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Nuclear factor of activated T-cells 1 increases sensitivity of v-*myb* transformed monoblasts to *all-trans* retinoic acid

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ABSTRACT

Nuclear factors of activated T-cells (NFATs) are important regulators of the cytokine gene expression in activated T-cells. In the last decade, NFATs have been shown to regulate cell cycle, differentiation and apoptosis in cells of various origins revealing their importance for cell homeostasis. In this study, we investigated the effects of NFAT1 on proliferation and differentiation of v-*myb*-transformed BM2 monoblasts. In contrast to many other leukemic cell lines, BM2 cells do not respond to retinoic acid. However, once overexpressing NFAT1, they became sensitive to *all-trans* retinoic acid (ATRA). The ATRA-treated BM2NFAT1 cells differentiated along monocyte/macrophage pathway as evidenced by changes in cell morphology, adherence, phagocytic and non-specific esterase activities, reactive oxygen species production, and vimentin expression. Furthermore, overexpressed NFAT1 either alone or in combination with the ATRA-driven signalling pathway deregulated cyclin A and retinoic acid receptor proteins in BM2 cells. Data presented in this study indicate that the NFAT1 and ATRA signalling pathways synergize in control of proliferation and differentiation of BM2 monoblasts.

1. Introduction

NFAT1, NFAT2, NFAT3, NFAT4 and NFAT5 are members of the family of nuclear factors of activated T-cells. These proteins have been extensively studied as important regulators of the cytokine gene expression in activated T-cells [1,2]. Multiple lines of evidence document the role of the NFAT proteins in hematopoiesis. First, early hematopoietic precursor cells exhibit high level of NFAT1 expression [3]. Second, NFATs participate in control of proliferation and selection of immature thymocytes and in antigen-induced differentiation of naive T helper (Th) cells into effector Th1/Th2 cells [4,5]. Third, NFAT2 acts as essential regulator of differentiation of bone-resorbing osteoclasts from myelomonocytic precursors [6]. In addition, the NFAT proteins regulate differentiation and development of cells of central nervous system, blood vessels, heart, kidney and skeletal muscle [7–9]. Generally, the NFAT transcription factors control expression of genes involved in regulation of cell cycle [10,11], differentiation [12], apoptosis [13] and invasiveness [14]. These features predispose the NFAT proteins to play an important role in cancerogenesis. There are data indicating that NFATs can act both as oncoproteins and tumor suppressors [15,16].

BM2 monoblasts constitutively express the v-*myb* oncogene of avian myeloblastosis virus [17]. v-*myb* is viral counterpart of the c-*myb* protooncogene that is highly expressed in immature cells of all hematopoietic lineages. The level of c-*myb* expression decreases as hematopoietic cells differentiate to terminal stages [18]. Unlike several other leukemic cell lines [19,20], BM2 cells do not respond to retinoids. However, their sensitivity to retinoids can be resumed by ectopic expression of retinoic acid receptor α (RARα) [21] or c-Jun [22]. Interestingly, interaction and cooperativity of RARα and NFAT1 proteins in macrophages and T-cells have been recently described [23,24]. The aim of this study was to evaluate the role of NFAT1 in control of differentiation and proliferation of v-*myb*-transformed monoblasts. We found that overexpressed NFAT1 reduced proliferation of BM2 cells and sensitized them to the differentiation-inducing effect of ATRA.

2. Materials and methods

2.1. Cell culture

BM2 monoblasts and their derivatives were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf

Abbreviations: BM2wt, BM2 wild type cells; BM2NFAT1, BM2 cells transfected with the pEGFP.N1-NFAT1 plasmid; BM2VIVIT, BM2 cells transfected with the pEGFP.N1-VIVIT plasmid; BM2mock, BM2 cells transfected with the pEGFP.N1 plasmid; ATRA, *all-trans* retinoic acid; CDK4, cyclin-dependent kinase 4; CsA, cyclosporin A; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); MFI, median of fluorescence intensity; NBT, nitroblue tetrazolium; NFAT, nuclear factor of activated T-cells; NSE, non-specific esterase; PBS, phosphate-buffered saline; PPARγ, peroxisome proliferator-activated receptor gamma; PVDF, polyvinylidene fluoride; RARα, retinoic acid receptor α; RLU, relative light units; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Th, T helper cell; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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serum, 5% heat-inactivated (1 h at 56 °C) chicken serum, 4.5 g/l glucose, 1 x non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 mg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere of 10% CO_2 at 37 °C. ATRA (Sigma-Aldrich, St. Louis, MI) to be used as differentiation inducer was diluted in dimethyl sulfoxide (DMSO) and added to cultivation media at the final concentration of 1 μM. The same amount of DMSO solvent was used as a negative control. ATRA in 1 μM concentration did not affect viability of BM2 cells during 5 days of treatment.

2.2. Cell transfection and transactivation assays

BM2NFAT1 and BM2VIVIT cells were derived from BM2 monoblasts by transfection with the pEGFP.N1-NFAT1 or pEGFP.N1-VIVIT plasmids [25,26] encoding the full-length wild-type murine NFAT1 protein (pEGFP.N1-NFAT1) and the NFAT-activation specific peptide inhibitor VIVIT fused with GFP (pEGFP.N1-VIVIT), respectively. Twenty-four hours after the transfection by lipofection using the Fugene HD transfection reagent (Roche Diagnostic, Indianapolis, IN), G418 (Life Technologies, Gaithersburg, MD) was added at the final concentration of 400 μg/ml. The pool of G418-resistant cells was selected and cloned by limiting dilution. Two independent (BM2NFAT1-A2, BM2NFAT1-E5) and (BM2VIVIT-A1, BM2VIVIT-C) clones were chosen for further experiments. Similarly, BM2mock cells to be used as negative controls were derived from BM2 cells by transfection with the vector alone.

To assess transactivation by NFAT1, we used the pNFAT-Luc (Stratagene, La Jolla, CA) reporter plasmid. BM2NFAT1, BM2VIVIT and BM2mock cells were transiently co-transfected with 1 μg of the reporter plasmid and 1 μg of control plasmid CMV-βgal using the Fugene HD transfection reagent. Twenty-four hours after the transfection, the cells were harvested, washed with PBS and processed for luciferase and β-galactosidase assays as described elsewhere [21]. Luciferase activity was expressed in relative light units and normalized for transfection efficiency according to the β-galactosidase activity.

2.3. Electrophoresis and immunoblotting

The cells were harvested and lysed by boiling in sodium dodecyl sulfate (SDS)-loading buffer containing 0.1 M Tris (pH 6.8), 16% v/v glycerol, 3.2% w/v SDS, 10% v/v β-mercaptoethanol and 0.005% w/v bromophenol blue. Sample loading was normalized according to the protein concentration determined by DC protein assay (Bio-Rad, Hercules, CA). Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The blots were probed with either anti-NFAT1 monoclonal antibody (556601, BD Pharmingen, San Diego, CA), anti-cyclin A monoclonal antibody (sc-53228, Santa Cruz Biotechnology, Santa Cruz, CA), anti-vimentin monoclonal antibody (V5255, Sigma-Aldrich, St. Louis, MI), anti-PPARγ polyclonal antibody (#2435, Cell Signaling Technology, Inc., Beverly, MA) or anti-RARα polyclonal antibody (sc-551, Santa Cruz Biotechnology, Santa Cruz, CA). Anti-actin (A5060, Sigma-Aldrich, St. Louis, MI) or anti-lamin B (sc-6216, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used as loading controls. The blots were developed with anti-mouse IgG or anti-rabbit IgG secondary antibodies conjugated with either horseradish peroxidase (A9041, A4914, Sigma-Aldrich, St. Louis, MI) or alkaline phosphatase (A3562, A3687, Sigma-Aldrich, St. Louis, MI) by standard procedures using Western Chemiluminiscent HRP Substrate (Millipore, Billerica, MA) or 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (NBT), respectively. Density of the bands was quantified by scanning densitometry using the ImageJ image analysis software (NIH, Bethesda, MD) and normalized according to the β-actin controls.

2.4. Proliferation and cell cycle analyses

To test proliferation rate, 3×10^4 of BM2NFAT1 and BM2mock cells were cultured in the presence of 1 μM ATRA or DMSO for 4 days. Living cells were counted daily using CASY cell counter (Roche-Innovatis, Basel, Switzerland). For cell cycle analyses, 2×10^5 of cells were treated with 1 μM ATRA or DMSO for 3 days, washed twice with 4 ml of ice-cold phosphate buffered saline (PBS), resuspended in 0.5 ml of ice-cold PBS and fixed with 4 ml of 70% ethanol for 24 h. Fixed cells were centrifuged, washed with PBS and stained with propidium iodide as described previously [27]. The DNA content was measured by the FACS CaliburTM system (Becton Dickinson, San José, CA). At least 15,000 cells were analyzed and frequency of cells in each phase of the cell cycle was determined using the ModFit 3.0 software (Verity Software House, Topsham, ME).

2.5. Oxidative burst analysis

The ability of cells to produce reactive oxygen species (ROS) upon treatment with ATRA was determined by NBT test [27]. 2×10^5 of cells were treated with ATRA or DMSO for 5 days. Next, 1×10^6 of cells were washed with PBS and resuspended in 400 μl of DMEM supplemented with 4.5 g/l glucose, 1x non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 mg/ml streptomycin and 100 U/ml penicillin. Next, 200 μl of NBT in PBS (1 mg/ml) and 400 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA) were added to cell suspension and incubated at 37 °C for 30 minutes. Then, the cells were centrifuged at 500 ×*g* for 10 min, resuspended in 1 ml of DMSO, boiled for 5 min and cooled. The mixture was clarified by centrifugation at 1000 ×*g* for 5 min and the optical density at 570 nm was determined by spectrophotometry using DMSO as a blank.

2.6. Non-specific esterase (NSE) activity assay

The NSE activity was determined as described previously [27]. 2×10^5 of cells were treated with ATRA or DMSO for 5 days. Then, 5×10^5 of cells were washed twice with the HEPES/NaCl buffer (150 mM NaCl, 25 mM HEPES, pH 7.6) and suspended in 250 μl of the HEPES/NaCl buffer containing 0.2 mg/ml α-naphthyl acetate and incubated at 37 °C for 30 min. The reaction was stopped by 100 μl of SDS buffer (4% SDS, 10 mM sodium-barbital, 100 mM sodium acetate, pH 8.0) with 0.2% Fast Blue BB salt. The samples were incubated at laboratory temperature for 10 min. Optical density was measured by spectrophotometry at 492 and 620 nm wavelengths.

2.7. Phagocytosis assay

2×10^5 of cells were treated with ATRA or DMSO for 4 days. Then, 3×10^7 of Dynabeads M-270 Epoxy (Dynal, Oslo, Norway) were added to media and cultured for next 24 hours. Then, the cells were washed with PBS, suspended in 1 ml of PBS and transferred to 2 ml of Histopaque-1077 (Sigma-Aldrich, St. Louis, MI). Following centrifugation, the cells were washed with PBS, suspended in 10 μl of PBS and dropped on a pre-stained slide (Testsimplets, Roche, Indianapolis, IN). The cells that engulfed at least one bead in population of 400 cells were enumerated by light microscopy.

2.8. Cell surface marker analysis

To assess the relative amount of the cell surface antigen Mo-1 [28], 2×10^5 of cells were treated with ATRA or DMSO for 5 days. Next, 5×10^5 of cells were centrifuged (200 ×g/5 min) and washed with ice-cold PBS containing 1% bovine serum albumin. The cells were incubated with the Mo-1-specific monoclonal antibody supernatant [28] for 40 min and subsequently with the FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MI) for 30 min. Then, the cells were