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# Hemocidins in a functional and structural context of human antimicrobial peptides

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## TABLE OF CONTENTS



## 1. ABSTRACT

Hemocidins are a recently discovered group of microbicidal peptides that emerge from heme-binding proteins, especially hemoglobin. Hemocidins have been obtained *in vitro* after the chemical or enzymatic fragmentation of globin molecules and have also been isolated from biological sources, such as insect guts and the tissues and excretions of the female reproductive tract. This work presents a concise review of contemporary studies concerning antibacterial peptides, especially those derived from humans, and against this broad structural and functional background discusses the properties of hemocidins.

## 2. INTRODUCTION

Antimicrobial peptides represent an ancient and ubiquitous famliy of effector molecules of the innate immune system. They have been found in both plants and in almost all species? of animals. To-date, the number of known representatives of these compounds approaches 900 (1). From an evolutionary point of view, the fact that microbicidal peptides are so widespread in itself testifies that they are not only highly efficient at fighting infections, but also have a great potential to bypass the constantly mutating defense mechanisms of microbes. From this reason and because there is a still increasing problem of pathogen resistance to common antibiotics, these molecules

are of exceptional interest for contemporary biomedical sciences (2, 3). There are additionally numerous potential advantages to using bactericidal peptides as drugs: i) they act quickly, in most cases causing irreversible and lethal perforation of bacteria membrane within minutes; ii) they are selective toward prokaryotic cells, but on the other hand, show a simultaneous affinity for many different microbes; iii) antimicrobial peptides do not have a specific molecular target so micro-organisms cannot easily acquire resistance to them; iv) due to multiple other biological activities, many of them act as immune-stimulants or general therapeutics, able, for example, to limit sepsis or to kill certain cancer cells; v) many antimicrobial peptides act synergistically - application of the one compound disproportionately amplifies the activity of the mixture of both endo- and exogenous bactericidal polypeptide factors. Unfortunately, there are also serious limitations that restrict the application of antimicrobial peptides: i) the high cost of chemical synthesis (gradually overcome by the application of recombinant DNA techniques); ii) problems with dosage (proteolytic degradation in the digestive tract in the case of oral administration, potential allergenic action); iii) cytotoxic activity toward certain eukaryotic cells (4). Moreover, according to the interesting observations of Ginsburg (5), cationic antimicrobial peptides mimicking classical bacteriolytic beta-lactam antibiotics and causing eventually a massive generation of pro-inflammatory factors such as lypopolysaccharide (LPS), teichoic acid (LTA), and peptidoglycan, can simultaneously induce the release of cytokines from phagocytes and activate complement as well as coagulation cascade. In effect, these events might cause lethal septic shock and multiple organ failure, especially in bacteremic patients. This fact may restrict the application of microbicidal peptides to the topical dosages. Nevertheless, the idea of utilizing one of the human body's own defense element to design more effective and safe antimicrobial drugs is extremely attractive and has inspired many researchers. Their attention has been focused both on numerous classical, specialized antimicrobial peptides as well as on lesser known bactericidal polypeptide fragments of certain proteins that often have primary functions other than killing micro-organisms (as e.g., lactoferrin, cathepsin G, or hemoglobin). The distinction between these two groups can sometimes be unclear but has a genetic base. The expression of the genes of specialized antimicrobial peptides is functionally related to immunity and takes place at sites of potential infection or in phagocytic cells. It should be briefly noted at this point, that many sources define this group of bactericidal peptides as "gene-encoded"; this definition, however, is not only misleading, but incorrect. Antimicrobial peptides that originate from the proteolytic degradation of other functional proteins do not have their separately expressed genes. The generation of these compounds at a specific physiological niche, in a certain cellular compartment or *in vitro* depends solely on the action of specific proteolytic enzymes. Recent studies suggest that hemoglobin is an especially good example of such a substrate for the generation of microbicidal fragments. The present study introduces antimicrobial peptides derived from hemoglobin in a broad, structural, and functional context of other known, especially human, microbicidal peptides.

## 3. TWO MAIN GROUPS OF ANTIMICROBIAL PEPTIDES

### 3.1. Classical antimicrobial peptides having their own genes

Antimicrobial peptides encoded by their own individual genes are produced both constitutively and in response to infection. They act as humoral factors as well as effector molecules of both epithelia and of cellular response. Most of them have several similar features: they count about 12 to 50 amino acids, have an amphipathic structure (physically separated hydrophobic and hydrophilic regions of molecule), possess a cationic net charge, and act mainly on bacterial membrane. On the other hand, antimicrobial peptides are quite diverse in their structure and are divided into three big subgroups according to these structural attributes.

The first subgroup comprises the alpha-helical linear peptides and currently counts about 150 members isolated from such diverse organisms as tunicates and humans (6). These cationic peptides are generally unstructured in solutions and can electrostatically bind to negatively charged microbial surfaces. This binding is facilitated by the interaction of peptides with LPS-rich external compounds present on the outer membrane leaflets of Gram-negative bacteria. In the case of Gram-positive species, binding is based on the interaction of peptides with negatively charged LTA and teichuronic acids, as well as with carboxyl groups of peptidoglycan. Direct contact of the peptide with the bacterial phospholipid membrane induces a rapid change in peptide structure to helical amphipathic conformation. Further membrane permeabilization occurs through four alternative mechanisms: insertion into the membrane and the formation of so called "barrel-stave" or toroidal pores as well as the accumulation of peptides parallel to membrane surface and disaggregation of the phospholipid bilayer in a detergent-like manner by forming micelle-like complexes ("carpet-like" model or aggregate model). It is also a characteristic fact that in some cases, the single peptide can act by two different modes of action, depending upon its concentration (7, 8). The only classic human alpha-helical linear antimicrobial peptide is LL-37 (9). This peptide is generated by neutrophil-derived proteinase 3 from a 18-kDa precursor protein named hCAP-18, the only human member of the so-called cathelicidin family. The gene of this protein is expressed mainly in neutrophils and in epithelial cells and is up-regulated by different bacterial and fungal products acting through the MAPK/ERK pathway (mitogen-activated protein kinase/extracellular signal-regulated kinase). LL-37 is able to kill a broad spectrum of micro-organisms and additionally has broad biological activities related to inflammation, including the ability to neutralize LPS and to promote wound repair as well as chemotactic and angiogenetic activity.

The second subgroup of antimicrobial peptides is often called defensins and comprises cysteine-containing antimicrobial peptides having beta-sheet sections in the molecule, stabilized by at least one intramolecular disulfide bridge (10). Apart from such similarities, defensins are an

extremely diverse subgroup of peptides; the number of disulfide bonds can vary from one to four, and the arrangement of the bridges between specific cysteine residues also varies. The best studied subgroup of these peptides consists of mammalian alpha- and beta-defensins, present mainly in the granules of neutrophils, in enteric Paneth cells, or secreted by epithelia. Both these subgroups are identified in humans: there are six known members of alpha-defensins (four neutrophil peptides named HNP-1 to HNP-4 as well as two enteric defensins, HD-5 and HD-6) and eleven beta-defensins (named HBD-1 to HBD-6 and HBD-25 to HBD-29) (11). Alpha-defensins are constitutively expressed and produced as pre-propeptides in cellular granules and enzymatically processed to mature forms during subsequent stages of cell maturation. The final bactericidal form fills specific granules of certain phagocytic cells and acts as one of the main oxygen-independent mechanisms for the killing of ingested micro-organisms. Beta-defensins have a similar way of maturation but with two important differences: mature peptides are not stored in the granules, but are secreted outside of the epithelial cells and the expression of these peptides is more precisely controlled and induced to a much higher level upon infection or inflammation. The action of defensins toward the microbial membrane is mainly electrostatically-driven in the first stages (as in the case of the alpha-helical peptides described above), but differs markedly in subsequent phases. Detailed studies on model phospholipid membranes suggest that defensins have a tendency to form oligomeric channels and that permeabilization occurs mainly by forming toroidal wormholes in the bilayer. The action of defensins on membranes is markedly inhibited by increasing concentrations of salt as well as divalent cations; these peptides thus act *in vivo* mainly in phagocytic vacuoles and in mucosal secretions, where low ionic strength is maintained. Apart from remarkable antibacterial, antifungal, and even antiviral activity, defensins demonstrate an unusually broad spectrum of other biological activities and in many features their functions overlap with chemokines (12). They are able to enhance phagocytosis and act as opsonins for macrophages and are chemotactic factors for many other types of cells. They induce the activation and degranulation of mast cells, regulate the transcription of interleukins by epithelial cells and monocytes, and govern the activation of the classical pathway of complement. Additionally, they inhibit fibrinolysis (by modulating tissue-type plasminogen activator) as well as ACTH-induced steroidogenesis. In other words, mammal defensins are multifunctional effector molecules that play an integrating role mainly in the innate immune response.

To the third and final subgroup of antimicrobial peptides that have their individually expressed genes belong linear polypeptides with one (or more) certain predominant amino acids (1). These compounds contain an elevated amount of histidine (as histatins, the only human members of this subgroup) (13); these are tryptophan-rich (such as bovine indolicidin), or tend to have predominant proline or proline and arginine residues (such as drosocins from insects or bovine peptide PR-39). Many of these peptides adopt atypical structures (such as polyproline type-II helix or wedge-shaped turns); some are even glycosylated or phosphorylated. Human histatins are represented by five peptides (named Hst1 to Hst5) that are constitutively produced by submandibular and sublingual glands and secreted to saliva. They play an important role in limiting eventual infections in the oral cavity and have a pronounced effect on fungi. Moreover, Hst5 inhibits host and bacterial enzymes involved in periodontal diseases. The peptide P-113, a 12-amino-acid long fragment of Hst5, is in phase I/II of clinical trials for the treatment of plaque and gingivitis.

### 3.2. Antimicrobial peptides emerging after proteolytic digestion of functional proteins

As was stated earlier, this group cannot always be sharply distinguished from the family described in the previous chapter. A typical example of a double-faced antimicrobial peptide is human cathelicidin hCAP18. On the one hand, this protein has its individual gene, expressed by phagocytic cells and epithelia, and this expression is up-regulated during inflammation. On the other hand, the bactericidal domain of this protein, the peptide LL-37, is released after contact with proteinase 3 produced by the key types of phagocytic cells, namely the neutrophils. One residue larger antimicrobial peptide, ALL-38, is also released from hCAP-18 in the acidic environment of the vagina by prostate serine proteinase – gastricsin (9). Thus some features of hCAP-18/LL-37 fit with classical antimicrobial peptides while others are a match for the bactericidal peptides released from the larger functional proteins.

One of the first discovered human proteins able to generate microbicidal peptides was bactericidal/permeability increasing protein (BPI), isolated in 1978 from the granules of polymorphonuclear leukocytes (14). This cationic 55-kDa protein belongs to the group of conserved, lipid-binding proteins and is expressed mainly in the bone marrow in the precursors of neutrophils and stored in their primary granules. In humans the expression in neutrophils is constitutive; however, recent studies suggest that the protein may also be produced by epithelial cells in response to stimulation upon endogenous anti-inflammatory metabolites of arachidonic acid. The studies using the recombinant BPI variants proved that for the primary antibiotic (especially toward Gram-negative bacteria) and endotoxin-neutralizing activities is responsible a N-terminal domain of the protein, named $rBPI_{21}$, spanning the residues 13-191 of the maternal protein. This domain is quite large (it is appropriately a small protein having a molecular mass of 21 kDa) and is not relevant to the subject of this work but for historical reasons has been roughly described. Last but not least, the active cationic microbicidal polypeptide from BPI is involved in numerous clinical trials, including a trial for the treatment of severe meningococcal sepsis. The results to-date, however, have not led to approved clinical application (15).

A second discovery of bactericidal domains in human protein took place in 1990 and was related to the intriguing observation that treatment of cathepsin G, a

lysosomal, about 23-kDa serine protease from neutrophils, with irreversible synthetic inhibitor did not diminished the antimicrobial activity of such a knocked-up enzyme (16). Subsequent studies using a library of synthetic peptides that covered an entire protein sequence proved that cathepsin G contained at least three antibacterial domains that demonstrated activity toward Gram-negative bacteria. The most potent form of these peptides, spanning residues 117-136 of the mature protein, also exerted a pronounced effect toward Gram-positive bacteria. This peptide was exposed on a surface of native cathepsin G molecule and the studies using modified synthetic peptides proved that to maintain its full bactericidal activity two key factors were essential: the presence of all four cationic arginine residues as well as the presence of certain hydrophobic amino acids at the N-terminal part of the peptide. The bactericidal properties of CatG117-136 peptide can also be significantly increased by the covalent linking of saturated linear fatty acids to N- or C-terminal amino acids (17). Interestingly, structural studies using large unilamellar liposomes as well as circular dichroism spectra measurements demonstrated that hybrid CatG117-136 synthetic peptides bearing specific fatty acid moieties (especially C-8, C-10 and C-12) had increased the ability of polypeptide backbone to form an alpha-helical conformation and at the same time markedly improved the bactericidal activity of the whole hybrid peptide molecule.

Lysozyme, apart from the above described cathepsin G, is the second and, to-date, the most recently discovered human enzyme having bacteria-killing capabilities independent of its catalytic properties. This common, about 14-kDa bactericidal protein, exceptionally widespread in many human (and animal) physiological secretions and fluids, has muramidase activity and by degrading the peptidoglycan layer of Gram-positive bacteria facilitates lysis of their cells. Relatively late, in 1992, Pellegrini *et al.* presented results proving that the partial denaturation of hen egg lysozyme did not diminished its bactericidal properties and suggested that this activity was related to the cationic properties of the protein (18). Further studies on heat-denatured lysozyme proved that partially unfolded, enzymatically inactive lysozyme formed dimers that had the ability to insert into and to disintegrate bacterial membranes. Similar properties had small synthetic peptides corresponding to the C-terminal part of this protein (19). Detailed studies on a peptide spanning residues 98-112 of hen egg lysozyme proved that this peptide was able to kill many species of Gram-negative and Gram-positive bacteria as well as *Candida albicans* (20). Interestingly, both in native protein and in solution, the peptide forms a helix-loop-helix structure and, moreover, studies on transformed *E. coli* cells proved that the peptide permeabilizes both outer and inner membranes of these bacteria, most probably, by the carpet-like mechanism. Similar studies were recently performed by Ibrahim *et al.* on human lysozyme (21). In elegant experiments, the authors demonstrate that pepsin, in conditions similar to that of a human newborn's stomach, is able to generate various bactericidal peptides from lysozyme. Two such bioactive peptides emerged from residues 1-38 of the maternal protein and, as with the previously described hen egg peptides, originated from a helix-loop-helix region of the lysozyme molecule. A third antimicrobial peptide from human muramidase spanned residues 18-56 and consisted of an alpha-helix and two-stranded beta-sheet. The human peptides, like their analogs from hen eggs, were also able to disrupt bacterial membranes in a similar way.

As was stated earlier, the bactericidal activity of cathepsin G and lysozyme is independent of their enzymatic activity. The situation with another bactericidal human protein, lactoferrin, is similar. This non-enzymatic iron-binding glycoprotein, having a molecular mass of about 80 kDa, is a common compound of milk and many other exocrine secretions. During inflammation, lactoferrin is also released to the blood serum by neutrophils (22). For a long time a strong iron-binding capacity was considered one of the principal factors in this protein responsible for limiting bacterial growth. These views changed in 1988, when Ellison *et al.* demonstrated that the bactericidal effect of lactoferrin is independent of its iron-binding properties and is also caused by the ability of lactoferrin to damage the outer membranes of Gram-negative bacteria (23). Further studies conducted by Bellamy *et al.* in 1992 proved that this membrane-directed effect is preserved after limiting acid or pepsin hydrolysis of the lactoferrin molecule (24). 15-18 amino acid long microbicidal peptide fragments of lactoferrin, known under the common name of lactoferricins, are located in the N-terminal region of the maternal lactoferrin molecule. These linear and cationic peptides were initially released *in vitro* but subsequent studies showed that they are also produced in stomach and in mucosal secretions through the proteolytic digestion of maternal protein by gastric enzymes, probably pepsin (25). Lactoferricins exhibit a broad range of antimicrobial activities and are able to kill many bacteria, fungi, and viruses. The exact mechanism of their action, however, is still unclear. In native maternal protein, lactoferricins domains are alpha-helical, but after releasing they adopt a beta-sheet amphipathic structure in solution. Some studies suggest that lactoferricins are able to form pores in bacterial membranes (26), but other detailed structural studies using model phospholipid membranes do not correlate well with the biological activities of these peptides, suggesting that they probably may also act intracellularly (27).

There is no doubt that, as described above, lactoferrin and lysozyme are common and important bactericidal factors present in mammalian milk, but factually the main proteinaceous compounds of this fluid are casein, alpha-lactalbumin, and beta-lactoglobulin. Because limited digestion of milk proteins represents an important mechanism in the generation of bioactive peptides, of special interest (particularly in this work), is the possibility of obtaining antimicrobial polypeptide fragments from just such three proteins. Numerous investigations in this field were first performed in 1960-1970 and were strengthened by a series of publications and patents documenting the generation of bactericidal peptides from cow and sheep casein, known as casosidins and isracedin (28). Several other bactericidal bovine casein domains were then discovered in 1999 by Reccio and

Visser (29), whereas in 2001, Liepke *et al.* documented obtaining antimicrobial peptides from human milk casein (30). Similarly, as in the case of the previously described studies on lysozyme, the bactericidal fragments of human casein were obtained in conditions similar to a newborn's stomach by treating the substrate with pepsin. The obtained peptide spanned residues 63-117 of the maternal protein and was a proline-rich cationic (pI=11.3) molecule, active toward both Gram-negative and Gram-positive bacteria as well as toward yeast. Interestingly, because of its high proline content, this linear peptide cannot adopt an alpha-helical structure. The mechanism of its action, to-date has not been investigated. In the case of the second essential milk protein, alpha-lactalbumin, initial reports on its bactericidal domains appeared in 1999 and concerned the discovery of three such peptides obtained after the digestion of bovine substrate by two gastric enzymes, trypsin and chymotrypsin (31). Digestion by pepsin appeared to be ineffective. The first from the discovered bactericidal fragments of bovine lactalbumin was a short linear pentapeptide while two other compounds, interestingly, were composed of two polypeptide chains linked by a single disulphide bridge. Subsequent studies by Malkoski *et al.* and also Lopez-Exposito *et al.* led to the characterization of several other antimicrobial bovine lactalbumin fragments, one of them a phosphorylated peptide, and this phosphorylation turned out to be essential for biological activity (32, 33). Microbicidal activity was also demonstrated in case of so called glycomacropeptide, a rich in sialic acids bovine kappa-casein fragment, spanning residues 106-169 (34, 35). Unfortunately, there is presently no information on human alpha-lactalbumin bactericidal domains. The situation is similar with regard to the last described milk protein, beta-lactoglobulin which is present in cow but not human milk. Numerous antibacterial fragments of bovine lactoglobulin were discovered by Pellegrini *et al.* in 2001 and found to have some interesting characteristics: almost all are negatively charged (pI=4.2 to 5.5) and, in consequence, were active toward Gram-positive bacteria only (36).

The other known microbicidal peptides that emerge after limited proteolytic degradation of functional proteins, especially in humans, are relatively less known and are currently cited only by single sources. It should also be noted that many of them were identified in maternal proteins according to certain specific biochemical criteria and were obtained only *in vitro*, in synthetic forms. Only some of these peptides were factually present and active in appropriate biological sources. Two such molecules, 7.5-kDa antimicrobial peptides, called thrombocidins TC-1 and TC-2, were isolated from human platelet granules (37). Both appeared to be fragments of human cytokines: the first peptide was a truncated form of neutrophil-activating peptide-2 (NAP-2), while the second emerged from connective tissue-activating peptide III (CTAP-III). While thrombocidins kill a broad spectrum of micro-organisms, the mechanism of this activity requires further study because current data has unequivocally proven that they do not disturb the bacterial membrane. Unknown also are the proteases involved in the truncation of maternal cytokines and the liberation of active thrombocidins. Pasupuleti *et al.* have demonstrated the antimicrobial activities of human peptide C3a, a pro-inflammatory multifunctional proteolytic fragment of the complement factor 3 digested during all pathways of complement activation (38). Moreover, performed phylogenetic and structural analyses showed a high degree of evolutional conservation of this concrete bactericidal domain; this suggests that it has a pivotal role in the innate immune system. Bactericidal activities were also demonstrated in other circulating human plasma components, for example, in angiogenin – a 14-kDa tumor-associated peptide induced also during inflammation (39) and in a 19 amino-acid-long fragment of domain 5 of high molecular weight kininogen (40). In two separate studies, the bactericidal peptide fragments of histones H1 and H2B were identified in acid extracts from human intestines (41, 42). The authors of these communications noted that these peptides were concurrently present with other classical antimicrobial peptides (as LL-37 and defensins), and speculated that all these factors are working synergistically as a barrier against bacterial invasion in intestinal mucosa. Andersson *et al.* have demonstrated the bactericidal properties of polypeptide domains that are able to bind glycosaminoglycans (as heparin and dermatan sulphate). These domains, also called Cardin and Weintraub motifs, are present in many endogenous proteins ( e.g., different isoforms of laminins, von Willebrand factor, vitronectin, protein C inhibitor, fibronectin) (43). The identical approach was applied in a study by Malmsten *et al.* who identified both heparin binding and microbicidal domains in many different growth factors, thus establishing a link between families of growth factors and of antimicrobial peptides, both of which are induced during tissue repair and remodeling (44).

Except for a description of hemoglobin, this presentation completes a concise review of human proteins known to be able to generate antimicrobial domains. At this point it should be recalled that the factual physiological role of such compounds in infection fighting is still unclear and requires further in-depth study. On the one hand, the involvement of the previously described family of peptides having their own individually expressed genes in innate immunity may be relatively easily verified by the studies on the mechanisms and the sites of their gene expression. On the other hand, the expression of such different proteins as casein or histones does not have a complete or direct association with the physiological mechanisms of bacteria killing. Nevertheless, under certain conditions, both these proteins can effectively generate bactericidal polypeptide domains. As was previously noted, such events can occur only in a specific physiological niche, containing definite proteinases as well as having specific physiochemical conditions (especially with regard to pH). This process of the generation of bioactive peptides is fundamental to the recent concept of a peptidergic mechanism for homeostasis regulation, based on the presence of a tissue-specific pool of regulatory peptides. This mechanism will be discussed further, after the introduction of one more protein - hemoglobin – a perfect proteinaceous substrate to the generation of bioactive, and in particular, antimicrobial peptides.

## 4. HEMOCIDINS - ANTIMICROBIAL PEPTIDES LIBERATED FROM HEMOGLOBIN

### 4.1. Discovery and biochemical characteristics

At first glance, hemoglobin is not a particularly promising candidate for a source of bactericidal polypeptides. Apart from its common nutrient role as an iron source for many micro-organisms, this protein is able to inhibit oxidative bactericidal mechanisms in macrophages, neutrophils, and cell-free systems; it can also bind antibiotics, simultaneously repressing their action (45, 46, 47). The first report documenting the existence of the second face of this protein - potentially bactericidal activity - appeared in 1958 and presented proof of the killing activity of both bovine and human hemoglobin toward Gram-negative bacteria (48). This activity manifested only in acidic conditions and in low ionic strength and was completely abolished by the binding of hemoglobin to haptoglobin and in the presence of divalent cations. The second, more contemporary biochemical study documenting the phenomenon of hemoglobin bactericidal activity appeared in 1999, when Fogaca *et al.* detected a peptide antimicrobial compound in the guts of the cattle tick, identifying it as a fragment of the bovine alpha-hemoglobin molecule, spanning residues 33-61 (49). The synthetic form of this peptide was highly efficient at killing Gram-positive bacteria and fungi, but was completely inactive toward Gram-negative bacteria. The molecular basis of this activity was not studied; the authors finished the work by concluding that they had discovered an interesting biological phenomenon, when the parasite – the tick – utilized a host protein to generate its own defense factor active against micro-organisms. The studies were continued *in vitro* by Froidevaux *et al.* who digested bovine hemoglobin with pepsin and also demonstrated the liberation of the bactericidal peptide (50). This bactericidal domain was different from the peptide characterized earlier and spanned residues 1-23 of alpha-globin. Unfortunately, as in the previous work, the biochemical and biological characteristics of the identified peptide were very limited.

The first complex and detailed study on bactericidal activity of hemoglobin appeared in 2000 when Mak *et al.* demonstrated that a principal operation that converts the hemoglobin molecule into a bactericidal polypeptide is to deprive it of heme, so that it partially unfolds and dissociates into individual globins (51). This discovery was in agreement with the above cited work from 1958, but went further, proving that not only partially denatured globin chains, but also denatured polypeptide chains of other heme-binding and structurally related, rich in alpha-helices proteins (as myoglobin and cytochrome c) were able to kill a broad spectrum of micro-organisms at micromolar concentrations. It was also suggested that these bactericidal properties were not a consequence of the presence of the specific microbicidal domain. The activity of the studied apohemoproteins was sustained after chemical and enzymatic digestion to approximately 50 amino-acid-long fragments; fragmentation into shorter pieces diminishes or completely eliminates the activity. The most active peptide identified in the course of the described studies was a cyanogen bromide fragment of myoglobin, spanning residues 56-131 (Mb56-131). Since it is relatively easy and cheap to obtain, this highly cationic peptide (net charge +10) was chosen for further in-depth study as a model and representative bactericidal peptide from hemoproteins. This peptide killed both Gram-negative and Gram–positive bacteria as well as fungi in micromolar concentrations. Its activity was inhibited in an elevated concentration of salt and divalent cations and its action was optimal in slightly acidic conditions (pH 6.0-7.0). The peptide was not hemolytic, even at high concentrations, yet did demonstrate a slight dose-independent cytotoxic effect toward eukaryotic cells. The peptide was able to perforate the *E. coli* membrane, but at the same time the dansyl polymyxin displacement assay as well as the LPS neutralization assay proved that it was not able to bind LPS. Circular dichroism spectra showed that the peptide has a disordered structure in solution but in phospholipid membrane-mimicking mixture forms predominantly alpha-helical structures. In a summary of the study, the authors proposed calling the bactericidal peptides emerged from heme-binding proteins, particularly from hemoglobin, ‘hemocidins’; they further speculated that the activities of these peptides are a consequence of an exceptional abundance of maternal proteins in amphipathic alpha-helical domains, characteristic simultaneously of other classical microbicidal peptides, as, for example, LL-37. In 2001, the results of Mak *et al.* were confirmed by an independent but quite similar study by Parish *et al.*, who additionally proved that bactericidal activities are characteristic of hemoglobins from many other animals (52). These results were further enriched in 2003 by Nakajima *et al.*, who, in a consecutive study concerning the analysis of the gut content of the soft tick, showed that this insect is able to generate two bactericidal peptides from rabbit hemoglobin, spanning residues 1-11 and 3-19 of the alpha-globin subunit (53).

### 4.2. Hemocidins in the female reproductive tract

As was previously stated, the first microbicidal hemoglobin peptides present in living organisms were of bovine or rabbit origin and have been identified in tick guts (49, 53). All subsequent studies on hemocidins were performed on the peptides obtained *in vitro*. The most convincing study documenting the origin of hemocidins in a human body appeared in 2004, when Mak *et al.* showed that the normal menstrual vaginal secretion of healthy females is exceptionally rich in bactericidal hemoglobin fragments (54). This study was a result of a search for a specific site within the human organism that would provide the specific conditions favorable to the generation of hemocidins and would also be under attack from microbial pathogens. The female vagina was a good candidate: due to menstrual bleedings this organ is periodically supplied with huge amounts of hemoglobin liberated from erythrocytes in its low pH. Moreover, this vaginal low pH caused hemoglobin molecules to dissociate and some hydrogen bonds to break, facilitating the action of the proteolytic enzymes predominant during menstruation, such as matrix metalloproteinases (MMPs) and neutrophil serine proteinases. Furthermore, this physiological niche is still exposed to the risk of infection. Detailed chromatographic analysis of menstrual secretion from three healthy women

completely confirmed these assumptions. Analyzed preparations appeared to be exceptionally rich in bactericidal polypeptide compounds and the identification of 44 different representatives confirmed their origin as human hemoglobin fragments. Most of the peptides had a molecular mass that varied between 2 500 and 4 000 Da; the majority of them (37 peptides) originated from alpha-globin, and the rest from beta-globin. There were no significant preferences concerning the localization of menstrual hemocidins within maternal proteins; both the N- and C-terminal parts of globin molecules were approximately equally represented. Interestingly, each analyzed peptide profile, obtained from three various patients, was different, suggesting that the proteolytic pathways of menstrual hemocidins generation are random. To a greater or lesser degree, all isolated peptides were able to kill *E. coli*. Detailed bactericidal activity was studied using two synthetic peptides identical to those found in menstrual secretion; one was a alpha-globin fragment spanning residues 35-56 (called HbA35-56) while the second was a beta-globin peptide spanning amino acids 115-146 (called HbB115-146). The estimated minimal inhibitory doses (MIC) of these peptides toward several Gram-negative and Gram-positive bacteria ranged from 27 to 293 micromol/L. Additionally, the significant synergistic effect of these two peptides was ascertained, suggesting that the identified menstrual hemocidins may establish an effective factor able to periodically sterilize the female reproductive tract and serve as an intriguing mechanism that helps maintain vaginal homeostasis during menstrual bleeding. This hypothesis was recently supported by the results of an epidemiologic study by Wicherek *et al.* who proved that patients with urogenital infections also had statistically shorter periods of menstrual bleeding (55). This shorter period is synonymous with a shorter exposure time to the bactericidal action of hemocidins and consequently results in a higher probability of eventual infection.

From a physiological point of view, menstrual hemocidins emerge in a process which is factually a miscarriage of an unfertilized egg (56). Therefore the bloody post-partum uterine excretions (so called 'lochia') might establish an ideal and relatively easily obtainable material to study the stages of hemocidins generation during menstruation. The results of such research were published by Mak *et al.* in 2006 (57). The authors of this study analyzed samples of uterine secretions from a group of healthy females who underwent cesarean delivery at term. Lochia was collected directly from the uterine cavity, at different stages of the birth. In all samples, both the level of the substrate – free hemoglobin in tetrameric form - as well as the level of peptide fraction was determined. Both these levels were the lowest in patients who underwent operations at the stage without symptoms of labor. On the other hand, the patients operated on in advanced stages of labor demonstrated the highest level of free hemoglobin, counting 0.12 g/dL, which is an exceptionally high value in comparison to the normal free hemoglobin level in human plasma, which stays below 0.005 g/dL. Patients in advanced labor were also characterized as having the highest level of the total peptide fraction. Identification of these peptide compounds, as in the case of menstrual secretions, confirmed their origin from human alpha- and beta-globins. Some of the lochia hemocidins were identical to the menstrual peptides while some were not. Interestingly, in contrast to the menstrual peptides, they had the same N-terminal start points and differed mainly at C-terminal end. This fact suggests that hemocidins from lochia are liberated from maternal protein by the same proteinases as in the case of menstrual hemocidins; however, their final differentiation is a result of the random action of carboxypeptidases. Studies on hemocidins present in uterine post-partum excretions displayed, first of all, that the substrate for hemocidins generation – hemoglobin - may emerge not from erythrocytes but from the uterine cavity - since the uterine cavity has a neutral pH, there is no possibility of the lysis of red blood cells. This finding agrees with the work of Beier and Beier-Hellwig, who have found that endometrial glands produce free hemoglobin in significant amounts (58). The second important conclusion from the studies on lochia hemocidins concerns the pathophysiology of endometritis and urinary tract infections, which occur more frequently after cesarean section than after normal vaginal delivery (59). A serious surgical procedure such as cesarean section surely affects the molecular mechanisms involved in bactericidal hemoglobin peptide generation and, in effect, might be responsible for the mentioned increase in the incidence of infection.

Microbicidal hemoglobin-derived peptides were also found in another type of tissue from the female reproductive tract – in the placenta (60). These peptides were detected in an acid extract of whole homogenized organs, obtained after parturition from healthy patients. One discovered peptide was a beta-globin, spanning residues 111-146, while the second, curiously enough, was a fragment of a fetal gamma-globin molecule, spanning amino acids 130-146. Synthetic peptides identical to those from the placenta, as in the case of menstrual hemocidins, were active in micromolar concentrations toward both Gram-negative and Gram-positive bacteria as well as toward fungi. Intriguingly, the beta-globin peptide had the ability to bind LPS. No hemolytic or cytotoxic activities were ascertained. The authors of the cited study speculate that the discovered peptides can be effectors of the innate immune response but, unfortunately, they do not discuss in what precise anatomic, cellular, or subcellular localization these act, nor do they say how this immune function could be realized in the placenta only.

**4.3. Biological activity**

Although periodic generation of hemocidins in the female reproductive tract is evident and the bactericidal activities of emerged hemoglobin fragments have been well documented, it is the activity of these peptides in the specific physiochemical conditions of the vagina (particularly in low pH) as well as their influence on the other specialized antimicrobial molecules operating in this organ that is of special interest. Currently this intriguing problem has been under investigation in only one study, published by Mak *et al.* in 2007 (61). The authors investigated the activity mentioned earlier of a model representative hemocidin, spanning residues 115-146 of

human beta-globin. This peptide was originally found in menstrual secretion and appears to be a truncated form of peptide 111-146 ascertained also by Liepke *et al.* in a previously described homogenate of the human placenta (60). In almost neutral pH, the HbB115-146 peptide showed MIC doses toward different bacteria in the range of tens of micromols/L, but studies using bacterial membrane permeability assay proved that lowering the pH to 4.4 increases the lytic activity of peptide almost 18-fold. This result demonstrates that this hemocidin is one of the most effective antimicrobials present in the vagina, where the normal, physiologic pH varies only from 4.0 to 4.5. Moreover, the studied peptide appeared to be completely insensitive to elevated salt concentration, demonstrating full bactericidal activity right up to 0.25 M NaCl. This observation contrasted with the properties of most classical microbicidal peptides, which, as mentioned before, exhibit a significant decrease in killing activity in the presence of salt. Interestingly, however, in another situation concerning the influence of divalent cations on the HbB115-146 hemocidin – the peptide was inhibited by these ions to a degree similar to classical microbicidal peptides.

The most curious characteristic of HbB115-146 hemocidin was a strong synergistic activity of mixtures of this peptide with the other known peptide microbicidal molecules acting in the female reproductive tract, namely, epithelial beta-defensin HBD-1, cathelicidin LL-37 as well as neutrophil defensin HNP-1. Mixtures of the hemocidin with these factors caused a much higher degree of *E. coli* cell lysis than the degree resulting from the simple sum of individual concentrations of the studied factors. This synergistic effect was preserved or even enhanced at a low pH and, with the exception of HBD-1, was observed also at physiological salt concentration. The synergistic effect was particularly well visible in the case of a mixture of HbB115-146 peptide with HNP-1 at pH 5.0, when the 50-fold molar excess of hemocidin over defensins was almost three times as active as the sum of activities of the individual peptides. The obtained results directly agreed with the other studies documenting the synergistic activities of the other classical human microbicidal peptides (62) and elegantly proved the significant role of the HbB115-146 hemocidin in the maintenance of vaginal homeostasis. Moreover, the HbB115-146 hemocidin demonstrated a noticeable amplifying effect toward both enzymatic and non-enzymatic activity of human lysozyme. Due to unclear molecular, probably steric, interactions, the presence of the peptide increases the muramidase activity of lysozyme by 15-20%. There was a much higher (5 to 8-fold increase of lytic activity toward *E. coli*) influence of the hemocidin on the non-enzymatic killing activity of the heat-denatured lysozyme. These results also confirmed the data obtained by other authors, who similarly reported a pronounced synergistic activity of many cationic antibacterial peptides with lysozyme (63, 64).

### 4.4. Mechanism of action

The results of the first preliminary mechanistic studies concerning action of the hemoglobin-derived microbicidal peptides on bacteria were described earlier in chapter 4.1 of this work. As was mentioned, these studies were performed using a convenient and easy to obtain model hemocidin - a cyanogen bromide fragment of myoglobin, spanning residues 56-131 (Mb56-131). These preliminary studies were taken further in a subsequent work by Mak *et al.*, published in 2001 (65). Key results were obtained during experiments with conductance measurements across an artificial planar lipid bilayer. The addition of the Mb56-131 peptide at 1 micromolar concentration to the solution contacting with one side of the membrane caused fluctuations of the measured noisy current. This fluctuating current gradually increased but without discrete conductance changes, followed by a sharp jump after about 60 s to the infinite readout, a symptom of membrane rupture. The same results were obtained for several different concentrations of the peptide; while there was a longer membrane lifetime at decrease in concentration of peptide, nevertheless the experiment ended each time with complete membrane disintegration. The bilayer incubated in the presence of native myoglobin solution was, however, entirely stable. The result obtained in Mb56-131 peptide presence was typical of the detergent- or carpet-like but not of the pore-forming microbicidal peptides. It should be remembered at this point that to the first, detergent-like group, belongs, as frequently mentioned earlier, human alpha-helical microbicidal peptide cathelicidin LL-37 (66). That the Mb56-131 hemocidin is similar to the described earlier in chapter 3.1 typical carpet-like-acting classical microbicidal peptides was also demonstrated during circular dichroism spectra measurements. The peptide had a disordered structure in aqueous solutions and folded gradually to form alpha-helical structures both in membrane-mimetic alcohols as well as in the presence of liposome suspensions.

Subsequently two interesting studies on the mechanism of action of hemocidins appeared in 2005 and 2007 when Sforca *et al.* as well as Machado *et al.* investigated the synthetic amidated forms of the bovine alpha-globin fragment, spanning region 33-61 (HbA33-61a) (67, 68). The non-amidated version of this peptide was originally found by Fogaca *et al.* in the guts of the tick, as described in the previously cited work *(*49). In contrast to the original, unmodified peptide, the C-terminally amidated form of the peptide demonstrated an increase in bactericidal activity. Moreover, amidation may potentiate resistance of the peptide to bacterial proteases. In fact, the HbA33-61a peptide exhibited significant antibacterial and antifungal activity, being practically completely inactive toward eukaryotic cells. The results of circular dichroism spectra measurements performed on solutions of this peptide proved that it is randomly structured in aqueous solutions but folds to form an alpha-helical conformation in membrane-mimetic mixtures. This data was highly consistent with the previously reported results of Mak *et al.* obtained for hemocidin from myoglobin. More detailed studies on the HbA33-61a structure using NMR spectroscopy, however, revealed a somewhat more complicated picture. First of all, these measurements proved that the peptide bound to the membrane-mimicking SDS-micelles reconstructs the main structural arrangements present in the corresponding segment of the maternal protein. The N-terminal part of the peptide forms a beta-

turn followed by a hinge region and then a longer alpha-helical stretch at the C-end. Hydrogen-deuteron exchange NMR experiments additionally proved that this alpha-helical C-terminal region is embedded in the SDS micelle while the N-terminal part is exposed to the solvent. One can speculate that, when bound to a membrane, the above membrane-penetrating the C-terminal region as well as the structural flexibility of a hinge region can cause, in effect, destabilization and the disruption of the phospholipid bilayer.

## 5. TISSUE-SPECIFIC POOL OF BIOACTIVE PEPTIDES AS A NOVEL SYSTEM OF HOMEOSTASIS REGULATION

In 1998 Karelin *et al.* proposed an interesting concept that helped, to some extent, to organize many chaotic reports concerning the phenomenon of the release of bioactive peptides from proteins that have some established functions *in vivo* (69). Naturally this conception concerned not only microbicidal peptides but an abundance of other immunomodulatory, mitogenic, neuromodulatory as well as vasoactive polypeptide compounds emerging from functional proteins. As has been stated here numerous times, these peptides can be obtained by proteolysis *in vitro* and have been identified in tissue-extracts or physiological fluids. This later group - the peptides found *in vivo* - often demonstrate biological activities functionally related to the source tissue, e.g., the family of 'hemorphins', comprising a group of hemoglobin-derived opioid-like peptides, was detected at significant concentrations only in human and animal brain extracts and cerebrospinal fluid (70); bone marrow was a source of isolation of an AcSDKP, a thymosin-derived inhibitor of hematopoietic stem cell proliferation (71); and finally, bactericidal hemocidins were identified at sites of potential infections: in insect guts and in human vaginal secretions. These facts may suggest that elimination of functional proteins in tissues is not only a random catabolic process leading ultimately to free amino acids but may constitute, under certain conditions, a complex set of proteolytic events leading to the formation of tissue-specific regulatory peptides. These regulatory molecules differ markedly from 'real' peptidergic neurotransmitters and hormones which are effectors of the classical neuro-, endo-, and paracrine regulatory systems. Neurotransmitters and hormones are produced from specific protein precursors by digestion at sharply defined sites and in strictly specified conditions. The concentrations of such emerging bioactive factors are quite low (0.001 to 1.0 nM/g of tissue) and have a high affinity for specific receptors. On the other hand, bioactive peptides from a tissue-specific regulatory pool do not have specific protein precursors – they are produced from functional proteins that establish proteinaceous substrates abundant at a specific physiological site. Production of these factors is not an effect of cutting at a unique and specific molecule region but is a result of a different and, to some extent random, action of tissue proteinases. On the one hand, the resulting peptides have a generally low affinity for eventual receptors or demonstrate a biological effect only at high concentrations. On the other hand, this weaker physiological activity is compensated for by the production of tissue-specific regulatory peptides at much higher (0.1 to 100 nM/g of tissue) levels than the hormones or neurotransmitters. Differences between these two systems of regulation, classical and tissue-specific pool, are also visible at the level of reaction time: hormones and neurotransmitters, due to their high affinity to receptors, act very quickly. Bioactive peptides from tissue-specific regulatory pool, on the other hand, act slowly. Thus these last factors should be considered rather as the molecules' involvement in long-term homeostasis regulation at the level of whole organs or organisms.

It is hard to find a better known protein substrate for the generation of tissue-specific regulatory peptides than hemoglobin, particularly the hemoglobin in central nervous system. As stated earlier, opioid-like peptides from this protein, called hemorphins, were primarily found in brain tissue extracts and in the cerebrospinal fluid of humans and many animals. On the other hand, recent studies prove that hemorphins and their truncated forms can be released from hemoglobin by macrophages, endogenous lysosomal proteases, pepsin, pancreatic elestase, and also by cathepsin D. In effect, different forms of these peptides were detected not only in the nervous system, but also in blood plasma, demonstrating a variety of other biological activities, such as analgesic, coronary-consistory activity, or the promotion of aggregation of platelets (70). The situation is similar with the generation of microbicidal hemoglobin fragments in the female reproductive tract. These peptides appear to be a subsequent ideal example of a tissue-specific pool of bioactive peptides, helping to maintain homeostasis in the vagina. Regular menstrual bleeding periodically supplies the vagina with large quantities of hemoglobin - a perfect substrate for generating bioactive peptides. Vaginal excretions are also rich in proteolytic enzymes such as MMPs and neutrophil serine proteinases. The action of these enzymes is probably facilitated by the partial denaturation of hemoglobin in acidic conditions maintained in the lower parts of the reproductive tract. In the case of vaginal excretions, the primary function of emerged bioactive peptides is killing bacterial pathogens and factually, menstrual excretions are quite abundant in these bactericidal factors and the total number of different proteolytic hemoglobin fragments in excretion from a single patient probably reaches into the tens. The detailed studies on a model menstrual hemocidin have clearly demonstrated the pronounced bactericidal activities of this peptide and proven that specific physiochemical properties of vaginal fluid promote its microbicidal action. Moreover, the studied peptide was able to act synergistically with other classical microbicidal polypeptides, constitutively produced by the epithelia of the reproductive tract. At the present time many questions concerning the details of hemoglobin cutting in menstrual secretions and concerning the biological activities of many other hemoglobin peptides present in this complex fluid remain. There is no doubt, however, that menstrual hemocidins ideally fulfill criteria that lie at the base of the concept of the tissue-specific pool of bioactive peptides.

## 6. PERSPECTIVES

Apart from the discovery of a subsequent example of regulatory peptides from a tissue-specific pool

discussed above, menstrual hemocidins appear to be a new biochemical factor throwing more light on the biological phenomenon of menstruation in humans. There are three classical barriers that protect the vagina from sexually transmitted pathogens while allowing it to tolerate sperm and a growing fetus: i) physical and physicochemical - epithelium, mucus, low pH and hydrogen peroxide, ii) biological - comensal microflora, antibodies, phagocytic cells and iii) proteinaceous - lysozyme, lactoferrin, secretory proteinase inhibitor, elafin, cathelicidin, alpha- and beta-defensins (72). However, there is presently no data on the functioning of this machinery during menstrual bleeding. This normal physiological process comprises the disintegration and excretion of the functionalis layer of the endometrium at the end of the secretory phase of the non-pregnant cycle as a result of progesterone withdrawal. Detailed molecular mechanisms involved in the initiation of menstruation are not fully understood, but are at present explained by two concurrent theories. The vasoconstrictor theory emphasizes the role of prostaglandins, while the inflammatory hypothesis underlines the role of leukocyte-mediated inflammatory processes (73, 74). As a result of these molecular mechanisms, the vagina is periodically flushed by an extremely complex mixture of the lysed functionalis layer of the endometrium, rich in inflammatory exudates, erythrocytes, and proteolytic enzymes, and finally, rich in hemocidins. These peptides might establish a base for a hypothesis that, independent of the reproductive aspects of menstruation, this physiological phenomenon may have still one important biological purpose: a periodic sterilization of lower parts of the female reproductive tract. This hypothesis has naturally already been proposed, not only for humans, but also for certain mammals and primates. Its underlying concept, however, was based mainly on a mechanistic action of a stream of the fluidic menstrual secretion (75). Discovery of hemocidins confers a reliable molecular foundation to this theory and deepens the physiological importance of menstruation.

Again, many questions still remain. The main source of the substrate for hemocidin production in the female reproductive tract is appropriately unknown. As has been previously stated, we can speculate that hemoglobin is liberated from erythrocytes in the low pH of the vagina; however, the study concerning postpartum uterine secretions suggests that the fragmentation of hemoglobin starts in the uterine cavity where a neutral pH is maintained. Therefore, it is possible that hemocidins from lochia and the peptides that emerge during menstruation have different origins. The first group may emerge from hemoglobin excreted from endometrial glands while the later group may be produced from hemoglobin liberated from erythrocytes in the vagina. The definite proteinases involved in the generation of hemocidins in the female reproductive tract are also unknown. As stated earlier, there are plenty of proteolytic enzymes involved both in menstruation and the initiation of labor, but their factual involvement in hemocidin generation has so far not been documented. The question of the full spectrum of the biological activities of the produced peptides is still open. In numerous cited studies concerning menstrual hemocidins, the emphasis was on a widely interpreted microbicidal effect. Still there is no doubt that these peptides may demonstrate other biological effects, such as an anti-inflammatory or vasoconstrictory effects, which are particularly 'useful' during reproductive tract bleeding. Discussed above against the broad background of other human bactericidal peptides are the results of the recent studies concerning hemocidins; these results will help to establish a starting point for future research.

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**Abbreviations:** ALL-37: human cathelicidin (long form), BPI: bactericidal/permeability increasing protein, CatG117-136: microbicidal cathepsin G fragment, CTAP-III: connective-tissue-activating peptide III, HbB115-146: microbicidal beta-hemoglobin fragment, HBD: human beta-defensin, hCAP18: precursor of human cathelicidin, HD: human enteric alpha-defensin, HNP: human neutrophil peptide (alpha-defensin), Hst: human histatin, LL-37: human cathelicidin, LPS: lypopolysaccharide, LTA: teichoic acid, MAPK/ERK: mitogen-activated protein kinase/extracellular signal-regulated kinase, Mb56-131: microbicidal myoglobin fragment, MIC: minimal inhibitory concentration, MMPs: matrix metalloproteinases, NAP-2: neutrophil-activating peptide 2, P-113: active fragment of Hst5, PR-39: bovine proline- and arginine-rich antibacterial peptide, $rBPI_{21}$: active domain of BPI, TC: thrombocidins

**Key Words:** Antibacterial, Antimicrobial, Cathelicidins, Defensins, Endometrium, Hemocidins, Hemoglobin, Innate, Microbicidal, Peptides, Reproductive, Review, Vagina

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