Thrombocidins, microbicidal proteins of human blood platelets

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SUMMARY

One of the major functions of blood platelets is their indispensable role in hemostasis. In addition, platelets contain several components varying from low molecular weight ions to high molecular weight glycoproteins which, once excreted or exposed at the cellular surface, mediate various processes in hemostasis and tissue repair. A relatively unknown feature of human platelets is the release of antimicrobial peptides after activation. This would suggest that platelets are part of the so-called innate immune system. In chapter 1 it is outlined briefly how platelets are activated and what role their antimicrobial peptides might play.

Antimicrobial peptides have been shown to provide an efficient defense in a great variety of microorganisms against microbial infections by directly interacting with and killing invading organisms. The several hundreds of antimicrobial peptides that have been isolated to date share a low molecular weight (2-4 kDa), a cationic nature, and generally activity against a broad spectrum of microorganisms. In humans such peptides have initially been isolated from neutrophils, but their presence has been demonstrated in increasing numbers of other cell types, including blood cells and epithelial cells. We termed microbicidal peptides from human blood platelets thrombocidins (TCs).

The presence of TCs has been demonstrated previously, but their physiological relevance, their number, identity, and specific activity are unknown. The aim of this thesis was to investigate the antibacterial effect of TCs in an in vivo infection model, to chemically characterize these peptides, and to assess their activity against a variety of microorganisms. Several of these studies were performed using TCs produced recombinantly in Escherichia coli.

An infectious disease in which platelets play a crucial role is infective endocarditis (IE). IE can develop after endocardial damage, resulting in deposition of a thrombus-like structure (vegetation) mainly consisting of platelets and fibrin. A normally harmless (transient) bacteremia can give rise to colonization of the vegetation, rapid multiplication of bacteria, malfunctioning of the affected valve, and heart failure which can be fatal. Although platelets are involved in the entrapment of bacteria in the vegetation, the concomitant release of antibacterial proteins from these cells at or near the vegetation could aid in removal of bacteria, as has been suggested in previous studies. To investigate the role of TCs in this process, rabbits were vaccinated with crude preparations of TCs, and the incidence of IE was monitored in vaccinated and control animals in an experimental IE model, as described in chapter 2. The sera from vaccinated rabbits contained antibodies recognizing TCs in a Western blot, and neutralized both human and rabbit platelet-released bactericidal activity in vitro. Thus, the bactericidal proteins in human and rabbit platelets are antigenically related and are most probably similar in structure. Development of (non-bacterial) endocarditis was induced in vaccinated and control animals, followed by challenge with TC-resistant or TC-susceptible viridans streptococci, common causative organisms of IE. Vaccinated rabbits appeared to have a higher incidence of IE due to TC-susceptible strains compared to non-vaccinated animals, while the incidence of IE due to a TC-resistant strain was not different in vaccinated and control animals. Apparently, antibodies against human TC neutralized rabbit platelet-derived bactericidal activity in
vaccinated animals. The finding that viridans streptococcal isolates causing IE were less susceptible to TC than viridans streptococcal isolates from non-IE patients suggested that TC could aid in the limitation in progression of IE in humans as well. Thus, decreased susceptibility to TC seems to be a virulence factor for viridans streptococci to cause IE.

In chapter 3 it is shown that human platelets contain at least 10 different bactericidal proteins as judged from the activity of platelet granular proteins fractionated by reversed phase HPLC. Two major proteins (TC-1 and TC-2) were purified to homogeneity using cation exchange chromatography and continuous acid urea gel electrophoresis, and their structure was determined by N-terminal sequencing and mass-spectrometrical analyses. TC-1 and TC-2 appeared to be variants of the CXC-chemokines neutrophil activating peptide-2 (NAP-2) and connective tissue activating peptide-III (CTAP-III), respectively, differing from these peptides by a C-terminal truncation of two amino acids. CTAP-III is a platelet-specific peptide with fibroblast mitogenic activity and thought to be involved in wound healing. NAP-2 is an N-terminal cleavage product of CTAP-III generated extracellularly by neutrophil-derived proteases, and is a potent neutrophil attractant and activator. These chemokines are characterized structurally by 4 conserved cysteines, of which the 2 located most N-terminally are separated by one residue, and generally by an α-helical conformation of the C-terminus. Although the differences between these chemokines and TCs are only small, NAP-2 and CTAP-III were not microbicidal, while TC-1 and TC-2 rapidly killed Bacillus subtilis, E. coli, Staphylococcus aureus, Lactococcus lactis, and the fungus Cryptococcus neoformans. The mechanism of several bactericidal peptides has been implicated to be the dissipation of membrane potential and/or pore formation in the bacterial membrane, but no such effect was observed in Lactococcus lactis exposed to either TC, suggesting that TCs act by a different mechanism.

Since structural differences between peptides with (TC-1 and TC-2) and without bactericidal activity (NAP-2 and CTAP-III) comprised only two residues in the C-terminal tail, structure-function studies were performed to identify domains in TCs responsible for their activity (chapter 4). Mapping of these regions was done using synthetic peptides, each 15 residues in length and overlapping each other by 10 residues, covering the entire sequence of CTAP-III. Only 2 of these peptides had microbicidal activity, and comprised regions around the N-terminally located CXC-motif and near (but not at) the C-terminus of CTAP-III/NAP-2. Fine-mapping of these 2 regions was done using peptides which were shifted by only 1 residue. The most active peptide in the N-terminal region (peptide L18) had minimal bactericidal concentrations (MBCs) varying from 1.9 μM for C. neoformans to 30 μM for S. aureus, while the MBCs of the C-terminal peptides never were below 30 μM, and were above 120 μM in the majority of cases. Apparently, the most active microbicidal domain in TCs is in the N-terminal part, while the C-terminal tail most likely is not involved in direct microbial killing. The role of the C-terminus in TCs could lie in an improved interaction with the target membrane due to the removal of an acidic residue in these molecules compared to NAP-2 and CTAP-III. In chapter 4 it was also shown that L18, the peptide representing the most active microbicidal domain, could be used as a lead compound for the development clinically applicable peptides with optimal activity, since L18 substituted at one position by lysine had increased microbicidal activity, even
exceeding that of natural TCs, and in addition had decreased hemolytic activity compared to L18.

The recombinant production of TCs in *E. coli* is described in chapter 5. Recombinant TCs were indistinguishable in structure from natural TCs in several analyses, but had slightly less micribicidal activity. Bactericidal activity of native and recombinant TC-1 was independent of peptide folding, while unfolded TC-2 was inactive. A variant of TC-1 C-terminally truncated by 5 residues retained bactericidal activity, showing that this activity is not critically dependent of the length of the C-terminal helix. rH-TC, a variant of TC-1 with a N-terminal tag consisting of 10 histidines, had enhanced microbicidal activity, and evidence was given that this was caused by these histidines by showing that peptides consisting of histidines alone also had microbicidal activity. Because of the potent activity of rH-TC, the activity and mechanism of action of this peptide was studied further (chapter 6). rH-TC was bactericidal for a large number of clinical isolates of Gram positive as well as Gram negative bacteria, and was fungicidal for *C. neoformans* with MBCs ranging from 0.5 to 2 μM. Activity of rH-TC against *E. coli* and *Staphylococcus epidermidis* was independent of environmental pH, and susceptibility of these organisms was independent of metabolic activity, was moderately dependent of the presence of a membrane potential and was severely decreased at low temperature. The latter observation could indicate that membrane fluidity would be involved in effective bacterial killing. An *E. coli* strain deficient in the synthesis of anionic phospholipids in the cytoplasmic membrane was as susceptible as wild type bacteria, indicating that activity of rH-TC is independent of the presence of these phospholipids. In contrast to natural TCs, rH-TC had no neutrophil chemotactic activity.

Peptides such as rH-TC offer attractive characteristics for further development towards a clinically applicable antiinfective compound. There is great interest in such therapeutics, especially since bacterial isolates are increasingly being found to have decreased susceptibility for or even be resistant against commonly used antibiotics. Antibacterial peptides in general offer an attractive source of such compounds, mainly because of their broad range activity and the low frequency of resistance that has been encountered so far. Several of such compounds are currently investigated in clinical trials with promising results in some cases. Most peptides suggested for clinical use are natural peptides, or have been modified to optimize activity. Although some peptide characteristics are known to be important for bactericidal activity, no systematic guidelines are available for the rational design of peptide antibiotics. In chapter 7 an overview of recent literature is given aiming to provide insight into the determinants of the most extensively studied peptide antibiotics for their bactericidal activity, as well as of some synthetic peptides. From such studies general concepts can be deduced which can be useful for the design of peptide antibiotics. Furthermore, the applicability of liposomes as model systems to test peptide membrane activity is discussed. Liposomes have allowed the (partial) elucidation of the mechanism of action of several peptides, but in most cases they do not contain LPS, an important membrane constituent of Gram-negative bacteria, and they are inert. The latter may be a concern since in some cases the versatility of microbial membranes has been demonstrated to result in resistance of the organism to peptide antibiotics.
In **chapter 8** it is discussed how platelets are an integrative part of the host immune system. This is exemplified by the ability of platelets and leukocytes to mutually activate each other. This causes the excretion of inflammatory mediators from all cells involved, and of TCs from platelets. It is likely that TCs contribute to an antinfective effect in this environment, and it is argued that this effect could even be enhanced by the local production of TCs generated from platelet-excreted CTAP-III through the action of various proteases released by both platelets and leukocytes.