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

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A B S T R A C T
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 polycarboxylates or collagen, silicon or nylon matrices, hyaluronic acid and ester films, fibrin-based materials and hydrogels. Materials implanted into the body of a 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
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-endotoxic characteristics. Therefore, we coated hAD with the throbmin 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 (GKYGFTYHVRFLKQIVY), GKY25 (GKYGFTYHVRFLKQIWQVQFGGE), 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 0.25 ± 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 chamber 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, and 32.4 nmol respectively. Polychymethylenediamine (PHMB) coated hAD-discs were prepared by adding 10 μl of 0.1% PHMB (Cosmocil FG Polyhexamidine, 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 × 10^8 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 H2O). 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 × 10^6 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 THP-1-Xblue™-CD14 reporter cells (here denoted THP-1 cells) (InvivoGen, France) according to the company’s instruction. Briefly, cells were cultured in RPMI 1640 with 10% (v/v) heat-inactivated FBS, 1% Anti–Aptin (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–Aptin. Subsequently, 500 μl/well were placed on 24-well plates. Peptide-coated hAD-discs were incubated with 20 μg 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% CO2, 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-dimethylthiazol-2-yl]-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 CO2 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). 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–Aldrich, USA) in 500 μl of RPMI medium at 37 °C for 1 h. Aliquots of the supernatants 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 CytoSen™ (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
quantified by measuring the absorbance at \( \lambda = 540 \) nm. Data are expressed as a percentage of TritonX-100 induced hemolysis.

2.13. Chromogenic substrate assay

Bacteria were grown to mid-logarithmic phase in TH medium. The microorganisms were then washed 3 times with 50 mM Tris–HCl (pH 7.5), re-suspended, and diluted to a final concentration of \( 2 \times 10^8 \) cfu/ml in 50 mM Tris–HCl/50 \( \mu \)M ZnCl\(_2\) buffer. A total of 100 \( \mu \)l of bacteria were incubated with peptide-coated discs for 10 min before the addition of 100 \( \mu \)l human citrate plasma. Samples were incubated for 30 min at 37°C with shaking. After centrifugation, pellets were washed twice in 50 mM Tris (pH 7.5), re-suspended in 100 \( \mu \)l 50 mM Tris–HCl/50 \( \mu \)M ZnCl\(_2\) buffer containing 2 mM of the chromogenic substrate S-2302 (Chromogenix), and incubated for 30–60 min at 37°C. The samples were centrifuged, and the absorbance of the supernatants was measured at \( \lambda = 405 \) nm. No endogenous proteolytic activity was measured when S-2303 was incubated with bacteria in the absence of plasma.

2.14. Data analysis

Values are shown as mean ± SD. For statistical evaluation of two experimental groups, the Mann–Whitney U-test was used. To compare more than two groups One-Way or Two-Way ANOVA with Bonferroni post-test were used. All statistical evaluations were performed using the GraphPad Prism software 6.0. with ns > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Fig. 1. Peptide release profile of hAD-discs coated with GKY20 and GKY25. (A) The release of antimicrobial peptides from hAD-discs was determined by evaluating the antimicrobial activity in RDA. For determination of effects, E. coli ATCC 25922 (4 \( \times \) 10⁶ cfu) was inoculated in 0.1%TSB agarose gel. Each 4 mm-diameter well was loaded with 6 \( \mu \)l of peptide collected at the indicated time points. The bar diagram in the upper panel indicates the zones of clearance obtained (in mm). These correspond to the inhibitory effect of released peptide after incubation at 37°C for 18–24 h (mean values ± SD are presented, n = 3). In the lower panel corresponding zones of clearance of one experiment are shown. (B) For comparison with the antimicrobial activity of peptide-coated hAD-discs and peptide alone, zones of clearance obtained with the indicated amount of peptide alone are shown. (C) Antimicrobial activity of peptides released from freshly prepared and 7-day-old (room temperature-preserved) peptide-coated discs determined by RDA (mean values ± SD are presented, n = 3).
3. Results

3.1. Antimicrobial peptide release profile of human acellular dermis coated with thrombin peptides

As mentioned above, GKY20 and GKY25, two C-terminal HDPs derived from human thrombin, have broad antimicrobial and immunomodulatory activities [25]. In order to determine antimicrobial effects of functionalized material, hAD-discs coated with increasing amounts of GKY20 and GKY25 were incubated in 10 mM Tris, 0.15 M NaCl, pH 7.4. At different time points (1 min-24 h), aliquots of the disc supernatant were collected. The release of active peptide was indirectly assessed by determining the antimicrobial activity of the samples using RDA against *E. coli* (Fig. 1A). As observed, the peptides were rapidly released. After 24 h of incubation, GKY20 released from hAD showed larger inhibition zones than those observed for GKY25. The antimicrobial activity was dose dependent and a minimum of 6.5 nmol of peptide (corresponding

Fig. 2. Antimicrobial effects of peptide-coated hAD-discs. (A) Antibacterial effects of peptide-coated discs against *E. coli* ATCC 25922 in viable count assays. 2 × 10⁵ cfu/ml of bacteria were incubated in 100 μl of 10 mM Tris, 0.15 M NaCl in the presence of hAD-discs with or without the indicated amount of peptides (mean values ± SD are presented, n = 3). (B) Visualization of bacteria adherent to peptide-coated hAD-discs. The discs were immersed in bacterial suspensions (2 × 10⁵ cfu/ml) and incubated for 2 h at 37 °C. The discs were transferred to fresh TH agar plates for determining viable bacterial colonies (image of one representative experiment is shown, n = 3).

Fig. 3. Peptide-coated human acellular dermis discs inhibit bacterial growth. Antibacterial effects of the peptide-coated discs on *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213 in viable count assays. 2 × 10⁵ cfu/ml of bacteria were incubated in 100 μl in the presence of hAD-discs with or without 32.4 nmol of the indicated peptide. The experiment was performed in 10 mM Tris, pH 7.4, 0.15 M NaCl. Bacteria incubated without hAD-disc were used as control. Bacterial growth was determined by plating appropriate dilutions of the bacterial solutions on TH agar plates. Plates were incubated at 37 °C over night and cfu were determined (image of one representative experiment is shown, n = 3).
to 20 μg GKY25) released during 24 h was required for detecting any antibacterial effects in the system used. For comparison, peptides alone were also tested for their antimicrobial activity (Fig. 1B). The antimicrobial activity at 0.65, 16.2 and 32.4 nmol was higher for peptides alone when compared to samples containing peptide released from impregnated discs (compare Fig. 1A and B). This difference suggested a certain amount of peptide binding to the hAD-discs. Therefore, in a separate experiment, the binding and release of GKY25 to hAD was assessed using discs impregnated with 32.4 nmol peptide, of which 2% was labeled with the fluorescent label TAMRA. TAMRA-GKY25 has similar characteristics as the endogenous GKY25 with respect to antimicrobial and anti-inflammatory activity [20] [Kalle et al., manuscript in preparation]. Analysis of both fluorescent and unlabeled peptide remaining on the discs after repeated washings in buffer, demonstrated that approximately 30–40% of the peptide amount was released after 6 h. Tracking of TAMRA-GKY25 demonstrated an initial peptide release of about 20–25% after 30 min, followed by a “leakage phase” during the 6 h wash period (Supplementary Fig. 1). The above observed reduction of antimicrobial effects of material eluted from the hAD (Fig. 1A), is thus compatible with peptide binding to the hAD. Finally, in order to test the stability of the peptides on hAD, the antimicrobial activity of coated discs, stored for 7 days at room temperature, was compared with the activity of newly prepared discs (Fig. 1C). Notably, there was no significant difference in the peptide release profile, as demonstrated by similar antimicrobial activity against E. coli in RDA.

### 3.2. Antibacterial effects of peptide-coated human acellular dermis

To investigate the antimicrobial efficacy, the peptide-coated hAD-discs were incubated with E. coli in physiological buffer conditions for 2 h. In concordance with the above results, only discs coated with GKY20 or GKY25 displayed antimicrobial activity (Fig. 2A). With 16.2 and 32.4 nmol peptide added, both peptides yielded complete killing of E. coli bacteria. Simultaneously, as presented in Fig. 2B, the presence of remaining viable adherent bacteria on the discs was investigated by placing the discs on sterile TH agar plates. The control disc was surrounded by bacterial growth. In accordance with the RDA and viable count data shown above, the two peptides inhibited bacterial growth on the discs, particularly at doses at or above 16.2 nmol per disc.

*P. aeruginosa* and *S. aureus* are frequently isolated from patients suffering from infected burns as well as chronic wounds [28]. Therefore, using viable count assays as above, the antimicrobial activity of peptide-coated discs (32.4 nmol peptide/disc) was determined against these pathogens and compared with the results on *E. coli* bacteria (Fig. 3). Both peptides completely eradicated the two Gram-negative bacterial strains *E. coli* and *P. aeruginosa*. A large proportion of the Gram-positive *S. aureus* bacteria population was killed by GKY25 and GKY20 (Fig. 3). Since it has been shown that AMPs may be inhibited in plasma [29], we also tested the activity of the peptide-coated discs in plasma as well as acute wound fluid. The results showed that the peptides largely retained their antimicrobial activity in these environments (Fig. 4).

### 3.3. Peptides protect human acellular dermis from degradation by bacteria

Scanning electron microscopy (SEM) images of the hAD-surface with and without peptide-coating, incubated with bacteria for 2 and 24 h are shown in Fig. 5. As seen in the control discs, the surface of the hAD is covered with *P. aeruginosa* (Fig. 5A) and *S. aureus* (Fig. 5B), and degradation of collagen fibers can be observed (lower
panels). hAD supplemented with GKY20 and GKY25 displayed significant bacterial inhibition at the two time points. Destruction of the collagen fibers was not observed.

3.4. Endotoxin-blocking effects of peptide-coated human acellular dermis

In addition to killing microbes directly, HDPs derived from thrombin have been shown to inhibit pro-inflammatory responses as well as excessive coagulation [30]. Thus we evaluated the immunomodulatory activity of peptide impregnated hAD in vitro using THP1-XBlue™-CD14 cells. To exclude the possibility of an LPS contamination of the hAD, an LAL test (limulus amoebocyte lysate test) was performed to detect the amount of endotoxin present. No detectable levels of bacterial endotoxins were found in the hAD material (data not shown). Thus, first, effects of peptides on LPS-induced NF-κB and AP-1 activation in THP1-XBlue™-CD14 cells were measured. Cells were incubated with E. coli LPS (10 ng/ml) and

Fig. 5. Visualization of antimicrobial effects on the surface of human acellular dermis. (A) SEM images of hAD-discs after incubation with bacteria for 2 and 24 h, respectively. (A) P. aeruginosa and (B) S. aureus. Upper panel shows extensive killing of bacteria on the surface of the hAD-disc after a 24 h incubation time. Lower panels illustrate collagen fiber degradation, which is prevented on peptide-coated discs. (Control: hAD alone, incubated for 24 h).
peptide-coated hAD-discs or control discs. After 18–24 h of incubation, NF-κB and AP-1 activation was assessed. The results in Fig. 6A show that discs coated with the two peptides blocked the NF-κB and AP-1 activation in a dose-dependent manner. However, complete blocking was only observed for hAD-discs coated with 32.4 nmol of peptide. To determine whether hAD itself could activate the cells, different batches of hAD-discs alone (3 discs from each batch), were incubated with THP-1 cells for 18 h and the NF-κB and AP-1 activation was studied. A minor, and variable activation was observed with different hAD batches (Fig. 6B), probably reflecting biological variability and material differences, as previously described for other acellular dermal matrices [31].

Next, as the results in Fig. 1A and Supplementary Fig. 1A and B indicated a certain degree of binding of the peptides to hAD, we undertook to investigate whether peptide-coated hAD still retained the anti-endotoxin effects after three repeated washing steps with buffer (10 mM Tris, 0.15 M NaCl, pH 7.4) as well as more extensive washing for 6 h (Supplementary Fig. 1C). Following washing, the discs were incubated with E. coli LPS (10 ng/ml) and later transferred to THP-1 cells to assess NF-κB and AP-1 activation. As seen, the washed discs retained their anti-endotoxin activity in these experimental setup (Fig. 6C, Supplementary Fig. 1C). Therefore, we evaluated the cytocompatibility of these peptides with erythrocytes and the human monocyte THP-1 cell line. As presented in Fig. 7A a dose-dependent permeation effect on erythrocytes was observed. Compatible with previous reports, the peptide GKY20 was less hemolytic [25]. Importantly, when the same amount of peptide was incorporated into hAD-discs, no hemolysis was observed (Fig. 7A), compatible with the observed scavenging of the peptides by the hAD matrix, reducing the amount of free peptide (Fig. 1A, Supplementary Fig. 1A and C). Further, potential toxicity of peptides towards the human mononuclear cell line THP-1 was evaluated using MTT and LDH assays (Fig. 7B and C) in the various indicated conditions. No peptide-mediated toxicity was observed after an incubation time of 18–20 h (Fig. 7B, C). In summary, these results suggest that, in line with previous reports [20,25,30], both GKY20 and GKY25 inhibited LPS-induced cellular responses without significantly affecting the cell viability.

3.5. Effect of peptide-coated human acellular dermis on contact activation

Previously, it was shown that GKY25 impairs the intrinsic pathway of coagulation in both human and murine plasma [30]. The contact system is initiated on bacterial membranes or negatively charged surfaces such as kaolin. This involves activation of FXII, which in turn activates plasma kallikrein (PK) and leads to FXI activation [34]. Depending on the type of surface, biomaterials may also induce this activation of the coagulation system [35]. Therefore we measured the PK activation at the surface of hAD alone and in combination with bacteria (P. aeruginosa) in order to mimic the conditions of a bacterial infection at an implantation site. As shown in Fig. 8, hAD, when in contact with human plasma activated the
contact system. The activation in the presence of bacteria was higher when compared to hAD alone. The decrease in PK activity was found to occur in a dose-dependent manner in the peptide-coated hAD samples (Fig. 8). These results thus demonstrated that exposure to hAD alone could induce activation of the coagulation system, that the initiation was increased in presence of bacteria, and that the two HDPs inhibited this activation.

3.6. Effects of GKY25 in combination with PHMB

Antiseptics such as polyhexamethylenebiguanide (PHMB) are commonly used in wound care. We hypothesized that PHMB, by killing bacteria, may lead to the release of proinflammatory endotoxins. In order to investigate whether GKY25 could block these endotoxins, hAD discs coated with GKY25, PHMB or combinations thereof were incubated with E. coli bacteria for 2 h. Following incubation, cfu were determined. More than 99% of the bacteria were killed by PHMB, GKY25, and PHMB with GKY25 (data not shown). Subsequently, the bacterial suspension including the discs was transferred to THP-1 cells to assess NF-κB and AP-1 activation. As demonstrated in Fig. 9 (upper panel), only hAD discs coated with GKY25 alone or in combination with PHMB blocked NF-κB and AP-1 activation. LDH and MTT assays (middle and lower panel), showed that the substances alone or in combination had minor or no permeation effect. These results demonstrate that GKY25 addition to PHMB inhibits the proinflammatory actions of endotoxins released after subjecting bacteria to the antiseptic.

4. Discussion

Despite several advances in prophylactic measures, infections still remain a significant complication related to biomaterials [1]. A technically successful operation is no guarantee against biomaterial-associated infections. It is estimated that during surgery in a standard operating theater environment, airborne bacterial wound contamination occurs at approximately 270 bacteria/cm² [36], and hence, most likely, sterile implant surgery is not possible. Although recent advancements in airflow and preventative techniques, particularly used in specialized surgery settings,
Simultaneously, LDH release and cell viability using an MTT assay was determined. measuring the production of SEAP (secreted embryonic alkaline phosphatase). diagram in the upper panel indicates % activation of NF-

Fig. 9. Anti-inflammatory effects of GKY25 in combination with PHMB. Discs coated with 32.4 nmol GKY25, 0.1% PHMB, or combinations thereof were incubated with E. coli (100 µl; 2 x 10⁶ cfu/ml) for 2 h. The bacterial suspension including the discs were transferred to THP1-XBlue™-cells to assess NF-κB and AP-1 activation. The bar diagram in the upper panel indicates % activation of NF-κB as determined by measuring the production of SEAP (secreted embryonic alkaline phosphatase). Simultaneously, LDH release and cell viability using an MTT assay was determined. (mean values ± SD are presented, n = 3).

may reduce this airborne contamination, the presence of a perioperative bacterial load, albeit reduced, is still a cause of infections worldwide. It is also notable that bacteria may be released into the wound from deeper layers in the skin [37,38]. In order to meet this problem, various antimicrobial coatings have been developed, particularly for use in revision surgery, and involving antibiotics released from implant coatings, or device fixation materials, such as antibiotic-loaded bone cements [39]. In other instances silver-impregnated coatings, or coatings that kill bacteria immediately upon adhesion to the coating are used for prevention of implant infection [1]. With respect to inflammation, anti-inflammatory surfaces are of high interest [2,4]. For example, Nilsson and colleagues showed that a factor H-binding peptide; and an ADP-degrading enzyme; using a PEG linker on both material and cellular surfaces inhibited complement attack [40]. Thierry et al. used an approach toward biomimetic surfaces based on the covalent immobilization of a carboxylate terminated PEGylated hyaluronan (HA-PEG) onto plasma functionalized NiTi alloy surfaces [41]. In other developments, synthetic polymer brushes are used as templates for further bioengineering of surfaces with enhanced biocompatibility [42].

In this perspective, the concept presented here, based on a thrombin-derived endogenous peptide, found in fibrin and released in wounds, represents a novel means of providing a new biological and endogenous antimicrobial functionality to an acellular dermal biomaterial. Previous studies on the two prototypic thrombin C-terminal peptides GKY20 and GKY25 demonstrated that these peptides exert a broad spectrum of antimicrobial and immunomodulatory activities that include inhibition of production of multiple pro-inflammatory cytokines, reduction of vascular leakage, blocking of contact activation and tissue factor-mediated pathways of the coagulation system during LPS-induced experimental shock and in animal models of P. aeruginosa sepsis [28,35,36,50]. For both LPS-induced shock and P. aeruginosa-induced sepsis, regulation of excessive cytokine levels is regarded as a relevant therapeutic target, and it is therefore notable that the peptides significantly dampen the pro-inflammatory cytokine response both in vitro and in vivo.

From the perspective of biomaterials however, the impact on cellular responses such as vasodilation, chemotaxis, and (importantly and specifically), local activation of the coagulation and complement systems may also play critical roles in modulating biocompatibility [2–5,35,43–46]. The inflammatory responses caused by implanted materials could be major factors leading to complications or degradation [35,47,48]. It is of note, that also endogenous biomaterials, such as dermal substitutes, although considered less proinflammatory, may exert some activating effects on monocytes in vitro [31]. In this perspective, the results in this present work indicate that coating with thrombin-derived peptides adds host defense functions to the human acellular dermis per se. Thus, in addition to a bactericidal effect, coating adds functionalities such as LPS-scavenging and reduction of bacteria-induced contact activation to hAD. In both cases, these effects could lead to advantageous inhibition of local inflammatory responses. Furthermore, the finding that the peptides were adsorbed by the hAD indicates that the cationic and amphipathic peptide may interact with the dermal matrix, consisting of collagen, hyaluronic acid, and other matrix components [49]. For example, peptide interactions with carboxyl-groups of hyaluronic acid, or matrix-derived heparan sulfate are likely to occur and may underlie the observed peptide-hAD binding. From the perspective of pharmacodynamics, analyses on peptide release, stability, as well as effects of serum components should be of importance for further therapeutic developments. For example, given the binding of the peptide to hAD, the relative contribution of bound and released peptide to the observed effects of the hAD matrix in vitro should be determined in more detail. Nevertheless, it is notable that peptide-coated and washed functionalised hAD blocked LPS responses in a serum environment.

Finally, the observation that the peptide blocked the proinflammatory effects of PHMB-killed E. coli is particularly relevant. Apart from extending the concept of endotoxin-scavenging to inhibition of responses to whole E. coli bacteria, the results imply that the strong antimicrobial potency of PHMB could be complemented with the anti-inflammatory effects of GKY25 in various therapeutic approaches.
Upon initial contact with blood or tissues, biomaterials may induce a dysfunctional immune response at the local level, which, as in systemic sepsis, may cause a dysfunctional host defense. If so, this reasoning implicates that the surroundings of a biomaterial may be infection-prone. In the case of experimental (systemic) sepsis, the thrombin peptides reduced coagulation factor consumption, and normalized coagulation and cytokine responses. With this as background, creating a situation in which proinflammatory mediators are controlled locally with immunomodulatory peptide coatings, enabling a preserved immune and coagulation response could be a new and attractive strategy for enhancing infection control for a given biomaterial. Clearly, future experiments using in vivo biomaterial models addressing not only antibacterial and anti-inflammatory effects on a wider range of gram-negative and gram-positive bacteria, but also pharmacodynamic aspects such as release, stability, and metabolism, are needed in order to explore potential therapeutic uses of such peptide-coated biomaterials.

5. Conclusions

Coating of a hAD with endogenous host defense peptides of thrombin adds antimicrobial and endotoxin-blocking properties to the biomaterial, as demonstrated using RDA, viable count assays, and SEM for analyses of antimicrobial activity, in combination with assays for LPS-binding activity, inhibition of NF-κB activation in monocytes, and reduction of contact activation in plasma. Of importance is the finding that the proinflammatory action of PHMB-treated bacteria is blocked using such peptide-functionalized hAD. Taken together, coatings with thrombin-derivated HDPs constitute an interesting strategy for the development of improved biomaterials with combined antimicrobial and anti-inflammatory functions. The present results based on in vitro studies, open up new possibilities to modulate unwanted biomaterial- and/or bacteria-induced reactions also in vivo.

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Appendix A. Supplementary data

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References


