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Abstract

Breast milk possesses inherent antimicrobial properties that have been attributed to several diverse molecules. Recently, antimicrobial peptides belonging to the cathelicidin gene family have been found important to the mammalian immune response. This antimicrobial is expressed is several tissues and increased in neonatal skin, possibly to compensate for an immature adaptive immune response. We hypothesized that the mammary gland could produce and secrete cathelicidin onto the epithelial surface and into milk. Human cathelicidin hCAP18/LL-37 mRNA was detected in milk cells by PCR. Quantitative real time PCR demonstrated an increase in relative expression levels at 30 and 60 days after parturition. Immunohistochemistry of mouse breast tissue identified the murine cathelicidin CRAMP in lobuloacinar and ductules but not large ducts. Western blot analysis of human milk showed LL-37 was secreted and present as in the mature peptide form. The antimicrobial activity of LL-37 against S. aureus, Group A Streptococcus, and enteroinvasive E.coli O29 in the milk ionic environment was confirmed by solution colony forming assay using synthetic peptide. These results indicate that cathelicidin is secreted in mammary gland and human milk, has antimicrobial activity against both Gram positive and Gram negative bacteria, and can contribute to the anti-infectious properties of milk.

Key Words:
Defensins, Cathelicidins, Breast Milk, Infection

Introduction

Antimicrobial peptides are found throughout nature and are important for the immune defense of plants, insects and animals (1). Most of these molecules have a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses. In mammals, peptides from the cathelicidin family assist the antimicrobial efficacy of neutrophils, macrophages, and mast cells (2-6). In addition, antimicrobial genes of the defensin and cathelicidin families are produced by epithelia in response to injury of lung, gut, urinary bladder, oral mucosa and skin (7-9). At the interface with the external environment, these molecules serve as a rapid first line defense for inhibition of microbial proliferation and invasion.

Breast milk contains various anti-infectious materials such as immunoglobulins, cellular components, and cytokines. It is believed that these molecules contribute to decreased morbidity and mortality of infants feed by breast milk (10, 11). Recently, peptide antibiotics have been isolated from human milk, providing additional evidence that multiple molecules can be involved in the immune defense of this material (12, 13). In other systems, the clinical consequences of antimicrobial peptide expression have been observed in patients with atopic dermatitis who are susceptible to infection and also lack the ability to increase the cathelicidin LL-37 and the defensin hBD-2 in response to inflammatory stimuli (14). Furthermore, patients with Kostmann syndrome, a rare inherited disorder characterized by frequent infections and neutrophil dysfunction, also have a deficiency in production and processing of the human cathelicidin LL-37(15). Such emerging clinical associations support the need to further explore the expression of antimicrobial peptides in specific aspects of the immune system.

The neonate produces increased levels of cathelicidin in the skin (16), suggesting that enhanced expression of a defense peptide may compensate for an immature immune defense system. In this investigation we evaluated expression of cathelicidins in milk to determine if the lactating breast expresses this molecule, thus providing the neonate with this natural antibiotic by an oral route and protecting mammary epithelia from infection. Our data show that cathelicidin is transcribed by cells of the mammary gland and that the human peptide LL-37 is secreted in its active form.

Materials and Methods

RNA and protein purification from human milk

Human milk was collected from healthy volunteers at Day-10, 15, 30, and 60 after parturition and cellular elements collected by centrifugation at 300g for 15 min at 4°C. For protein purification, 20 ml of human milk was mixed with 20 ml of 1% trifluoroacetic acid (TFA) in 60% acetonitrile (ACN), incubated overnight at 4°C, then spun 1000g for 15 min at 4°C for collection of supernatant. Supernatant was filtered (0.20 μm, low binding, Fisher, CA) and applied on to Sep-Pak C18 cartridges (Waters, Milford, MA) equilibrated with 0.1% TFA in doubly glass distilled water (DDW). Column was washed with 0.1% TFA, and bound material eluted with 0.1% TFA in 80% ACN. Eluted fractions were concentrated by lyophilization and resuspended in 100 μl of DDW. All samples were stored at –20˚C until use. Total protein concentration of the collected milk was evaluated by BCA assay (protein assay reagent, Pierce, Rockford, IL). Milk collection was done with informed consent and under approval of UCSD institutional review board protocol#021060XT.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from fresh cells and tissues using RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was prepared from 1μg of total RNA using RETROscript kit (Ambion, Woodward Austin, TX). PCR protocol for amplification of LL-37, human beta-defensin 1 (hBD-1), and human beta-defensin 2 (hBD-2) cDNA was as previously described (17). PCR amplification of LL-37 was performed with the forward primer (5'- GATAACAAGAGATTTGCCCTGCTG -3') and the reverse primer (5'- TTTCTCAGAGCCCAGAAGCCTG -3') for a 173 bp product. Amplification of 18S rRNA was done in parallel for all samples with QuantumRNA classic 18S internal standard kit (Ambion, Woodward Austin, TX). PCR protocol; denaturation 90°C for 10 min, followed by 35 cycles of amplification with denaturation at 94°C for 30 sec, primer annealing at 50°C for 30 sec, and extension at 72°C for 1 min.

Real-time quantitative PCR

Quantitative PCR was performed using a GeneAmp® 7700 Sequence Detection System from Perkin Elmer. 1 μl RT reaction was used in 23.5 μl SYBR® Green PCR Master Mix (Applied Biosystems,

Warrington, UK) and 0.25 µl of each10 µM primer described above. Thermal profile: 50 degrees 2 min, 95 degree 10 min, 40x (95°C 15 sec, 60°C 1 min). Results were analyzed using the Comparative Ct Method, where Ct is the number of cycles required to reach an arbitrary threshold (16).

Tissue sampling

For localization of CRAMP (mouse cathelicidin homologue) by immunohistochemistry, C57BL6 mice (Jackson Laboratory (Bar Harbor, ME)) were housed in a pathogen free barrier facility. Animal use was approved by the San Diego VA Subcommittee on animal studies, protocol 02-037. Following CO_2 euthanasia, tissue was immediately embedded in OCT compound. Mammary glands were microdissected for extraction and immunostaining. All specimens were stored it at –80˚C until use.

Immunohistochemistry

For CRAMP protein, rabbit anti CRAMP polyclonal antibody was used as previously described (18). 10 µm, tissue sections were immersed in PBS after 4% paraformaldehyde fixation for 10 min, and endogenous peroxidase activity blocked with a 30 min incubation in 0.3% H_2O_2 in methanol. Sections were blocked with 2% goat serum in PBS for 30 min, and incubated with primary antibody, rabbit anti CRAMP polyclonal antibody (0.8 µg/ml) in PBS, 3% bovine serum albumin for over night at 4°C. Signal was detected with Vectorstain ABC Elite Rabbit kit (Vector Laboratories, Birlingame, CA) and processed using an ABC kit (Vector Laboratories, Burlingame, CA). As a negative control, normal rabbit preimmune IgG diluted with PBS containing 3% BSA was used at the same protein concentration as the primary antibody. Sections were incubated in 0.02% diaminobenzidine with 0.05% H_2O_2 in PBS for 2 minutes and counterstained with Mayer's hematoxylin for 10 sec. The specificity of the primary antibody reaction has been confirmed in separate experiments by absorption of the anti-CRAMP with excess amounts of synthetic peptide.

Immunoprecipitation and Western-blot analysis

Rabbit anti LL-37 polyclonal antibody for immunostaing was derived against full-length LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES, QED Bioscience Inc, CA). Stock concentrations of antibody was 0.73 mg/ml. Chicken polyclonal antibodies against LL-37 mature form

and cathelin domain (CATH) were also used for re-probing, as previously described (19).

For immunoprecipitation, 100 µl of milk was mixed with 20 µl of anti LL-37 rabbit antibody, and incubated for 60min at 4°C, and then 20 µl of protein A-Agarose (Roche, Germany) added and incubated overnight at 4 °C. After centrifuging at 1000g for 2 min. the supernatant was removed and washed with 3 times with 120 µl of PBS (pH 6.5). Beads were then resuspended in 50 µl of tris glycine sample buffer with 10% β-mercaptoethanol, denatured at 85°C for 10 min, then centrifuged at 1500g for 5 min. SDS-PAGE was on a 4-20% tris-glycine polyacrylamide gel electrophoresis system. (Express gels, ISC BioExpress, Kaysville, VT). Gels were transferred to nitrocellulose membrane (OSMONICS, Westborough, MA), blocked (0.1% TTBS: 5% nonfat milk in 0.1% Tween 20 / tris buffered saline (TBS: 150 mM NaCl, 10 mM Tris Base, pH 7.4)) for 60 min at R.T., and probed with rabbit anti LL37 polyclonal antibody (1:5000 in the blocking solution) over night at 4°C. After washing membrane 3x with 0.1% TTBS, horseradish peroxidase labeled anti rabbit goat polyclonal antibody (1:5000 in the blocking solution) was incubated with the membrane for 60 min at R.T. Signal was developed in ECL solution (Western Lightning Chemiluminescence Reagents Plus, New Lifescience Products, Boston, MA) and exposed for 60 sec to X-ray film (Kodak).

For confirmation of the identity of the band detected by western blot, filters were stripped of antibody in (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM β-melcaptoethanol) at 50˚C for 30 min, then reacted with a chicken anti cathelin antibody (1:10000) and HRP-anti chicken IgY goat antibody (1:10000, Aves Labs, OR).

Measurement of LL-37 concentration in human milk

To estimate the concentration of LL-37, quantitative dot-blot analysis was performed. 10 µl of milk sample (Day-10) was compared to a standard curve of various concentrations of synthetic peptide applied onto nitrocellulose filters(14). LL-37 was detected as described for Western blot above.

Antimicrobial assay in milk

The antimicrobial activity of LL-37 was evaluated in an ionic environment similar to human milk by colony-forming unit assays performed with S. aureus (clinical isolate), Group A Streptococcus (NZ131, GAS), and enteroinvasive E. coli O29 as described (20). To duplicate milk but denature nature proteins, human milk (Day-10) was autoclaved, centrifuged at 10,000g rpm for 15 min, and supernatant

collected. This process was repeated 5 times until the color of the milk preparation was clear light yellow (autoclaved milk supernatant: AMS). Before analysis, concentration of the bacteria in culture was determined directly by plating different bacterial dilutions (At $A_{600,}$ 1.0 corresponded to $3.5x10^9$ / ml for S. aureus, $2.5x10^8$ and GAS, and $3.5x10^8$ for E. coli). Bacteria were washed twice with 10mM phosphate buffer and diluted to a concentration of $2x10^6$ cells/ml (S. aureus, GAS) or $2x10^5$ cells/ml (E. coli) in AMS and incubated for 2 hrs at 37˚C with various concentrations of LL-37 in 50 µl of 1x AMS or 0.1x AMS using wells of a 96 well round bottom tissue culture plate (Costar 3799, Corning inc., NY). Bacteria in AMS was then diluted from 10x to 10^5x, and each of 10 µl of those solutions was plated in triplicate on Todd Hewitt broth plate, for direct determination of CFU.

Results

Human cathelicidin hCAP-18/LL-37 and hBD-1 mRNA is present in human milk.

To determine if transcripts for the antimicrobial peptides hCAP18/LL-37, hBD-1, and hBD-2 were present in cellular elements of human milk, RT-PCR was performed on cells derived from breast milk collected at 10 to 60 days after parturition **(Figure 1)**. In these preparations, LL-37 and hBD-1 were detectable but hBD-2 was not.

Next, to quantitatively evaluate and confirm the expression of LL-37 seen in Figure 1, LL-37 mRNA levels were examined by quantitative real time RT-PCR. Compared to expression at Day-10, the abundance LL-37 in Day-30 and Day-60 milk cells showed a 12- and 15-fold increase. hBD-1 mRNA expression in Day-30 was about 5-fold higher compared to the level at Day-10 **(Figure 2)**.

Cathelicidin proteins are localized in mouse mammary glands and secreted in human milk

To further explore and define cathelicidin protein expression in the mammary gland, breast tissue from mice was examined for CRAMP by immunohistochemistry, and human breast milk peptides evaluated by Western blot. Abundant CRAMP was detected in the lactating lobular units of the mouse **(Figure 3a)**. The signal was diffusely located in the cytoplasm of lobuloacinar and ductules, but not in ducts **(Figure 3c)**. No signal was detected in tissues using IgG controls. **(Figure 3b, d)**.

The detection of cathelicidin protein in the murine breast tissue cell suggested that this antimicrobial peptide might also be present and secreted in active form in human milk. To study this, human milk (Day-10) was evaluated by Western blot and quantified by dot blot analyses with two antibodies independently derived against distinct domains of the cathelicidin proprotein; anti-LL-37 and anti-CATH. After immunoprecipitation, LL-37 was detected in human milk **(Figure 4a)**. In specific immunoprecipitates, a band at ca. 4.5 kDa corresponding to mature processed form of LL-37 was detected. Large molecular weight immunoreactive material was seen predominantly in unbound washed material from immunoprecipitates, possibly due to non-specific reactivity of rabbit IgG with

milk proteins, aggregation of LL-37 in a form not precipitable, or unprocessed and aggregated hCAP18/LL-37 also in a form not precipitable. To confirm a lack of the unprocessed precursor cathelicidin in milk, membranes were stripped and reprobed with chicken antibody specifically against the cathelin immature domain (CATH). No specific band was detected at the expected sized of 18 Kda for hCAP18/LL-37 **(Figure 4b)** although reactivity was detectable at high molecular weight in the immunoprecipitate. To quantitatively evaluate the concentration of LL-37 in milk, dot blot analysis was performed. LL-37 abundance was approximately 1 μmoles/21.7 μg of total milk protein (milk protein concentration: 764 μg/ml) or 35 μM (Figure 4c)..

LL-37 is a functional antimicrobial peptide in milk

To evaluate if LL-37 has antimicrobial activity in the ionic environment of the human milk, bactericidal assays were performed in an autoclaved milk supernatant (AMS) solution. GAS, S. aureus or E. coli incubated for 2 hrs at 37˚C in AMS **(Figure 5)**. GAS, S. aureus and E. coli were first incubated in AMS without addition of synthetic LL-37 peptide, to evaluate if AMS alone was antimicrobial. After 2 hrs incubation, 75% of GAS, 98% of S. aureus, 100% of E. coli survived in AMS, but did not proliferate. Since existing antibody to LL-37 does not neutralize bioactivity it was not feasible to directly evaluate the contribution of LL-37 to the antimicrobial activity observed in AMS. Furthermore, the lack of bacterial growth in this solution prevented determination of inhibitory activity. Therefore, the effect of supplemental addition of synthetic LL-37 on direct bacterial killing was tested **(Figure 5a)**. LL-37 was an effective antimicrobial in the milk environment but demonstrated increased cytotoxic activity against test microbes when AMS was diluted. 32 μM of LL-37 killed 77% of GAS in 1x AMS and 100% in 0.1x AMS **(Figure 5b)**. Against S. aureus, 32 μM of LL-37 killed 40% in 1x AMS and 99% in 0.1x AMS **(Figure 5c)**. Against E.coli, 32 μM of LL-37 killed 17% in 1x AMS and 100% in 0.1x AMS **(Figure 5d)**.

Discussion

Peptides with antimicrobial activity are found throughout nature (1). First isolated from mammalian granulocytes (21, 22), peptides such as the defensins and cathelicidins are present in intracellular granules where they can facilitate microbial killing by phagocytic cells such as neutrophils and mast cells (6, 23). The ability of these antibiotic gene products to inhibit microbial proliferation is also associated with the immune defense strategy of epithelia from organs such as skin, lung, gut and kidney. At the epithelial interface, production of antimicrobial peptides is thought to provide an important first-line of defense against infection. In this study we investigated if the cathelicidin antimicrobial peptide LL-37 is present in human milk and if the murine gene homologue CRAMP is expressed in lactating mouse mammary tissue. Results show that human cathelicidin hCAP18/LL-37 mRNA can be detected in cells shed into human milk for at least 60 days after parturition. This expression is coincident with the presence of hBD-1, a distinct mammalian antimicrobial peptide previously described in mammary epithelia (13, 24). Importantly, the hCAP18/LL-37 gene product was also demonstrated in soluble form in milk and present in its activated form as the peptide LL-37. This peptide was directly shown to have antimicrobial activity in aqueous solution derived from human milk. These observations suggest that production of cathelicidins by mammary tissue may be an important component of innate immune defense during lactation.

The detection of cathelicidin mRNA in the cellular elements of human milk was based on a sensitive RT-PCR approach. Milk leukocytes, mammary epithelial cells, or epithelial cells from the surface of the breast could conceivably contribute to the detection of transcripts by this technique. A previous study of milk epithelial cells has shown hBD-1 but not hBD-2 mRNA is present in these cells (24). Our findings are consistent with these results. Since hBD-2 was not detected, but is likely present in the surface keratinocytes of breast skin (25); it is unlikely that the origin of the detectable transcript is from surface skin contamination. Previously, hBD-2 mRNA was found in breast tissue by RNA dot-blot and in-situ hybridization (26). However, as in a prior study of hBD-1 expression in milk cells (24), we failed to detect the presence of hBD-2 transcripts. This apparent conflict may be due to instability of hBD-2 message. In addition, it is possible that the mammary expression of antimicrobial

peptides occurs at both a constitutive and inducible level. Under the present conditions, it appears hBD-1 and LL-37 are each constitutive products of the lactating mammary gland. Other epithelia show a large induction of cathelicidins and defensins in response to injury or bacterial challenge (18), a phenomenon that may have contributed to expression of hBD-2 in prior studies.

Lactating mice were used as an animal model to further explore the expression of cathelicidins in breast tissue. The mouse cathelicidin CRAMP is a close homologue of human hCAP18/LL-37 and has been found to correlate with human expression patterns in prior investigations (16, 18, 19). Furthermore, the availability of a knockout mouse deficient in CRAMP has enabled investigation of the biologic significance of cathelicidin expression in vivo (23). In the present study, CRAMP expression was found in the cytoplasm of lobuloacinar and ductule cells but not in ducts. This is distinct from prior reports of the expression of hBD-1 protein and hBD-2 mRNA in ductule epithelium. Unique expression patterns for defensins and cathelicidins are consistent with that observed in other tissues and may suggest distinct stimuli and targets for their gene products. In addition, cathelicidins and defensins have synergistic action as antimicrobials (27). Observation that both are expressed in the lactating mammary gland enables colocalization and the potential for enhanced immune defense.

In human milk, cathelicidin was detected exclusively in its activated peptide form. When first synthesized as a precursor of approximately 18 kDa, the hCAP18/LL-37 protein is thought to be inactive. Processing of full-length precursor cathelicidins has been associated with several different serum proteases including elastase and proteinase 3(28). After processing, the N-terminal cathelin protein has activity as both an antimicrobial and a protease inhibitor (29). This precursor protein was not detectable in human milk, although techniques used for detection of the precursor protein was not optimized in this system. Further work is required to confirm that unprocessed or the cathelin protein alone is not secreted from mammary tissue. On the other hand, LL-37 appeared relatively abundantly in milk at concentrations approaching 32μM. At this concentration, LL-37 synthetic peptide is a potent antimicrobial against a wide range of microbes. However, antimicrobial activity reported for natural occurring peptides is highly dependent on environmental conditions. Factors such as pH, sodium chloride concentration and binding to other macromolecules in the soluble solution are critical

determinants of the ability of antimicrobial peptides to interact with the microbial target membrane (30, 31). In order to assess antimicrobial activity in the ionic environment of milk, a heat-denatured milk solution was produced and antimicrobial activity against Staph. aureus or E. coli determined. Our results suggest that LL-37 has direct bacteriocidal activity on these bacteria in milk solution. Actual activity in vivo may be most relevant for bacterial growth inhibition or further augmented by the synergistic presence of other natural antimicrobial molecules in human milk such as IgA, lactoferrin and casein-k (12). Molecules such as lactoferrin are inactivated by heat sterilization (32), thus underestimating the potential antimicrobial effect of LL-37 observed here. Inhibitory activity from native LL-37, hBD-1 or other heat-resistant molecules in AMS may also have contributed to the growth inhibitory activity observed for AMS alone. Further work to specifically evaluate milk in the absence of these molecules will be required to definitively identify their contribution to milk's antimicrobial properties.

The presence of antimicrobial molecules in human milk may be important for protection of the mammary gland against infection and development of mastitis, protection of milk from microbial proliferation following secretion, or protection of the infant due to ingestion of natural occurring antibiotics(33). The ability of cathelicidins and defensins to directly confer protection against bacterial colonization of epithelial surfaces has been shown in gut, lung and skin (34). Therefore, it is reasonable to propose that these molecules may also provide a protection against mastitis. Possible protection to the neonate was suggested in studies of neonatal rat pups reared by artificial formula feeding. These pups have an increase in gut Enterobacteriaceae compared to those fed breast milk (35). This increase in colonization and bacterial translocation may be a consequence of a direct antimicrobial action of milk. Identification of additional candidate genes such as cathelicidins in breast milk provides further information for study of this important system. These data support the importance of antimicrobial peptides to immune defense and suggest further research is required to delineate the biological role of small cationic peptides in the protection of the mammary gland, the infant or both.

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References

1. Zasloff M 2002 Antimicrobial peptides of multicellular organisms. Nature 415:389-395.
2. Zaiou M, Gallo RL 2002 Cathelicidins, essential gene-encoded mammalian antibiotics. J Mol Med 80:549-561.
3. Panyutich A, Shi J, Boutz PL, Zhao C, Ganz T 1997 Porcine polymorphonuclear leukocytes generate extracellular microbicidal activity by elastase-mediated activation of secreted proprotegrins. Infect Immun 65:978-985.
4. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jornvall H, Wigzell H, Gudmundsson GH 2000 The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 96:3086-3093.
5. Tomasinsig L, Scocchi M, Di Loreto C, Artico D, Zanetti M 2002 Inducible expression of an antimicrobial peptide of the innate immunity in polymorphonuclear leukocytes. J Leukoc Biol 72:1003-1010.
6. Di Nardo A, Vitiello A, Gallo RL 2003 Cutting Edge: Mast Cell Antimicrobial Activity Is Mediated by Expression of Cathelicidin Antimicrobial Peptide. J Immunol 170:2274-2278.
7. Schutte BC, McCray PB, Jr. 2002 [beta]-defensins in lung host defense. Annu Rev Physiol 64:709-748.
8. Bals R 2000 Epithelial antimicrobial peptides in host defense against infection. Respir Res 1:141-150.
9. Ganz T 2002 Epithelia: not just physical barriers. Proc Natl Acad Sci U S A 99:3357-3358.
10. Lonnerdal B 2003 Nutritional and physiologic significance of human milk proteins. Am J Clin Nutr 77:1537S-1543S.
11. Goldman AS, Chheda S, Garofalo R 1998 Evolution of immunologic functions of the mammary gland and the postnatal development of immunity. Pediatr Res 43:155-162.
12. Liepke C, Zucht HD, Forssmann WG, Standker L 2001 Purification of novel peptide antibiotics from human milk. J Chromatogr B Biomed Sci Appl 752:369-377.
13. Jia HP, Starner T, Ackermann M, Kirby P, Tack BF, McCray PB, Jr. 2001 Abundant human

beta-defensin-1 expression in milk and mammary gland epithelium. J Pediatr 138:109-112. t&artType=abs&id=a109375&target=.

14. Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, Gallo RL, Leung DY 2002 Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med 347:1151-1160.
15. Putsep K, Carlsson G, Boman HG, Andersson M 2002 Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. Lancet 360:1144-1149.
16. Dorschner RA, Lin KH, Murakami M, Gallo RL 2003 Neonatal skin in mice and humans expresses increased levels of antimicrobial peptides: innate immunity during development of the adaptive response. Pediatr Res 53:566-572.
17. O'Neil DA, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T, Kagnoff MF 1999 Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. J Immunol 163:6718-6724.
18. Dorschner RA, Pestonjamasp VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, Agerberth B, Gudmundsson GH, Gallo RL 2001 Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. J Invest Dermatol 117:91-97.
19. Murakami M, Ohtake T, Dorschner RA, Gallo RL 2002 Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. J. Dental Research 2002; 81: 845-850..
20. Porter EM, van Dam E, Valore EV, Ganz T 1997 Broad-spectrum antimicrobial activity of human intestinal defensin 5. Infect Immun 65:2396-2401.
21. Lehrer RI, Ladra KM, Hake RB 1975 Nonoxidative fungicidal mechanisms of mammalian granulocytes: demonstration of components with Candidacidal activity in human, rabbit, and guinea pig leukocytes. Infect. Immun. 11:1226-1234.
22. Weiss J, Elsbach P, Olsson I, Odeberg H 1978 Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. J Biol Chem 253:2664-2672.
23. Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, Gallo RL 2001 Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414:454-457.

24. Tunzi CR, Harper PA, Bar-Oz B, Valore EV, Semple JL, Watson-MacDonell J, Ganz T, Ito S 2000 Beta-defensin expression in human mammary gland epithelia. Pediatr Res 48:30-35.
25. Harder J, Bartels J, Christophers E, Schroder JM 1997 A peptide antibiotic from human skin. Nature 387:861.
26. Bals R, Wang X, Wu Z, Freeman T, Bafna V, Zasloff M, Wilson JM 1998 Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. J Clin Invest 102:874-880.
27. Nagaoka I, Hirota S, Yomogida S, Ohwada A, Hirata M 2000 Synergistic actions of antibacterial neutrophil defensins and cathelicidins. Inflamm Res 49:73-79.
28. Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, Borregaard N 2001 Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood 97:3951-3959.
29. Zaiou M, Nizet V, Gallo RL 2003 Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence. J Invest Dermatol 120:810-816.
30. Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM 1997 Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. Cell 88:553-560.
31. Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI 1998 Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. Antimicrob Agents Chemother 42:2206-2214.
32. Raptopoulou-Gigi M, Marwick K, McClelland DB 1977 Antimicrobial proteins in sterilised human milk. Br Med J 1:12-14.
33. Langhendries JP 2002 [Human milk: a perpetual (re)appreciation]. Arch Pediatr 9:543-548.
34. Boman HG 2003 Antibacterial peptides: basic facts and emerging concepts. J Intern Med 254:197-215.
35. Nakayama M, Yajima M, Hatano S, Yajima T, Kuwata T 2003 Intestinal adherent bacteria and bacterial translocation in breast-fed and formula-fed rats in relation to susceptibility to infection. Pediatr Res 54:364-371.

Figure Legends

Figure 1.

HCAP18/LL-37 and hBD-1 mRNA is detectable in milk cells by RT-PCR

1 µg of total RNA was used for each RT reaction. Samples analyzed were obtained from breast milk samples at 10,15,30, and 60 days after delivery. HCAP18/LL-37 and hBD-1 mRNA was detected in all milk samples, but not hBD-2. The expected sizes of PCR products were 272 bp, 254 bp, and 173 bp for hBD-1, hBD-2, and LL-37, respectively. Internal 18S was amplified as a loading control, and Distilled water (DW) used as a negative control. The ladder (m) is the 10 µl of phi-X 174/HaeIII size marker. Data shown are representative of 4 sample preparations each from 3 individuals.

Figure 2.

Measurement of hCAP18/LL-37 and hBD-1mRNA in milk cells by real time PCR

To compare the relative expression of hCAP18/LL-37 or hBD-1 mRNA in milk cells over time after delivery, quantitative real time RT-PCR was performed. Transcript abundance at Day 10, 15, 30, and 60 are shown normalized to the relative abundance of each gene product at day 10. hBD1 demonstrated a 6-fold increase at Day 30 relative to day 10. hCAP18/LL37 increased 12 fold by day 30 and expression persisted up to day 60. Data are triplicate measurements ±SEM representative of 2 experiments.

Figure 3.

Mouse Cathelicidin is expression in mammary glands

To define cathelicidin expression in breast tissue, mammary glands from lactating mice were examined for CRAMP expression by immunohistochemistry. Abundant CRAMP was detected in the lactating lobular unit **(a)**. The signal was diffusely located in the cytoplasm of lobuloacinar (arrow) and ductules (arrow with asterisk), but not large secretory duct **(c)**. Rabbit IgG used for negative control showed no signal detected in these tissues **(b, d)**. Original magnification 100x (a, b) and 400x (c, d)

Figure 4.

Human milk contains the mature cathelicidin peptide LL-37

Breast milk collected at Day-10 was evaluated by immunoprecipitation and Western blot with antibody specific to LL-37 (a). Far left lane (milk) shows single band by western blot migrating at a size consistent with the expected 4.7 kDa mature form of LL-37 and similar to 100 pmol of LL-37 synthetic peptide loaded in far right lane (LL-37 syn pep). Lanes 1,2,3,4 show immunoreactive material successively washed but not immunoprecipitated, representing aggregates of LL37 and milk proteins, unprocessed hCAP18/LL37 or non-specific binding. Membrane was stripped and reprobed with antibody against the cathelin immature domain present only in unprocessed hCAP18/LL37 (b). No specific band was detected at the expected size of 18KDa. Dot blot analysis was showed that LL-37 abundance was approximately 1 μmoles/21.7 μg of total milk protein or 35 μM (Figure 4c).

Figure 5.

LL-37 synthetic peptide kills E. coli, S. aureus, and GAS in milk solutions.

The antimicrobial activity of LL-37 was evaluated by solution killing assay in a heat-denatured solution prepared from breast milk (AMS). Bacteria did not proliferate and AMS had weak antimicrobial activity alone, thus determination of the minimal inhibitory concentration (MIC) was not possible in this solution (a). To determine minimal bacteriocidal concentrations (MBC) LL-37 at concentrations between 0 and 32μM were incubated for 2 hr with GAS (b), S. aureus (c), and E. coli (d) in AMS (1X) or AMS diluted with water 10 fold (0.1x). Bacteriocidal activity was observed for all organisms but maximal in diluted AMS. Values are percentage of living bacteria (% live) compared with the control (no synthetic peptide). Data shown are representative of triplicate determinations from three independent experiments.