Differentiation 80 (2010) 195–203

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Role of NFKB2 on the early myeloid differentiation of CD34+ hematopoietic stem/progenitor cells ☆

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ARTICLE INFO

Article history:
Received 24 February 2010
Received in revised form
2 June 2010
Accepted 24 July 2010

Keywords:
Hematopoietic stem/progenitor cells
Bone marrow
Myeloid differentiation
NFKB2
siRNA

ABSTRACT

To better understand the early events regulating lineage-specific hematopoietic differentiation, we analyzed the transcriptional profiles of CD34+ human hematopoietic stem and progenitor cells (HSPCs) subjected to differentiation stimulus. CD34+ cells were cultured for 12 and 40 h in liquid cultures with supplemented media favoring myeloid or erythroid commitment. Serial analysis of gene expression (SAGE) was employed to generate four independent libraries. By analyzing the differentially expressed regulated transcripts between the un-stimulated and the stimulated CD34+ cells, we observed a set of genes that was initially up-regulated at 12 h but were then down-regulated at 40 h, exclusively after myeloid stimulus. Among those we found transcripts for NFKB2, RELB, IL1B, LTB, LTBR, TNFRSF4, TGFB1, and IKBKA. Also, the inhibitor NFKBIA (IKBA) was more expressed at 12 h. All those transcripts code for signaling proteins of the nuclear factor kappa B pathway. NFKB2 is a subunit of the NF-κB transcription factor that with RELB mediates the non-canonical NF-κB pathway. Interference RNA (RNAi) against NFKB1, NFKB2 and control RNAi were transfected into bone marrow CD34+HSPC. The percentage and the size of the myeloid colonies derived from the CD34+ cells decreased after inhibition of NFKB2. Altogether, our results indicate that NFKB2 gene has a role in the early commitment of CD34+HSPC towards the myeloid lineage.

1. Introduction

Hematopoiesis is a highly regulated process resulting in the formation of all blood lineages from the multipotential hematopoietic stem/progenitor cells (HSPCs) which possess the potentials of self-renewal, proliferation, and differentiation towards different lineages of blood cells(Mao et al., 1998).

The CD34 antigen is a surface glycophosphoprotein expressed on developmentally early lymphohematopoietic stem and progenitor cells, endothelial cells and embryonic fibroblasts and it is a hallmark of HSPC (Mayani and Lansdorp 1998; Krause et al., 1996). The earliest multilineage stem cell is CD34+, MDR-1+, c-kit+, and CD45RO+, but negative for CD38 and HLA-DR (Burt, 1999). CD34 antigen is found on progenitor cells that are already committed towards lineage specificity; within this CD34+ population resides a subset of multipotent stem cells capable of myeloid, lymphoid, erythroid, or megakaryocyte commitment (Sutherland et al., 1993). Progenitor cells expressing the surface antigen CD34 are also capable of long-term B lymphopoiesis and myelopoiesis *in vitro* and mediate T, B, and myeloid repopulation of human tissues implanted into SCID mice (DiGiusto et al., 1994; Gao et al., 2001).

Although much knowledge about hematopoietic stem cells has been gained, we still do not know much about the genetic mechanisms determining their development. One of the major questions in stem cell biology remains that the identification of the mechanisms by which a multipotent cell selects a particular differentiation pathway.

Several studies have been carried out to address the role of hematopoietic cytokines in this process (Phillips et al., 2000). However, it has been shown that the whole process of hematopoietic differentiation is orchestrated at the molecular level by a complex network of transcription factors that act by regulating the expression of a large set of target genes (Testa, 2004). As the phenotype of any given cell is ultimately the

☆This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Processos 2002/08273-1 and 1998/14247-6) and CNPq, Brazil.
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doi:10.1016/j.diff.2010.07.004

product of the genes, which is or has been expressed during the course of its development, describing the complete gene expression programs of self-renewing and differentiating cells is an approach that addresses how self-renew and differentiation are regulated (Liu et al., 2007).

Efforts to understand the molecular mechanisms underlying the differentiation of hematopoietic stem/progenitor cells into mature blood cells have focused so far on late events that largely reflect the differentiated state of the cell (Ferrari et al., 2007; Komor et al., 2005). In order to evaluate early changes of the gene expression profile of HSPC subjected to differentiation stimuli, we used serial analysis of gene expression (SAGE) to generate transcriptional profiles of immuno-magnetically sorted bone marrow CD34+ HSPC, before and after 12 and 40 h of culture in conditions favoring either myeloid or erythroid commitment. We observed a set of genes that was initially up-regulated at 12 h but then it was down-regulated at 40 h, exclusively after myeloid stimulus. Functional studies confirmed the early involvement of the NF-κB pathway in the commitment of CD34+ cells towards the myeloid differentiation.

2. Materials and methods

2.1. CD34+ hematopoietic and progenitor cells

Bone marrow iliac crest aspirates from healthy adult donors were collected after an informed consent was obtained, approved by the local Institutional Review Board. CD34+ cells were isolated to $>95\%$ purity by MACS Indirect CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec), following the manufacturer's instructions. Cell purity was determined by flow cytometry using anti-CD34-PE and anti-CD45-PerCP (BD Pharmingen) and data were acquired on a FACScan system (Becton Dickinson).

2.2. Differentiation assay

We used two different culture conditions to induce the differentiation process. For myeloid differentiation we used StemSpan (SteamCell Technologies, No. 09600) supplemented with 20% FBS, SCF (100 ng/ml), Flt-3lig (50 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml) and GM-CSF (20 ng/ml), whereas for erythroid differentiation we used StemSpan supplemented with 20% FBS, IL-3 (0.1 ng/ml), GM-CSF (1 ng/ml) and erythropoietin (3 U/ml). After 12 and 40 h of liquid culture, cells were collected and an aliquot of each sample was cultivated for 14 days in MethoCult-GF H4434 (StemCell Technologies, No. 04444) in order to examine the proportion of each type of colonies formed: BFU-E (burst forming unit-erythroid), CFU-GM (colony forming unit-granulocytic/monocytic) and CFU-Mix (mixed colony). All colonies containing more than 30 cells were scored as a colony-forming unit.

2.3. RNA extraction

The remaining cells, collected at 12 or 40 h of differentiation culture assays, were submitted to RNA extraction to construct the SAGE libraries. Four distinct libraries were constructed: myeloid for 12 h (M12) and 40 h (M40), erythroid for 12 h (E12) and 40 h (E40). Total RNA was prepared using TRizol® LS Reagent (Invitrogen Corporation), following manufacturer's instructions; RNA was obtained from a total of 8×10^6 polled cells for each library. Additionally, RNA obtained from CD34+ cells without any differentiation stimulus was used as a control (Panepucci et al., 2007).

2.4. SAGE transcriptomes

Twenty-eight micrograms of total RNA was used for each SAGE library, carried out by the I-SAGE™ Kit (Invitrogen Corporation, Cat no. T5001-01), following the manufacturer's instructions. Tag frequency tables were obtained from sequences by the SAGE™ analysis software with minimum tag count set to 1 and maximum ditag length set to 28 bp; the other parameters were set as default. The annotation was based on CGAP SAGE Genie (http://cgap.nci.nih.gov/SAGE). A SAGE library of un-stimulated CD34 BM cells, previously constructed in our laboratory (Panepucci et al., 2007), was used as a reference to compare the stimulated CD34 samples. For the analyses, the number of tags in each library were normalized to a total count of 300,000 tags. Statistical analyses were carried using the software SAGEstat (Ruijter et al., 2002) and a maximum p-value of 0.01 was used to consider a gene as differentially expressed. The SAGE data were further analyzed, through the use of PathwayStudio 4.0 (Ariadne Genomics Inc.) to search for meaningful biological interactions.

2.5. RNA-interference (RNAi) assay

siRNA mediating inhibition of gene expression was carried out using Stealth RNAi duplex oligoribonucleotides for NFKB1 and NFKB2 genes (Invitrogen corporation, Cat. no. 1299003—set of three oligos for each gene) and Stealth RNAi Negative Control (Invitrogen corporation, Cat. no. 12935-200); transfection was performed using Lipofectamine 2000 (Invitrogen Corporation, Cat. no. 11668-019). All the procedures were carried out following the manufacturer's instructions.

siNFKB1, siNFKB2 and negative control siRNA were transfected into CD34+ HSPC. RNAi assay was carried out in duplicate. Cells submitted to transfection with siRNAs and intact cells were cultured in the same condition that stimulates the myeloid differentiation process; after 72 h of culture they were subjected to evaluation by real-time PCR and an aliquot of the cells were cultured on methylcellulose.

2.6. Quantitative polymerase chain reaction

To confirm SAGE data and to measure gene expression of the siRNA experiments, real-time polymerase chain reaction was performed. Total RNA (0.5 μg) was processed directly to cDNA by reverse transcription using High Capacity cDNA Archive Kit (Applied Biosystems) following manufacturer's instructions.

Real-Time PCR to validate the SAGE libraries was performed in duplicates for 13 genes and, were carried out with TaqMan probes and Master Mix (Applied Biosystems). To normalize sample loading, the differences of threshold cycles (ΔCt) were derived by subtracting the Ct value for the internal reference (GAPDH), from the Ct values of the evaluated genes. The relative fold value was obtained by the formula $2^{-\Delta\Delta Ct}$ using the ΔCt value of un-stimulated CD34+ sample as a reference, ΔΔCt was calculated by subtracting the reference ΔCt from the ΔCt values of the samples.

Real-time PCR to confirm the knock-down efficiency was performed for NFKB1 and NFKB2 genes. TaqMan probes and Master Mix (Applied Biosystems) were also employed as well as the $2^{-\Delta\Delta Ct}$ method and GAPDH as internal reference. The ΔCt value of CD34+ without siRNA transfection was used as a reference, ΔΔCt was calculated by subtracting the reference ΔCt from the ΔCt values of the siRNA transfected samples. Statistical significance of difference between the siRNA samples and the control siRNA was determined by unpaired Student's t-test.