro differentiation. *BAALC* transcripts were virtually not expressed in unselected blood cells while unsorted bone marrow cells showed only very low levels of *BAALC* expression. To address the question whether this results from weak expression in many bone marrow cells, or high expression in a subpopulation of cells, we analyzed *BAALC* in different cell fractions. Our results show that *BAALC* expression is restricted to CD34$^+$ hematopoietic progenitor cells. Further quantitative analysis of *BAALC* expression in different subsets revealed high levels in uncommitted CD34$^+$/CD38$^-$ cells, a small subclass, which is enriched for primitive hematopoietic cells [13], as well as in committed CD34 progenitor cells. Lymphoid-committed cells including T-cell (CD34$^+$/CD7$^+$) and B-cell progenitors (CD34$^+$/CD19$^+$/CD10$^+$ pro-B cells) also revealed high levels of *BAALC* expression, as did myeloid-committed CD34$^+$/CD33$^+$ and erythroid CD34$^+$/CD71$^+$/CD45$^-$ progenitor cells [14–17]. In contrast, none of the subsets of CD34$^-$ bone marrow cells analyzed showed detectable *BAALC* expression. Immunocytochemistry confirmed the presence of BAALC protein in morphologically immature CD34$^+$ cells with a granular, cytoplasmic localization of the protein. In accord with the absence of *BAALC* transcripts in CD34$^-$ cells, staining of CD34$^-$ cells showed no detectable BAALC protein. These results strengthen our hypothesis that *BAALC* is a marker of both lineage-uncommitted and lineage-committed hematopoietic progenitor cells.

CD34 is a useful marker of human hematopoietic progenitor cells, and in transplantation studies CD34$^+$ enriched cells have been sufficient for reconstitution of the hematopoietic system [18]. More recent studies have provided experimental evidence demonstrating the existence of CD34$^-$ hematopoietic stem cells [19,20]. We can neither exclude nor confirm that a very small subset of CD34$^-$ cells with hematopoietic stem cell potential expresses *BAALC*.

To study *BAALC* during differentiation using a dynamic model, we analyzed *BAALC* expression at different time points using various cytokines to induce lineage-specific maturation. Suspension cultures of CD34$^+$ cells are a useful system to mimic and analyze hematopoietic differentiation in vitro [16]. Our data show that in response to the differentiation stimuli of lineage-specific cytokines *BAALC* was promptly downregulated. This response did not appear to be cytokine specific. Although *BAALC* expression is closely related to the CD34 phenotype, expression analyses of *BAALC* and *CD34* mRNA levels revealed differences: *BAALC* expression is sustained in maintenance culture, whereas *CD34* expression becomes downregulated. While *BAALC* expression seemed to be restricted to the compartment of CD34 positive cells, the transcriptional regulation of *BAALC* and *CD34* appeared to be independent.

Additional observations allow us to propose that *BAALC* expression represents a new marker of hematopoietic progenitor cells. First, whereas *BAALC* expression is absent in leukemic cells from CLL and CML patients, high levels of *BAALC* transcript can be detected in subsets of AML and ALL cases [10]. In AML, *BAALC* expression is predominantly seen in the French-American-British (FAB) subtypes M0/M1/M2, whereas high expression levels are less frequent in the monocytic FAB subtype M5 [10,11]. Second, while no expression is found in myeloid progenitors and mature myeloid in chronic phase CML cases, patients with CML in blast crisis, either myeloid or lymphoid, show higher levels of *BAALC* (unpublished observation). The predominance of high *BAALC* expression in the acute leukemias, its

absence in the chronic leukemias, and its restriction to $CD34^+$ hematopoietic progenitor cells point to a stage-specific expression.

A common model of hematopoiesis is based on the existence of three distinct lineages including lymphoid, myeloid, and erythro-megakaryocytic progenitors, all arising from a pluripotent hematopoietic stem cell [1,21]. Recently a new model proposed a common myeloid-lymphoid and a common myeloid-erythroid-megakaryocytic pathway, in which myeloid potential accompanies early stages of T- and B-cell as well as erythroid development [22,23]. The shared expression of certain genes in different lineages does not prove the existence of a common progenitor cell; however, the identification of involved genes could help to elucidate the regulatory network involved. *BAALC*, a gene with a progenitor-specific expression pattern, appears to be a novel marker common to myeloid, lymphoid, and erythroid pathways. Whereas this specific expression of *BAALC* may support the existence of a myeloid-lymphoid and a myeloid-erythroid pathway, it may also be a feature of the pluripotent hematopoietic stem cell maintained throughout the progenitor stage. We are currently investigating potential molecular mechanisms by which *BAALC* participates in hematopoiesis and leukemogenesis.

Acknowledgments

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