REGULATION AND CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES IN MAN AND MICE

Jenny Karlsson

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Kvark, elektron, proton, atomkärna, kolatom,
vattenmolekyl, peptid, protein, virus,
bakterie, cell, vävnad, jag, jorden,
vintergatan, galax, en evighet
av möjligheter....
Illustrations by Helen Karlsson, 2007
Abstract

The gene-encoded antimicrobial peptides have emerged as an important component of innate immunity. In mammals, antimicrobial peptides are either directly secreted from cells or found in cell granules, which can be released after stimuli. This thesis aims at investigating antimicrobial peptides stored in granules in neutrophils and in Paneth cells of the small intestine.

The condition severe congenital neutropenia (SCN) illustrates the pivotal requirement for neutrophils as primary defence cells since affected individuals die in infancy. In this thesis I show that all patients with SCN, independent of inheritance pattern, genetic mutations or G-CSF responsiveness, display a deficiency of the antimicrobial peptide LL-37 and its pro-form in plasma and in neutrophils, even after G-CSF treatment. This deficiency points at a common mechanistic denominator for all patients with SCN. The LL-37 phenotype may be used as a diagnostic method to discriminate the lifelong, and without treatment, life threatening disease SCN from the more benign forms of chronic neutropenia. Granulocytic precursors from patients with SCN were here demonstrated to produce pro-LL-37 after in vitro stimulation with the hormonal form of vitamin D₃. The induction of pro-LL-37 in plasma did not take place in vivo following oral treatment of one patient with a low dose vitamin D₃.

Neutrophils and Paneth cells are strikingly similar in their repertoire of granule derived antimicrobial peptides. Paneth cells reside at the base of the crypt of the small intestine of most mammals, where they secrete high concentrations of antimicrobial substances that sterilize the crypt and shape the profile of the resident microflora. Here I show that the mouse Paneth cell arsenal is broadened by the presence of cryptdin related sequence (CRS) peptides. The antimicrobial spectrum is increased further by their ability to form both homo- and heterodimers, which have distinct but overlapping antimicrobial spectra. In addition, different repertoires of CRS peptides in different mouse strains, suggest a highly efficient positive selection pressure. Two groups of transcripts coding for CRS peptides could be separated based on expression profile in the small intestine. One group was expressed at a consistently high level along the small intestine, while the other group increased strongly through the length of the small intestine. A similar increase, although not as prominent, has also been demonstrated by others for the enteric antimicrobial peptide human defensin 5, which is involved in the pathomechanism of Crohn’s ileitis. Elucidating the regulatory pattern in mice may thus be useful in unravelling the regulation of human enteric antimicrobial peptides.
This thesis is based on the following papers, which are referred to in the text by their Roman numerals

Low plasma levels of the protein pro-LL-37 as an early indication of severe disease in patients with chronic neutropenia
Br J Haematol., 2007, 137(2), 166-169

II Andersson M, Karlsson J, Carlsson G, Pütsep K.
Expression of granule-associated proteins in neutrophils from patients with severe congenital neutropenia
Blood, in press

III Karlsson J, Carlsson G, Andersson M, Pütsep K.
Vitamin D3 induces pro-LL-37 expression in myeloid precursors from patients with severe congenital neutropenia
Manuscript

IV Hornef MW, Pütsep K, Karlsson J, Refai E, Andersson M.
Increased diversity of intestinal antimicrobial peptides by covalent dimer formation
Nat Immunol., 2004, 5(8), 836-843

V Karlsson J, Pütsep K, Chu H, Kays R, Bevins CL, Andersson M.
Regional variations in Paneth cell antimicrobial peptide expression along the mouse intestinal tract
Manuscript
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<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>1,25-dihydroxy-vitamin D$_3$</td>
</tr>
<tr>
<td>AIN</td>
<td>autoimmune neutropenia</td>
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<td>AML</td>
<td>acute myeloid leukemia</td>
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<td>AMP</td>
<td>antimicrobial peptide</td>
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<td>ANC</td>
<td>absolute neutrophil count in blood</td>
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<td>ATRA</td>
<td>all-trans retinoic acid</td>
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<tr>
<td>CAMP</td>
<td>cathelicidin antimicrobial peptide</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
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<tr>
<td>CRS</td>
<td>cryptdin-related sequence</td>
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<td>cryptdin</td>
<td>crypt α-defensin in mouse</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>Gfi1</td>
<td>growth factor independent 1</td>
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<tr>
<td>HAX1</td>
<td>HCLS1-associated protein X1</td>
</tr>
<tr>
<td>HD5</td>
<td>human defensin 5</td>
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<tr>
<td>HD6</td>
<td>human defensin 6</td>
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<tr>
<td>HNP1-4</td>
<td>human neutrophil peptides 1-4</td>
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<tr>
<td>HSCT</td>
<td>hematopoietic stem cell transplantation</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
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<tr>
<td>IN</td>
<td>idiopathic neutropenia</td>
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<tr>
<td>LEF</td>
<td>lymphoid enhancer-binding factor</td>
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<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
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<tr>
<td>MDS</td>
<td>myelodysplastic syndrome</td>
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<tr>
<td>MMP7</td>
<td>matrix metalloproteinase 7, matrilysin</td>
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<td>MPO</td>
<td>myeloperoxidase</td>
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<tr>
<td>NE</td>
<td>neutrophil elastase</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
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<tr>
<td>NOD2</td>
<td>nucleotide oligomerization domain 2</td>
</tr>
<tr>
<td>PGRP</td>
<td>peptidoglycan recognition protein</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear cell, neutrophil</td>
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<tr>
<td>pro-LL-37</td>
<td>human cationic antimicrobial pro-peptide, hCAP18</td>
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<td>PRRs</td>
<td>pattern-recognition receptors</td>
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<td>SCN</td>
<td>severe congenital neutropenia</td>
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<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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INTRODUCTION

This thesis aims to characterize mammalian antimicrobial peptides derived from granulated cells, both from the white blood cell type neutrophil and from the small intestinal Paneth cell. Throughout the text both the cathelicidins and defensins of antimicrobial peptides will be discussed in more detail. These two families of peptides are central in the mammalian innate defense.

Below follows an overview of innate immunity followed by sections describing neutrophil and Paneth cell derived defense, with focus on the contribution of antimicrobial peptides.

WHAT KEEPS US HEALTHY?

How can we live in a surrounding of potential pathogens?

Humans have co-evolved with microorganisms. Most, but not all, of the body surfaces have resident microbes; the natural microflora, which is tolerated by the host defense system and necessary for our well-being (Guarner 2006). Different anatomical sites are associated with different repertoires of microbial species. There is however, a higher risk of infection at epithelial surfaces of the body, such as the respiratory-, gastrointestinal-, urogenital tract, and the skin, all facing and separating us from the external environment and resident microbiota. Both transient-, as well as our resident microorganisms can cause infection. It is mainly under specific circumstances, such as inherited or acquired defects in immune system or physiology, that the resident microbiota is able to overcome our protective responses and cause infection.

Vertebrates, including humans, have adopted a highly sophisticated system based on B and T cells to combat infectious agents (Janeway and Bottomly 1994). The system is inducible, specific- and acquires an immunological memory towards the infectious agent. Both B and T cells needs to be primed and it takes several days from the time the infectious agent is recognized until an adaptive response can be elicited. The host is however confronted with infectious agents that may replicate every 20 minutes and an
Immediate response is therefore crucial. In contrast to the adaptive defense, the innate defense is instantaneous, independent of priming, and hence an ever present arm of host defense. The cooperation between the innate- and the adaptive defense is needed for proper control of our resident microflora.

The challenges and countermeasures of epithelia

The epithelial lining, at many locations represented by a single cell layer, fend off most insults. If the epithelial lining is breached the innate defense factors are rapidly activated/attracted. Cellular recognition of the infectious agent leads to the release of cytokines that orchestrate an inflammatory and anti-infectious response consisting of innate- and adaptive immune factors (Gillitzer and Goebeler 2001).

**Figure 1**

The recognition molecules for microbial components, present on epithelial cells and leucocytes, are referred to as pattern recognition receptors (PRRs) (figure 1) (Eckmann 2004). As the name implies, they recognize broad but distinct types of microbial molecules. One example being the intracellular protein NOD2 (nuclear oligomerization domain 2) that recognize muramyl...
dipeptide, a cell wall component of gram-positive and gram-negative bacteria.

In higher organisms the epithelial cells are both creating a physical barrier and a "chemical barrier". This chemical barrier consists of secreted anti-infectious substances, such as proteins/peptides that directly kill microbes, reduce nutrition levels, degrade microbial virulence factors and so forth (Lievin-Le Moal and Servin 2006).

Antimicrobial peptides as an integral part of the innate defense mechanism

Antimicrobial peptides, AMPs, were first characterized in 1981 by Håkan Steiner et al (Steiner, et al 1981), even though an inducible defense mechanism of the silk moth *Hyalophora cecropia* after bacterial challenge was demonstrated already in 1972 (Boman, et al 1972). The antibacterial activity of the gene encoded antimicrobial peptides is in the same range as classical antibiotics, *i.e.*, often low micromolar range. Almost 900 eukaryotic peptides have been described in the AMPs database (http://www.bbcm.univ.trieste.it/~tossi/pag2.htm). Alongside their direct antibacterial properties, many are also active against fungi, protozoan, viruses and tumor cells (Lichtenstein, et al 1986, Selsted, et al 1985).

Most of the antimicrobial peptides are positively charged and rapidly disintegrate membranes as described in figure 2 (Shai 2002). Their three-dimensional structures are very diverse; like the α-helical structure represented by LL-37 in figure 2A in contrast to the defensins (α-, β- and θ-group) characterized by a β-sheet configuration (Trabi, et al 2001).

The specificity towards microbial- but not to host cell membranes is attributed to the cationic, amphipathic nature of the antimicrobial peptide in three dimensional space (figure 2A). The negatively charged bacterial cell wall creates a hydrophobic and electrostatic attraction towards the two faces of the peptide (Figure 2) (Shai 2002) while eukaryotic cell membranes contain neutral zwitterions and cholesterol on the outer leaflet, which partly repels the peptides and therefore protects against the action of endogenous AMPs.
The epithelial derived antimicrobial peptides can be secreted in a constitutive manner, induced after PRR signaling (figure 1), or alternatively by other means. Antimicrobial peptides are synthesized as inactive precursors and activated by proteolytic processing either before storage or after release from the cell. The AMPs retain their activity in the c-terminal part of the protein, while the pro-part most likely is present to induce folding and prevent autotoxicity (Wu, et al 2007).

In addition to the direct antimicrobial function of AMPs, they also alter the host immune response through receptor-dependent

**Figure 2** Three proposed mechanisms of direct killing of bacteria by antimicrobial peptides. A helical wheel diagram of the human antimicrobial peptide LL-37 (red; positive, yellow; negative, blue; non-charged amino acids). The hydrophilic (red) and the hydrophobic (blue) separation in three dimensional space is shown in A). After electrostatic interactions between the positively charged amino acid residues and the negatively charged bacterial cell wall, the peptides associate with the membranes B), leading to a destabilization of the membrane and subsequent cell death. C) Carpet model, D) barrel stave pore, and E) toroidal pore. Figure adopted from (Shai 2002).
interactions at sub-bacteriolytic concentrations. Examples of these types of activities are angiogenesis, wound healing, and chemotaxis (Zanetti 2005). Both neutrophils and Paneth cells secrete antimicrobial peptides in concentrations that are clearly antibacterial.

In recent years, the bactericidal components of the innate immune system have received increased attention because of the emergence of antibiotic resistance. Both administration of in vitro synthesized AMPs and endogenous stimulation of the innate system are potential candidates for antibacterial therapy (Hancock and Sahl 2006).
NEUTROPHIL DERIVED DEFENSE

Ilya Mechnikov was the first to report on phagocytic cells already in 1883 (Metchnikoff 1883), and for that he was awarded the 1908 Nobel Prize in Physiology or Medicine. After Mechnikov’s first discovery of phagocytic cells, much attention has been devoted to understanding their biology. Out of the phagocytic cells in human blood the neutrophils are the dominating type. A healthy individual has 2 to 7 \times 10^9 neutrophils per litre blood, referred to as the absolute neutrophil count (ANC) (Boxer and Dale 2002). Neutrophils outnumber the second most numerous phagocytic cell type, the monocyte/macrophage, in blood about 10 fold. Neutrophils are also named polymorphonuclear cell (PMN) due to their characteristic multi-lobed nuclei.

Neutrophils are pivotal in innate immunity. This can be demonstrated in patients with severe neutropenia (i.e., abnormally low number of neutrophils in circulation) who are at substantial risk of life threatening bacterial infections (Boxer and Dale 2002). Neutrophils are the first leucocytes recruited to a site of infection, where the main task is engulfment of the infectious agent by phagocytosis and release of antimicrobial substances and immune mediators (Lentsch and Ward 2000, van Wetering, et al 2005). In addition, neutrophils can actively extrude granule proteins and chromatin that together form extracellular antimicrobial fibers, named neutrophil extracellular traps (NETs) (Brinkmann, et al 2004).

Neutrophils are short-lived cells, with an average half life of six hours in circulation, and die after one to two days in tissues in the absence of inflammation. Alternatively, they become activated in the presence of cytokines, and attracted by a gradient of chemotactic factors (Akgul and Edwards 2003, Gillitzer and Goebeler 2001). The down side of neutrophil activation is the development of tissue destruction following neutrophil release of inflammatory mediators and proteases (Lentsch and Ward 2000).

Myelopoiesis

Myelopoiesis is the process of the proliferation and differentiation of granulocytes; neutrophils, eosinophils, basophils, and monocytes/macrophages in the bone marrow.
Figure 3 The differentiation to neutrophils goes through promyelocyte-, myelocyte-, metamyelocytes-, and band cell stages in the bone marrow prior to release into the blood stream. The commitment to a terminally differentiated neutrophil is a process that takes 10-12 days (Figure 3) (King-Smith and Morley 1970).

The commitment to a certain hematopoietic cell in the bone marrow is directed by the presence of several cytokines and chemokines that activate transcription factor programs, illustrated in figure 4A (Borregaard, et al. 2001). The signalling through the cognate receptors of colony-stimulating factors (CSFs) and vitamins, orchestrate the proliferation and differentiation (Barreda, et al. 2004, Bastie, et al. 2004). Neutrophil maturation is dependant mainly on G-CSF (granulocyte), GM-CSF (granulocyte/macrophage) and vitamin A, while monocyte maturation is influenced by M-CSF (macrophage), GM-CSF and vitamin D₃.
Neutrophil granule constituents

Many of the factors used to combat infectious agents e.g., nitric oxide, oxygen radicals, enzymes, and antimicrobial peptides are contained in neutrophil granules (Faurschou and Borregaard 2003, Theilgaard-Monch, et al 2005). Four different types of granules; primary, secondary, tertiary and secretory, can be distinguished based on their content. The proteins are produced and packed in granules in a gradual fashion during neutrophil maturation in the bone marrow. Targeting of proteins to certain granule subsets is primarily by timing and not by sorting mechanisms (Borregaard, et al 2001). Many of the genes coding for granule proteins are regulated by the transcription factors that are required for neutrophil maturation (figure 4).

Figure 4

**Figure 4** A) Neutrophil maturation is dependant on the gradual influence of several transcription factors including C/EBPα, -δ, -ε, PU.1 and CDP. B) The primary granules are produced in the myeloblasts to myelocyte stage of neutrophil maturation. The secondary granules contain pro-LL-37, lactoferrin, NGAL among others, and are produced in myelocyte to metamyelocytes stage. Tertiary and secretory granules are mainly produced in the band cell stage. They contain gelatinase, the integrin CD11b, as well as other receptors vital for attachment and for recognition of antigens (Theilgaard-Monch, et al 2005).
Phagocytosis of microbes and secretion of antimicrobial- and pro-inflammatory mediators are hallmarks of neutrophil functions. Mainly primary-, but also to some extent secondary-, granules fuse with the phagosome, creating a microbicidal milieu. Tertiary and secretory granules are most easily mobilized and secreted after stimuli.

The most abundant human primary granule constituents are the neutrophil α-defensins (Ganz, et al 1985). Human neutrophils produce four α-defensins named human neutrophil peptide 1-4 (HNP1-4) (Wilde, et al 1989). In addition to α-defensins, myeloperoxidase (MPO), serine proteases and other potential antimicrobial substances (Theilgaard-Monch, et al 2005) are also stored in primary granules. The neutrophil-derived serine proteases include neutrophil elastase (NE), cathepsin G (CATG), proteinase 3 (PR3) and azurozidin (AZ). The enzymatic activity of the proteins not only degrades bacterial proteins and decrease bacterial viability, but additionally activates cytokines and AMPs (Wiedow and Meyer-Hoffert 2005).

The secondary granule protein pro-LL-37 (human cationic antimicrobial pro-peptide, hCAP18, cathelin-LL-37) is the only member of the cathelicidin family of peptides found in humans. The cathelicidin family have been identified in several mammalian species and are characterized by a conserved pro-peptide. LL-37 was first identified in blood cells (Agerberth, et al 1995), but has, since then, been found at numerous locations in the body. Neutrophil derived pro-LL-37 is processed extracellularly by proteinase 3 (Sorensen, et al 2001). LL-37 has been assigned several other functions besides being a broad-spectrum antibacterial peptide, as described in figure 5.
Some proteins are expressed throughout several neutrophil maturation stages and end up in both primary and secondary granules, like lysozyme. Once reaching the blood stream the mature neutrophils are equipped with all four types of granules.

Severe congenital neutropenia

Chronic neutropenia is characterized by recurrent bacterial infections. The condition is termed severe if neutrophil numbers in circulation persistently stays below ANC 0.5 x 10^9/L. Several rare diseases are associated with congenital hereditary neutropenia, the most frequent being severe congenital neutropenia (SCN, Kostmann syndrome). Göran Carlsson, Md PhD, has published a presentation
of the different forms of congenital neutropenia, which is recommended for the clinically oriented reader (Carlsson 2004).

Severe congenital neutropenia has a penetrance of one-two cases per 1 000 000 (Welte, et al 2006). This disease was first described by the Swedish pediatrician Rolf Kostmann in his thesis in 1956, where he named the new syndrome infantile genetic agranulocytosis (agranulocytosis infantilis hereditaria) (Kostmann 1956). Dr Kostmann assigned it as being a recessive trait, but since then patients with autosomal dominant- as well as sporadic forms have been characterized (Welte, et al 2006). From here on the disorder will be referred as SCN, independent of genetic inheritance.

The disease is manifested as early onset of severe bacterial infections. Without treatment, the outcome of these infections is often death within the first year of life. Treatment with a personalized dosage of G-CSF restores neutrophil levels to normal in most cases (Dale, et al 1993). In spite of treatment these patients frequently experience recurrent infections and periodontal diseases including gingivitis and periodontitis (Carlsson, et al 2006, Defraia and Marinelli 2001). The higher infection rate may, in part, be due to a deficiency of the antibacterial peptide LL-37 in neutrophils, plasma and in saliva (Putsep, et al 2002).

Patients with SCN are at a high risk of developing myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML). The only curative measure for patients with SCN is hematopoietic stem cell transplantation (HSCT) (Zeidler, et al 2000).

The neutropenia is caused by a maturation arrest in the bone marrow at the promyelocyte- to myelocyte stage (Welte, et al 2006). The pathophysiologic mechanisms leading to the maturation arrest have not been fully elicited but the bone marrow precursors display an elevated rate of mitochondrial associated apoptosis (Carlsson, et al 2004). A lower level of the transcription factor LEF-1 (lymphoid enhancer-binding factor 1) in the precursors may also have a role in this defect (Skokowa, et al 2006).

Genetic linkage analysis has revealed mutations in the gene coding for the mitochondria-associated protein HAX1 (HCLS1- associated protein X1) as a common denominator of SCN, with recessive inheritance patterns both in patients from northern Sweden described by Dr. Kostmann, and in patients with Kurdish descent.
A high proportion of patients with SCN have \textit{ELA2} mutations (40-60%), the gene coding for neutrophil elastase (Bellanne-Chantelot, \textit{et al} 2004, Karlsson, \textit{et al} 2007). In addition, mutations in \textit{ELA2} have close to 100% penetrance in patients with cyclic neutropenia. Surprisingly, several of the identified \textit{ELA2} mutations can cause either disease (Bellanne-Chantelot, \textit{et al} 2004). Also \textit{Gfi1} (growth factor independent 1) and other gene defects has been linked to SCN. Still, patients with severe congenital neutropenia without known mutations can be found (Karlsson, \textit{et al} 2007, Skokowa and Welte 2007). Several hypothetical mechanisms linking \textit{ELA2} and \textit{HAX1} to maturation arrest has been proposed (Benson, \textit{et al} 2003, Klein, \textit{et al} 2007, Kollner, \textit{et al} 2006).

In conclusion, neutrophils are rapidly attracted to a site of infection where they kill microbes by several means, including direct killing by antimicrobial peptides. Patients with SCN display an increased bacterial susceptibility, despite G-CSF treatment which restores neutrophil numbers in circulation to normal. Patients with the recessive form of the disease display a deficiency of the antimicrobial pro-peptide pro-LL-37 in G-CSF mobilized neutrophils, which suggests a role for neutrophil derived LL-37 in protecting against bacterial infection.
Small intestinal control of resident and transient microbes

The small intestine is a heterogeneous tissue with longitudinal differences in nutrient absorption capacity and resident microbiota (Hao and Lee 2004). The ileum, i.e., the distal part of the small intestine, has a higher pH, slower peristalsis, and lower oxidation-reduction potential. The ileum can thereby maintain a more diverse microflora as compared to the duodenum (proximal to the gut) and jejunum (the medial segment of the small intestine). Host defense factors in the small intestine include Paneth cell constituents, rapid turnover of epithelial cells and the mucus barrier.

**Figure 6**

Paneth cells (bottom) reside in the base of the crypt (right) of the small intestine (left). The stem cells are positioned adjacent to the Paneth cells. Paneth cells secrete high concentrations of antimicrobial substances, including defensins. Adopted from an illustration by D. Schumick (Porter, et al. 2002).
Paneth cells are specialized secretory cells containing antimicrobial substances including antibacterial peptides (figure 6) (Porter, et al 2002). They reside at the base of the small intestinal crypts of many mammals.

Paneth cells, together with enterocytes, enteroendocrine cells and goblet cells make up the monolayer of epithelial cells. The epithelia is covered with mucus, which is made up of highly interlinked glycosylated proteins (mucins) (Hao and Lee 2004). The mucosal layer physically protects the epithelia from bacterial attachment. Enterocytes build up a chemical defense barrier through the production of antibacterial compounds, for example the β-defensins and LL-37 (Eckmann 2004). Human β-defensin 1 is constitutively expressed while human β-defensin 2 and 3 are regulated by the transcription factor nuclear factor κB (NF-κB) (O’Neil, et al 1999). NF-κB is a downstream transcription factor of PPRs and certain interleukin receptors (Winkler, et al 2007).

Paneth cell characteristics

Secretion of Paneth cell granules are dependant on bacterial products (Ayabe, et al 2000) and on cholinergic agonists (Ayabe, et al 2002b). The granule products are released into the small volume of the crypt base measured in picolitres (figure 6).

The most abundant of the Paneth cell granule constituents are the α-defensins. In humans these are represented by human defensin 5 and 6 (HD5 and HD6, respectively) (Jones and Bevins 1993). Mice have six α-defensins in their Paneth cells, represented by cryptdin (crypt defensin) 1-6, although additional mRNA coding for cryptdins has been found (Huttner, et al 1994). Mouse cryptdins secreted into the crypt lumen reaches at least an estimated 2.5 mM (10 mg/ml if estimating 4 kDa) (Ayabe, et al 2000), several orders of magnitude higher than the general minimal inhibitory concentration for the peptides.

An additional family of peptides in mice, the cryptdin related sequences (CRS), with potent antimicrobial activity have been identified at a protein level (Paper IV, (Hornef, et al 2004)). In addition to the α-defensins and CRS peptides, Paneth cell granules contain a number of other peptides/proteins including lysozyme, phospholipase A2, TNF-α, metalloproteinase 7 (MMP7, matrilysin) and Reg3γ (regenerating protein 3γ, (Cash, et al 2006)). In mice,
MMP7 is responsible for the activation of cryptdins (Ayabe, et al 2002a).

Several experiments demonstrate that Paneth cell α-defensins are important for intestinal resistance to transient pathogens. One such experiment includes MMP7−/− mice that were more susceptible to oral *Salmonella* and to dextrane sulfate induced colitis (Shi, et al 2007, Wilson, et al 1999).

In the normal setting, antimicrobial substances secreted from Paneth cells and from epithelial cells are believed to sterilize the lower parts of the crypt and thereby shape the microflora (Salzman, et al 2007). This view has been supported by the demonstration that the luminal content of microbes was shifted in transgenic mice having an extra enteric antimicrobial peptide (Wehkamp, et al 2005). The intrinsic self-regulatory function of the microflora should not be underestimated. The enteric AMPs are probably also present to inhibit bacteria from attaching to the epithelial monolayer.

**Differentiation of Paneth cells**

All small intestinal epithelial cells originate from the small intestinal stem cells, located just above the Paneth cells in the crypt (figure 6). Four to six stem cells per crypt regenerate the entire villus epithelium every three to five days (Potten and Loeffler 1990). Wnt/β-catenin mediated regulation determines the cell fate (Ahuja, et al 2006, Andreu, et al 2005). This system is dependant on the extracellular signal Wnt that bind the membrane receptor frizzled. Cytoplasmic β-catenin is stabilized after receptor binding, and migrates to the nucleus where it can act together with LEF/TCF (T-cell specific, HMG-box) group of transcription factors. The complex regulates both autocrine Wnt, as well as other genes that are executors of proliferation. β-catenin, together with TCF-4, regulate, for example, the expression of Eph (ephrin receptor) B2 and B3, which in turn control the positioning of Paneth cells within the crypt villous axis (Andreu, et al 2005). Paneth cell derived α-defensins are also regulated by TCF-4 (Andreu, et al 2005, van Es, et al 2005).

Colon tumours with uncontrolled Wnt signalling are frequently associated with metaplastic Paneth cells (Andreu, et al 2005). Moreover, metaplastic Paneth cells have been demonstrated in the gut during human *Helicobacter Pylori* infection (Shen, et al 2005).
Diseases associated with Paneth cell dysfunction

The significance of human Paneth cell \( \alpha \)-defensins was, for the first time, described by Jan Wehkamp and co-workers in 2004 (Wehkamp, et al 2004). They demonstrate that Crohn’s ileitis patients, a form of relapsing inflammatory bowel disorder (IBD), express lower amounts of Paneth cell derived HD5. The decrease in expression was not seen for the other type of IBD named ulcerative colitis (Wehkamp, et al 2005). Crohn’s disease is most often defined to the distal part of the small intestine, hence the name Crohn’s ileitis.

A general view of the pathology of IBD is that commensal bacteria associate with the epithelium, induce an aberrant immune response and thereby inflammation (Swidsinski, et al 2005). By which mechanism this is conducted is not known, but one hypothesis is that the mucus barrier is not able to separate the bacterial from the epithelial monolayer (Einerhand, et al 2002).

Approximately 35% to 45% of patients with Crohn’s ileitis carry at least one mutation in the \( \text{CARD15} \) gene coding for the pattern recognition protein NOD2, although not all with \( \text{CARD15} \) mutations will develop the disease. However, Scandinavian, Irish and Scottish patients with Crohn’s disease have an association with \( \text{CARD15} \) that is much lower than expected, and there is no association in the Chinese or Japanese population. Other gene defects have also been associated with incidence of Crohn’s disease (Vermeire and Rutgeerts 2005).

Haematopoietic stem cell transplants are emerging as a new therapy for Crohn’s disease (Leung, et al 2006). The mechanism behind the recovery is not known, but HSCT recovery is associated with \( \text{CARD15} \) mutation frequency in both donor and recipient (Holler, et al 2004). I believe that one plausible explanation could be that certain intestinal cells are replaced with genetic material of the donor. Mice have been demonstrated to replenish both myofibroblasts (Brittan, et al 2002), laying just beneath the epithelial cells, as well as the small intestinal epithelial cells (Krause, et al 2001) with the donors genetic material after HSCT. Notably, myofibroblasts have been known to express both Wnt (Andoh, et al 2005) and NOD2 (Otte, et al 2003).

Lessons from the crypt; \( \alpha \)-defensins are key factors in the healthy interplay between microflora and intestinal barrier. Both cryptdins
and HD5/6 are regulated by Wnt/β-catenin mediated signalling. The mouse is hence a good model to further characterize the regulatory mechanism leading to lower defensin levels in the human setting during Crohn’s disease.
COMMON DENOMINATORS OF PANETH CELLS AND NEUTROPHILS

Neutrophils and Paneth cells are strikingly similar when it comes to the production of granule derived antimicrobial peptides e.g., α-defensins, lysozyme and sPLA2 (Keshav 2006), where they are constitutively expressed. Antimicrobial peptides often work in synergy (Yan and Hancock 2001), and granule release of a cocktail of peptides from neutrophils or Paneth cells may therefore have a higher capacity of bacterial killing than the sum of each isolated component. The occurrence of expression/release of several different antimicrobial molecules at the same time/site is frequently seen in higher organisms. It facilitates a broad potential of microbial killing and possible defective expression of one molecule is in part compensated for by the others. This is probably a reason for the relatively few examples of human conditions where a single antimicrobial peptide dysregulation displays a strong phenotype.

Both intestinal and hematopoietic stem cells rely on Wnt and Notch signalling for development and differentiation. This system is primarily active during hematopoietic stem cell renewal, while determining cell fate in the small intestine (Moore and Lemischka 2006).

In this thesis two examples of association between AMPs and human disease are highlighted. The first being patients with the recessive form of severe congenital neutropenia who lack the antimicrobial peptide LL-37 in neutrophils, which probably explains their higher susceptibility to bacterial infections and periodontal diseases. The second being patients with Crohn’s ileitis, having decreased expression of the antimicrobial peptide HD5 and consequently an increased susceptibility to gastrointestinal inflammation. The two examples of peptides represent the broad groups of antimicrobial peptides in humans, cathelicidins and defensins respectively. In both settings they are constitutively expressed. Of note is that both cathelicidins and defensins are inducible in other locations, such as in intestinal epithelial cells (O'Neil, et al 1999) and keratinocytes (Midorikawa, et al 2003).
AIMS OF THE THESIS

One aim of the studies on neutrophils was to elucidate the association between severe neutropenia and LL-37 expression in order to understand the contribution of antimicrobial peptides in disease outcome. The other aim was to examine how the expression of LL-37 can be modulated, to be able to regulate this peptide expression both in severe congenital neutropenia, but also in other conditions.

We sought to characterize the mouse enteric antimicrobial peptide repertoire and especially the novel cryptdin related sequence peptides, in order to explore the peptide bactericidal capacity, antimicrobial repertoire, expression profile and regulatory patterns. Studying the regulatory pattern of antimicrobial peptides in mouse will increase the understanding of the interplay between peptide expression, infection/inflammation and microflora in both mouse and man.
RESULTS AND DISCUSSION

FROM IDENTIFICATION OF COMMON DENOMINATOR FOR SEVERE CONGENITAL NEUTROPENIA TO A POTENTIAL CANDIDATE FOR ELEVATING PRO-LL-37 LEVELS IN THE PATIENTS

Paper I

Since patients with the recessive form of severe congenital neutropenia (SCN) exhibit low levels of pro-LL-37 in neutrophils, plasma and saliva (Putsep, et al 2002), we sought to investigate this association in patients with sporadic and dominant inheritance patterns. In addition to SCN patients, we investigated the pro-LL-37 levels in plasma from patients with cyclic neutropenia, autoimmune neutropenia (AIN), and idiopathic neutropenia (IN). Cyclic neutropenia is a rare disease manifesting as a cyclic nadir in ANC every third week (Boxer and Dale 2002) while AIN, which result from neutrophil destruction due to auto-antibodies directed against neutrophils, is more common with an incidence of at least 1/100,000 newborns (Lyall, et al 1992). For idiopathic neutropenia the underlying mechanisms are unknown, and the unexplained reduction in circulating neutrophils can be found among both children and adults (Boxer and Dale 2002).

15 patients with SCN were enrolled in this study, representing a majority of the patients diagnosed in Sweden. In this material, seven (47%) had ELA2 mutations, three HAX1 mutations and six patients presented without any known mutations. The percentage of patients with ELA2 defects is in accordance with the findings from the French neutropenia register (Bellanne-Chantelot, et al 2004).

In this study we could demonstrate decreased/absent pro-LL-37 levels in plasma from all patients with SCN independent of inheritance pattern, ANC, G-CSF treatment or genetic mutations (see below). This was in contrast to patients with immune- or idiopathic neutropenia that exhibited plasma levels within the
variation of healthy individuals. Plasma levels of pro-LL-37 in patients with cyclic neutropenia correlated to the drop of circulating neutrophil numbers every third week.

Relative levels of plasma pro-LL-37 based on the densitometry readings of immunoblots. The readings in the graph represent the percentage of the mean value of the two reference controls (indicated by arrows). *** = P < 0.001. Filled shapes denote G-CSF administration.

The general absence of pro-LL-37 indicates that the multi-genetic disease SCN has a common mechanistic denominator. The defect could hence be used to identify such common traits.

At present there is no simple method to distinguish the lifelong severe disease SCN that is associated with high risk of developing MDS/AML, from the other, less severe forms of chronic neutropenia (Welte, et al 2006). Considering that chronic neutropenia patients are often identified in infancy and that the current procedure, bone marrow analysis, is not without risk for the young patient, other methods are desired. Genetic analysis has emerged as a parallel diagnosis method. At present, genetic analyses will require full length sequences of at least three genes (ELA2, HAX1, Gfi1). Since not all patients with SCN have mutations in these genes, the method on its own is not sufficient. Plasma level of pro-LL-37 could be a complementary method for differential diagnosis of chronic neutropenia.
Paper II

Recent data clearly demonstrates that SCN is a multi-genetic disease (Carlsson, et al 2007), but it is not known if variants in genetic defects results in differences in neutrophil protein expression patterns. We included five patients with SCN out of which one had undergone a hematopoietic stem cell transplant rendering him a chimera, still dependant on a low dose of G-CSF, but in this context served as a control. The other patients had dominant *ELA2*-, sporadic *ELA2*-, recessive *HAX1* mutation, or were without known gene defect. The patients were treated with daily administration of G-CSF during the course of the study.

We could conclude that pro-LL-37 protein was low in neutrophils from all patients studied, paralleling the plasma levels (Paper I). No other granule proteins had a reduction comparable to that of pro-LL-37 (see above). Interestingly, we noted lower levels of \( \alpha \)-defensins in two patients, none of which had *ELA2* mutations. The HNP1-3 pattern cannot be explained by lower mRNA levels (see
results in Paper III). One possibility for the reduced protein levels could be secretion from pre-activated neutrophils, documented by Elsner et al. after G-CSF administration (Elsner, et al. 1992). The results differ from a recent report (Donini, et al. 2007), but this is most likely a result of patient group selection. At present, we cannot link the genetic mutation or pathology to a certain neutrophil granule expression pattern. Our results support the hypothesis that decreased immune defense is in part attributed to lower pro-LL-37 in these patients.

Paper III

Our aim was to assess whether neutrophil precursors from patients with SCN have the ability to produce pro-LL-37 at all, and if they could be induced to produce pro-LL-37 by external means.

The regulatory mechanism of pro-LL-37 in neutrophil bone marrow progenitors has not been elucidated. C/EBPε however has been implicated as a regulator since induced expression of C/EBPε32 in the myeloid leukemia cell line U937 induces pro-LL-37 mRNA expression (Gombart, et al. 2001).

Recently it was demonstrated that the hormonal form of vitamin D₃, 1,25(OH)₂D₃, is a strong inducer of pro-LL-37 expression in monocytes/macrophages (Gombart, et al. 2005, Wang, et al. 2004).

Many cell lines respond to 1,25(OH)₂D₃ with an increase in transcripts for LL-37 (Gombart, et al. 2007). Both 1,25(OH)₂D₃ and vitamin A (all trans-retinoic acid; ATRA) are differentiators of hematopoietic cells (Bastie, et al. 2004). A major function of 1,25(OH)₂D₃ is to control calcium and phosphorus homeostasis and thereby skeletal development (DeLuca 2004). The observation that a number of patients with SCN experience osteoporosis/osteopenia (Carlsson and Fasth 2001, Welte, et al. 2006) urged us to investigate whether 1,25(OH)₂D₃ may have a role in the defective pro-LL-37 synthesis.

We describe here that the lack of protein could not be explained by gene or promoter mutations. The abrogated protein levels were accompanied by low transcript levels, pointing at suppressed transcription or decreased mRNA stability. Epstein-Barr virus (EBV) transformed B-cells from one patient with SCN and one healthy sibling was included to investigate if the cells from the patient were responsive with regard to pro-LL-37 synthesis. B-cells are known to produce low levels of pro-LL-37 transcripts (Agerberth, et
al 2000), even though they do not employ the same transcription factor programs as that governing granulocytic maturation. Both 1,25(OH)\textsubscript{2}D\textsubscript{3} and ATRA induced expression of pro-LL-37 in the cell lines, demonstrating that the gene for LL-37 (CAMP) is functional in patients with SCN.

The CAMP gene can respond to other differentiation signals in other cell types, for instance to sodium butyrate in epithelial cells of the colon (Hase, et al 2002). We could however not find any elevated LL-37 synthesis in sodium butyrate incubated EBV transformed B-cells (unpublished observations).

In vitro stimulation was similarly performed using bone marrow derived granulocytic precursors from patients and from a healthy individual. The granulocytic precursors did not produce pro-LL-37 in the absence of vitamins, as opposed to the control cells (see below). 1,25(OH)\textsubscript{2}D\textsubscript{3} augmented the expression of pro-LL-37 in cells from patients with SCN to the same level as that of control cells. ATRA did not, in contrast, stimulate pro-LL-37 production. A combination of ATRA and 1,25(OH)\textsubscript{2}D\textsubscript{3} abrogated the 1,25(OH)\textsubscript{2}D\textsubscript{3} induction.

The systemic levels of 1,25(OH)\textsubscript{2}D\textsubscript{3} originates from conversion of 25(OH)D\textsubscript{3} by 25(OH)D\textsubscript{3}-1α-hydroxylase that takes place in the kidney and ends up in the blood stream. In addition, 1,25(OH)\textsubscript{2}D\textsubscript{3} can also be produced locally (Stoffels, et al 2006). Stimulation of granulocytic precursors with inactive 25(OH)D\textsubscript{3} induced pro-LL-37, demonstrating that the local conversion to the hormonal form is functional as well. In addition, the concentration of 25(OH)D\textsubscript{3} and 1,25(OH)\textsubscript{2}D\textsubscript{3} systemically was not altered in patient serum although it was in the lower range, which may have an impact on skeletal development. A low dose of the hormonal form of vitamin D\textsubscript{3} was
orally administered to one patient with SCN but no induction of pro-LL-37 could be detected in plasma after treatment.

Our current interpretation of the data is that 1,25(OH)\(_2\)D\(_3\) drives the granulocytic precursors towards monocytes and thereby LL-37 production as monocytes are known to respond to 1,25(OH)\(_2\)D\(_3\) by pro-LL-37 production (Gombart, et al 2005, Wang, et al 2004). ATRA, in contrast, overruns 1,25(OH)\(_2\)D\(_3\) signaling and directs precursors towards neutrophil lineage (Bastie, et al 2004). The neutrophil precursors can probably not respond to 1,25(OH)\(_2\)D\(_3\) stimulus. Accordingly, we were not able to identify "classic" neutrophil precursors producing LL-37 among the stimulated granulocytic precursors.

EBV transformed B-cells, but not granulocytic precursors, responded to ATRA with upregulation of pro-LL-37. ATRA probably induces a differentiation signal in B-cells which results in pro-LL-37 as a secondary effect of the differentiation, and not by the vitamin D receptor (VDR) binding to the vitamin D binding site (VDRE) present in the promoter of CAMP. In line with this idea is that the cell line U937 fail to respond to ATRA with an upregulation of pro-LL-37, while 1,25(OH)\(_2\)D\(_3\) stimulation resulted in a strong induction (unpublished results).
FROM THE IDENTIFICATION OF A NEW FAMILY OF MURINE ANTIMICROBIAL PEPTIDES TO THE FUTURE IMPLICATIONS IN THE HUMAN SETTING

Paper IV

The first cryptdin (mouse α-defensin) was isolated at protein level in 1992 (Ouellette, et al. 1992), and since then a total of six cryptdin peptides has been identified. In 1990 Andre Ouellette and John Lualdi described a new family of transcripts coding for putative antimicrobial peptides in mouse small intestine (Ouellette and Lualdi 1990). They named the mRNA that they found "cryptdin related sequences", based on the homology to the transcripts of cryptdins (Ouellette, et al. 1989). Deduced protein sequences from CRS and cryptdin mRNA revealed a highly homologous pro-part but a highly diverged c-terminal part.

We separated protein extracts from whole small intestine of C3H/HEN mice based on hydrophobicity and identified fractions with antibacterial activity which could not be assigned to known antimicrobial peptides. Six different CRS4C peptides were detected in the region, at comparable protein amounts to the cryptdins, by means of MALDI-TOF mass spectrometry and Edman degradation analysis. Isolated CRS4C peptides contained nine cysteines contrary to the six making up the three disulfide bonds characteristic for defensins. CRS4C peptides were found in both homo- and hetero dimer form (see below). The peptides were assigned CRS4C-1, -2 and -3, based on amino acid divergence found in the processed peptide. CRS peptides of two different lengths were detected, most probably as a result of MMP7 cleavage, similar to the cryptdins (Wilson, et al. 1999).
Cloning of CRS4C peptides from C3H/HEN mice did not reveal six transcripts that could code for the peptides found, but rather 19 closely related mRNA sequences. The deduced sequences coded for 17 different pro-CRS4C sequences. Many of the sequences varied only in the pro-region. This pattern is similar to that for the cryptdins, where many more transcripts than proteins have been identified (Huttner, et al 1994). The wealth of cryptdin mRNA and CRS mRNA without corresponding peptides has however remained an enigma.

Covalent dimers of antimicrobial peptides are rare in vertebrates, but have been demonstrated in guinea pig (Yomogida, et al 1996), the tree frog *Phyllomedusa distincta* (Batista, et al 2001) and mRNA for one β-defensin in C57/BL6 mouse has been found that only codes for five cysteines (Morrison, et al 2002). In addition, mammalian PGRPs (peptidoglycan recognition proteins) that naturally form dimers, were recently identified as being antimicrobial (Cho, et al 2005, Lu, et al 2006). Many antimicrobial peptides create non covalent dimers in solution (Hill, et al 1991), and in such cases, dimerization has been suggested to aid in the formation of multimeric pores (figure 2) (Hristova, et al 1996).

The high content of cysteines in CRS peptides probably creates a high proteolytic stability (Maemoto, et al 2004). This was evident from our unsuccessful attempts to cleave the oxidized form of the peptide, when trying to elucidate the cysteine linkage (data not shown). We could demonstrate that different homo- and heterodimers had different antimicrobial spectra, suggesting for the first time that dimerization is also a way to increase the repertoire of intestinal antibacterial peptides.
CRS sequences can be divided into groups based on sequence similarities; CRS1C, CRS4C-1/2/3 and CRS4C-4/5/6, which in turn each have several members. The distinction is made on cysteine pattern and primary sequence. CRS1C peptides have 11 cysteines compared to CRS4C that have nine (or in one occasion 10).

<table>
<thead>
<tr>
<th>CRS1C</th>
<th>CRS4C-1/2/3</th>
<th>CRS4C-4/5/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRS1C-1/2/3</td>
<td>CRS4C-1a/e/f/h/j</td>
<td>CRS4C-1b/d/g/i</td>
</tr>
<tr>
<td>CRS1C-3/4</td>
<td>CRS4C-2a/b/c</td>
<td>CRS4C-3a/b/c</td>
</tr>
<tr>
<td>CRS1C-5</td>
<td>CRS4C-4/5</td>
<td>CRS4C-6</td>
</tr>
</tbody>
</table>

Different mouse strains express mRNA for different CRS peptides. In addition, many of the transcripts differ only in the sequence which is not coding for the processed peptide. This is the reason why several transcripts are grouped together for example CRS4C-1a/e/f/h/j.

In this table the CRS1C - and CRS4C-4/5/6 sequences are grouped together for convenience even though they all code for distinct peptides. Sequences from outbred swiss mice, 129/SVJ- and C57/BL6 mice are adopted from (Huttner, et al 1994, Ouellette and Lualdi 1990, Patil, et al 2004).

In paper IV, CRS4C-1/2/3 was detected both on mRNA and at a protein level in C3H/HeN mice. In this paper we describe cloning of CRS peptides from FVB mice identifying novel CRS4C-1/2/3 and CRS1C sequences. As displayed in the table above, different CRS transcripts have been detected in different mouse strains. This is in accordance with the positive selection pressure that has been described for the defensin locus (Maxwell, et al 2003, Patil, et al 2004). Wild mice from different parts of the world would be of interest to study when it comes to CRS repertoire in relation to microbial challenge.

A hypothesized reason for the divergence between species is that mice has complemented the lack of α-defensins in neutrophils (Eisenhauer and Lehrer 1992) by expressing an additional family of antimicrobial peptides from Paneth cells to broaden the antimicrobial effect. The divergence of the defensins may also have relevant implication in human disease as a correlation between β-defensin gene copy number and colonic Crohn’s disease has been demonstrated (Fellermann, et al 2006).
A real-time quantitative PCR assay was developed that measures the absolute copy number of different CRS peptides. The mRNA expression levels were compared to that of other Paneth cell products and the results obtained with qPCR were confirmed at a protein level. The results demonstrate that CRS peptides are expressed in the same high abundance as the cryptdins. The results were in line with the results obtained using Edman degradation from Paper IV.

Unexpectedly, CRS4C-1/2/3 and CRS1C were found to be differently regulated in the gut. The CRS1C transcripts were expressed at a high level throughout the small intestine while CRS4C-1/2/3 increased more than 10 000 fold going from the duodenum to the ileum. Cryptdin 4 displayed a 10 fold difference using the same assay. The gradual expression pattern for cryptdin 4 has been noted previously (Ouellette, et al 1999). The local concentration of the antimicrobial peptides may have implications for the local homeostasis of the microbiota, considering the vicinity to the highly colonized caecum.

Andreau and Van Es demonstrated that the Paneth cell α-defensins are regulated by the transcription factor TCF-4 (Andreu, et al 2005, van Es, et al 2005). Since then, the general idea has been that all Paneth cell α-defensins are regulated in the same way. The divergence in expression pattern between CRS4C (and cryptdin 4), as opposed to the other cryptdins, points at yet another regulatory mechanism. A bioinformatic approach to analyze promoter sequences for CRS and cryptdin sequences did not however reveal a confirmative result, grouping CRS4C and cryptdin 4 in one group and the other cryptdins and CRS1C in another group (unpublished results).

Recently, HD5/6 was demonstrated to have a gradual increase in the small intestine (Wehkamp, et al 2006), although the difference was not as drastic as that for CRS4C. Elucidating the regulatory factors governing CRS4C expression is therefore of potential interest when elucidating the pathomechanism of Crohn’s ileitis displaying suppressed HD5 expression.
FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Paneth cell derived antimicrobial peptides probably play an important role in limiting and shaping the normal flora in addition to maintaining a sterile environment in the small intestinal crypt. The microflora of a healthy individual in turn protects against potential pathogens by secretion of antibacterial compounds and it physically occupies areas that otherwise would be available for colonization.

We have demonstrated that the Paneth cells of the distal mouse small intestine express substantially higher amounts of antimicrobial peptides as compared to the proximal part. The difference was most prominent for the CRS4C group of transcripts. The higher distal production of antimicrobial peptides most likely limits ascending bacteria from the heavily colonized colon. In addition to direct bactericidal mechanisms, CRS peptides may have other potential protective functions including anti-viral- or anti-fungal effects, wound healing/angiogenic roles, inducers of mucin release, modulation of Chloride ion secretion and immuno-stimulatory effects.

The gradual increase in expression indicates that novel regulatory factors are present, governing Paneth cell antimicrobial peptides. Transcription factors and factors inhibiting transcription that have longitudinal expression are now of potential interest in our search for regulators. The elucidation of the regulatory mechanisms can potentially, in addition, unravel the underlying defects resulting in Crohn’s ileitis, which has been associated with reduced Paneth cell antimicrobial peptides.

The question still remains whether CRS1C is present on the protein level, if they forming dimers, or if they are merely pseudo-genes. The dimeric nature of the CRS peptides in three dimensional space is of interest since it can aid in the elucidation of their precise mechanism of killing and in the search for potential other functions.
We have further noted that different mouse strains exhibit different repertoires of CRS peptides, which many lead to alterations in the diversity of the microflora. This may in turn explain differences in susceptibility of different mouse strains to microbial challenge. The association between antibacterial compounds and microflora will get one step closer to being elucidated by the rapidly emerging availability of total DNA sequencing.

Neutrophil infiltration is a hallmark of the gingival epithelium, especially during infection/inflammation. In the addition to the beneficial anti-pathogenic effects of their presence, they also cause tissue destruction. The balance between the two sides is probably of importance both for the composition of the oral microflora and for the anti-infectious and anti-inflammatory response.

Patients with SCN, independent of inheritance pattern, frequently present with severe juvenile periodontitis and elevated infection frequency, which most likely arises as a consequence of defects in their antibacterial capacity. The SCN phenotype, deficiency of pro-LL-37 in neutrophils and plasma, points at a common mechanistic denominator resulting in SCN. It remains to be seen if the lack of LL-37 is a direct- or indirect effect of genetic alterations. As, for instance, the transcription factor LEF-1 has been reported deficient in a cohort of German patients and its association to pro-LL-37 deficiency is of potential interest.

We aimed at modulating the expression of LL-37 in granulocytic precursors which as an end-result would increase expression. The pro-LL-37 expression in bone marrow precursors could in vitro be augmented by vitamin D₃ but oral administration of a low dose of vitamin D₃ to one patient did not increase the pro-LL-37 level in plasma. The 1,25(OH)₂D₃ induction of CAMP, could in theory respond in the same way as that for G-CSF where physiological administrated concentrations are not enough to have a stimulatory effect, but rather need pharmacological concentrations. The vitamin D₃ administration could therefore still be considered a potential complement to G-CSF treatment.
METHODOLOGY

Immuno-identification

Proteins, i.e., antigens, can be detected by many means. We have used Western blot, ELISA (enzyme-linked immunosorbent assay), immunohistochemistry and flow cytometric analysis (FACS, see next paragraph). Western blot was mainly used since it identifies the presence of an antigen and predicts the weight of the molecule. This method was used in paper I, II, III and V. The advantage of an ELISA lies in the number of samples that can be processed simultaneously. This method is optimal if the identity of the molecule is known and the assay is well established. ELISA was used in paper IV when macrophage inflammatory protein (MIP-2, the IL-8 homologue of mouse) was measured. Immunohistochemistry was applied in paper IV to identify the tissue distribution of the CRS4C antigen.

Cell population separations

Cell population separation was applied to hematopoietic cells. An initial dextrane sedimentation step was included to reduce the number of erythrocytes from blood and bone marrow. Subsequently, Lymphoprep and Percoll were used to separate different forms of leucocytes in blood and bone marrow, respectively. Granulocyte precursor (GP) cell populations were enriched for by Percoll density centrifugation in Paper III. GP cells were immediately either seeded into wells or subjected to FACS. Monoclonal antibodies against CD11b and CD13 surface antigens were used to identify four stages of myeloid differentiation; CD13+/CD11b- (promyelocytes), CD13-/CD11b- (myelocytes), CD13+/CD11b+ (metamyelocytes), and CD13+/CD11b+ (band cells/granulocytes) as in (Orfao, et al 2004). Cells were collected in a CD11b low and a CD11b high population and subjected to RNA isolation.

Gene amplification

Total RNA was enriched using both differential centrifugation techniques (paper IV and V) and membrane based (paper III) methods. Both techniques efficiently remove traces of DNA that can interfere in the subsequent analyses. Reverse transcriptase PCR (RT-PCR), cloning and sequencing was performed with standard procedures. The clones generated were used both to assess the identity of the cDNA and as an internal standard for quantitative PCR. Quantification of gene products were, in this thesis, accessed using four different methods mentioned in the order of increasing accuracy; 1) standard PCR, 2) semi-quantitative PCR with cyber green using laser detection, 3) semi-quantitative PCR with probe binding and laser detection (Taqman©) 4) quantitative PCR using laser detection and internal standards. Semi-quantitative PCR with a probe based method, as opposed to other methods, increase the gene specificity by a third hybridization molecule. The first three
methods were accessed with external standards i.e., a house-keeping gene. The fourth method, using internal standards, is the most accurate. This method provides copy numbers of the cDNA / 10 ng of total RNA. The disadvantage is the time consuming cloning step for each product. The fourth method was used in paper V when the different gene products were compared.

Cell cultures

The Epstein-Barr virus (EBV) can infect human B-cells in vitro, transforming them into permanently growing lymphoblastoid cell lines. Lymphoblastoid cell lines from one patient with SCN and a healthy sibling was a generous gift from Hans Boman and Ingemar Ernberg, Karolinska Institutet. Stimulation of lymphoblastoid cell lines and bone marrow cells with vitamins was usually performed for four or five days since protein signal was not seen earlier than three days. Bone marrow cells were cultured in the presence of autologous bone narrow plasma.

The intestinal epithelial cell line m-ICCl2 was grown, as in (Bens, et al 1996), to a polarized and differentiated state for six days before experiments. Cytotoxicity-, immunostimulation - and LPS neutralization experiments were performed on m-ICCl2 cells. Standard lactate dehydrogenase release assay (Invitrogen), hemoglobin - and MIP-2 concentration was used as read out systems.

Protein extraction/ protein modifications

Neutrophils were lysed by sonication in the presence of a high concentration of a protease inhibitor cocktail (10x of Complete, Roche). The lysed neutrophils were frozen at -80 °C together with Western blot sample buffer until further use.

Ground mouse small intestine was enriched for peptides and small proteins by 60 % acetonitrile-1% trifluoroacetic acid (TFA) precipitation at four degrease. The extract was thoroughly homogenated, cleared by centrifugation, lyophilized, rehydrated in 20 % ethanol, cleared by centrifugation and the extract was frozen at -80 °C until further use.

Presence of CRS4C dimers in the small intestinal extract was confirmed using dithiotreitol (DTT) as reducing agent. Extraction, as above, in the presence of iodacetamide was performed to exclude that the extraction procedure induced the dimerization. Dimerization of synthetic CRS4C was induced with 5 min of oxygen gas followed by 24 hours at 22 °C with constant stirring in 50 mM tris-HCl pH 8.

Reverse phase high performance liquid chromatography

Small intestinal extract was initially separated based on hydrophobicity using a reverse phase column (C18, Vydac) in a gradient of acetonitrile. Peptides/proteins were detected at 214 nm, collected fractions and antimicrobial activity was measured with the indicator strain *Bacillus*
megaterium in a thin layer assay, see below. A re-chromatographic step was with another brand of C18 (ACE), utilizing the fact that difference in performance between brands of reverse phase columns can be as prominent as changing to a C4/C8 column. Separation of synthetic CRS4C peptides was with C18 (ACE) using a steeper gradient.

Mass spectrometry

MALDI-TOF (matrix assisted laser desorption/ionization time of flight) mass spectrometry and Edman degradation was used to assess CRS4C mass identities. Edman degradation N-terminally identifies proteins, and in addition computes the protein concentration by means of quantification of released amino acids.

Antibacterial assays

A colony forming assay and an inhibition zone assay were used to determine the bacterial killing capacity of the peptides. The antibacterial activity in terms of lethal concentration was measured from serial dilutions in a thin agar plate assay, as in Hultmark et al (Hultmark, et al 1983).
LITERATURE CITED


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≈Jenny