Heparan sulfate proteoglycan-dependent neutrophil chemotaxis toward PR-39 cathelicidin

Angela Djanani1, Birgit Mosheimer1, Nicole C Kaneider1, Christopher R Ross2, Giovanni Ricevuti3, Josef R Patsch1 and Christian J Wiedermann*1

Address: 1Laboratory of Medical Intensive Care, Division of General Internal Medicine, Department of Medicine, Medical University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria, 2Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, Coles Hall 228, 1600 Denison Avenue, Manhattan, KS 66506-5602, USA and 3Department of Internal Medicine and Therapeutics, Section of Internal Medicine, University of Pavia, Viale Liberta, I-27100 Pavia, Italy

Email: Angela Djanani - angela.djanani@uibk.ac.at; Birgit Mosheimer - birgit.mosheimer@uibk.ac.at; Nicole C Kaneider - nicole.kaneider@uibk.ac.at; Christopher R Ross - ross@vet.k-state.edu; Giovanni Ricevuti - giovanni.ricevuti@unipv.it; Josef R Patsch - josef.patsch@uklibk.ac.at; Christian J Wiedermann* - christian.wiedermann@asbz.it

* Corresponding author

Abstract

Cathelicidins are mammalian proteins containing a C-terminal cationic antimicrobial domain. Porcine PR-39 cathelicidin affects leukocyte biology. Mechanisms of action may involve alteration of heparan sulfate proteoglycan-dependent functions in inflammatory cells. It was tested whether PR-39 affects human neutrophil migration and if such effects involve heparan sulphate proteoglycans. Neutrophils were from forearm venous blood of healthy donors. Migration was tested in modified Boyden chamber assays. Involvement of heparan sulfate proteoglycans was tested by their chemical modification and by the use of specific antibodies. PR-39 induced migration in neutrophils in a concentration dependent manner. Modification of heparan sulfate proteoglycans with sodium chlorate inhibited migration whereas chemotaxis toward the chemoattractant formyl-Met-Leu-Phe was not affected. Removal of heparan sulfates or chondroitin sulfates from the surface of neutrophils by heparinase or chondroitinase inhibited migration toward PR-39. In conclusion, antimicrobial PR-39 stimulates human neutrophil chemotaxis in a heparan sulfate proteoglycan-dependent manner. Involvement of syndecans is likely as both heparinase and chondroitinase were abrogating. Data suggest active participation of heparan sulfate proteoglycans of neutrophils in cathelicidin peptide-mediated regulation of the antimicrobial host defense.

Findings

Peptides with in vitro antimicrobial activity have been identified from several gene families. Two major antimicrobial peptide families in mammals are the defensins [1] and the cathelicidin peptides [2-4]. It is known that the defensin structure is based on a common beta sheet core, which is stabilized by three disulfide bonds [1,2] but cathelicidins are highly heterogeneous. Their conserved cathelin domain sequence has been used as a genetic probe enabling the discovery of numerous new members of this family [4-10]. Circulating neutrophils, myeloid bone marrow cells and epithelial surfaces are an impor-
tant source of cathelicidine expression [7,11-14]. The cathelicidin, prolin-arginine-rich 39 peptide (PR-39), first isolated from the porcine small intestine [15] was also identified in porcine neutrophils [16]. Different forms occur and PR-39 isolated from porcine small intestine is slightly different in composition from that isolated from porcine neutrophils [17]. PR-39 posseses antibacterial activity [18] and has the ability to induce syndecan expression in wounds in animal studies [12]. PR-39 kills bacteria by a mechanism that stops protein and DNA synthesis after a lag period of about 8 min [18]. PR-39 is an inhibitor of neutrophil function in injured tissue, is involved in metastatic activity of human tumor cells, and can induce angiogenesis [1-3,19,20]. These observations suggest that the efficacy of PR-39 is not species specific.

Heparan sulfate proteoglycans (HSPG) from endothelium and leukocytes interact with P-selectin, an important adhesion molecule regulating leukocyte adhesion and migration [21]. HSPG localize to granules of myeloid cells including monocytes and neutrophils [22], and expression of mRNA for syndecan core protein has been detected in different types of leukocytes including neutrophils [23,24]. As PR-39 is abundantly expressed in mammalian tissues and is best investigated among cathelicidins, our motivation in this project was to determine if porcine PR-39 affects human neutrophil migration and whether such effects involve HSPG.

Heparinase I and chondroitinase ABC were from Sigma Chemical Corp. (St. Louis, MO, USA). PR-39 was synthesized by solid-phase method with greater than 90% purity [25]. Antibodies against the core-protein of syndecan-4 (D-16) and the ectodomain of this proteoglycan (SC9) were affinity purified goat polyclonal antibodies raised against peptides mapping with the respective regions of the human syndecan-4 protein (both Santa Cruz Inc., Wiltshire, England). According to the manufacturers instructions, suitability of its use has been demonstrated for detection of syndecan-4 as well as for use as control antibodies in siRNA studies. Other reagents not further specified were also from Sigma. Neutrophils were obtained from forearm venous blood of healthy volunteers, anticoagulated with 1.6 mg EDTA/mL of blood. Neutrophil preparation was performed as described [26]. Cell preparations yielded > 95% neutrophils (by morphology in Giemsa stains) and > 99% viability (by trypan dye exclusion). Chemotaxis of neutrophils into cellulose nitrate to gradients of soluble attractants was measured in RPMI 1640/0.5% BSA using a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD, USA) in which a 5 μm pore-sized filter (Sartorius, Göttingen, Germany) separates the upper and lower chamber as previously described [26]. When PR-39 was used as attractant, concentrations ranging from 10 nmol/L to 1 nmol/L were tested. As positive control chemotactic agent in the lower chamber fMLP was used. For some experiments cells were pretreated with heparinase I, an enzyme that cleaves highly sulfated regions of heparan sulfate-like glycosaminoglycans at 2-O-sulfated uronic acids, for 50 min. Thereafter cells were washed twice before testing for chemotaxis. For other experiments cells were pretreated with chondroitinase ABC also for 50 min that cleaves chondroitin sulfate side chains of cell surface proteoglycans. Since it is known that sodium chlorate is able to modify proteoglycan sulfation, we tested PR-39 chemotaxis after pre-treatment of cells for 20 min with sodium chlorate. As neutrophil migration toward PR-39 might be mediated via syndecan-4, chemotaxis experiments were performed in the presence of monoclonal antibodies toward the core-protein of syndecan-4 and a side chain of this proteoglycan. Cells were incubated with these antibodies for 20 min, washed twice and allowed to migrate toward PR-39.

Data are expressed as mean and standard error of the mean (S.E.M.). Means were compared by Kruskal-Wallis analysis of variance and by Mann-Whitney u-test for non-parametric samples (Abacus Concepts, Berkley, CA). A difference with p < 0.05 was considered to be significant.

To determine whether PR-39 induces human neutrophil chemotaxis, we tested in vitro migration of the cells at a wide range of concentrations [10 nmol/L to 1 nmol/L]. PR-39 induced human neutrophil chemotaxis in a concentration dependent manner with a maximum effect at 10 μmol/L (data not shown). To investigate the role of intact HSPG on the surface of neutrophils in PR-39-induced cell migration, neutrophils were pretreated for 50 min with heparinase I or chondroitinase [both, 50 nU/mL to 50 μL/mL] at 37°C, followed by two washing steps. As glypicans carry heparan sulfate side chains but not chondroitin sulfate side chains, whereas syndecans carry both [27], experiments were performed with both heparinase I and chondroitinase. Results showed a concentration-dependent reduction of migration by removal of these two substrates from the cell surface, whereas chemotactic effects of fMLP [10 nmol/L] were not affected (Fig. 1). To investigate the effect of sodium chlorate which is known to modify sulfation of proteoglycans and sulphated proteins in cell culture, neutrophils were pretreated with sodium chlorate [10 mmol/L to 40 mmol/L], washed, and then allowed to migrate towards PR-39. Neutrophil chemotaxis to PR-39 was significantly inhibited by sodium chlorate whereas chemotaxis toward the chemokine IL-8 used as alternative control attractant was not affected (data not shown).

Because PR-39-induced chemotaxis was inhibited by chondroitinase and heparinase I, we suggested the
involvement of syndecans but not glypicans by PR-39. Moreover, in vivo expression of syndecans has been reported as being affected by PR-39 [12]. Therefore, chemotaxis of neutrophils toward PR-39 was tested in the presence of migration-blocking monoclonal antibodies to syndecan-4 core protein or syndecan-4 side chain because this pathway was shown to affect the cell’s motility. Neutrophils were again pre-treated with either of the two antibodies or an isotype-matched IgG, and then allowed to migrate toward PR-39 [10 μmol/L]. The antibodies specifically inhibited neutrophil migration toward PR-39 (Fig. 1).

In the present study, PR-39 stimulated human neutrophil chemotaxis in a bell-shaped dose-response curve. PR-39 is known to induce chemotaxis in porcine neutrophils [25]. As PR-39 is effective in improving survival in animals models of severe sepsis [28,29], the observation that PR-39 affects neutrophil function across different species may be of relevance if PR-39 is further developed for potential use in severe sepsis. In porcine leukocyte chemotaxis, peak responses occurred at 0.5 to 2 micromoles per liter [25] which correlates well with PR-39 effects on human neutrophils (data not shown). This finding suggests that exogenous PR-39 may play a role not only in animal but also in human inflammation.

For LL-37, the only human cathelicidin identified so far, it was proposed that chemotactic responsiveness of leukocytes involves formyl peptide receptor-like 1, and activation of this formyl peptide receptor-like 1 cross-deactivates LL-37 responsiveness [30]. In mast cell responses to cathelicidins, there may be two types of receptors involved, a high affinity receptor responsible for chemotaxis, and a low affinity receptor with undefined function [31]. Detailed biochemical mechanisms or a biochemical characterization of these binding sites, however, remained unknown.

Antithrombin-III, a prototypical glycosaminoglycan ligand, has only recently been identified to exert direct effects on cells of the innate immune system via HSPG [26,32]. Already two decades ago, consensus sequences for glycosaminoglycan recognition were determined as [-X-B-B-X-B-X-] and [-X-B-B-X-B-X-B-X-] where B is the probability of a basic residue and X is a hydrophobic residue, which form potential nucleation sites for the recognition of polyanions in proteins [33]. As antimicrobial peptides including defensins and cathelicidins contain consensus sequences for HSPG recognition [34], we further explored the roles of HSPG in mediating PR-39 effects in neutrophil migration. Heparan sulfate chains abound on syndecans and glypicans which can bind a rep-

Figure 1
Effects of heparinase, chondroitinase and anti-syndecan 4 antibodies on PR-39-induced chemotaxis of neutrophils. Heparinase I (left panel) or chondroitinase (middle panel) was added to neutrophils. After an incubation (humidified, 37°C/5% CO2) period of 50 min, cells were washed twice and chemotaxis assays were performed. PR-39 [10 μmol/L] was used as chemoattractant and fMLP [10 nmol/L] served as control attractant. Data are expressed as percent of medium control (pre-treatment with medium), with a distance of migration toward fMLP of 79.8 ± 3.1 μm, towards PR-39 of 66.8 ± 1.3 (n = 4). Statistical analysis: Mann-Whitney U-test versus medium control, *, p < 0.05 after Kruskal-Wallis test, p < 0.05. Effects of antibodies to syndecan-4 core protein or syndecan-4 chain epitopes (right panel) were tested by preincubation of neutrophils for 20 minutes (humidified 37°C/5% CO2). After washing, cells were allowed to migrate toward PR-39 [10 μmol/L] in modified Boyden chambers using nitrocellulose micropore filters. Isotype matched IgG served as control. Results are given as mean ± SEM of the chemotaxis index, which is the ratio between the distance of migration toward attractant and that toward control. Distance of random migration was 40.0 ± 2.78 (n = 4). Statistical analysis: Mann-Whitney U-test versus no antibody, * p < 0.05 after Kruskal-Wallis test p < 0.05.
The syndecan family of cell surface proteoglycans has been implicated in a number of biological processes, including blood coagulation, cell adhesion, signal transduction and wound repair [38]. Originally found on epithelial cells, syndecans were later shown to be present in several other mesenchyma-derived cell types, including fibroblast, smooth muscle cells, and neutrophils [28].

PR-39 has been shown to interact with a domain within the integrin mediated signaling protein, namely p130(Cas) [39], an assembling molecule of actin filaments which promotes cell movement, cell migration, and cell spreading in fibroblasts [40]. Identification of p130(Cas) as a mediator of focal adhesion kinase-promoted cell migration [41] fits well to its interaction with PR-39, given the finding that syndecan-4 modulates focal adhesion kinase phosphorylation [42] and may be activated by PR-39 as suggested by our observed inhibition of PR-39-induced chemotaxis of neutrophils with antibodies to syndecan-4. This hypothesis, however, requires biochemical confirmation.

The data provided do not permit the conclusion that only syndecan-4 mediates the chemotaxis of human neutrophils to PR-39. Other syndecans or glypicans may also participate in coordinating the chemotactic response, as additional cell surface proteoglycans harboring HS and/or CS might also be involved.

To summarize, besides proposed involvement of formyl peptide receptor-like 1, no signalling pathway for cathelicidins in neutrophils had been identified so far. Our results provide strong evidence for interactions of PR-39 with proteoglycans on the surface of leukocytes. Biochemical and functional tests identify syndecan-4 as a putative acceptor site for PR-39 which contains consensus sequences for glycosaminoglycan recognition. Our findings may be of particular relevance if PR-39 proofs have a therapeutic potential in neutrophil-mediated inflammatory diseases.

Abbreviations
BSA – Bovine serum albumin
fMLP – formyl-Met-Leu-Phe
HSPG – Heparan sulfate proteoglycan
PR-39 – Prolin-arginine-rich 39 peptide

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
AD carried out the chemotaxis experiments and drafted the manuscript. BM and NCK carried out the enzyme digestion and antibody inhibition studies, respectively. CRR, GR and JRP participated in the design of the study. CJW conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements
The study was partly supported by the „Verein zur Förderung von Forschung und Fortbildung in Klinischer Kardiologie und Intensivmedizin – Innsbruck, Österreich“.

References
1. Lehrer RI, Ganz T: Defensins of vertebrate animals. *Curr Opin Immunol* 2002, 14:96-102.
2. Lehrer RI, Ganz T: Cathelicidins: a family of endogenous antimicrobial peptides. *Curr Opin Hematol* 2002, 9:18-22.
3. Zaiou M, Gallo RL: Cathelicidins, essential gene-encoded mammalian antibiotics. *J Mol Med* 2002, 80:549-61.
4. Zanetti M, Gennaro R, Romeo D: Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett* 1995, 374:1-5.
5. Zanetti M, Del Sal G, Storici P, Schneider C, Romeo D: The cDNA of the neutrophil antibiotic Bac5 predicts a pro-sequence homologous to a cysteine proteinase inhibitor that is common to other neutrophil antibiotics. *J Biol Chem* 1993, 268:522-6.
6. Bagella L, Scocchi M, Zanetti M: cDNA sequences of three sheep myeloid cathelicidins. *FEBS Lett* 1995, 376:223-8.
Gallo RL, Kim KJ, Bernfeld M, Kozak CA, Zanetti M, Merluzzi L, Gen- naro R: Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *J Biol Chem* 1977, 272:3088-93.

Agerberth B, Gunne H, Odberg J, Kogner P, Boman HG, Gudmund- son GH: FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *Proc Natl Acad Sci USA* 1995, 92:195-9.

Storici P, Scocchi M, Tossi A, Gennaro R, Zanetti M: Chemical syn- thesis and biological activity of a novel antibacterial peptide deduced from a pig myeloid cDNA. *FEBS Lett* 1994, 337:303-7.

Tossi A, Scocchi M, Zanetti M, Storici P, Gennaro R: MPAM-37, a novel antibacterial peptide from pig myeloid cells. cDNA cloning, chemical synthesis and activity. *Eur J Biochem* 1995, 228:941-6.

Zanetti M, Litteri L, Gennaro R, Horstmann H, Romeo D: Bacteriocins, defense polypeptides of bovine neutrophils, are generated in granules stored in the large granules. *J Cell Biol* 1991, 111:1363-71.

Gallo RL, Ono M, Povsic T, Page C, Eriksson E, Klagsbrun M, Bernfeld M: Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from bovine neutrophils. *Proc Natl Acad Sci USA* 1994, 91:1616-9.

Frohm NM, Sandstedt B, Sorensen O, Weber G, Borregaard N, Staale-Backdahl M: The human cationic antimicrobial peptide (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. *Infect Immun* 1995, 63:2561-6.

Sorensen O, Arniljots K, Cowland JB, Bainton DF, Borregaard N: The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood* 1997, 90:2796-803.

Agerberth B, Lee YP, Bergman T, Carlquist M, Boman HG, Must V, Jornvall H: Amino acid sequence of PR-39. Isolation from pig intestin e of a new member of the family of proline-arginine- rich antibacterial peptides. *Eur J Biochem* 1991, 202:849-54.

Shi J, Ross CR, Chengappa MM, Blecha F: Identification of a proline-arginine-rich antibacterial peptide from neutrophils that is analogous to PR-39, an antibacterial peptide from the small intestine. *J Leukoc Biol* 1994, 56:807-11.

Shi J, Ross CR, Chengappa MM, Sylte MJ, McVey DS, Blecha F: Anti- bacterial activity of a symmetrical peptide (PR-26) derived from PR-39, a proline-arginine-rich neutrophil antibacterial peptide. *Antimicrob Agents Chemother* 1996, 40:115-21.

Boman HG, Agerberth B, Boman A: Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestinal mucosa. *Infect Immun* 1993, 61:2978-84.

Ohtake T, Fujimoto Y, Iikata K, Saito H, Ohhira M, Ono M, Kohgo Y: Proline-rich antibacterial peptide, PR-39 gene transcription altered invasive activity and actin structure in human hepa- tocellular carcinoma cells. *Br J Cancer* 1999, 80:393-403.

Li J, Post M, Volk R, Gao Y, Li M, Matias C, Sato K, Tsai J, Aird W, Rosenberg RD, Hampton TG, Sellke F, Carmeliet P, Simons M: PR39, a peptide regulator of angiogenesis. *Nat Med* 2000, 6:49-55.

Koenig A, Norgard-Sumnicht K, Linhardt R, Varki A: Differential interactions of heparin and heparan sulfate glycosaminogly- cans with the selectins. Implications for the use of unfrac- tionated and low molecular weight heparins as therapeutic agents. *Clin Invest* 1998, 101:877-89.

Parmley RT, Hurst RE, Takagi M, Spicer SS, Austin RL: Gly- cosaminoglycans in human neutrophils and leukemic myeloblasts: ultrastructural, cytochemical, immunologic, and biochemical characterization. *Blood* 1983, 61:257-66.

Yeaman M, Raperae AC: Membrane-anchored proteoglycans of mouse macrophages: P388D1 cells express a syndecan-4-like heparan sulfate proteoglycan and a distinct chondroitin sulfate form. *J Cell Biol* 1993, 127:43-52.

Yeaman M, Raperae AC: Post-transcriptional regulation of syndecan-1 expression by cAMP in peritoneal macrophages. *J Cell Biol* 1993, 122:941-50.

Huang HJ, Ross CR, Blecha F: Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. *J Leukoc Biol* 1997, 61:624-9.

Dunnendorfer S, Kaneider N, Rabensteiner A, Meierhofer C, Reinsch C, Romisch J, Wiedermann CJ: Cell-surface heparan sulfate pro- teoglycan-mediated regulation of human neutrophil migra- tion by the serpin antithrombin III. *Blood* 2001, 97:1079-85.

Mertens G, Casimian JF, Van den Bergh E, Vemulapalli D, Ji David G: Cell surface heparan sulfate proteoglycan from human vascular endothelial cells. Core protein characterization and anti- thrombin III binding properties. *J Biol Chem* 1992, 267:20435-43.

James PE, Madhani M, Ross C, Klei L, Barchowsky A, Swartz HM: Tis- sue hypoxia during bacterial sepsis is attenuated by PR-39, an antibacterial peptide. *Adv Exp Med Biol* 2003, 530:645-52.

Madhani M, Barchowsky A, Klei L, Ross CR, Jackson SK, Swartz HM, James PE: Antibacterial peptide PR-39 affects local nitric oxide and preserves tissue oxygenation in the liver during septic shock. *Biochim Biophys Acta* 2002, 1582:232-40.

De Y, Chen Q, Schmidt AP, Anderson GM, Wang J, Wooters J, Oppenheim JJ, tertor O: LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemottract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* 2000, 192:1069-74.

Nyonsaba F, Ibawuchi K, Someya A, Hirata M, Matsuda H, Ogawa H, Nagaoa I: A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology* 2000, 106:206-6.

Kaneider NC, Egger P, Dunzendorfer S, Wiedermann CJ: Syndecan-4 as antithrombin receptor of human neutrophils. *Biochem Biophys Res Commun* 2001, 287:42-6.

Cardin AD, Weintraub HJ: Molecular modeling of protein-gly- cosaminoglycan interactions. *Antimicrob Agents Chemother* 1989, 32:1-3.

Andersson E, Rydengard V, Sonesson A, Morgelin M, Bjoerk L, Schmidtchen A: Antimicrobial activities of heparin-binding peptides. *Eur J Biochem* 2004, 271:1219-26.

Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Linemme J, Zander J: Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 1999, 68:729-77.

Wadstrom T, Ljung A: Glycosaminoglycan-binding microbial proteins in tissue adhesion and invasion: key events in micro- bial pathogenicity. *J Med Microbiol* 1999, 48:223-33.

Kjellen L, Lindahl U: Proteoglycans: structures and interac- tions. *Annu Rev Biochem* 1991, 60:443-75.

Bernfield M, Kokkeny R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EF: Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu Rev Cell Biol* 1992, 8:365-93.

Chen YR, Gallo RL: PR-39, a syndecan-inducing antimicrobial peptide, binds and affects p130(Cas). *J Biol Chem* 1999, 273:28978-85.

Honda H, Nakamoto T, Sakai R, Hiris H: p130(Cas), an assem- bling molecule of actin filaments, promotes cell movement, cell migration, and cell spreading in fibroblasts. *Biochem Biophys Res Commun* 1999, 262:25-30.

Cary LA, Han DC, Polte TR, Hanks SK, Guan JL: Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *J Cell Biol* 1998, 140:21-21.

Wilcox-Adelman SA, Denzeh F, Gootsch PK: Syndecan-4 modu- lates focal adhesion kinase phosphorylation. *J Biol Chem* 2002, 277:32970-7.