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Proteolytic degradation of hemoglobin *in vivo*. Role in formation of tissue specific peptide pool

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Abstract: Analysis of the composition of peptides formed inside human erythrocytes and released by erythrocyte primary culture was carried out. The patterns of generation of relatively long (containing more than 28 amino acid residues) hemoglobin fragments inside the erythrocytes were established. The intraerythrocyte proteolysis is followed by the next degradation step coupled with excretion of newly formed shorter peptides from red blood cells. Both, the primary and the secondary proteolysis products are subjected in animal tissues to further stepwise C- and N-terminal chain shortening, giving rise to families of closely related peptides. Amino acid sequences of hemoglobin fragments secreted by the erythrocytes were established. Most of these peptides were related to seven families grouped according to the patterns of proteolytic splitting of globin chains. Eight of the peptides released by erythrocytes showed biological activity in various tests systems, while the other overlapped with well-known biologically active hemoglobin fragments. On the basis of the presented facts a concept of "tissue specific peptide pool" describing a novel system of peptidergic regulation, complementary to the conventional hormonal and nervous systems was formulated.

INTRODUCTION

Over a hundred of endogenous hemoglobin fragments have been isolated from different mammalian tissues and about a half of them was shown to be active in various biological tests both *in vitro* and *in vivo*. Different groups of hemoglobin-derived peptides exhibit hormone releasing, coronaro-constrictory, neuroregulatory, cardiotropic, immune-regulatory activities, antitumor effect or regulated the activity of enzymes. A large group of β-globin fragments, hemorphins and the related peptides were shown to exhibit opioid effects. On that basis it was assumed that hemoglobin fragments participate in regulation of metabolic processes in the organism (for review see ref. 1).

The abundance of hemoglobin fragments in various tissues raises a question of the mechanism of their formation and delivery. Since the globin genes can be expressed not only in red blood cells the respective peptides might be formed directly within any cell (ref. 2). However, the huge amount of hemoglobin circulating inside the erythrocytes suggests itself as the most probable precursor of these peptides.

The formation of relatively long (about 30 amino acid residues) hemoglobin fragments inside the erythrocytes was reported earlier (ref. 3). The generation of these peptides does not depend on blood group, Rhesus factor, age and sex of healthy donors, while notable changes occurred in the erythrocytes of patients with Hodgkin's disease (ref. 4). Therefore at least the initial stages of hemoglobin fragmentation take place inside the erythrocytes. At the same time, most of the biologically active hemoglobin fragments, isolated from different sources are 3-17 amino acid residues in length (ref. 1). We suggested that the short hemoglobin fragments might be formed by consecutive degradation of long intraerythrocytic peptides and secreted by these cells.

FORMATION OF HEMOGLOBIN FRAGMENTS INSIDE ERYTHROCYTES

Analysis of the peptide content in human erythrocytes demonstrated that intensive proteolytic degradation of hemoglobin takes place inside the erythrocytes. The overall amount of identified peptides comprised ca. 0.5 % of the hemoglobin content. With the exception of neokyotorphin and its des-C-terminus derivative (TSKY) the intraerythrocytic peptides have notably longer sequences than most of endogenous hemoglobin fragments (ref. 3).

In this work a more complete analysis of peptides present in the lysate of human erythrocytes was carried out. The result of chromatographic analysis of low molecular weight fraction of erythrolysate is given in Fig. 1.Twenty five peptides were isolated and sixteen hemoglobin fragments containing more than 30 amino acid residues were identified (Table 1).

Analysis of the established amino acid sequences shows that the identified peptides overlap a considerable part of both globin chains. These findings suggest that at least two stages (discussed below) of proteolytic digestion of the globin chains take place inside the erythrocytes. The data obtained show also that several long hemoglobin fragments isolated from other tissues (ref. 5,6) must form inside the erythrocytes.

SECRETION OF HEMOGLOBIN FRAGMENTS BY ERYTHROCYTES

Most of the hemoglobin-derived peptides isolated from different sources are less than 20 amino acid residues in length (ref. 1). We have demonstrated above that such peptide are practically absent inside the erythrocytes. Therefore we suggested that short hemoglobin fragments might be released by these cells immediately upon their formation. To prove this suggestion we examined the composition of the substances produced by the primary culture of human erythrocytes. Red blood cells were incubated in the isoosmotic buffer for 1.5 hours at 37° C, the obtained supernatant was separated by RP-HPLC (Fig. 2). The fractions corresponding to main peaks were rechromatographed and isolated substances were analysed by Edman technique. As a result, 36 amino acid sequences of isolated peptides were established. 32 peptides were identified as hemoglobin fragments (Table 2), the remaining four are derived from other proteins (β-actin, fructose-biphosphate aldolase A and keratin). All identified peptides were less than 20 amino acid residues in length.

Eight novel hemoglobin fragments were identified. Two substances (the fragments of α-chain (84-95) and (84-88)) form a novel family of hemoglobin-derived peptides, while the peptides corresponding to β-chain (12-31) segment form the pattern of hemoglobin degradation different from that of tissues (ref. 1).

Since erythrocytes release neokyotorphin but not the fragment of neokyotorphin (1-4) while both these peptides are equally represented inside the red blood cells, the secretion of hemoglobin fragments should be considered as a specific process (ref. 15).

We believe that long peptides containing more than 30 amino acid residues and found in tissue extracts come from the intraerythrocyte pool, while the shorter peptides are either released by erythrocytes or generated by the action of proteolytic enzymes of other cells.

PROTEOLYTIC DEGRADATION OF HEMOGLOBIN

Identification of intracellular peptides overlapping more than half of the sequences of both globin chains allows to suggest some principles of hemoglobin degradation inside the erythrocytes. We considered that formation of the longest peptides, in particular N-terminal fragments of α-chain (1-48?) and that of β-chain (1-44?), correspond to the earlier stage of hemoglobin degradation, while the fragments of α-chain (1-33) and (34-68) as well as that of β-chain (1-41) are generated at a later stage of proteolysis.

C-terminal fragments of both globin chains were also assumed to result from a later proteolytic step. This suggestion was supported by the fact of isolation of a much longer β-chain (89-132?) segment from human cerebellum (ref. 16). Striking similarity of degradation patterns of both globin chains points to similar areas of primary splitting, positioned at the 65-90 site of each chain,

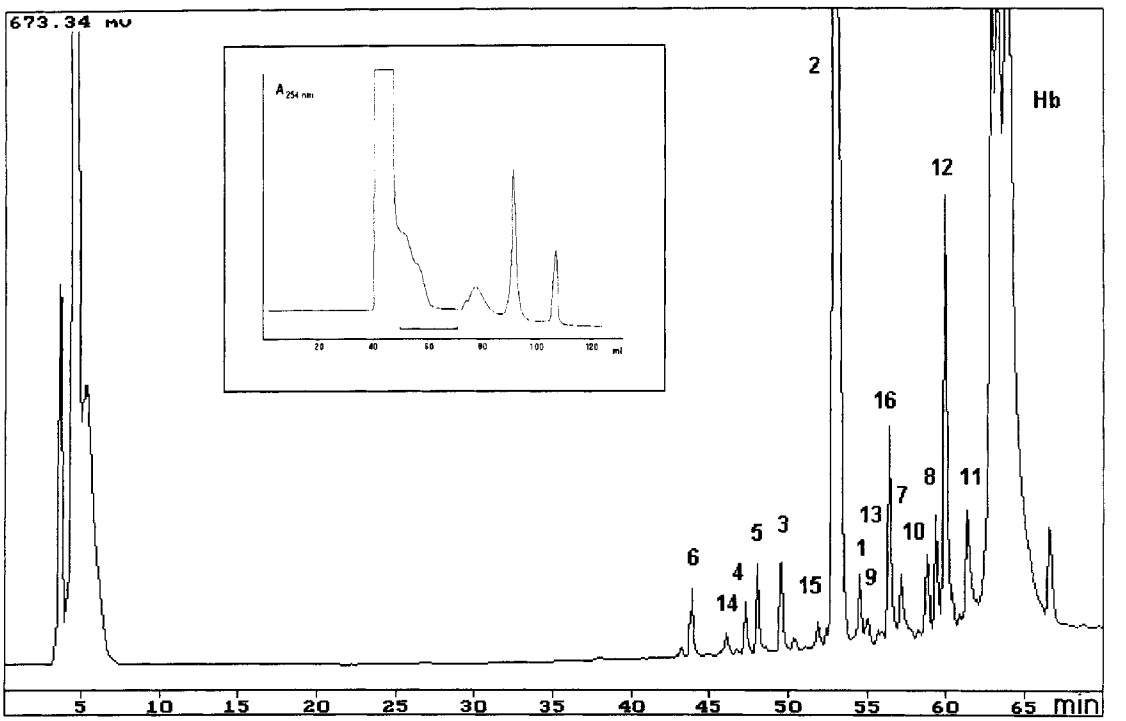

Fig. 1. RP-HPLC of the fraction the lysate of human erythrocytes obtained after size-exclusion separation on Sephadex G-25 sf (the respective chromatogram is given as insertion). Absorbance range is given in mV (1800 mV = 2.56 AUFS). The peaks containing peptides with established amino acid sequences are marked with numbers. The obtained sequences are given in Table 1.

Table 1. Peptides isolated from human erythrolysate.

#	Sequence	Position
1	VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKYFPHFDLS...	α 1-48-?
2	VLSPADKTNVKAAWGKVGAHAGEYGAEALERMF	α 1-33
3	VLSPADKTNVKAAWGKVGAHAGEYGAEALERM	α 1-32
4	VLSPADKTNVKAAWGKVGAHAGEYGAEALER	α 1-31
5	VLSPADKTNVKAAWGKVGAHAGEYGAEALE	α 1-30
6	VLSPADKTNVKAAWGKVGAHAGEYGAEAL	α 1-29
7	LSFPTTKTYFPHFDLSHGSAQVKGHGKKVADALTNAVAH	α 34-68
8	LVTLAAHLPAEFTPAVHASLDKFLSVSTVLTSKYR	α 106-141
9	VTLAAHLPAEFTPAVHASLDKFLSVSTVL	α 107-136
10	AAHLPAEFTTPAVHASLDKFLSNVSTVLTSKYR	α 110-141
11	VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESF...	β 1-45-?
12	VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRF	β 1-41
13	VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLV	β 1-33
14	VHLTPEEKSAVTALWGKVNVDEVGGEALGR	β 1-30
15	VHLTPEEKSAVTALWGKVNVDEVGGEALG	β 1-29
16	VCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHRYH	β 111-146

The peptides corresponding to α-chain (1-32), α-chain (1-31) and β-chain (1-29) segments were isolated from bovine brain (ref. 5) and β-chain (1-41) segment was isolated from human pituitary gland (ref. 6).

Question marks indicate unfinished sequences.

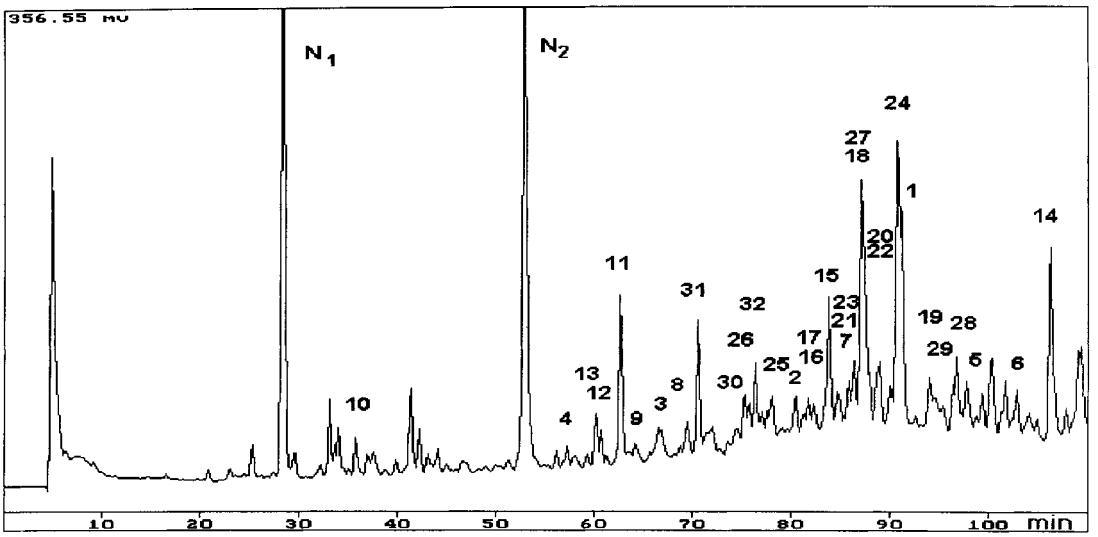

Fig. 2. RP-HPLC of the supernatant of primary culture of human erythrocytes. Absorbance range is given in mV (1800 mV = 2.56 AUFS). The peaks corresponding to peptides with established amino acid sequences are marked with numbers. The obtained sequences are given in Table 2.

Table 2. Hemoglobin fragments released by human erythrocytes.

Peptides released by human erythrocytes			Identical or similar peptides from other sources		
#	Sequence	Position	Sequence	Activity	Ref.
1	VLSPADKTNVKAAWGKV	α 1-17	VLSAADKGNVKAAWGK	Hemopoietic	5,7
2	VLSPADKTNVKA	α 1-12	VLSAADKGNVKAAWG	Antigonadotropic	8
3	VLSPADKTNV	α 1-10	VLSAADKGNVKAA	Hemopoietic	5,7
4	VLSPADKTN	α 1-9			
5	AEALER	α 26-31	GAEALER	Hemopoietic	5,7
6	FPHFDL	α 43-48	FLGFPTTKYFPHF	ACTH-releasing	9
7	ALSAL	α 79-83	LPGALSE	Hemopoietic	5,7
8	SDLHAHKLRVDP	α 84-95	-		
9	SDLHA	α 84-88	-		
10	TSKYR	α 137-141	TSKYR	Neuromodulatoric	10
11	VHLTPEEKSAV	β 1-11	MLTAEEKAAVT	Hemopoietic	5,7
12	VHLTPEEKSA	β 1-10	MLTAEEKAAV	Hemopoietic	5,7
13	VHLTPEEK	β 1-8	VHLSAEEKEA	GH-releasing	11
			MLTAEEKAA	Hemopoietic	5,7
14	TALWGKVNVDEVGGEALGRL	β 12-31	-		
15	TALWGKVNV	β 12-19	-		
16	TALWGKVN	β 12-20	-		
17	TALWGKV	β 12-20	-		
18	ALWGKVNV	β 13-20	-		
19	NVDEVGGEALGRL	β 19-31	GKVKVDEVGGEALGRL	Hemopoietic	5,7
20	GGEALGRL	β 24-31	GKVN	GH-releasing	11
21	GGEALGR	β 24-30	DEVGGEALGR	Hemopoietic	5,7
22	GEALGRL	β 25-31	ALG	Hemopoietic	5,7
23	GEALGR	β 25-30			
24	VYPWTQRF	β 34-41	YPW	Opioid	12
25	VYPWTQ	β 34-39	YPWTQRF	Opioid	13
26	VYPW	β 34-37			
27	YPWTQRF	β 35-41			
28	SDGLAHLDNLKGTF	β 72-84	SNGMKGLDDLK	Hemopoietic	5,7
29	SDGLAHLDNLK	β 72-82			
30	TLSEL	β 87-91			
31	VVAGVANALAHRYH	β 133-146	VVAGVANALAHRYH	Unknown	14
32	VAGVANALAHRYH	β 134-146			

the concrete sites of primary splitting require identification of peptides overlapping the middle segments of hemoglobin sequence. The secondary splitting results in formation of α-chain (1-33), (34-68), (106-141) and β-chain (1-41) and (111-146) fragments.

Overlap of some long peptide sequences (for instance, of α-chain fragments (1-33) and (34-68) or (1-32) and (33-55?) (ref. 16)) allows to consider that proteolysis takes place at several adjacent peptide bonds rather then at strictly defined single pairs of amino acid residues. The following degradation of these peptides leads to generation of short hemoglobin fragments released by erythrocytes.

As soon as the β-chain fragmentation takes place at Val^{34}, rather that at Leu^{32}, the composition of hemorphin-related peptides detected in the supernatant of the erythrocytes differs significantly from that formed in other biological sources. LVV- and VV-hemorphines, present in high amounts in tissue extracts were not detected in the secreted material. At the same time, erythrocytes contained V-hemorphin-7 (VYPWTQRF) and V-hemorphin-5 (VYPWTQ), as well as VYPW ,i.e., three hemorphin-related peptides, that were not identified earlier. Erythrocytes contain also hemorphin-7 that appears in blood plasma of donors after marathon running (ref. 13). LVV- and VV-hemorphins were also not found in bovine bone marrow extracts (ref. 5) while these peptides are well represented in the other tissues (> 1 nmol/g of tissue) (ref. 17).

The available quantitative data although rather fragmentary on the content of the other hemoglobin derived peptides in various samples also speak of tissue specificity of their formation. For instance, the level of neokyotorphin in rat lung is 4-5 times higher than in erythrocytes (ref. 18). In other words, in spite of arguments favouring the role of erythrocytes as a common primary source of hemoglobin fragments each tissue has its own, specific set of hemoglobin derived peptide components.

We suggest that peptides released from the erythrocytes are either accumulated in the tissue surrounding the blood vessel or are further degraded by tissue specific proteases. The observed level of peptides in the tissue extract is a sum of the contributions from the erythrocytes always present in blood vessels and from the rest of the tissue.

These findings suggest also that, in addition to secretion of hemoglobin fragments by erythrocytes, there must be another mechanism leading to generation of these peptides in tissues. Proteolytic enzymes of other cells might participate in this process. For instance, macrophages release LVV-hemorphin after interaction with hemoglobin *in vitro* (ref. 19). This peptide has been found in high amounts in tissues (ref. 17) but was not detected in the supernatant of erythrocytes.

Summarising the facts given above we suggest that both primary and secondary steps of proteolysis of hemoglobin take place exclusively inside the erythrocytes while all following steps predominantly occur on crossing the erythrocyte membrane and later - within the tissue.

The obtained results clearly speak of a non-random, stepwise manner of generation of hemoglobin fragments both present inside the erythrocytes and released by these cells. Different families of peptides bearing the same structural segment but varying in N- and C- terminal fragments might be formed from the common peptide precursor. The localisation of primary and secondary splitting areas might depend on conformation of globin chains or the resultant products of primary cleavage, while the formation of the groups of related peptides might be carried out mainly by amino- and carboxypeptidases. Combined action of the two mechanisms leads to generation of structurally related families of the peptides, exhibiting similar spectrum of biological effects (ref. 1).

BIOLOGICAL ACTIVITY OF THE SECRETED PEPTIDES

Erythrocytes release a number of well-established biologically active peptides. Hemophin-7 (ref. 13) and VYPW (ref. 12) exhibit opioid effects, neokyotorphin shows cardiotrophic (ref. 20), antihibernatic (ref. 20) and the other activities (ref. 10,18). Other peptides overlap hemoglobin segments responsible for hormone-releasing (ref. 9), immunoregulatory (ref. 21) and hemopoetic activities (ref. 7) (Table 2). Therefore we can expect that these peptides will exhibit in higher or lower extent, the similar effects.

Supernatant of erythrocytes was enriched with the fragments (1-11) and (134-146) of the β-chain (about 3 nmol/ml of cell suspension). The former peptide overlaps the hemoglobin segment responsible for

antigonadotropic and hemopoetic activities, while the function of the other fragment is not established. At the same time, we can suggest that increased production of these peptides might be of functional significance *in vivo*.

We have examined the influence of the peptides released by the erythrocytes on proliferation of tumor cells (Table 3). Two novel peptides corresponding to β-globin (12-20) segment significantly suppress the number of live tumor cell, while neokyotorphin increased proliferation of the transformed cells. In accordance with the earlier established antitumor effect of opioid receptor ligands (ref. 22), both hemorphin-related peptides also exhibite reliable antiproliferative activity. To examine the influence of N-terminal fragments of α-chain (the peptides exhibiting hemopoetic activity (ref. 5,7)) on proliferation of tumor cells we studied antiproliferative activity of the longest peptide of this structural family: the fragment (1-17).

Table 3. Influence of the peptides released by the erythrocytes on proliferation of L929 murine tumor cells.

Peptide	Concentration (M)			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
TALWGKVNV	- 45 ± 5	- 35 ± 4	- 36 ± 4	- 27 ± 2
TALWGKVN	- 44 ± 1	- 24 ± 8	- 19 ± 8	- 31 ± 10
TSKYR	-	+ 35 ± 6	+ 32 ± 4	+ 30 ± 5
VLSPADKTNVKAAWGKV	- 37 ± 7	- 32 ± 2	- 19 ± 6	- 18 ± 3
VYPWTQRF	-	- 33 ± 3	- 32 ± 3	- 31 ± 6
YPWTQRF	- 20 ± 1	- 25 ± 4	- 32 ± 3	- 31 ± 6

Influence of the test substances on proliferation of tumor cells i.e. the change of viable cell number as compared with the negative control is calculated as the difference between the live cell number in the control and the same parameter in the sample. The dead (Trypan blue-stained) and the live (non-stained) cell numbers were determined visually under the microscope (ref. 23). The values of proliferative activity are marked with "+"; those of antiproliferative activity are marked with "-".

Since the peptide was active in this test, we can expect similar effects from the other members of this group. Thus, erythrocytes release a number of peptides exhibiting various activities, including both stimulation and inhibition of proliferation of tumor cells.

HEMOGLIBIN FRAGMENTS AS COMPONENTS OF TISSUE SPECIFIC PEPTIDE POOLS

It has been generally accepted that the proteins normally present in the tissue were digested by proteolytic enzymes after completion of their function. At the same time, the broad spectrum of biological effects exhibited by endogenous hemoglobin derived peptides allows to make a conclusion that proteolytic degradation of hemoglobin carries out an important *in vivo* regulatory function. This concept originally formulated in 1992 was further supported by the above described data on intensive formation in and release from the erythrocytes of active peptides subsequently found in various tissues (ref. 5).

We suggest that protein elimination does not occur by random hydrolysis directly leading to amino acids that are further utilised in metabolic reactions. Instead it represents a specific process regulated by the level of tissue specific proteinases and the availability of their substrates. As a result of that process a large number of peptides is formed which can be defined as "peptide buffer", "peptide background", or more accurately as "tissue specific peptide pool". The properties of that pool depend both on the concrete set of peptide components and on individual levels of those components.

In contrast to peptides derived from functional proteins most of the signal peptide molecules of the nervous tissue (neurotransmitters) or endocrine system (hormones and parahormones) are released from a narrow group of specific precursors for which no other than being a precursor function is known. Some of the components of the peptide pool are known to bind to the same receptors as neurotransmitters or hormones and to exhibit similar effects. While binding affinities of the pool peptides are by several orders of magnitude lower than for hormones or neurotransmitters, these peptides are found in higher amounts and typically occur as families of related molecules rather than single representatives. Hemorphins and closely related peptides with their opioid receptor binding ability consistently found in most of the

investigated sources provide a good example of that tendency. Since the intensity of proteolytic processes depends on such relatively conservative parameter as metabolic state of the organism we suggest that the tissue specific peptide pool predominantly controls long-term processes, i.e. is responsible for maintenance of tissue homeostasis. The latter includes cell proliferation, differentiation and cell death. Prevention of cell transformation and lysis of tumor cells also fall into that category. The data presented above as well as the recent finding that hemoglobin fragments induce death of transformed cells (ref. 23,24) and regulate proliferation and differentiation of normal cells (ref. 5,7,24) provides a convincing argument favouring our concept.

There is a growing evidence of changes in tissue composition of hemoglobin-derived peptides accompanying various pathologies, such as human lung carcinoma (ref. 25), Alzheimer's disease (ref. 16), brain ischemia (ref. 26) and Hodgkin's disease (ref. 4). Notwithstanding the clinical and biochemical differences, all above mentioned pathologies have a common principle feature - they involve changes in tissue homeostasis or metabolic state of the cells, whatever the origin of the disease: cell transformation (carcinoma), tissue atrophy (Alzheimer's disease and ischemia) or impaired lymphoproliferation (Hodgkin's disease). Although it is not known whether the above mentioned changes in peptide composition are the cause or the consequence of the disease these changes are in full accord with our views of the biological role of tissue specific peptide pool.

More data should be accumulated before the concept of tissue specific peptide pool finds its final shape. However, regardless of these developments there is little doubt that besides the oxygen transport (and possibly the transport of nitrogen oxide (ref. 27)) hemoglobin serves as a rich source of biologically active peptides. In that case the function of erythrocytes might be compared with the function of endocrine gland and the participation of hemoglobin fragments in regulation of homeostasis provides an example of a novel peptide mediated regulatory pathway complementary to traditional hormonal or neuropeptide mechanisms.

Endogenous fragmentation of the hemoglobin and the properties of respective peptides are studied much better than analogous processes with other functional proteins. Still, identification of active peptides derived *in vivo* from cytochrome *c* oxidase (ref. 28), immunoglobulins (ref. 5,29-31), albumins (ref. 32), fibrinogen (ref. 33) and other functional proteins (ref. 5,34) allows to conclude that regulatory role of hemoglobin derived peptides should not be considered as a unique, isolated phenomenon. On the contrary, it is only one of the components (although an important one) of the general system of peptidergic regulation of tissue homeostasis.

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