The Extracellular Adherence Protein (Eap) of Staphylococcus aureus Inhibits Wound Healing by Interfering with Host Defense and Repair Mechanisms

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A.N.A.: performed research
M.E.: performed research
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H.W.: performed research
H.G.A.: performed research
S.A.E.: performed research
U.S.: provided vital reagents
T.L.: performed research
P.P.N.: participated in designing research
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Abstract

Staphylococcus aureus is a major human pathogen interfering with host cell functions. Impaired wound healing is often observed in S. aureus infected wounds, yet, the underlying mechanisms are poorly defined. Here, we identify the extracellular adherence protein (Eap) of S. aureus to be responsible for impaired wound healing. In a mouse wound healing model wound closure was inhibited in the presence of wildtype S. aureus and this effect was reversible when the wounds were incubated with an isogenic Eap-deficient strain. Isolated Eap also delayed wound closure. In the presence of Eap, recruitment of inflammatory cells to the wound site as well as neovascularization of the wound were prevented. In vitro, Eap significantly reduced ICAM-1-dependent leukocyte-endothelial interactions and diminished the consequent activation of the pro-inflammatory transcription factor NF\(\kappa\)B in leukocytes associated with a decrease in expression of tissue factor. Moreover, Eap blocked \(\alpha_v\)-integrin-mediated endothelial cell migration and capillary tube formation, and neovascularization in matrigels in vivo. Collectively, the potent anti-inflammatory and anti-angiogenic properties of Eap provide an underlying mechanism that may explain the impaired wound healing in S. aureus infected wounds. Eap may also serve as a lead compound for new anti-inflammatory and anti-angiogenic therapies in several pathologies.
Introduction

*Staphylococcus aureus*, and especially strains with resistance to antimicrobial agents, is an unabated challenge in community-acquired and nosocomial infections ranging from wound infections or osteomyelitis to life-threatening endocarditis, or septic shock (1-2). Wound infection with *S. aureus* is frequently associated with impaired healing yet, interference of *S. aureus* with wound healing mechanisms is poorly understood (3,4). *S. aureus* expresses a number of bacterial cell wall-anchored adhesins mediating its adherence to host extracellular matrix (ECM) components (5). Moreover, *S. aureus* produces and secretes proteins with ECM binding properties, such as coagulase (6), the extracellular fibrinogen binding protein (5), as well as the extracellular adherence protein (Eap), also designated Map (MHC class II analogous protein), a 60 kDa protein with a broad repertoire of interactions to host ECM components (7-9). Recently, we demonstrated direct interactions of Eap with the host adhesive proteins ICAM-1, fibrinogen (FBG) and vitronectin (VN), resulting in the disruption of integrin-dependent leukocyte recruitment *in vitro* and *in vivo*, thereby serving as a potent anti-inflammatory factor (10). While Eap was also shown to exert immunomodulatory actions by interfering with T-cell functions (11), a possible interference of Eap with host wound healing is not defined.

Wound healing is a well-organized sequence of events involving an inflammatory, proliferative, and maturation phase (12,13). (i) The inflammatory phase requires initial recruitment of neutrophils and later of macrophages that besides defending against invasive microbes, also release pro-inflammatory cytokines important for the subsequent activation of fibroblasts, keratinocytes and endothelial cells in the proliferative phase of wound healing (12,13). Leukocyte recruitment requires a coordinated sequence of multistep adhesive events including selectin-mediated rolling, leukocyte activation leading to integrin-mediated firm adhesion and
diapedesis (14). During firm leukocyte adhesion to endothelial cells, leukocyte β2-integrins LFA-1 and Mac-1, as well as β1-integrins interact with endothelial counterligands such as ICAM-1, or VCAM-1 (14-15). The activation of infiltrating leukocytes is linked to the modulation of gene expression of pro-inflammatory and procoagulant factors, such as interleukin-6 or tissue factor (17,18) mediated at least in part by the activation of the transcription factor NFκB (19,20), which is a critical molecular bridge between cell exposure to inflammatory/infectious stimuli and gene expression (21). (ii) During the proliferative phase, the migration and proliferation of keratinocytes, fibroblasts and capillary endothelial cells results in re-epithelialization and tissue granulation in close context with neovascularization. Repair angiogenesis depends on the interplay between growth factors, such as the vascular endothelial growth factor (VEGF), as well as on adhesive contacts of the endothelial cells with the ECM (22,23). Emerging evidence also points to a crosstalk between inflammatory cells and angiogenesis, and particularly both, pro-angiogenic and anti-angiogenic activities of neutrophils and macrophages have been described (24). (iii) In the maturation/remodelling phase, the completion of tissue repair is related to the proteolytic balance that regulates the degradation of excess collagen in the wound site (12,13).

Wound healing is frequently delayed in S. aureus infected wounds (3,4). Our previous observations that identified Eap as an inhibitor of leukocyte recruitment (10), prompted us to investigate, whether Eap can influence the wound healing process. We found Eap to exert anti-inflammatory actions by inhibiting leukocyte recruitment and activation, and strikingly, Eap was identified as a potent anti-angiogenic factor in vitro and in vivo. Thus, by interfering with major steps of the wound healing process, Eap appears to constitute a major bacterial component responsible for impaired wound healing.
Methods

Materials The following reagents were provided from these sources: Blocking monoclonal antibody (mAb) IB4 against human CD18 (Alexis, Grünberg, Germany); mAb against tissue factor (W. Ruf, La Jolla, CA); mAb LM609 against αvβ3 and mAb 6S6 against CD29 (Chemicon, Hofheim, Germany); mAb against ICAM-1 (DAKO, Hamburg, Germany); rabbit polyclonal antibody against tissue factor (American Diagnostica, Heidelberg, Germany); rabbit anti-mouse myeloperoxidase (MPO) for western blot analysis (Dunn Labortechnik, Asbach, Germany); rabbit anti-mouse MPO for immunohistochemistry (Abcam, Cambridge, MA); rat anti-mouse F4/80 (Acris, Hiddenhausen, Germany); rat anti-mouse CD31 (Pharmingen, Hamburg, Germany); respective isotype-matched control antibodies and secondary-conjugated antibodies (DAKO, Carpinteria, CA); recombinant ICAM-1, tumor necrosis factor-α (TNF-α) and VEGF (R&D Systems, Wiesbaden, Germany); purified αvβ3-integrin and α5β1-integrin (Chemicon, Hofheim, Germany). FBG, fibronectin (FN), S. aureus protein A and monocyte chemoattractant protein-1 (MCP-1) were from Sigma (Munich, Germany). VN was purified from human plasma and converted into the multimeric form as described (10). Polyclonal antibody against Eap was previously described (10).

Bacteria and purification of Eap S. aureus strain Newman and the isogenic Eap-deficient strain AH12 were previously described (10). Eap from strain Newman was purified as described with modifications (10). Briefly, S. aureus strain Newman was harvested following a 20hr incubation in BHI medium (4x500 ml) at 37°C. The resultant 1M lithium chloride-treated extract was dialysed against PBS, concentrated and adsorbed onto SP Sepharose (Amersham-Pharmacia, Freiburg, Germany) in
loading buffer (30mM phosphate buffer, pH 7.0, 200mM NaCl) at 4°C overnight. After stepwise elution with increasing NaCl concentrations, the pooled eluted fractions (between 0.6 and 0.8M NaCl) were dialysed against 1:4 diluted PBS at 4°C and concentrated by ultracentrifugation using Millipore centricron centrifugal filter devices (MWCO 30kD, Millipore, Bedford, MA). The rententate (containing 2mg/ml Eap) was further purified by cation exchange chromatography on Mono S 10/100 GL tricorn column using an ÄKTA fast performance liquid chromatography system (Amersham-Pharmacia), operated with 10mM Tris/HCl pH 8.0 and an increasing linear NaCl gradient (0-1 M NaCl). Eap-containing fractions were purified on a Superdex 75 HR 10/30 gelfiltration column, equilibrated with TBS at pH 7.4, and Eap-positive fractions were pooled, concentrated by ultracentrifugation (1-1.5 mg/ml), sterile filtered and snap frozen at -80°C until further use. Eap was devoid of detectable endotoxin.

Cell culture Myelo-monocytic THP-1 cells and human umbilical vein endothelial cells (HUVEC) were cultivated as described (25). Human neutrophils were isolated from peripheral blood as described (25).

Leukocyte adhesion to endothelial cells and transendothelial migration Adhesion of THP-1 cells or neutrophils to HUVEC monolayers and neutrophil transendothelial migration was tested exactly as described (25,26,27).

Endothelial cell migration Endothelial cell migration was tested using Transwell membranes (8-µm pore size and 6.5-mm diameter; Corning Costar) that were coated with VN, FBG, FN or BSA (each at 10µg/ml) exactly as described (28).
In vitro ligand-receptor interactions  Binding of FBG, VN or FN to immobilized integrins (10µg/ml) was performed as described (25,29). Briefly, binding of biotin-FBG or VN (each at 2µg/ml) to immobilized αvβ3-integrin or binding of FN to immobilized α5β1-integrin was performed in TBS containing 0.3% BSA, 0.05% Tween-20, 1mM Ca2+ in the absence or presence of Eap. After incubation for 2hr at 22°C in each case, the respective anti-ligand antibodies (rabbit anti-VN and rabbit anti-FN) followed by addition of appropriate secondary peroxidase-conjugated antibodies were used. For detection of biotin-FBG, peroxidase-coupled streptavidin was utilized. After extensive washing the substrate ABTS was added, and binding was quantitated at 405 nm. Nonspecific binding to BSA-coated wells was used as blank and was subtracted to calculate specific binding.

In vitro angiogenesis assay  In vitro angiogenesis in collagen gels was quantitated using endothelial cell spheroids as described previously (30). In brief, spheroids containing 400 cells were generated overnight after which they were embedded into collagen gels. A collagen stock solution was prepared prior to use by mixing 8 vol. acidic collagen extract of rat tails (equilibrated to 2mg/ml, 4°C) with 1 vol. 10x EBSS (Gibco BRL, Eggenstein, Germany), and approx. 1 vol. 0.2 N NaOH to adjust the pH to 7.4. This stock solution (0.5 ml) was mixed with 0.5 ml room temperature medium (ECBM, Endothelial cell basal medium [PromoCell, Heidelberg, Germany] with 40% FCS [Biochrom, Berlin, Germany]) containing 0.5% (w/v) methylcellulose (to prevent sedimentation of spheroids prior to polymerization of the collagen gel) and 50 spheroids. The spheroid containing gel was rapidly transferred into pre-warmed 24 well plates and allowed to polymerize for 30 min after which 0.1 ml ECBM with the corresponding test substance was laid on top of the gel. The gels were incubated at 37°C, 5% CO2, and 100% humidity. After 24hr, in vitro angiogenesis was digitally
quantitated by measuring the length of the sprouts that had grown out of each spheroid using the digital imaging software analysis (Soft imaging system, Münster, Germany) analyzing at least 10 spheroids per experimental group and experiment.

**Endothelial cell proliferation** Endothelial cell proliferation was determined by measuring total cell number exactly as described (31).

**Matrigel plug assay** After thawing on ice, matrigel (BD Bioscience, Bedford, MA) containing 30 IU/ml heparin (Roche, Germany), in the absence or presence of 100ng/ml VEGF without or with Eap in a total volume of 400 µl was injected subcutaneously into the laterodorsal abdominal region of 8-week old male C57Bl/6 mice. The matrigel samples were recovered five days after implantation for photo documentation. To quantitate neovascularization the haemoglobin concentration in matrigel homogenisates was measured as described (32).

**Generation of cutaneous wounds** Wound healing experiments were performed as described (27,33,34). Ten week-old female mice were anesthetized and two round cutaneous wounds (diameter 14mm) were generated at both sides of the lower dorsal trunk after shaving the hair and disinfecting the skin. A full-thickness excisional wound was created by removal of the skin and panniculus carnosus. The wounds were separated by more than 1cm skin. A semipermeable transparent dressing (Tegaderm; 3M Health Care, St. Paul, MN) was placed over the wound. The different treatments or the inoculated bacteria (2.5X10^9/wound) were applied directly under the dressing at a volume 0.1 ml. This protocol was approved by the Govermental Office Karlsruhe, Germany. To evaluate wound size, the vertical and horizontal diameters of each wound were recorded immediately after wounding at day 0 and at
subsequent days thereafter in anesthetized mice. The mean of the wound diameters was used in the calculation of each wound area and wound size was calculated relative to the size at day 0.

In other experiments, wounds were extracted at days 0, 1, 3, and 5 after generation and processed for immunohistochemistry or western blot analysis. For the preparation of total wound extract, wounds were homogenized using a pestle in liquid nitrogen and were then lysed in a buffer containing 50mM Tris-HCl, pH 8.0, 150mM NaCl, 0.02% Sodium azide, 1% Triton X-100, 2mM Benazamidin, 20µg/ml soybean inhibitor, 1mM leupeptin, 1µg/ml aprotinin, 0.5mM PMSF. After centrifugation (4000 rpm, 2 min., 4°C), lysates were cleared from insoluble material, followed by four freeze (-80°C)/thaw (37°C) cycles. Lysates were centrifuged (15,000 rpm, 5 min, 4°C), the supernatant was collected and centrifuged again (4000 rpm, 1 min, 4°C). The supernatant was used for Western blot analysis after determining and adjusting protein concentration. Western Blot analysis for the detection of MPO or tissue factor was performed. The same protocol was used for the detection of Eap in S. aureus-infected or non-infected wounds from human subjects.

**Western Blot analysis** Western Blot analysis for the detection of tissue factor on THP-1 cells, or for the detection of tissue factor or MPO in wound extract was performed as described (27).

**Immunohistochemistry** At the time of sacrifice, immediately after final tracing of the wound edges, the entire wound was excised down to the fascia. Tissue was snap-frozen and stored at -80°C. For detection of neutrophils, macrophages and vessels, antibodies to mouse MPO, F4/80 and PECAM-1 were used, respectively. Ten µm thin cryostat sections were prepared from day 1 or day 5 wounds and were collected.
on slides. After fixing with ice cold acetone for 10 min, blocking and washings, sections were incubated with a rabbit-anti-MPO (diluted 1:100), anti-mouse F4/80 (diluted 1:200), or biotinylated rat anti-mouse CD31 (diluted 1:200) for 2hr at room temperature. Sections were then reacted with appropriate peroxidase-coupled secondary antibodies. Peroxidase activity was detected with AEC substrate. Consecutive sections that stained without first antibodies were used as negative controls. Slides were coverslipped and examined using a Zeiss microscope at a magnification of 100x and 200x. For the evaluation of blood vessels present in the wounds at day 5, 7 microscopic fields/wound and 8 wounds/treatment group were analyzed and neovascularization was expressed as number of blood vessels per microscopic field.

For the detection of Eap in human wounds, biopsies were taken from S. aureus- infected or non-infected wounds; tissues were processed as above and Eap detection was performed with sheep polyclonal antibody against Eap. Written consent was obtained from all patients.

Quantification of blood flow using microspheres For the measurement of blood flow, Fluospheres™ (Molecular Probes, Leiden, The Netherlands) were used as described (35). Briefly, at day 5 following wound generation mice were anesthetized, the chest was opened, followed by injection of $1.5 \times 10^6$ microspheres (diameter 10μm), dissolved in 0.5 ml of 0.9% NaCl into the left ventricle. Mice were sacrificed 3 min after injection, and the wounds were extracted, weighed and digested using 4M KOH for 24hr. Fluorescence per weight tissue sample in each wound specimen was determined according to the manufacturer´s protocol.
Electrophoretic mobility shift assay (EMSA) Nuclear proteins from cells were harvested and assayed for transcription factor binding activity using the NFκB consensus sequence 5'-GTTGAGGGGACTTTCCAGGC-3 as described (27). Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabeled consensus oligonucleotides and by supershift experiments.

Statistical analysis Data were compared using ANOVA with post-hoc analysis as appropriate; P values of < 0.05 were regarded as significant.

Results
Inhibition of wound healing by Eap

S. aureus-infected wounds often display impaired wound healing. As shown in Fig.1A and 1B, Eap was detected in S. aureus-infected wounds from patients but not in non-infected wounds by immunohistochemistry and by western blot. We have previously shown that Eap binds endothelial ICAM-1 and inhibits leukocyte recruitment (10). As inflammatory cell infiltration is crucial in wound healing, we continued to define the functional role of Eap in wound healing. Excisional wounds were placed on the dorsal skin of mice. In the presence of wildtype S. aureus a dramatic delay in wound healing was observed (Fig.1C). A significant delay in wound closure was observed already after the second day. In the presence of S. aureus wound closure was observed after 14-15 days, whereas control wounds healed after 10-11 days (Fig.1C). In order to define the role of Eap in the inhibition of wound healing by S. aureus, we engaged an isogenic Eap-deficient strain. The inhibitory effect of S. aureus in wound healing was reverted when the Eap-deficient strain was used (Fig.1C), indicating that Eap contributes to the S. aureus-mediated delay in wound closure. Moreover, we applied
isolated Eap locally to the wound at the days 0, 1, 3 and 5. In Eap-treated wounds a significant delay in closure was observed after the second day as compared to buffer-treated wounds. Thereafter, the wound areas of Eap-treated mice remained significantly larger until day 13 and healed with a delay of 3 days as compared to buffer-treated wounds (Fig.1D). Inhibition of wound healing by Eap was also dose-dependent (10, 20 and 30 μg/wound were used, data with 20 μg/wound are shown). In contrast, S. aureus protein A did not affect wound healing (Fig.1D). Moreover, co-incubation of Eap with an antibody against Eap neutralized the inhibitory effect of Eap (not shown). These data indicate that Eap inhibits macroscopic wound healing.

Reduced leukocyte infiltration in wounds and leukocyte activation by Eap

In the inflammatory phase of wound healing, neutrophils and macrophages migrate into the wound site at different times following injury (12,13). One day after injury neutrophil infiltration at the wound sites of Eap-treated mice was markedly lower, as compared to control mice (Fig.2A and 2B). The detection of MPO by western blot analysis was also decreased in Eap-treated mice (Fig.2C), indicating less infiltrated neutrophils in these wounds. Using the anti-F4/80 antibody that recognizes macrophages, the number of macrophages at day 5 was found to be significantly lower in Eap-treated wounds as compared to buffer-treated wounds (Fig.2D and 2E). In contrast, in protein A-treated wounds no decrease in neutrophil or macrophage recruitment was observed (not shown). These data indicate that Eap inhibits leukocyte infiltration to wound sites. The infiltrating leukocytes in wounds release pro-inflammatory and pro-coagulant factors, such as tissue factor, that contribute to the repair mechanism. We therefore compared the expression of tissue factor in Eap- and buffer-treated wounds. At days 3 and 5 after injury reduced levels of tissue factor
were found by western blot analysis in Eap-treated wounds as compared to control wounds (Fig.2F, data for day 3 not shown).

We previously demonstrated that Eap inhibits the interaction between ICAM-1 and its leukocyte counter-receptors Mac-1 and LFA-1 (10). As Eap-treated wounds displayed reduced inflammatory cell infiltration, we investigated whether Eap interferes with the adhesion of peripheral blood neutrophils or THP-1 cells to endothelial cells. The presence of Eap significantly reduced the β2-integrin-dependent adhesion of neutrophils to endothelial cells to the level of inhibition obtained with blocking mAb against ICAM-1 (Fig.3A). MCP-1-stimulated neutrophil transendothelial migration was also blocked by Eap (Fig.3B). Inhibition of neutrophil transendothelial migration by Eap could be due to its antiadhesive effect. In order to study, whether Eap directly interferes with transendothelial migration, experiments were performed in two ways: (i) Neutrophils and inhibitors were co-incubated during the whole course of the transmigration experiment; (ii) neutrophils were incubated on endothelial cells for 20 min in the absence of competitors in order to facilitate their initial attachment on the endothelial surface, and thereafter inhibitors were added into the wells. CD18 and ICAM-1 participate in both neutrophil-endothelial adhesion and neutrophil transendothelial migration (36). Therefore, anti-CD18 and anti-ICAM-1 inhibited neutrophil diapedesis in both settings, i.e. when co-incubated with the neutrophils during the whole course of the transmigration assay, and when added to the wells after initial neutrophil attachment. Unsurprisingly, the degree of inhibition of transmigration by anti-CD18 and anti-ICAM-1 was slightly lower in the latter situation. Contrastingly, anti-CD29 blocked transmigration only when co-incubated with the neutrophils, but not when added after the initial neutrophil attachment, suggesting a role of β1-integrin primarily in neutrophil adhesion. Consistent with our previous studies that identified Eap to block ICAM-1 (10), Eap behaved similar to anti-CD18 or
anti-ICAM-1, which suggests that Eap interferes with both neutrophil adhesion and transmigration (Fig.3B). The effect of Eap on HUVEC-related neutrophil adhesion and their transmigration was also dose-dependent (not shown). No effect of protein A on neutrophil-endothelial adhesion and neutrophil transmigration was found (Fig.3A and B). Similar results were obtained with THP-1 cells (not shown).

The transcription factor NFκB links inflammatory stimuli and gene expression in leukocytes and endothelial cells (21), and ligation and activation of leukocyte integrins as it takes place during adhesive interactions can result in the activation of NFκB (19,20). We therefore tested whether the anti-inflammatory effect of Eap extends towards differences in NFκB binding activity, as assessed by EMSA in THP-1 cells. As shown in Fig.3C, ligation of β2-integrins with recombinant ICAM-1 increased TNF-α–induced NFκB activity. This effect was prevented by Eap, but not by protein A (not shown). NFκB is central in the regulation of the expression of inflammatory mediators in leukocytes such as tissue factor, and TNF-α-induced tissue factor expression in monocytes is upregulated upon β2-integrin ligation with ICAM-1 (18). Co-incubation of THP-1 cells with Eap prevented the ICAM-1-induced tissue factor expression in THP-1 cells (Fig.3D). Taken together, these data clearly demonstrate that Eap exerts anti-inflammatory properties by blocking ICAM-1-dependent leukocyte adhesion to and transmigration through the endothelium and reducing the ICAM-1-mediated upregulation of the activity of NFκB with respective consequences for the expression of pro-inflammatory and pro-coagulant molecules in vitro and in vivo.
Impaired angiogenesis in Eap-treated wounds

During the proliferative phase of wound healing, neovascularization is indispensable for the generation of granulation tissue. Immunohistochemical inspection of Eap-treated wounds displayed a dramatic inhibition of neovascularization, as the number of CD31-positive vessels at day 5 after injury was reduced in Eap-treated wounds as compared to buffer control (Fig.4A-B). No effect of protein A in wound neovascularization was observed (not shown). In order to assess and quantify angiogenesis in more detail, we examined the perfusion of wounds using fluorescent microspheres. In line with the previous findings, Eap treatment significantly reduced blood flow in wounds by 40-50% (Fig.4C). Thus, Eap exerts strong anti-angiogenic activity, impairing neovascularization in the proliferative phase of wound repair.

Inhibition of $\alpha_v$-integrin-dependent endothelial cell migration by Eap

As Eap binds to different ECM proteins, we hypothesized that it might interfere with interactions between integrins and matrix proteins that are crucial in angiogenesis. Endothelial cells engage different integrins to migrate onto matrix proteins such as VN, FBG or FN. Whereas endothelial cell migration towards VN and FBG is dependent predominantly on $\alpha_v$-integrins, $\alpha_5\beta_1$-integrin mediates migration towards FN, as shown by blockade with specific antibodies (Fig. 5A). Eap, but not protein A, dose-dependently blocked $\alpha_v$-integrin-dependent migration towards VN or FBG, both under basal conditions or under stimulation with VEGF (only data in the presence of VEGF are shown in Fig.5A). Only a very moderate but not significant inhibitory effect of Eap on the $\alpha_5\beta_1$-integrin-dependent migration of endothelial cells towards FN was observed.
In agreement with our previous observations that Eap directly interacts with VN or FBG (10), Eap was found to block the binding of both ECM proteins to immobilized αvß3-integrin in a dose-dependent manner. In contrast, although Eap also binds to FN (10), the interaction between FN and α5ß1-integrin was not affected by Eap (Fig. 5B-D), indicating that Eap specifically affects αv-integrin-dependent interactions that are crucial for angiogenesis (37).

Inhibition of endothelial proliferation, capillary sprout formation and in vivo angiogenesis by Eap

VEGF-stimulated increase in endothelial cell proliferation plays a crucial role during pathological neovascularization in wound healing. VEGF-stimulated HUVEC proliferation was inhibited in the presence of Eap (Fig. 6A) in a dose-dependent manner, whereas protein A was not effective (not shown). In a capillary-like sprout formation in vitro assay that examines the invasive and migratory potential of endothelial cells in a 3D cell spheroid system, Eap dose-dependently reduced the VEGF-induced capillary-like sprouting (Fig. 6B). The IC₅₀ of Eap in the 3D angiogenesis assay is 10.7 µg/ml [95% confidence interval from 5.9 to 19.6 µg/ml], very well within the range of Eap found in the supernatants of S. aureus in culture (8). Moreover, Eap inhibited sprout formation to the same extent as established αv-integrin-inhibitors, such as the mAb LM609 or cyclic RGD peptides do, whereas protein A had no inhibitory effect (Fig. 6C).

Finally, the anti-angiogenic activity of Eap in vivo was investigated using the matrigel plug assay. Matrigel in the absence or presence of Eap was injected subcutaneously into the laterodorsal abdominal region of mice and recovered 5 days later for analysis. VEGF-induced new vessel formation was inhibited in matrigels exposed to Eap (Fig. 7A), indicating that Eap exerts a strong anti-angiogenic activity.
in vivo. By determining the haemoglobin concentration in the matrigels we found that 10 μg and 30 μg of Eap inhibited angiogenesis in the matrigel assay by 40 and 70 %, respectively (Fig. 7B).

Discussion

Impaired wound healing is frequently seen in S. aureus-infected chronic wounds. The present study clearly demonstrates for the first time that S. aureus Eap delays wound closure due to its potent anti-inflammatory and anti-angiogenic activities. The ability of Eap to interact with adhesion molecules and especially with endothelial ICAM-1 as well as with adhesive proteins in the ECM thereby blocking integrin-mediated adhesive and migratory interactions of both inflammatory and endothelial cells provides the common pathway for the anti-inflammatory and anti-angiogenic actions of Eap. To our knowledge, the here described inhibition of neovascularization by Eap acting in concert with the anti-inflammatory properties of the molecule defines an entirely novel mechanism of inhibition of wound healing by S. aureus.

Upon skin injury, initial clot formation and local inflammation characterized by infiltration of neutrophils and macrophages is followed by the proliferative phase, in which neovascularization of the granulation tissue is indispensable (12,13). The discovered features demonstrate that Eap specifically interferes with the inflammatory phase and blocks angiogenesis in the proliferative phase: (i) In the presence of isolated Eap or wildtype S. aureus wound closure was markedly delayed. The delay in wound healing due to S. aureus presence was reversible when an isogenic Eap-deficient strain was engaged, clearly demonstrating that inhibition of wound healing in S. aureus infected wounds may be at least in part attributed to Eap. However, our findings also imply that other S. aureus factors may be involved in delayed wound healing. (ii) The subsequent analysis of Eap-treated wounds demonstrated reduced infiltration of neutrophils and macrophages, and
consequently, reduced expression of the pro-inflammatory and procoagulant tissue factor. These data were supported by in vitro findings that Eap inhibits ICAM-1-dependent leukocyte endothelial adhesion and transendothelial migration and the ICAM-1-mediated upregulation of NFκB activity and the consequent expression of tissue factor. Thus, Eap blocks ICAM-1-dependent leukocyte-endothelial interactions and ICAM-1-dependent leukocyte activation and the present data clearly extend previous findings by our group and others (10,38). (iii) Further analysis of wound tissue surprisingly revealed that Eap-treated wounds displayed dramatically impaired neovascularization. This profound reduction in neovascularization by Eap may explain the prolonged inhibitory effect of Eap on wound repair. Of note, the concentrations of Eap engaged in our studies lie very well within the range of the amounts of Eap found in the supernatants of S. aureus in culture (8).

During angiogenesis, ECM associated proteins such as VN or FBG are incorporated into a provisional adhesive fibrillar network that regulates diverse endothelial cell functions including growth and migration, and cellular adhesion-dependent signals are transmitted predominantly through αv-integrins (37). While Eap efficiently blocked endothelial cell migration primarily by interfering with αv-integrins and their ligands VN and FBG, α5β1-dependent migration in response to FN was hardly affected by Eap. These findings demonstrate a specific role of Eap as potent inhibitor of the αv-integrin-ligand-system, very reminiscent of the blocking function of other αv-integrin inhibitors. This anti-αv-integrin activity of Eap thereby influences different angiogenesis-related functions of endothelial cells as was evidenced in Eap-mediated inhibition of capillary-like tube formation in three-dimensional matrices or the blockade of neovascularization in matrigel plug assay in vivo. Furthermore, Eap dose-dependently inhibited VEGF-stimulated endothelial cell
proliferation, however the underlying mechanism is not revealed by our present results and should be addressed in a future study.

Eap may inhibit angiogenesis through a combination of several complementary mechanisms culminating in blockade of $\alpha v$-integrin-function that were identified in this study. As inflammatory cells are fine regulators of angiogenesis by providing growth factors and matrix metalloproteinases (24), the anti-inflammatory role of Eap may indirectly contribute to its anti-angiogenic action as well. Moreover, tissue factor is not only a major initiator of haemostasis following vascular injury, but also a well-recognized pro-angiogenic factor (17). Eap-mediated inhibition of tissue factor expression can thereby downregulate its angiogenic potential. Tissue factor expression in leukocytes depends in great parts on the activity of the transcription factor NF$\kappa$B. In line with the Eap-mediated downregulation of tissue factor expression, NF$\kappa$B activation in monocytes was inhibited by Eap as well. Thus, the anti-inflammatory action of Eap extends to events downstream to integrin-dependent adhesion, such as blockade of NF$\kappa$B activity. To our knowledge this is the first demonstration that an $S.\ aureus$ product may prevent NF$\kappa$B activation, and Eap might counteract the effects of peptidoglycan that stimulates NF$\kappa$B activation via toll-like receptor 2 (39) and promotes wound repair (40).

In addition to blocking leukocyte extravasation, Eap also interferes with T-cell functions, thereby affecting the course of chronic infections such as arthritis or osteomyelitis (11). Other immuno-modulating properties of $S.\ aureus$ may add to the here described function of Eap. For example, Eap may cooperate with the chemotaxis-inhibitory protein of $S.\ aureus$ that blocks chemoattractant-induced neutrophil and monocyte migration into the infection site (41). However, these previous studies were limited to the immuno-modulatory actions of Eap (10,11), but
are extended here to inhibition of crucial endothelial cell functions by Eap. The
importance and implications of the observed anti-angiogenic function of Eap are
manyfold. Besides being responsible for the impaired wound healing in chronically
*S. aureus*-infected wounds or ulcers (3,4), as clearly demonstrated in the present
study, the anti-angiogenic properties of Eap may enable the development of a novel
class of anti-angiogenesis compounds to be engaged against uncontrolled
neovascularization in cancer and other pathologies and studies are in progress to
test these concepts.

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References


Legends to figures

Figure 1: Detection of Eap in wounds and effect of Eap on wound healing. (A) Eap detection in *S. aureus* infected wounds. (B) Eap was detected by Western blot analysis in extracts of non-infected (lane 1) or *S. aureus* infected wounds (lane 2). Molecular mass markers are indicated along the right margin. (C) The course of wound closure is shown in the absence (buffer; filled circles), or in the presence of wildtype *S. aureus* (filled squares) or the Eap-deficient strain (open squares). *: P<0.05 as compared to buffer treated wounds; #: P<0.05 as compared to wounds that received the Eap-deficient strain; **: P<0.05 as compared to buffer-treated wounds. (D) The course of wound closure is shown in the absence (filled circles), or in the presence of 20 µg Eap (filled squares) or protein A (open triangles). *: P<0.05 as compared to buffer treated wounds. Wound closure is expressed relative to the wound size of each wound at day 0 that represents 100 %. Data are expressed as mean +/- SD (n=8).

Figure 2: Inhibition of leukocyte infiltration into wounds by Eap. (A, B) Typical photomicrographs of immunohistochemical staining for the detection of neutrophils (anti-MPO) at day 1 following wound generation in the absence (A, buffer) or presence of 20 µg Eap (B) is shown. (C) The expression of MPO at day 1 following wound generation as assessed by Western blot is shown in the absence (open bar) or in the presence of Eap (filled bars). The insert demonstrates a typical blot with staining for MPO. Densitometric data are expressed relative to control (buffer-treated wounds), and are mean +/- SD of three separate experiments. (D, E) Typical photomicrographs of immunohistochemical staining for the detection of macrophages (anti-F4/80) at day 5 following wound generation in the absence (D, buffer) or presence of Eap (E) is shown. (F) The expression of tissue factor at day 5 following wound generation as assessed by Western blot analysis is shown in the absence.
Figure 3: Influence of Eap on leukocyte-endothelial cell interactions and on the activity of NFκB. (A) Adhesion of human neutrophils to endothelial cells is shown in the absence (-) or presence of blocking mAb to CD18, ICAM-1, CD31 as indicated (each 20 µg/ml), Eap or protein A (each 20 µg/ml). Cell adhesion is expressed relative to control (in the absence of competitor). Data are mean+/±SD (n=3) of a typical experiment, similar results were obtained in three separate experiments. (B) The trans-endothelial migration of human neutrophils in response towards 50 ng/ml MCP-1 is shown in the absence (-) or presence of mAb to CD18, mAb to ICAM-1, mAb to CD29 (each 20 µg/ml), Eap or protein A (each 20 µg/ml). Neutrophils and inhibitors were coincubated during the whole course of the transmigration experiment (open bars) or neutrophils were incubated on the endothelial cells for 20 min in the absence of competitors in order to facilitate their initial attachment on the endothelial surface, and inhibitors were added thereafter into the wells (filled bars). Transmigration is presented as percent of control (in the absence of competitor). Data are mean+/±SD (n=3) of a typical experiment, similar results were obtained in three separate experiments. (C) The DNA binding activity of NFκB without or with TNFα or TNFα + Eap in THP-1 cells is shown in the absence (open bars) or presence of ICAM-1 (10 µg/ml, gray bars), as indicated. The insert demonstrates a typical EMSA for NFκB DNA binding activity (1: control, 2: TNF-α, 3: TNF-α+Eap, 4: ICAM-1, 5: ICAM-1+TNF-α, 6: ICAM-1+TNF-α+Eap). (D) The expression of tissue factor without or with TNFα or TNFα + Eap in THP-1 cells is shown in the absence (open bars) or presence of ICAM-1 (10 µg/ml, gray bars). Densitometric data are expressed relative to control (buffer-treated wounds), and are mean+/±SD of three separate experiments.
expressed relative to control (100% control is represented in the absence of stimuli or competitors), and are mean+/-SD of two separate experiments. *:P<0.05.

Figure 4: Eap inhibits neovascularization in wound healing. (A) Typical photomicrographs (100x magnification) of immunohistochemical staining for the detection of PECAM-1 (CD31) in association with blood vessels at day 5 following wound generation in the absence (buffer) or presence of 20 µg Eap is shown. Negative control (first antibody omitted) is also shown. (B) Statistical evaluation of the density of CD31-positive blood vessels (expressed as number of vessels/field) in wound sections at day 5 following wound generation in the absence (buffer) or presence of 20 µg Eap is shown. Evaluation of 7 fields / wound from 8 wounds / treatment group is shown. (C) Mice were anesthetized and fluorescent microbeads were injected. The recovered fluorescence / weight of wound tissue is shown, and data expressed relative to control (buffer-treated wounds) are mean+/-SD of 10 wounds. *: P< 0.01 as compared to control.

Figure 5: Eap interferes with αvβ3-integrin-dependent cell function. (A) VEGF (20 ng/ml)-stimulated migration of HUVEC towards VN, FBG or FN (each 5 µg/ml) is shown in the absence (filled bars) or presence of the blocking mAb against αvβ3-integrin, LM609 (for VN and FBG), the blocking mAb against β1-integrin, 6S6 (for FN) (20 µg/ml, open bars), or in the presence of Eap (gray bars, 20 µg/ml), or protein A (hatched bars, 20 µg/ml). Cell migration is expressed relative to control, which is represented as cell migration in the absence of any stimulus or competitor. Data are mean±SD of 4 experiments performed in triplicates. *:P<0.05 as compared to VEGF-stimulated migration in the absence of competitor. (B-D) Binding of VN to immobilized αvβ3-integrin (B), binding of FBG to immobilized αvβ3-integrin (C) and binding of FN to immobilized α5β1-integrin (D), was performed in the absence (-; filled bars) or presence of blocking mAb against, αvβ3-integrin, LM609 (for VN and FBG), the
blocking mAb against β1-integrin, 6S6 (for FN) (20 µg/ml, open bars), or increasing concentrations of Eap (gray bars) as indicated. Specific binding is expressed as absorbance at 405 nm. Data are mean±SD (n = 3) of a typical experiment; similar results were observed in three separate experiments.

Figure 6: Effect of Eap on endothelial cell proliferation and capillary sprout formation. (A) HUVEC proliferation; HUVEC were incubated without (open bar) or with VEGF (filled bars; 10 ng/ml) in the absence (-) or in the presence of increasing concentrations of Eap as indicated. Proliferation of HUVEC is expressed as % of control, defined as cell proliferation in the absence of any stimulus or competitor. Data are Mean±SD (n=3) of one experiment typical of three separate experiments so performed. (B-C) Capillary sprout formation; (B) HUVEC were incubated for 24 h in the absence (-; open bar) or presence of VEGF (25 ng/ml; filled bars) without or together with increasing concentrations of Eap, as indicated. Capillary-like tube formation is expressed as capillary sprout length in µm. (C) HUVEC were incubated for 24 h in the presence of VEGF (25 ng/ml) without (-) or together with Eap, protein A (each 20 µg/ml), cRGD (20 µg/ml) or the mAb against αvβ3-integrin, LM609 (20 µg/ml), as indicated. Capillary-like tube formation is expressed relative to control, which is represented as sprout formation in the presence of VEGF and in the absence of any competitor. Data are mean±SD (n=10) of a representative experiment. Similar results were obtained in three independent experiments.

Figure 7: Inhibition of angiogenesis by Eap in the in vivo matrigel plug assay. (A) Neovascularization in the matrigel plug assay was performed without (buffer) or with VEGF (100 ng/ml) in the absence or presence of 10 µg or 30 µg Eap as indicated. Photographs and of a typical experiment performed in triplicates are shown; similar results were obtained in three separate experiments. (B) The quantitation of neovascularization in the matrigels was performed by measuring the haemoglobin
concentration. Haemoglobin concentration was expressed as mg haemoglobin / g wet tissue. Data are expressed as % of the maximum (VEGF treatment in the absence of competitors). Data are mean±SD (n = 3) of a typical experiment; similar results were observed in three separate experiments.
The Extracellular Adherence Protein (Eap) of Staphylococcus aureus Inhibits Wound Healing by Interfering with Host Defense and Repair Mechanisms

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