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Continuous-release or burst-release of the antimicrobial peptide human lactoferrin 1-11 (hLF1-11) from calcium phosphate bone substitutes

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***Objectives*: In order to identify possible drug delivery systems against resistant bone infection, we determined the release of the antimicrobial peptide (AMP) human lactoferrin 1-11 (hLF1-11) from commercially available bone substitutes.**

***Methods*: We combined six calcium phosphate cements and six granule-types with 5 mg/g hLF1-11 and measured its availability and release *in vitro* from cements (7 days) and granules (3 days). The integrity and antimicrobial activity of the hLF1-11 that was released during the first 24 h were measured, using mass spectrometry, and a killing assay on methicillin-resistant *Staphylococcus aureus* (MRSA).**

***Results*: Most of the cements showed burst release followed by low-level continuous release, whereas the coated granules showed high burst release for 24 h. After release the peptide was active (in nine of 12 materials) and intact.**

***Conclusions*: Different release profiles may be obtained by choosing the appropriate carrier, which supports the feasibility of biodegradable carriers releasing AMPs against resistant infections.**

Keywords: bone infections, human lactoferrin, biodegradable, carriers

Introduction

Antimicrobial resistance will probably complicate future treatment of bone infection, treatment of which requires local and systemic antibiotics and often several surgical interventions. The non-degradable polymethylmethacrylate beads which are used currently as antibiotic carriers require operative removal and may induce resistant bacteria.[1]

We aimed to address this increasing problem by combining an antimicrobial peptide (AMP) of human origin (hLF1-11) with biodegradable carriers—which obviates the need for operative removal—and analysing its availability and release. These carriers, which consist of (a combination of) calcium-phosphate ceramics, e.g. tricalcium phosphate, $Ca_3(PO_4)_2$, are slowly replaced by ingrowing bone after implantation.[2] AMPs form a novel class of antimicrobial agents of natural origin that have been identified in virtually all forms of life as part of the antimicrobial defence system. These positively charged peptides kill by forming pores in the negatively charged bacterial cell-membrane and targeting intracellular organelles, without development of resistance.[3] Moreover, they seem to have an immunomodulating effect, killing microorganisms at lower concentrations *in vivo* (ng/mL) than *in vitro* (μg/mL).[4,5]

Materials and methods

Peptide

hLF1-11-peptide (GRRRRSVQWCA, 1375 Da) was manufactured by solid-phase peptide synthesis using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry as described previously.[6] Reanalysis of peak fractions by reversed phase HPLC resulted in one major peak revealing at least 90% purity. The authenticity was confirmed by electrospray-ionization quadrupole-time-of-flight mass-spectrometry (Q-TOF MS, Micromass Inc., Manchester, UK). Thermal stability in solution, adhesion to polystyrene and solubility were tested as described previously.[7]

Release experiment

After mixing cement powder and liquid containing 5 mg hLF1-11 per gram of powder (liquid/powder ratio according to the manufacturer), cylindrical specimens hardened overnight at 37°C in 6 × 5 mm moulds. The cements were: Biobon (Biomet Merck Biomaterials, Darmstadt, Germany), Calcibon (Biomet Merck Biomaterials), Biofil, (experimental, DePuy CMW, Blackpool, UK), Bonesource (Stryker-Leibinger, Freiburg, Germany), Chronos Inject, (experimental, Mathys, Bettlach, Switzerland) and Norian SRS (Mathys).

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Table 1. hLF1-11 released by the carriers and antimicrobial activity against MRSA

Carrier	hLF1-11 (mg/g)			Killing		
	released	biologically available	extracted after release	(%)	hLF1-11 (log cfu)	control (log cfu)
Cements						
Biobon	0.57 ± 0.10	3.25 ± 0.18	2.47 ± 0.07	98	4.1	5.8
Biofil	0.57 ± 0.19	2.70 ± 0.13	1.96 ± 1.08	72	6.3	6.9
Bonesource	0.10 ± 0.05	2.69 ± 0.06	2.65 ± 0.45	97	4.4	6.0
Calcibon	0.22 ± 0.02	2.89 ± 0.27	2.25 ± 0.45	88	5.1	6.0
Chronos	1.69 ± 0.30	3.43 ± 0.07	2.19 ± 0.24	92	4.8	6.0
Norian	0.12 ± 0.10	2.65 ± 0.12	2.66 ± 0.30	83	6.2	6.9
Granules						
Allogran	2.52 ± 0.38	2.64 ± 0.57	0.00	99	3.8	6.2
Bicalphos	4.25 ± 0.30	4.31 ± 0.60	0.07	99	3.8	6.4
Biosorb	2.53 ± 0.68	2.63 ± 0.84	0.00	99	3.8	6.2
Bonesave	1.58 ± 0.21	1.83 ± 0.40	0.00	99	3.8	6.2
Cerasorb	1.50 ± 0.20	1.52 ± 0.18	0.11	99	3.8	6.2
Vitoss	4.43 ± 1.46	4.61 ± 1.00	0.00	99	3.8	6.2

The amount of hLF1-11 available immediately after production (biologically available) and after the release experiment (extracted after release) was determined by fine grinding and addition of 1 M NaCl. The values represent the mean ± S.D. from three experiments, totalling at least 12 samples per carrier-material. Sterile cultures were considered 99% killing, the detection limit.

Granules were immersed in 1.0 mL of dH_2O containing 5 mg of hLF1-11 per gram of material and lyophilized. After removal of the granules, the residual hLF1-11 in the vessel was measured. The granules were: Bonesave (Stryker-Leibinger), Biosorb (Science for Biomaterials, Lourdes, France), Allogran-R (Orthos, Bristol, UK), Vitoss (Orthovita Malvern, PA, USA), Cerasorb (Curasan, Kleinostheim, Germany) and Bicalphos (Medtronic, Memphis, TN, USA).

Specimens were immersed in 500 µL of dH_2O and kept in sealed polystyrene 48-well plates (Costar) at room temperature on a shaking device (180 rpm). The water was replaced at regular intervals: 30, 90 and 180 min on day 1 and then 24 hourly for 7 days (cements) or 3 days (granules) and stored at –20°C.

After production and after release, three specimens per group were finely ground and suspended in 5 mL dH_2O containing 1 M NaCl and the initial and residual hLF1-11 were determined. The hLF1-11 remaining in the vessel after lyophilization of the granules was also determined.

The hLF1-11 concentration was measured using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) and read at 540 nm (Bio-Rad) (hLF1-11 serial dilutions were used as a reference, the detection limit was 2.5 µg). Accuracy of the assay was calculated as 6.5% (mean error of true value), precision 3.5% (coefficient of variance). Control samples without hLF1-11 were used for background correction. The authenticity of the hLF1-11 of the first day release samples was analysed by Q-TOF MS and sequencing of the 1375 Da peak in one of the samples.

Antimicrobial activity

Samples of 500 µL release medium (taken after 24 h) were lyophilized (without carrier material) and 10^6 cfu of an MRSA clinical isolate (ATCC BAA-811) in PBS containing 0.01% Brain Heart Infusion was added. After 60 min at 37°C, these were plated on blood agar ($n = 6$), and colonies were counted after 18 h at 37°C. The percentage of killing was calculated: [1 – (cfu in sample/cfu in control)] × 100% (>90% was considered active). Samples without hLF1-11 were used as controls, samples from the first day were used because these all contained adequate amounts of peptide.

Results

Tests of hLF1-11 confirmed stability at 37°C in water for 26 weeks, solubility up to 150 mg/mL and no adhesion to polystyrene. Sample weight showed only a small variation (<0.01 g in samples of 0.150 g). Table 1 shows the amount of hLF1-11 that was initially available for dissociation, the quantity that was actually released from the carrier, and the part that was left in the carrier. From the cements, less hLF1-11 was extractable (2.65–3.43 mg/g) than had been incorporated (5 mg/g). For the granules, the amount of available peptide varied (1.5 g–4.6 mg/g), the rest was recovered from the vessel after lyophilization.

All cements displayed a sustained release profile for several days, with Biobon, Biofil and Chronos releasing significantly more hLF1-11 than the three other cements (Figure 1). All granules had *burst release* profiles in the first day only; Bicalphos, Bonesave and Vitoss released significantly more peptide than the other three granule types (not shown).

After release, a 1375 Da peak on MS-QTOF of the correct amino-acid sequence confirmed the integrity of hLF1-11 (control samples had no 1375 Da peak). The antimicrobial activity was >90% in nine of 12 carriers (Table 1).

Discussion

Low systemic toxicity and high local drug concentrations are the main advantages of local antibiotic delivery systems. These can be used both for treatment of chronic bone infection, requiring prolonged high antibiotic concentrations and for prevention, in which a short duration local antibiotic peak concentration may suffice.[8]

Using methods described by Kühn, adapted for biodegradable specimens, we determined the available hLF1-11 in samples before and after release.[9] Not all hLF1-11 added to *cement* was available for extraction, even after fine grinding and adding high salt concentra-

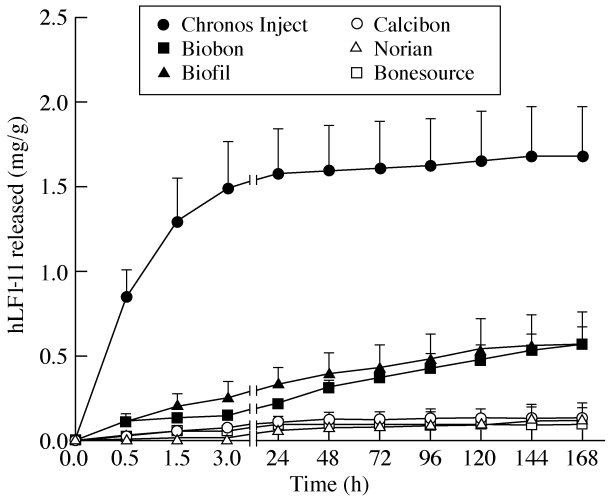

Figure 1. The cumulative release of hLF1-11 from cements is expressed in mg hLF1-11 per gram carrier material. The increments between the different time points represent the release for that specific interval, the *x*-axis is interrupted between 3.0 and 24 h. Total release from *high release* cements was: 33.8% for Chronos Inject (filled circles), 11.5% for Biobon (filled squares) and 11.4% for Biofil (filled triangles). This was significantly higher ($P < 0.05$, Student's *t*-test) than from *low release* cements: 2.7% for Calcibon (open circles) and 2.5% for Norian (open triangles) 1.9% for Bonesource (open squares).

tions to decrease charge-dependent binding. This suggests that part of the hLF1-11 strongly binds to the hardening cement leading to sequestration and unavailability for release, but it might still become available after osteoclastic resorption.

A variable amount of hLF1-11 attached to the *granules* during lyophilization, and the rest was detected in the coating vessel. High-porosity granules (Vitoss) bound most hLF1-11 indicating that the surface area of the carrier material might determine the loading capacity.

Thus, two different release profiles were observed in this study, which support a two-phase explanation of the release process. The initial burst-release is predominantly determined by the release of the drug from the surface of the carrier; the second phase shows the more gradual diffusion of the drug from deeper layers, determined by the porosity of the carrier.[9] The hLF1-11 released in the first day was active in nine of the 12 materials. Of these, Chronos was the highest releasing cement-type and Vitoss the highest releasing granule-type (Table 1). The amount of hLF1-11 released may be relevant *in vivo,* as Nibbering and co-workers demonstrated that hLF1-11 concentrations as low as 0.1 ng/mL kill MRSA *in vivo*.

Limitations of this study can be found in the extrapolation of the *in vitro* results to *in vivo* concentrations in bone and surrounding tissues. Instead of using simulated body fluid as the release medium, the experiment was carried out using small volumes of water. This allowed the use of a simple protein assay for the detection of hLF1-11. Disadvantages of this method are: (i) pH and ionic content were different from the *in vivo* situation, (ii) proteases that could degrade the hLF1-11 were not present, (iii) any positive interaction of AMP with the adaptive immune system could not be included.[5] The strength of *in vitro* release studies of this kind lies in a qualitative comparison of different carrier materials, allowing the identification of the most suitable materials for *in vivo* testing. In spite of the *in vitro* shortcomings, a positive correlation between *in vivo* and *in vitro* results has been reported by several authors.[10]

To conclude, in nine of the 12 combinations, basic requirements for designing a drug delivery system (controlled release of an active substance) have been met.[8] At present, investigations using animal models to study the *in vivo* efficacy of AMP-releasing drug delivery systems are under way.

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References

1. van de Belt, H., Neut, D., Uges, D. R. *et al.* (2000). Surface roughness, porosity and wettability of gentamicin-loaded bone cements and their antibiotic release. *Biomaterials* **21**, 1981–7.

2. Blokhuis, T. J., Termaat, M. F., den Boer, F. C. *et al.* (2000). Properties of calcium phosphate ceramics in relation to their in vivo behavior. *Journal of Trauma* **48**, 179–86.

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

4. 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.

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

6. 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.

7. 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.

8. Henry, S. L. & Galloway, K. P. (1995). Local antibacterial therapy for the management of orthopaedic infections. Pharmacokinetic considerations. *Clinical Pharmacokinetics* **29**, 36–45.

9. Kühn, K.-D. (2000). *Bone Cements: Up-To-Date Comparison of Physical and Chemical Properties of Commercial Materials*. Springer, Berlin, London.

10. Baro, M., Sanchez, E., Delgado, A. *et al.* (2002). In vitro–in vivo characterization of gentamicin bone implants. *Journal of Controlled Release* **83**, 353–64.