Leukemia Research 37 (2013) 705–712

Contents lists available at SciVerse ScienceDirect

Leukemia Research

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

Bone morphogenetic proteins regulate differentiation of human promyelocytic leukemia cells

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ARTICLE INFO

Article history:
Received 26 December 2012
Received in revised form 1 March 2013
Accepted 3 March 2013
Available online 23 March 2013

Keywords:
Acute promyelocytic leukemia
Bone morphogenetic proteins
All-trans retinoic acid
Differentiation
ID genes
Cell lines
Patients

ABSTRACT

We investigated the role of bone morphogenetic proteins (BMPs) in suppression of all-trans retinoic acid (ATRA)-mediated differentiation of leukemic promyelocytes. In NB4 and HL60 cell lines, BMPs reduced the percentage of differentiated cells, and suppressed PU.1 and C/EBPε gene expression induced by ATRA. BMP and ATRA synergized in the induction of ID genes, causing suppression of differentiation. In primary acute promyelocytic leukemia bone-marrow samples, positive correlation of PML/RARα and negative of RARα with the expression of BMP-4, BMP-6 and ID genes were found. We concluded that BMPs may have oncogenic properties and mediate ATRA resistance by a mechanism that involves ID genes.

1. Introduction

Acute promyelocytic leukemia (APL) is characterized by the accumulation of abnormal promyelocytes unable to differentiate into granulocytes and specific translocation t(15;17)(q22;q21) [1,2]. Translocation results in the formation of PML/RARα (promyelocytic leukemia/retinoic acid receptor α) fusion gene that plays a central role in leukemogenesis of APL by blocking the function of RARα and PML proteins responsible for differentiation and apoptosis of APL cells [3]. All-trans retinoic acid (ATRA) is used for treatment of APL patients with high rate of complete remission when used alone or in combination with standard cytotoxic treatment [4]. Retinoic acid (RA) acts as morphogen through two subfamilies of nuclear receptors (RAR and RXR), ligand inducible transcription factors, in order to regulate the expression of genes containing retinoid response elements [5]. ATRA has been shown to induce terminal differentiation of APL cells by activation of RARα, and by inducing degradation of PML/RARα [2]. However, prolonged ATRA treatment often results in relapse due to the development of ATRA resistance by leukemic cells [4]. Although early studies suggested that an adaptive hypercatabolic response to pharmacological doses of ATRA is the principal mechanism of resistance, recent observations suggest that molecular disturbances in APL cells have a predominant role [6].

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor β (TGF-β) superfamily. Besides their role in bone physiology, they also regulate proliferation, differentiation, morphogenesis and apoptosis of hematopoietic cells. BMP-2 can induce hematopoietic environment after subcutaneous implantation and regulate apoptosis in various B-cell lines and primary myeloma cells [7–9]. BMP-4 has a crucial instructive role for the induction and formation of blood cell precursors [10] and induces formation of embryonic hematopoietic tissues [11]. BMP-6, produced by bone-marrow (BM) cells, participates in the regulation of proliferation, apoptosis and differentiation of B-cell lineage [12]. Dosen-Dahl et al. showed that treatment with TGF-β or BMP-6 protects leukemic cells against chemotherapy-induced cell death and allow them to escape therapy [13].

BMPs are known to exert their effect through two different types of serine-threonine kinase receptors known as receptors type I (BMP receptor (BMPR)IA, BMPRIB and activin receptor (ActR)IA)

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http://dx.doi.org/10.1016/j.leukres.2013.03.002

and type 2 (BMPRII, ActRIIA and ActRIIB), and intracellular SMAD (small mothers against decapentaplegic) proteins, which play a central role in BMP signaling [14]. Important direct target of SMAD pathway is the gene family of DNA binding inhibitors (ID) [15]. ID genes encode a family of proteins that block basic helix-loop-helix transcription factors to bind to DNA, thus interrupting their regulatory role in many developmental and differentiation processes. Different studies on human tumors proved their oncogenic properties in regulating cell-cycle progression, migration and invasiveness [15–19].

Our previous study has shown that BMP-2, BMP-4 and BMP-7, and their receptors were strongly expressed in patients with APL, paralleled by the expression of PML/RARα oncogene. Absence of BMP expression correlated with clearance of the tumor molecular marker [20]. In this study, we aimed to investigate molecular mechanisms by which BMPs suppress differentiation of APL cells induced by ATRA, using myeloid cell lines NB4 and HL60, and primary samples from APL patients. Since RA/RAR pathway functionally interacts with BMP/SMAD signaling [21], we proposed that BMP signal interfere with ATRA-induced differentiation of APL cells. Our findings may add to the understanding of the mechanisms of resistance to ATRA in APL patients.

2. Materials and methods

2.1. Patient samples and cell lines

Human myeloid leukemia cell lines NB4 and HL60 (DSMZ cell line collection, Braunschweig, Germany) were grown in RPMI 1640 with 10% fetal calf serum (FCS) (Gibco, Invitrogen Ltd., Grand Island, NY, USA), 2 mM glutamax and 100 U/mL penicillin/streptomycin, in a 5% CO_2 at 37 °C. APL cell line NB4 carries the specific t(15;17) translocation, whereas HL60, a myeloblastic leukemia cell line, lacks t(15;17) translocation but differentiates along granulocytic pathway upon ATRA treatment. To induce differentiation, cells (0.3×10^6/mL in 25 cm^2 flasks) were treated with ATRA (0.5 μM for NB4 and 2 μM for HL60). Recombinant human (rh)BMP-2, rhBMP-4 or rhBMP-6 (all from R&D Systems, Abingdon, UK), and soluble BMP antagonist NOGGIN (NOG) (PeproTech, London, UK) were added as indicated in each experiment.

After obtaining approval from the institutional Ethics Committee and informed consent from participants, we analyzed BM and peripheral blood (PBL) samples from 5 APL patients collected as a part of routine clinical assessment, at diagnosis and during the clinical follow-up. BM specimens were obtained by sterile punction of the iliac crest. PBL was drawn by standard venipuncture, followed by mononuclear cell separation using Histopaque (Sigma, St. Louis, MO, USA; density 1.077 g/mL). Diagnosis was established by morphological characterization, cytochemistry, and cytogenetic- and immuno-phenotyping according to the criteria of French–American–British classification for AML-M3 [22]. Presence of PML/RARα fusion gene as a tumor molecular marker was confirmed by polymerase chain reaction (PCR) analysis. Patients were treated with the combination of oral ATRA (45 mg/m^2 per day), divided into 2 doses, and idarubicin (12 mg/m^2 on days 2, 4, 6 and 8) as described previously [20].

2.2. May-Grünwald Giemsa staining

Cytospin preparations for standard May-Grünwald Giemsa staining were made after ATRA treatment, as indicated in each experiment, to detect morphological changes associated with granulocyte differentiation (pale cytoplasm and mulitlobulated segmented nucleus) [23].

2.3. Flow-cytometric analysis

Myeloid leukemia cell lines NB4 and HL60 were analyzed by flow cytometry using a FACS Calibur instrument and Cell-Quest software (BD Biosciences, San Jose, CA, USA). Cells were analyzed for the phenotypic evidence of differentiation (expression of granulocyte surface markers CD11b and CD11c) by standard protocols [24]. Viability and apoptosis were evaluated by annexin V/propidium iodide (PI) staining (BD Biosciences) according to the manufacturer's instructions. Cells were classified on dot-plots as dead (PI^+), apoptotic (PI^-/annexin V^+) or viable (double negative). For cell-cycle analyses, cells were resuspended in DNA staining solution (0.01 M Tris, 10 mM NaCl, 700 U/L RNAse, 7.5×10^{-5} M PI, 0.01% Nonidet P-40). Cell-cycle distribution was presented as histograms (G_0/G_1, S, G_2/M phases) [8].

2.4. Gene expression analysis

Total RNA was extracted (TriPure; Roche Mannheim, Germany) from primary APL BM and PBL samples, or NB4 and HL60 cells, reversely transcribed to cDNA (MuLV Reverse Transcriptase; Applied Biosystems, Foster City, CA) and amplified by quantitative (q)PCR using specific TaqMan assays (Supplementary Table 1) in an ABI Prism 7000 Sequence Detection system (Applied Biosystems). Each reaction was performed in triplicate in a 25 μL reaction volume as previously described [8]. The relative quantities of unknown samples for the each gene were interpolated from the six-point serial dilution standard curve of the calibrator sample (BM cells or cell lines). To equalize samples according to the amount of input cDNA, the relative quantity of the target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.03.002.

2.5. Statistics

All experiments were repeated at least three times. Gene expression triplicates in treated cell lines were expressed as mean±standard deviation (SD) and compared using analysis of variance with Student–Newman–Keuls *posthoc* test. Gene expressions in primary samples were correlated to clinical data using rank correlation and Spearman's coefficient of rank correlation rho (ρ) with its 95% confidence interval. Statistical analysis was performed using MedCalc software-package (Mariakerke, Belgium). For all experiments, α-level was set at 0.05.

3. Results

3.1. ATRA induced differentiation of NB4 and HL60 cell lines

We confirmed ATRA-induced differentiation of myeloid leukemia cell lines NB4 and HL60 by the expression of differentiation makers (CD11b/CD11c), and changes in cell proliferation and apoptosis. Differentiation was followed up to 4 days, with increase in the expression of CD11b and CD11c in both cell lines with the time of ATRA treatment (Fig. 1A). Differentiated cells showed morphological changes characteristic for granulocytic differentiation, containing pale cytoplasm and multilobulated segmented nucleus (Fig. 1A). At the representative time-point (day 3 of ATRA treatment for NB4 and day 4 for HL60, according to the percent of CD11b/CD11c-positive cells), we observed the suppression of cell-cycle progression (Fig. 1B). Furthermore, ATRA decreased the percentage of apoptotic NB4 cells, but increased the percentage of apoptotic HL60 cells, indicating that proapoptotic effect is not associated with t(15;17) rearrangement [25]. Finally, we determined the expression of PML/RARα oncogene in NB4 cells by qPCR, which was further increased by ATRA treatment together with the expression of total RARα (Fig. 1C). We also confirmed the responsiveness of cell lines to BMPs by detecting gene expression for BMP receptors (ActRIA, BMPRIA, BMPRIB, BMPRII, ActRIIA, ActRIIB), BMP-signaling related molecules (SMAD5, SMAD6, NOG, bone morphogenetic and activin membrane-bound inhibitor (BAMBI)) and BMP-target genes (ID1, ID2, cKit), with and without ATRA treatment (Fig. 1C). The major difference between two analyzed cell lines, that could influence the functional response of those cells to BMP treatment, is huge induction of ID genes in NB4 cells by ATRA and constitutively lower expression of cKit in HL60 cells.

3.2. BMPs suppressed differentiation of ATRA-treated NB4 and HL60 cell lines

Since our previous study on APL primary BM samples showed that treatment with ATRA was associated with the suppression of BMP expression [20], we aimed to reveal the effect of BMPs on ATRA induced differentiation of NB4 cells as an in vitro model for APL. HL60 cell line, not bearing the specific t(15;17)(q22;q21) translocation, was used in addition to test if the BMP effect is PML/RARα specific. Cells were treated with the combination of ATRA and BMP-2, BMP-4 or BMP-6. BMP alone did not affect the expression of differentiation markers, but in combination with ATRA reduced the percent of cells expressing CD11b and CD11c by 40 to 60% respectively, with stronger effect in NB4 cells (Fig. 2A). This effect was