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

Kasetty, Gopinath; Kalle, Martina; Mörgelin, Matthias; Brune, Jan Claas; Schmidtchen, Artur

Published in:
Biomaterials

DOI:
10.1016/j.biomaterials.2015.02.111

2015

Biomaterials 53 (2015) 415–425

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

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

Gopinath Kasetty [a, b, *], Martina Kalle [a], Matthias Mörgelin [c], Jan C. Brune [d], Artur Schmidtchen [a, e]

[a] Division of Dermatology and Venereology, Department of Clinical Sciences, Lund University, Biomedical Center, Tornavägen 10, SE-22184 Lund, Sweden
[b] Division of Respiratory Medicine and Allergology, Department of Clinical Sciences, Lund University, Biomedical Center, Tornavägen 10, SE-22184 Lund, Sweden
[c] Division of Infection Medicine, Department of Clinical Sciences, Lund University, Biomedical Center, Tornavägen 10, SE-22184 Lund, Sweden
[d] Deutsches Institut für Zell- und Gewebeersatz gGmbH, Berlin, Germany
[e] Lee Kong Chian School of Medicine, Nanyang Technological University, 308232, Singapore

ARTICLE INFO

Article history:
Received 22 September 2014
Received in revised form
20 February 2015
Accepted 24 February 2015
Available online 18 March 2015

Keywords:
Host defense peptides
Epiflex
Human acellular dermis
Antimicrobial
Inflammation

ABSTRACT

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.

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

* Corresponding author. Tornavägen 10, SE-22184 Lund, Sweden. Tel.: +46 46 2227118; fax: +46 46 157756.
E-mail address: gopinath.kasetty@med.lu.se (G. Kasetty).

http://dx.doi.org/10.1016/j.biomaterials.2015.02.111

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

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 2×10^6 cfu/ml in 50 mM Tris–HCl/50 μM $ZnCl_2$ buffer. A total of 100 μl of bacteria were incubated with peptide-coated discs for 10 min before the addition of 100 μl 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 μl 50 mM Tris–HCl/50 μM $ZnCl_2$ 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 Bonferoni post-test were used. All statistical evaluations were performed using the GraphPad Prism software 6.0. with ns > 0.05, $^*p \leq 0.05$, $^{**}\leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

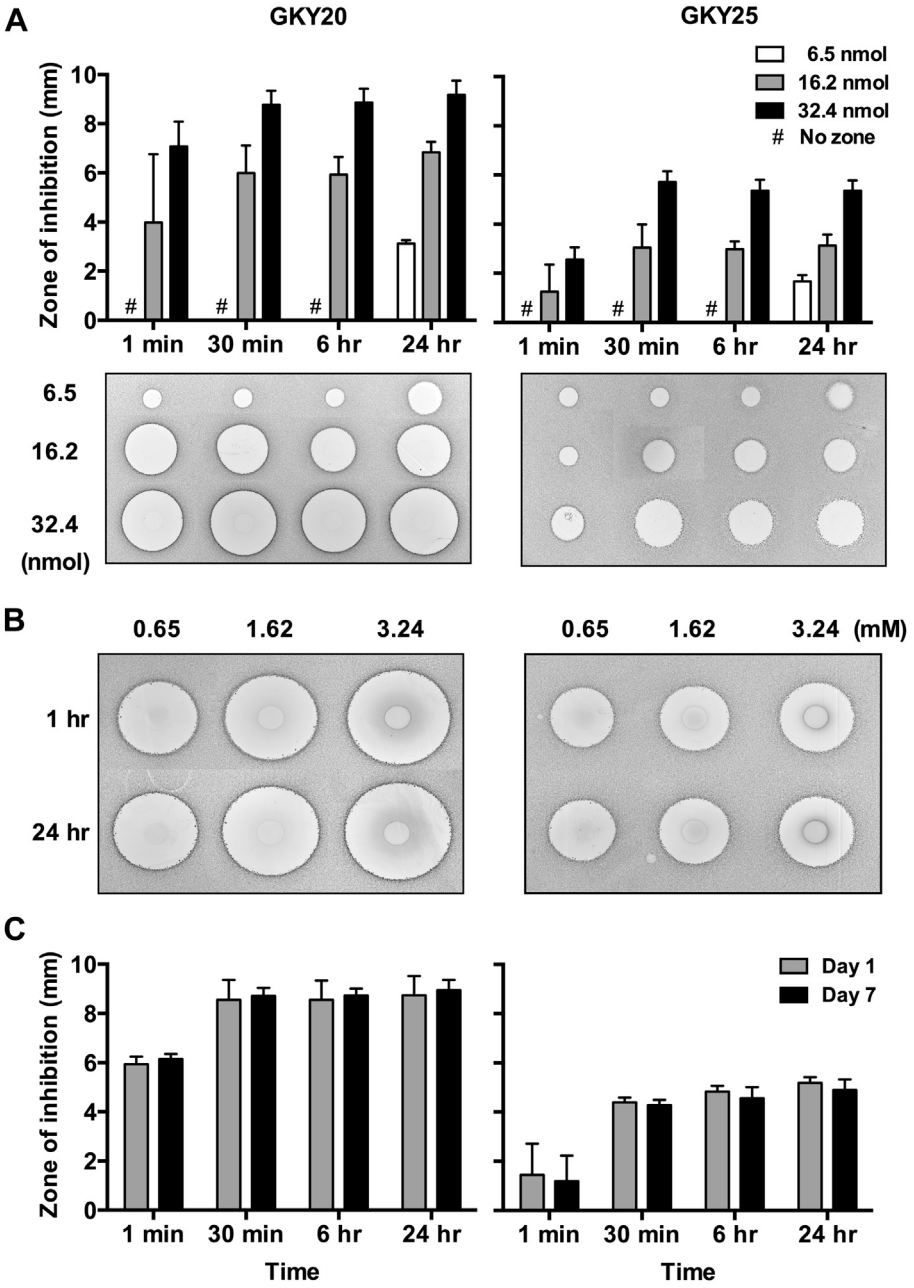

Fig. 1. Peptide release profile of hAD-discs coated with GKY20 and GKY25. (A) The release of antimicrobial peptides from hAD-discs was determined by evaluating the antimicrobial activity in RDA. For determination of effects, *E. coli* ATCC 25922 (4×10^6 cfu) was inoculated in 0.1%TSB agarose gel. Each 4 mm-diameter well was loaded with 6 μl 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).

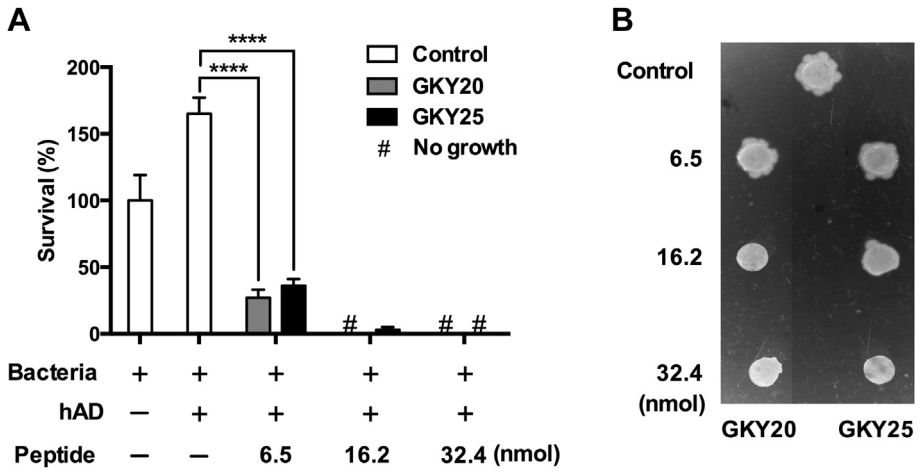

Fig. 2. Antimicrobial effects of peptide-coated hAD-discs. (A) Antibacterial effects of peptide-coated discs against *E. coli* ATCC 25922 in viable count assays. 2×10^5 cfu/ml of bacteria were incubated in 100 μl of 10 mM Tris, 0.15 M NaCl in the presence of hAD-discs with or without the indicated amount 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 (2×10^5 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).

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

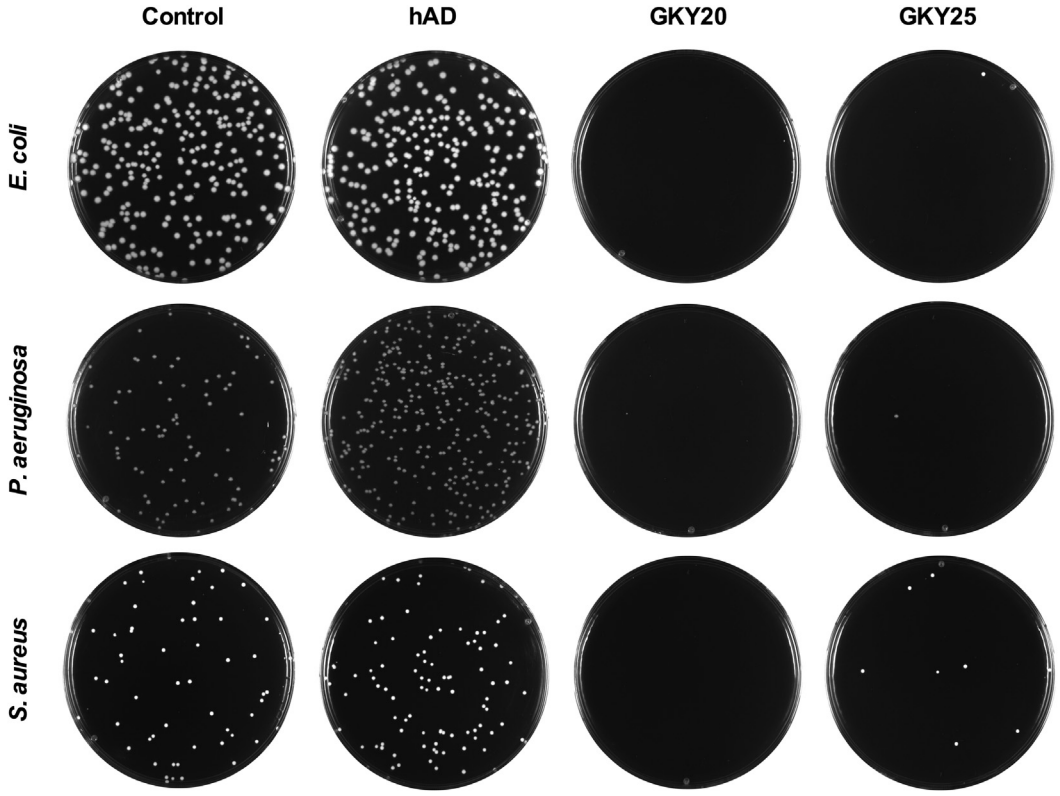

Fig. 3. Peptide-coated human acellular dermis discs inhibit bacterial growth. Antibacterial effects of the peptide-coated discs on *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213 in viable count assays. 2×10^5 cfu/ml of bacteria were incubated in 100 μl in the presence of 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 microcopy (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

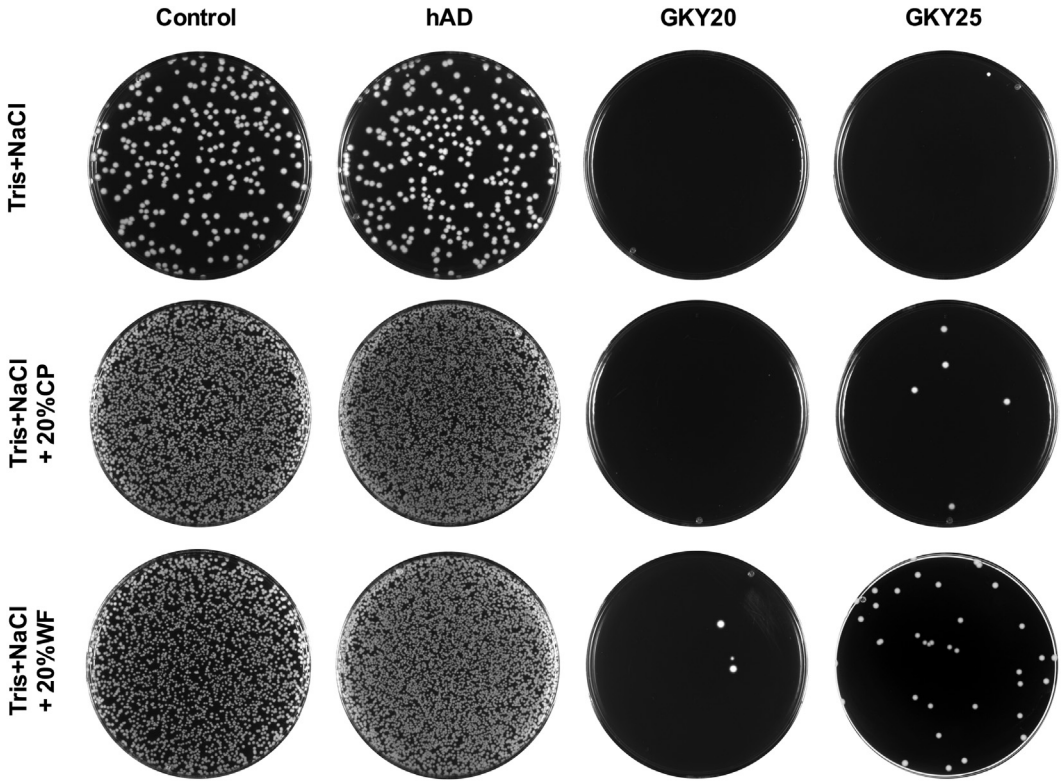

Fig. 4. Antimicrobial activities at physiological conditions. *E. coli* ATCC 25922 bacteria were incubated with peptide-coated discs (32.4 nmol) in 10 mM Tris pH 7.4, 0.15 M NaCl or the same buffer containing 20% human plasma or 20% acute wound fluid. To quantify bactericidal activity appropriate dilutions of reaction mixtures were plated on TH broth agar followed by incubation over night at 37 °C and the number of cfu was determined (image of one representative experiment is shown, n = 3).

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

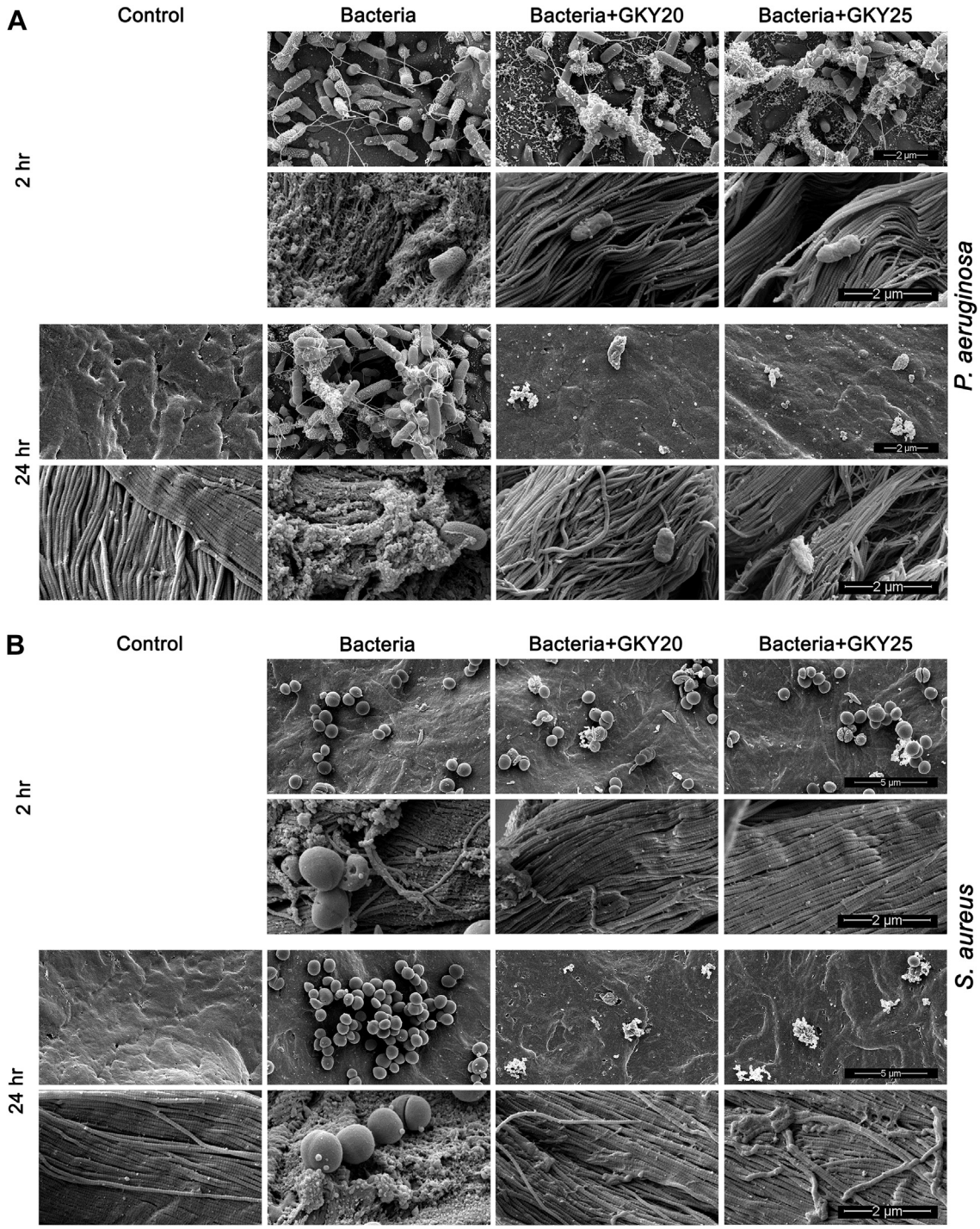

Fig. 5. Visualization of antimicrobial effects on the surface of human acellular dermis. (A) SEM images of hAD-discs after incubation with bacteria for 2 and 24 h, respectively. (A) *P. aeruginosa* and (B) *S. aureus*. Upper panel shows extensive killing of bacteria on the surface of the hAD-disc after 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]. Further, 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).

Next, we also determined if the peptide-impregnated hAD-discs could inhibit the LPS-induced production of pro-inflammatory cytokines in whole blood. Human blood was stimulated with 10 ng/ml *E. coli* LPS in the absence and presence of hAD-discs with or without peptides. In accordance with previous data, both peptide-coated discs significantly inhibited the production of the proinflammatory cytokines TNF-α, IL12p40, but also the anti-inflammatory cytokine IL-10 (Fig. 6D). Many antimicrobial peptides may also permeabilize human cells *in vitro* [32,33], effects which often are abrogated after addition of plasma or blood [15]. 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 monocytic 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

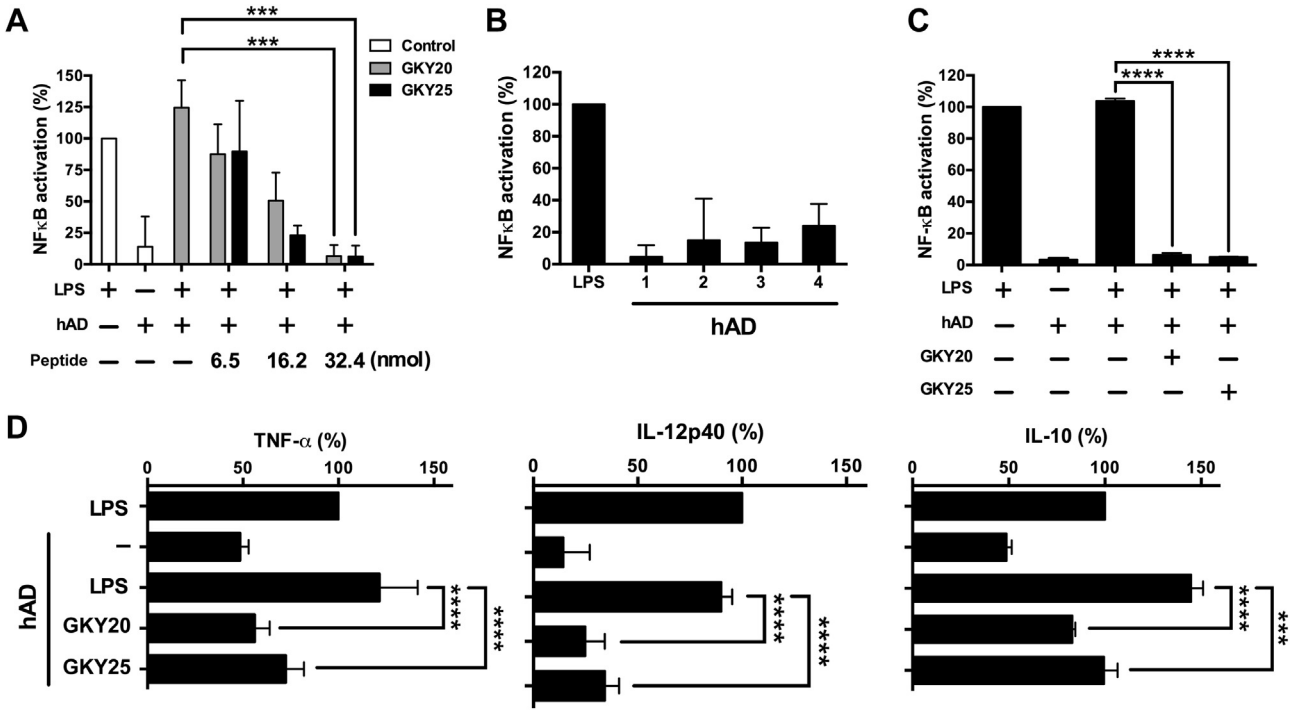

Fig. 6. Anti-inflammatory effects of peptide-coated hAD-discs. (A) THP1-XBlue™-CD14 cells were stimulated with 10 ng/ml *E. coli* LPS in presence of peptide-coated discs. The bar diagram indicates the activation of NF-κB as determined by measuring the production of SEAP (mean values ± SD are presented, n = 3). (B) THP1-XBlue™-CD14 cells were incubated with discs from different batches of human acellular dermis and the NF-κB activation was evaluated (mean values ± SD are presented, n = 3). (C) THP1-XBlue™-CD14 cells were stimulated with 10 ng/ml *E. coli* LPS in presence of washed peptide-coated discs and the NF-κB activation was evaluated. The peptide-coated discs (32.4 nmol) were washed 3 times for 10 min each in 1 ml Tris, 0.15 M NaCl, pH 7.4 and subsequently incubated with *E. coli* LPS for 1 h. The whole mixture was added to THP1 cells to determine the % activation of NF-κB (mean values ± SD are presented, n = 3). (D) Human blood was stimulated for 18 h with 10 ng/ml *E. coli* LPS alone, or in presence of hAD-discs (alone or coated with peptide). The levels of TNF-α, IL-12p40, and IL-10 are presented as percentage in relation to LPS alone which is defined as 100% (mean values ± SD are presented, n = 5).

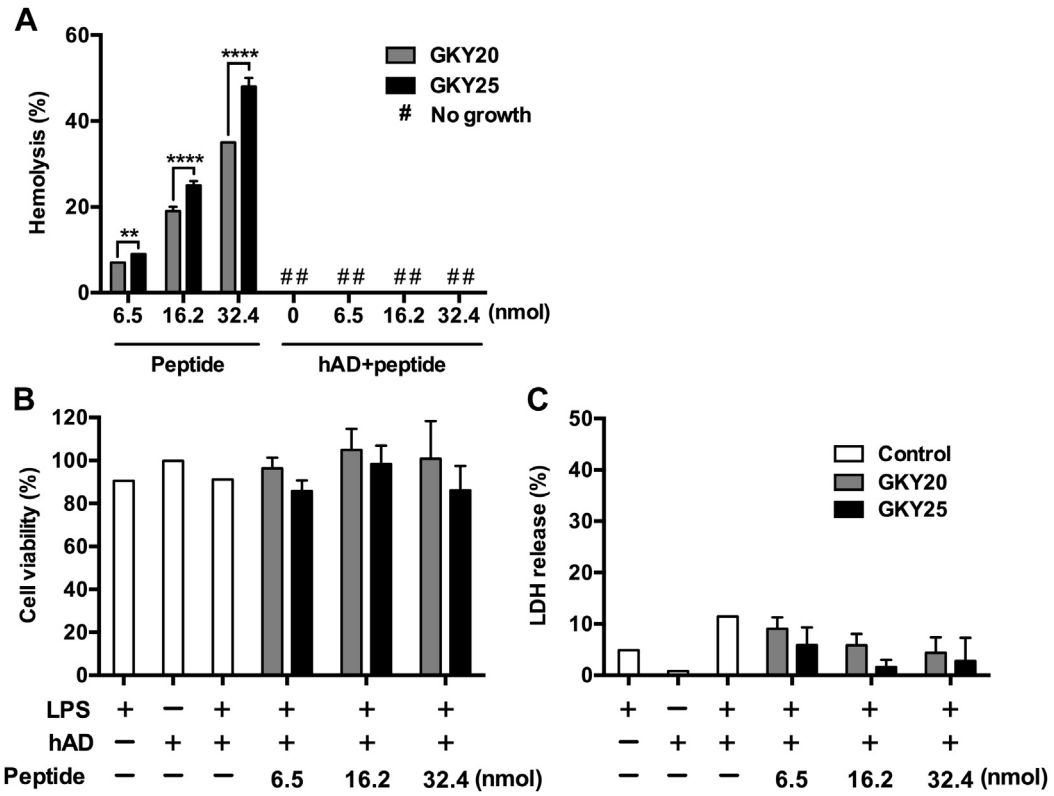

Fig. 7. Effects of peptide-coated discs on cell viability. (A) Hemolytic effects were investigated by incubating erythrocytes with peptides alone (Peptide) or peptide-coated hAD-discs (mean values ± SD are presented, n = 3). (B) The percentage of viable cells was quantified with an MTT assay (mean values ± SD are presented, n = 3). (C) LDH release in supernatants of THP1-XBlue™-CD14 cells was determined. Values are presented in relation to the positive control (defined as 100%) (mean values ± SD are presented, n = 4).

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.

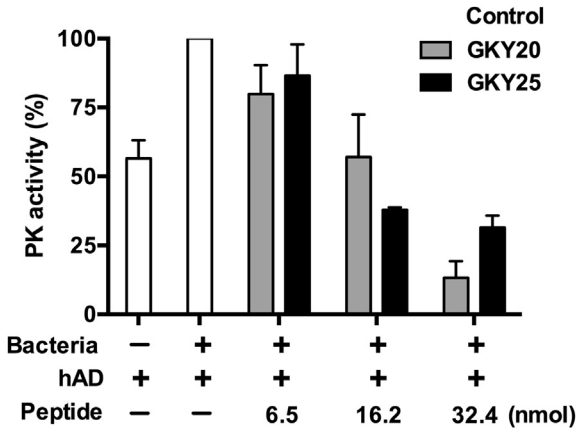

Fig. 8. Effects on contact activation of peptide impregnated hAD-discs in absence and presence of bacteria. hAD-discs with or without peptide were incubated with *P. aeruginosa* ATCC 27853. After 10 min, human plasma was added and incubated for additional 30 min. The plasma was then removed by centrifugation and the pelleted bacteria were washed and re-suspended in substrate buffer. After 15–30 min of incubation, plasma kallikrein activity was measured in a substrate assay. Data are presented as percentage activity compared to the control (mean values ± SD, n = 2).

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/cm^2 [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,

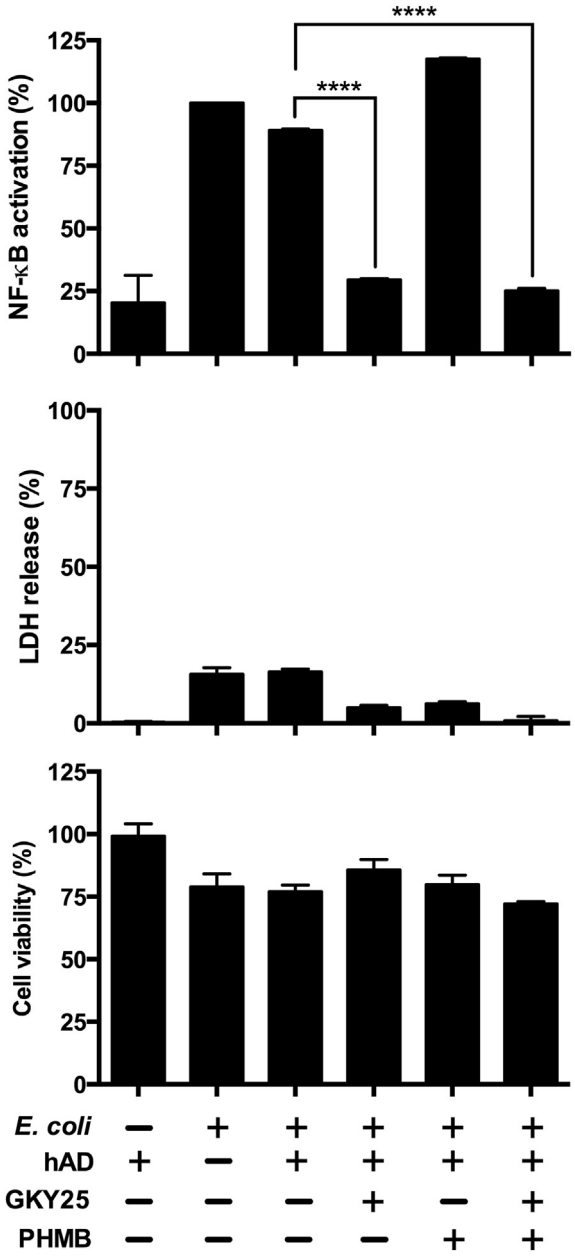

Fig. 9. Anti-inflammatory effects of GKY25 in combination with PHMB. Discs coated with 32.4 nmol GKY25, 0.1% PHMB, or combinations thereof were incubated with *E. coli* (100 μl; 2×10^6 cfu/ml) for 2 h. The bacterial suspension including the discs were transferred to THP1-XBlue™-cells to assess NF-κB 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 temaplates for further bioengineering of surfaces with enhaced 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 [20,25,30]. 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-derived 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*.

Acknowledgments

We thank Dr. Mark D. Smith from the German Institute for Cell and Tissue Replacement (DIZG) for support, stimulating discussions, and valuable input and comments on the final manuscript. This work was supported by grants from the Swedish Research Council (project 2012–1883), Vinnova, Torsten Söderberg, the Welander-Finsen, Knut and Alice Wallenberg, Crafoord, Österlund, Kock Foundations, and The Swedish Government Funds for Clinical Research (ALF).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.02.111.

References

[1] Busscher HJ, van der Mei HC, Subbiahdoss G, Jutte PC, van den Dungen JJ, Zaat SA, et al. Biomaterial-associated infection: locating the finish line in the race for the surface. Sci Transl Med 2012;4(153). 153rv10.
[2] Ekdahl KN, Lambris JD, Elwing H, Ricklin D, Nilsson PH, Teramura Y, et al. Innate immunity activation on biomaterial surfaces: a mechanistic model and coping strategies. Adv Drug Deliv Rev 2011;63(12):1042–50.
[3] Tang L, Hu W. Molecular determinants of biocompatibility. Expert Rev Med Devices 2005;2(4):493–500.
[4] Franz S, Rammelt S, Scharnweber D, Simon JC. Immune responses to implants – a review of the implications for the design of immunomodulatory biomaterials. Biomaterials 2011;32(28):6692–709.
[5] Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Seminars Immunol 2008;20(2):86–100.
[6] Badylak SF. Xenogeneic extracellular matrix as a scaffold for tissue reconstruction. Transpl Immunol 2004;12(3–4):367–77.
[7] Bosman FT, Stamenkovic I. Functional structure and composition of the extracellular matrix. J Pathology 2003;200(4):423–8.
[8] Rossner E, Smith MD, Petschke B, Schmidt K, Vitacolonna M, Syring C, et al. Epiflex((R)) a new decellularised human skin tissue transplant: manufacture and properties. Cell Tissue Bank 2011;12(3):209–17.
[9] Wainwright DJ. Use of an acellular allograft dermal matrix (AlloDerm) in the management of full-thickness burns. Burns 1995;21(4):243–8. Journal of the International Society for Burn Injuries.
[10] Orenstein SB, Qiao Y, Kaur M, Klueh U, Kreutzer DL, Novitsky YW. Human monocyte activation by biologic and biodegradable meshes in vitro. Surg Endosc 2010;24(4):805–11.
[11] Hill JL, Wong L, Kemper P, Buseman J, Davenport DL, Vasconez HC. Infectious complications associated with the use of acellular dermal matrix in implant-based bilateral breast reconstruction. Ann Plastic Surg 2012;68(5):432–4.
[12] Weichman KE, Wilson SC, Saadeh PB, Hazen A, Levine JP, Choi M, et al. Sterile "ready-to-use" AlloDerm decreases postoperative infectious complications in patients undergoing immediate implant-based breast reconstruction with acellular dermal matrix. Plastic Reconstr Surg 2013;132(4):725–36.
[13] Liu DZ, Mathes DW, Neligan PC, Said HK, Louie O. Comparison of outcomes using AlloDerm versus FlexHD for implant-based breast reconstruction. Ann Plastic Surg 2014;72(5):503–7.
[14] Wehkamp J, Schmid M, Stange EF. Defensins and other antimicrobial peptides in inflammatory bowel disease. Curr Opin Gastroenterology 2007;23(4):370–8.
[15] Kasetty G, Papareddy P, Kalle M, Rydengard V, Walse B, Svensson B, et al. The c-terminal sequence of several human serine proteases encodes host defense functions. J Innate Immun 2011;3(5):471–82.
[16] Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curr eye Res 2005;30(7):505–15.
[17] Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 2003;3(9):710–20.
[18] Elsbach P. What is the real role of antimicrobial polypeptides that can mediate several other inflammatory responses? J Clin Investigation 2003;111(11):1643–5.
[19] Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. J Leukoc Biol 2004;75(1):39–48.
[20] Papareddy P, Rydengard V, Pasupuleti M, Walse B, Morgelin M, Chalupka A, et al. Proteolysis of human thrombin generates novel host defense peptides. PLoS Pathog 2010;6(4). e1000857.
[21] Nordahl EA, Rydengard V, Morgelin M, Schmidtchen A. Domain 5 of high molecular weight kininogen is antibacterial. J Biological Chem 2005;280(41):34832–9.
[22] Papareddy P, Kalle M, Kasetty G, Morgelin M, Rydengard V, Albiger B, et al. C-terminal peptides of tissue-factor pathway inhibitor are novel host defense molecules. J Biological Chem 2010;285:28387–98.
[23] Nordahl EA, Rydengard V, Nyberg P, Nitsche DP, Morgelin M, Malmsten M, et al. Activation of the complement system generates antibacterial peptides. Proc Natl Acad Sci U. S. A 2004;101(48):16879–84.
[24] Andersson E, Rydengard V, Sonesson A, Morgelin M, Bjorck L, Schmidtchen A. Antimicrobial activities of heparin-binding peptides. Eur J Biochem 2004;271(6):1219–26.
[25] Kasetty G, Papareddy P, Kalle M, Rydengard V, Morgelin M, Albiger B, et al. Structure-activity studies and therapeutic potential of host defense peptides of human thrombin. Antimicrob Agents Chemother 2011;55(6):2880–90.
[26] Lehrer RI, Rosenman M, Harwig SS, Jackson R, Eisenhauer P. Ultrasensitive assays for endogenous antimicrobial polypeptides. J Immunol Methods 1991;137(2):167–73.
[27] Lundqvist K, Herwald H, Sonesson A, Schmidtchen A. Heparin binding protein is increased in chronic leg ulcer fluid and released from granulocytes by secreted products of Pseudomonas aeruginosa. Thrombosis Haemostasis 2004;92(2):281–7.
[28] Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev 2001;14(2):244–69.
[29] Yeaman MR, Gank KD, Bayer AS, Brass EP. Synthetic peptides that exert antimicrobial activities in whole blood and blood-derived matrices. Antimicrob Agents Chemother 2002;46(12):3883–91.
[30] Kalle M, Papareddy P, Kasetty G, Morgelin M, van der Plas MJ, Rydengard V, et al. Host defense peptides of thrombin modulate inflammation and coagulation in endotoxin-mediated shock and Pseudomonas aeruginosa sepsis. PloS One 2012;7(12). e51313.
[31] Orenstein S, Qiao Y, Kaur M, Klueh U, Kreutzer D, Novitsky Y. In vitro activation of human peripheral blood mononuclear cells induced by human biologic meshes. J Surg Res 2010;158(1):10–4.
[32] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 2003;55(1):27–55.
[33] Tossi A, Sandri L, Giangaspero A. Amphipathic, alpha-helical antimicrobial peptides. Biopolymers 2000;55(1):4–30.
[34] Oehmcke S, Herwald H. Contact system activation in severe infectious diseases. J Mol Med 2010;88(2):121–6.
[35] Tang L, Eaton JW. Inflammatory responses to biomaterials. Am J Clin Pathology 1995;103(4):466–71.
[36] Fitzgerald Jr RH. Microbiologic environment of the conventional operating room. Archives Surg 1979;114(7):772–5.

[37] Dobbins BM, Kite P, Kindon A, McMahon MJ, Wilcox MH. DNA fingerprinting analysis of coagulase negative staphylococci implicated in catheter related bloodstream infections. J Clin Pathology 2002;55(11):824–8.
[38] Schommer NN, Gallo RL. Structure and function of the human skin microbiome. Trends Microbiol 2013;21(12):660–8.
[39] Alessandro Bistolfi GM, Verne Enrica, Masse Alessandro, Davide Deledda SF, Miola Marta, Galetto Fabrizio, et al. Antibiotic-loaded cement in orthopedic surgery: a review. ISRN Orthop 2011;2011(290851):8.
[40] Nilsson PH, Ekdahl KN, Magnusson PU, Qu H, Iwata H, Ricklin D, et al. Autoregulation of thromboinflammation on biomaterial surfaces by a multicomponent therapeutic coating. Biomaterials 2013;34(4):985–94.
[41] Thierry B, Winnik FM, Merhi Y, Griesser HJ, Tabrizian M. Biomimetic hemocompatible coatings through immobilization of hyaluronan derivatives on metal surfaces. Langmuir. ACS J Surfaces Colloids 2008;24(20):11834–41.
[42] Yu K, Mei Y, Hadjesfandiari N, Kizhakkedathu JN. Engineering biomaterials surfaces to modulate the host response. Colloids Surfaces B, Biointerfaces 1 December 2014;124:69–79.
[43] Tang L, Jennings TA, Eaton JW. Mast cells mediate acute inflammatory responses to implanted biomaterials. Proc Natl Acad Sci U. S. A 1998;95(15):8841–6.
[44] Gorbet MB, Sefton MV. Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. Biomaterials 2004;25(26):5681–703.
[45] Tang L, Eaton JW. Fibrin(ogen) mediates acute inflammatory responses to biomaterials. J Exp Med 1993;178(6):2147–56.
[46] Gretzer C, Emanuelsson L, Liljensten E, Thomsen P. The inflammatory cell influx and cytokines changes during transition from acute inflammation to fibrous repair around implanted materials. J Biomaterials Sci Polym Ed 2006;17(6):669–87.
[47] Sutherland K, Mahoney 2nd JR, Coury AJ, Eaton JW. Degradation of biomaterials by phagocyte-derived oxidants. J Clin Investigation 1993;92(5):2360–7.
[48] Lindman JP, Talbert M, Zhang W, Powell B, Accortt NA, Rosenthal EL. Promotion of acellular dermal matrix resolution in vitro by matrix metalloproteinase-2. Archives Facial Plastic Surg 2006;8(3):208–12.
[49] Roessner ED, Vitacolonna M, Hohenberger P. Confocal laser scanning microscopy evaluation of an acellular dermis tissue transplant (Epiflex(R)). PloS One 2012;7(10). e45991.