First Line of Defense in Early Human Life

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Innate antimicrobial peptides are considered to play an important role in host defense against microbial invasion. They are expressed in a wide variety of organisms. In the case of human beings, defensins and the cathelicidin LL-37 appear to be the major microbicidal peptides. With respect to human neonates, only few investigations have been performed in this context, revealing the presence of α-defensins and LL-37 in neutrophils and vernix caseosa. In addition, β-defensins are present in tracheal aspirates and breast milk, whereas LL-37 has been detected in the skin of the newborn baby. During recent years, immunomodulatory activities such as chemotaxis have emerged as important functions of antimicrobial peptides. Thus, these innate effectors may work synergistically to provide a first line of defense against infection, as well as to promote interactions between the innate and adaptive immunity in newborn infants.

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The transition of the fetus from the womb into the open air is not only one of the most stressful events during life, but is also usually associated with the first challenge of microbial invasion. Antimicrobial peptides are of ancient evolutionary origin and exert key influences on host defense. These peptides have been isolated from a variety of human tissues, including neonatal tissues probably supporting newborn infants to thrive in the extrauterine world. Recent experimental evidence concerning the significance of antimicrobial peptides associated with primary host defense in vivo is convincing. Of the antimicrobial peptides identified to date, the defensins are the most abundant in humans.

The newborn infant requires efficient host defense against infection as it emerges from the sterile environment in the womb into a world full of potential hostile pathogens. There has been limited knowledge on the primary immune and anti-infective systems in fetus and newborn, but the adaptive immune system, relying on B and T lymphocytes, is known to be immature due to shortage of antigen exposure. However, passive transport of immune components, including immunoglobulins, from the mother is well documented.

Recent studies confirm the presence of innate defense effectors in the fetus and the newborn infant, and these innate components may represent the main defense capacity of neonates. The present review focuses primarily on the antimicrobial peptides defensins and LL-37 as effectors of innate immunity in human neonates.

Human Antimicrobial Peptides

Mammalian antimicrobial peptides are classified into two large families: the defensins and the cathelicidins. The defensins, which can further be divided into two main subgroups, α- and β-defensins, are a family of 3- to 4-kDa cationic peptides, forming a triple-stranded β-sheet structure with three intrachain disulfide bridges, where α- and β-defensins differ in the bridging of their conserved cysteines.

Six human α-defensins have been characterized, including human neutrophil peptide 1 to 4 (HNP1-4) mainly found in neutrophils and human defensins 5 to 6 (HD5-6) expressed by Paneth cells in the small intestine. The four β-defensins (HBD1-4) so far characterized are expressed primarily by epithelial cells.
Genomic analysis has recently revealed 28 putative genes for human β-defensins, although it is not yet known whether all of them are functional.\textsuperscript{17, 18}

The cathelicidins have been thought to be expressed only in mammals.\textsuperscript{7} However, recently, a cathelicidin was identified in hagfish.\textsuperscript{19} This family of highly variant antimicrobial peptides contains a conserved proregion designated cathelin with a variable antimicrobial C-terminal domain, which is cleaved off by processing enzyme(s), liberating the active antimicrobial peptide.\textsuperscript{7} LL-37 is a 37-residue peptide with an amphipathic α-helical conformation, which is critical for its broad antimicrobial spectra and is the only member of the cathelicidins present in humans.\textsuperscript{20, 21}

**Mechanism of Action**

The cationic properties of antimicrobial peptides are essential for their affinity to the negatively charged microbial membranes. Studies in vitro have shown that the peptides are able to kill Gram-positive and Gram-negative bacteria, fungi, parasites such as trypanosomes and plasmodia, certain enveloped viruses, and even cancer cells.\textsuperscript{22} The interaction of antimicrobial peptides with models of bacterial membranes has been extensively studied. Most of these peptides have affinity to the bacterial outer membrane with negatively charged phospholipid headgroups, and it has been suggested that their hydrophobic properties can integrate into the bacterial membranes, resulting in destabilization of the membrane with subsequent lysis of the bacteria.\textsuperscript{23} In contrast, the outer layer of the membrane of the host cells is composed primarily of lipids with no net charges, leading to weaker affinity between these membranes and antimicrobial peptides.\textsuperscript{2}

**Expression and Function of Antimicrobial Peptides in Neonates**

Multiple antimicrobial peptides are expressed by many organs in the human body (Table 1). α-Defensins are present primarily in neutrophils, but also in Paneth cells of the small intestine. The β-defensins are expressed primarily in epithelial tissues such as the skin, the respiratory and gastrointestinal tracts, the urogenital system, but also in the kidney, the pancreas, and the placenta.\textsuperscript{13, 24-25} The α-defensins and β-defensin-1 (HBD1) are constitutively expressed, whereas the three β-defensins, HBD2 to 4, are inducible.\textsuperscript{4} LL-37 is encoded by the CAMP (cathelicidin antimicrobial peptide) gene on chromosome 3p21.3,\textsuperscript{26} and is widely distributed throughout the epithelial linings.\textsuperscript{27} In addition, LL-37 is stored in both neutrophils and specific mononuclear cells,\textsuperscript{28} and works synergistically with lactoferrin and lysozyme.\textsuperscript{29, 30} Although the distribution of these microbicidal peptides in infants may be similar to that in adults, relatively

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<td><strong>Peptides</strong></td>
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<td>α-Defensins</td>
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few investigations have been performed in human neonates.

**Neutrophils**

Neutrophils are the first cells recruited to sites of infection and inflammation, where they play a major role in host defense, utilizing a variety of first-line antimicrobial action. Of the different peptides stored in the granules of human neutrophils, α-defensins (HNP-1-3) appear to be the major bactericidal components, accounting for 5-7% of the total cellular proteins in these cells. Lactoferrin, secretory phospholipase A2, lysozyme, and LL-37 are examples of other bactericidal protein/peptides constituents of neutrophils. The fourth α-defensin (HNP-4) is also found in azurophile granules of neutrophils but at lower concentrations than HNP1 to 3. In response to stimulation of neutrophils, these effectors are activated within vacuoles of the cell or released into the bloodstream and cooperate in killing microbes.

Recruitment of neutrophils usually occurs at the time of birth. Thus, the neutrophil function is very important for newborn infants at birth. Although there are many reports on neonatal neutrophilic activity, including immature adherence, chemotaxis, and phagocytosis, little is presently known about the activity of bactericidal peptides of these cells in neonates. However, a 55-kDa protein, the cationic bactericidal/permeability-increasing protein (BPI), exhibits remarkably specific cytotoxic activity toward Gram-negative bacteria, reflecting its high affinity for bacterial lipopolysaccharides. The average BPI level in neutrophils derived from cord blood of full-term newborns has been demonstrated to be much lower than in adult blood, although with some individual variations. In another study, the plasma levels of BPI in newborns with clinical sepsis were found to be higher than those of both healthy term infants and adults, whereas preterm infants had lower capacity to release BPI. The binding capacity of neutrophils, originating from neonatal cord blood, to lipopolysaccharide (LPS) was also weaker than that of neutrophils derived from adults. These findings suggest that some newborns, especially preterm infants, are at high risk to develop serious sepsis or meningitis, when exposed to Gram-negative bacteria. However, further investigations, including prospective clinical studies, are required to establish the contribution of BPI in the protection against Gram-negative bacterial infection at birth. Recently, administration of recombinant BPI to cord blood proved to exert a beneficial effect on newborns against Gram-negative bacterial infections.

LL-37 is expressed not only by human neutrophils, but also by B cells, NK cells, γδT cells, and monocytes. LL-37 has been detected in neutrophils that have migrated into the skin of the newborn, indicating an active role of this peptide in primary host defense for newborn infants.

**Skin**

Several antimicrobial peptides are expressed in adult human skin, where LL-37 and β-defensins appear to be important effectors for cutaneous defense against microbial invasion. HBD1 is produced constitutively, while HBD2, HBD3, and LL-37 are induced in inflamed skin, where LL-37 is upregulated in keratinocytes of the epidermis in certain inflammatory skin disorders. Both HBD1 and HBD2 exhibit bactericidal activity predominantly against Gram-negative bacteria such as *E. coli* and *P. aeruginosa*, whereas HBD3 is highly effective against the Gram-positive bacteria *S. aureus*. Skin, respiratory, and gastrointestinal tracts are sites for microbial invasion also in newborns. Interestingly, the expression of both LL-37 and HBD2 in the newborn foreskin is strong when compared with adult skin. These two peptides exhibit bactericidal activity against group B streptococcus (GBS), a frequent neonatal pathogen.

Erythema Toxicum Neonatorum (ETN), which is a well known benign, inflammatory skin disorder of neonates at early days after birth, contains several immune cells such as dendritic cells and eosinophils, expressing LL-37, while there is low expression in the normal skin of the newborns. At present, the etiology and physiological significance of ETN is unknown. However, these data suggest that, even though it might be in localized skin area, these cells and
LL-37 may serve as active skin protectors of newborn infants after birth.

Vernix caseosa (vernix) is a lipid-rich white substance that covers the skin of the fetus and the newborn. The integral composition and the physiologic role of vernix is gradually becoming evident. The characteristic composition of vernix suggests limited interactions with hydrophilic liquids which suggest that in uterus vernix acts as a hydrophobic barrier, waterproofing the fetal skin against amniotic fluid. Recently, we discovered that vernix contains several antimicrobial peptides such as HNP1 to 3, lysozyme, LL-37, and psoriasin. Notably, the mucous plug that is formed at the cervix also contains some of the same antimicrobial peptides. Furthermore, in amniotic fluid, similar microbicidal peptides are present, ie, HNP1 to 3, lysozyme, and LL-37, that partly could be derived from the fetal skin detachments and respiratory elements. These findings suggest that vernix works as a natural biofilm for obstruction of microbial passage, interacting with amniotic fluid, and hence contributes to skin defense of the fetus and the newborn against microbial invasion at birth.

Airway

The first mammalian epithelial antimicrobial peptide was isolated from bovine tracheal mucosa, and subsequently characterized as a β-defensin with inducible properties. Several investigations have focused on antimicrobial peptides in the respiratory tract. HBD1 to 3 and LL-37 are found in the epithelial lining of the airway and play a pivotal role in intrinsic mucosal immune defense. HBD4 transcripts have also been detected in human lung, however with a low expression.

Pulmonary infection is common in newborns and often takes a serious clinical course with ventilator care. In general, the more premature the newborn is, the stronger severity of disease. The direct activity of antimicrobial peptides to microbes as the first line of defense in neonatal respiratory system is thought to be essential, but little is known about its intrinsic role in the newborn infant. There is limited knowledge about the developmental regulation of antimicrobial peptides in the human fetus. Although HBD1 is developmentally regulated postnatally in lung parenchyma, the expression of HBD1 could not be detected by the sensitive RNase-protection assay in samples from fetal tissue at 15 and 22 weeks of gestation. In another study, it was demonstrated that HBD1 to 2 and LL-37 were detected by an antigen capture assay in neonatal tracheal aspirates during ventilator support of term and preterm infants. The concentrations of the peptides were similar in all infants studied, and the levels of the peptides were enhanced on infection and correlated with inflammatory parameters such as IL-8 and TNF-α. Recently, we have detected bactericidal activity by an inhibition zone assay in bronchoalveolar lavage of infected newborns after birth, and this activity was shown to correlate positively with systemic inflammation. These data suggest that rapid response or production of antimicrobial peptides seems to occur after microbial challenge after birth.

Gastrointestinal Tract

Human enteric α-defensins (HD5 and HD6) are expressed in Paneth cells. Both HD5 and HD6 seem to be developmentally regulated, since their transcripts are detected by PCR at the gestation of 13.5 weeks in the small intestine and for HD5 mRNA also in the colon. At 17 weeks gestation, the expression of HD5 is only located in the small intestine. By Northern blot analysis and immunohistochemistry, the expression of both these enteric peptides was detectable at 24 weeks gestation, confirming the presence of HD5 to 6 in the Paneth cells of the fetus. At 24 weeks gestation, both the number of Paneth cells and the level of mRNA are significantly lower than those in adult, which may predispose preterm newborns to serious enteric infection. Necrotizing enterocolitis (NEC) is a critical cause of morbidity and mortality among preterm infants. The etiology is still unclear and enteric infections are generally involved and associated with a premature local innate defense. The increase in both enteric defensin mRNA expression and Paneth cell number was demonstrated in subjects with NEC compared with a control group. The upregulation of the expression of enteric defensins may be a consequence of the pathologic process of NEC. In colonic biopsies
derived both from adult and children with *Shigella* infection, epithelial downregulation of both LL-37 and HBD1 was demonstrated. Interestingly, LL-37 is upregulated in colon epithelial cells by short chain fatty acids, including butyrate, which may have potential in therapeutic use for this disorder. Butyrate in the colon originates from fermentation of fibers and hence the normal flora is responsible for its production. These results suggest that antimicrobial peptides might be key effectors in a complicated situation in the human enteric tract in which pathogens and commensal bacteria interact with each other.

**Human milk**

It is well known that breast milk contains several antiinfective components, such as lactoferrin, the bifidus factor, lysozyme, lactoperoxidase, and oligosaccharides, which are suggested to protect newborns against a variety of infection. Lactoferrin, an iron-binding protein abundant in human milk, absorbs enteric iron in the presence of IgA and bicarbonate, thus preventing microbes from obtaining the iron needed for survival. Recently, the expression of HBD1 was found in human breast milk and mammary gland epithelia, which could provide mucosal defenses in both mother and newborn. The HBD1 expression is usually documented to be constitutive. However, higher HBD1 immunoreactivity was demonstrated in breast tissue during lactation compared with that during nonlactation. Furthermore, enhanced expression was detected in the urinary tracts of pregnant women. These data suggest that the expression of HBD1 may be upregulated by hormones during pregnancy. Although HBD1 is highly effective against Gram-negative bacteria, it may work synergistically with other antibiotic peptides against a broad range of microbes such as *Staphylococcus aureus*, which is a main cause of lactational mastitis. Further studies remain to be conducted to clarify the regulation of HBD1 expression in breast milk, and the influence of breastfed-mediated antimicrobial peptides in newborn during or after lactation.

**Multifunction and Link to Disease**

Additional functions for antimicrobial peptides other than direct microbicidal activity have been established in recent years, such as chemotaxis, histamine release from mast cells, wound repairing, and apoptosis (Fig 1). Chemotactic activity for T cells has been reported for HNP1 to 2, with induction of IL-8 synthesis. The HBD2 expression is induced by IL-1α, IL-1β, and TNFα stimulation, with activation through NF-κB (nuclear factor κB), and HBD1 to 2 attracts CD4 T cells and dendritic cells through interactions with the chemokine receptor CCR6. LL-37 is able to attract CD4 T cell, monocytes, and neutrophils via the formyl peptide receptor-like 1 (FPRL-1). Thus, antimicrobial peptides are multifunctional with activi-
ties that enhance our defense barrier with reference to both adaptive and innate defenses.

It is clinically important to investigate the alteration of antimicrobial peptides expression associated with disease. The knock-out mouse for the CRAMP gene, the homologue to the human CAMP gene encoding LL-37 is very sensitive to skin infections by Streptococcus group A. In human, the Kostmann syndrome, which is a severe congenital neutropenia from the first day of life and is usually fatal due to serious bacterial infections, can be treated with infusions of granulocyte-colony stimulating factor (G-CSF). A recent study revealed that LL-37 was missing both in granulocytes and saliva of Kostmann patients. One patient, who after a bone-marrow transplantation restored LL-37, did not exhibit severe periodontal disease, a common feature in Kostmann patients. LL-37 might be one relevant component to the defects in Kostmann syndrome and can be considered for therapeutical use. Atopic dermatitis is a common allergic skin disorder, often manifested with cutaneous infections by Staphylococcus aureus. Decreased expressions of both HBD2 and LL-37 in the skin lesions of atopic dermatitis were demonstrated compared with the inflamed lesions of psoriasis. This finding may explain the high susceptibility of S. aureus in the skin of atopic dermatitis.

It is likely that antimicrobial peptides are intrinsic components of barrier defenses in early human life like they are in adults. Future research must consider defects in expression and activity of innate antimicrobial peptides as one possible cause for immunological disorders that affect the fetus or the newborn infant. There is limited knowledge on variations in the levels of antimicrobial peptides related to innate defenses. The peptides represent first line defenses, and low expression levels might be linked to susceptibility to infections that are often fatal for newborns. Furthermore, the immune modulatory activities of the peptides might be connected to adjuvant activity, where the peptides might be initiators of important immune functions to train the system for later challenges.

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First Line of Defense in Early Life

309
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