# The human cathelicidin hCAP-18

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## 1. INTRODUCTION

The microbicidal mechanisms of neutrophils have been divided into those who are oxygen-independent and those that are oxygen-dependent. The oxygen-dependent mechanism of microbicidal activity relies on the ability of polymorphonulear leukocytes (PMNs) to generate free oxygen radicals through the NADPH oxidase (1, 2). Defects in the NADPH oxidase cause chronic granulomatous disease (CGD) characterized by increased severity and number of bacterial infections (3). Probably due to CGD, the oxygen-dependent mechanism of bacterial killing for many years attracted a lot of research regarding the bacteriocidal mechanisms of neutrophils. However, it is now evident that PMNs contain a lot of antibiotic proteins of utmost importance for the ability of PMNs to kill microbes (4). Most of these antibiotic proteins are localized to granules, and many in particular to azurophil granules (5). Apart from lysozyme identified by Alexander Flemming in 1922 (6), the first protein identified from neutrophils involved in bacterial killing was Batericidal/Permeability Increasing Protein (BPI) (7). Five years later the first defensins - abundant antimicrobial peptides of the azurophil granules - were identified in rabbits (8) and humans (9).

Antimicrobial peptides are defined as small peptides less than 100 aa residues. They are important effector molecules in the innate immunity from insects to man (10, 11). The antibacterial mechanism of antimicrobial peptides varies, and while many peptides kill through destabilization of the bacterial membranes, some peptides have intracellular targets (11).

In mammals there are two large families of antimicrobial peptides, the defensins and the cathelicidins. The defensins are divided into the α-defensins, found in neutrophils, macrophages, and Paneth cells in the small intestine, and the β-defensins, which are found widespread in epithelial cells. The cathelicidins are mainly found in the peroxidase negative granules of neutrophils (12), however the human cathelicidin hCAP-18 has also been found in various epithelial sites (13-19), mast cells (19) and subpopulations of monocytes and lymphocytes (20). The cathelicidins share a highly conserved N-terminus of 12 kDa, named cathelin after a putative *cathe*psin *L in*hibitor isolated from porcine neutrophils (21). However, the active antimicrobial C-terminal domain of the cathelicidins varies greatly in amino acid sequence and structure.

Antimicrobial peptides are synthesized as preproproteins and (with the exception of defensins in neutrophils) stored as inactive proproteins (22). In order to become biologically active, the peptides must be liberated from the proproteins by proteolytic cleavage. This is exemplified with the cathelicidins in neutrophils. The cathelicidins are stored as proproteins in the peroxidase negative granules and processed to antimicrobial peptides after exposure to proteases from azurophil granules either following exocytosis (23, 24) (IV) or degranulation into the phagolysosome (23). In the case of the cathelicidins the antimicrobial activity seems to reside both in the antimicrobial peptide and in the cathelin domain (25).

The proteolytic generation of antimicrobial peptides is very important for the clearance of bacteria at sites of infection. In mice, α-defensins from Paneth cells in the small intestine of mice are generated by matrilysin-mediated cleavage of prodefensins, and matrilysin knock-out mice have increased susceptibility to intestinal infections (26). Inhibition of activation of the porcine neutrophil cathelicidins by elastase impairs clearance of bacteria from wounds in vivo (27).

The only human cathelicidin, hCAP-18, was identified independently by three groups in 1995 (28-30). The C-terminal 37 amino acid antimicrobial peptide, LL-37, liberated from hCAP-18 exerts broad antimicrobial activity (31). However, this peptide also has other important biological effects, which include neutralizing the biological effects of lipopolysaccharide (32), stimulation of angiogenesis (33) and chemotaxis of neutrophils, monocytes and T-cells (34).

This thesis describes further studies of hCAP-18 in plasma, neutrophils, seminal plasma, and in epithelial cells.

## 2. MATERIALS AND METHODS

### 2.1. ISOLATION OF NEUTROPHILS AND BONE MARROW CELLS

Human neutrophils were isolated from buffy coats or whole blood from healthy volunteers. Following sedimentation with Dextran, the neutrophils were separated from the mononuclear cells by density centrifugation on Lymphoprep® (35). The remaining erythrocytes were removed by hypotonic lysis. Except for dextran sedimentation all steps were carried out at 4 °C.

Exudate neutrophils, i.e. neutrophils that have left the circulation and migrated out in the tissue, were obtained from skin window chambers (36, 37). Skin windows were made by elevation of the dermis by suction. The resulting dermal blisters were unroofed by scissors. This method is painless, bloodless and very reproducible (37). Local inflammation was induced by autologous serum applied to the chambers for 18 hours. The chambers were emptied, washed and filled with autologous plasma. Neutrophils were then allowed to accumulate in the chambers for 7 hours. By using this approach neutrophils were stimulated to diapedese through the endothelium, and by collecting the cells in plasma rather than in serum further stimulation of the neutrophils by agonists generated in serum was avoided (36).

Bone marrow cells were obtained by aspiration from the posterior superior iliac crest. Following sedimentation of the erythrocytes with Dextran the leukocyte rich supernatant was layered on top of a two-layer Percoll gradient (1.065/1.080) as described (38, 39). Centrifugation resulted in separation of bone marrow cells into three bands. Starting at the bottom, the bands were designated band 1, which contains mainly band cells and segmented cells (together with some erythrocytes); band 2 which contains myelocytes and metamyelocytes; and band 3, which contains mainly myeloblasts and promyelocytes (38, 39).

### 2.2. SUBCELLULAR FRACTIONATION

Neutrophils were disrupted by nitrogen cavitation. This was followed by centrifugation to pellet unbroken cells and nuclei. The postnuclear supernatant was loaded either on a two-layer Percoll gradient (1.05/1.12 g/ml) (40) or on a three-layer Percoll gradient (1.05/1.09/1.12 g/ml) (41) and centrifuged for 30 minutes at 37,000g. On a three-layer gradient this resulted in four visible bands. Starting at the bottom, the bands were designated the α-band, which contains the azurophil granules; the $\beta_1$-band, which contains the specific granules; the $\beta_2$-band, which contains the gelatinase granules; and, the γ-band, which contains the plasma membranes and the secretory vesicles. On a two layer gradient only three bands

were visible, the α-band, the β-band, and the γ-band, where the β-band contains both the specific- and gelatinase granules.

2.3. STIMULATION OF NEUTROPHILS

Neutrophils were stimulated to either exocytosis or to phagocytosis by various secretagogues (fMLP, ionomycin, serum treated zymosan (STZ)) or to phagocytosis with STZ or immunoglobulin coated latex beads. The extent of exocytosis was subsequently determined by ELISA's of marker proteins from the different subcellular compartments. Phagocytosis was quantified by immunoelectron microscopy.

2.4. PURIFICATION OF hCAP-18

***2.4.1. Neutrophil-derived hCAP-18***

The β-band from the subcellular fractionation contains specific and gelatinase granules. This was harvested either manually or by a fraction collector. Percoll was removed by ultra centrifugation. The granules were lysed and applied to a column with the anti-hCAP-18 antibodies described in section 3.1.1. The column was washed extensively and the bound hCAP-18 eluted by low pH. The eluted hCAP-18 was used for cleavage experiments to ascertain the identity of the C-terminal peptide generated by cleavage with proteinase 3. For some experiments the affinity purified hCAP-18 was repurified by cation exhange chromatography on a MonoS column using ÄKTA-FPLC. hCAP-18 was subsequently eluted with 10 mM NaOH, 140 mM NaCl.

For all other cleavage experiments, the isolated specific granule proteins were subjected to cation exchange chromatography on a MonoS column using ÄKTA-FPLC and hCAP-18 was eluted with NaOH as stated above. This preparation was used for cleavage experiments with hCAP-18, where the cleavage subsequently was visualized by immunoblotting.

***2.4.2. Epididymis-derived hCAP-18***

hCAP-18 from samples of seminal plasma was affinity purified on an anti hCAP-18 antibody column followed by cation exchange chromatography as described in section 2.4.1

2.5. PRODUCTION OF RECOMBINANT PROTEINS

***2.5.1. Production of recombinant hCAP-18***

Recombinant hCAP-18 was produced in bacteria as a fusion protein with γ-glutamyl S-transferase (GST) joined by a cleavage site. The cDNA for hCAP-18 was PCR-amplified from a human bone marrow cDNA library and was cloned into the pGEX-vector. The sequence of the construct was checked by DNA-sequencing. The recombinant protein was purified by affinity chromatography on a glutathione column, and the GST was removed by cleavage with thrombin.

***2.5.2. Production of recombinant cathelin***

A recombinant form of cathelin, the N-terminal part of hCAP-18, was produced using the Baculovirus Expression Vector System. The cDNA for the cathelin part of hCAP-18 was PCR-amplified from a human bone marrow cDNA library and was cloned into the pAcGP67(b) vector. The sequence of the construct was checked by DNA-sequencing. Recombinant protein was produced by *Spodoptera frugiperda* (Sf9) cells after co-transfection of the cells with recombinant pAcGP67(b) and BaculoGold DNA. The recombinant protein was harvested from the supernatant of the infected Sf9 cells and purified by affinity chromatography on a anti-hCAP-18 antibody column.

2.6. ISOLATION OF LIPOPROTEINS AND DELIPIDATION OF PLASMA

VLDL-LDL was precipitated from plasma by precipitation with dextran sulfate and $MgCl_2$ as described (42). Lipoproteins were furthermore isolated by ultracentrifugation of plasma after adjustment of the density by solid potassium bromide (43). At a density of 1.215 the top fraction was enriched in lipoproteins while the bottom fraction represented the delipidated plasma. The quality of the procedures was subsequently determined by ELISA's for marker proteins (immunoglobulins, albumin and apolipoproteins).

2.7. QUANTITATION OF PROTEINS

***2.7.1. hCAP-18 ELISA***

Anti-hCAP-18 antibodies were purified from anti-hCAP-18 serum by affinity chromatography on a protein A using FPLC. These antibodies were both used as capture antibodies and biotinylated and used as detecting antibodies. Capture antibodies were diluted in carbonate buffer and applied to the wells of flat bottom immunoplates. Nonspecific binding was blocked with incubation with buffer containing 1% BSA and the samples and the standards of recombinant hCAP-18 were then applied. The hCAP-18 was detected by incubation with biotinylated polyclonal anti-hCAP-18 antibodies followed by horseradish peroxidase labeled avidine. Color was developed with o-phenyl-diamine as substrate, and absorbance measured at 492 nm, using a 96-well microtiter plate spectrophotometer.

***2.7.2. Other proteins***

IgG, IgM, IgA, apolipoprotein A-I (a marker of HDL), apolipoprotein B (a marker of VLDL and LDL), and albumin were quantitated by a semi-quantitative ELISA. The samples were diluted in carbonate buffer and applied to the wells of flat bottom immunoplates. Nonspecific binding was blocked with buffer containing 1% BSA and the antigen was detected by application of polyclonal antibodies against the above-mentioned markers followed by horseradish peroxidase labeled goat secondary antibodies. Color was developed with o-phenyl-diamine as substrate, and absorbance measured at 492 nm, using a 96-well microtiter plate spectrophotometer.

Elastase, cathepsin G, proteinase 3, and $\alpha_1$-antitrypsin were quantitated by semiquantitative ELISAs as above but with biotinylated primary antibodies and the secondary antibody replaced by avidin-horse radish peroxidase.

2.8. IMMUNOELECTRON MICROSCOPY

Cells were fixed in a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde. The cells were then single or double immunogold labeled for investigation of subcellular localization of hCAP-18.

2.9. INDUCTION OF hCAP-18 AND OTHER ANTIMICROBIAL PROTEINS/PEPTIDES IN HUMAN KERATINOCYTES

***2.9.1. Growth and stimulation of primary human keratinocytes***

Primary keratinocytes were grown in serum free keratinocyte medium from clonetics, supplemented with transferrin, hEGF, hydrocortisone, gentamycin, amphotericin B, epinephrine but without insulin. Cells were stimulated only after complete confluence was achieved. Cells and medium was harvested 0h, 3h, 6h, 12, 24h, and 48h after stimulation with 100 ng/ml IGF-I, 50 ng/ml TGF-α, IGF-I /TGF-α, 10 ng/ml TGF-$\beta_1$, 100 ng/ml bFGF, 20 ng/ml IL-1β, and 20 ng/ml TNF-α. For some experiments cells and medium was harvested afte 0h, 24h, 48h, 72h, and 98h. The induction of antimicrobial peptides by growth factors was also tested in primary epidermal cultures containing human epidermal keratinocytes grown on collagen-coated membranes according to the instructions by the manufacturer. In epidermal cultures EGF and insulin was removed from the medium 48 hours prior to stimulation.

***2.9.2. Analysis of mRNA-levels following stimulation of primary keratinocytes***

Total RNA was puried with Trizol according to the manufacturer's recommendations. The purified RNA was run on an agarose gel and blotted onto a Hybond-N membrane. The filters were subsequently hybridized with specific $^{32}$P-labeled probes. The blots were developed and quantified by a phosphoimager. For quantitative assessments the intensities of the obtained signals were normalized to the hybridization intensity from a probe against the housekeeping gene β-actin.

## 3. RESULTS

### 3.1. ANTIBODIES AND IMMUNOLOGICAL ASSAY FOR hCAP-18

#### *3.1.1. Antibodies*

hCAP-18 was produced recombinantly in bacteria. The recombinant protein was used for generation of specific polyclonal rabbit antibodies against hCAP-18. By immunoblotting of plasma samples and neutrophil homogenate these antibodies were found to be specific for hCAP-18. Only one band of the appropriate molecular weight was observed, and the reactivity in western blotting of both plasma and neutrophil samples was abolished with an excess of recombinant hCAP-18 together with the primary antibodies.

Immunoblotting of exocytosed material from neutrophils revealed three bands at 18 kDa, 14 kDa and 4-5 kDa corresponding to the holoprotein (hCAP-18), the cathelin domain, and LL-37, respectively. Immunoblotting with excess recombinant hCAP-18 abolished binding to all three bands observed in the exocytosed material while immunoblotting with excess recombinant cathelin abolished binding to the 14 kDa band of cathelin. Thus, the polyclonal antibodies recognized both the cathelin domain of hCAP-18 and the C-terminal antimicrobial peptide (**Figure 1**).

#### *3.1.2. ELISA*

The polyclonal antibodies were used to set up a specific ELISA for hCAP-18 with a detection limit of 0.08 ng/ml. The sensitivity of the ELISA allowed 1000 fold dilutions of plasma samples and 10,000 fold dilution of subcellular fractions. These dilutions decrease the risk of proteolysis and of the interference of other proteins with the assay (44).

### 3.2. hCAP-18 IN PLASMA

Prior to our studies, expression of hCAP-18 had only been found in the bone marrow and testis and only at the RNA level (30). Our measurements of plasma samples revealed a protein concentration of hCAP-18 of about 1.2 μg/ml which correlated to approximately 20% of the concentration of hCAP-18 found in circulating neutrophils (I). No difference was found in the plasma level of hCAP-18 between women and men, thus excluding testis as a major source of

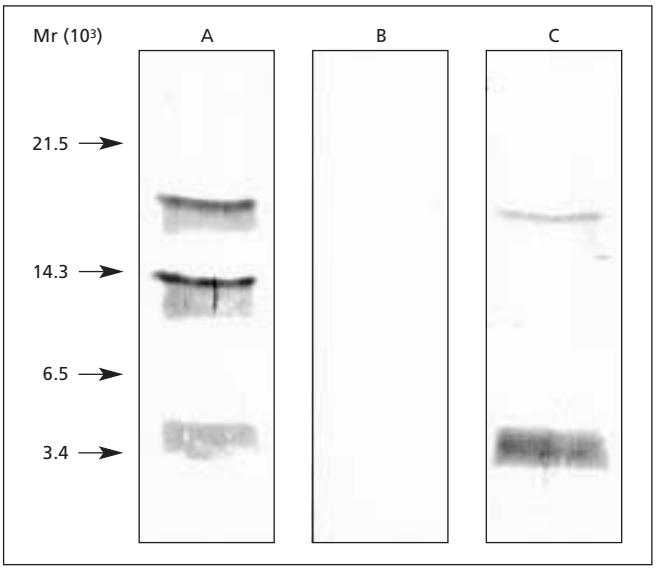


**Figure 1.** Immunoblotting of exocytosed material from neutrophils. Exocytosed material from neutrophils was subjected to SDS-PAGE and subsequent immunoblotting with anti-hCAP-18 antibodies. *Lane A*, normal immunoblotting; *lane B*, immunoblotting with an excess of recombinant hCAP-18; *lane C*, immunoblotting with an excess of recombinant cathelin. The faint band of hCAP-18 in C is due to the fact that the antibodies binding to the cathelin part of the molecule are blocked by recombinant cathelin. © The American Society for Biochemistry and Molecular Biology. Published with permission.

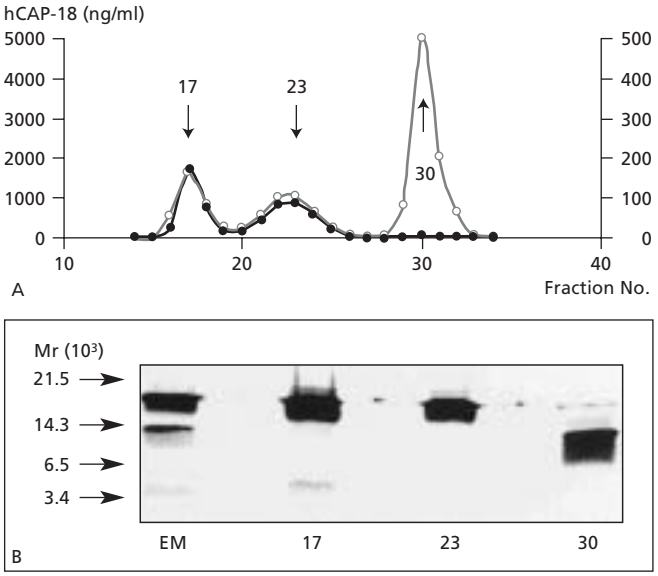


**Figure 2.** Gel filtration of plasma with and without incubation with exocytosed material from neutrophils. *A*, concentrations of hCAP-18 in plasma before (●) and after (○) incubation with exocytosed material from neutrophils. The *right axis* shows the concentration of hCAP-18 in plasma, and the *left axis* shows the concentration of hCAP-18 in plasma after incubation with exocytosed material from neutrophils. *B*, SDS-PAGE followed by immunoblotting of peak fractions of hCAP-18 obtained by gel filtration after incubation of exocytosed material (*EM*) from neutrophils with plasma. Fractions 17, 23, and 30 are shown. © The American Society for Biochemistry and Molecular Biology. Published with permission.

hCAP-18 in plasma. The relative high level of hCAP-18 could not be explained as a consequence of degranulation during sampling since other neutrophil specific granule proteins do not have a similar high plasma level (45). The hCAP-18 in plasma was found to originate from the bone marrow (I) as does lysozyme, a neutrophil granule protein with a similar high plasma level (46). This was demonstrated by the fact that the level of hCAP-18 in plasma strictly paralleled the neutrophil count in a patient undergoing allogeneic bone marrow transplantation. Furthermore, the hCAP-18 level in plasma was unaffected when the level of circulating neutrophils was transiently raised by methylprednisolone without affecting their rate of production of neutrophils in the bone marrow (I).

Surprisingly, in contrast to lysozyme, hardly any hCAP-18 was excreted in the urine (I). Gel filtration experiments of plasma demonstrated that the hCAP-18 in plasma was present in high molecular weight forms (I) (also in contrast to lysozyme). To identify the nature of these high molecular weight forms hCAP-18 was isolated from plasma by affinity chromatography using an anti-hCAP-18 antibody column. The eluate contained several proteins including hCAP-18 and apolipoproteins (II). The high molecular weight forms of hCAP-18 co-eluted with lipoproteins in gel filtration experiments and the association with lipoproteins was validated by the co-fractionation of hCAP-18 with lipoproteins using two different methods for isolation of lipoproteins from plasma (II). The lipoproteins had a large capacity for additional binding of hCAP-18 and the binding to lipoproteins was mediated by the antimicrobial C-terminus of hCAP-18 (II) by a hydrophobic interaction (**Figure 2**).

The interaction of the antimicrobial C-terminus of hCAP-18, LL-37, with plasma proteins was also observed by Johansson et al. (47), who found the conformation of LL-37 was altered in the presence of serum with a subsequent loss of antibacterial activity. By affinity chromatography using immobilized LL-37, Wang et al. identified apolipoprotein A-I as the protein responsible for binding and inhibiting the antibacterial activity of LL-37 in plasma (48). This proposed binding between LL-37 and apolipoprotein A-I was abolished by lowering the pH to 5 indicating an ionic interaction between apolipoprotein A-I and LL-37 (48).

The binding of hCAP-18 solely to apolipoprotein A-I by an ionic

interaction was not supported by our data (II), and can be explained by different experimental approaches. We primarily investigated the endogenous hCAP-18 present in plasma, where only the holoprotein was detected. Our experiments clearly demonstrated that hCAP-18 in plasma was bound to lipoproteins containing mainly apolipoprotein B but also to a certain extent apolipoprotein A-I. Furthermore, we were not able to dissociate the hCAP-18/lipoprotein complexes by lowering the pH to 4.5 but by disintegration of the lipoproteins by detergent.

We found a hydrophobic interaction between the antimicrobial domain of hCAP-18 and VLDL, LDL and HDL (II). hCAP-18 is a very hydrophobic molecule and partitions mainly in the hydrophobic phase during Triton X-114 phase separation (28). The different classes of lipoproteins contain different composition of proteins (apolipoproteins) and Turner et al. demonstrated that LL-37 binds to liposomes (31) devoid of proteins. Thus, it seems likely that the antimicrobial domain of hCAP-18 associates with the membrane of lipoproteins – probably much the same way the C-terminus of hCAP-18 is believed to insert into the phospholipid bacterial membrane and cause bacterial lysis.

Synthetic amphipatic α-helical peptides have previously been found to associate themselves with lipoproteins (49). It is, however, not known whether other α-helical antimicrobial peptides or their proproteins are associated with lipoproteins in plasma.

LL-37 is antibacterial and, at higher concentrations, cytotoxic towards mammalian cells depending on the α-helical conformation of the peptide (47). Both the antibacterial and cytotoxic effects of LL-37 are abolished by the binding to plasma proteins (47). Thus, lipoproteins may serve either to preserve high plasma levels of promicrobicidal substance or to protect against the potential harmful effect of LL-37.

## 3.3. ULTRASTRUCTURAL LOCALIZATION AND BIOSYNTHESIS OF hCAP-18 IN NEUTROPHILS

### *3.3.1. Ultrastructural localization*

hCAP-18 was identified by Cowland et al. as a protein present in specific granules of human neutrophils (28). At the same time, the protein was cloned from human bone marrow (29, 30). Following the generation of specific polyclonal antibodies we further examined the subcellular localization of hCAP-18 in mature human neutrophils (III). Using the three-layer Percoll gradient in subcellular fractionation experiments as described (41, 50), we found that hCAP-18 was strictly co-localized with lactoferrin as a matrix protein in specific granules (III) (**Figure 3**), and was not present in other granules. This was confirmed by immunoelectron microscopy (**Figure 4**). By measuring the amount of hCAP-18 and lactoferrin in the subcellular fraction, hCAP-18 was found to be as abundant as lactoferrin in specific granules on a molar basis. Using secretagogues of different potency we could demonstrate that the co-localization with lactoferrin was accompanied by co-mobilization (III) (**Table 1**).

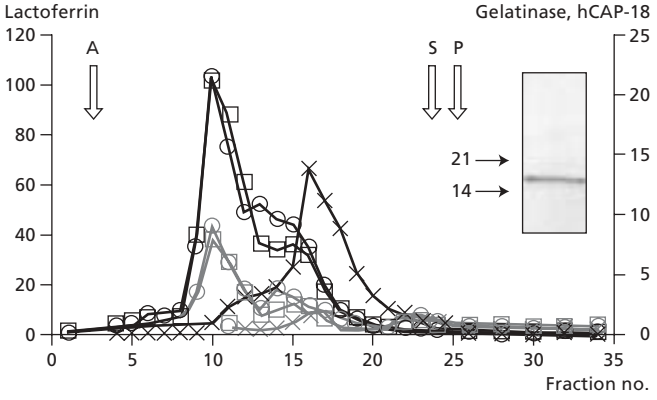


**Figure 3.** Subcellular fractionation of neutrophils. Isolated neutrophils, either unstimulated (black lines) or stimulated by 1.0 μmol/L ionomycin (gray lines) were cavitated and fractionated on a 3-layer Percoll density gradient. Fractions were collected from the bottom of the centrifuge tube and assayed for the following proteins: Myeloperoxidase (azurophil granules. Peak indicated by open arrow marked A). Lactoferrin (specific granules, □). Gelatinase (gelatinase granules, X). Latent alkaline phosphatase (secretory vesicles. Peak indicated by open arrow marked S). HLA (plasma membranes. Peak indicated by open arrow marked P) and hCAP-18 (○). Values are given as μg/mL. Insert: Immunoblot of control cells with anti-hCAP-18 antibody. MW marker indicated by thin arrows. © the American Society of Hematology. Published in accordance with the Rights & Permission policy of Blood.

### *3.3.2. Biosynthesis*

The sorting of granule proteins in the different granule subsets has been proposed to be regulated by timing (also called the "targeting by timing hypothesis"), i.e. granule proteins synthesized at the same time localize to the same granules (51). Therefore, we investigated the biosynthesis of hCAP-18 in neutrophil precursors from the bone marrow. We found that hCAP-18 was synthesized in myelocytes and metamyelocytes along with lactoferrin (III), thus supporting the timing by targeting hypothesis. Our finding was later supported by others (52). By analyzing a large number of transcripts for human

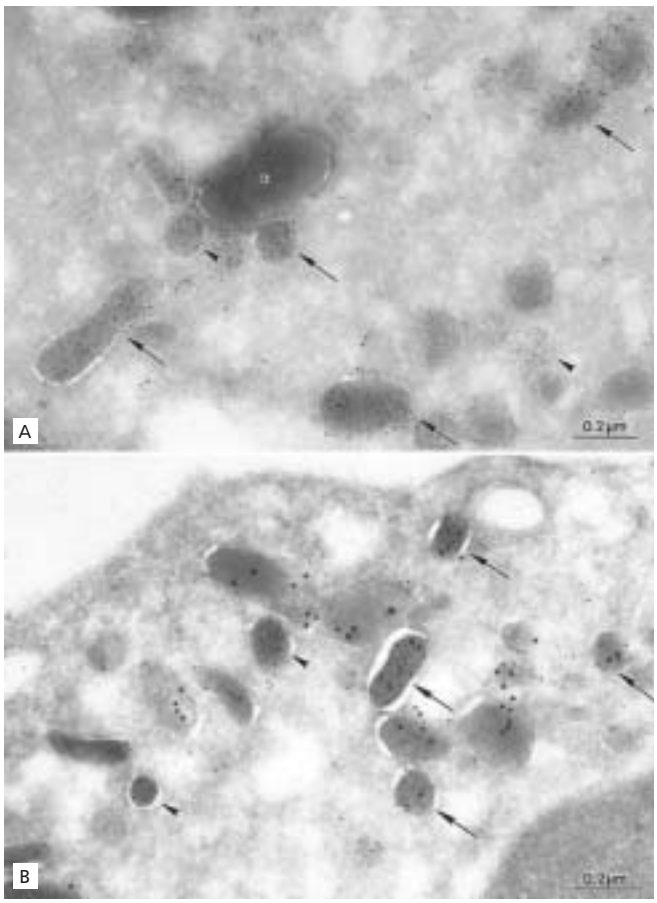


**Figure 4.** Localization of hCAP-18 in resting human neutrophils by immunogold labeling of ultrathin cryosections. *A*, hCAP-18 (large gold particles) is shown to colocalize with lactoferrin (small gold particles) in many of the specific granules (arrows). Some lactoferrin positive granules did not label for hCAP-18 (arrowheads). The large dense peroxidase-positive granules (*) were rarely labeled with either antibody. Biotinylated hCAP-18 antibodies were labeled with streptavidin gold (10 nm) and lactoferrin antibodies were labeled with goat antirabbit gold (5 nm). The cells were reacted enzymatically for peroxidase before being frozen to identify the azurophil granules (*), while the specific granules were identified by immunogold labeled lactoferrin. Original magnification [OM] × 78,000. *B*, anti-hCAP-18 antibody was labeled with goat antirabbit gold (15 nm) and biotinylated antilactoferrin antibody was labeled with streptavidin gold (10 nm). No peroxidase reaction was performed before immunogold labeling. Arrows point to specific granules with both sizes of gold, and arrow heads to granules that label only for lactoferrin. OM × 77,500. © the American Society of Hematology. Published in accordance with the Rights & Permission policy of Blood.

**Table 1.** Exocytosis of granule constituents in response to stimulation of neutrophils. © the American Society of Hematology. Published in accordance with the Rights & Permission policy of Blood.

| | Gelatinase (%) | hCAP-18 (%) | Lactoferrin (%) | Myeloperoxidase (%) |
|---|---|---|---|---|
| No addition ..... | 4.7 | 2.3 | 2.5 | 1.5 |
| fMLP, $10^{-8}$ mol/L ..... | 20.2 | 2.9 | 3.0 | 1.5 |
| Ionomycin 1 μmol/L .. | 89.5 | 58.8 | 53.6 | 21.6 |
| STZ, 1 mg/ml ..... | 22.9 | 7.6 | 8.8 | 3.6 |

neutrophil granule proteins, Cowland and Borregaard could demonstrate that the biosynthetic window during neutrophil maturation indeed determines the subcellular localization of human neutrophil granule proteins (53).

There are some differences in the biosynthesis and subcellular localization of the neurophil cathelicidins between different species. The bovine members of the cathelicidin family are synthesized at the same time point as hCAP-18 in neutrophil maturation (54). However, in the bovine neutrophils the granule subsets are organized differently than in the human counterpart (55, 56). Bovine cathelicidins are stored in a subpopulation of peroxidase negative granules named large granules (54). This subpopulation of granules is not found in human neutrophils. The guinea pig cathelicidin, CAP11, is predominantly synthesized in metamyelocytes and subsequently localized in gelatinase granules (57). The cathelicidins are therefore located in different subtypes of peroxidase negative granules in various species. Since the various subpopulations of granules are mobilized to varying degrees, the differences in subcellular localization might have functional implications for the different members of the cathelicidin family.

Likewise, the subcellular localization of the α-defensins in neutrophils has also been shown to vary between species. While defensins in human neutrophils are stored in azurophil granules (9) separated from the human cathelicidin, which is stored in specific granules, the bovine neutrophil defensins are stored in the large granules together with the bovine cathelicidins (58).

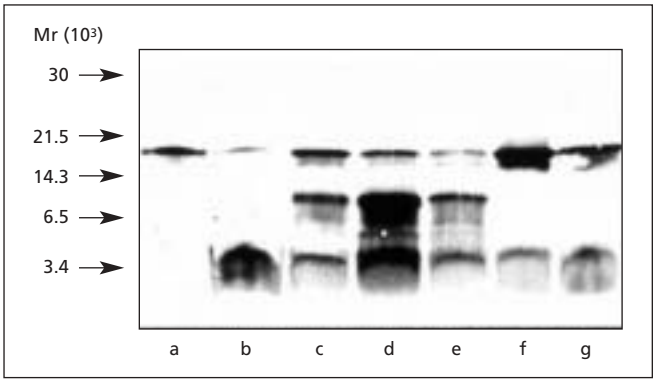


**Figure 5.** Processing of hCAP-18 in exocyted material from neutrophils. All samples were run on SDS-PAGE followed by immunoblotting with monoclonal anti-LL-37 antibody. *Lane a*, purified hCAP-18; *lane b*, exocytosed material from ionomycin-stimulated neutrophils; l*ane c*, purified hCAP-18 incubated with azurophil granule proteins; *lane d*, elastase; *lane e*, cathepsin G; *lane f*, proteinase 3; *lane g*, with exocytosed material from ionomycin-stimulated neutrophils after depletion of the endogenous hCAP-18. © the American Society of Hematology. Published in accordance with the Rights & Permission policy of Blood.

Bovine cathelicidins:
***ELQSV*** RIRR (Bac7)
***ELQSV*** RFRP (Bac5)

Porcine cathelicidin:
***EVQSV*** RGGG (protegrin 3)

Human cathelicidin:
***NKRFA*** LLGDF (hCAP-18)

**Figure 6.** Cleavage sites of cathelicidins. Cleavage sites between the cathelin part and the antimicrobial peptide of the bovine and porcine cathelicidins, cleaved by elastase, compared with the cleavage site of hCAP-18. Cathelin parts are shown in boldface italics. © the American Society of Hematology. Published in accordance with the Rights & Permission policy of Blood.

### 3.4. PROCESSING OF hCAP-18 TO ACTIVE ANTIMICROBIAL PEPTIDES

#### *3.4.1. Processing of neutrophil-derived hCAP-18*

hCAP-18 and other cathelicidins need to be proteolytically processed to become biologically active. Cathelicidins from peroxidase negative granules of bovine and porcine neutrophils are processed to the active antimicrobial peptides following exposure to the proteases from azurophil granules (23, 24) following degranulation either to the exterior (23, 24) or to the phagolysosome (23). hCAP-18 is stored in the specific granules as a holoprotein (III). Thus, our initial hypothesis was that the protease responsible for liberation of the C-terminal antimicrobial peptide was located in a different granule subset than hCAP-18. Indeed, we found that serine proteases from azurophil granules were capable of cleavage of hCAP-18 (IV), as is the case with the bovine and porcine cathelicidins. Phagocytosis experiments were performed both with neutrophils harvested from peripheral blood and exudated neutrophils harvested from skin windows. Neutrophils harvested from skin windows represent the closest possible experimental correlate to neutrophils that have migrated from the bloodstream into the tissues (36, 37). Both serum-treated zymosan and immunoglobulin-coated latex beads were used to stimulate phagocytosis. Even though significant amounts of hCAP-18 were found in the phagolysosome together with azurophil granule proteins, cleavage of hCAP-18 was not detected in the neutrophil phagolysosome (IV). This is in contrast to what is found in the bovine neutrophils (23), where the cathelicidins are processed in the phagolysosome. However, apart from in bovine and human neutrophils the processing of the cathelicidins in the phagolysosome has not been studied.

Immunoblotting of exocytosed material from neutrophils demonstrated that hCAP-18 was cleaved following exocytosis (**Figure 5**). Immunoblotting of the exocytosed material with monoclonal anti-LL-37 gave rise to a single band of the size of LL-37. No other fragments were detected by the monoclonal antibody. By immunoblotting with polyclonal anti-hCAP-18 antiserum an additional band of 14 kDa was observed (IV). This corresponded to a specific cleavage of the exocytosed hCAP-18 into the cathelin domain and the antimicrobial peptide. Thus, hCAP-18 was cleaved to liberate the antimicrobial peptide from the cathelin part only following exocytosis (IV).

The bovine and porcine cathelicidins are cleaved by elastase in vivo and in vitro (23, 24, 60), and are not susceptible to in vitro cleavage by cathepsin G (24, 60). The cleavage site of hCAP-18, however, is different from the cleavage sites of the bovine and porcine cathelicins (**Figure 6**). Most notably, the basic arginyl residue after the cleavage site is replaced by the small aliphatic leucyl residue, and the traditional valyl residue before the cleavage site is replaced by an alanyl residue. Leukocyte elastase preferentially cleave after a valyl rather than at an alanyl residue (61). This suggested that elastase was not likely responsible for the cleavage of hCAP-18 following exocytosis.

However, we found that elastase, as well as the other serine proteases present in azurophil granules (cathepsin G and proteinase 3) was capable of cleaving hCAP-18 in vitro. By examining the in vitro cleavage of hCAP-18 by these serine proteases both with polyclonal anti-hCAP-18 antibodies and a monoclonal anti-LL-37-antibody, it was clear that only the cleavage with proteinase 3 resulted in a specific cleavage between the cathelin domain and the antimicrobial peptide as that observed following exocytosis (Figure 5). Since biological cleavage of hCAP-18 was observed after exocytosis, we chose

the exocytosed material as an experimental model to identify the serine protease responsible for cleavage of hCAP-18 in vivo. The endogenous hCAP-18 fragments in the exocytosed material were removed by affinity chromatography on an anti-hCAP-18 antibody column. The hCAP-18-depleted exocytosed material was capable of cleaving purified hCAP-18 in a manner similar to that originally observed in the exocytosed material (fig 5). This cleavage was inhibited by the elastase inhibitor CMK but not by chymostatin (an inhibitor of cathepsin G) or secretory leukocyte protease inhibitor (SLPI) (an inhibitor of cathepsin G and elastase). This pattern of inhibition corresponded to that obtained with purified proteinase 3. Furthermore, a specific immunoprecipitation of proteinase 3 from the exocytosed material abolished the cleavage of hCAP-18 (**Figure 7**)(IV). The antimicrobial peptide LL-37 and the corresponding cathelin domain were also isolated from exocytosed material from human neutrophils (62) (II), and by amino acid sequence analysis we were able to demonstrate that cleavage of hCAP-18 by proteinase 3 generated LL-37. Based on these data, we concluded that proteinase 3 was responsible for cleavage of hCAP-18 following exocytosis (IV). Even though we could demonstrate that elastase and cathepsin G are present as active enzymes in the exocytosed material, contrary to our in vitro data with purified enzymes, they did not cleave hCAP-18 following exocytosis. Thus, hCAP-18 is a specific substrate for proteinase 3 – indeed the first specific physiological substrate for proteinase 3 identified.

The cleavage of hCAP-18 by proteinase 3 is contrary to that of the bovine and porcine cathelicidins, which are cleaved by elastase as mentioned above (24, 60). Cleavage of the bovine cathelicidins has been demonstrated in the phagolysosome (23). A possible explanation for the lack of cleavage of hCAP-18 in the phagolysosome might be that proteinase 3, in contrast to elastase, is presumably not active in the intraphagosomal milieu (63).

The cleavage of hCAP-18 by proteinase 3 demonstrates that the cleavage site is a functional variable part of the cathelicidins together with the antimicrobial domain. The cathelicidin genes are comprised of 4 exons and 3 introns (62, 64-68). The first three exons encompass the signal peptide and the conserved cathelin domain, while the fourth exon encompasses the putative cleavage site and the antimicrobial peptide. The first three exons are very conserved between all members of the cathelicidin family, while there is no homology in the fourth exon, which encodes the cleavage site and the antimicrobial peptide, between the different cathelicidins even within the same species. Thus, during evolution the variable biological functions of the cathelicidins have changed solely by alterations in the fourth exon.

The processing of neutrophil derived hCAP-18 to LL-37 by proteinase 3 was the first detailed study to describe the enzymatic generation of a human antimicrobial peptide from a proprotein. Likewise, it was demonstrated that generation of active antimicrobial peptides from common proproteins occurs differently in related species. This species-dependent generation of antimicrobial peptides has also later been shown for the processing of the α-defensins in the gut, where the prodefensins in mice are processed to mature defensins by matrilysin (26), while this processing in humans are mediated by trypsin (69).

### *3.4.2. Processing of epididymis-derived hCAP-18*

In addition to being present in the neutrophils hCAP-18 is expressed at various epithelial sites (13-15, 70). Since proteinase 3 is expressed only in myeloid cells and not in epithelial cells, this protease is most likely not responsible for the cleavage of hCAP-18 expressed by epithelial cells.

hCAP-18 is synthesized in the epithelial cells of the epididymis and is present in large amounts in seminal plasma (15). In order to gain insight into the processing of epithelially derived hCAP-18, we chose to study the processing of hCAP-18 in seminal plasma. Prolonged incubation of seminal plasma at neutral pH did not give rise to cleavage of hCAP-18 (V). However, at the pH level found in the vagina (pH 4) cleavage of hCAP-18 was observed with liberation of the antimicrobial domain from the cathelin part (as found in neutrophils) (**Figure 8**) (V). The cleavage of hCAP-18 in seminal plasma at pH 4 was characterized by analysis of both the cathelin part and the antimicrobial domain. We found that hCAP-18 was cleaved to

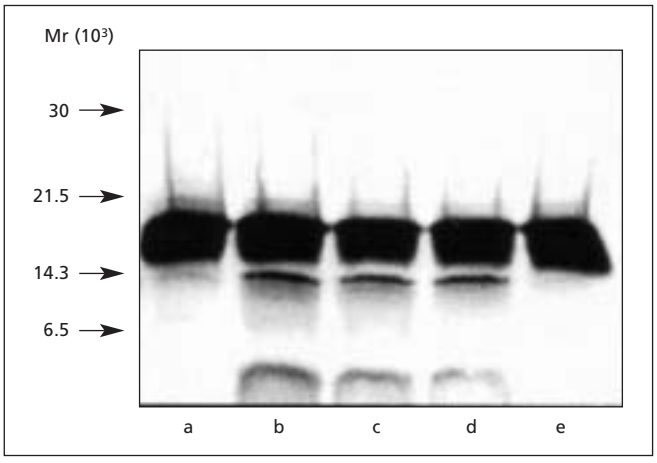


**Figure 7.** Cleavage of hCAP-18 by exocytosed material after the immunoprecipitation of individual serine proteases. Endogenous hCAP-18 fragments were deleted from the exocytosed material. Individual serine proteases were removed from the exocytosed material by immunoprecipitation before incubation with purified hCAP-18. Samples were run on SDS-PAGE followed by immunoblotting with anti-hCAP-18 antibodies. Purified hCAP-18 (*lane a*) was incubated with the exocytosed material after immunoprecipitation with preimmune rabbit antibodies (*lane b*), anti-elastase antibodies (*lane c*), anti-cathepsin G antibodies (*lane d*), and anti-proteinase 3 antibodies (*lane e*). © the American Society of Hematology. Published in accordance with the Rights & Permission policy of Blood.

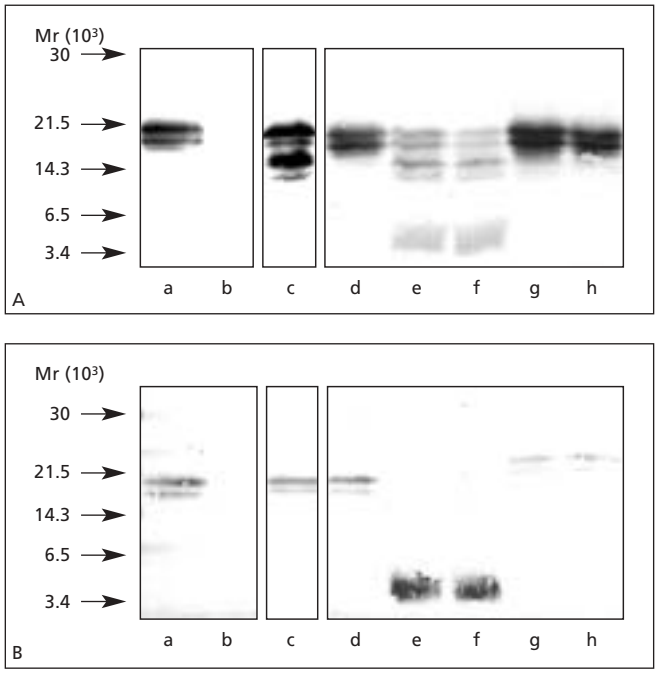


**Figure 8.** Processing of seminal plasma hCAP-18. Samples were subjected to SDS-PAGE and immunoblottings were performed with either polyclonal anti-hCAP-18 antibodies (*A)* or monoclonal anti-LL-37 antibody (*B*). *Lane a*: seminal plasma. *Lane b*: vaginal fluid. *Lane c*: vaginal sample collected 10 hour after sexual intercourse. *Lane d*: vaginal fluid and seminal plasma following incubation at 37 °C. *Lane e*: vaginal fluid and seminal plasma following incubation at 37 °C at pH 4. *Lane f*: seminal plasma following incubation at 37 °C and at pH 4. *Lane g*: vaginal fluid and seminal plasma with the addition of pepsinostreptin following incubation at 37 °C at pH 4. *Lane h*: seminal plasma with addition of pepsinostreptin following incubation at 37 °C and at pH 4. © The American Society for Biochemistry and Molecular Biology. Published with permission.

generate the antimicrobial peptide ALL-38 consisting of the 38 C-terminal amino acids of hCAP-18 in seminal plasma (V). The antibacterial activity of ALL-38 was equivalent to that of LL-37 towards the organisms tested (V). To characterize the protease responsible for this cleavage, the pH in the seminal plasma was lowered in the presence of various protease inhibitors. In the presence of pepstatin A and pepsinostreptin, the cleavage of hCAP-18 at low pH was inhibited. Pepstatin A is an aspartic protease inhibitor and pepsinostreptin is a specific inhibitor of the aspartic protease pepsin. These results indicated that hCAP-18 was cleaved by gastricsin (pepsin C), the only aspartic protease described in human seminal plasma (71). Specific immunoprecipitation of gastricsin from seminal plasma abolished the cleavage of hCAP-18 in seminal plasma at pH 4. Cleavage of hCAP-18 with purified gastricsin gave rise to a similar cleavage pattern of hCAP-18 judged by immunoblotting as that observed in seminal plasma at low pH.

To demonstrate that hCAP-18 in seminal plasma was processed following sexual intercourse, a post coital vaginal sample, containing seminal plasma and vaginal fluid, was collected and analyzed. The post coital sample had a low pH (pH <5) and here hCAP-18 was found in its processed form, similar to that which is found in seminal plasma at low pH (Figure 8). Control experiments ascertained that vaginal fluid did not contain protease activity towards hCAP-18, and that low pH was necessary for processing of seminal plasma-derived hCAP-18 in the vagina. Thus, the observed post coital processing of hCAP-18 was similar to that observed in seminal plasma at low pH.

Accordingly, both in vivo and in vitro data of processing of hCAP-18 in seminal plasma are in agreement with the known activation pattern of gastricsin activation (72, 73).This enzymatic activation of an antimicrobial substance in seminal plasma following exposure to the vaginal milieu represents a novel mechanism to prevent infection following sexual intercourse.

### 3.5. REGULATION OF THE EXPRESSION OF hCAP-18 AND OTHER ANTIMICROBIAL (POLY)PEPTIDES IN KERATINOCYTES

hCAP-18 has been described to be expressed at various epithelial sites, i.e. in keratinocytes during psoriasis (13) and cutaneous injury (74), squamous epithelia (14), the lung (70), sweat glands (18), salivary glands (17), and in the epididymis (15). How the hCAP-18 expression in epithelial cells was regulated had not been determined. Since hCAP-18 was clearly inducible in keratinocytes (13, 74), we chose primary keratinocytes as an experimental model to study the induction of the expression of hCAP-18 in epithelial cells. The expression of other human antimicrobial (poly)peptides has been shown to be induced by proinflammatory cytokines (75-79). However, since psoriasis is a disease of hyperproliferation and hCAP-18 was also induced during cutaneous injury we chose also to investigate the possible role of growth factors of importance in wound healing and psoriasis in the induction of hCAP-18 and other antimicrobial (poly)peptides in keratinocytes.

In primary human keratinocytes, the expression of hCAP-18 was induced by IGF-I (VI) (**Figure 9**). Apart from hCAP-18 the

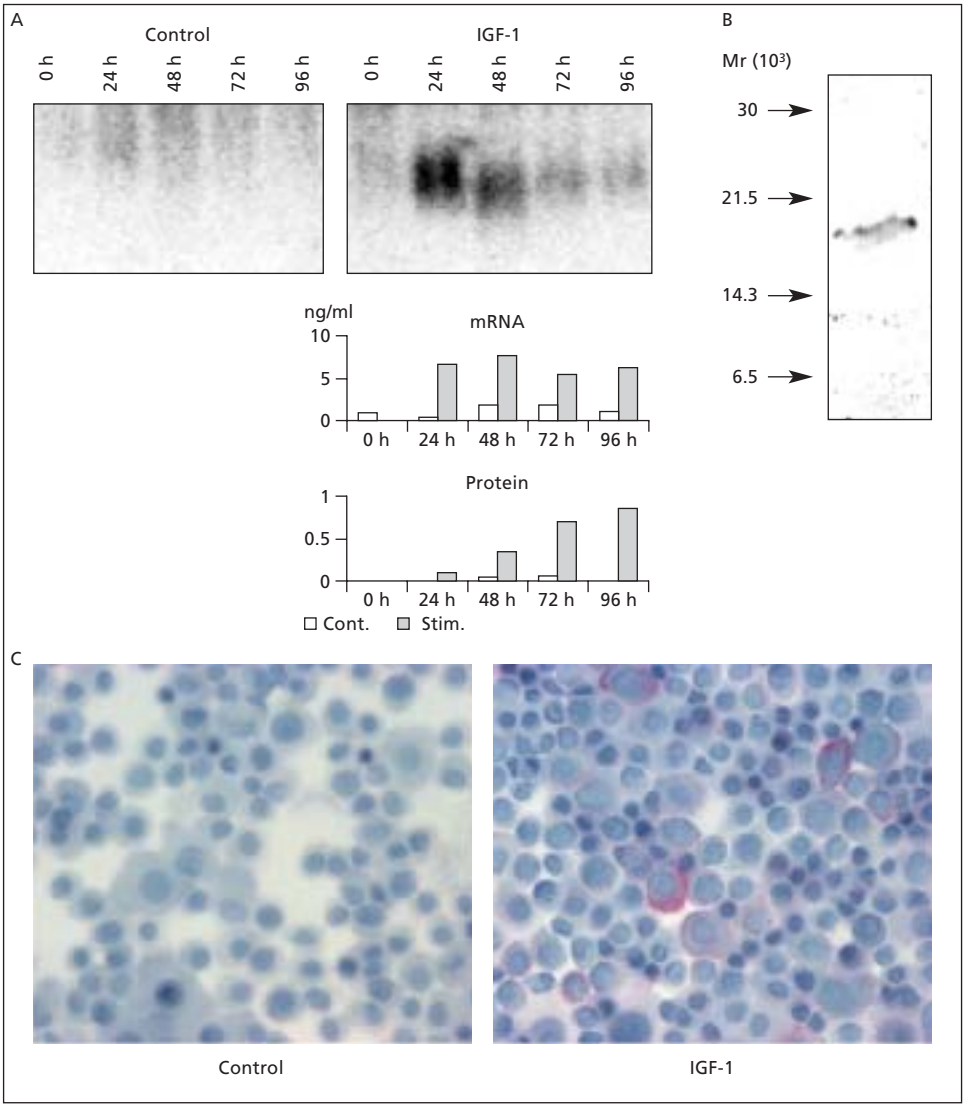


**Figure 9.** Expression of hCAP-18 in keratinocytes. *A,* Northern Blot of total RNA from control cells and IGF-I stimulated cells. The blot was hybridized with probes for hCAP-18 and β-actin (β-actin hybridization not shown). Below the Northern Blot is a schematic presentation of the expression of hCAP-18 normalized to the expression of β-actin. The basal expression was given the value 1. hCAP-18 was measured by ELISA in the media from control and stimulated keratinocytes. The concentration of hCAP-18 is shown in ng/ml. A schematic presentation of these measurements is shown below the Northern Blot data. *B*, material from stimulated keratinocytes was run on SDS-PAGE followed by immunoblotting with anti-hCAP-18 antibodies. A band of the appropriate molecular size was seen. *C, k*eratinocytes were either stimulated with IGF-1 or left unstimulated. After 48 hours of stimulation the cells were trypsinated and cytospins were made followed by immunostaining with anti-hCAP-18 antibodies. Copyright 2003. The American Association of Immunologist, Inc. Shown with permission.

**Table 2.** Expression of antimicrobial peptides/polypeptides in human keratinocytes in response to growth factors and proinflammatory cytokines. 

| | NGAL | SLPI | hCAP-18 | hBD-1 | hBD-2 | hBD-3 | hBD-4 |
|---|---|---|---|---|---|---|---|
| IGF-I . . . . | + | + | + | - | | | |
| TGF-α . . . | + | + | | - | | + | |
| TGF-β . . . | - | - | | - | | | |
| bFGF . . . | - | - | | - | | | |
| IL-1β . . . . | + | + | | - | + | | |
| IL-6 . . . . . | - | - | | - | | | |
| TNF-α . . . | - | + | | - | | | |

+: induced/increased expression of a peptide/protein in response to the factor/cytokine;
-: no induction or increase in the expression;
A blank space denotes no detectable expression.

(poly)peptides NGAL, SLPI and hBD-3 (VI), all of which possess antimicrobial activity (76, 80, 81) were induced by IGF-I and/or TGF-α. Both growth factors have been shown to be important in wound healing (82). The growth factors IGF-I and TGF-α furthermore had a synergistic or additive effect on the expression of hBD-3, NGAL, and SLPI (VI) (**Table 2**). TGF-α and IGF-I also has a synergistic effect in promoting growth of keratinocytes (83).

The induction of hCAP-18 and other antimicrobial (poly)peptides in response to growth factors defines an immunological role for growth factors in wound healing Indeed, an immunological function has previously been proposed for TGF-α because of the central role this factor plays in wound healing (84). Saliva from humans and other animals contain IGF-I and TGF-α and this has been postulated to be of importance when animals lick their wounds, and thereby apply growth factors to stimulate the wound healing. These growth factors may aid the sterilization of the wound by induction of antimicrobial (poly)peptides. From a clinical point of view it is well known that keeping a wound free of infection is a prerequisite for proper wound healing. It is noteworthy that hCAP-18 and SLPI are induced in keratinocytes following cutaneous injury and wounding (14, 74). The study of the induction of antimicrobial (poly)peptides has so far focused on the setting of inflammation and infection (and thereby on proinflammatory cytokines). In this contest it is noteworthy that TGF-α is present and reportedly exocytosed from inflammatory cells (85, 86). Thus release of growth factors may contribute to the induction of antimicrobial (poly)peptides in areas of inflammation.

The proinflammatory cytokines IL-1 and TNF-α induced the expression of hBD-2, NGAL and SLPI as previously described (77-79). Our studies revealed that each antimicrobial (poly)peptide has an individual pattern of induction in response to growth factors and proinflammatory cytokines, and that each of the factors and cytokines has an individual profile of induction of antimicrobial (poly)peptides (Table 2). As a result, keratinocytes may respond to different pathological stimuli by distinct patterns of expression of antimicrobial effector molecules. This is true even for the structurally and genetically closely related β-defensins. Because of the variable antimicrobial specificity of the (poly)peptides, the ability to vary the defensive repertoire may be of functional importance. Their ability to generate a differentiated "innate immune response" also underscores the importance of the keratinocyte as an immunocompetent cell in the host defense.

## 4. PERSPECTIVES

Antimicrobial (poly)peptides are important effector molecules of innate immunity present both in professional phagocytes (i.e. macrophages and neutrophils) and in epithelial cells. Animal studies have started to demonstrate the importance of these molecules in the clearance of bacteria in vivo (26, 27, 87). However, there is a great redundancy of antimicrobial (poly)peptides in various body fluids, and removal of just one of these may not seem to have great apparent impact in an experimental in vivo setting. Another problem is the very large variation among the species regarding the particular antimicrobial (poly)peptides present. Thus, the findings in a knock-out mouse may not apply to a human setting.

Defining the exact role for a single antimicrobial (poly)peptide in a human setting has proved to be challenging. When it comes to cathelicidins in neutrophils, this thesis has elucidated that the activation of antimicrobial peptides varies among different species. The same has later been shown for α-defensin in the intestine. The generation of antimicrobial peptides in vagina through a pH dependent activation of proteolytic activity can only take place in a few species, since low vaginal pH is only found in a few species. Though it seems conceivable that effectors of innate immunity are generated following sexual intercourse in other species, the detailed mechanism will need to be different. Hence, in order to define the role of specific effector molecules or mechanisms in innate immunity in humans, experiments will need to be performed in a human setting.

Looking at the number of potential infectious microbes a human being encounters each day, overt infection is the rare exception. Thus, the main role of the (innate) immune system is not only to combat established infections but rather to prevent infections from first developing. Indeed, body fluids (e.g. human airway fluid (88)) contain antimicrobial substances even in absence of infection. On the other hand, in some situations infections are more likely to develop, for example after wounding. To prevent infection it would be beneficial to the host if "risk situations" alerted the innate immune system. This thesis elucidated aspects of the innate immune response in two high-risk situations for infection, sexual intercourse and wounding. In the latter we demonstrated that growth factors that are released during wounding indeed induced the expression of antimicrobial (poly)peptides in the absence of infection. Similarly, we found that antimicrobial peptides could be generated in the vagina by a mechanism independent of infection. Moreover, neutrophils (which contain an abundance of antimicrobial peptides including hCAP-18) are known to be present in large quantities in the blood even in the disease-free state and are recruited to wounds and sites of inflammation also in the absence of microbes. Thus, studies of the role of antimicrobial peptides in the immune response should not be narrowly limited to settings of overt infection and microbial colonization.

## ABBREVIATIONS

aa: Amino acid
bFGF: Basic fibroblast growth factor
BSA: Bovine serum albumin
CAP: Cationic antimicrobial protein
CGD: Chronic granulomatous disease
CMK: *N*-methoxy-succinyl-ala-ala-pro-val chloromethyl ketone
EGF: Epidermal growth factor
ELISA: Enzyme-linked immunosorbent assay
fMLP: Formyl-methionyl-leucyl-phenyl-alanine
FPLC: Fast performing liquid chromatography
GST: γ-glytamyl S-transferase
hBD: human β-defensin
hCAP-18: Human cationic antimicrobial protein of 18 kDa
HDL: High density lipoprotein
HPLC: High pressure liquid chromatography
IGF-I: Insulin-like growth factor I
IL-1β: Interleukin-1β
IL-6: Interleukin 6
kDa: Kilo Dalton
LDL: Low density lipoprotein
MW: Molecular weight
NADPH: Nicotinamide adenine dinucleotide phosphate
NGAL: Neutrophil gelatinase-associated lipocalin
PMN: Polymorphonuclear leukocyte
SLPI: Secretory leukocyte protease inhibitor
STZ: Serum treated zymosan

TGF-α: Transforming growth factor α
TGF-$\beta_1$: Transforming growth factor $\beta_1$
TNF-α: Tumor nekrosis factor α
VLDL: Very low density lipoprotein

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