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EXPERIMENTAL STUDIES

SYSTEM ANALYSIS OF SURFACE CD MARKERS DURING THE PROCESS OF GRANULOCYTIC DIFFERENTIATION

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Plasma membrane proteins with extracellular-exposed domains are responsible for transduction of extracellular signals into intracellular responses, and their accessibility to therapeutic molecules makes them attractive targets for drug development. In this work, using omics technologies and immunochemical methods, we have studied changes in the content of markers of clusters of differentiation (CD markers) of neutrophils (CD33, CD97, CD54, CD38, CD18, CD11b, CD44, and CD71) at the level of transcripts and proteins in NB4, HL-60, and K562 cell lines, induced by the treatment with all-*trans*-retinoic acid (ATRA). Transcriptomic analysis revealed the induction of CD38, CD54, CD11b, and CD18 markers as early as 3 h after the addition of the inducer in the ATRA-responsive cell lines HL-60 and NB4. After 24 h, a line-specific expression pattern of CD markers could be observed in all cell lines. Studies of changes in the content of CD antigens by means of flow cytometry and targeted mass spectrometry (MS) gave similar results. The proteomic profile of the surface markers (CD38, CD54, CD11b, and CD18), characteristic of the NB4 and HL-60 lines, reflects different molecular pathways for the implementation of ATRA-induced differentiation of leukemic cells into mature neutrophils.

Key words: surface CD markers; transcriptomics; proteomics; immunochemistry; SRM/SIS analysis; all-*trans*-retinoic acid (ATRA)

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INTRODUCTION

Plasma membrane (PM) proteins are of particular interest for fundamental and applied research due to their unique signaling functions providing interaction of cells with the environment [1, 2]. PM proteins containing domains exposed in the extracellular space are responsible for converting extracellular signals into intracellular responses. This group of proteins includes members of important protein families such as G protein-coupled receptors, receptor tyrosine kinases, integrins, etc. [3, 4]. The location of proteins on the cell surface makes them accessible to therapeutic molecules, and their ability to control cellular responses makes them attractive targets for drug development.

A separate group of PM proteins, involved in processes of cell differentiation, is called CD (cluster of differentiation) antigen/markers [5]. From the name CD antigen it is clear that immunochemical methods are used to determine this type of protein. CD molecules are widely used as cellular markers for identification, evaluation of differentiation stage, and isolation of leukocyte populations.

Determination of cell surface proteins by means of antibodies is a powerful tool for studying the processes of cell differentiation [6]. However, this method is not without drawbacks, mainly associated with the difficulty of obtaining antibodies, with unclear antibody specificity and the difficulty of determining the concentration of the studied protein in the cell [7].

Currently, mass spectrometry (MS) methods are widely used for the detection and quantification of proteins in biological fluids, tissues and cells. Two main approaches are used for MS identification of proteins. The first approach includes a group of methods known as shotgun proteomics. This approach is used to search for qualitative and quantitative changes in the proteome of the studied biological object. The second method belongs to the category of targeted proteomics, aimed at studying proteins previously determined by a researcher. Using this method it is possible to achieve high sensitivity (several protein molecules per cell) and high specificity of the analysis [8]. The use of a unique peptide mapped to a protein for targeted measurements and its synthetic isotope-labeled analogue as an internal standard allows to detect the protein of interest with high specificity and at the same time to determine its absolute concentration.

HL-60 and NB4 cells are popular model objects for studying the molecular nature of granulocytic differentiation. The myeloid HL-60 and NB4 cells differ at the molecular genetic level: the NB4 cells carry the chimeric *PML-RARα* gene, formed due to the t(15;17) translocation between chromosomes 15 and 17; HL-60 cells carry

the wild-type *RARα* gene, but they are characterized by deletion of the *p53* gene and multiple amplification of the *c-MYC* gene. Cells of both lines, exposed to all-*trans*-retinoic acid (ATRA), acquire a mature granulocyte (neutrophil) phenotype characterized by segmented morphology and expression of the surface markers CD11b and CD38 [9]. Induced differentiation causes a cascade of changes in HL-60 and NB4 cells at the mRNA level and subsequently at the protein level. This includes changes in the set of detectable CD markers that characterize the transition of myeloid cells into mature granulocytes and neutrophils [10].

In this study, we have measured the concentrations of eight CD markers (CD33, CD97, CD54, CD38, CD18, CD11b, CD44, and CD71) during the ATRA-induced differentiation of cell lines HL-60, NB4, and the control cell line K562 by MS-SRM analysis and compared the data obtained with the results of flow cytometry using antibodies to the same markers, as well as with data on the relative changes in their mRNA levels.

MATERIALS AND METHODS

Cultivation of HL-60, NB4, and K562 Cell Lines and Induction of Granulocytic Differentiation

The HL-60 and K562 cell cultures were obtained from the cryobank "Collection of Vertebrate Cell Cultures" of the Institute of Cytology, Russian Academy of Sciences (St. Petersburg, Russia). The NB4 cells were obtained from CLS Cell Lines Service GmbH (CLS Cell Lines Service GmbH, Germany). The identity of all cell lines was confirmed by the DNA profile (short tandem repeat analysis, STR). The HL-60 cells (acute myeloid leukemia, AML) and NB4 cells (acute promyelocytic leukemia, APL) are ATRA-sensitive and during treatment with this inducer they acquire the phenotype of mature neutrophils. Chronic myeloid leukemia cell line K562, which is not responsive to ATRA, was used as a negative control for ATRA-induced differentiation.

After thawing, the cells were washed 3 times to remove DMSO in RPMI-1640 growth medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; v/v) by centrifugation (at 300 *g* for 5 min at 25°C). All the cells were cultivated in RPMI-1640 growth medium supplemented with 10% FBS (v/v), 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine (all reagents from Gibco) in a CO_2 incubator under standard conditions. (37°C, 5% CO_2, 80% humidity) for 7 days. After reaching a concentration of 1 million cells/ml, the cell cultures were seeded at a ratio of 1:3. Cells were counted in a Goryaev chamber.

For differentiation, HL-60, NB4, and K562 cells (0.3 million cells/ml) were placed in 15 ml of growth medium and ATRA (Sigma Aldrich, USA) was added to a final concentration of 10 μM.

For transcriptome and proteome analyses, cells from each line were induced with ATRA in two biological replicates. Differentiation experiments were carried out within 15–20 passages after cell recovery after cryopreservation.

For proteome and transcriptome analyses, the HL-60 and NB4 cells were taken at 0 h, 3 h, 12 h, 24 h, and 72 h after the ATRA addition; K562 cells were taken at 0 h, 24 h, and 72 h after the ATRA addition. Cells (~1 million per sample) were then pelleted by centrifugation and the sediment was washed twice with phosphate-buffered saline (PBS) (Sigma-Aldrich).

Measurement of the Level of Surface Markers Using Flow Cytometry

HL-60, NB4, and K562 cells were taken for the measurement at 0 h, 24 h, and 72 h after the addition of ATRA and washed twice by centrifugation (5 min, 300 *g*), followed by resuspension of the cell sediment in PBS (Sigma-Aldrich). Cells were then fixed in 4% paraformaldehyde in PBS for 1 h, washed twice with PBS, resuspended in 1% bovine serum albumin (BSA) in PBS to block nonspecific binding, and incubated for 20 min at 25°C. After sedimentation, the cells were resuspended in 100 μl solutions (containing 1% BSA in PBS) of the following monoclonal antibodies, diluted according to the manufacturer's recommendations: antibody to CD11b and CD18, labeled with fluorescein isothiocyanate (FITC) (Elabscience Biotechnology, China), antibody to CD38, labeled with allophycocyanin (APC) (Elabscience Biotechnology), and antibody to CD71 phycoerythrin-labeled (PE) (Cloud-Clone Corp., China). The resultant samples were incubated at 4°C. Isotype antibodies labeled with an appropriate fluorescent label (Becton Dickinson, USA) were used as a negative control.

After 60 min, cells were sedimented, washed twice with PBS, and resuspended in 500 μl PBS for subsequent analysis. The study was performed on a ZE5 flow cytometer (BioRad, USA).

The expression level of the marker under study was assessed using a histogram of the fluorescence intensity of the label conjugated to specific antibodies. Registered events were recorded (50 thousand events) at a cell suspension flow rate of 1 μl/s. To exclude registration of noise, as well as objects with sizes smaller than a cell, the registration threshold for direct light scattering was set to 20,000.

Further analysis of the obtained data was carried out using Floreada.io software. To compare the fluorescence intensity of the test sample and the isotype control, the results of the test and control samples were superimposed in the histogram mode, and using the geometric mean fluorescence intensity (gMFI), the relative fluorescence intensity (RFI) was calculated by the formula:

$$RFI = \frac{gMFI_{\text{ATRA treated cells}} - gMFI_{\substack{\text{isotypic control} \\ \text{for ATRA treated cells}}}}{gMFI_{\text{control cells}} - gMFI_{\substack{\text{isotypic control} \\ \text{for control cells}}}}.$$

Chip Based Transcriptome Analysis

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's recommendations. The quality of the resulting RNA was determined on an Agilent 2100 Bioanalyser instrument (Agilent Technologies, USA) using RNA 6000 Nano LabChip chips (Agilent Technologies). For further analysis, only samples with a RIN (RNA Integrity Number) of at least 8 were used. The RNA concentration and efficiency of fluorescent label incorporation were determined spectrophotometrically on a NanoDrop ND-1000 instrument (Thermo Fisher Scientific, USA).

RNA preparation for hybridization was performed using a LowInput QuickAmp Labeling Kit (Agilent Technologies) according to the One-Color Microarray-Based Gene Expression Analysis protocol (version 6.9.1). Since the analysis was carried out in a single-color hybridization format, fluorescently labeled 3-CTP nucleotides (PerkinElmer, USA) were used as a label. Also, according to the standard protocol, appropriate amounts of RNA from the Agilent RNA Spike-In Kit (Agilent Technologies) were added to the samples. Fluorescently labeled cRNA was fragmented, hybridized with 4x44K G4112F Agilent whole-genome expression chips (Agilent Technologies), and scanned on an Agilent G2505B confocal laser scanner. Data extraction and primary statistical processing were performed using Feature Extraction 10.10.1.1 software (Agilent Technologies). After extraction, the data processing was performed using the open programming language R version 4.3.0 and the Limma library (3.56.2). Expression data on genes encoding plasma membrane proteins (CD33, CD97, CD54, CD38, CD18, CD11b, CD44, and CD71) measured after ATRA exposure are given in the Supplementary materials (Table S1, CD markers transcriptome sheet).

Selection of Proteotypic Peptides Mapping to Plasma Membrane Proteins and Synthesis of Isotope-Labeled Internal Standards (SIS)

PM membrane proteins were selected for analysis on the basis of literature data on their ATRA-induced changes in AML cells (CD33, CD97, CD54, CD38, CD18, CD11b, CD44, and CD71) [11–17]. Proteotypic peptides were selected from the NextProt database, and the uniqueness of the amino acid sequence of the peptide within a biological species was checked using the Peptide unicity checker resource. The conditions for selecting candidate peptides for synthesis were: a predominant length of 10 or more residues, the absence of glutamine (Q) and glutamic acid (E) residues at the N-terminus of the amino acid sequence of the peptide, the absence of methionine (M) and the predominant absence of cysteine (C). As a result, it was possible to select 13 peptides that met all the criteria. Peptides are listed in Table S1 of the Supplementary materials (the Proteotypic peptides sheet).

Solid-phase peptide synthesis was carried out on an automatic peptide synthesizer Overture (Protein Technologies, UK) according to the method described previously [8]. During synthesis of isotope-labeled peptides, the isotope-labeled amino acids Fmoc-Lys-OH-13C6.15N, or Fmoc-Arg-OH-13C6.15N (Cambridge Isotope Laboratories, USA) were used instead of the usual lysine or arginine, respectively.

To introduce the modification (carbamidomethylation of cysteine), an aqueous solution containing 10 μg of the SIS peptide, LGTQTVPCNK (CD38), was dried, then redissolved in 20 μl of triethylammonium bicarbonate buffer (TEAB, pH 8.5) and incubated in the presence of 50 mM Tris(2-carboxyethyl)phosphine (TCEP) and 80 mM 2-chloroacetamide (CAA) at 80°C for 40 min. The reaction mixture was then dried again and redissolved in 0.1% formic acid in a volume corresponding to the original volume of the SIS aqueous solution. The resulting solution was then used to prepare a mixture of SIS peptides for SRM analysis.

Hydrolytic Digestion of Proteins for MS Analysis

For sample preparation for MS analysis a 200 μl-aliquot of lysis buffer containing 1% SDS and cOmplete™, Mini Protease Inhibitor Cocktail (Sigma-Aldrich) in 100 mM Tris HCl (pH 8.5) was added to HL-60, NB4, and K562 cells collected at various time-points after incubation with ATRA (0 h, 3 h, 12 h, 24 h, 72 h). After sonication performed by a Bandelin Sonopuls ultrasonic disintegrator with a probe at 50% power for 1 min in ice the samples were centrifuged at 14,000 *g* for 15 min at 10°C to sediment the foam. In the resulting samples (supernatant), the concentration of total protein was determined by a colorimetric method using a commercial Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) in accordance with the manufacturer's recommendations.

Hydrolytic digestion of proteins was carried out according to the FASP (Filter-Aided Sample Preparation) protocol [18] with some modifications. Cysteine disulfide bonds were reduced and alkylated by adding 20 mM TCEP and 32 mM CAA (final concentrations) to cell lysate samples containing 100 μg of total protein. The samples were incubated at 80°C for 40 min and then applied to concentrating filters with a cutoff of 30 kDa and washed 3 times with a buffer containing 8 M urea in 0.1 M Tris HCl (pH 8.5) by centrifugation at 11,000 *g* for 15 min at 20°C. Then the samples were washed 3 times with a buffer

containing 0.1 M Tris HCl (pH 8.5) and 2 times with trypsinolysis buffer (50 mM TEAB, pH 8.5), by centrifugation at 11,000 *g* for 15 min at 20°C. Then 50 μl of 50 mM TEAB (pH 8.5), a trypsin solution with a concentration of 0.5 μg/μl was added to each sample (trypsin-to-total protein mass ratio of 1:50). The samples were incubated overnight at 37°C. After the incubation peptides were eluted by centrifugation at 11,000 *g* for 15 min at 20°C and the filter was washed with 100 μl of 5% formic acid. Peptides were dissolved in 40 μl of a solution containing 0.1% formic acid and 13 SIS standards at quantity of 50 fmol/μg total protein.

Mass Spectrometry Analysis in the SRM Mode (MS-SRM)

Each experimental sample was analyzed in 3 technical replicates.

Chromatographic separation was carried out using an Agilent 1200 series system (Agilent Technologies) connected to a TSQ Quantiva triple quadrupole mass analyzer (Thermo Scientific). A 3.5 μl-sample containing 15 μg of native peptides and SIS standards was separated using a ZORBAX SB-C18 analytical column (150×0.5 mm, particle diameter 5 μm, Agilent Technologies) in an acetonitrile gradient with a flow rate of 20 μl/min. The column was first equilibrated with 5% solution B (80% acetonitrile in 0.1% formic acid) and 95% solution A (0.1% formic acid) for 5 min, then the concentration of solution B was linearly increased to 60% over 30 min. After that the concentration of solution B was increased to 99% in 1 min, and the column was washed with 99% solution B for 5 min, then within 1 min the concentration returned to the initial conditions and the column was equilibrated for 9 min. MS analysis was carried out in the dynamic selected reaction monitoring (dSRM) mode, using the following MS detector settings: capillary voltage — 4000 V, drying gas rate (nitrogen) — 7 L/min, axillary gas rate (nitrogen) — 5 L/min min, capillary temperature — 350°C, isolation window for the first and third quadrupole — 0.7 Da, scan cycle time — 1.2 s, gas pressure (argon) in the collision cell — 1.5 mTorr. The retention time window on the reverse phase column was 1.8 min for each precursor ion. During the analysis, transitions, listed in Table 1S were monitored (Supplementary materials, Table 1S, the SRM transitions sheet). For SRM analysis of target proteins, 2-4 transitions per precursor were used. For quantitative assessment, all monitored transitions were used in Skyline software.

Additional calibration was carried out before serial analysis of samples. After calibration and before starting the analysis, one injection of a blank sample and one injection of a mixture of peptide standards in 0.1% formic acid were performed. All samples were measured in one series. Between every three technical replicates for experimental samples, a blank sample was injected. It represented aqueous solution of 0.1% formic acid used in the same volume and under the same chromatography-mass spectrometric conditions as in the experimental sample. At the end of the serial analysis of samples, a mixture of peptide standards was injected in 0.1% formic acid.

The results were loaded into Skyline MacCoss Lab Software (version 4.1.0) to compare the chromatographic profiles of the endogenous peptide and the corresponding SIS standard. The peak area ratio for the endogenous peptide and the corresponding SIS standard was automatically calculated in Skyline. To determine the amount of protein, the ratio calculated in Skyline was multiplied by the known content of each SIS standard. The arithmetic mean value of the content calculated based on the results of SRM analysis in three technical replicates was taken as the measurement of each protein.

RESULTS

Differential Expression of Surface CD Markers at the Transcriptome Level in HL-60, NB4, and K562 Cells in Response to ATRA Treatment

Analysis of changes in mRNA levels for selected genes encoding CD markers was carried out using chip-based transcriptome analysis. Data on the dynamics of changes in the transcript content during induced differentiation in cells of the HL-60, NB4, and K562 lines compared to control (0 h) are given in Supplementary materials (Table S1, the CD markers transcriptome sheet). The first significant changes in the expression of CD markers (p-value<0.05, fold change (FC) >2) in ATRA-responsive cell lines were observed at the transcriptome level at 3 h after ATRA addition: the content of the CD38 marker increased by 8 and 4 times in HL-60 and NB4 cell lines, respectively; the content of CD markers CD54 and CD11b in HL-60 cells increased by 4.4 and 3 times, respectively. In addition, at 3 h after the addition of ATRA, the content of the CD18 transcript in NB4 cells increased by 2.2 times. In the control K562 cell line, no significant changes in the expression of mRNA encoded by the CD marker genes (*CD11b*, *CD18*, *CD33*, CD38, *CD44*, *CD54*, *CD71*, and *CD97*) were detected at 3 h after ATRA addition.

The most pronounced effect in the ATRA-responsive cells (HL-60 and NB4) was observed for transcripts CD38, CD54, CD11b, and CD18; their content increased significantly at 24 h after ATRA addition. Figure 1 shows the transcriptomic signature for surface CD markers (CD38, CD54, CD11b, CD18, CD44, and CD71) (FC>2, p-value<0.05) in HL-60, NB4, and K562 cells at 24 h after ATRA treatment.

As can be seen from the presented graphs, ATRA addition to the incubation medium increased expression of genes encoding surface CD markers selected for analysis. In HL-60 cells the 63.1-, 5.2-, 3.1-, and 3.3-fold increase was observed

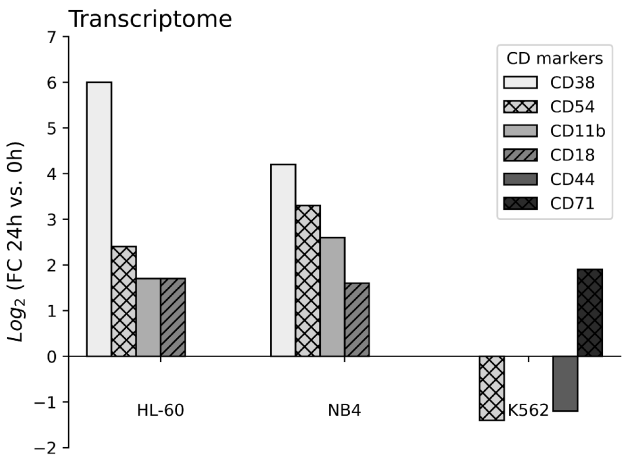

Figure 1. Transcriptomic signature for surface CD markers (CD38, CD54, CD11b, CD18, CD44, and CD71) in HL-60, NB4, and K562 cells at 24 h after ATRA addition. The y-axis shows the relative change in the transcript content (FC>2, p-value<0.05) at the time point of 24 h compared to the control (0 h), logarithmized to base 2.

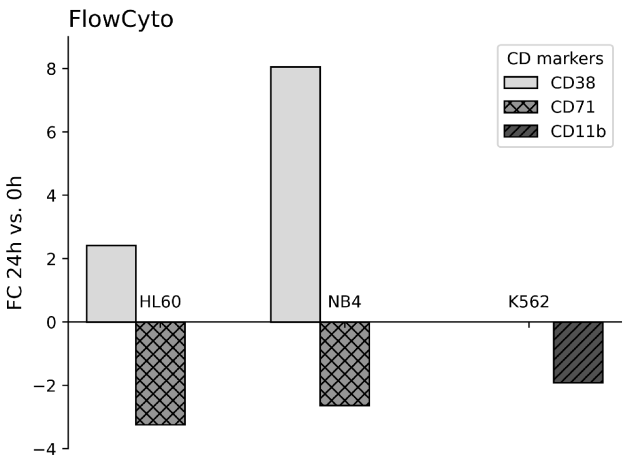

Figure 2. Cytofluorimetric signature for surface CD markers (CD38, CD11b, and CD71) in HL-60, NB4, and K562 cells at 24 h after ATRA addition. The ordinate axis shows the change in relative fluorescence intensity (RFI) for CD markers (FC>1.5, p-value<0.05) at the time point of 24 h compared to the control (0 h) without transformation.

for markers CD38, CD54, CD11b, and CD18, respectively. In NB4 cells the fold change increase was 18.4, 9.6, 6.1, and 3.0, respecively. At the same time, in the control K562 cell line, the expression of mRNA encoded by the CD54 and CD44 genes decreased (2.6 and 2.3 times, respectively), while the content of the CD71 transcript demonstrated a 3.8-fold increase.

Dynamics of the Response of CD Markers to ATRA Treatment, Determined by Flow Cytometry

The level of the CD markers CD38, CD11b, CD18, and CD71 in HL-60, NB4, and K562 cells treated with ATRA was assessed using flow cytometry. Data on the dynamics of changes in the expression levels of CD38, CD11b, CD18, and CD71 at time points 0 h, 24 h, and 72 h after ATRA addition are shown in Supplementary materials (Fig. 1S); the signature for surface CD markers (CD38, CD11b, CD18, and CD71) (FC>1.5, p-value<0.05) at 24 h after ATRA induction is shown in Figure 2.

In the ATRA-responsive cells HL-60 and NB4, the expression of CD38 increased by 2.4 and 8 times at 24 h after ATRA addition, while the content of the surface marker CD71 demonstrated a 3.2- and 2.6-fold decrease, respectively. In the control cell line K562, the content of the CD11b marker decreased by 1.9 times.

The Proteomic Profile of CD Markers Obtained Using Targeted Mass Spectrometric Analysis in the SRM mode

Using targeted mass spectrometry, 13 peptides were measured in samples of the HL-60, NB4, and K562 cells and mapped to eight CD markers (CD33, CD97, CD54, CD38, CD18, CD11b, CD44, and CD71). Proteomic profiling was performed in HL-60 and NB4 cells at 0 h, 3 h, 12 h, 24 h, and 72 h after ATRA treatment. Data on the dynamics of changes in the content of proteins CD11b, CD18, CD33, CD38, CD44, CD54, CD71, and CD97 at the time points 3 h, 12 h, 24 h, and 72 h after ATRA addition are given in Supplementary materials (Table 1S, the sheet CD markers proteome, Figure 2S). Expression levels of CD markers were also measured in the control K562 cells insensitive to ATRA treatment, at 0 h, 24 h, and 72 h after ATRA addition (Fig. 3).

Figure 3 shows that all eight CD markers studied were detected in HL-60, NB4, and K562 cells. Treatment with the differentiation inducer for 24 h increased the CD38 protein content in both ATRA-sensitive cells from the level below the detection limit to 123±18 thousand molecules per cell (HL-60), and from 35±4 thousand molecules to 346±3 thousand molecules per cell (p<0.001) (NB4). The CD18 protein content increased in both ATRA-responsive lines, from 79±9 thousand molecules to 135±31 thousand molecules per cell (HL-60), and from 84±15 thousand molecules to 286±12 thousand molecules per cell (p=0.00569) (NB4). In HL-60 cells the CD54 protein content was below the detection limit at all time points. In NB4 cells, the amount of CD54 protein increased from 22±4 thousand molecules to 371±5 thousand molecules per cell (p<0.001). The CD11b protein content increased in the NB4 cells from the level below the detection limit to 76±4 thousand molecules per cell 24 h after treatment with ATRA, while CD11b expression was detected at a level of 45±13 thousand molecules per cell, starting from 3 h after ATRA addition.

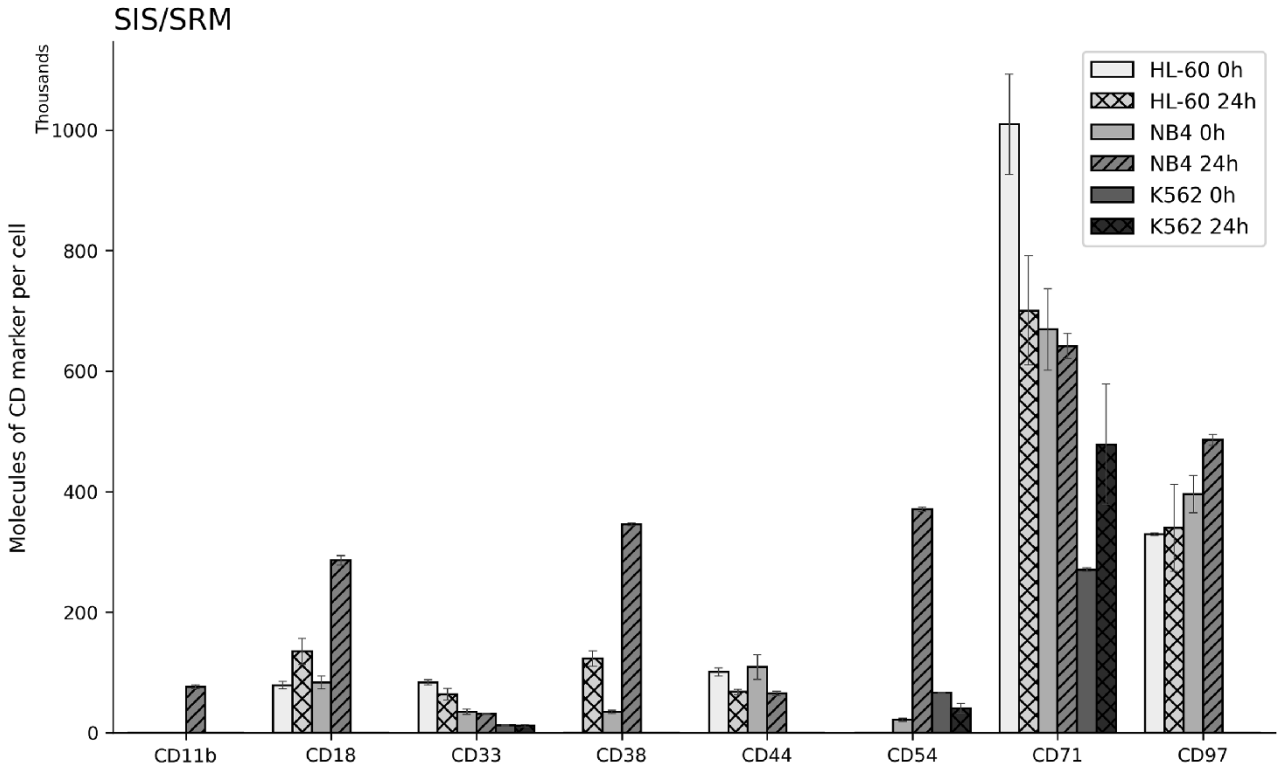

Figure 3. The content of surface markers CD33, CD97, CD54, CD38, CD18, CD11b, CD44, and CD71 measured using the SRM/SIS method in HL-60, NB4, and K562 cells at 0 h and 24 h after ATRA addition. Because the total number of cells (~1 million cells per sample) and total protein levels in samples used for SRM/SIS analysis are known, we have calculated the protein concentration as the number of thousands of marker molecules per cell.

In the chronic myeloid leukemia cells K562, only markers CD33, CD71, and CD54 were detected. The content of the CD71 marker showed a tendency to increase from 271±4 thousand molecules to 479±141 thousand molecules per cell 24 h after ATRA addition.

The signature for significantly changed surface markers (CD38, CD54, CD11b, and CD18) (FC>2, p-value<0.01) in the ATRA-responsive cells HL-60 and NB4 is shown in Figure 4.

At 24 h after ATRA addition the proteomic profile for significantly changing surface markers (CD38, CD54, CD11b, and CD18) (FC>2, p-value<0.01) was different for NB4 and HL-60 cells.

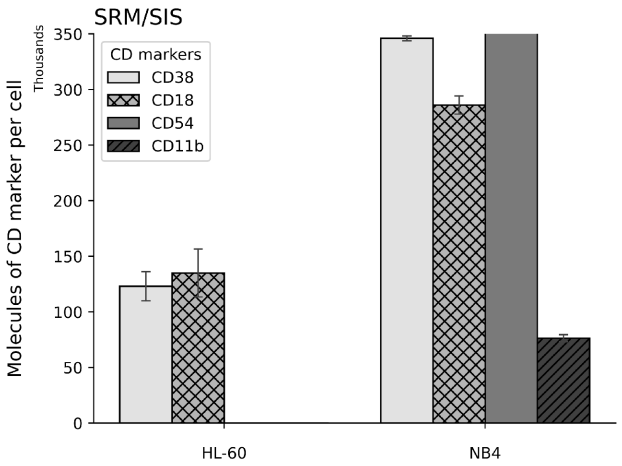

Figure 4. The proteomic signature for surface CD markers (CD38, CD18, CD54, and CD11b) in HL-60 and NB4 cells treated with ATRA for 24 h.

DISCUSSION

The search for new cancer markers that can be used for early diagnostics, histological analysis, prognosis, and also as targets for therapeutic treatment of oncology is one of the key areas of academic and pharmaceutical research in recent decades.

Phenotypic changes associated with malignant transformation, including cell proliferation, adhesion, and migration, are often mediated by PM proteins [13–16]. Malignant transformation of cells is undoubtedly associated with genetic changes, but the difference between the proteomes of cancer and normal cells is often recorded only in changes in the content of a number of PM proteins. In addition, pharmacological interventions highly selectively stimulate or, on the contrary, inhibit the expression of PM proteins, which may reflect responsiveness to treatment.

Traditionally, the expression of PM proteins and clusters of differentiation (CD markers) is assessed using immunochemical methods, particularly flow cytometry and Western blot. Measuring concentrations

by immunochemical methods is difficult due to the lack of calibration, which requires the use of protein standards for each individual antibody-antigen pair. Fluorescence intensity or fold change (FC) is used as a quantitative parameter, which is justified within one experiment, but it is hardly applicable to other studies [19]. In addition, assessment of the expression of one or two CD markers does not allow obtaining comprehensive biological information. At the same time, omics technologies are characterized by high throughput and a high degree of multiplexity, or the ability to analyze simultaneously tens or hundreds of analytes.

Transcriptomic technologies are a highly sensitive tool for detecting changes in the level of gene transcription and for searching for disease markers. However, changes in mRNA levels not necessarily correlate with changes in the protein content [8, 20]. In the case of our data, on the one hand, the content of the surface receptor CD71 increased in the K562 cells at the mRNA and protein levels, and the expression of CD38 and CD18 markers similarly increased in the ATRA-responsive HL-60 and NB4 cells at the transcriptomic and proteomic levels. On the other hand, the activation of CD54 expression at the transcriptomic level for HL-60 cells was not confirmed at the proteome level, and the reduced expression of the CD11b marker, recorded in K562 cells by flow cytometry, was not detected in the transcriptomic experiment. It is possible, that the decrease in CD11b content in K562 cells is achieved due to activation of protein degradation rather than inhibition of transcription of the corresponding gene. Thus, to obtain relevant biological information, it is important to know the dynamics of CD markers at the proteomic level.

Using transcriptomic analysis it is possible to register changes in gene expression within the first hours after treatment of cells with a pharmacological agent [21]; this is important for determining the molecular mechanisms underlying the early stages of granulocytic differentiation. In our study, increased expression of CD38 was noted in the ATRA-responsive HL-60 and NB4 cells at 3 h after ATRA addition. The CD38 marker is used to diagnose various types of cancer and also as a target for immunotherapy. Therapeutic antibodies to CD38, such as daratumumab and isatuximab, are already used to treat leukemia [22]. Also, a high level of CD38 expression is a prognostic indicator in leukemia [23]. In our experiments, transcripts identified as differentially expressed at 3 h after ATRA addition, may be involved in alterations of protein-protein interactions with CD38 in HL-60 and NB4 cells (Fig. 5).

Components of such potential network may be involved in the response to stress and in the regulation of developmental/differentiation processes in HL-60 cells. Moreover, the network also contains the CD11b marker (ITGAM), as well as important regulatory transcription factors (TFs) (SMAD3, RUNX3, and NOTCH2).

On the other hand, a decrease in the concentration of certain PM proteins may also indicate the development of a pathological process. For example, a decrease in the expression of CD18 (integrin β_2), the main cell adhesion molecule, correlates with a decrease in the level of proliferation, metastasis, and infiltration of myeloid cells into the liver in *in vivo* experiments on mice [24]. On the other hand, knockout of CD11b (integrin αM) inhibits the trafficking of myeloid cells thus slowing tumor growth [25], while an increase in its concentration in myeloid cells promotes metastasis in breast cancer [26].

Some combinations of markers are a powerful diagnostic/prognostic test. For example, blood cells demonstrating a simultaneous increase in $CD44^+$ $CD54^+$ indicate the presence of gastric cancer stem cells [27].

Regarding the HL-60 cells we selected, it was shown that ATRA addition to the incubation medium, along with activation of CD54, CD18, CD38, and CD11b, caused a significant increase in the content of transcripts for such CD markers as CD4, CD38, CD49e, CD50, CD52, CD53, CD58, CD63, CD68, CD82, CD242, CD300A, CD300C, CD300LB, and CD300L (Supplementary Material Table S1, the Annotation sheet). At the same time, the content of CD44, CD151, and CD320 transcripts decreased. In the case of NB4 cells, ATRA caused a significant concentration increase of the following CDs (shown in descending order): CD52, CD38, CD54, CD24, CD11b, CD101, CD300A, CD11c, CD68, CD93, CD274, CD1D, CD300LF, CD300C, CD300LB, CD18, and CD302, as well as a decrease of CD151, CD248, CD3D, CD79B, CD82, CD83, CD8A, CD9, and CD99. The induction of these CD antigens is consistent with the known differentiation of these leukemic cells into abnormal neutrophils [19]. Approximately half of the ATRA-induced antigens on NB4 cells are adhesion molecules, including CD11a, CD11b, CD11c, and CD54; this is consistent with the increased leukemia cell adhesiveness observed in PML patients treated with ATRA. In HL-60 cells, ATRA selectively induced expression of CD4, CD53, CD58, CD63, CD82, CD49e, and CD242 markers. It should be noted that according to the SignaLink interactome database (version 3.0), expression of all these markers is under the control of ETS1 TF, which also affects the expression of cytokine and chemokine genes. In NB4 cells containing the chimeric *PML-RARα* gene, ATRA caused a noticeable increase in CD antigens, which was not observed in HL-60 cells: CD24 (by 7.3 times), CD101 (by 6.0 times), CD93 (by 4.1 times), CD274 (by 4.0 times), and CD302 (by 3.0 times). Expression of these antigens can be regulated by PML-RARα in the presence of ATRA. Such CD antigens may be targets for synergistic treatment of APL with therapeutic antibodies after ATRA treatment [28].

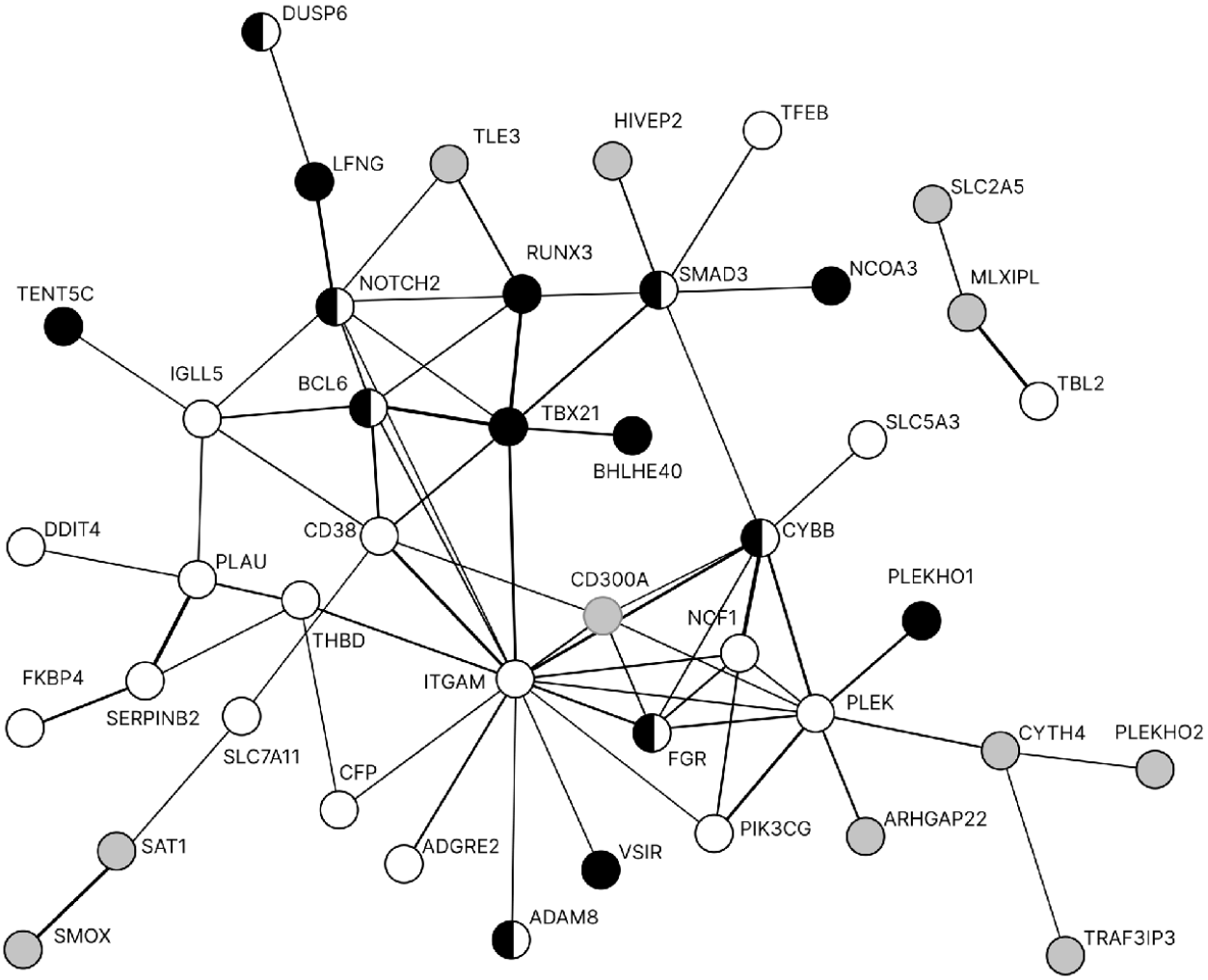

Figure 5. The scheme of protein-protein interactions for protein products of genes differentially expressed in HL-60 cells treated for 3 h with ATRA and including the CD38 marker (STRING, version 12.0). Molecules involved in the response to stress (white) and in the regulation of developmental/differentiation processes (black) are highlighted. Molecules involved in other processes are shown in gray.

In proteomics, the Aebersold group implemented the idea of constructing PM protein profiles by using the SWATH (Sequential Windowed Acquisition of all THeoretical fragments) MS method. Using this method it is possible to perform registration and semi-quantitative evaluation of proteins [29]. Targeted MS-SRM is also a good alternative for protein quantitation. The use of the SRM method and an internal isotope-labeled standard allows measurements of absolute protein concentrations. Protein concentrations can be expressed as a number of molecules per cell, when the number of cells sampled is known, or as moles per mg of total protein in the sample. Data on the number of molecules in a cell suggest the stoichiometry of protein complexes. For example, β_2 integrin (CD18) is a heterodimer consisting of a β_2 subunit and one α subunit, which could be either CD11a, or CD11b, or CD11c. Based on our quantitative data on the CD18/CD11b ratio, it is clear that one-third of all CD18 molecules are occupied by CD11b (Fig. 4), therefore, the remaining 2/3 should be occupied by CD11a and/or CD11c. Since the data are expressed in absolute values, they can be used by other researchers and this is an advantage of the SRM method.

Potential markers of differentiation are not limited to membrane proteins. Increased expression of specific enzymes, such as alpha enolase (ENO) in the case of neutrophils, may indicate the acquisition of a functional phenotype by leukemia cells.

AML is characterized by the presence of tumor cells that are at different stages and follow a certain direction of differentiation. The use of methods capable of recognizing a specific differentiation pathway can be very useful in research and in the clinical diagnostics of leukemia. Although this study have used only targeted methods of analysis of eight surface proteins expressed in various cell lines, this work demonstrates the potential of a MS strategy to determine AML subclasses. In the future, the panel of studied proteins can be expanded, based on their literature data or our own proteomic data, up to a complete list of 400 known CD proteins [8, 30].

Since the synthesis of new peptide SIS standards for the SRM/SIS method is low cost and simple compared to the production of new monoclonal antibodies, it is possible in the future to expand the existing panel of protein analytes in accordance with new tasks.

CONCLUSIONS

Using a combination of omics approaches, we have evaluated the dynamics of the expression of surface CD markers CD33, CD97, CD54, CD38, CD18, CD11b, CD44, and CD71 during ATRA-induced granulocytic differentiation. Transcriptomic analysis revealed activation of genes encoding surface CD marker (CD38, CD18, CD11b, and CD54) in the ATRA-responsive HL-60 and NB4 cells already at 3 h after ATRA addition. Early activation of CD38 and CD18 may be involved in stress response and developmental/differentiation processes. Transcriptomic and proteomic analyses also resulted in determination of the lineage-specific expression pattern for surface CD markers in the ATRA-responsive HL-60 and NB4 cells at 24 h after ATRA addition. Based on this result, a panel of CD markers could be developed in the future for typing leukemia patients to assess response to therapy, which is consistent with the personalised medicine paradigm. The results of omics experiments correlate with immunohistochemistry data, while transcriptomic and proteomic analysis is applicable for simultaneous quantitative analysis of a larger number of analytes during the initial stages of ATRA exposure.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

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СИСТЕМНЫЙ АНАЛИЗ ПОВЕРХНОСТНЫХ CD-МАРКЕРОВ В ПРОЦЕССЕ ГРАНУЛОЦИТАРНОЙ ДИФФЕРЕНЦИРОВКИ

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Белки плазматической мембраны с доменами, экспонированными в сторону внеклеточного пространства, отвечают за преобразование внеклеточных сигналов во внутриклеточные ответы, а их доступность для терапевтических молекул делает их привлекательными мишенями для разработки лекарств. В данной работе с применением омиксных технологий и методов иммунохимии мы исследовали изменение содержания маркеров-кластеров дифференцировки (CD-маркеров) нейтрофилов (CD33, CD97, CD54, CD38, CD18, CD11b, CD44 и CD71) на уровне транскриптов и белков в клетках линий NB4, HL-60 и K562 под действием полностью-*транс*-ретиноевой кислоты (ATRA). Транскриптомный анализ выявил индукцию маркеров CD38, CD54, CD11b и CD18 уже через 3 ч после добавления индуктора к ATRA-восприимчивым клеточным линиям HL-60 и NB4. Через 24 ч во всех клеточных линиях можно было наблюдать линия-специфичный паттерн экспрессии CD-маркеров. Исследования изменений содержания CD-антигенов методами проточной цитофлуориметрии и таргетной МС показали однонаправленные результаты. Протеомный профиль поверхностных маркеров (CD38, CD54, CD11b и CD18), характерный для линий NB4 и HL-60, отражает различные молекулярные пути реализации ATRA-индуцированной дифференцировки лейкозных клеток в зрелые нейтрофилы.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: поверхностные CD-маркеры; транскриптомика; протеомика; иммунохимия; SRM/SIS анализ; полностью-*транс*-ретиноевая кислота (ATRA)

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