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Short Communication

Cathelicidin production and release by mammary epithelial cells during infectious mastitis

Tiziana Cubeddu[a], Carla Cacciotto[b], Salvatore Pisanu[b], Vittorio Tedde[b], Alberto Alberti[a], Marco Pittau[a], Simone Dore[c], Agnese Cannas[c], Sergio Uzzau[b,d], Stefano Rocca[a], Maria Filippa Addis[b,e,*]

[a] Università degli Studi di Sassari, Dipartimento di Medicina Veterinaria, Via Vienna 2, 07100 Sassari, Italy
[b] Porto Conte Ricerche, SP 55 Porto Conte/Capo Caccia, Km 8.400, Loc. Tramariglio, 07041 Alghero, Italy
[c] C.Re.N.M.O.C., Centro di Referenza Nazionale per le Mastopatie degli Ovini e dei Caprini – Istituto Zooprofilattico Sperimentale della Sardegna, Via Duca degli Abruzzi 8, 07100 Sassari, Italy
[d] Università degli Studi di Sassari, Dipartimento di Scienze Biomediche, Viale S. Pietro 43/B, 07100 Sassari, Italy
[e] Università degli Studi di Milano, Dipartimento di Medicina Veterinaria, Via Celoria 10, 20133 Milano, Italy

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ABSTRACT

Cathelicidins are well-characterized antimicrobial peptides (AMPs) that are present in significant amounts in mastitic milk. Neutrophils are believed to be the main producers of these AMPs, while the role of mammary epithelial cells (MECs) in their production and release is still unclear. In this work, cathelicidin production patterns were investigated in mammary tissues of ewes infected by *Staphylococcus aureus*, *Streptococcus uberis*, or *Mycoplasma agalactiae*, with a combined approach including immunohistochemistry, immune-colocalization, and fluorescent *in situ* hybridization. Our results confirm that MECs produce and release cathelicidins in response to different mastitis pathogens. As opposed to neutrophils, however, MECs do not seem to store the preformed protein precursor in their cytoplasm, but appear to synthesize and release it only upon exposure to the microorganisms. Cathelicidin production by MECs appears to occur before leukocyte influx in the milk, suggesting a role for these cells in the initial response of the mammary epithelium to microbial infection. Once in the milk, infiltrating neutrophils release massive amounts of cathelicidin by degranulation and production of neutrophil extracellular traps, acting as the main contributor for cathelicidin abundance in mastitic milk. Taken together, our results support the active contribution of MECs to cathelicidin production and release, and reinforce the value of cathelicidins as sensitive and pathogen-independent mastitis markers.

1. Introduction

Mastitis is an inflammatory disease of the mammary gland mainly caused by an intramammary infection (IMI). In ewes, the most common etiological agents are gram-positive bacteria, including different environmental staphylococci and streptococci and contagious pathogens such as *Staphylococcus aureus* (*S. aureus*) and *Mycoplasma agalactiae* (*M. agalactiae*) (Dore et al., 2016). In the mammary gland, recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) triggers the release of effectors including antimicrobial peptides (AMPs) such as cathelicidins. The cathelicidin family of AMPs is quite widespread in nature, and it was initially identified in the secondary granules of leukocytes where it is stored as a precursor. Upon stimulation, cathelicidins are released outside the cell and cleaved by proteases to produce the C-terminal antimicrobial peptide (Zanetti et al., 1995).

The role of mammary epithelial cells (MECs) in the release of AMPs is not clearly defined, and conflicting data have been published in the latest years. It was demonstrated that murine lactating lobular units produce cathelicidins (Murakami et al., 2005). Tomasinsig and co-workers also demonstrated the constitutive expression of cathelicidin mRNA in the mammary gland of healthy cows, but they did not detect up-regulation of gene transcription during mastitis (Tomasinsig et al., 2010). Recently, a higher level of cathelicidin mRNA was detected in healthy tissues when compared to *Staphylococci* infected tissues (Kościuczuk et al., 2014). Accordingly, Smolensky and co-workers suggested that cathelicidins found in mastitic milk are derived by degranulating neutrophils recruited in the inflamed mammary alveolus (Smolensky et al., 2011). In previous studies conducted by our group, several AMPs and immunity-related proteins were identified among the

* Corresponding author at: Università degli Studi di Milano, Dipartimento di Medicina Veterinaria, Via Celoria 10, 20133 Milano, Italy.
E-mail address: filippa.addis@unimi.it (M.F. Addis).

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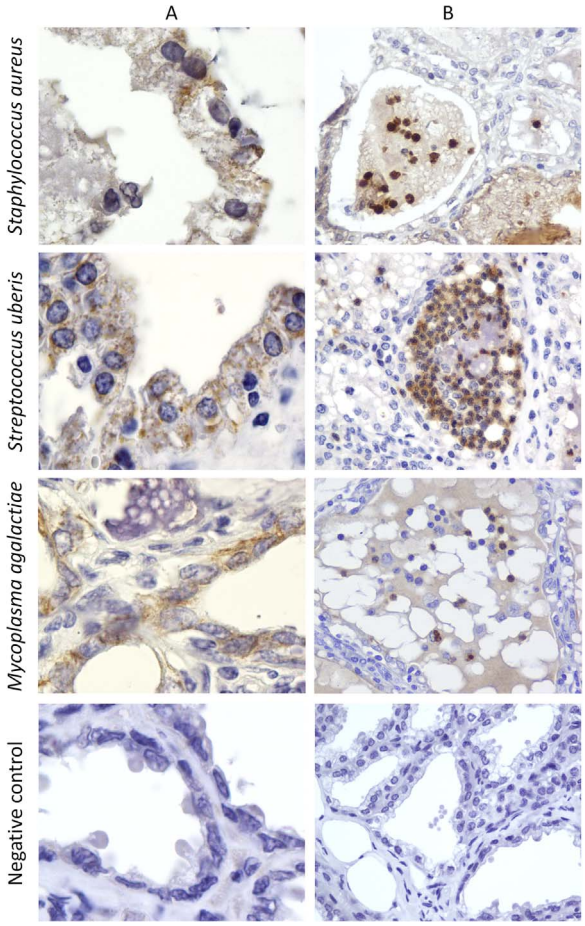

Fig. 1. Patterns of cathelicidin reactivity in sheep mammary gland tissues by immunohistochemistry. A: areas of tissue with visible epithelial lining. Magnification: 1000x. B: areas of tissue with acute inflammatory lesions. Magnification: 400x.

proteins that increase in milk upon infection (Addis et al., 2011, 2013). In preliminary IHC and immune co-localization experiments, cathelicidin positivity was observed in MECs of ewes experimentally infected with *S. uberis* (Addis et al., 2013), indicating the ability of these cells to produce and release cathelicidins.

In this study, cathelicidin production and release patterns were further investigated in mammary gland tissues of ewes naturally infected by *S. aureus*, *S. uberis*, and *M. agalactiae*. To this aim, different antibody-based techniques, including IHC, immune co-localization by confocal immunomicroscopy, and bacterial localization by fluorescent in-situ hybridization were applied. Results demonstrated that MECs produce and release cathelicidin in response to bacterial infections.

2. Materials and methods

2.1. Animal selection and tissue collection

In the context of a screening program for improvement of mammary health in sheep, several animals from different farms with mastitis problems due to *S. aureus*, *S. uberis*, or *M. agalactiae* were identified by physical examination, bacteriological tests, pan-cathelicidin ELISA, or western immunoblotting. When animals were selected for culling, tissues were retrieved at the slaughterhouse and brought to the laboratory, where milk and tissue samples were collected. Tissues were fixed in 10% neutral-buffered formalin, processed through graded concentrations of alcohol and xylene, and embedded in paraffin wax with a HISTO-PRO 200 vacuum tissue processor (Histo-Line, Milan) for histopathological and immunofluorescence examination, as previously described (Cacciotto et al., 2016).

2.2. Microbial culture

Milk samples collection and bacteriological examination of milk were carried out following standard procedures, according to the standards of the National Mastitis Council (1999), as previously described (Watts, 1990; Addis et al., 2016a,b). Briefly, 10 μL of milk were streaked onto 5% sheep blood agar plates and aerobically incubated at 37 °C. After 24 h and 48 h plates were examined and colony forming units (CFU) were counted. Isolated bacteria were classified for colony morphology, hemolytic activity, and Gram staining (Gram stain kit, Becton-Dickinson Co., Franklin Lakes, NJ). Gram-positive cocci were screened for catalase activity. BBL Coagulase Plasma Rabbit (with EDTA, BD), Staphylase Test kit (Oxoid, Thermo Scientific), and API Staph (bioMérieux) were used to identify suspected staphylococci. All commercial tests were performed according to the manufacturers' instructions. For *M. agalactiae* isolation, milk was plated onto blood agar base supplemented with 20% heat-inactivated horse serum and 500 μg/mL ampicillin and plates were controlled up to 14 days, as previously described (Cacciotto et al., 2010, 2013). Mycoplasma species was confirmed by means of specific FS1/FS2 PCR, as previously described (Tola et al., 1996).

2.3. Immunoassays

The presence of cathelicidin in milk samples was assessed with an in-house pan-cathelicidin sandwich ELISA developed in our laboratories, as previously described (Addis et al., 2016a,b). For immunohistochemical analysis, 3 μm sections of mammary tissues were used. Prior to incubation with antibodies, tissue sections were dewaxed in xylene, rehydrated through graded alcohol series, and incubated with 0.3% hydrogen peroxide in PBS for 30 min to block endogenous peroxidases. After treatments, sections were incubated with a rabbit anti-CAMP (anti-cathelicidin, Sigma-Aldrich), diluted 1:1500. Reaction was revealed with the ImmPRESS™ Universal Antibody (anti-mouse IgG/anti-rabbit IgG, Peroxidase) (Vector) and ImmPACT™ DAB Peroxidase (Vector) following the manufacturer instructions. Nuclei was lightly counterstained with hematoxylin. IHC images were acquired with a Nikon Eclipse 80i microscope equipped with a Nikon DS-Fi1 camera (Nikon Instruments Inc.).

2.4. Co-localization assays

2.4.1. Streptococcus uberis, Staphylococcus aureus and cathelicidin

An integrated approach of immunofluorescence (IF) and fluorescent *in situ* hybridization (FISH) was used to co-localize cathelicidin with *S. uberis* and *S. aureus*. Three μm sections of mammary tissues were mounted on Superfrost™ slides (Thermo Scientific), dewaxed in xylene, rehydrated through graded alcohol series. Upon digestion with pepsin for 30 min (0.8% in 0.2 N HCl, 37 °C in PBS), sections were postfixed with an alcohol ascending scale (from 50% to 98%) and air dried. Sections were incubated for 2 h at 55 °C with 200 μL of prehybridization solution (50% of Hybridization Solution II [43% deionized formamide, 7% nuclease-free water]; Fluka). Then, slides were incubated with 200 μL of hybridization solution containing alternatively 1 nM biotynilated probes for *S. uberis* (5′-Btn-GCGAAGTGGGACATAAAGTTA-3′) or *S. aureus* (5′-Btn-AGGTA TGCAATTTGATCGTGGTTATCAATCACCGTATATGGTTACTGATTC-3′), denatured for 8 min at 98 °C, and incubated for 16 h at 55 °C in a ThermoBrite StatSpin. Tissue sections were washed at room temperature for 5 min with 2X, 1X, 0.5X, and 0.1X SSPE (1X SSPE is 0.18 M