Thrombocidins, microbicidal proteins of human blood platelets

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Chapter 8

General Discussion
Antimicrobial proteins in humans

The existence of cationic antimicrobial proteins in serum has been known since the end of the 19th century, and soon after their discovery it became clear that these 'substances' originated from granulocytes (62). It then took over 50 years before the notion emerged that the cytoplasmic granules of these cells were the source of antimicrobial proteins, and that degranulation resulted in their release in the extracellular environment (34,78). The first chemically characterized proteins were those from rabbit PMNs, which appeared to be rich in arginine accounting for their cationic nature, and cysteine (79). Studies in later years have identified and characterized several antimicrobial peptides from these cells, including bactericidal and permeability increasing protein (BPI) (72) and defensins (58).

PMNs have proven a rich source of antimicrobial peptides in many organisms (44,45), including humans (25,43,59) providing these cells with an efficient means to kill engulfed bacteria (59). This has often been regarded as a specialized system of these cells. However, in humans alone numerous antimicrobial proteins have been isolated and characterized from multiple sources, including T cells (63), bronchoalveolar lavage (55), plasma (10), wound fluid (24) and various organs (80). Furthermore, over the past few years a range of antimicrobial proteins have been found in epithelial tissue of airways (6,20,26,61), urogenital tissue (68), skin (32), and intestine (36,37,48). These findings suggest that host defense by means of antimicrobial proteins might be more general than was assumed initially.

The sites of expression of antimicrobial peptides mentioned above are all locations possibly subject to microbial encounter (body fluids, mucosal surfaces). However, only limited data are available indicating that these proteins are actually functional in situ. For instance, expression of some antimicrobial proteins is induced upon contact with LPS or bacteria (32,56,64). In addition, defensin activity in lungs of cystic fibrosis patients most likely is impaired due to the increased concentration of salt. This might account for the frequent infections encountered in these patients (7,26,39).

Microbicidal proteins in human platelets: thrombocidins

The presence of antimicrobial proteins in rabbit blood platelets has been implicated for almost thirty years (22,73). We have shown (chapter 3) that human platelets contain at least ten antimicrobial peptides, which we named thrombocidins (TCs). Rabbits vaccinated with crude TCs were more susceptible for development of viridans streptococcal IE than non-vaccinated animals. When challenged with streptococci resistant to TCs, development of IE was equally frequent in vaccinated and non-vaccinated rabbits (chapter 2). These results implicate a protective role for TCs in the development of IE due to TC-susceptible streptococci. Furthermore, since the level of resistance of viridans streptococci isolated from blood of IE-patients was higher than of isolates from bacteremic patients without IE, we concluded that bacterial resistance to TCs can be considered a virulence factor for IE-causing organisms. Similar results have been obtained with rabbit platelet microbicidal proteins (PMPs) in studies showing that S. aureus (19)and C. albicans (77) resistant to PMPs produced more severe IE than PMP-susceptible microorganisms. Conversely, bacteremic isolates from IE patients had higher levels of resistance for PMPs.
than isolates from non-IE patients (8,76). TCs might also come into play in a much more frequently occurring condition in which platelet activation is involved, that of hemostasis at sites of lesions. Release of antimicrobial substances at sites of injury could be very beneficial in the prevention of wound infections.

The elucidation of the structure of TCs revealed their descent from CXC-chemokines. Although microbicidal activity may be a novel trait in this class of proteins (see below), the structural building blocks of TCs are not uncommon to antimicrobial proteins. N-terminally, TCs consist of three antiparallel β-sheets stabilized by intramolecular disulfide bridges, and the C-terminal part is an α-helix. Classification of antimicrobial peptides has been based on secondary structures, and two important groups are the α-helical (magainins, cecropins) and β-sheeted, disulfide-stabilized peptides (defensins, protegrins) (30). Thus, structurally TCs are novel among the antimicrobial peptides in that they have combined α-helical and β-sheeted parts in one molecule. This has been investigated by synthesizing pentadecameric overlapping peptides covering the entire length of TCs, and testing these for microbicidal activity (chapter 4). We identified functional domains in both the β-sheeted and the α-helical parts of TCs indicating that these elements could indeed participate in microbicidal activity. Furthermore, replacement of positively charged residues by (neutral) leucine in the peptide derived from the β-sheeted domain decreased microbicidal activity, while replacement of neutral residues by (positively charged) lysine increased this activity (chapter 4). This suggests that basic residues play a crucial role in the microbicidal activity of TCs.

Chemokines with microbicidal activity

Thrombocidins 1 and 2 are derived from neutrophil activating peptide-2 (NAP-2) and connective tissue activating peptide-III (CTAP-III), respectively, by a C-terminal truncation of two residues (chapter 2). NAP-2 and CTAP-III belong to the family of the chemokines, and more specifically to the CXC-chemokines according to the spacing of the two C-terminal cysteines, which are separated by one residues. Other classes are the CC- and CXXC chemokines, although the latter consists of only one member (5). Traditionally, chemokines have been considered to be leukocyte attractants and activators, CXC-chemokines being more or less specific for neutrophils, and CC-chemokines for monocytes (1,4,53). Chemokine activity (and specificity) is mediated by G-protein-coupled chemokine receptors. However, many of the chemokines recognize more than one receptor, and each receptor can be activated by several chemokines (2,18). With the ever-increasing number of chemokines identified, the net response of a cell exposed to several chemokines at a time is hard to predict, but it has become clear that chemokines play an important role in the recruitment of and communication between inflammatory cells (4,9,53).

Within the large number of chemokines known to date also additional biological functions for these proteins are being uncovered. Angiogenesis was among the first processes noted to be regulated by chemokines. CXC-chemokines containing a conserved ELR-triad were potent angiogenic factors, while those without this sequence were angiostatic (16,38,65). This activity has been shown to influence the course of tumor formation (3,49). Furthermore, chemokines direct vascular (66,81) and neuronal development (47,81). Chemokine receptors have been shown to act as coreceptor for HIV
cell-entry (11,21), a discovery which has boosted the research on chemokines. The identification of CXC-chemokine derivatives TC-1 and TC-2 with microbicidal activity has not only added two new members to the chemokine family, their biological activity is novel for this class of protein.

**Microbicidal proteins with chemokine activity**

TCs have microbicidal activity, but also have chemotactic activity for neutrophils (chapter 5). This activity may be a concern for possible clinical application of such compounds as antiinfective agents, but it can be questioned whether this activity distinguishes them from other antimicrobial proteins. For instance, human defensins are chemoattractants for monocytes (67) and are mitogenic for epithelial cells and fibroblasts at concentrations at which they also have microbicidal activity (51). Another neutrophil microbicidal peptide, PR-39, was a chemoattractant for neutrophils (35).

Combined antibacterial and chemoattractant activities in one molecule may effectuate elimination of invading microorganisms both by direct killing and by enhancing phagocytosis by recruited cells. It may even be questioned which activity, microbicidal or chemoattractant, prevails in vivo, since in a mouse infection model a causative relationship was implied between the presence of neutrophil defensin HNP-1 (present in concentrations well below its MIC), the accumulation of leukocytes, and a decrease in severity of the infection (74).

**Platelets as inflammatory cells**

The close collaboration between cellular and acellular host defense systems can be exemplified by a number of events involving platelets and thrombocidins, which also will make clear that platelets are truly inflammatory cells. As has been discussed earlier, platelets become activated when they contact bacteria or subendothelial matrix molecules. Degranulation is an important event in the activation process, leading not only to release of the granule content but also to exposure of previously buried glycoprotein receptors on the cell surface. One of these receptors is P-selectin (GMP-140, PADGEM, CD62), in resting platelets an integral α-granular membrane protein, which is redistributed to the cellular membrane upon degranulation (28,42). P-selectin is also stored in Weibel-Palade bodies of endothelial cells, and is exposed after activation. Neutrophils can bind to P-selectin (42), either from platelet or endothelial origin (23), and subsequently are activated (28). This is followed by the release of several hydrolytic enzymes from neutrophil azurophilic granules. One of these proteins is cathepsin G, which can activate platelets (46) probably through a specific receptor (57), and is one of the proteolytic enzymes responsible for the proteolytic cleavage of platelet-excreted CTAP-III, generating NAP-2 (12,14). In addition, P-selectin can bind monocytes triggering these cells to secrete chemokines (75) and proteases with cathepsin G-like specificity also generating NAP-2 from CTAP-III (13,70). NAP-2 can attract more neutrophils, partly by increasing vascular permeability (69), and further activates these cells (71).
These events show that platelets, neutrophils and monocytes mutually activate each other in order to propagate the inflammatory reaction. In this process TCs will be excreted from platelets and add to the antiinfective potential of locally released antimicrobial proteins. Furthermore, it could well be possible that the combined release of CTAP-III from platelets, and the variety of proteases from their lysozomal granules and from other cell types will lead to the local generation of TCs. Given the considerable amount of CTAP-III present in platelets, the potentially available TCs could contribute substantially to antimicrobial host defense at sites of platelet activation.
Application of microbicidal peptides in antiinfective therapy

During the last decade, the emergence of multiple-drug resistant bacteria has increased dramatically (15,17,50). Therefore, the need to search for alternative antibiotics is widely accepted. Microbicidal peptides may provide an attractive source of such compounds, and therefore their designation as 'peptide antibiotics' seems appropriate.

The possible applicability of peptide antibiotics is favored by mainly three arguments, which are closely linked. First, their mode of action is different from conventional antibiotics. Most peptides act by disturbing membrane integrity, thereby killing the target microorganism. Second, many peptide antibiotics have a broad spectrum activity, which is assumed to be due to the fact that their cidal mechanism does not involve specific receptors. Finally, induction of microbial resistance is generally low, again resulting from their property to affect membrane integrity against which no defense would be readily available.

In chapter 7 a review was presented on the current status in the development of peptide antibiotics. At present, several peptides have been synthesized which have been derived from naturally occurring peptides. In many cases modification of natural peptides was needed either to improve microbicidal activity or to decrease cytotoxicity and thus increase selectivity for microorganisms. Systemic application of such peptides may not be feasible yet, but topical use is being investigated in several clinical trials (29,31).

One form of topical application of peptide antibiotics is their delivery from matrices implanted at sites where infection can occur. The work presented in this thesis was performed in collaboration with the Department of Biomedical Technology at Twente University, Enschede, The Netherlands, aimed to develop a biodegradable matrix containing TCs integrated in prosthetic heart valves. Upon implantation of such devices, controlled release of TC would eliminate circulating bacteria locally, thereby preventing bacterial attachment to the implant and the surrounding endocardium and thus prevent prosthetic valve endocarditis (PVE). A gelatin-derived matrix was developed, specifically designed to store and release small, cationic proteins (40,41). A peptide potentially applicable in this system could be rH-TC (chapters 5 and 6). This derivate of TC-1 has broad range microbicidal activity and is more potent than native TC-1 itself (chapter 6). Alternatively, microbicidal peptides derived from TCs (chapter 4) could be tested. Another recent example of the development of such a release system is the incorporation of histatins, salivary fungicidal peptides, in a xanthan matrix for oral antiinfective therapy (54).

Although naturally occurring microbicidal peptides or closely related derivatives thereof are the primary source for the development of clinically applicable peptide antibiotics, ideally such compounds should be synthesized by rational design. Before this can be achieved, the microbicidal mechanism of these peptides should be revealed in detail first. In this respect, major progress has been made in recent years by varying peptide characteristics such as charge and hydrophobicity, but rational design has not been proven successful yet (chapter 7).

Another point of concern may be the assumed absence or low level of microbial resistance against peptide antibiotics. Several bacterial species are intrinsically resistant to cationic bactericidal proteins, including *Proteus* (60) while resistant *Klebsiella* and
Escherichia strains have been reported as well (33,52). Resistance to peptide antibiotics appeared to be due to identical modifications (acylation, incorporation of aminoarabinose or ethanolamine) in the lipid A moiety of LPS in each of these species, which also have been shown to cause peptide resistance in Salmonella (27). Although the impact of these modifications for clinical practice are not fully clear at this moment, the assumed difficulties microorganisms might have adapting to the membrane activity of peptide antibiotics may still be overcome. Thus, the development of new peptide antibiotics should not only include activity testing using standard test strains or liposomes, but should also take into consideration the possible increase in resistance due to modifications in LPS.

REFERENCES


