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Cutting Edge: Mast Cell Antimicrobial Activity Is Mediated by Expression of Cathelicidin Antimicrobial Peptide

Anna Di Nardo,*† Antonella Vitiello,‡ and Richard L. Gallo2*

Cathelicidins (caths)3 comprise a family of antimicrobial peptides (AMPs) that have been identified in epithelial tissues and some myeloid cells of humans and animals (1). Caths consist of a putative N-terminal signal peptide, a highly conserved cathelin-like domain, and a less conserved C-terminal domain corresponding to the AMP domain. Enzymatic processing of full-length procathepsin releases and activates the C-terminal cath AMP (2). About 30 different caths have been described in mammals, but so far only one has been identified in humans and one in mice, LL-37 (3) and cathelin-related AMP (CRAMP) (4), respectively. As antimicrobials, caths have activity against a wide range of bacteria, fungi, enveloped viruses, and protozoa. The human and mouse peptides are cationic and amphipathic, properties that promote favorable interactions with biological membranes. Through these membrane interactions, the peptides disrupt specific microbial membranes and kill sensitive organisms. The presence of these molecules is essential for defense against invasive bacterial infection in skin, as targeted deletion of cath CRAMP in mice results in increased susceptibility to group A Streptococcus (GAS) (5), and increased AMP expression can confer disease resistance in lung models (6). Caths also have other properties that interact with the host immune system, such as promoting neutrophil chemotaxis (7) and mast cell (MC) recruitment (8). The multifunctional properties of caths suggest they have a vital role in mediating innate immune defenses and influencing adaptive immune responses.

MCs originate from hemopoietic bone marrow precursors, circulate in the peripheral blood as immature progenitors, and complete their differentiation in the mucosal and connective tissues in a microenvironment-characteristic manner. Independent of their location, all mature MCs are mononuclear cells characterized by the presence of abundant cytoplasmic granules and systemic allergic responses. Recently, the presence of an AMP was described in fish MCs, suggesting that this cell type and surface expression of c-kit and FceRI. Functionally, MCs are known for their key role in initiating and maintaining local and systemic allergic responses. Recently, the presence of an AMP was described in fish MCs, suggesting that this cell type can be an additional source for AMPs (9). Because MCs are distributed along the interface with the external environment at the portals of entry of many infectious agents, the presence of AMPs in MCs could signify an essential first line of defense (10) (11). In support of this hypothesis, in vitro studies have shown that MCs, upon contact with bacteria, release a variety of molecules including TNF-α and leukotriene B4 factors that play a

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3 Abbreviations used in this paper: cath, cathelicidin; MC, mast cell; AMP, antimicrobial peptide; BMD-MC, bone marrow-derived MC; TLR, Toll-like receptor; GAS, group A Streptococcus; LTA, lipoteichoic acid; Ct, cycle threshold; PG, peptidoglycan; TSB, tryptic soy broth; mBD-4, murine β-defensin-4; CRAMP, cathelin-related AMP.
critical role in host defense against Gram-negative bacterial infection (12, 13). In addition, MCs can kill opsonized bacteria and also recognize bacteria in the absence of opsonins (10). This trait is likely mediated in part through the cell surface pattern recognition receptors, such as Toll-like receptors (TLRs) –2, –4, –6, and –8, and the FimH receptor CD48 for Escherichia coli fimbriae (14). Finally, MC-deficient mice are less efficient in surviving experimentally induced infections (15). The location and immune functions associated with MCs, and observations of AMPs in MCs of lower vertebrates, led us in the present study to hypothesize that mammalian MCs contain AMPs and that these peptides directly participate in the MC immune response.

Materials and Methods

Cell culture

Primary mast cells were produced from murine bone marrow by extracting bone marrow cells from the femurs of 5- to 8-week-old mice and culturing cells in RPMI 1640 (Life Technologies, Tualsa, OK) supplemented with 10% inactivated FCS (Life Technologies), 4 mM glutamine, penicillin/streptomycin, and 50 μg/ml 2-ME. Recombinant murine IL-3 (1 ng/ml) and recombinant murine stem cell factor (20 ng/ml), both shown to support the in vitro growth and differentiation of the MC precursor, were included (16). After 4 wk, MCs were consistently generated as confirmed by the expression of CD117 and FcεRI (see Fig. 1) and by staining with toluidine blue. MC cultures were derived from BALB/c, 129/SVJ, and CRAMP-deficient Cnlp+/− mice in the 129/SVJ background (5). For antimicrobial assays, cells were grown in antibiotic-free medium.

FACS analysis

MCs were processed for staining with FITC-labeled rat anti-mouse CD 117 (BD Pharmingen, San Diego, CA) and rat anti-mouse IgG-R (BD Pharmingen). For evaluating CRAMP expression, cells were permeabilized with 0.02% saponin (Sigma-Aldrich, St. Louis, MO) then incubated with affinity purified rabbit anti-CRAMP IgG (or rabbit IgG control) at 0.8 μg/ml (17). The secondary Ab was goat anti-rabbit FITC (Cappel Research Products, Durham, NC) at 80 μg/ml. For flow cytometry, FACSscan (BD Biosciences, San Jose, CA), equipped with CellQuest software, was used.

Immunohistochemistry

Human sections. Sections from human skin were deparaffinized and hydrated with toluene and decreasing concentrations of ethanol in PBS (138 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 7H2O, and 1.5 mM KH2PO4, pH 7.3). Sections were rinsed in PBS, endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in water, and LL-37 staining was determined by previously described (17). Briefly, samples were blocked with 2% goat serum in PBS for 20 min, incubated for 60 min with primary Ab diluted in 3% BSA in PBS. Primary Ab was rabbit anti-LL-37 coupled to a rabbit serum (a generous gift of Dr. B. Agerberth, Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden) at 5 μg/ml. Sections were then washed with PBS, goat anti-rabbit biotinylated Ab (Vector Laboratories, Burlingame, CA) was applied for 30 min, washed again and developed with diaminobenzidine substrate (Sigma-Aldrich), and counterstained with Gill’s hematoxylin (Vector Laboratories). For detecting tissue MCs with toluidine blue, sections were stained with a 0.2% solution of toluidine blue in water at 70°C.

Mouse mast cell cultures. Mouse mast cell cultures were cytospun on glass slides, fixed in cold methanol for 10 min (−20°C), and rinsed in PBS. Samples were blocked with 2% goat serum in PBS and incubated for 60 min with primary Ab diluted in 3% BSA in PBS. Primary Ab was affinity purified rabbit anti-CRAMP IgG (with rabbit IgG control) at 0.8 μg/ml (17) or affinity purified rabbit anti-murine β-defensin-4 (mBD-4) at 10 μg/ml (Alpha Diagnostic International, San Antonio, TX). The secondary Ab was goat anti-rabbit FITC (Cappel Research Products) at 80 μg/ml.

RT-PCR

RNA was extracted from mouse bone marrow-derived MC (BMD-MC) cultures, adult mouse skin, or mouse femur bone marrow with the RNA-Easy kit (Qiagen, Valencia, CA). For RT-PCR, 1 μg of extracted RNA was used with the Retroscript kit (Ambion, Austin, TX). A previously described protocol (17) was used for qualitative PCR with the following oligonucleotides: CRAMP, F1 (AGGAGATCTTCGGAACCATGAGTT) and R1 (GCAGATCTACT GCTCCGGTGAG) and mBD-4 F, AACAGCTATCACAATTG; and mBD-4 R, TCTAGTCCGGTTCTCTATCT. The GeneAmp 5700 System Detection system (PerkinElmer, Foster City, CA) was used for real-time quantitative PCR, with 18S Classic Primers (Ambion) used to amplify 18S RNA and CRAMP-F (CTTACACCGAGTCTGCTACAGA) and CRAMP-R (TCCAGGTCAGGAGCGTA) primers to amplify CRAMP cDNA. Of the reverse transcriptase reaction, 1 μl was used in 25 μl SYBR Green PCR Master Mix (Applied Biosystems, Warrington, U.K.) and 0.25 μl of each 10 μM primer. The thermal profile consisted of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 10 s. Results were analyzed by the comparative cycle threshold (Ct) Method, where Ct is the number of cycles required to reach an arbitrary threshold. The efficiency of CRAMP Ct was confirmed by real-time PCR of triplicate samples derived from various amounts of RNA from murine bone marrow. For each starting concentration of mRNA, ΔCt = CtA − CtR, (R = reference and G = target) was plotted against the log of the starting mRNA concentration. The slope of the resulting plot was <0.1, indicating no requirement for a correction factor. The ΔΔCt for samples were calculated as ΔCtG = ΔCtE−ΔCtC, and CPE = experimental and CBE = baseline relative expression is calculated as 2ΔΔCt to account for exponential PCR amplification.

Western blots

BMD-MCs were harvested and washed with PBS, and protein was extracted with 1 ml of a solution of 1 M HCl and 1% trichloroacetic acid. Samples were lyophilized and resuspended with distilled water. Protein concentration was calculated by the bicinchoninic acid assay. From each sample, 10 μg of protein was loaded onto a 16.5% Tris-tricine gel (Ready Gel; Bio-Rad, Hercules, CA) for electrophoresis. Proteins were transferred to a nitrocellulose membrane, and the membrane was blocked in 5% nonfat milk in 0.1% Tween TBS and then incubated in primary Ab diluted in 5% nonfat milk. The primary Ab was rabbit anti-mouse CRAMP (0.16 μg/ml) against the CRAMP mature peptide (17) and chicken anti-mouse cath (Avex Laboratories, Tigard, OR) against the cathe- lin domain (precursor (8), and the secondary Ab was peroxidase-conjugated goat anti-rabbit (DAKO, Carpinteria, CA) or goat anti-chicken (Avex Laboratories), respectively. Immunoreactive proteins were detected by ECL.

MC activation

MCs were seeded in 6-well plates to a concentration of 2 × 10^5 in 2 ml of medium. The following bacterial components were added: 200 ng/ml LPS (from E. coli); 1 μg/ml lipopolysaccharide (LTA) (from Streptococcus faecalis); 1 μg/ml lipoprotein A; 1 μg/ml peptidoglycan (PG), and 1 μg/ml LT (from Staphylococcus aureus) (all from Sigma-Genosys, Houston, TX). Cells were harvested after 3 h at 37°C and RNA was collected as described.

MC antibacterial assay

GAS strain NZI 31 is a serotype isolated from a patient who developed acute poststreptococcal glomerulonephritis. A single colony was resuspended in 4 ml of tryptophan broth (TSB) and incubated overnight. Of this solution, 100 μl was added to fresh TSB and grown for 2–4 h to early log phase. Bacterial numbers were assessed at OD_600, based on predetermined evaluations that determined CFUs per OD_600. The equivalent of 85 CFUs of bacteria in 15 μl of TSB medium was added to 35 μl of MC culture medium alone or containing 2.5 × 10^7 MCs derived from age- and sex-matched wild-type or CRAMP−/− mice. After 2–3, and 5-h incubations, 15 μl of the medium was plated, and CFUs were counted by serial dilutions and manual colony counts. Statistical significance between groups was determined by Student’s t-test.

Results and Discussion

Caths are expressed in murine BMD-MCs.

On the basis of prior observations of MC location and function, we hypothesized that MCs contain cath AMPs and that the activity of these peptides enables MC antimicrobial action. To explore the cath content of MCs, we cultured murine BMD-MCs that most closely resembled connective tissue MCs (12) and used a well-characterized Ab specific to CRAMP for immunohistochemical analysis (Fig. 1, A and B). Immunostaining showed that 90% of the cells in culture were immunofluorescent and that these cells had granular cytoplasmic fluorescence consistent with localization of CRAMP in MC granules. The high percentage of cells directly observed to stain positively for CRAMP and a simultaneously high proportion of cells confirmed to be MCs by FACS analysis and by toluidine blue staining (not shown) confirmed that murine BMD-MCs, and not an undetected minor cell-contaminating population, express CRAMP.
Because CRAMP is processed from a larger propeptide by proteolytic release of the active mature peptide from the cathelin domain (2), it was critical to determine the size of the cath protein present in these cells. Two independently derived Abs were used to evaluate MC protein extracts by Western blot: a rabbit anti-CRAMP Ab specific to the peptide domain and a chicken Ab specific to the cathelin domain alone (18). Anti-CRAMP detects both the 18-kDa propeptide and the mature 5-kDa CRAMP peptide. Anti-cathelin only detects propeptide. The propeptide at 18 kDa was easily detected in BMD-MC protein extracts by both Abs, but processed CRAMP at 5 kDa was not seen on standard gel exposures. Longer, 30-min exposures of extracts by both Abs, but processed CRAMP at 5 kDa was not seen by immunofluorescence or Western blot. Therefore, additional studies on MC AMPs focused exclusively on cath. Comparison of mBD-4 expression in Cnlp−/−-derived MCs showed no apparent change in the level of expression of this transcript despite the lack of detectable CRAMP (data not shown).

Human MC contain caths

To determine whether human MCs also express caths, we used skin sections from a human mastocytoma lesion for immunohistochemical analysis with a well-characterized Ab specific to the human cath peptide LL-37. LL-37-specific staining was seen in the cytoplasm of several cells in the dermis (Fig. 2A) that were identified as MCs by the metachromatic counterstaining of adjacent sections with toluidine blue (Fig. 2B). These observations suggest that cath expression is common to both human and murine MCs. Because culture and functional analysis of human MCs is technically difficult, additional experiments focused on murine BMD-MCs exclusively. Nonetheless, the presence of cath in both species suggests that the mouse is a relevant species for comparison to humans. This similarity between AMPs in MCs might be limited only to caths, because in neutrophils, defensin expression is absent in mice but abundant in humans (19).

MC cath expression is inducible

Effector mechanisms of innate immunity are often responsive to pathogen (pattern) recognition events. LPS is known to be a strong activator of such events in MCs, inducing TLR-4 stimulation and TNF-α and IL-6 production (20). MCs are also

with the highest abundance of CRAMP mRNA (bone marrow) (Fig. 1E). BMD-MCs showed high levels of CRAMP transcripts, with levels comparable to those seen in bone marrow and ~100-fold higher than seen in normal mouse skin.

We also investigated the presence of β-defensin transcripts in BMD-MCs to determine whether additional families of AMPs are present in these cells. We generated oligonucleotide probes specific to mBD-1, -3, and -4 and confirmed their sensitivity and specificity by amplifying cDNA derived from mouse tissues known to contain these transcripts. These RT-PCR products were cloned and sequenced to verify identity. Only mBD-4 was detected in BMD-MCs (Fig. 1F). mBD-4 shows greatest homology with human β-defensin-2, an AMP abundantly present in inflamed skin. However, unlike CRAMP, the mBD-4 protein could not be detected by immunofluorescence or Western blot. Therefore, additional studies on MC AMPs focused exclusively on cath. Comparison of mBD-4 expression in Cnlp−/−-derived MCs showed no apparent change in the level of expression of this transcript despite the lack of detectable CRAMP (data not shown).

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known to contain several additional pattern recognition receptors, including TLR-2, -6, and -8 (14). To determine whether synthesis of CRAMP was also responsive to activation by microbial products, we challenged BMD-MCs with LPS, lipid A, LTA, and group G Streptococcus components and measured CRAMP mRNA after 3 h (Fig. 3). LPS increased expression of CRAMP mRNA 6-fold compared with the control (Fig. 3A). This difference was also visible with qualitative PCR (Fig. 3A, inset). To evaluate whether CRAMP protein expression also increased in MCs after microbial stimulation, we analyzed MCs by FACS after incubating MCs for 48 h with LPS or LTA (Fig. 3B). CRAMP protein expression increased after exposure to these bacterial cell membrane products. This increase in CRAMP suggests that MCs not only store preformed AMPs but also increase their production in response to appropriate stimuli. Differences in the relative stimulation of CRAMP might reflect cooperative activation of multiple TLRs (such as TLR-2 and TLR-4).

MCs are dependent on CRAMP for antimicrobial activity

MCs can recognize and kill bacteria through specific membrane receptors that interact with bacterial surfaces (10, 14, 20). However, the effector mechanisms for MC antimicrobial activity have not been completely identified. Because cath is present in MCs, but is stored as a precursor form that requires activation to kill bacteria, it was necessary to test whether caths were active as antimicrobials in MCs. To examine the ability of MCs to kill bacteria, we added live bacteria to cultures of BMD-MCs and quantified bacterial growth over time. GAS was chosen because it is very sensitive to CRAMP or LL-37 action under standard culture conditions (17), and resistance to GAS skin infections in vivo is dependent on CRAMP expression (5).

BMD-MCs were generated from wild-type 129/SvJ mice (MC<sup>w/t</sup>) and age- and sex-matched Cnlp<sup>−/−</sup> in the 129/SvJ background (MC<sup>−/−</sup>). FACS analysis confirmed generation of the MC phenotype in culture for MC<sup>−/−</sup> (Fig. 4A), and PCR confirmed the lack of CRAMP and similar levels of mBD-4 in both MC cultures (Fig. 4B). Comparing bacterial growth in culture medium alone in the absence of any MCs to growth in the presence of MC<sup>w/t</sup> demonstrated that these cells significantly suppress the rate of GAS growth (Fig. 4C). In the absence of CRAMP, GAS grew more rapidly than with MC<sup>w/t</sup>, thus indicating that MC<sup>−/−</sup> had a significantly decreased ability to suppress GAS proliferation. Addition of synthetic CRAMP peptide to these cultures at a final concentration of 32 μM completely suppressed GAS proliferation and abolished the difference between MC<sup>−/−</sup> and MC<sup>w/t</sup>.

The observations that MCs in coculture with bacteria are able to reduce growth and that they lose this capacity when they lack CRAMP suggests that the presence of cath in mammalian MCs provides a potent mechanism that enables MCs to participate in defense against infection. It is unclear at present whether the...
antimicrobial activity observed for cultured BMD-MCs is due to activation of procath and release of processed CRAMP into the medium or to intracellular killing of GAS after uptake and fusion with intracellular granules containing active CRAMP. A lack of detectable cath in culture medium argues against the former, although GAS could be effectively killed if CRAMP was added extracellularly. Prior observations that MCs are able to kill opsonized bacteria and can recognize bacteria in the absence of opsonins support the latter hypothesis (10). If antimicrobial activity depends on uptake, then the lack of complete elimination of GAS growth as shown in Fig. 4 for MCw/t might be due to inefficient GAS phagocytosis under the conditions of this assay. Residual antimicrobial action seen in MCs −/− might also reflect the activity of other AMPs, such as mBD-4, observed in these cells. Finally, the difference in antimicrobial activity of MC −/− possibly reflects a secondary function of CRAMP. Lack of cath might influence autocrine-signaling events necessary for efficient bacterial killing.

The significance of caths in MCs might explain observations that MC-deficient mice are more susceptible to infection (11). In addition, LL-37 expression is active as a leukocyte chemoattractant through binding of human formyl peptide receptor like-1/lipoxin-A receptor (7). This function might also be critical to MC action, as it could combine with the chemotactic activity previously demonstrated for TNF-α and leukotriene B4 produced by the MC (13). Furthermore, human LL-37 has recently been shown by gene-chip analysis to influence the expression of over 40 genes by macrophages, and many of these gene products include chemokines, such as IL-8, and chemokine receptors, such as CCR2 and IL8RB (21). The multifunctional activity of caths supports their importance to normal MC function.

We have shown that patients with atopic dermatitis lack appropriate expression of LL-37 and human β-defensin-2, a homologue of mBD-4 (22). The susceptibility of these patients to a variety of microbial pathogens could be explained by this deficiency in AMP production by the epidermis. However, because MCs are thought to play a vital role in the development of atop dermatitis and are expected to reside in the skin of these patients, MCs in atopics might also have diminished AMP production. It will be of interest to directly measure the expression of LL-37 in MCs of atopics to determine whether the contribution of MC AMPs could also explain infection in atop dermatitis. Overall, the current findings suggest that cath expression is essential to the biology and normal function of MCs.

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