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Osteomyelitis prevention in rabbits using antimicrobial peptide hLF1-11- or gentamicin-containing calcium phosphate cement

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***Objectives*: The efficacy of prophylactic treatment with human lactoferrin 1–11 (hLF1-11), a broad-spectrum antimicrobial peptide, was studied in a rabbit model of femur infection.**

***Methods*: Calcium phosphate cement with 50 mg/g hLF1-11 or gentamicin was injected into the femoral canal, after inoculation with *Staphylococcus aureus*. Three weeks later, slices of the proximal femora were sawn for quantitative bacterial culture and histology.**

***Results*: Treatment with hLF1-11 ($P < 0.038$) or gentamicin ($P < 0.008$) caused a reduction of cfu compared with the untreated control rabbits. The number of sterile cultures was higher in hLF1-11- (3/7) and gentamicin- (5/6) treated animals than in controls (1/7). Radiological and histological analysis showed early bone ingrowth into the cement cracks, and only moderate pathological changes in rabbits with positive cultures.**

***Conclusions*: Local prophylaxis with hLF1-11 effectively reduced development of osteomyelitis in a rabbit model, but gentamicin resulted in a larger number of sterile femora.**

Keywords: biodegradable carriers, drug delivery, intramedullary infections, peptide antibiotics, animal models

Introduction

In spite of increasing diagnostic and treatment options, bone infection remains one of the most serious complications in orthopaedic surgery. Its high impact is suffered equally in terms of increased morbidity and comprehensive use of expensive healthcare resources.[1] *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) have been the main causative organisms; during the last two decades a shift toward more CoNS and resistant strains has been reported.[2,3] Current treatment of osteomyelitis usually includes a thorough debridement and placement of polymethyl-methacrylate (PMMA) beads releasing an antibiotic e.g. gentamicin. However, the induction of gentamicin-resistant bacteria in patients treated with genta-beads has recently been reported.[3] Therefore new treatment options, including novel antibiotics which are slowly released from carrier-matrices, are being explored.[4–10]

Antimicrobial peptides (AMPs), short positively charged peptides, have been found in virtually all forms of life as a first line of defence against invading microorganisms.[11,12] They combine pore formation in negatively charged bacterial cell membranes with intracellular killing events and have a minimal propensity to induce resistance.[13–15] Their fast killing effect on a broad range of microorganisms offers a great opportunity for the development of new antibiotic agents.[14] These peptides have been proposed as a novel class of natural antibiotic agents, and might be used clinically to treat infections caused by resistant bacterial strains.[11] Potent antimicrobials have been produced by using natural peptides as a template, and by taking active domains of larger proteins.[16–18]

The antimicrobial peptide hLF1-11 consists of N-terminal amino acids 1–11 of human lactoferrin, and has a broad antimicrobial spectrum.[5,19,20] It has shown *in vivo* bactericidal activity after systemic administration against a variety of microorganisms, including methicillin-resistant *S. aureus* (MRSA).[20] Biodegradable calcium phosphate (CaP) cements have been studied as a carrier for the controlled release of many different antibiotics.[6] We previously shown that hLF1-11 was still able to kill MRSA after being released from CaP carriers *in vitro*.[5] The present study describes the efficacy of hLF1-11-loaded

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and gentamicin-loaded CaP cement for the prevention of osteomyelitis in a well-characterized rabbit model.[7–9]

Materials and methods

Bacterial strain

S. aureus (Wood 46, ATCC 10832) was used to inoculate the rabbit femora (gentamicin MIC 8 mg/L by broth dilution method; hLF1-11 LC_{50} 6.25 mg/L by cfu assay adapted for salt-sensitive AMPs).[5,20] After culture in brain heart infusion, a stock of aliquots for single use was frozen. Pre-operatively, samples containing $\sim 10^7$ cfu/mL PBS were prepared; a volume of 0.1 mL (10^6 cfu) was injected into the femoral canal. Previous studies have shown this dosage to result in a high rate of infection in the presence of PMMA cement.[9] Post-operatively, the inoculum was serially diluted and plated on blood agar; colony count after 18 h at 37°C confirmed accurate bacterial content.

Antimicrobial agents

The hLF1-11-peptide (GRRRRSVQWCA, 1375 Da) was manufactured by solid-phase peptide synthesis using Fmoc (9-fluorenylmethoxycarbonyl) chemistry, as described previously.[21] Reanalysis of peak fractions by reversed phase HPLC resulted in one major peak, revealing at least 90% purity; the authenticity of the peptide was confirmed by electrospray ionization quadrupole time-of-flight mass spectrometry (Q-TOF MS, Micromass Inc., Manchester, UK). Gentamicin sulphate powder (GS) was a gift from Biomet Merck BioMaterials (Darmstadt, Germany).

Animals

Twenty-six healthy (specific pathogen free) adult female New Zealand White rabbits (Harlan, Horst, the Netherlands) of 3.23 ± 0.24 kg were kept in group housing. They were assigned to the following treatments: hLF1-11 ($n = 7$), gentamicin ($n = 7$), control (infection, no antibiotic agent, $n = 9$) and sham (operated on but not infected, $n = 3$).

The guidelines according to the Dutch *Act on Animal Experiments* (1985) have been observed.

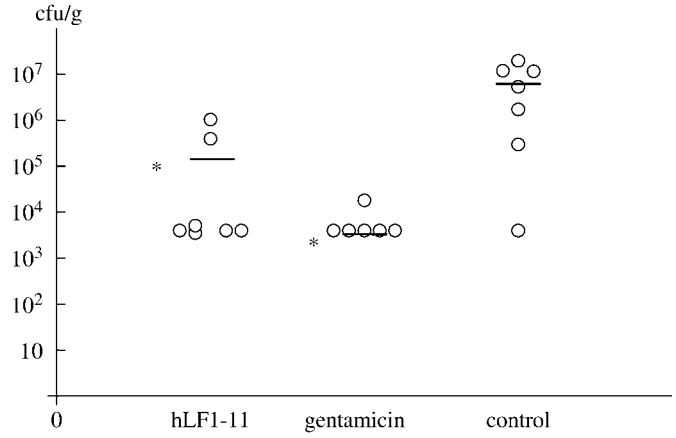

Figure 1. Bone cultures show a significant reduction in bacteria for both the hLF1-11 ($P < 0.038$) and the gentamicin ($P < 0.008$) groups compared with the control group (indicated by *). The average bacterial counts per group are shown as a horizontal line.

Operative procedure

The operation was performed as described by Nijhof *et al.*[7] After sedation, blood samples were drawn for leucocyte count (WBC) and erythrocyte sedimentation rate (ESR) determination. The trochanter tertius of the right femur was surgically exposed and a hole to the medullary canal drilled. Subsequently, 0.1 mL of bacterial suspension (hLF1-11, gentamicin and control groups) or PBS (sham group) was injected, followed by injection of the cement paste. The cement was prepared immediately before inoculation of 1 g of Bonesource cement powder (Stryker-Leibinger, Freiburg, Germany), 50 mg of hLF1-11 (hLF1-11 group) or GS (gentamicin group) and 0.40 mL of mixing solution (Stryker-Leibinger). The control and sham groups received plain cement without antibiotic agent. The cement syringe was weighed before and after injection, to determine the amount of injected cement.

As a baseline image, radiographs of the right femora were made post-operatively. Additional blood samples were drawn on days 7 and 21.

Autopsy and sample acquisition

After 21 days, the animals were killed with a pentobarbital overdose; both femora were aseptically excised and radiographed.[7] Signs of osteomyelitis were scored using Norden's system in which a maximum score of seven corresponds with maximal pathogenic changes.[22]

Of the proximal femoral diaphysis, two transverse slices of ± 0.5 cm were sawn; the proximal sample was used for histological and the distal for bacteriological determination. Proximal bone sections were decalcified, embedded in paraffin, H&E stained and mounted on slides. Signs of infection were scored using Smeltzer's system, in which a maximum score of 16 signifies severe osteomyelitis.[23] After careful removal of the cement from the distal sample, it was weighed and ground in 50 mL of PBS. This suspension was serially diluted and plated on blood agar; the colony count after 18 h at 37°C was calculated to represent cfu/g bone. Negative cultures were conservatively calculated as 4000 cfu/g (the average detection limit). A bacterial load greater than 10^5 cfu/g (arbitrarily set at 10% of the inoculum dose) was considered a sign of full-blown infection.

Statistics

The samples from all animals that completed the 21 day follow-up period were randomized in a blinded manner and evaluated by two observers. The differences between groups were analysed using a two-tailed Mann–Whitney *U*-test; $P < 0.05$ was considered significant (values are means ± SD).

Results

Three animals were excluded due to post-operative complications. One rabbit in the control group died immediately post-operatively; autopsy revealed massive pulmonary embolisms. Two animals sustained a femoral fracture during the follow-up period, one in the gentamicin group and one in the control group. The other 23 animals recovered well and appeared to slightly increase in weight (0.20 ± 0.27 g) without showing signs of systemic infection.

Quantitative culture results indicated that the addition of gentamicin ($P = 0.008$) or hLF1-11 ($P = 0.038$) resulted in a significant decrease in viable bacteria (Figure 1). Table 1 summarizes the results for all treatment groups: cultures, amount of injected

Table 1. Characteristics of *S. aureus*-inoculated rabbit femora after prophylactic treatment with cement containing 50 mg/g hLF1-11 or gentamicin compared with controls

Group	Culture ($\log_{10}$ cfu/g)	Histology (0–16)	Cement weight (g)	Radiology (0–7)	Weight change (kg)
hLF1-11	4.2 ± 1.1^a	7.5 ± 2.8	0.89 ± 0.50	2.9 ± 2.9	$+0.21 \pm 0.29$
Gentamicin	3.7 ± 0.3^b	3.9 ± 1.1^c	0.82 ± 0.23	1.6 ± 1.7	$+0.12 \pm 0.27$
Control	6.2 ± 1.3	6.8 ± 2.8	0.78 ± 0.28	2.8 ± 1.5	$+0.04 \pm 0.20$

[a] $P < 0.038$.
[b] $P < 0.008$.
[c] $P < 0.045$.

cement, body-weight change, histological score and radiological score. Bacteria were cultured from 11 of the 23 operated femora: four of seven in the hLF1-11 group, one of six in the gentamicin group and six of eight in the control group. The non-inoculated sham group and the un-operated left femora had sterile cultures. The amount of cement injected during the operation was 0.83 ± 0.34 g; there was no correlation between amount of injected cement and culture result.

The pathological changes of infected rabbit femora included intramedullary abscess formation, bone necrosis and periostal bone formation. However, not every femur with positive bacterial cultures had all histological signs of infection. The biodegradability of the cement resulted in early remodelling by ingrowing bone in some of the samples. The group of femora with positive bone cultures had a significantly higher histological score (7.5 ± 2.5, $P = 0.028$) than those with sterile cultures (4.7 ± 2.5). The gentamicin-treated rabbits had a significantly lower score ($P = 0.045$) than controls; hLF1-11 treatment did not result in a statistical difference from controls (Table 1).

Overall, the femora with positive culture results had a higher average radiographic score (3.4 ± 1.2, $P = 0.006$) than sterile femora (2.1 ± 1.7). However, this did not result in significant radiological differences between the treatment groups (Table 1). The ESR and WBC values showed no significant post-operative changes within animals or between groups (data not shown).

Discussion

To our knowledge, this is the first study that describes the efficacy of a locally released AMP, hLF1-11, for prevention of osteomyelitis. Injection of calcium phosphate cement containing hLF1-11 or gentamicin into a previously inoculated femoral canal significantly reduced the bacterial load compared with plain cement. Although gentamicin resulted in more sterile bone cultures than hLF1-11, this difference was not statistically significant.

The *in vivo* killing effect of natural and synthetic AMPs has been demonstrated on a variety of microorganisms, but the number of published animal studies is still relatively small.[20,24–26] Intravenous injection of low concentrations (ng/mL) of hLF1-11 effectively reduced infection by resistant *S. aureus* strains in a mouse model of thigh-muscle infection.[20] Synthetic and bovine analogues of hLF1-11 have demonstrated antimicrobial activity in mouse models by decreasing *Escherichia coli* bladder infection, and *Toxoplasma gondii*-induced brain cysts.[24,25] Furthermore, intradermal injection of 1–6 mg Novispirin G10 effectively reduced infection in rats with *Pseudomonas aeruginosa*-infected burns.[26] Finally, a number of clinical trials underlines the potential of future AMP-based drugs for treatment of superficial infections, oral mucositis and paediatric sepsis.[27–29]

Besides direct antimicrobial killing activity through pore formation and intracellular targeting, AMPs are claimed to have an immunomodulating effect.[15,20] This results in higher *in vivo* than *in vitro* antimicrobial activity by specific activation of signalling cascades in the host immune system.[11,20] The *in vivo* antimicrobial activity after intravenous administration of hLF1-11 at minimal (ng/mL) concentrations supports the role of an immunomodulating effect of this human-derived peptide.[20] Although some AMPs, like the bee venom mellitin, are toxic to mammalian cells, exposure of human erythrocytes to high concentrations of hLF1-11 up to 200 μg/mL did not indicate *in vitro* cytotoxicity of the peptide (unpublished data). Furthermore, human allergic reactions would not be likely since hLF1-11 consists of a small fragment of the human lactoferrin protein.

Rabbits are among the most frequently used animals for experimental bone infection studies.[30] Most studies report complications similar to those described in the current study, either related to infection, to surgery or to cement injection.[9,10] Animal models of osteomyelitis prevention require a high rate of infection in the (untreated) control animals in order to detect differences with the treated animals. In the presented study, infection was established in 85% of the (untreated) control animals; similar rates of infection have been reported in other rabbit infection studies.[9,31] The infection rate in this study compares well with other studies, and modification of several factors could further improve results and decrease the number of complications.[23,30,32] Virulence of the bacterial strain, inoculum dose, presence of a biomaterial to which bacteria preferentially adhere, surgical technique and local tissue trauma all influence the infection rate in untreated animals.[8,31,33]

The presence of tissue necrosis or a foreign material is essential in the development of osteomyelitis in animal models.[8,32] The competition between host cells and bacteria to establish predominance on the implant surface (the race for the surface) determines whether or not the implant becomes infected.[34] The result of this conflict depends highly on the material properties of the implant surface. Although speculative, injectable CaP cement may influence bacterial growth by its setting reaction and subsequent osteoconductive properties, which might explain why no infection was detected in one of seven control animals (infected but untreated).

In the present study, the rabbits' clinical results showed mild to moderate signs of infection; the small increase in body

weight, the minor variation in ESR and WBC and the absence of signs of systemic illness all indicate that the induced infection was contained within the femur. Furthermore, the relatively small number of pathological changes recorded on histological and radiological observation could be consistent with an infection of intermediate severity. The cement in the proximal femur could have obscured some of the pathological signs on radiographs; nevertheless, the low histological scores appear to confirm the contained and limited nature of the femur infection. However, the short follow-up period (3 weeks) may not have been sufficient for both the disappearance of tissue reaction due to the surgical trauma and the full development of signs of infection in all rabbits.

We used an established technique for histology and culture, but a sample error may still have occurred, which could have resulted in missed cases of infection.[7] The difference in infection reduction between hLF1-11 and gentamicin was not significant; adaptation of the drug delivery system may increase the cure rate. Future improvements could include alterations to the AMP dose, the peptide structure and the carrier material to optimize release kinetics *in vivo*. We previously demonstrated that part of the added hLF1-11 may bind to the cement during the setting reaction, which could limit release. More complete release occurred when higher amounts were added to the cement.[4,5] These experiments support using a high dose of hLF1-11; however, there are practical limitations to the scale of experimental peptide production and purification methods.[21]

In conclusion, both hLF1-11 and gentamicin reduced the number of infected animals and the bacterial count in infected femora. The recent observation of resistant bacterial strains on explanted gentamicin-containing PMMA beads (in 18 of 20 patients) highlights the clinical impact of antibiotic resistance in orthopaedic infections.[3] Our results, using a non-resistant strain, support the concept of using hLF1-11 in the prevention of osteomyelitis and thus warrant pre-clinical studies of bone infection caused by resistant strains, comparing the efficacy of AMPs with current antibiotic agents.

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References

1. Sculco, T. P. (1993). The economic impact of infected total joint arthroplasty. *Instructional Course Lectures* **42**, 349–51.

2. Ostendorf, M., Malchau, H., Dhert, W. J. *et al.* (2001). 749 Revisions for infection from the Swedish National Hip Registry. In *Programs and Abstracts of the Sixty-eighth Annual Meeting of the American Academy of Orthopaedic Surgeons, 2001*, Abstract 749. American Academy of Orthopaedic Surgeons, Rosemont, IL, USA.

3. van de Belt, H., Neut, D., van Horn, J. R. *et al.* (1999). Antibiotic resistance—to treat or not to treat? *Nature Medicine* **5**, 358–9.

4. Faber, C., Stallmann, H. P., Lyaruu, D. M. *et al.* (2003). Release of antimicrobial peptide Dhvar-5 from polymethylmethacrylate beads. *Journal of Antimicrobial Chemotherapy* **51**, 1359–64.

5. Stallmann, H. P., Faber, C., Slotema, E. T. *et al.* (2003). Continuous-release or burst-release of the antimicrobial peptide human lactoferrin 1–11 (hLF1-11) from calcium phosphate bone substitutes. *Journal of Antimicrobial Chemotherapy* **52**, 853–5.

6. Castro, C., Sanchez, E., Delgado, A. *et al.* (2003). Ciprofloxacin implants for bone infection. In vitro-in vivo characterization. *Journal of Controlled Release* **93**, 341–54.

7. Nijhof, M. W., Stallmann, H. P., Vogely, H. C. *et al.* (2000). Prevention of infection with tobramycin-containing bone cement or systemic cefazolin in an animal model. *Journal of Biomedical Materials Research* **52**, 709–15.

8. Nijhof, M. W., Dhert, W. J., Fleer, A. *et al.* (2000). Prophylaxis of implant-related staphylococcal infections using tobramycin-containing bone cement. *Journal of Biomedical Materials Research* **52**, 754–61.

9. Nijhof, M. W., Fleer, A., Hardus, K. *et al.* (2001). Tobramycin-containing bone cement and systemic cefazolin in a one-stage revision. Treatment of infection in a rabbit model. *Journal of Biomedical Materials Research* **58**, 747–53.

10. Vogely, H. C., Oosterbos, C. J., Puts, E. W. *et al.* (2000). Effects of hydroxyapatite coating on Ti-6A1-4V implant-site infection in a rabbit tibial model. *Journal of Orthopaedic Research* **18**, 485–93.

11. Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389–95.

12. van't Hof, W., Veerman, E. C., Helmerhorst, E. J. *et al.* (2001). Antimicrobial peptides: properties and applicability. *Biological Chemistry* **382**, 597–619.

13. Groisman, E. A. (1994). How bacteria resist killing by host-defense peptides. *Trends in Microbiology* **2**, 444–9.

14. Hancock, R. E. (1997). Peptide antibiotics. *Lancet* **349**, 418–22.

15. Helmerhorst, E. J., Breeuwer, P., van't Hof, W. *et al.* (1999). The cellular target of histatin 5 on *Candida albicans* is the energized mitochondrion. *Journal of Biological Chemistry* **274**, 7286–91.

16. Nekhotiaeva, N., Elmquist, A., Rajarao, G. K. *et al.* (2003). Cell entry and antimicrobial properties of eukaryotic cell-penetrating peptides. *FASEB Journal* **18**, 394–6.

17. Helmerhorst, E. J., van't Hof, W., Veerman, E. C. *et al.* (1997). Synthetic histatin analogues with broad-spectrum antimicrobial activity. *Biochemical Journal* **326**, 39–45.

18. van der Kraan, M. I. A., Groenink, J., Nazmi, K. *et al.* (2004). Lactoferrampin: a novel antimicrobial peptide in the N1-domain of bovine lactoferrin. *Peptides* **25**, 177–83.

19. Lupetti, A., Danesi, R., 't Wout, J. W. *et al.* (2002). Antimicrobial peptides: therapeutic potential for the treatment of Candida infections. *Expert Opinion on Investigational Drugs* **11**, 309–18.

20. Nibbering, P. H., Ravensbergen, E., Welling, M. M. *et al.* (2001). Human lactoferrin and peptides derived from its N terminus are highly effective against infections with antibiotic-resistant bacteria. *Infection and Immunity* **69**, 1469–76.

21. van't Hof, W., Driedijk, P. C., van den Berg, M. *et al.* (1991). Epitope mapping of the *Dermatophagoides pteronyssinus* house dust mite major allergen Der p II using overlapping synthetic peptides. *Molecular Immunology* **28**, 1225–32.

22. Norden, C. W., Myerowitz, R. L. & Keleti, E. (1980). Experimental osteomyelitis due to *Staphylococcus aureus* or *Pseudomonas aeruginosa*: a radiographic-pathological correlative analysis. *British Journal of Experimental Pathology* **61**, 451–60.

23. Smeltzer, M. S., Thomas, J. R., Hickmon, S. G. *et al.* (1997). Characterization of a rabbit model of staphylococcal osteomyelitis. *Journal of Orthopaedic Research* **15**, 414–21.

24. Isamida, T., Tanaka, T., Omata, Y. *et al.* (1998). Protective effect of lactoferricin against *Toxoplasma gondii* infection in mice. *Journal of Veterinary Medical Science* **60**, 241–4.

25. Haversen, L. A., Engberg, I., Baltzer, L. *et al.* (2000). Human lactoferrin and peptides derived from a surface-exposed helical region reduce experimental *Escherichia coli* urinary tract infection in mice. *Infection and Immunity* **68**, 5816–23.

26. Steinstraesser, L., Tack, B. F., Waring, A. J. *et al.* (2002). Activity of novispirin G10 against *Pseudomonas aeruginosa* in vitro and in infected burns. *Antimicrobial Agents and Chemotherapy* **46**, 1837–44.

27. Giles, F. J., Miller, C. B., Hurd, D. D. *et al.* (2003). A phase III, randomized, double-blind, placebo-controlled, multinational trial of iseganan for the prevention of oral mucositis in patients receiving stomatotoxic chemotherapy (PROMPT-CT trial). *Leukemia and Lymphoma* **44**, 1165–72.

28. Isaacson, R. E. (2003). MBI-226. Micrologix/Fujisawa. *Current Opinion in Investigational Drugs* **4**, 999–1003.

29. Levin, M., Quint, P. A., Goldstein, B. *et al.* (2000). Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. *Lancet* **356**, 961–7.

30. Cremieux, A. C. & Carbon, C. (1997). Experimental models of bone and prosthetic joint infections. *Clinical Infectious Diseases* **25**, 1295–302.

31. Mayberry-Carson, K. J., Tober-Meyer, B., Lambe, D. W., Jr. *et al* (1992). Osteomyelitis experimentally induced with *Bacteroides thetaiotaomicron* and *Staphylococcus epidermidis*. Influence of a foreign-body implant. *Clinical Orthopaedics* **280**, 289–99.

32. Norden, C. W. & Niederriter, K. (1988). Treatment of experimental chronic osteomyelitis due to *Staphylococcus aureus* with LY146032. *Infection* **16**, 27.

33. van de Belt, H., Neut, D., Schenk, W. *et al.* (2001). *Staphylococcus aureus* biofilm formation on different gentamicin-loaded polymethylmethacrylate bone cements. *Biomaterials* **22**, 1607–11.

34. Gristina, A. G., Oga, M., Webb, L. X. *et al.* (1985). Adherent bacterial colonization in the pathogenesis of osteomyelitis. *Science* **228**, 990–3.