

## Tissue-specific peptide pools. Generation and function\*

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**Abstract:** Systematic analysis of several tissue extracts for peptide components followed by bioactivity studies leads to formulation of the concept of “tissue-specific peptide pools”. According to that concept the endogenous proteolysis of proteins with well-established functions, such as hemoglobin, actin, and cellular enzymes in tissues leads to formation of the sets (or pools) of bioactive peptides. The sets are tissue-specific on one hand and conservative in a given tissue at normal conditions on the other. The content and the composition of pool components are sensitive both to pathologies linked with alterations of tissue metabolism and to prolonged physiological changes. *In vivo* formation of fragments of functional proteins includes several consecutive proteolytic stages inside the cells and further release of bioactive compounds into the surrounding medium. The effects of pool components take place predominantly at tissue and cellular levels, their effects being related to stimulation or inhibition of cell growth, induction of cell differentiation, and death. The above-mentioned features lead to the proposal that the main *in vivo* function of components of tissue-specific peptides is maintenance of tissue homeostasis, i.e., the normal ratio of functional, dividing, differentiating, and dying cells of tissues. Components of tissue-specific peptide pools display several features distinguishing them from “classical” peptide hormones and neuromediators. Summarizing, a novel peptidergic regulatory system is considered.

### INTRODUCTION

The very fast development of genomic studies results in a wealth of information on amino acid sequences of proteins involved in all areas of functioning of biological systems. At present, the scientific and the medical perspectives of application of the information on genome structures are being actively considered. At the same time, the huge achievements of genomic research do not allow predicting the actual levels (i.e., the relative intensities of synthesis and degradation), the post-expression modifications, and functions of protein products of gene expression *in vivo*. On the other hand, it is well known that proteolysis is essential both for generation of the majority of regulatory proteins and peptides and for fragmentation of molecules that already completed their function. At present, a strong interest is attracted to regulatory proteolytic enzymes (the hits are proteasomes and caspases) [1,2], while the biological activity of the peptides formed by these enzymes is not yet established. Since 1990, we have been carrying out detailed studies of various peptides present in mammalian tissues. As a result, about 500 peptides were sequenced and evaluated for bioactivity. The results of our experiments, together with the available literature data, provided considerable information on the patterns of generation and biological properties of these peptides. The data obtained allowed to formulate the concept of “tissue-specific peptide pools” [3–5].

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## MAIN CHARACTERISTICS OF TISSUE-SPECIFIC PEPTIDES

Endogenous peptides present in tissue extracts are proteolytically derived from a limited group of functional proteins such as hemoglobin, myelin basic protein, fibronectin,  $\gamma$ -globulins, cellular enzymes, or from undefined protein precursors. The major source of such peptides is hemoglobin. The overall content of these peptides comprises 0.5–6.5% of the tissue weight, (i.e., it is several orders of magnitude higher than that of the hitherto-described classical peptides such as hormones or neuromediators). The cellular and the tissue extracts contain 50–800 individual peptides with the content 100 pmol/g of tissue or higher [6]. Analysis of the samples of rat brain, heart, and lung extracts, and the lysate and the supernatant of human erythrocytes have shown that the content and the composition of the fragments of functional proteins is highly stable and independent of individual differences in healthy donors or animals kept at standard conditions [5,7–10]. Comparative analysis of the peptide sets of rat and bovine brain, as well as of the lysate of human and rat erythrocytes have shown them to be quite similar in the same tissue of different species. On the other hand, comparative analysis of the peptide sets in various mammalian tissue extracts have shown that the content and the composition of the fragments of functional proteins vary significantly in different tissues. These differences were due both to the presence of the fragments of tissue-specific proteins and to differences in hemoglobin fragmentation (i.e., the sets of fragments of functional proteins are tissue-specific) [6,11].

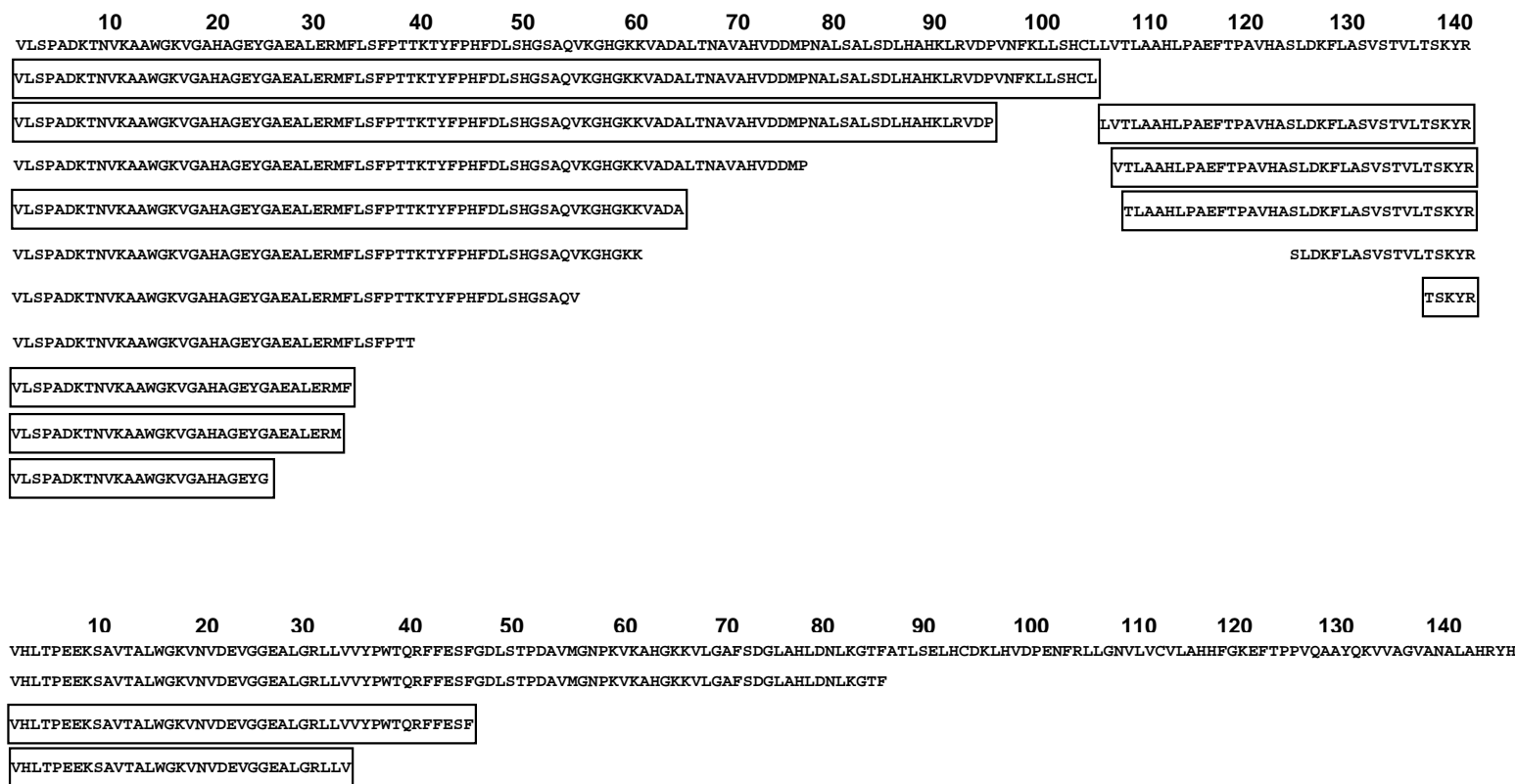
## THE PATTERNS OF PROTEOLYTIC FORMATION OF POOL COMPONENTS

Using hemoglobin, the major source of components of tissue-specific peptide pools, as an example, we have studied the patterns of proteolytic degradation of that protein in the erythrocytes. In communications [3,12–14] amino acid sequences were reported for 9, 16, and 32 hemoglobin fragments found in the lysate of human erythrocytes. The majority of these components are relatively long peptides built of more than 30 amino acid residues. However, in the course of these studies it became increasingly clear that standard chromatographic procedures involving acidification of the solution up to pH 3–5 gives rise to changes in the structures and levels of the peptides initially present inside the cell at neutral conditions. Inhibitory analysis leads to the conclusion that the enzyme responsible for that process must be an unknown aspartate proteinase resembling cathepsin E and inhibited by pepstatin and activated at pH < 5.

The post-lytic proteolysis is avoided if the lysate is fractionated at neutral pH. Under these conditions peptides were isolated and sequenced by automatic gas-phase sequencer and MALDI mass spectrometry that we believe adequately represent the *in vivo* endogenous content of peptides within the erythrocytes. The respective structures are shown in Fig. 1. The majority of those peptides [ $\alpha$ -(1-105),  $\alpha$ -(1-95),  $\alpha$ -(1-65),  $\alpha$ -(1-33),  $\alpha$ -(1-32),  $\alpha$ -(1-25),  $\alpha$ -(106-141),  $\alpha$ -(107-141),  $\alpha$ -(108-141),  $\alpha$ -(137-141),  $\beta$ -(1-45), and  $\beta$ -(1-33)] as expected are also present in the previously studied samples. Fragments  $\alpha$ -(1-78),  $\alpha$ -(1-61),  $\alpha$ -(1-55),  $\alpha$ -(1-40), and  $\beta$ -(1-86) serve as exceptions apparently resulting from fast post-lytic acid-activated proteolysis. The most striking result of activation of the above-mentioned proteinase is sharp increase in the level of the peptides  $\alpha$ -(1-32) (ca. 30-fold) and especially  $\alpha$ -(1-33) (ca. 1000-fold).

It follows from Fig. 1 that primary splitting of  $\alpha$ -globin takes place at the Leu<sup>105</sup>-Leu<sup>106</sup> site. After that, the N-terminal fragment of the  $\alpha$ -globin is stepwise shortened from the C-terminal and the C-terminal fragment from the N-terminus. Similar steps could take place with the  $\beta$ -globin as well, although more data is needed to confirm such sequence of events.

The overall yield of peptides in the fresh erythrolysate comprises 0.1–0.3% from the hemoglobin mass. Incubation of the lysate at pH 7.2 for 8 h at 20 °C practically leads to no change in the peptide content and composition. However, analogous incubation with 6N guanidinium chloride results in a



**Fig. 1** Generation of hemoglobin fragments in human erythrocytes. The peptides were isolated from the lysate of the erythrocytes prepared at pH 7.2 by means of RP-HPLC (sample buffer, PBS; pH 7.2; eluent, 20–60% of acetonitril in 0.1% TFA) and the structures of homogenous compounds were determined by means of gas-phase sequencing and MALDI mass spectrometry. The peptides marked as boxes have been isolated from lysate of human erythrocytes treated with 0.1 M acetic acid (final pH 3.0) [13].

sharp (up to 2%) rise in the total peptide level. We believe that this fact could indicate that part of hemoglobin in erythrocytes exists and possibly functions in a “nicked” state. In 6N guanidinium chloride the “nicked” chains dissociate, releasing the peptides that are detected at the chromatograms.

The process of degradation of hemoglobin fragments does not stop at the stage of long peptides shown above in Fig. 1. In refs. 3, 12, and 13 we demonstrated that primary culture of erythrocytes releases into the surrounding medium a series of shorter (4–20 membered) fragments of  $\alpha$ -globin (10 peptides) and  $\beta$ -globin (22 peptides). Significantly, excretion of peptides in the absence of energy source (glucose) acquires the maximal speed at the beginning of incubation and levels off after 40–80 min [13].

This fact indirectly confirms the statement that release of peptides is not linked with nonspecific leakage of peptides from the erythrocytes through the damaged membrane. We prefer to view the process as the function of membrane-associated proteinase(s) interacting with the intraerythrocyte peptide substrates and transporting the resultant degradation products across the membrane.

The data obtained provide additional information on the proteolytic processes involved in generation of components of tissue-specific peptide pools. While a great number of pool components are already identified and the possibility of generation of such peptides by cell cultures (macrophages, lymphocytes, tumor cells) and individual enzymes *in vitro* is demonstrated, little is known about the enzymes involved in their production *in vivo*. The literature data suggest several patterns of generation of pool components. First, the peptides can be formed inside the viable cells of tissues or biological fluids and further released into surrounding medium [12]. In that case, the proteinases, responsible for utilization of proteins already completed their function, or participating in processing of peptide hormones and cytokines, or in the proteolytic regulation of the activity of intracellular regulatory proteins (proteasome, calpain, caspase-1) can be directly involved in generation of pool components [1,2,15]. Second, the peptides can be formed extracellularly by means of the proteolytic enzymes present in the surrounding medium, such as matrix proteinases or cathepsins [16,17]. Third, the peptides can be formed in dying cells and further released extracellularly after cytolysis. Caspases, directly involved in apoptosis could participate in that process [18]. We believe that all above-mentioned enzymes can contribute to formation of biologically active peptides from functional proteins.

### **THE COMPONENTS OF TISSUE-SPECIFIC PEPTIDE POOLS: SPECTRUM OF BIOLOGICAL EFFECTS AND PROPOSED *IN VIVO* FUNCTION**

Tissue-specific peptides exhibit a variety of behavioral and physiological activities, their effects being realized predominantly at the tissue and cellular level. The spectrum of the activities both *in vitro* and *in vivo* includes: (1) inhibition or stimulation of proliferation of normal and tumor cells [12,19,20]; (2) promotion of differentiation [21]; (3) induction of cytolysis [22]; (4) regulation of angiogenesis [23]; (5) supporting the cell viability in the absence of growth factors [24]; (6) protection of the cells from the toxic effects of cytostatic drugs and radiation [25–27].

In the course of our studies, more than 100 of the fragments of functional proteins isolated from various tissue extracts, the lysate and the supernatant of the erythrocytes were subjected to primary screening in tumor cells. The test system applied allowed identification of the peptides (1) stimulating proliferation, (2) inhibiting proliferation, and (3) inducing cytolysis of tumor cells. As a result, the majority of the peptides were shown to display at least one of these above-mentioned activities. Thus, in a broad sense, their activity is closely linked with regulation of tumor cell number.

The nature of the activity of several groups of hemoglobin fragments depends strongly on concentration on one hand and is sensitive to subtle structural differences on the other. As seen from Table 1, consecutive cleavage of the C-terminal amino acid residues starting from the fragment of  $\alpha$ -globin  $\alpha$ -(1-33) strongly influences the activity:  $\alpha$ -(1-33) slightly decreases the tumor cell number, the

**Table 1** The change of L929 cell number induced by intraerythrocytes hemoglobin fragments [28]. Decrease of cell number is marked with (-).

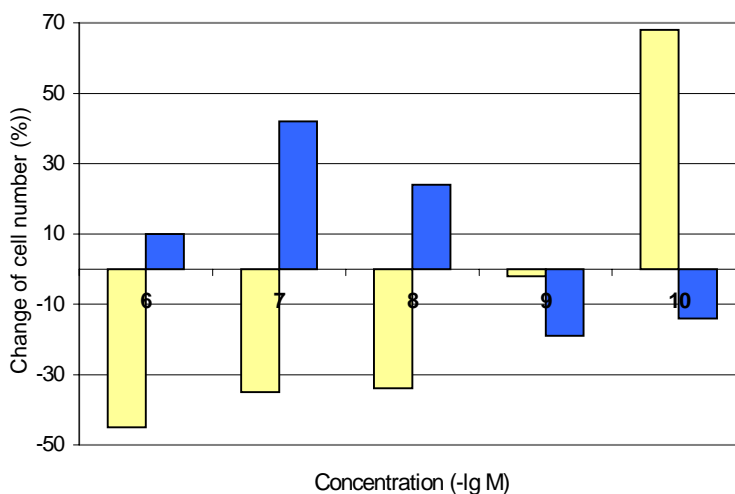
Hemoglobin fragment	Concentration (M)				
	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>	10 <sup>-10</sup>
$\alpha$ -globin 1-33	21 ± 10	-7 ± 7	-13 ± 5	6 ± 6	7 ± 3
$\alpha$ -globin 1-32	10 ± 4	42 ± 8	24 ± 9	-19 ± 3	-14 ± 2
$\alpha$ -globin 1-31	13 ± 8	42 ± 3	12 ± 5	-18 ± 1	-16 ± 2
$\alpha$ -globin 1-29	10 ± 1	8 ± 3	9 ± 3	15 ± 8	0 ± 6
$\alpha$ -globin 1-25	8 ± 5	4 ± 4	6 ± 3	4 ± 3	ND

$\alpha$ -(1-31),  $\alpha$ -(1-32), and  $\alpha$ -(2-32) peptides exhibit reliable proliferative activity (30% or more), while the  $\alpha$ -(1-29) was not active. The nature of the effects of  $\alpha$ -(1-32) and  $\alpha$ -(1-31) depends strongly on concentration: both peptides showed a quite pronounced (40%) proliferative activity in 10<sup>-6</sup>–10<sup>-8</sup> M concentration range, while both compounds exhibit a modest antiproliferative effect at lower concentration [28].

In contrast, short hemoglobin fragments isolated from rat spleen show the opposite tendency. As seen from Table 2,  $\alpha$ -(12-25) and  $\alpha$ -(12-24) induce 35–45% decrease of live tumor cell number at 10<sup>-6</sup>–10<sup>-7</sup> M. At the same time, these peptides stimulate proliferation at 10<sup>-10</sup> M, up to 60% in the case of  $\alpha$ -(12-25). Effects of the peptides belonging to this family are strongly sequence-dependent. Thus, removal of the C-terminal Gly residue from the peptide  $\alpha$ -(12-25) lowers the proliferative activity and, to some extent, the ability to reduce the L929 cell number, the further C-terminal shortening gives rise to nonactive peptides, such as  $\alpha$ -(12-23),  $\alpha$ -(13-25)  $\alpha$ -(12-25) and, finally, N-terminal shortening of  $\alpha$ -(12-25) results in suppression of both proliferative and antiproliferative effects. At the same time, the peptide  $\alpha$ -(1-17), released by erythrocytes, exhibits a pronounced antiproliferative effect in tumor cells [12]. Summarizing, the proteolysis of  $\alpha$ -(1-32) gives rise to a group of peptides exhibiting opposite activities (Fig. 2). The effects of such peptides suggest that regulation of cell number *in vivo* can be performed by change of their content in the organisms, i.e., by the activity of the enzymes directly involved in their formation and degradation. While the *in vivo* function of the peptides inducing cytolysis or inhibiting growth of tumor cells seems rather clear, i.e., they can participate in antitumor defense of the organism [16,27], the role of the stimulators of proliferation of tumor cells is not obvious. The experiments in the primary cultures of normal cells suggest the possible biological function of the

**Table 2** The change of L929 cell number induced by hemoglobin fragments (The experiments were carried out according to procedure described in ref. 12,28). Decrease of cell number is marked with (-).

Hemoglobin fragment	Concentration (M)					
	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>	10 <sup>-10</sup>
$\alpha$ -globin 12-25	ND	-45 ± 3	-35 ± 5	-34 ± 8	-2 ± 1	68 ± 14
$\alpha$ -globin 12-24	ND	-38 ± 4	-23 ± 6	-25 ± 7	-21 ± 11	14 ± 4
$\alpha$ -globin 12-23	ND	-13 ± 2	-8 ± 6	-11 ± 4	-10 ± 4	-14 ± 6
$\alpha$ -globin 13-25	ND	-14 ± 7	-16 ± 3	-6 ± 5	-25 ± 4	-18 ± 4
$\alpha$ -globin 17-23	ND	ND	7 ± 0	-5 ± 5	-8 ± 0	0 ± 0
$\alpha$ -globin 1-17	-37 ± 7	-32 ± 2	-19 ± 6	-18 ± 3	ND	ND
Epirubicin	ND	-53	ND	ND	ND	ND



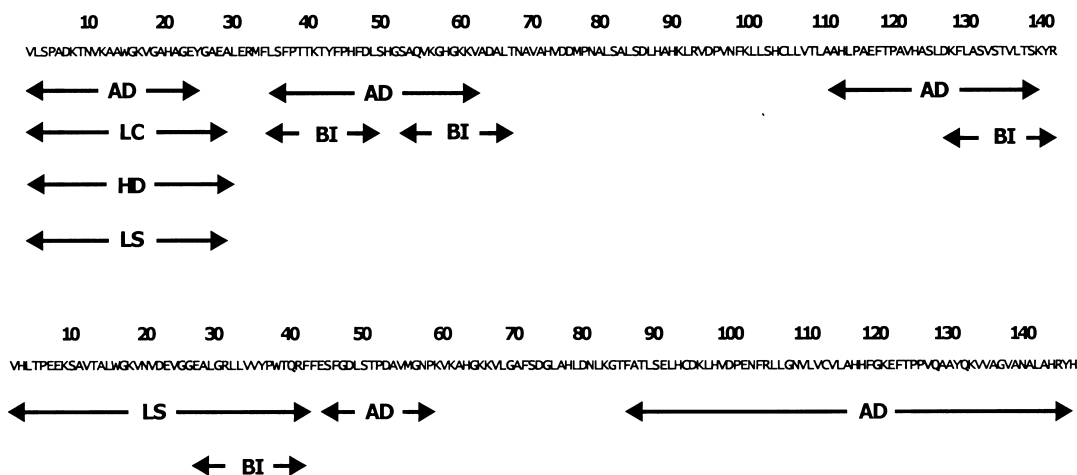
**Fig. 2** Concentration-dependent effects induced by  $\alpha$ -globin 1-32 (shaded columns) and  $\alpha$ -globin 12-25 (light columns) in L929 tumor cells.

peptides stimulating tumor growth [20,28]. Neokytorphin,  $\alpha$ -(137-141), was shown to support survival of brown preadipocytes [24] and murine splenocytes in the absence of growth factors. The study of the mechanism of action suggests that neokytorphin replaces growth factors and maintains the proliferative rate, thereby protecting the cells from apoptosis. These effects of neokytorphin suggest its participation in the support of viability of the cells in tissues during pathological conditions linked with alterations in the levels of growth factors. Intraerythrocyte hemoglobin fragments  $\alpha$ -(1-32),  $\alpha$ -(2-32), and  $\alpha$ -(1-31) are another group of proliferative peptides probably involved in restoration of cell number *in vivo*. These peptides restore proliferation of red bone marrow cells treated with epirubicin. For example, they can participate in the regeneration processes required after death of a large part of cell population induced by introduction of toxic compounds (chemiopreparations in our case) or in the course of pathologies accompanied with tissue-degenerative processes.

The examples given above, together with the available literature data on the activities of the fragments of functional proteins *in vitro* and *in vivo*, suggest that components of tissue-specific peptide pools participate in maintenance of tissue homeostasis (i.e., in the support of appropriate ratio of functional cells in a given tissue). The latter implies all aspects of the control of cell number, i.e., prevention of abnormal proliferation on one hand and restoration of cell number in the case of extensive cell death.

### **SUGGESTED BIOLOGICAL ROLE OF PEPTIDE POOLS AND THEIR PLACE AMONG OTHER PEPTIDERGIC REGULATORY SYSTEMS**

As follows from the data given above, the composition and the content of the components of peptide pools are quite stable at normal conditions and are considered an important characteristic of a normal tissue. On the other hand, the levels of individual peptides derived from hemoglobin, myelin basic protein, calmodulin, etc. change significantly during pathologies related to tissue degeneration (Alzheimer's disease, brain ischemia) [10], proliferation disorders (Hodgkin's disease) [8] and cancer (lymphosarcoma [29], lung cancer [29,30]). These diseases correspond to two major types of alterations of tissue homeostasis, i.e., degeneration and death of the cells of tissue (Alzheimer's disease and ischemia) and proliferation disorder and transformation (Hodgkin's disease and cancer). Tissue-degenerative



**Fig 3** Alterations in level of hemoglobin fragments linked with Alzheimer's disease (AD) [10], brain ischemia (BI) [10], lymphosarcoma (LC) [29], Hodgkin's disease (HD) [8], and lung cancer (LC) [29]. The segments of hemoglobin sequence sensitive to these pathologies are marked with arrows. C-terminal pentapeptide  $\alpha$ -(137-141) found in lung carcinoma tissue [30] is not marked in the figure.

processes are known to be accompanied by increase of the activity of proteolytic enzymes. Alzheimer's disease, Parkinson's disease, Duchenne muscular dystrophy, etc. are accompanied by a reliable increase in the activity of calpain [15]. Caspase 3 is suggested to participate in development of Alzheimer's disease [18]. The proteinase is involved in formation of a cytotoxic fragment of the  $\beta$ -amyloid precursor protein, the latter peptide being putatively considered a direct cause of apoptosis of neurones during Alzheimer's disease. On the other hand, the pathologies due to tissue-degenerative processes are accompanied by accumulation in the damaged tissue of several groups of hemoglobin fragments (Fig. 3). We suggest several possibilities of biological background of the alterations in the levels of these peptides. First, the peptides can be a consequence of extensive cell death taking place during these pathologies, which is known to be accompanied by protein degradation carried out by immune cells. On the other hand, such peptides overlap bioactive hemoglobin fragments, particularly those involved in restoration of hemopoiesis after treatment by cytostatic drugs or radiation ( $\alpha$ -(1-17) and  $\alpha$ -(1-10) [25,26]) and in the support of cell viability in serum deprivation conditions ( $\alpha$ -(137-141) [24]). It can be suggested that such hemoglobin fragments are involved in restoration of the damaged tissue. Summarizing, the alterations of the activity of proteolytic enzymes are linked with tissue degeneration processes, and could lead to significant changes in the level of bioactive components of tissue-specific peptide pools.

On the other hand, accumulation of the peptides stimulating growth of tumor cells is shown to accompany proliferation disorders and cancer. Hodgkin's disease, lymphosarcoma, and lung cancer are characterized by significantly increased levels of peptides  $\alpha$ -(1-32),  $\alpha$ -(1-31), and  $\alpha$ -(1-30) inside the erythrocytes of patients, as compared with those of healthy donors [8,29]. It is logical to suggest that the alterations in the levels of these peptides are due to the increase in the activity of pH-regulated intraerythrocyte proteinases, described above. We can suggest that proteolysis of  $\alpha$ -globin at the site Met<sup>33</sup>-Phe<sup>34</sup> is significantly increased in the erythrocytes of the patients in a mode similar to that taking place at pH 3.0, giving rise to considerable amounts of  $\alpha$ -(1-33), which is rapidly degraded to  $\alpha$ -(1-30),  $\alpha$ -(1-31), and  $\alpha$ -(1-32). Since these peptides exhibit proliferative effect at high concentrations only (see below), the increase of their content might lead to stimulation of tumor growth. The peptides  $\alpha$ -(1-32) and  $\alpha$ -(1-31) were identified earlier in bovine brain extract [6], their content being much higher than that

of  $\alpha$ -(1-33). These peptides are not excreted by the erythrocytes, so the mechanism of their presence in tissues is not known, while they can be released after hemolysis of the erythrocytes, or generated by macrophages from hemoglobin [28]. The alterations in levels of pool components during cancer are not linked exclusively to  $\alpha$ -globin fragmentation inside the erythrocytes. As seen from Fig. 3, the content of  $\beta$ -(1-41) increases inside the erythrocytes of patients with lymphosarcoma, and for neokytorphin,  $\alpha$ -(137-141), accumulation takes place in lung carcinoma tissue. The latter example speaks of the possibility of the enhanced production of proliferative peptides directly in the "affected" tissue [30].

Generally, alterations in formation of pool components linked with the pathologies due to shifts of tissue homeostasis seem to originate from the changes in the activity of the enzymes involved in their formation. Generation of pathology-associated peptides can be both the reason of the disease and the mechanism of normalization of tissue state.

Tissue-specific peptides belong to the same chemical class as typical regulatory peptides exhibiting hormonal, neurotransmitter, and parahormonal effects. At the same time, there are clear differences between the two groups of compounds. First, classical regulatory peptides are formed from specific and usually inactive precursors by means of strictly specific proteolysis. In contrast, the pool components are generated from functional proteins and evolve as families of closely related species. In general, formation of fragments of functional proteins is much less specific than that of regulatory peptides. Second, production of regulatory peptides as a rule is carried out by specialized cells. In contrast, fragments of functional proteins are formed by a variety of different cells in tissues and biological fluids (erythrocytes, macrophages, lymphocytes, tumor cells) processing well-defined functions apparently not related to peptide production. Generation of fragments of functional proteins, in contrast to that of 'classical regulatory peptides', is not directly dependent on the activity of the nervous system. Third, regulatory peptides act typically through receptoric mechanisms. In contrast, only restricted groups of fragments of functional proteins have receptor targets on cell surface [31]. Several peptides act by other than receptoric mechanisms, such as modulation of ion channels [32], or intracellular and extracellular enzymes [5], as well as by regulation of intracellular signal transduction and DNA transcription [21]. Finally, there is an important difference in the *in vivo* function of the hormones and peptide neurotransmitters on one hand and the fragments of functional proteins on the other. The hormones and the neurotransmitters, as a rule, are seen as compounds inducing rapid changes in tissue states, while the function of the components of tissue-specific pools might be due to reversion of the tissue to the stationary condition. The latter implies an appropriate ratio of cells able to maintain the function of the tissue. Summarizing, components of tissue-specific peptide pools are suggested to form a novel peptidergic regulatory system, complementary to nervous, endocrine, immune, and others.

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