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Effects of retinoic acid and histone deacetylase inhibitor Bml-210 on protein expression in NB4 cells

V. V. Borutinskaite[1, 2*],

K.-E. Magnusson[2],

R. Navakauskienë[1]

[1] *Department of Developmental Biology, Institute of Biochemistry, LT-08662 Vilnius, Lithuania*

[2] *Division of Medical Microbiology, Department of Molecular and Clinical Medicine, Linkoping University, SE-581 85 Linkoping, Sweden*

The acute promyelocytic leukemia cell line NB4 can differentiate to granulocytes in response to retinoic acid (RA) or to monocytes / macrophages in response to 1.25 dihydroxyvitamin D_3 (1.25 D_3) and phorbol esters. We have examined changes in protein levels during granulocytic differentiation of NB4 cells treated with RA alone or in combination with the histone deacetylase inhibitor (HDACI), Bml-210. By MALDI-TOF MS analysis we identified proteins that displayed significant changes in the expression level with indicated treatments. One of the identified proteins whose expression levels underwent significant changes is β-dystrobrevin, a splice isoform of α-dystrobrevin.

We further demonstrated changes in the expression of α-dystrobrevin in total soluble and insoluble protein fractions of NB4 cells treated with RA or Bml-210 alone or in combination. We found increased levels of α-dystrobrevin in the insoluble fraction of NB4 cells exposed to RA or in combination with Bml-210 for 24–48 h. The current results indicate an association between the increased level of dystrobrevin expression and induction of differentiation by RA. In the present work we also characterised some proteins that are present in proliferating leukemia cells and may be involved in the differentiation process in response to RA and combination of RA with HDAC inhibitor. Our results suggest that HDACI Bml-210 may be a promising agent in leukemia therapy.

Key words: differentiation, NB4 cells, dystrobrevin, histone deacetylase inhibitor

INTRODUCTION

Acute promyelocytic leukemia (APL) is a well-defined entity among acute leukemias, cytogenetically characterized by a t (15; 17) (q22; q11–12) translocation [1]. The NB4 cell line has become one of the most important cell culture models for studying differentiation therapy in acute promyelocytic leukemia [2, 3]. It undergoes neutrophilic or monocytic differentiation in response to all-trans retinoic acid (ATRA), 1.25 dihydroxyvitamin D_3 (1.25 D_3) and phorbol esters, respectively [4].

The promyelocytic leukemia (PML) and retinoic acid receptor alpha (RARalpha) fusion product recruits histone deacetylase (HDAC) and DNA methyltransferase (DNMT) effects on retinoic acid (RA)-target promoters causing their silencing and a differentiation block. Pharmacological doses of RA induce epigenetic modifications at its target loci and restore myeloid differentiation of APL blasts [3]. APL cells containing the PLZF-RARα are resistant to RA, but transcriptional repression mediated by HDAC can be released with HDAC inhibitors [5]. The addition of HDAC inhibitors (HDACI) has been shown to inhibit proliferation and to stimulate differentiation and apoptosis in transformed cells *in vitro* and *in vivo* [6]. The implications of HDACI for differentiation therapy are currently under extensive investigation.

RA-mediated transcription can be facilitated by other differentiation inducers or HDACI, such as sodium butyrate or phenyl butyrate; these inhibit histone deacetylase activity and are currently used in the clinic. Bml-210 is a novel agent which inhibits histone deacetylase activity. Several observations have predicted that histone deacetylase inhibitors can antagonize oncogenic fusion proteins by partially decreasing transcriptional repression of crucial genes involved in cell growth, differentiation and apoptosis. The identification of site-specific histone deacetylase

* Corresponding author. Department of Developmental Biology, Institute of Biochemistry, Mokslininku 12, LT-08662 Vilnius, Lithuania. E-mail: veronika.borutinskaite@one.lt

inhibitors may open the way for a more rational development of differentiation therapy [7]. Thus, it is important to know more about the specific proteins that are involved in these all processes, and changes of their expression level.

In this study, we characterised some proteins that are present in proliferating leukemia cells and may be involved in the differentiation process in response to RA and a combination of RA with the HDAC inhibitor Bml-210. Moreover, we have investigated the properties of dystrobrevins in association with NB4 cell granulocytic differentiation as mediated by RA alone or in combination with Bml-210.

MATERIALS AND METHODS

Cell culture

The human APL cell line NB4 was cultured in RPMI media (Gibco, Invitrogen, Sweden) containing 10% of fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Invitrogen, Sweden), and 20 mM glutamine at 37 °C in humidified air and 5% CO_2. The cultures were seeded at a density of 4 × 10^5 cells/ml and (at maximum 1.54 × 10^6 cells/ml) subsequently transferred to a fresh medium.

Cell treatment with all-trans retinoic acid and Bml-210

On the day of induction, cells were seeded at a density of 4 × 10^5 cells/ml in the above medium and treated with or without 1 μM RA or 10 μM Bml-210, or in combination – at 1 μM and 5 μM, respectively, for different periods of time (2, 4, 8, 24, 48, 72, 96 h). The level of differentiation was determined by nitro blue tetrazolium (NBT)-reduction assay.

Isolation and SDS-PAGE fractionation of total soluble and insoluble protein cell fractions

Briefly, cells were harvested and washed twice in PBS (pH 7.5), resuspended to 3 × 10^7 cells/ml in lysis solution (150 mM NaCl, 10 mM Tris/HCl (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 5 mM NaF, 1 mM Na_3VO_4, and 1 × protease inhibitor cocktail (Roche). The lysates were homogenized and incubated for 15 min at 0 °C and then centrifuged at 15,000 g for 30 min. The supernatants were collected as soluble protein fraction. The pellet was dissolved by adding 1/10 volumes of benzonase (Merck, Germany) and a 2 × SDS loading buffer (Fermentas, Lithuania) and was called insoluble protein fraction.

For SDS-PAGE, to the protein samples equal volumes of 2 × SDS loading buffer (Fermentas, Lithuania) we added and boiled for 5 min. The supernatants were immediately subjected to electrophoresis or frozen at –76 °C. For the former technique, we used an 8–16% polyacrylamide gradient gel in Tris-glycine electrophoresis buffer. After SDS-PAGE, gels were stained with Brilliant Blue G-Colloidal (Sigma) or immunoblot analysis was performed.

Immunoblot analysis

After SDS-PAGE, proteins were transferred to Immobilon™ PVDF membranes (Millipore) and blocked by incubating with 3% BSA dissolved in PBS supplemented with 0.1% Tween-20 at 4 °C overnight. Then the membranes were incubated for 1 h at room temperature with antibodies against α-dystrobrevin [8] at a concentration of 2 μg/ml in PBS containing 0.1% Tween-20, 0.35 M NaCl, and 1% BSA. The membranes were subsequently washed 3 × 10 min with PBS-Tween-20 and then incubated with a horseradish-peroxidase-conjugated secondary antibody (DAKO, Copenhagen, Denmark) diluted 1:2000 in PBS-Tween-20 for 1 h at room temperature. Thereafter, the filters were washed as described above, and immunoreactive bands were detected by enhanced chemiluminescence (ECL™ Western blotting detection reagents, Amersham Biosciences, Sweden) according to the manufacturer's instructions. Reprobing of the membranes, if needed, was done according to the standard ECL Western blotting protocols (Amersham Biosciences, Sweden).

In-gel tryptic digestion and MALDI-TOF MS

The areas of the gel that were deemed to be of interest were cut out and subjected to in-gel tryptic digestion overnight [9], the gel slices were dehydrated with 50% acetonitrile and then dried completely using a centrifugal evaporator (DNA Mini, Eppendorf). The protein spot was rehydrated in 20 μl of 25 mM ammonium bicarbonate (pH 8.3) containing 20 μg/ml modified trypsin (Promega). Once this solution was fully absorbed by the gel, a trypsin-free buffer was added just enough to cover the slice, and the samples were incubated overnight at 37 °C. The tryptic peptides were subsequently extracted from the gel slices as follows. Any extraneous solution remaining after the digestion was removed and placed in a fresh tube. The gel slices were first subjected to an aqueous extraction and then to organic extraction with 5% trifluoracetic acid in 50% acetonitrile, shaking occasionally. The digestion and extract solutions were then combined and evaporated to dryness. For MALDI-TOF (Matrix-Assisted Laser Desorption / Ionization Time-Of-Flight) analysis, the peptides were redissolved in 3 μl of 30% acetonitrile and 0.01% trifluoracetic acid and were then prepared with a matrix (α-cyano-4-hydroxicinnamic acid) on the target plate. The analysis was performed on a Voyager MALDI-TOF MS (Perspective Biosystems Inc., Town State) and externally calibrated using synthetic peptides with known masses. The spectra were obtained in the positive ionization mode at 25 kV. The mass information generated from the composite spectrum was submitted to a search performed with

the Protana or EXPASY database, using the MS-Fit algorithm or the PeptIdent search engine.

RESULTS AND DISCUSSION

The present study was designed to determine changes in protein expression during differentiation of acute promyelocytic leukemia cells after treatment with the differentiation inducer RA and HDAC inhibitor Bml-210. NB4 cells were treated with RA or histone deacetylase inhibitor Bml-210 alone, or in combination. The changes in protein expression after different times of treatment (2, 4, 8, 24, 48, 72, 96 h) were observed. Our previous data demonstrate that the highest amount of differentiated cells after RA treatment was noticed at 72–96 h when the proportion of differentiated cells in cell population had reached up to 70% and 80%, respectively [10]. Our latest results (unpublished data) showed that combinative RA and Bml-210 treatment significantly enhances granulocytic differentiation of NB4 cells.

To investigate changes in protein expression in the proliferating and NB4 cells treated with RA and Bml-210 the total soluble and insoluble protein fractions were fractionated by SDS-PAGE and then stained with Brilliant Blue G-Colloidal (Fig. 1A, B). Bands of interest were cut from the SDS-PAGE gels (Fig. 1B), digested with trypsin and analyzed by MALDI-TOF. Tryptic digestion of proteins leads to peptides of different length and abundance, depending on the distribution of corresponding tryptic cleavage sites (arginine and lysine).

Protein identification was performed by searching in a protein sequence database (SwisProt) using the PeptIdent and MS-Fit programs. The following parameters were used for database searches: monoisotopic mass accuracy 50–100 ppm, missed cleavages 0–1, and complete carbamidomethylation of cysteines. The list of proteins identified by PeptIdent program is presented in Table.

Most of the identified proteins are known to be present in cancerous cells and are membrane or membrane-associated proteins. Some proteins (Fig. 1B, band No. 02) are expressed only in the insoluble fraction of NB4 cells treated with RA or Bml-210 alone or in combination after 24 h. We have identified that 26 kDa protein (Fig. 1B, band No. 02) matches with Rab2B and US6NL proteins. Rab2B belongs to the largest Ras-like small GTPase family, the Rab family involved in cellular transport functions and cell motility. In its GTP-binding active form, Rab proteins are membrane-associated; however, in its GDP-binding inactive form they are mainly distributed in plasma. Rab GDP dissociation inhibitor (GDI3) forms a soluble complex with Rab proteins and thereby prevents the exchange of GDP for GTP during cell differentiation [11, 12]. Rab5 can be activated by receptor tyrosine kinases (RTK). Also, Rab5 interacts with actin, F-actin binding protein and actinin-4 [13]. It is known that another member of the Rab family, Rab7b, is a lysosome-localized monocytic cell-specific small GTPase, and is involved in PMA-induced APL cell differentiation and possibly in the regulation of monocyte functions [14]. We found that after RA and Bml-210 stimulation of NB4 cells, the expression of Rab2B in insoluble fraction is greatly decreased after 8 h of treatment, concomitantly with the increase in total soluble protein fraction during the same time of treatment, indicating that Rab2B expression may be associated with granulocytic differentiation of NB4 cells.

In the same band (Fig. 1B, No. 02), we identified USP6NL protein. This protein acts as a GTPase activating pro-

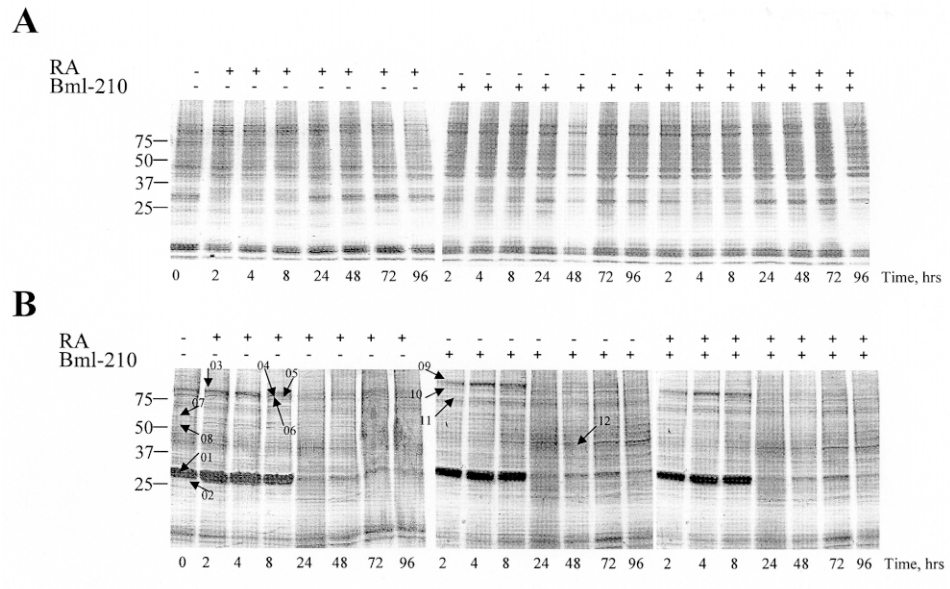

Fig. 1. Expression of proteins in NB4 cells treated with RA and Bml-210
Total soluble (A) and insoluble (B) protein fractions were isolated from control and NB4 cells treated with 1 μM RA and 10 μM Bml-210 alone or in combination (1 μM RA and 5 μM Bml-210) indicated period of time. The isolated proteins were fractionated by SDS-PAGE on an 8–16% acrylamide gradient gel and then stained with brilliant Blue G-Colloidal. Migration of the molecular size marker proteins is indicated to the left (kDa values). The position of bands that were cut to MALDI-MS analysis is designated by arrows and numbers in the images. Migration of the molecular size marker proteins is indicated to the left (kDa values)

Table 1. **Proteins identified using PeptIdent program and EXPASY database. experimental (gel position) and calculated Mw and pI. MOWSE SCORE and sequence coverage (PeptIdent) for each protein were shown**

Band No.	Calculated		Experimental	PeptIdent		Protein name
	pI	Mw(kDa)	Mw(kDa)	Score	Seq.cover. (%)	
01	8.65	28.029	31	0.20	16.2	Caspase -7
	6.50	30.269	31	0.20	18.0	Nesprin-2
02	4.7	26.101	29	0.08	24.5	Rab
	12.41	22.305	29	0.07	40.5	USP6NL protein
03	6.65	97.775	100	0.11	14.9	Vav-3
04	8.13	100.198	100	0.10	32.1	ADAMTS-19
	4.86	99.174	100	0.08	22.8	Lipoprotein receptor-related protein
05	5.60	78.542	90	0.05	28.2	ADAM-17
	8.88	87.644	90	0.05	23.1	Actin-binding LIM protein 1
06	6.66	64.256	80	0.11	20.1	Caldesmon (CDM) splise isoform 3.
	5.6	93.560	80	0.13	16.8	Caldesmon
07	6.41	57.984	63	0.11	19.1	Calpain 10
	8.82	64.424	63	0.13	14	Beta-dystrobrevin
08	5.06	53.520	60	0.28	18.5	Vimentin
09	7.77	96.496	105	0.09	22.7	ADAMTS-17
	8.73	101.173	105	0.06	17.2	GEF-H1
10	5.37	79.096	90	0.33	21.7	Calpain 9
11	5.49	81.890	85	0.11	24.8	Calpain 1
12	5.29	41.605	43	0.42	28.3	Actin
	7.61	35.024	43	0.33	8.7	F-actin capping protein alfa-subunit

1st column – the number of band in gels (Fig. 1B); 2nd column – the protein Mw and pI calculated from the amino acid sequence in ExPASy database; 3rd column – the calculated Mw according to their migration in SDS-PAGE; 4th column – MOWSE scores and sequence coverage (%); 5th column – the list of identified proteins.

tein for Rab5A and is involved in receptor trafficking [13].

We have also identified actin (Fig. 1, band No. 12) and several actin-associated proteins: calpain (band No. 10, 11, 07), nesprin-2 (band No. 01), actin-binding LIM protein (band No. 05), vimentin (band No. 08) and caldesmon (band No. 06). It has been shown by Shan and others [15] that vimentin intermediate filaments are colocalized with a variety of intracellular structures such as actin filaments and plasma membrane. The synthesis of vimentin increased after 12–24 h of differentiation induction to macrophages, suggesting a participation of vimentin filaments in the maldistribution of nuclei in M1 cells during differentiation [16].

Protein bands No. 04 and 09 (Fig. 1) were identified as ADAMTS-19 and 17 proteins, respectively. ADAMTS (A Disintegrin-like and Metalloprotease with Thrombospondin motifs) proteins have a homology with the metalloprotease region of the ADAM proteases, but also have at least one of the Thrombospondin type 1 Sequence Repeat (TSR) motifs that are common in extracellular matrix proteins [17].

In band 09 we identified ADAM-17. ADAMs (A Disintegrin and Metalloprotease) are multifunctional, membrane-bound cell surface glycoproteins, which have numerous functions in cell growth, differentiation, and motility [18]. The increased level of ADAM-17 both in Bml-210 and Bml-210 with RA treated cells for the period 2–8 h shows that this protein could be important in the signalling cascades caused by such treatments.

Using MALDI-TOF MS analysis, we have also identified β-dystrobrevin in the 63 kDa protein band (Fig. 1, band No. 07). In our previous studies we have shown [8] that α-dystrobrevin and its splice isoforms are present in human leukemia cell lines. Moreover, during granulocytic differentiation induced by RA they undergo tyrosine phosphorylation. Consequently, we decided to examine changes of α-dystrobrevin and its splice isoform levels both in soluble and insoluble protein fractions of NB4 cells treated with RA or Bml-210 alone or in combination.

The dystrobrevins are a family of dystrophin-related as well as dystrophin-associated proteins being localized at the inner surface of cell membranes [19].

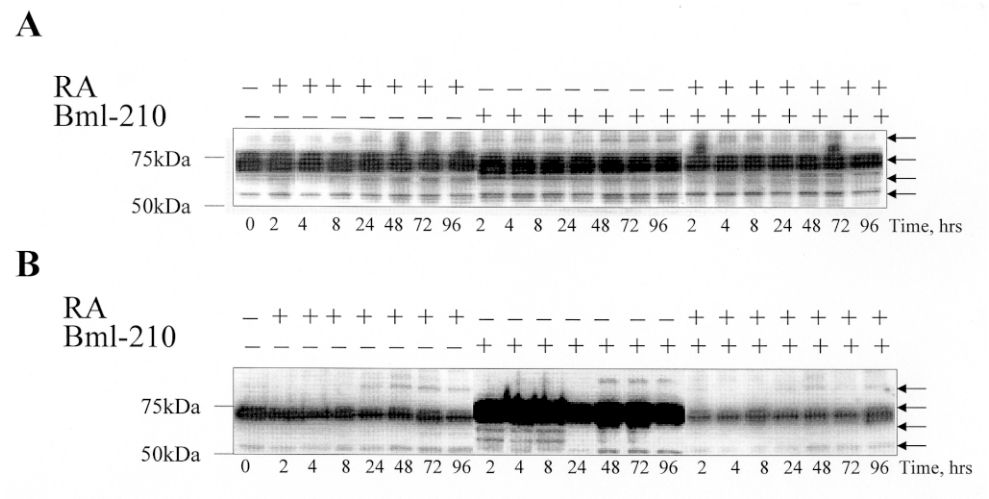

Fig. 2. Expression of a-dystrobrevin in NB4 cells after treatment with RA and Bml-210 alone or in combination

Total soluble (A) and insoluble (B) proteins were isolated from NB4 cells treated with 1 mM RA. 10 mM Bml-210 or in combination (1 mM RA and 5 mM Bml-210) for different periods of time. The proteins were fractionated by SDS-PAGE on an 8–16% acrylamide gel gradient and transferred onto Immobilon™ PVDF membrane. The membranes were analyzed with polyclonal antibodies against human a-dystrobrevin and developed with an enhanced chemiluminescence detection system. The positions of a-dystrobrevin slice isoforms are designated by arrows to the right. Migration of the molecular size marker proteins is indicated to the left (kDa values)

Dystrobrevins are highly homologous protein products of two different genes: α- and β-dystrobrevin, each of which produces multiple transcripts via alternative splicing and/or alternative initiation sites [20, 21]. The alternative splicing of the α-dystrobrevin transcript generates multiple isoforms, which differ by molecular weight and are named as α- (75 kDa), β- (62 kDa), γ- (58 kDa), δ- (42 kDa) dystrobrevin [8].

We further fractionated the total soluble and insoluble protein fractions of NB4 cells by SDS-PAGE and transferred to PVDF Immobilon-P membranes after cell treatment with RA and Bml-210 alone or in combination. Total expression of dystrobrevins was assessed using the produced rabbit polyclonal antibody against α-dystrobrevin [8] (Fig. 2 A, B). In the total soluble protein fraction, the antibody revealed bands with molecular masses of approximately 84 kDa, 75 kDa, 65 kDa and 58 kDa bands (Fig. 2, A). Their level varied and depended on the time of treatment. By contrast, in the insoluble protein fraction we identified five different isoforms of proteins that varied in size from 58 to 84 kDa (Fig. 2, B) during late hours of RA and Bml-210 treatment or co-treatment. In the controls and during early times of treatment we observed only two different splice isoforms in the insoluble fraction. Thus, the decrease of α-dystrobrevin isoform level in the soluble protein fraction and the increase in insoluble protein fraction in response to RA alone or cotreatment with Bml-210 are paralleled with the induction of differentiation.

Our results demonstrate that different isoforms of α-dystrobrevin are expressed in the acute promyelocytic leukemia cell line NB4. Using antibodies raised against human α-dystrobrevin, we identified the isoforms of dystrobrevin in soluble cell compartments and in insoluble-membrane fraction. There was furthermore a correlation between changes of the expression level of dystrobrevin isoforms and differentiation of NB4 cells. Using MALDI-TOF MS we identified proteins in the insoluble fraction of NB4 cells, the expression level of which changed in the very beginning of NB4 cell differentiation. These changes could be related to early changes of chromatin structure during cell treatment with Bml-210 with the following granulocytic differentiation induced by RA. In summary, our results suggest that Bml-210 may be a promising agent in leukemia therapy.

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RETINOINËS RÛGÐTIES IR HISTONØ DEACETILAZIØ INHIBITORIAUS BML-210 POVEIKIS NB4 LÀSTELIØ BALTYMØ RAIÐKAI

Santrauka

Þmogaus ûmios promielocitinës leukemijos NB4 làsteliø linijai bûdinga t(15;17) chromosominë translokacija. NB4 làstelës gali diferencijuotis á granuliocitus arba monocitus atitinkamai paveikus retinoine rûgðtimi arba vitaminu D3 bei forbolo esteriais. Ðiuose tyrimuose nustatydami baltymus mes naudojome SDS elektroforezæ, Westernblotà ir MALDI-TOF MS analizæ.

Apdorojus MALDI-TOF MS analizës rezultatus kompiuterine PeptIdent programa, buvo apibûdinti keli baltymai, esantys tirpioje ir netirpioje (membraninëje) làsteliø frakcijoje po indukcijos su RA arba Bml-210 bei jø deriniu. Buvo aptiktas aktinas, su juo sàveikaujantys kalpainas, nesprinas, vimentinas, taip pat Rab2A ir USP6NL baltymai. Visi ðie baltymai bûdingi leukeminëms làstelëms ir gali bûti svarbûs diferenciacijai. Taip pat mes nustatëme ir apibûdinome α-dystrobrevino ir jo izoformø raiðkos pokyèius tirpioje ir netirpioje làsteliø frakcijose. Ðie pokyèiai, o bûtent α-dystrobrevino kiekio padidëjimas, gali bûti siejami su diferenciacija leukeminëse NB4 làstelëse.