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Anti-endotoxic and antibacterial effects of a dermal substitute coated with host defense peptides

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Biomaterials used during surgery and wound treatment are of increasing importance in modern medical care. In the present study we set out to evaluate the addition of thrombin-derived host defense peptides to human acellular dermis (hAD, i.e. *epiflex*®). Antimicrobial activity of the functionalized hAD was demonstrated using radial diffusion and viable count assays against Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* bacteria. Electron microscopy analyses showed that peptide-mediated bacterial killing led to reduced hAD degradation. Furthermore, peptide-functionalized hAD displayed endotoxin-binding activity *in vitro*, as evidenced by inhibition of NF-κB activation in human monocytic cells (THP-1 cells) and a reduction of pro-inflammatory cytokine production in whole blood in response to lipopolysaccharide stimulation. The dermal substitute retained its anti-endotoxic activity after washing, compatible with results showing that the hAD bound a significant amount of peptide. Furthermore, bacteria-induced contact activation was inhibited by peptide addition to the hAD. *E. coli* infected hAD, alone, or after treatment with the antiseptic substance polyhexamethylenebiguanide (PHMB), yielded NF-κB activation in THP-1 cells. The activation was abrogated by peptide addition. Thus, thrombin-derived HDPs should be of interest in the further development of new biomaterials with combined antimicrobial and anti-endotoxic functions for use in surgery and wound treatment.

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1. Introduction

Over the past years, the use of biomaterial implants and tissue transplants has increased in areas of orthopedics, cardiology, ophthalmology, surgery, and dermatology, to name a few of many uses [1]. Biomaterials may include polymers such as polysaccharides or collagen, silicon or nylon matrices, hyaluronic acid and ester films, fibrin-based materials and hydrogels. Materials implanted into the body of human or an animal must be sterile to minimize risk for subsequent infection, and given bacterial presence, potential endotoxins should be controlled. Infection sensitivity and risk for chronic infections are significant biomaterial-associated problems [1]. The exact mechanisms underlying biomaterial-related infections have only been partly elucidated, mainly with a current focus on adhesion and biofilm formation. However, in the initial contact with blood or tissues, various biomaterials also induce an excessive inflammatory and coagulative response [2–5]. In this perspective, novel biologically-oriented strategies, providing coatings which comprise multiple features such as control of inflammation and coagulation, and blocking of bacteria and endotoxins should be of value.

Recently, scaffolds derived from xenogenic and allogenic extracellular matrices have been developed for tissue engineering applications including musculoskeletal, cardiovascular, urogenital and integumentary structures [6]. These bioscaffolds are composed of structural and functional proteins that are part of the native mammalian extracellular matrix that provides structure, mechanical properties, anchorage and communication sites for cells, factors

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of importance for adequate integration and tissue remodeling [7]. Over the past few years, allogenic human acellular dermis (hAD) has been found to have several applications in reconstructive surgery [8,9]. Concerns have been raised regarding acellular dermal matrices as a separate risk factor for developing surgical-site infection or inflammation [10,11]. However, current data describe a relatively low rate of infection when sterile hAD is used [12,13].

Antimicrobial peptides (AMP) are important components of innate immunity [14]. AMPs are short, cationic and amphipathic peptides, displaying broad spectrum activity against various microorganisms, such as bacteria, fungi, and viruses [15,16]. AMPs may also exert roles in modulating various immune responses [17–19], motivating the designation host defense peptides (HDP). Research on novel aspects of the innate immune response activated during infection and wounding has demonstrated that proteolytic cascades generate several novel bioactive HDP with antimicrobial and anti-inflammatory activities *in vitro* and *in vivo* [20–25]. In particular, C-terminal peptides of human thrombin are generated in wounds and fibrin in response to infection and inflammation [20]. Considering the functions of these HDPs, and their presence in the endogenous "biomaterial" fibrin, we aimed to develop an hAD matrix coated with such thrombin-derived HDPs and to explore its potential bactericidal and anti-endotoxic characteristics. Therefore, we coated hAD with the thrombin peptides GKY25 and GKY20 and evaluated their *in vitro* activity against various bacterial strains characteristic for skin wounds, their effects on lipopolysaccharide (LPS)-mediated inflammatory responses, and activation of coagulation-inducing kallikrein.

2. Materials and methods

2.1. Peptides

The peptides GKY20 (GKYGFYTHVFRLKKWIQKVI), GKY25 (GKYGFYTHVFRLKKWIQKVIDQFGE), and tetramethylrhodamine (TAMRA) labeled GKY25 were synthesized by Biopeptide Co., San Diego, CA. The purity (95%) of these peptides was confirmed by mass spectral analysis (MALDI-ToF Voyager).

2.2. Microorganisms

The microorganisms used in the tests for antimicrobial activity were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 obtained from the American Type Culture Collection.

2.3. Carrier material

Human cell-free (acellular) dermis allograft (*epiflex*®, DIZG, Deutsches Institut für Zell- und Gewebeersatz gemeinnützige GmbH) was prepared as described previously [8]. A biopsy punch was used to fabricate discs with a diameter of 4 mm and a thickness of 0.55 ± 0.25 mm to be used as a carrier material for peptides GKY25 and GKY20.

2.4. Disc coating

Peptides were solubilized in sterile water and stock solutions were prepared with concentrations of 0.647, 1.62 and 3.24 mM. Each hAD-disc was coated with 10 μl of peptide solution from an appropriate stock solution. After addition of the peptide solution, discs were incubated in a moisture camber for 1 h at room temperature and then freeze dried for 1 h. The final amount of peptide per 4 mm hAD-disc were 6.5, 16.2, 32.4 nmol respectively. Polyhexamethylenebiguanide (PHMB) coated hAD-discs were prepared by adding 10 μl of 0.1% PHMB (Cosmocil PG Polyhexanide, ARCH UK biocides, United Kingdom) solution to the discs, followed by freeze drying.

2.5. Peptide release from discs

To measure the rate at which peptides were released from hAD, peptide-coated discs were incubated in 100 μl of Tris buffer containing NaCl (10 mM Tris, pH 7.4, 0.15 M NaCl) at 37 °C under shaking (500 rpm). Aliquots of 10 μl were taken after 1 min, 30 min, 6 h, and 24 h. Peptide activity was measured by a microbiological assay (Radial Diffusion Assay, RDA) using *E. coli* ATCC 25922. A similar disc without peptide served as control.

2.6. Radial diffusion assay

RDA was performed as described previously [26]. Briefly, bacteria were grown to mid-logarithmic phase in 10 ml of full-strength (3% w/v) tryptic soy broth (TSB) (Becton–Dickinson, Cockeysville, MD). The bacteria were washed once in 10 mM Tris, pH 7.4 and subsequently, 4×10^6 bacterial colony forming units (cfu) were added to 15 ml of the underlay agarose gel consisting of 0.03% (w/v) TSB, 1% (w/v) low electro endosmosis type (EEO) agarose (Sigma, St Louis MO) and 0.02% (v/v) Tween 20 (Sigma). The underlay was poured into a Ø 144 mm petri dish. After agarose had solidified, 4 mm-diameter wells were punched and 6 μl of test sample was added to each well. Plates were incubated at 37 °C for 3 h to allow diffusion of the peptides. The underlay gel was then covered with 15 ml of molten overlay (6% TSB and 1% Low-EEO agarose in distilled H_2O). Antibacterial activity of a peptide is visualized as a clear zone around each well after 18–24 h of incubation at 37 °C. The activities of the peptides are presented as clear zone-well diameter (excluding the 4 mm well).

2.7. Viable-count analysis

E. coli ATCC 25922 and *S. aureus* ATCC 29213 bacteria were grown to mid-logarithmic phase in Todd-Hewitt (TH) medium (Becton and Dickinson, Maryland, USA) and *P. aeruginosa* ATCC 27853 bacteria were grown in TH medium overnight. The bacteria were washed and diluted with 10 mM Tris, pH 7.4, containing 0.15 M NaCl. Following this, bacteria (100 μl; 2×10^5 cfu/ml) were incubated at 37 °C for 2 h, with peptide-coated discs in 10 mM Tris, 0.15 M NaCl, with or without 20% human citrate-plasma or 20% acute wound fluid [27]. To quantify the bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar, followed by incubation at 37 °C overnight and the number of cfu was determined. One hundred percent survival was defined as total survival of bacteria in the same buffer and under the same condition in the absence of peptide. The discs were transferred to another TH agar plate to determine the number of viable adherent bacteria on the disc.

2.8. NF-κB/AP-1 assay

NF-κB and AP-1 activation was assessed in THP1-Xblue™-CD14 reporter cells (here denoted THP-1 cells) (InvivoGen, France) according to the company's instructions. In brief, cells were grown in RPMI 1640 with 10% (v/v) heat-inactivated FBS, 1% Anti–Anti (Invitrogen), 100 μg/ml G418, and 200 μg/ml of Zeocin. Cells were centrifuged at 250 × g for 5 min and re-suspended at 2×10^6 cells/ml in RPMI supplemented with 10% heat-inactivated serum and 1% Anti–Anti. Subsequently, 500 μl/well were placed on 24-well plates. Peptide-coated hAD-discs were incubated with 20 ng of *E. coli* (0111:B4) LPS (Sigma–Aldrich, USA; approximate 500.000 endotoxin units/mg) in 500 μl of RPMI medium at 37 °C for 1 h. After incubation the whole mixture including the disc was transferred to 500 μl of THP-1 cells and incubated. After an 18–22 h incubation at 37 °C and 5% CO_2, activity was determined in 20 μl of supernatant by using 180 μl QUANTI-Blue substrate (InvivoGen). Plates were incubated at 37 °C and the level of secreted embryonic alkaline phosphatase (SEAP), an indicator of activation of transcription factors NF-κB and AP-1, was measured after 1–2 h at OD 600 nm.

2.9. MTT assay

Sterile filtered MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide; Sigma–Aldrich) solution (5 mg/ml in PBS) was stored protected from light at −20 °C until use. 180 μl of THP1 cells were transferred to 96-well plates (Costar) and 20 μl of the MTT solution was added to each well. Plates were incubated for 3 h in CO_2 at 37 °C. After incubation the plate was centrifuged at 300 × g for 10 min and MTT containing medium was removed by aspiration. The blue formazan product generated was dissolved by the addition of 100 μl of 100% DMSO (Applichem, Germany) per well. The plates were then gently swirled for 30 min at room temperature to dissolve the precipitate. The absorbance was measured at 550 nm, and results given represent mean values from triplicate measurements.

2.10. Lactate dehydrogenase (LDH) assay

One hundred μl of each of the supernatants from the above experiment were transferred to 96 well plates for determining LDH release. The LDH based TOX-7 kit (Sigma–Aldrich, St Louis, USA) was used for quantification of LDH. Results given represent mean values from triplicate measurements. Results are given as fractional LDH release compared to the positive control consisting of 1% Triton X-100 yielding 100% LDH release.

2.11. Whole blood stimulation and cytokine analysis

Peptide-coated hAD-discs were incubated with 20 ng of *E. coli* LPS (Sigma) in 500 μl of RPMI medium at 37 °C for 1 h. After incubation the whole mixture including the disc was transferred to 500 μl of lepirudin-blood aliquoted in 24-well plates. After 18–20 h incubation, plasma was collected and stored at −20 °C. Cytokine release was measured using BioSource CytoSet™ (Invitrogen) according to the manufacturer's instructions.

2.12. Hemolysis assay

EDTA-blood was centrifuged at 800 g for 10 min, and plasma and buffy coat were removed. The erythrocytes were washed three times and re-suspended in 5% PBS, pH 7.4. The cells were then incubated (with end-over-end rotation) for 1 h at 37 °C in the presence of peptide-coated discs or peptides alone at indicated concentrations. Samples containing 2% Triton X-100 (Sigma–Aldrich) served as positive control. The samples were then centrifuged at 800 g for 10 min. The hemoglobin release was

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