Peptidomics: A modern approach to biodiversity of peptides*

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Abstract: Murine myelomonocytes WEHI-3 and human erythroid leukemia K562 cells were shown to release peptides into the surrounding medium. Four N-terminal sequences of the peptides derived from unknown protein precursors were identified in the conditioned medium of WEHI-3 culture. Twelve N-terminal sequences of the peptides released by K562 cells were established. K562 cells were shown to release long hemoglobin α- and ε-chains fragments, as well as peptides derived from carbonic anhydrase XI, melanophilin, cadherin EGF LAG G-type receptor 2, aldolase A, melastatin 1, peptide homologous to ryanodine receptor fragment, and a few peptides derived from the proteins with unknown function.

Comparative analysis of the peptides released by the intact erythroid leukemia K562 cells and the same cells after differentiation (induced by guanosine monophosphate, GMP, and accompanied by start of hemoglobin biosynthesis), has demonstrated that the level of six components, including all established hemoglobin fragments, carbonic anhydrase fragment, and the fragment of the unknown protein significantly increased in the course of differentiation. The spectrum of the peptides released by K562 cells strongly differed from that of the mature erythrocytes: (1) in contrast to the mature erythrocytes, K562 cells release long α-globin fragments and the fragment of ε-globin; (2) erythrocytes almost exclusively release the products of hemoglobin proteolysis; in the case of K562 cells the fragments of enzymes, receptors, and other functional proteins were found. So, the concentration and the content of the individual peptides released by the cells depend on the differentiation state.

The comparative analysis of the dynamics of peptide secretion by human erythrocytes performed in the presence and the absence of the 3 % glucose has shown that the rate of the peptide release strongly depends on cell metabolism. In summary, peptidomic studies of cell cultures provide valuable information on the mechanisms of peptide pool formation in tissues and the whole organism.

Keywords: peptidomics; cell cultures; erythrocytes; peptides; hemoglobin.

INTRODUCTION

Rapid development of modern analytical techniques provided conditions for total screening of biological samples for any given class of biomolecules participating in metabolism. As a result, several new

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branches of chemical biology have emerged that deal with such analysis, carrying in their name a common suffix "omics", such as proteomics, lipidomics, metabolomics, etc. The terms "peptidome" and "peptidomics" appeared in the literature in 2000–2001 as a logical extension to proteome and proteomics, and were simultaneously employed by several independent groups (see ref. [1] and refs. therein). Respective studies deal with structure and biological properties of complete sets of peptides present in biological tissues, cells, or fluids. A broad range of biological samples, mostly of animal origin, have been subjected in the past 5–10 years to peptidomic analysis by chromatographic, mass-spectral, and Edman degradation techniques. Hundreds of new peptides were discovered and sequenced (Table 1). It was found that specialized protein precursors as well as proteins with other functions (enzymes, structural proteins, transport proteins, etc.) contribute to the formation of peptidomes. Peptides comprising the peptidome participate in a variety of regulatory functions in nervous, endocrine, and other systems. In our preceding publications, we formulated a concept according to which tissue peptidomes (also called tissue-specific peptide pools) generally regulate long-term processes and participate in maintenance of the homeostatic balance (i.e. cell differentiation, proliferation, and specific elimination) [21,22].

Table 1 Main objects and short summary of the results of peptidomic studies.

Tissue/organ/cell culture	Organism	Number of identified peptides	Ref.
Whole brain	Human	20	[2]
Whole brain	Bovine	107	[3]
Whole brain	Rat	61	[4]
Whole brain	Mouse	105	[5]
Whole brain	Mouse	430	[6]
Whole brain	Pig	48	[7]
Whole brain	Ground squirrel	25	[8]
Hypothalamus	Rat	41	[9]
Hypothalamus	Mouse		
Pituitary	Mouse	79	[5]
Pancreas	Frog	21	[10]
Pancreas	Newt		
Entire body	Hydra	300	[11]
Entire body	Hydra	427	[12]
B-lymphocytes	Human	200	[13]
Erythrocytes	Human	51	[14]
Neurons	Drosophila	28	[15]
Neurons	Locust	44	[16]
Neurons	Crab	28	[17]
Blood plasma	Human	340	[18]
Urine	Human	96	[19]
Cerebrospinal fluid	Human	35	[20]

In this paper, we present new data demonstrating that individual cell populations serve as a specific source of peptides to surrounding medium, each contributing thereby its share to the resultant peptide pools.

The ability of normal and tumor cells to release into culture medium bioactive low-molecular substances has been demonstrated in a number of publications [23–27]. However, in most cases the chemical nature of those factors remained unclear. R. V. Petrov, A. A. Mikhailova et al. found that pig bone marrow cells produce a family of short peptides displaying a broad range of immunomodulatory activities [28]. In our studies of peptides generated by human erythrocytes, we found that hemoglobin serves in the surviving red blood cell cultures as a rich source of the peptides (up to 94 amino acid residues

long) located within the cells and of shorter peptides (5–20 residues long) released to the medium [14]. Most of these peptides are active in a variety of biological test systems [14,29,30]. In other words, a growing amount of evidence was pointing to individual cell populations as specific sources of functionally important peptides in vivo. In order to provide more information on that apparently fundamental process, we studied in this work: (i) generation of peptides by two unrelated tumor cell lines (murine myelomonocytic leukemia cells WEHI-3 and human erythroid leukemia cells K562); (ii) the impact on peptide release of one differentiation step in K562 cells (the initial step of their transformation to mature erythrocytes); and (iii) dependence of peptide release from human erythrocytes on cell metabolism.

RESULTS AND DISCUSSION

Two nucleated cell lines of myeloid origin were studied as a source of peptides released to culture medium: WEHI-3 murine transformed myelomonocytes and K562 human erythroid leukemia cells, representing monocyte- and erythrocyte-like types, respectively. The time dependence of peptide secretion was investigated. The peptides secreted by non-induced and partially differentiated K562 cells were analyzed. K562 cells are the polypotent tumor cells that may be differentiated into the monocyte- and erythrocyte-like cell lines [31]. Non-stimulated K562 cells contain mainly the embryonal P and G hemoglobin and correspondingly only the embryonic γ- and ε-globin chains [31]. Induction with various agents leads to the increase of hemoglobin expression level [31]. We used GMP (guanosine monophosphate)-induced K562 cells that are partially differentiated into the erythrocyte-like cell line producing hemoglobin [32]. We compared the components released by the intact and the GMP-induced cells to find out if partial differentiation of K562 cell accompanied by the increase of hemoglobin level would affect the sets of peptides released by these cells. Besides, the peptide sets released by K562 cells were compared with those secreted by erythrocytes. To analyze the dependence of the peptide release on the cell metabolic conditions, we compared the dynamics of peptide secretion by erythrocytes in the presence and in the absence of glucose.

Peptides released by WEHI-3 murine myelomonocyte leukemia cells

To study the dynamics of the peptides released by the WEHI-3 cells, the cells (15.000.000–21.000.000 cells per 30 ml flack per time point) were incubated in isotonic solution for 1, 2, and 3 h. Cell viability was checked by Trypan blue inclusion test. Dead cell percentage was below 4 % at all time points (i.e., spontaneous release of intracellular peptides into the conditioned medium was minimized). After completion of the incubation, the cells were sedimented and the supernatants were collected and subjected to solid-phase extraction followed by reverse-phase high-performance liquid chromatography (RP-HPLC). The cells were shown to release comparatively low amounts of peptide material with the MM 500–12 000 Da. The obtained chromatograms are given in Fig. 1 (A–C). Time dependence of the secretion of the peptides marked on the chromatogram with numbers is given in Fig. 3A. As follows from Fig. 1, the level of the pentapeptide [1] increased in the course of cell incubation in the isotonic solution. The level of the components [2–5] did not change significantly after the first hour of incubation. The major components were subjected to matrix-assisted laser desorption ionization (MALDI) mass spectrometry and sequencing. The pentapeptide KAKVT and 3 N-terminal sequences of the peptides with the MM from 12 237 to 546 Da (mass spectrometry) were identified (Table 2). The direct search in a peptide protein data bank (PIR, Non-Redundant Reference Protein Database) did not allow us to establish their precursors, probably owing to insufficient protein sequence information on murine genome/proteome.
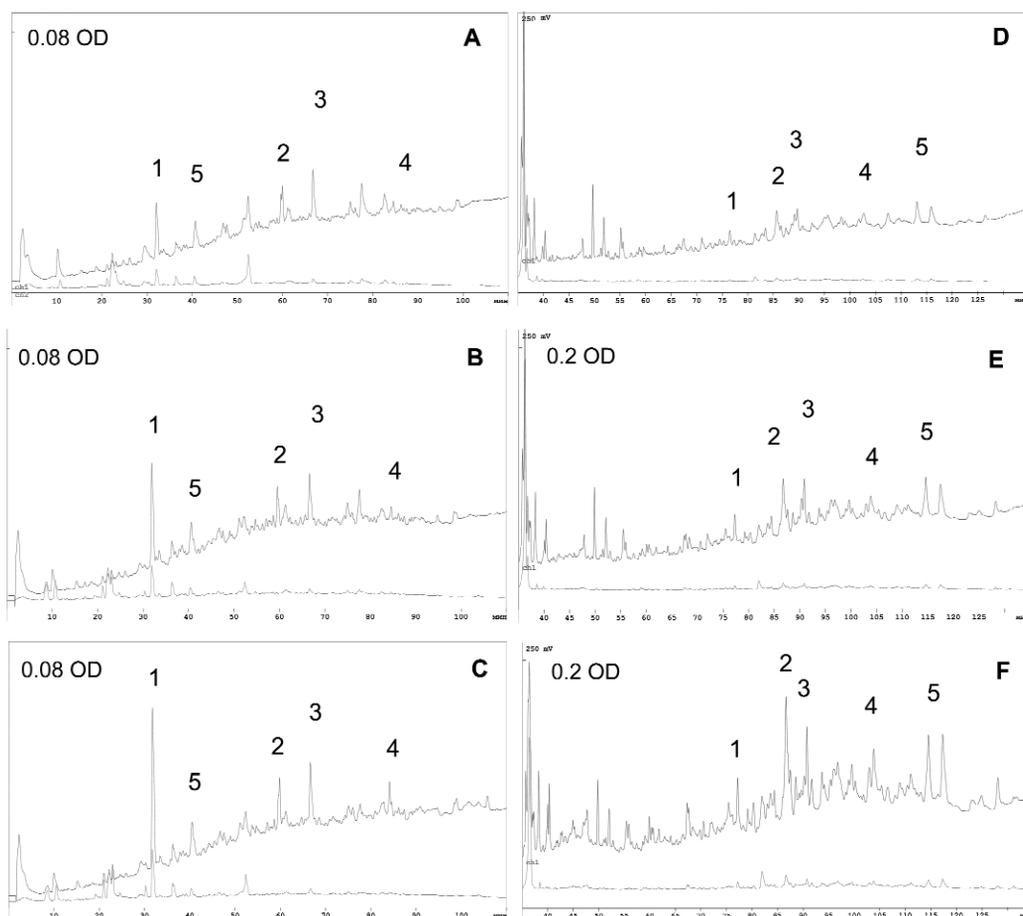
Fig. 1 Dynamics of peptide release. Chromatograpgic analysis of WEHI-3 (A–C) and K562 (D–F) supernatants (A: 1 h incubation; B: 2 h incubation; C: 3 h incubation; D: 0.5 h incubation; E: 1.5 h incubation; F: 4.5 h incubation). The peaks corresponding to identified components are marked by numbers.

Table 2 The peptides secreted by WEHI-3 cells.

Peak #	MM	Sequence	Precursor
1	546	KAKVT	No homology
2	12 237	DEKEKGFVKE…	No homology
3	7899	DQESXKIREE…	No homology
4	3257	Not determined	Not applicable
5	1794	GEFMKNYVXK…	No homology

Peptides released by non-induced K562 human erythroid leukemia cells

The cells were incubated in serum-free RPMI-1640 medium. The cells were incubated for 0.5, 1, or 4.5 h in 75-cm^2 culture flasks, 250 000 cells/ml, 30 ml per flask. The supernatants were collected and subjected to chromatographic fractionation. The obtained chromatograms, corresponding to each time point, are given in Fig. 1 (D–F) and the dynamics of the secretion of peptide components marked on the chromatogram by numbers is shown in Fig. 3B. As follows from Fig. 1, the level of peptides, in con-

trast to the WEHI-3 cells, linearly increased in the course of incubation. The obtained components were subjected to MALDI mass spectrometry and sequencing. As a result, 12 sequences have been obtained (Table 3), among them three peptides correspond to long hemoglobin fragments, two of them being derived from α-globin (1–94 and 1–105), and one corresponding to ε-globin fragment (1–88). This fact contrasts with the behavior of long hemoglobin fragments in human erythrocytes where all such fragments remain inside the cell. The factors responsible for such difference and apparently related to specific membrane properties of the two cell types remain unclear. In contrast to erythrocytes, a number of non-hemoglobin-derived fragments were also released, namely carbonic anhydrase XI (278–327), melanophilin (339–390), cadherin EGF LAG G-type receptor 2 (502–?), aldolase A (1–67), melastatin 1 (319–408), a peptide homologous to ryanodine receptor fragment (4377–4443), and two peptides derived from proteins with unknown function. Most of the non-hemoglobin fragments isolated from K562 supernatant are derived from the proteins essential for tumor cell metabolism. Aldolase A is involved in glycolysis and endocytosis process [33]. Carbonic anhydrase XI is an enzyme-like CNS-specific protein overexpressed in some tumors [34]. It seems to play a significant role in tumor cell homeostasis as its inhibition was shown to decrease tumor cell growth [34]. Melanophilin is a myosin Va-targeting molecule that links myosin Va with the cargo vesicles. Melanophilin directly activates the actin-activated ATPase activity of myosin Va and thus its motor activity [35]. The activity of this protein is believed to be regulated by proteolysis. The melanophilin fragment 339–390 found in K562 cells supernatant is cleaved from myosin Va-binding domain located between sites most susceptible to proteolysis that were shown to be targeted by Ca^{2+}-sensitive calpains [36]. Melastatin (TRPM1), a member of the TRPM ion channel subfamily, was first detected in melanoma tumor cell lines, and its down-regulation was correlated with higher potential for melanoma metastasis [37]. Heterologously expressed melastatin was shown to induce Ca^{2+} entry, and the channel appeared to be regulated by a cytoplasmic isoform [38]. These results considerably broaden the spectrum of non-specialized protein precursors contributing their peptide fragments to experimentally observed peptide pools.

Table 3 Peptides secreted by K562 cells.

Peak #	MM	Sequence	Precursor	Fragment	Homology
4	7597	SQIFGFDL...	Ryanodine receptor 2	4377–4443	SDIFGLDL... (75 %)
5	9992	SQIFV...	Melastatin 1	319–408	100 %
7*	11 668	The peptide with MM = 11668 Da			
10	7486	PYQYPALTPE…	Aldolase A (E.C.4.1.2.13)	1–67	100 %
14*	14 510	SLXXVAXGNA…	No sequence in NREF	ND	No significant homology by BLAST
15	?	AEVNVN…	Unnamed protein product gi|10434198|dbj|BAB14167.1|	46–…	FEVNVN… (83 %)
16	?	APIFVXT…	Cadherin EGF LAG seven-pass G-type receptor 2	502–…	APIFVST…(100 %)
19*	10 042	VLSPADXTNV…	α-Globin	1–94	100 %
20	7519	SLFTVEQ…	Hypothetical protein	5–68	SVFTVEQ… (86 %)
21*	11 307	VLSPADXTNV…	α-Globin	1–105	100 %
22*	9590	VHFXAEEGAA…	ε-Globin	1–88	VHFTAEEGAA… (90 %)

Table 3 (*Continued*).

Peak #	MM	Sequence	Precursor	Fragment	Homology
24	5790	SQIFE…	Melanophilin	339–390	100 %
26*	5772	SQIFXSL…	Carbonic anhydrase XI	278–327	100 %

*Peptides whose concentration has grown 3-fold or more after induction of differentiation with GMP.

Peptides released by GMP-induced K562 human erythroid leukemia cells

K562 cells were daily treated with 50 mcM GMP for 4 days. On day 6, the percentage of cells expressing hemoglobin was determined by staining with benzidine, and the percentage of benzidine-stained (hemoglobin-containing) cells was 77 %. The cultures obtained on day 6 were transferred into the serum-free RPMI-1640 culture medium and incubated for 4.5 h. Figure 2 shows the chromatographic profiles obtained with the intact (A) and the partially differentiated cultures (B) of K562 cells. The peaks whose area increases threefold or more after differentiation are marked by arrows in Fig. 2 and by asterisks in Table 3. These are all detected hemoglobin fragments (19, 23, and 24), the fragment (26) of carbonic anhydrase, and the fragment (14) of an unknown protein.
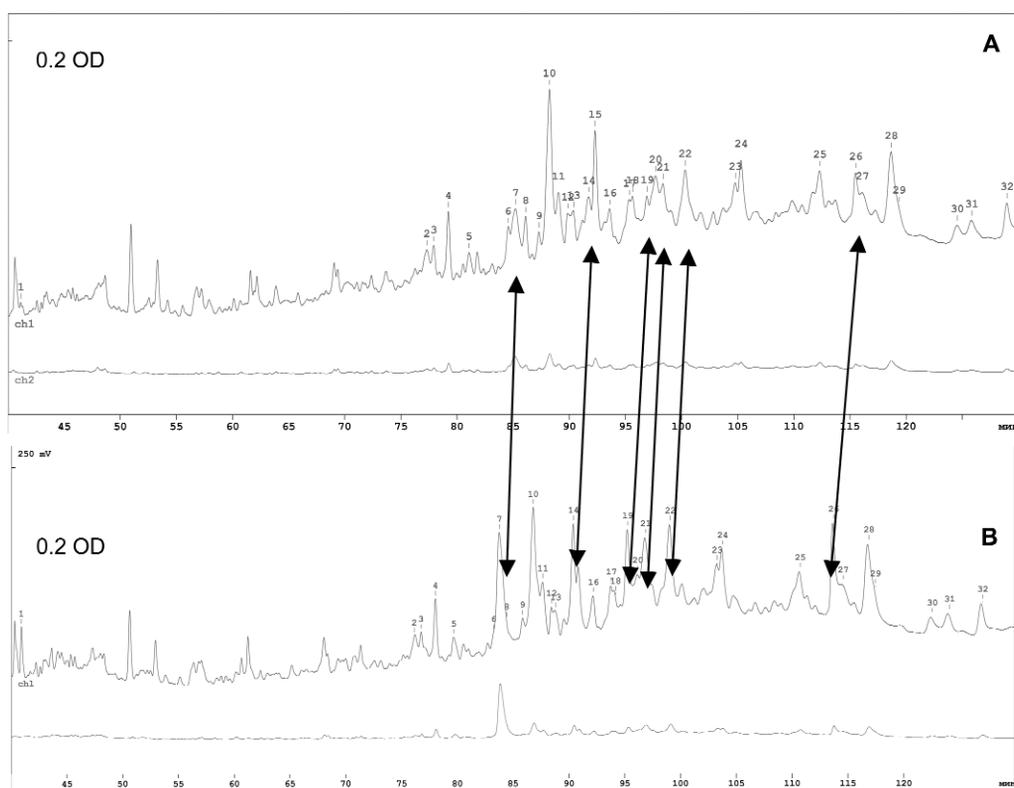

Fig. 2 Chromatographic analysis of K562 supernatants: (A) intact cell culture, (B) cell culture subjected to differentiation in the presence of GMP. Arrows indicate peptides with 3-fold or higher concentration growth after treatment with GMP.
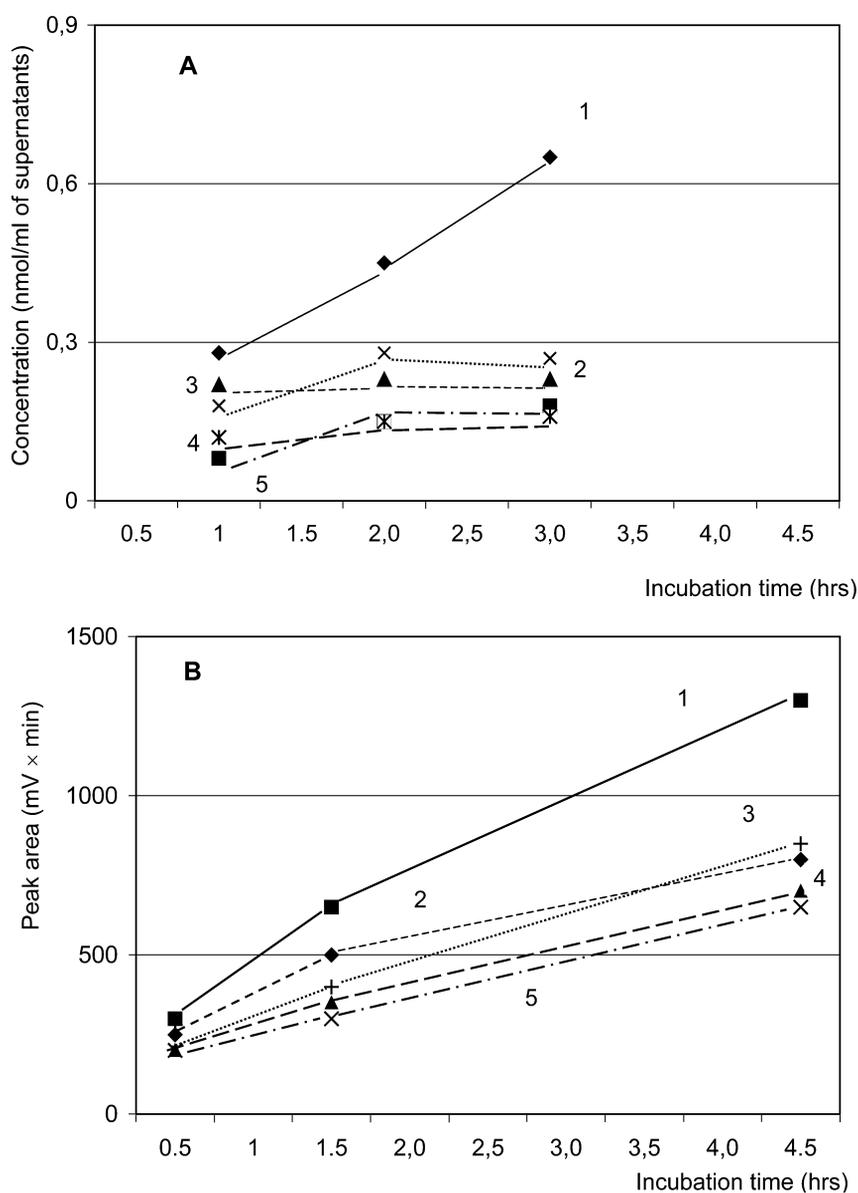
Fig. 3 Dynamics of peptide release: **A**: peptides isolated from the supernatant of WEHI-3 cells; **B**: peptides isolated from the supernatant of K562 cells. Numbering of the plots corresponds to the peaks marked on the chromatographic profiles (Fig. 1, A–C) and Table 2 for WEHI-3 cells; and to the peaks marked on the chromatographic profiles (Fig. 1, D–F) for K562 cells.

Release of peptides by human erythrocytes in the presence of 3 % glucose

The process of peptide secretion by human erythrocytes in isotonic buffer was described previously [29]. As glycolysis is the main energy source in the erythrocyte metabolism, we compared the dynamics of peptide secretion by human erythrocytes incubated in isotonic solution without and in the presence of 3 % glucose.

Erythrocytes were incubated for 0.5, 1.5, and 4.5 h, after that the supernatants were harvested and analyzed. As follows from Fig 4, the release of peptides by erythrocytes in glucose-free buffer levels off with time. In the presence of glucose, the dynamics of peptide secretion was not saturable even after 4.5 h of incubation. On the basis of the results obtained, we concluded that peptide secretion by erythrocytes is an active, cellular metabolism-dependent process. The dynamics of peptide secretion by WEHI-3 cells, incubated in the isotonic solution in most cases is close to that of the erythrocytes incubated in the same concentrations. In contrast, the rate of peptide release by 562 cells incubated in cell culture medium was similar to that of the peptide release by the erythrocytes in the 3 % glucose solution. The deficiency of the components essential for normal cell survival leads to alterations of cell metabolism and, as a result, decreases the levels and affects the dynamics of the release of the peptides.
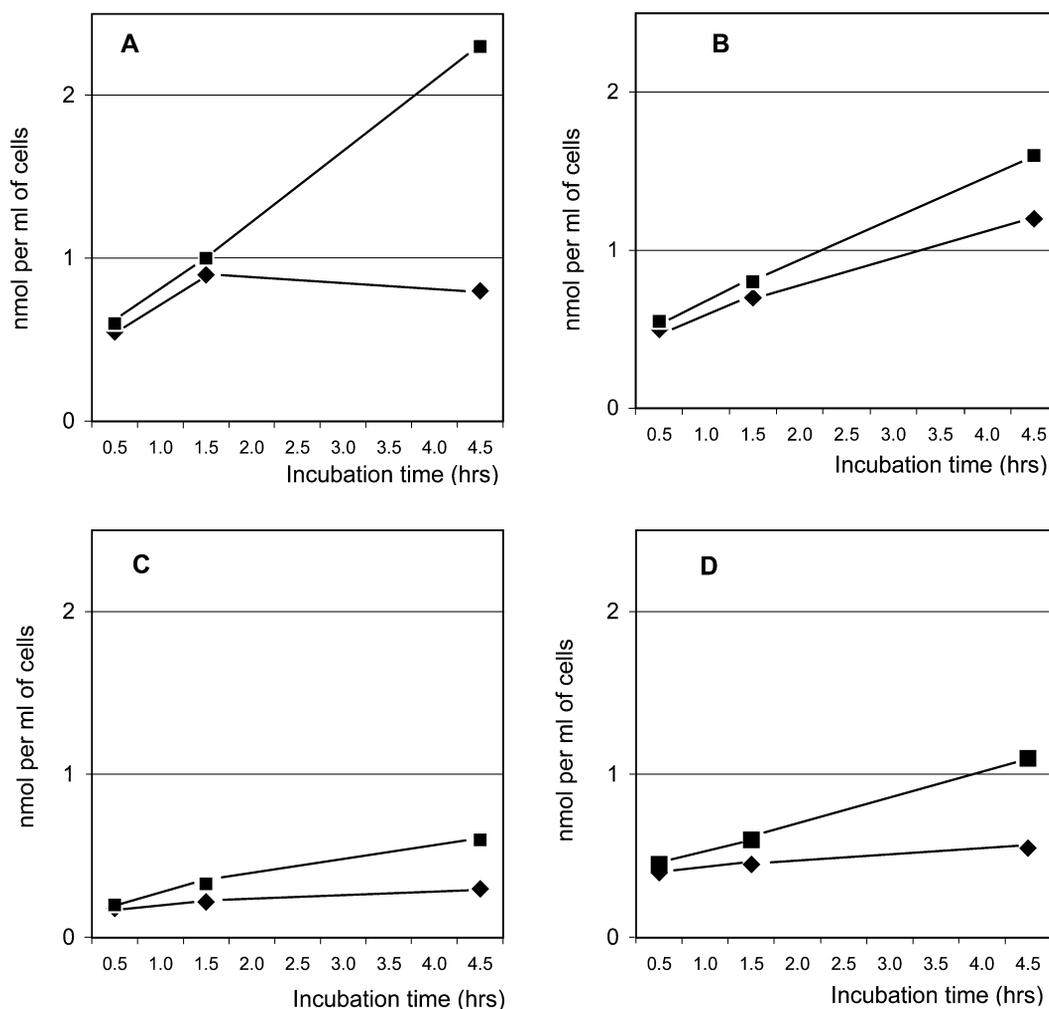

Fig. 4 Dependence of the rate of release of individual peptides from human erythrocytes on the presence of 3 % glucose (30 mg/ml) in cultural media. (♦) erythrocytes incubated in the isotonic solution; (■) erythrocytes incubated in the presence of 3 % glucose. A: β-globin 1-11; B: β-globin 134–146; C: hemorphin-7; D: β-globin 12-31.

CONCLUSION

The results obtained confirm the view on peptide release from animal cells as a general phenomenon. The process is cell-specific and sensitive to differentiation and metabolic state of the cells. It cannot be considered as a nonspecific flow (leaking) through the cellular membrane. The results obtained in vitro demonstrate that peptides generated by individual cell types (i.e., cell peptidomes) could be divided into two principal populations, namely, peptides released to the culture medium and peptides residing inside the cells.

On the basis of these data, one can suggest that the patterns of the peptide pool formation in vivo are similar to those established with cell cultures: peptide pools of biological fluids are mainly formed by the components released by cells, while tissue-specific peptide pools comprise the sum of all (both extracellular and intracellular) peptides generated by cells forming the tissue.

On the basis of the available data, we suggest the following links between genome, proteome, peptidome, and peptide pools.

In contrast to genome, which is mutual for all cell populations present in a given organism, the proteomes (i.e., the set of proteins corresponding to the particular cell type) strongly vary in different cell populations. Formation of a particular proteome depends on cell metabolism. In its turn, composition of the proteome is the major factor responsible for metabolic processes within the cell and therefore can be considered as a main attribute of the metabolic state of a homogenous cell population.

The peptidomes are directly derived from individual cellular proteomes and consequently reflect the function of individual proteomes. Accordingly, the term "peptidome" should be appropriately applied to peptide multitudes generated by individual cell populations. The overall sets of peptides present in biological fluids, tissues, and organs and combined from different peptidomes are conveniently defined as peptide pools. When peptides are formed in the course of proteolysis by enzymes released by different cell types, peptide pools of tissues or biological fluids result from interactions of different proteomes.

Changes in the metabolic state of an organism, including introduction of pharmacologically active compounds [39] as well as the changes related to some diseases (Alzheimer's disease, cancer [2,40]) are directly reflected in the protein content, which in turn affects the peptidome.

As a consequence, the analysis of peptide pool components in the case of pathology can be used for evaluating the proteins and the biochemical mechanisms involved in the disease. Further steps of our investigation will include broadening the list of sequenced peptides generated by cell cultures as well as screening of their biological properties.

MATERIALS AND METHODS

Cell culturing

WEHI-3 murine myelomonocytic leukemia and K562 human erythroid leukaemia cells were grown in RPMI-1640 culture medium (Sigma) containing 10 % of FBS (Sigma), 2 mM of L-glutamine (Sigma), 10 % standard vitamin solution (Sigma), 100 u/ml of penicillin G, 0.1 mg/ml of streptomycin sulfate and 0.25 µg/ml of amphotericin B (Sigma). The cells were subcultered every 48–72 h. For subculturing, K562 cells were thoroughly mixed with 10 ml pipette and subcultured to the concentration at least 250 000 cells/ml. WEHI-3 cells were suspended in versen solution (0.02 % EDTA in PBS) before subculturing.

Determination of cell number and the percentage of dead cells

The concentration of live cells (N of cells/ml) was determined by visual cell count in the presence of trypan blue dye. The percentage of dead cells was calculated as follows: $C(\%) = (N_{dead}/N_{total}) \times 100$ %;

where N_{dead} = number of stained cells per 25 squares of Goryaev chamber; N_{total} = number of stained and transparent cells per 25 squares of Goryaev chamber.

K562 differentiation

The cells were placed in 75 cm^2 culture flasks, 250 000 cells/ml, 30 ml per flask. For the first 4 days, the cells were daily treated with 50 mcM GMP. The incubation period was 6 days, during that period the cells were subcultured once (day 3). On day 6, the percent of the cells expressing hemoglobin was determined by staining with benzidine and visual cell counting as described in ref. [41]. For staining, 1 ml of cell suspension was mixed with 1 ml of 0.2 % benzidine solution in 0.5 % AcOH and 15 µL of 90 % H_2O_2. Hemoglobin catalyzes the oxidation of benzidine with hydrogen peroxide, thus the cells containing hemoglobin accumulate the dark violet particles of oxidized benzidine and are well distinguished from transparent hemoglobin-free cells. Differentiation (D, %) in 5 independent samples was calculated using the formula: $D(\%) = (N_{stained}/N_{total} \times 100)$, where $N_{stained}$ = number of stained cells per 25 squares of Goryaev chamber; N_{total} = number of stained and transparent cells per 25 squares of Goryaev chamber. The mean D was obtained using the values obtained. CV was less than 10 %.

Preparation of WEHI-3 supernatants

The cells were grown in 75 cm^2 culture flasks to high cell density. Culture medium was removed, and the adhered cells were washed 4 times with PBS containing 0.1 g/l of $MgCl_2 \cdot 6H_2O$ and 0.1 g/l of $CaCl_2$. 10 ml of the same buffer were added to each flask and the cells were incubated 60, 120, or 180 min at 37 °C. The cells were removed by centrifugation in 50-ml conical tubes (1500 rpm, 20 min, 15 °C, Jouan BR-4i centrifuge, S40 rotor) and the supernatants were collected. The batch solid-phase extraction of the peptide material was carried out at Separon SGX C_{18} column, using 0.1 % TFA in H_2O as precipitating buffer and 80 % acetonitrile, 0.1 % TFA in H_2O as eluting buffer, flow rate 1 ml/min. Elution was controlled with Dual λ 2487 UV detector (Waters) at λ_1 = 226 nm and λ_2 = 280 nm. The fraction obtained (14 ml) was lyophilized.

Chromatographic fractionation of WEHI-3 supernatants

The extract samples were redissolved in 0.1 % TFA in H_2O and separated by RP-HPLC at Nucleosil 120-5C_{18} column (4.0 × 250 mm) (Macherey–Nagel) in the linear gradient of acetonitrile (0–60 %, 120 min, 0.1 % TFA, flow rate 1 ml/min). The peaks were detected at λ_1 = 226 nm and λ_2 = 280 nm (Dual λ 2487 UV detector, Waters). Fractions corresponding to predominant chromatographic peaks were collected manually and lyophilized.

Preparation of K562 supernatants

The cells were grown in 75 cm^2 culture flasks to high cell density. Culture medium was removed by centrifugation in 50-ml conical tubes (1500 rpm, 12 min, 25 °C, Jouan BR-4i centrifuge, S40 rotor), cells were collected in 15-ml conical centrifuge tubes (about 3 ml of the sedimented cell mass, that corresponds to about 700 millions of nondifferentiated K562 cells and about 350 millions of 75 % differentiated K562 cells) and washed 5 times with 12 ml of serum-free RPMI-1640 culture medium. Cells were suspended in 3 volumes (12 ml) of culture medium, divided into 3 equal portions (4 ml of suspension each) and incubated for 0.5, 1.5, and 4.5 h respectively at 37 °C with gently shaking. The cells were removed by centrifugation (1500 rpm, 20 min, 15 °C, Jouan BR-4i centrifuge, S40 rotor) and the supernatants were collected and frozen at –20 °C.

Chromatographic fractionation of K562 supernatants

The supernatant aliquots (1 ml) were separated by RP-HPLC at Nucleosil $300\text{-}5C_{18}$ column (4.6 × 250 mm) (Macherey–Nagel) in the linear gradient of acetonitrile (0–24 % 20 min, 24–64 % 80 min, 64–80 % 20 min, 0.1 % TFA, flow rate 0.75 ml/min). The peaks were detected at $\lambda_1 = 226$ nm and $\lambda_2 = 280$ nm (Dual λ 2487 UV detector, Waters). Peaks generated by culture medium components were excluded using the control sample (1 ml of RPMI-1640 medium). Fractions corresponding to predominant chromatographic peaks were collected manually and lyophilized.

Isolation of erythrocytes and preparation of the supernatant

Peripheral venous blood was obtained from healthy volunteers by venipuncture at the Research Hematological Center (Russian Academy of Medical Sciences, Moscow). Blood samples (40 ml) were placed into 50-ml conical centrifuge tubes containing sodium citrate buffer (to final concentration of 0.25 %). Cells were separated from plasma by centrifugation (1500 rpm, 20 min, 4 °C, Jouan BR-4i centrifuge, S40 rotor). The obtained pellets were washed 5 times by phosphate-buffered balanced salt solution (PBS), with following centrifugation under the same conditions. The samples were discarded if hemolysis was detected spectrophotometrically (Hitachi U-2800 spectrophotometer, $\lambda = 412$ nm). To obtain secreted peptides, 10 ml of washed erythrocytes were suspended in 40 ml of PBS (with or without 3 % glucose) and incubated for 0.5, 1.5, and 4.5 h (37 °C, gently shaking). The samples were discarded if hemolysis took place in the course of incubation. At the end of incubation, the cells were pelleted (1500 rpm, 20 min, 25 °C, Jouan BR-4i centrifuge, S40 rotor); supernatants were collected and frozen at –20 °C.

Chromatographic fractionation of the erythrocyte supernatant

The supernatant aliquots (1 ml) were separated by RP-HPLC at Nucleosil $300\text{-}5C_{18}$ column (4.6 × 250 mm) (Macherey-Nagel) in the linear gradient of acetonitrile (0 % 5 min, 0–60 % 80 min, 0.1 % TFA, flow rate 0.75 ml/min). The peaks were detected at $\lambda_1 = 226$ nm and $\lambda_2 = 280$ nm (Dual λ 2487 UV detector, Waters). Fractions corresponding to predominant chromatographic peaks were collected manually and lyophilized.

Chromatographic data analysis

The amounts of peptides in samples were estimated according to area calculation under corresponding peaks, and confirmed by sequencing data. Peak areas were calculated by area normalization methods using the program "MultiChrom", version 2.67 (Ampersand, Russia). The relationship between the peak area at 226 nm and the corresponding peptide quantity was calibrated using the purified fragment 1–33 of the hemoglobin α-chain.

Peptide identification

Molecular masses of isolated peptides were determined using MALDI-TOF mass-spectrometer Vision 2000 (Thermo Bioanalysis Corp., UK). N-terminal amino acid sequences of isolated peptides were determined using gas-phase sequencer Model 447A (Applied Biosystems, USA). PIR-NREF - PIR Non-Redundant Reference Protein Database (PIR - Protein Information Resource) was used for the identified peptides searching.

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