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ПРОТЕОМНОЕ ПРОФИЛИРОВАНИЕ МОНОЦИТОПОДОБНЫХ КЛЕТОК ЛИНИИ THP-1 И ПРОДУЦИРУЕМЫХ ИМИ МИКРОВЕЗИКУЛ С ПОМОЩЬЮ MALDI-МАСС-СПЕКТРОМЕТРИИ

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Резюме. Отделяющиеся от плазматической мембраны клетки экстраклеточные везикулы принимают активное участие в межклеточной коммуникации, транспортируя широкий спектр молекул, среди которых важное функциональное значение придается белкам, липидам, нуклеиновым кислотам и сахарам. Одним из важных этапов в понимании дистантной коммуникации клеток и механизмов ее регуляции является изучение протеома различных экстраклеточных везикул, в том числе микровезикул и экзосом. Синтезируемые моноцитами провоспалительные цитокины и отдельные компоненты системы комплемента играют ключевую роль в осуществлении их специфических функций. Целью данного исследования явилось изучение протеомного состава моноцитоподобных клеток линии THP-1 и продуцируемых ими микровезикул. В результате MALDI-масс-спектрометрического анализа электрофоретических белковых фракций лизата клеток и микровезикул идентифицировано 107 белков, выполняющих различные функции. Среди 19 функциональных групп наибольшие по численности группы образуют белки-регуляторы транскрипции и белки с неизвестными функциями, домены. Наименьшие по численности функциональные группы представлены белками-регуляторами клеточной дифференцировки и морфогенеза, белками иммунного ответа и воспаления, рецепторами и их регуляторами, транспортными белками и белками-регуляторами транспорта, белками-регуляторами клеточной адгезии и процессинга белков, белками убиквитин-протеасомной системы деградации белков, белками внутриклеточной сигнализации, белками-регуляторами аутофагоцитоза и экзоцитоза, белками структуры хроматина, белками-регуляторами гемостаза, гормонами. Промежуточное

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положение занимают цитокины и факторы роста, ферменты, белки цитоскелета, структурные и моторные белки, белки-регуляторы трансляции, транскрипции и процессинга РНК. С помощью последующего кластерного анализа (DAVID Functional Annotation Clustering) идентифицированы наиболее широко представленные группы белков, распределенных по молекулярной функции, биологическому процессу и по положению в клетке. Отдельно в микровезикулах идентифицированы среди прочих белковых молекул белки иммунного ответа и воспаления, цитокины и факторы роста, белки внутриклеточной сигнализации, белки-регуляторы клеточной дифференцировки и морфогенеза, белки-регуляторы клеточной адгезии. Полученные данные о частичном протеоме моноцитоподобных клеток линии THP-1 и продуцируемых ими микровезикул расширяют имеющиеся представления о дистантной коммуникации клеток и указывают на новые механизмы взаимодействия моноцитов/макрофагов и их микроокружения.

Ключевые слова: иммунный ответ, моноциты, макрофаги, микровезикулы, воспаление, протеомный анализ, MALDI-масс-спектрометрия

MALDI-TOF MASS SPECTROMETRIC PROTEIN PROFILING OF THP-1 CELLS AND THEIR MICROVESICLES

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Abstract. Extracellular vesicles that are shed from the plasma membranes take an active part in intercellular communication, transporting a wide range of molecules, including proteins, lipids, nucleic acids and carbohydrates, being of great functional importance. One of the steps to better understanding of distant communications of cells and their regulatory mechanisms is a proteomic study of various extracellular vesicles, including microvesicles and exosomes. Pro-inflammatory cytokines produced by monocytes and individual complement system components play a key role in their specific functioning. The aim of this work was to study proteomic composition of THP-1 monocyte-like cells and their microvesicles. The MALDI-mass spectrometric analysis of electrophoretic protein fractions of cell lysates and microvesicles allowed for identifying 107 proteins that perform various functions. Among 19 determined functional groups, the largest ones comprise transcription regulators and proteins with unknown functions. The smallest functional groups include regulators of cell differentiation and development, proteins participating in immune response and inflammation, cellular receptors and their regulators, transporter and transport regulatory proteins, as well as cell proteins mediating adhesion and matrix structures, processing regulators, proteins of ubiquitin-proteasome system, intracellular signaling, autophagy and exocytosis regulators, chromatin structural proteins, hemostatic regulators, and peptide hormones. An intermediate position is occupied by cytokines and growth factors, enzymes, cytoskeleton and motor proteins, as well as RNA processing and translation regulators. The subsequent DAVID Functional Annotation Clustering analysis allowed for identifying the most common groups distributed by their molecular function, biological processes, and cellular component. Separately, in the microvesicles derived from THP-1 monocyte-like cells, proteins of the immune response and inflammation, cytokines and growth factors, intracellular signaling proteins, cell differentiation regulators and developmental proteins, as well as cell adhesion and matrix proteins were identified among other protein molecules. The data obtained on the partial proteome of THP-1 monocyte-like cells and their microvesicles extend the existing knowledge on distant communications between the cells and suggest new mechanisms of interaction between monocytes/macrophages and their microenvironment.

Keywords: immune response, monocytes, macrophages, microvesicles, inflammation, proteomics, MALDI-TOF mass spectrometry

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Introduction

Microvesicles (MVs) are subcellular structures that are shed from the plasma membrane and may participate in intercellular communication. MVs transfer a variety of proteins, nucleic acids, lipids, and sugars from cell to cell [23] and are involved in the regulation of numerous biological processes, including angiogenesis, placentation, regeneration, and malignancy [32].

Of particular interest among various MV sources are monocytes, which are the most active phagocytes of peripheral blood. They carry out antitumor, antiviral, antimicrobial, antifungal and antiparasitic immunity, as well as participate in the specific immune response [27, 35]. There is evidence of the ability of monocytes to produce MVs, since those with the CD14 phenotype (LPS-R) have been found in peripheral blood plasma [34, 37].

The activity of exosomes derived from inactivated and activated THP-1 cells against THP-1 differentiated macrophages and THP-1 undifferentiated monocytes, as well as other types of cells, has been shown in a number of studies devoted to the isolation and description of various types of extracellular MVs produced by the THP-1 monocyte-like cell line. According to the authors, this activity was due to the activation of ERK1/2 and p38 kinases and the increased secretion of proinflammatory cytokines (TNFα, IL-8, IL-12) [10, 33], as well as the presence of such monocyte effector molecules in those exosomes as chitinase-3-like protein 1, acidic mammalian chitinase, C-C motif chemokine 5, interleukin 4-induced 1, vimentin, cell division control protein 42 homolog, RhoC, Rap1-GTP-interacting adaptor molecule, integrin-linked kinase [33], thyroid hormone receptor-associated protein 3, HLA-DRA, deoxynucleoside triphosphate triphosphohydrolase SAMHD1, STAT1, STAT2, interferon-induced protein with tetratricopeptide repeats 1, ubiquitin-like protein ISG15, interferon induced protein 44 like, and other proteins [40]. Other researchers have shown a similar activity of MVs produced by infected macrophages against intact macrophages *in vitro* and *in vivo* [39], as well as an activity of MVs derived from infected THP-1 monocyte-like cells against intact THP-1 cells [14]. The authors of these studies consider promising the use of proteomic technologies for elucidating the mechanisms of interaction of immune cells with their microenvironment in response to infection and are considering the possibility of using extracellular MVs as an alternative to existing therapeutic drug delivery systems.

Previously, using various modifications of gel electrophoresis and mass spectrometry, there was shown that the MVs produced by the THP-1 monocyte-like cell line contain cytoskeleton proteins, cell adhesion receptors, signaling molecules, heat shock proteins, protein biosynthesis and energy metabolism enzymes, components of the ubiquitin-proteasome system, nuclear proteins [4], as well as proteins involved in MV formation, vesicular transport, and the immune response [4, 14]. Given the incompleteness of available information on the proteome of the source cells [12, 17, 18] and extracellular MVs produced by them, this study was aimed at expanding the existing knowledge of the proteomic profile of THP-1 cells and their MVs. To undertake this, direct MALDI-TOF mass spectrometry assay was used for identification of tryptic peptides in gel strips obtained after the one-dimensional gel electrophoresis analysis. Data on protein profiling of MVs produced by monocytes/macrophages will allow for assessing previously unknown mechanisms of interaction between these cells and their microenvironment under physiological and inflammatory conditions.

Materials and methods

Cells and cell culture

The cells of the THP-1 monocyte-like cell line (American Tissue Culture Collection, USA) obtained from the peripheral blood of a 1-year-old human male with acute monocytic leukemia were cultured in a suspension culture in accordance with the manufacturer's recommendations at a concentration of 0.7-1.0 × 10^6 cells/ml using the complete cell culture medium based on RPMI-1640 (Sigma-Aldrich Chem. Co., USA) containing 10% fetal calf serum (Invitrogen, USA). The medium was inactivated at 56 °C for 30 min, depleted of its own MVs using membrane filters with a pore diameter of 0.1 μm, and supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich Chem. Co., USA). The cells were cultured using standard cell culture procedures under the damp atmosphere at 37 °C and 5% CO_2. Using the trypan blue solution, the cell vitality was evaluated, which was not less than 96%.

Isolation of biomaterial

One day before the isolation of MVs in the flasks containing the cell culture, the culture medium was completely replaced with a dilution required to achieve a concentration of 1.0 × 10^6 cells/ml. The next day, the cell vitality was evaluated, after which the culture media from the flasks were centrifuged at 200 g (22 °C, 10 min) to separate the cells.

Because of no single standard protocol available for the isolation and characterization of MVs, a variety of methodological approaches are currently used to obtain MV fractions with a proper degree of purity and enrichment [22]. Therefore, the MVs separated from THP-1 cells were isolated by the modified step-wise centrifugation method in Hanks's solution without Ca^{2+} and Mg^{2+} (Sigma-Aldrich Chem. Co., USA), for which the supernatants were sequentially centrifuged at 500 g (4 °C, 10 min) and 9 900 g (4 °C, 10 min). After the second centrifugation, the pellet was washed twice with cold phosphate buffer solution (PBS; Sigma-Aldrich Chem. Co., USA) and was recentrifuged at 19 800 g (4 °C, 20 min). The supernatant was discarded, with the pellet washed several times with cold PBS, each time precipitating the MVs by centrifugation at 19 800 g (4 °C, 20 min). The purified pellet was resuspended in MilliQ deionized water, the protease inhibitor mixture (cOmplete, EDTA-free; Roche Diagnostics GmbH, Germany) being added at the concentration specified by the manufacturer, and was then stored at -80 °C until being analyzed. This protocol allows for isolating MVs with a diameter of 100-200 nm with sufficient purity and minimal losses of the biomaterial, while the MVs are sequentially separated from coarse particles of cellular debris and large apoptotic bodies, as well as from exosomes [21].

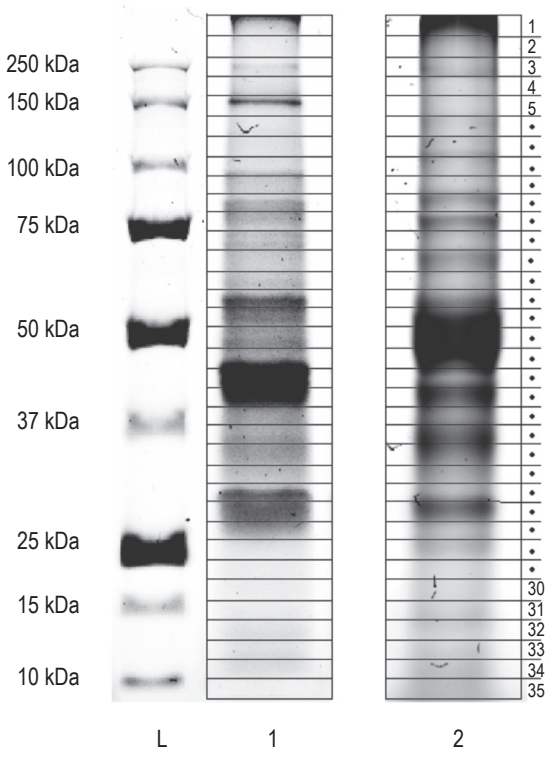

Figure 1. One-dimensional gel electrophoregrams showing 35 excised segments: L. Ladder; 1. Lysate of THP-1 cells; 2. Lysate of microvesicles produced by THP-1 cells (Coomassie G250 staining, 40 μg protein load in the both lysates)

Laser correlation analysis of microvesicles

The granulometric analysis of the MVs was performed by the dynamic light scattering method described in [20]. The MV diameter was calculated using Zetasizer Software 7.11 (Malvern Instruments, UK). The dimensions of the isolated MVs were shown to lie in the 170-410 nm range, which corresponds to the diameter of ectosomes (100-1000 nm), with the peak size amounting to 195 nm. The granulometric data obtained by us were consistent with the results of other researchers who evaluated the size of MVs produced by a number of cells [38].

Biomaterial preparation

The frozen cells and their MVs were thawed and subjected to repeated "freeze-thaw" cycles five times, and were then intensively homogenized in a glass homogenizer for 5 min. The debris was removed by centrifugation at 16 000 g (4 °C, 10 min), with the supernatant collected for further investigation.

Spectrophotometric analysis

The analysis of protein content in the cell and MV lysates was performed through the Bradford protein assay using the NanoDrop One spectrophotometer and NanoDrop One Viewer software (Thermo Scientific, USA).

One-dimensional gel electrophoresis analysis

Cell and MV proteins (the protein content was 40 μg in the both lysates) were fractionated in the Laemmli SDS electrophoresis system in 10% polyacrylamide gel under denaturing conditions in accordance with the manufacturer's protocol (Bio-Rad Laboratories, USA). Fractionated proteins in the gel were visualized by Coomassie G250 staining, after which 35 stained segments, an equal amount for the cells and their MVs, were excised from the gel (Figure 1).

MALDI-TOF mass spectrometric analysis

To remove the dye and SDS, the excised gel strips were crushed and washed three times in 50% acetonitrile (Sigma-Aldrich Chem. Co., USA) in 30 mM Tris buffer solution (pH 8.2) within 15 min at room temperature. After discarding the solution, the pieces of gel were dehydrated by incubation for 10 min in acetonitrile, and then, after removing the latter, the samples were dried for 40 min at 4 °C.

Thereafter, 10 μl of modified bovine trypsin solution (Promega, USA) in 50 mM ammonium bicarbonate with a concentration of 20 ng/ml were added to the dried samples and incubated for 1 h on ice until the gel was completely rehydrated. After that, the excess trypsin solution was removed, and 50 μl of 30 mM Tris buffer solution (pH 8.2) were added to the samples, and those were incubated for 16-18 h at 37 °C. Mixtures of tryptic peptides were extracted three times

from the gel with 50% acetonitrile solution in 30 mM Tris buffer solution (pH 8.2) containing 0.1% formic acid (Sigma-Aldrich Chem. Co., USA) in an ultrasonic bath for 20 min. The peptides in the resulting solutions were dried up in the air at 4 °C and frozen at -80 °C until being analyzed.

On the day of the analysis, the dried-up mixtures of tryptic peptides were dissolved in 50 μl of 50% acetonitrile-water solution (Sigma-Aldrich Chem. Co., USA) containing 0.1% trifluoroacetic acid (Sigma-Aldrich Chem. Co., USA). The contents of the tubes were thoroughly mixed on a Vortex shaker until being completely dissolved. The solutions were then applied to standard steel target plates for MALDI analysis based on the following protocol: 2 × 0.5 μl of the matrix solution and 5 × 0.5 μl of a protein sample solution (in order to concentrate it on the substrate as much as possible). 2,5-dihydroxybenzoic acid at a concentration of 10 mg/ml in 10 mM sodium chloride (Sigma-Aldrich Chem. Co., USA) was used as the matrix. The mixtures were dried up in the air.

MALDI-TOF mass spectra of tryptic peptides were acquired on an Axima Resonance MALDI mass spectrometer (Shimadzu/Kratos Analytical Ltd., UK) in the range of 200-3000 m/z with mass accuracy of all measurements within 0.01 m/z unit, selecting the laser power which is optimal for achieving good results. The measurements were carried out in the positive ion mode.

Proteins were searched against the UniProt/SwissProt database (https://www.uniprot.org) and the NCBI database (https://www.ncbi.nlm.nih.gov) with a taxonomic restriction for the species *Homo sapiens* using the Mascot search engine (www.matrixscience.com) by peptide mass fingerprinting. Parallel search was performed using the database of inverted and random amino acid sequences (decoy). After the peptides were identified, they were checked for their compliance with their actual positions on the gel.

Functional analysis

The identified proteins were divided into groups depending on molecular function, cellular component, and biological process. It should be noted that such a division is largely arbitrary, since many of the established proteins show multiple functions in the cell, and in most cases, this division corresponds to the division principle accepted in the SwissProt and NCBI databases. The functions of the proteins and their localizations in the cells were also determined using the GeneGO database with the algorithms of the DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov).

Results

The total protein content in THP-1 cells and their MVs was found to amount to 82.4±5.86 μg/10^6 cells and 0.15±0.036 μg/10^6 source cells, respectively. These data subsequently allowed for calculating the protein load of the gel in order to obtain valid results.

The MALDI-TOF mass spectrometric analysis determined a total of 107 proteins that have a variety of functions (Table 1, Table 2).

The subsequent manual functional analysis showed that among 19 determined functional groups, the largest (> 15% of the total) ones comprise transcription regulators (20 entries) and proteins with unknown functions (19 entries). The smallest (< 5% of the total) functional groups include cell differentiation regulators and developmental proteins (5 entries), proteins of the immune response and inflammation (4 entries), receptors and receptor regulators (4 entries), transport proteins and transport regulatory proteins (4 entries), cell adhesion and matrix proteins (3 entries), protein processing regulators (3 entries), ubiquitin-proteasome system proteins (3 entries), intracellular signaling proteins (3 entries), autophagy regulators (2 entries), exocytosis regulators (1 entry), chromatin structural proteins (1 entry), hemostatic regulators (1 entry), and hormones (1 entry). An intermediate position (5-15% of the total) is occupied by cytokines and growth factors (10 entries), enzymes (9 entries), cytoskeleton and motor proteins (8 entries), and RNA processing and translation regulators (6 entries).

The identified entries were also distributed into functional groups by the DAVID GO analysis algorithm, with the proteins showing different functions in the cell appearing simultaneously in several groups. The subsequent cluster analysis (DAVID Functional Annotation Clustering) allowed for combining similar functional groups under one broader concept. Thus, the most common groups were obtained, being distributed by molecular function, biological process, and cellular component (Figure 2).

The analysis of the identified proteins distributed by molecular function showed that the bulk of the clusters is involved in sequence-specific DNA (8.5% of the total) and cytoskeletal protein (7.5% of the total) binding, as well as growth factor activity (4.7% of the total), while the minor components altogether account for only 7.5% of the total number of the identified proteins and include clusters involved in myosin heavy chain binding, transmembrane receptor protein serine/threonine kinase binding, BMP receptor binding, and NADP-retinol dehydrogenase activity (Figure 2A).

The distribution of proteins by biological process showed that the most representative (6.6-8.5% of the total) clusters are involved in positive regulation of transcription and transcription from RNA polymerase II promoter, while clusters involved in positive chemotaxis, adult locomotory behavior, anterior/posterior pattern specification, positive regulation of dopamine secretion, organ induction, and cellular re-

TABLE 1. MALDI-TOF MASS SPECTROMETRIC PROTEOME PROFILING OF THP-1 CELLS (p < 0.05)

Protein	UniProtKB / NCBI(*) entries	Gene	MW, kDa	pI	Number of peptides (% AAC)
Cytoskeleton and motor proteins					
Actin-binding LIM protein 2 isoform 1	Q6H8Q1	ABLIM2	67.8	8.29	11 (10)
Coronin-1A	P31146	CORO1A	51.0	6.25	7 (6)
Coronin-1B	Q9BR76	CORO1B	54.2	5.61	7 (4)
Dynein light chain 2, cytoplasmic	Q96FJ2	DYNLL2	10.3	6.81	7 (14)
RNA processing regulators					
tRNA (cytosine(38)-C(5))-methyltransferase isoform a	O14717	TRDMT1	44.6	8.78	6 (5)
Protein processing regulators					
Beta-1,3-galactosyltransferase 2	O43825	B3GALT2	49.2	9.50	10 (6)
Glycoprotein endo-alpha-1,2-mannosidase	Q5SRI9 / NP_078917(*)	MANEA	53.6	9.14	11 (7)
UDP-N-acetyl-alpha-D-galactosamine polypeptide N-acetylgalactosaminyl-transferase 13 isoform b	X5DRI3 / AHW56697(*)	GALNT13	7.4	9.39	6 (19)
Enzymes					
Iduronate 2-sulfatase	O60597 / AAC05984(*)	IDS	19.5	8.80	6 (11)
Iduronate 2-sulfatase (Hunter syndrome) isoform CRA_a	EAW61280(*)	IDS	19.5	9.21	6 (11)
Retinol dehydrogenase 13 isoform 1	Q8NBN7	RDH13	35.9	8.23	6 (7)
Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3 isoform 1	Q9P035	HACD3	43.1	9.04	6 (4)
Receptors. Receptor regulators					
Olfactory receptor 1L6	Q8NGR2	OR1L6	39.5	9.60	6 (6)
Prostate and testis expressed protein 4	P0C8F1	PATE4	11.4	8.97	6 (22)
Hormones					
Prolactin-releasing peptide	P81277	PRLH	9.6	11.66	6 (13)
Proteins of the immune response and inflammation					
C-type lectin domain family 2 member B	Q92478	CLEC2B	17.3	9.02	6 (13)
Protein phosphatase 1B isoform beta-1	O75688	PPM1B	52.6	4.95	8 (5)
Protein phosphatase 1 regulatory subunit 14B	Q96C90	PPP1R14B	15.9	4.75	5 (10)

Таблица 1 (продолжение)
Table 1 (continued)

Protein	UniProtKB / NCBI(*) entries	Gene	MW, kDa	pI	Number of peptides (% AAC)
Cytokines. Growth factors					
Bone morphogenetic protein 8A	Q7Z5Y6	BMP8A	44.8	9.06	6 (2)
Bone morphogenetic protein 8B isoform 1	P34820	BMP8B	44.7	8.76	6 (3)
Stromal cell-derived factor 1 isoform alpha precursor	P48061	CXCL12	10.1	9.72	5 (16)
Stromal cell-derived factor 1 isoform beta	P48061	CXCL12	10.7	9.93	5 (16)
Stromal cell-derived factor 1 isoform theta	P48061 / ABC69273(*)	CXCL12	11.4	9.67	5 (15)
Exocytosis regulators					
Synaptotagmin-8 isoform 4	Q8NBV8	SYT8	44.1	9.65	7 (6)
Transcription factors					
Cyclin-dependent kinase 9 isoform 1	P50750	CDK9	42.8	8.97	9 (8)
Flt3-interacting zinc finger protein 1	Q96SL8	FIZ1	52.0	8.59	8 (3)
Homeobox protein Hox-A7	P31268 / NP_008827(*)	HOXA7	25.3	5.26	6 (6)
Homeobox protein Hox-D12 isoform 1	P35452	HOXD12	29.0	9.82	6 (6)
Pirin	O00625	PIR	32.1	6.42	11 (7)
Putative transcription factor ovo-like protein 3	O00110 / AAB51180(*)	OVOL3	24.3	10.08	9 (11)
Putative transcription factor ovo-like protein 3 isoform X2	XP_011525553(*)	OVOL3	13.2	9.77	6 (13)
Putative zinc finger protein 840	A6NDX5	ZNF840P	83.2	9.69	13 (3)
Transcription initiation factor TFIID subunit 8 isoform 1	Q7Z7C8	TAF8	34.2	6.03	6 (5)
Zinc finger and SCAN domain-containing protein 9 isoform 2	O15535 / NP_001186408(*)	ZSCAN9	51.6	8.07	9 (8)
Zinc finger protein GLIS2	Q9BZE0	GLIS2	55.7	9.08	6 (4)
Cell differentiation regulators. Developmental proteins					
Neuronatin isoform alpha	Q16517	NNAT	9.2	10.17	4 (18)
Outer dense fiber protein 3 isoform 1	Q96PU9	ODF3	27.7	9.90	5 (9)
Vexin isoform 1	Q8TAG6	VXN	22.6	10.05	9 (6)

Таблица 1 (окончание)
Table 1 (continued)

Protein	UniProtKB / NCBI(*) entries	Gene	MW, kDa	pI	Number of peptides (% AAC)
Cell adhesion and matrix proteins					
Sperm acrosome membrane-associated protein 3 isoform CRA_b	Q8IXA5 / EAW80218(*)	SPACA3	15.1	8.93	5 (12)
Proteins with unknown functions. Domains					
C2 domain-containing protein 2, partial	Q9Y426 / CAB43307(*)	C2CD2	19.8	5.97	6 (8)
Capsid scaffold protein	A0A126LB05 / AMD82185(*)	U53.5	27.6	7.06	7 (6)
cDNA FLJ39825 fis, clone SPLEN2012175, highly similar to Nicotinamide riboside kinase 1	B3KUG3 / BAG53425(*)	N/A	21.7	5.44	6 (7)
JHDM1D protein	A0JNV9 / AAI27008(*)	JHDM1D	9.8	11.46	7 (16)
Nicotinamide riboside kinase 1 isoform 3	Q5W125 / NP_001317607(*)	NMRK1	23.8	5.33	6 (6)
Nuclear pore complex-interacting protein family member B7	O75200	NPIPB7	47.7	10.35	6 (3)
Outcome predictor in acute leukemia 1, partial	Q1EG69 / AAV68560(*)	OPAL1	3.3	11.53	5 (33)
Putative uncharacterized protein B3GALT5-AS1 isoform 1	P59052	B3GALT5-AS1	15.7	9.21	4 (4)
Testis-expressed protein 50	A0A1B0GTY4	TEX50	20.8	9.36	6 (8)

Note. Abbreviations: AAC, amino acid coverage; MW, molecular weight; pI, isoelectric point; N/A, not applicable or not available.

TABLE 2. MALDI-TOF MASS SPECTROMETRIC PROTEOME PROFILING OF MICROVESICLES PRODUCED BY THP-1 CELLS ($p < 0.05$)

Protein	UniProtKB / NCBI(*) entries	Gene	MW, kDa	pI	Number of peptides (% AAC)
Cytoskeleton and motor proteins					
Costars family protein ABRACL	Q9P1F3	*ABRACL*	9.1	5.86	5 (17)
Myosin regulatory light chain 12A	P19105	*MYL12A*	19.8	4.67	7 (10)
Myosin regulatory light chain 12B	O14950	*MYL12B*	19.8	4.71	7 (10)
Myosin regulatory light polypeptide 9 isoform 1	P24844	*MYL9*	19.8	4.80	7 (10)
RNA processing and translation regulators					
cDNA FLJ35275 fis, clone PROST2006282, weakly similar to Translation initiation factor IF-2	Q8NAJ1 / BAC03923(*)	N/A	27.4	11.90	10 (10)

Таблица 2 (продолжение)
Table 2 (continued)

Protein	UniProtKB / NCBI(*) entries	Gene	MW, kDa	pI	Number of peptides (% AAC)
28S ribosomal protein S11 isoform 1, mitochondrial	P82912	MRPS11	20.6	10.82	7 (11)
39S ribosomal protein L12, mitochondrial	P52815	MRPL12	21.3	9.04	7 (7)
Mitochondrial assembly of ribosomal large subunit protein 1	Q96EH3	MALSU1	26.2	5.32	7 (7)
Ribonuclease P protein subunit p20	O75817	POP7	15.6	9.09	8 (14)
Ubiquitin-proteasome system proteins					
RING finger protein 175 isoform X3	XP_011530183(*)	RNF175	33.9	9.16	7 (5)
RING finger protein 175 isoform CRA_a, partial	EAX04950(*)	RNF175	34.1	9.15	7 (5)
UBX domain containing 5 isoform CRA_d	EAX07827(*)	UBXN5	21.5	8.42	7 (12)
Enzymes					
Acyl-coenzyme A thioesterase 13 isoform 1	Q9NPJ3	ACOT13	15.0	9.23	8 (16)
cDNA FLJ61119, highly similar to Developmentally-regulated GTP-binding protein 2	B4DIG2 / BAG58474(*)	N/A	15.5	9.27	8 (24)
Cytosolic 5'-nucleotidase 1A	Q9BXI3	NT5C1A	41.0	6.11	7 (6)
Dehydrogenase/ reductase SDR family member 4 isoform 2	Q9BTZ2 / NP_001269916(*)	DHRS4	20.3	10.32	11 (20)
Valacyclovir hydrolase isoform 1	Q86WA6	BPHL	32.5	9.20	6 (7)
Receptors. Receptor regulators					
Oestrogen receptor, partial	Q13262 / AAB35900(*)	7 ER	4.6	6.45	6 (13)
Paired immunoglobulin-like type 2 receptor beta isoform 3	AAG17224(*)	PILRB	29.6	9.84	6 (11)
Proteins of the immune response and inflammation					
TLR4 interactor with leucine rich repeats	Q7L0X0	TRIL	88.7	9.70	21 (3)
Cytokines. Growth factors					
Astrocyte-derived trophic factor 2	AAB33494(*)	GDNF	14.7	9.30	9 (16)
Fibroblast growth factor 10	O15520	FGF10	23.4	9.61	7 (9)
Fibroblast growth factor 10, partial	O15520 / CAG46489(*)	FGF10	23.4	9.67	7 (9)

Таблица 2 (продолжение)
Table 2 (continued)

Protein	UniProtKB / NCBI(*) entries	Gene	MW, kDa	pI	Number of peptides (% AAC)
Fibroblast growth factor 10, partial	Q8NFI9 / AAM46926(*)	FGF10	19.2	9.94	7 (12)
Glial cell line-derived neurotrophic factor isoform 1	P39905	GDNF	23.7	9.26	15 (9)
Autophagy regulators					
Microtubule-associated proteins 1A/ 1B light chain 3B	Q9GZQ8	MAP1LC3B	14.7	8.89	5 (11)
Microtubule-associated proteins 1A/ 1B light chain 3 beta 2	A6NCE7	MAP1LC3B2	14.6	8.74	5 (11)
Intercellular signaling proteins					
BTB/ POZ domain-containing protein KCTD20 isoform 2	Q7Z5Y7 / NP_001273508(*)	KCTD20	28.9	5.25	8 (7)
Casein kinase II subunit alpha isoform 1	P68400	CSNK2A1	45.1	7.29	7 (4)
Guanylyl cyclase-activating protein 3 isoform 1	O95843	GUCA1C	23.8	4.95	7 (8)
Transport proteins. Transport regulatory proteins					
ATP synthase H+ transporting mitochondrial F1 complex delta subunit isoform CRA_a	EAW69531(*)	ATP5F1D	14.7	11.83	7 (14)
Biogenesis of lysosome-related organelles complex 1 subunit 6 isoform 1	Q9UL45	BLOC1S6	19.7	6.01	7 (11)
Sesquipedalian-1 isoform 1	Q8N4B1	PHETA1	27.2	9.18	9 (6)
Vacuolar protein sorting-associated protein VTA1 homolog isoform 1	Q9NP79	VTA1	33.9	5.87	5 (3)
Hemostatic regulators					
Endothelin-2 isoform 2 preproprotein	NP_001289198(*)	EDN2	16.5	10.19	7 (11)
Transcription factors					
cDNA FLJ38903 fis, clone NT2NE2001252, highly similar to Homeobox protein Hox-B8	Q8N8T3 / BAC04730(*)	N/A	27.7	8.18	7 (7)
GA-binding protein subunit beta-1 isoform 1	Q06547	GABPB1	42.5	4.77	6 (5)
Homeobox protein BarH-like 2	Q9UMQ3	BARX2	31.2	8.65	5 (4)
Homeobox protein Hox-B8	P17481 / NP_076921(*)	HOXB8	27.6	8.48	7 (7)
Homeobox protein Hox-C8	P31273	HOXC8	27.7	6.57	6 (5)

Таблица 2 (окончание)
Table 2 (continued)

Protein	UniProtKB / NCBI(*) entries	Gene	MW, kDa	pI	Number of peptides (% AAC)
Homeobox protein MOX-1 isoform 1	P50221	MEOX1	28.0	7.79	6 (5)
PAXIP1-associated glutamate-rich protein 1	Q9BTK6	PAGR1	27.7	4.40	6 (4)
THAP domain-containing protein 8	Q8NA92	THAP8	30.1	10.24	8 (6)
ZZ-type zinc finger-containing protein 3, partial	Q8IYH5 / BAB84945(*)	ZZZ3	58.1	4.96	11 (6)
Chromatin structural proteins					
Histone H4	P62805	H4C1	11.4	11.36	11 (22)
Cell differentiation regulators. Developmental proteins					
Netrin-4 isoform 1	Q9HB63	NTN4	70.0	8.44	10 (4)
Netrin-4, partial	Q9HB63 / BAB14964(*)	NTN4	37.9	8.40	13 (9)
Cell adhesion and matrix proteins					
LIM and senescent cell antigen-like-containing domain protein 2 isoform 2	Q7Z4I7 / NP_060450(*)	LIMS2	41.5	8.71	18 (6)
LIM and senescent cell antigen-like-containing domain protein 2 isoform X2	XP_011509755(*)	LIMS2	36.6	8.92	18 (7)
Proteins with unknown functions. Domains					
cDNA FLJ75546	A8K1F9 / BAF82563(*)	N/A	13.3	10.88	5 (11)
Chromosome 11 open reading frame 58 isoform CRA_c	E9PRZ9 / EAW68457(*)	C11orf58	10.6	4.09	5 (15)
Divergent protein kinase domain 1C isoform 1	Q0P6D2	DIPK1C	46.4	6.38	6 (4)
Leucine-rich repeat and IQ domain-containing protein 4	A6NIV6	LRRIQ4	63.9	8.43	8 (6)
Protein CEI isoform 1	Q86SI9	C5orf38	15.1	11.42	7 (9)
Small acidic protein	O00193	SMAP	20.3	4.57	6 (8)
Testis-expressed protein 50	A0A1B0GTY4	TEX50	20.8	9.36	5 (6)
Testis-expressed protein 51	A0A1B0GUA7	TEX51	18.8	7.59	5 (7)
Testis-expressed protein 51 isoform X12	XP_011510580(*)	TEX51	18.6	6.43	5 (8)
Uncharacterized protein C5orf47	Q569G3	C5orf47	19.2	10.48	6 (11)
Uncharacterized protein DKFZp762B162	Q69YQ6 / CAH10612(*)	DKFZp762B162	18.5	6.51	12 (12)

Note. As for Table 1.

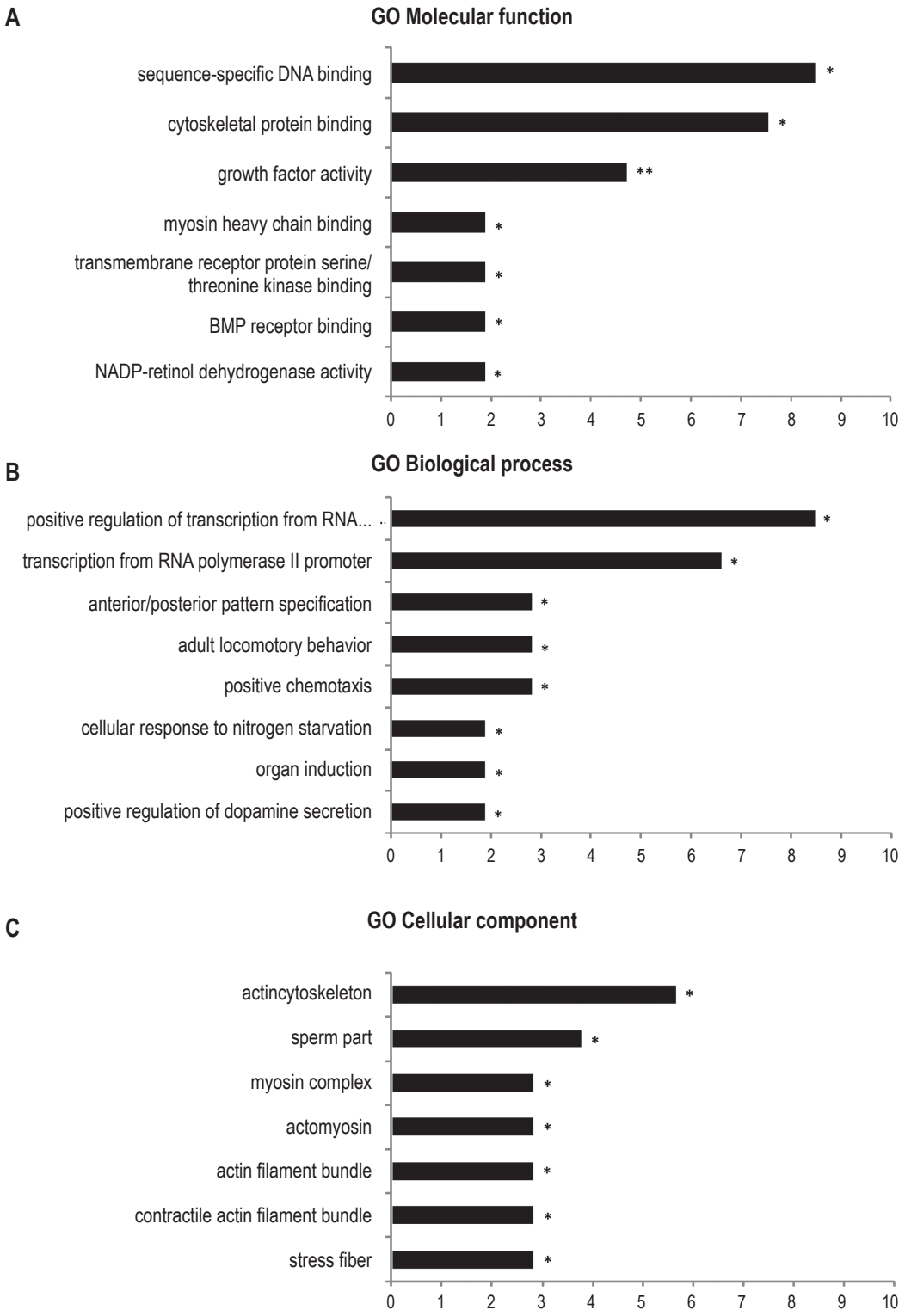

Figure 2. Most common clusters of proteins obtained from lysates of THP-1 cells and their microvesicles: A, molecular function; B, biological process; C, cellular component (percentage of the total number of identified proteins; * p < 0.05, ** p < 0.005; DAVID 6.8)

sponse to nitrogen starvation are minor components, which account for only 14.2% of the total number of the identified proteins (Figure 2B).

In contrast, the functional groups distributed by cellular component were found to be more uniform. Approximately equal proportions (2.8-3.8% of the total) were obtained for the clusters associated with sperm part, myosin complex, actomyosin, actin filament bundle and contractile actin filament bundle, and stress fiber. At the same time, the most representative cluster, which accounts for only 5.7% of the total number of the identified proteins, was formed by proteins associated with the actin cytoskeleton as a whole (Figure 2C).

Among 16 functional groups of proteins identified separately in the MVs, the largest (> 15% of the total) ones are represented by proteins with unknown functions (11 entries) and transcription regulators (9 entries). The smallest (< 5% of the total) functional groups comprise receptors and receptor regulators (2 entries), autophagy regulators (2 entries), cell differentiation regulators and developmental proteins (2 entries), cell adhesion and matrix proteins (2 entries), proteins of the immune response and inflammation (1 entry), hemostatic regulators (1 entry), and chromatin structural proteins (1 entry). An intermediate position (5-15% of the total) is occupied by RNA processing and translation regulators (5 entries), enzymes (5 entries), cytokines and growth factors (5 entries), cytoskeleton and motor proteins (4 entries), transport proteins and transport regulatory proteins (4 entries), ubiquitin-proteasome system proteins (3 entries), and intracellular signaling proteins (3 entries) (Table 2).

Discussion

THP-1 cells used by us allow for *in vitro* analyzing the main biochemical processes that occur in monocytes *in vivo*, including those leading to spontaneous secretion of their MVs into the extracellular space. The data obtained using MALDI-TOF mass spectrometry indicate that the studied cells and their MVs are characterized by a wide range of proteins with various functions and properties, providing the possibility of multilateral regulation of cell metabolism.

In recent years, international research teams have analyzed the proteome of the THP-1 monocyte-like cell line [12, 17, 18]. However, the most detailed study only mentioned approximately 5 400 proteins, which is significantly less than the number of proteins contained in any eukaryotic cell. That strongly indicates the incompleteness of the monocyte-like cell proteome described to date. Nevertheless, the main functional groups of proteins presented in the above works matched those found in our study (Table 1). However, from the list of proteins presented in the work [4] devoted to the protein profiling of the MVs derived from the THP-1 monocyte-like cell line, only 1 entry (histone H4) matched those found by us in these MVs (Table 2). Meanwhile, the remaining 59 entries of the proteins identified by us in the studied MVs give new information on the proteome of the MVs produced by monocyte-like cells.

In concordance with the biogenesis pathways of the studied MVs, their proteome constitutes a set of protein molecules of cellular origin. Proteomes of extracellular MVs produced by platelets, mature lymphocytes, endotheliocytes, mast and some other types of cells are currently studied in relative detail, and data from these studies are presented by many authors, in particular [8, 11, 26]. Thanks to these works, it is now known that MVs derived from blood and vascular cells contain both non-specific proteins characteristic of any type of cells and specific proteins involved in the functioning of a particular cell. Proteins specific for cells of a certain type are, for example, a T cell receptor expressed primarily on T lymphocytes, platelet P-selectin, and other proteins capable of being involved in the immune response [2]. Regardless of their cellular origin, MV proteins are most often involved in the very formation of vesicles. Undoubtedly, such proteins are tetraspanins (CD9, CD63, CD81, and CD82), heat shock proteins (HSP70, HSP90), cytoskeletal elements, enzymes of various metabolic pathways, adhesion molecules, receptors, as well as proteins of the main histocompatibility complex (MHC) [36].

In the MVs studied by us, proteins belonging to the above classes were also found, in particular cytoskeleton and motor proteins, enzymes, cell adhesion and matrix proteins, and receptors. As well, as common proteins should be classified protein processing regulators and ubiquitin-proteasome system proteins identified in this study. Besides, we found some specific proteins, in particular those involved in the implementation of defense mechanisms, such as proteins of the immune response and inflammation, cytokines and growth factors, as well as their receptors.

Among the proteins identified by us in the studied MVs, cytokines, receptors, and regulatory proteins (Table 3) should be especially distinguished for two reasons. First, most of them have been found in monocytes/macrophages isolated from both tissues or peripheral blood and the transplantable monocyte-like cells. Secondly, the expression of such proteins in the MVs can have a certain signaling or regulatory function in relation to the microenvironment.

For example, fibroblast growth factor 10 (FGF10) identified by us in the studied MVs, which was not previously found there by other researchers, can exert multiple effects on cells of the microenvironment, activating the ERK signaling cascade and thus affecting the proliferation, survival and motility of various types of cells, as well as taking part in the activation of T cells, proliferation, migration and differentiation of

TABLE 3. SEVERAL FUNCTIONS OF PROTEINS OF MICROVESICLES PRODUCED BY THP-1 CELLS

Protein	Function (according to the SwissProt/UniProt and NCBI databases)	Presence in monocytes/ macrophages or THP-1 cells [references]
Fibroblast growth factor 10 (FGF10)	a growth factor; plays an important role in the regulation of embryonic development, cell proliferation and cell differentiation; required for normal branching morphogenesis; may play a role in wound healing; activates ERK1/2 cascades	[15] (*FGF10* gene expression)
Glial cell line-derived neurotrophic factor (GDNF), same as Astrocyte-derived trophic factor (ATF)	a neurotrophic factor; enhances survival and morphological differentiation of dopaminergic neurons and increases their high-affinity dopamine uptake; negatively regulates extrinsic apoptotic signaling pathway in absence of ligand; positively regulates cell differentiation and cell population proliferation; binds various TGF-beta receptors leading to recruitment and activation of SMAD family transcription factors that regulate gene expression	[7]
TLR4 interactor with leucine rich repeats	a component of the TLR4 signaling complex; mediates the innate immune response to bacterial lipopolysaccharide leading to cytokine secretion	[6]
Oestrogen receptor	an estrogen receptor, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription; recruited to the NF-kappa-B response element of the CCL2 and IL8 promoters and can displace CREBBP; present with NF-kappa-B components RELA/p65 and NFKB1/p50 on ERE sequences	[30]
Paired immunoglobulin-like type 2 receptor beta	paired receptors; consist of highly related activating and inhibitory receptors and are widely involved in the regulation of the immune system; thought to act as a cellular signaling activating receptor that associates with ITAM-bearing adapter molecules on the cell surface	[31]
LIM and senescent cell antigen-like-containing domain protein 2	an adapter protein in a cytoplasmic complex linking beta-integrins to the actin cytoskeleton; bridges the complex to cell surface receptor tyrosine kinases and growth factor receptors; plays a role in modulating cell spreading and migration	[31]
BTB/POZ domain-containing protein KCTD20	an intracellular signaling protein; promotes the phosphorylation of AKT family members	[19]
Casein kinase II subunit alpha	an intracellular signaling protein; regulates numerous cellular processes, such as cell cycle progression, apoptosis and transcription, as well as viral infection; required for p53/TP53-mediated apoptosis; phosphorylates the caspases CASP9 and CASP2 and the apoptotic regulator NOL3 (phosphorylation protects CASP9 from cleavage and activation by CASP8, and inhibits the dimerization of CASP2 and activation of CASP8); phosphorylates and regulates numerous transcription factors including NF-kappa-B, STAT1, CREB1, IRF1, IRF2, ATF1, SRF, MAX, JUN, FOS, MYC, and MYB; during viral infection, phosphorylates various proteins involved in the viral life cycles of EBV, HSV, HBV, HCV, HIV, CMV, and HPV; regulates Wnt signaling by phosphorylating CTNNB1 and the transcription factor LEF1	[29]
Endothelin-2 isoform 2 preproprotein	a member of the endothelin protein family of secretory vasoconstrictive peptides; is processed to a short mature form which functions as a ligand for the endothelin receptors that initiate intracellular signaling events; is involved in a wide range of biological processes, such as hypertension and ovulation; regulates growth in several cell types and may also affect differentiation, inflammation, and angiogenesis	[3]

endothelial cells during angiogenesis. FGF10 can also regulate synaptic plasticity and phosphorylation of the transcription factor p53, as well as activate granzyme B cleavage of the FGFR1 receptor [28]. The *FGF10* gene expression was shown in tumor associated macrophages [15]. Moreover, the role of FGF10 in the development of chorionic villi was elucidated, and, as currently established, the factor is expressed by both decidual cells and the cytotrophoblast [1]. The data obtained by us on FGF10 being present in the studied MVs may become a reason for further studies, in particular, of placental macrophages, which will possibly expand existing knowledge of their role in cell communication in the uteroplacental contact area.

Glial cell line-derived neurotrophic factor, also identified by us in the studied MVs, is able to protect from degeneration dopaminergic neurons in the substantia nigra of the midbrain and the terminals of tyrosine hydroxylase-positive axons in the striatum [7]. It can be assumed that TLR4 interactor with leucine rich repeats (a component of Toll-like receptor 4), which is also widely present in the brain and other organs and tissues, when transferred to microenvironmental cells (in particular, peripheral blood mononuclear cells and glial cells), will increase their production of cytokines in response to bacterial infection [6]. Similarly, LIM and senescent cell antigen-like-containing domain protein 2 (an adapter protein in a cytoplasmic complex linking beta-integrins to the actin cytoskeleton) is able to modulate the process of cell migration during tumor development [31].

Furthermore, it was found that the studied MVs contain receptors and their regulators, namely the estrogen receptor and paired immunoglobulin-like type 2 receptor beta. Taking into account the fact that these MVs are able to transmit their receptors to the membranes of recipient cells [25], we can assume the ability of those cells to respond to signals that were previously inaccessible to them, provided that the cells have ready intracellular signal transduction pathways for these receptors [30, 31].

The specific effects of monocytes/macrophages on the surrounding cells and tissues can also be contributed to by the broad-spectrum signaling molecules identified by us, such as casein kinase II subunit alpha, which regulates cell survival at different levels – it promotes DNA repair, affects the NF-κB, Wnt, PI3K/ACT and JAK-STAT signaling cascades, interacts with chaperones, activates antiapoptotic proteins and inactivates proapoptotic ones, including caspases [29], as well as endothelin-2 isoform 2 preproprotein, which plays a key role in blood vessel homeostasis [3], and BTB/POZ domain-containing protein KCTD20, which activates AKT, a key enzyme of the PI3K/AKT signaling pathway involved in the regulation of cell proliferation, growth and survival [19].

The molecular mechanisms of MV formation suggest that MVs should include components of the actin network adjacent to the cell membrane and other cytoskeletal elements [9]. The data obtained by us allow suggesting that the studied MVs may have an ectosomal nature. Thus, we identified proteins of the actin-myosin system and one regulator of the cytoskeleton dynamics, namely myosin and costars family protein ABRACL, that are involved in MV formation and budding from the cell membrane [24, 32]. Various isoforms of cytoskeletal proteins have not been found in exosomes yet according to the available literature [5, 13].

The performed functional and cluster annotation analyzes altogether revealed the predominant molecular functions of the proteins identified in the studied MVs, and showed possible participation of these proteins in biological processes (Figure 2). It was found that the dominant clusters characterizing the molecular function of the identified proteins are associated with sequence-specific DNA and cytoskeletal protein binding, as well as growth factor activity. The association of the proteins with the actin cytoskeleton is also indicated by the distribution of functional groups by the localization of the identified proteins in the cell and their belonging to a variety of cellular parts. Some of these actin-binding proteins are actively involved in the reorganizing of actin filaments in response to the effects of various growth factors, cytokines and chemoattractants and may play a key role in the development of a number of pathologies in the human body [16]. At the same time, the distribution of the proteins by biological process showed that the most representative clusters comprise proteins involved in the regulation of gene transcription, while the groups of proteins responsible for positive chemotaxis, the spatial organization of differentiating cells, and a cellular response were minor components. The results obtained by us are consistent with the biological nature of the studied MVs and are in line with the data collected by other researchers [12, 17, 18].

To date, a number of good proteomic research strategies have been described that are based on a combination of different approaches and techniques. Unfortunately, protein identification is a rather complex multi-stage process and does not always lead to reproducible results. The scheme proposed in this study can lead to good results with a qualitative description of the protein profile of the MVs released from THP-1 cells. However, the search for the target protein using this approach poses significant difficulties, mainly due to the extremely low yield of the total protein. For a more detailed analysis of the MV proteome using mass spectrometric methods, it would be advisable to include in the study protocol additional preparation steps at the stage of the isolation of the MVs, such as preserving accumulation, ultracentrifugation and im-

munoprecipitation. It is also worth noting that the data obtained by us in this study should also be further verified using the immunoblotting method, in both THP-1 cells and macrophages isolated from a variety of tissues, as well as at different stages of macrophage differentiation.

Conclusion

Summarizing the data obtained, we can conclude that MVs produced by the THP-1 monocyte-like cell line, along with common proteins, also contain proteins of the immune response and inflammation, cytokines and growth factors, with the help of which these MVs can contribute to the specific effects of monocytes/macrophages on the surrounding cells. Our data on the proteome of the studied MVs will expand the existing knowledge of distant communication of cells and indicate new mechanisms of interaction between monocytes/macrophages and their microenvironment. The presented results will be useful for further proteomic studies of MVs produced by cells involved in an immune response under physiological and inflammatory conditions.

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