The FASEB Journal article fj.09-137588. Published online August 3, 2009.

Structure-activity analysis of histatin, a potent wound healing peptide from human saliva: cyclization of histatin potentiates molar activity 1000-fold

Menno J. Oudhoff,*[,1] Kim L. Kroeze,[†] Kamran Nazmi,* Petra A. M. van den Keijbus,* Wim van 't Hof,* Mar Fernandez-Borja,[‡] Peter L. Hordijk,[‡] Susan Gibbs,[†] Jan G. M. Bolscher,* and Enno C. I. Veerman*

*Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, Amsterdam, The Netherlands; [†]Department of Dermatology, VU University Medical Center, Amsterdam, The Netherlands; and [‡]Department of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

ABSTRACT **Wounds in the mouth heal faster and with less scarification and inflammation than those in the skin. Saliva is thought to be essential for the superior oral wound healing, but the involved mechanism is still unclear. We have previously discovered that a human-specific peptide, histatin, might be implicated in the wound-healing properties of saliva. Here we report that histatin enhances reepithelialization in a human full-skin wound model closely resembling normal skin. The peptide does not stimulate proliferation but induces cell spreading and migration, two key initiating steps in reepithelialization. Activation of cells by histatin requires a G-protein-coupled receptor that activates the ERK1/2 pathway. Using a stepwise-truncation method, we determined the minimal domain (SHREFPFYGDYGS) of the 38-mer-parent peptide that is required for activity. Strikingly, N- to C-terminal cyclization of histatin-1 potentiates the molar activity ~1000-fold, indicating that the recognition of histatin by its cognate receptor requires a specific spatial conformation of the peptide. Our results emphasize the importance of histatin in human saliva for tissue protection and recovery and establish the experimental basis for the development of synthetic histatins as novel skin wound-healing agents.—Oudhoff, M. J., Kroeze, K. L., Nazmi, K., van den Keijbus, P. A. M., van 't Hof, W., Fernandez-Borja, M., Hordijk, P. L., Gibbs, S., Bolscher, J. G. M., Veerman, E. C. I. Structure-activity analysis of histatin, a potent wound healing peptide from human saliva: cyclization of histatin potentiates molar activity 1000-fold. *FASEB J.* 23, 000–000 (2009). www.fasebj.org**

Key Words: antimicrobial peptides · cell migration · cyclic peptide · domain mapping · reepithelialization

IT IS GENERALLY ASSUMED that saliva plays a critical role in maintaining oral health. The importance of saliva is best recognized by patients that have reduced salivary flow, for example, after radiation of head and neck cancer or patients with Sjögren syndrome. Many of these patients suffer from conditions such as mucositis or xerostomia, and they have increased risk for oral infections as well as trouble eating, tasting, and sometimes even talking (1). Saliva is a very versatile fluid secreted by the different salivary glands; according to the latest count, it contains >1000 different proteins (2). Saliva is thought to be essential for the phenomenon of superior oral wound healing as compared with skin (3–5). The wound-healing effects of human saliva have been attributed to the presence of growth factors, such as epidermal growth factor (EGF) and nerve growth factor (6, 7). However, this finding was based on rodent studies that found particularly high concentrations of these two growth factors in saliva (17–899 μg/ml, depending on type of stimulation; refs. 8, 9). We have demonstrated that members of the salivary histatin peptide family, histatin-1 (Hst1), Hst2 [or (Hst1(12–38)], and Hst3, stimulate epithelial cell migration and might therefore be the prime agents that mediate the wound-healing activity of human saliva (10). Although histatins in saliva readily are degraded by proteases, the steady-state concentrations are well within the range needed for cell activation (11). Our finding revealed a new and important activity of this histatin peptide family, which for >3 decades had been primarily regarded as antimicrobial peptides implicated in the innate immunity of humans and higher primates (12, 13).

The two activities of histatins, *i.e.*, antimicrobial and cell-stimulating, are very distinct in their modes of action, structural requirements, and selectivity. The antimicrobial activity occurs through disruption of the phospholipid membrane of the target cell and is independent of the chirality of the peptide (10, 14–16).

[1] Correspondence: Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. E-mail: mj.oudhoff@vumc.nl

doi: 10.1096/fj.09-137588

This is in contrast to its stimulating activity on host cells, which involves a stereospecific interaction with a putative membrane receptor (10). In addition, Hst1 and Hst1(12–38) are the most potent enhancers of *in vitro* wound closure, whereas Hst5, the most potent antimicrobial of the histatin family, is virtually inactive (10).

Building on our previous finding that Hst enhances *in vitro* cell migration (10), in the present study we demonstrate that Hst enhances reepithelialization in a tissue-engineered epidermal-skin equivalent, which closely resembles the human skin (17). Rather than proliferation, Hst stimulated cell spreading and migration, which was inhibited by pertussis toxin (PTx). We mapped the active domain by stepwise truncation and explored the effect of cyclization of the peptide. This revealed that the minimal biologically active domain encompasses a 13-aa stretch of Hst1 and that cyclization of Hst1 results in a 1000-fold increase in activity on a molar basis. The data suggest that the interaction with a G-protein-coupled receptor (GPCR) requires a specific 3-dimensional conformation of the peptide. This work identifies histatin as a potent wound-healing agent, present in human saliva, which may form the basis of a novel skin wound-healing medication.

MATERIALS AND METHODS

Peptide synthesis

Linear peptides were synthesized by solid-phase peptide synthesis using Fmoc chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Biosearch, Bedford, MA, USA) and purified by RP-HPLC, and their authenticity was confirmed by mass spectrometry, as described before (15). The amino acid sequence of Hst1 is DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN; that of the truncated versions can be deduced from this sequence [*e.g.*, Hst1(12–38) represents aa 12 to 38]. The cyclic peptide was synthesized using Fmoc-Glu-NovaSyn TGA-ODmab (NovaBiochem, Läufelfingen, Switzerland), a dedicated support, in which the first amino acid is coupled to the resin by its side-chain carboxyl group, while its α-carboxyl group is protected by the semiorthogonal Dmab group (18). After completion of the sequence, the N-terminal Fmoc was removed with 20% piperidine in *N*-methylpyrrolidone, and subsequently the C-terminal ODmab was removed by 2% hydrazine in *N,N*-dimethylformamide (DMF). On-resin head-to-tail cyclization was achieved by prolonged reaction (72 h) with 1 eq PyBOP, 1 eq HObt, and 1 eq DIEA in DMF containing 20% DMSO and 2% DCM. After cleavage from the resin and purification by RP-HPLC, cyclization was confirmed by mass spectrometry, which showed that the molecular mass of cHst1 was 18 Da less than that of the linear Hst1, 4830 Da instead of 4848 Da, respectively, in accordance with the formation of a lactam bond. The recovery was 1.3%, which is a reasonable yield for on-resin synthesis of a cyclic peptide of this length.

Epidermal skin equivalent culture, wound model, and reepithelialization measurements

Epidermal keratinocytes were isolated from neonatal foreskins, essentially as described earlier (19). The study was approved by the VU Medical Center Medical Ethical Committee. Second-passage cultures of keratinocytes were seeded on deepidermized dermis. After culturing for 1 wk in medium containing DMEM (ICN Biomedicals, Irvine, CA, USA)/Ham's F-12 (ICN Biomedicals; 3:1), 1% UltroSerG (BioSepra SA, Cergy-Saint-Christophe, France), 1% penicillin/streptomycin (Life Technologies, Inc., Paisley, UK), 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 2 ng/ml keratinocyte growth factor (KGF), and 0.5 ng/ml EGF, cultures were lifted to the air-liquid interface and cultured for a further 2 wk in air-exposed culture medium [DMEM/Ham's F-12 (3:1), 0.2% UltroSerG, 1% penicillin/streptomycin, 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 2 ng/ml KGF, 0.5 ng/ml EGF, 10 μM L-carnitine, 10 mM L-serine, 1 μM DL-α-tocopherol acetate with a lipid supplement containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid, and 24 μM BSA]. Culture medium was refreshed 2×/wk. Unless otherwise stated, all culture additives were obtained from Sigma (St. Louis, MO, USA).

Full-thickness wounds were made in epidermal skin equivalents after 2 wk of air-exposed culture (20). Wounds were created using freeze burning, which resulted in cell death of the entire region of the epidermis that was treated with a device cooled to −196°C in liquid nitrogen for 10 s. During freeze wounding, the area of the device in contact with the epidermal skin equivalent was 2 cm long and 2 mm wide. Two wounds separated by ≥0.5 cm were introduced into each culture. The experiment was performed from 3 independent donors in duplicate.

Directly after wounding, epidermal equivalents were further cultured in air-exposed medium without EGF and KGF (see above) and supplemented with 72 μM Hst1(12–38) or D-Hst1(12–38). Medium supplemented with 10 ng/ml recombinant human (rh)EGF was used as positive control, medium without supplementation as negative control. Medium was changed every 2 d. After 6 d, reepithelialization was analyzed on hematoxylin and eosin (H&E)-stained paraffin sections (5 μm) with the aid of a Nikon microscope (Nikon, Tokyo, Japan) and Osteomeasure software (Osteometrics, Atlanta, GA, USA). Reepithelialization was measured as the distance the newly formed epidermis had migrated into the wound bed. Reepithelialization after exposure to Hst1(12–38), D-Hst1(12–38), or rhEGF-supplemented medium was compared with control medium and given relative to control. As reepithelialization occurred from both wound margins, duplicate readings were obtained for each wound.

Cell-line culture and *in vitro* wound-scratch assay

The human buccal epithelial cell line HO-1-N-1 was provided by the Japanese Collection of Research Bioresources (Osaka, Japan) and maintained as described previously (10). *In vitro* wound-scratch experiments were performed as described previously (10). In short, in a confluent layer of cells that was serum starved in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) for 6 h, a scratch was made with a sterile tip. The width of the scratch was measured at the beginning and after 18 h using microscopic images (Leica DM IL, Leica DFC320 camera; Leica Microsystems, Wetzlar, Germany). Relative closure was calculated by dividing the closure of treated wound (DMEM/F12+peptide) by that of the untreated wound (DMEM/F12 only). Final concentration of synthesized peptides in the assays was 10 μM, except for the dose-response experiments. As a positive control, 10 ng/ml rhEGF (Invitrogen) was used. PTx (Invitrogen) concentration was applied to the cells 1 h before the scratch was made and during the experiment at a final concentration of 200 ng/ml. U0126 (5 μM; LC Laboratories, Woburn, MA, USA) was used during incubation (no preincubation) as a specific inhibitor of MEK, thereby inhibiting phosphorylation of ERK1/2.

Proliferation assay

Serum-starved (24 h) HO-1-N-1 cells were trypsinized and seeded in 96-well culture plates (1×10^4 cells/well). After 6 h of adherence, the medium was changed with DMEM/F12 supplemented with 0.1, 1, 10, or 100 μM Hst1(12–38), 10 ng/ml rhEGF, or without anything (control). Medium was refreshed after 24 h. After 48 h, cells were fixed with paraformaldehyde (3.7%; Merck, Whitehouse Station, NJ, USA) in PBS for 20 min and permeabilized with Triton X-100 (0.25%) in PBS for 5 min. Cells were then stained with propidium iodide (PI; 10 μM; Invitrogen), and PI fluorescence was measured at excitation and emission wavelengths of 544 and 620 nm, respectively, in a Fluostar Galaxy microplate fluorimeter (BMG Labtechnologies, Offenburg, Germany). Fluorescence was proportionally increased with the cell number as was tested separately. Representative microscopic (Leica DM IL equipped with Leica DFC320 camera) images were taken to illustrate differences in cell numbers.

Cell-spreading assay

HO-1-N-1 cells that were serum starved for 24 h were trypsinized and subsequently seeded at low density so that a single-cell population prevailed. The cells were seeded with or without 10 μM Hst1(12–38), and after 16 h they were fixed with paraformaldehyde (3.7%; Merck) in PBS for 20 min and permeabilized with Triton X-100 (0.25%) in PBS for 5 min. Then the cells were incubated with Alexa Fluor 488 phalloidin (Invitrogen) for 1 h for F-actin staining and DAPI for 10 min for nuclei staining. Images were taken (Leica DM IL equipped with Leica DFC320 camera), and the area of

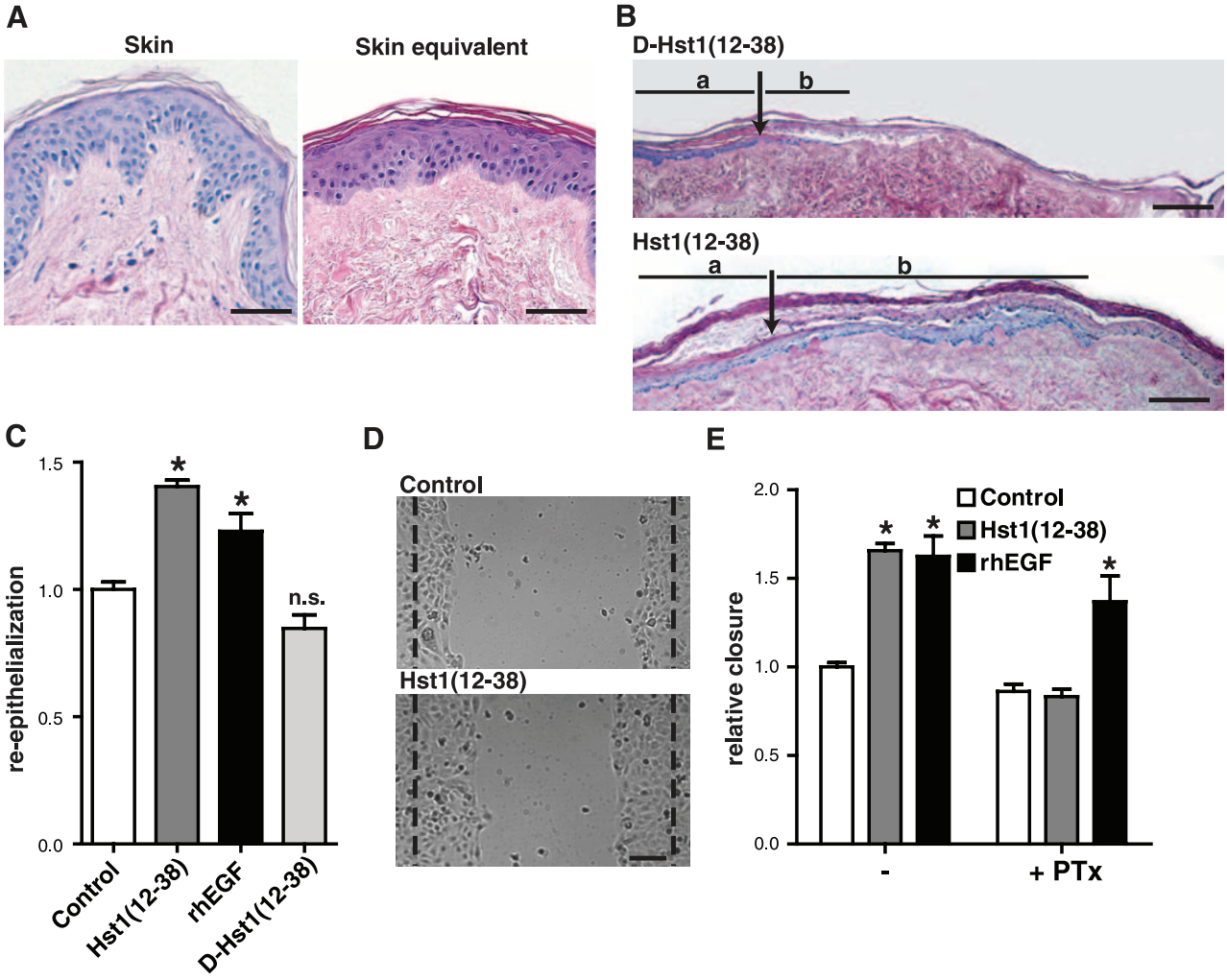

Figure 1. Histatin enhances reepithelialization in skin equivalent and is PTx sensitive. *A*) Comparison of human skin and tissue-engineered skin model. Tissue sections were stained with H&E for histological analysis. *B*) Representative micrographs of D-Hst1(12–38)- and Hst1(12–38)-treated wounds 6 d after wounding. *a*) Healthy epidermis. *b*) Ingrowing epidermis. Arrow indicates start of wound area. *C*) Wounds were introduced in the skin model. After 6 d, cultures were harvested, and reepithelialization was studied on H&E-stained paraffin sections. Reepithelialization was quantified as the distance the newly formed epidermis (blue) had migrated into the wound bed after D-Hst1(12–38), Hst1(12–38), and rhEGF supplementation relative to unsupplemented control cultures. Two wounds were introduced in each culture; duplicate readings were obtained from each wound. Data represent means ± SD; $n = 3$. $^*P \leq 0.01$. *D*) Representative micrographs of Hst1(12–38)-enhanced wound closure in the *in vitro* wound-scratch assay after 18 h. Dotted lines in images represent wound edges at $t = 0$ h. *E*) Relative closure, by measuring wound width from images in *D*, was calculated by dividing wound closure of treated wounds by that of the control. Wounds were treated with Hst1(12–38) or rhEGF, with and without PTx. Data represent means ± SD; $n = 12$. $^*P < 0.01$ *vs.* appropriate control. Scale bars = 100 μm (*A*, *D*); 250 μm (*B*).

individual cells (n=35/treatment, of phase-contrast images) was calculated using ImageJ software (21).

Electric cell-substrate impedance sensing (ECIS)

For ECIS-based cell-spreading experiments (22), ECIS electrodes (Applied Biophysics, Troy, NY, USA) were coated with L-cysteine for 30 min and with fibronectin (Sigma) in 0.9% NaCl overnight at 37°C. Serum-starved (24 h) HO-1-N-1 cells were seeded (2×10^5 cells/well) in DMEM/F12 (400 μl) with or without 10 μM Hst1(12–38). Immediately after seeding, the chamber slide was placed in its holder. ECIS was continuously monitored for up to 6 h.

Statistical analysis

Data were analyzed using 1-way ANOVA with additional least significant difference test to determine significance between samples. P values are as described in the figure legends.

RESULTS

Histatin accelerates wound reepithelialization in a human-skin model through a PTx-sensitive pathway

In a previous study (10), we demonstrated that histatins stimulate cell migration in an artificial wound-scratch model. In this model, a wound is made mechanically by applying a scratch in a monolayer of epithelial cells cultured on a plastic substrate (see also **Fig. 1*D***). In the present study, to test whether histatins have potential applicability as general wound-healing agents, their activity was examined in a tissue-engineered epidermal-skin equivalent that closely resembles the native healthy skin (17). The epidermal-skin equivalent consists of reconstructed epidermis on human acellular dermis. Due to the air-exposed culture method, complete epidermal differentiation occurs. This results in the formation of a compact basal layer, spinous layer, granular layer, and stratum corneum, which is similar to that found in native healthy skin (Fig. 1*A*). Full-thickness wounds were introduced by cold injury, as described earlier (20). The degree of reepithelialization of the wound was studied after 6 d.

Representative images of Hst1(12–38)-treated and D-Hst1(12–38) [the D-enantiomer of Hst1(12–38)]-treated wounds show that reepithelialization was enhanced when treated with Hst1(12–38) (Fig. 1*B*). In contrast, D-Hst1(12–38) had no effect, indicating that stereospecific interactions underlie the histatin-induced enhancement of reepithelialization. Quantification of the reepithelialization by analyzing a series of images represented by the one shown in Fig. 1*B* confirmed that reepithelialization was significantly enhanced by Hst1(12–38) with comparable rates as rhEGF, which we included as a positive control (Fig. 1*C*). These data indicate that histatins stimulate reepithelialization of the skin after wounding and build on our previous work showing that histatins are key modulators of the superior wound-healing properties of the mouth (10).

Since the D-enantiomer of Hst1(12–38) is not able to activate cells (Fig. 1*B*, *C*), we suspected that histatin-mediated activation of cells is a receptor-mediated process. In a first attempt to define which class of receptors may be involved in this effect, cells were treated with PTx during an *in vitro* wound-scratch experiment. PTx is known to specifically inhibit signaling through GPCRs that couple to heterotrimeric proteins of the $G\alpha_i$ subtype. We applied a scratch in a confluent layer of epithelial cells and measured the relative closure of the wound at $t = 0$ and $t = 18$ h (Fig. 1*D*). Hst1(12–38) enhanced wound closure to the same extent as rhEGF, but contrary to rhEGF, its activity was inhibited by PTx (Fig. 1*E*). This implies the involvement of a $G\alpha_i$-linked GPCR in the activation of cell migration by histatins. Previously, we have determined that the activation of epithelial cells by histatins is not mediated by the EGF receptor (10), which is a common receptor utilized for transactivation by different cellular stimuli.

Histatin promotes cell spreading and cell migration but does not enhance cell proliferation

Histatin promotes cell migration (Fig. 1*D*, *E*). However, since reepithelialization is also enhanced by cell prolifer-

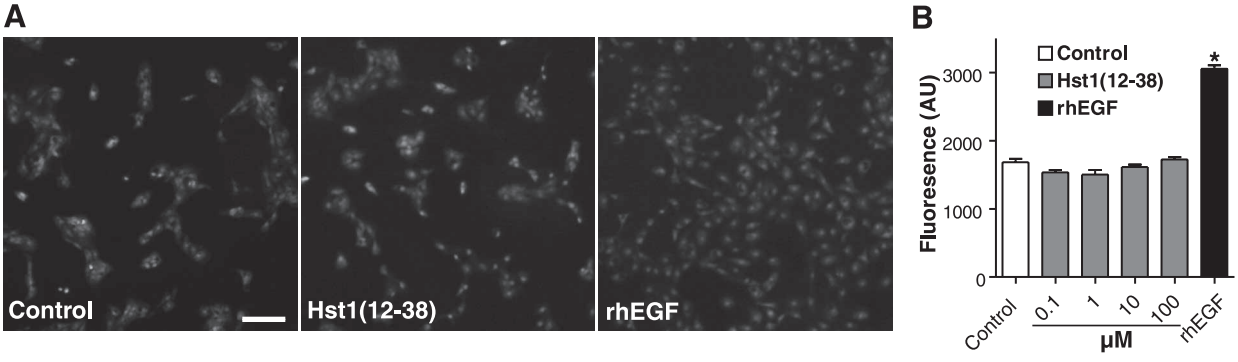

Figure 2. Histatin does not promote cell proliferation. *A*) Representative micrographs illustrating the number of cells per well after incubation in the presence or absence of Hst1(12–38) at concentrations ranging from 0.1–100 μM or rhEGF at 10 ng/ml. HO-1-N-1 cells were seeded (1×10^4 cells/well); 48 h later, cells were fixed and permeabilized, and subsequently DNA was stained with PI, a red dye that becomes fluorescent on binding to DNA. Scale bar = 100 μm. *B*) Total cell numbers were quantified by measuring PI fluorescence of DNA. Hst1(12–38) does not enhance proliferation, whereas rhEGF does. AU, arbitrary units. Data represent means ± SD; $n = 12$. *$P < 0.01$ *vs.* control.

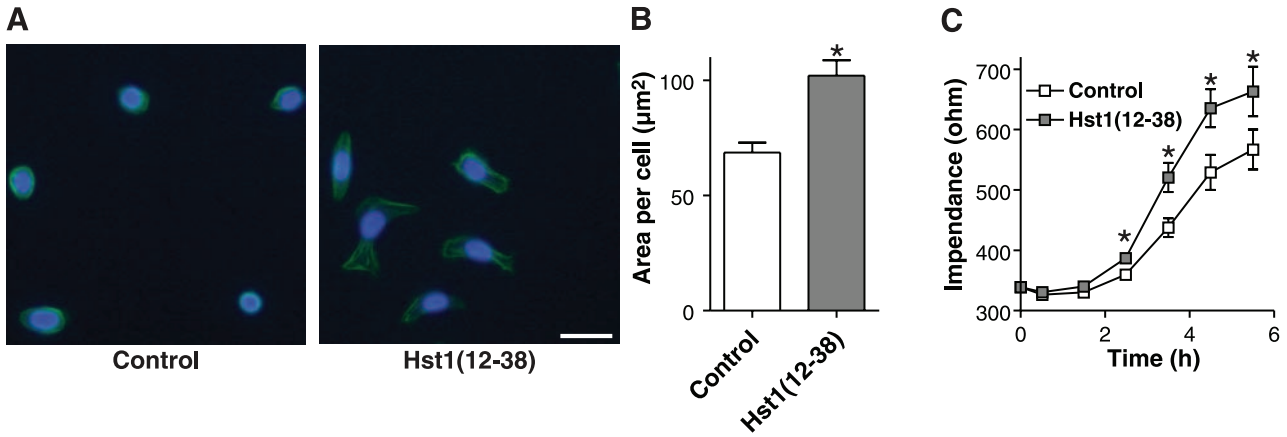

Figure 3. Histatin enhances cell spreading. *A*) Representative micrographs of HO-1-N-1 cell spreading 16 h after seeding in the presence or absence (control) of Hst1(12–38). Green, actin; blue, nuclei. Scale bar = 20 μm. *B*) Average surface area per cell was quantified from images similar to those in *A*. Data represent means ± SD; $n = 35$. *C*) Hst1(12–38)-mediated cell spreading in real time by ECIS. HO-1-N-1 cells were seeded on fibronectin-coated golden ECIS electrodes, and attachment (0–30 min) and spreading (1–6 h) were monitored continuously; $n=4$. $^*P < 0.01$ *vs.* control.

ation, we tested the effect of histatin on cell division. The dose-response effect of Hst1(12–38), or of rhEGF as a positive control, on proliferation was examined by quantifying the amount of DNA after 48 h of incubation. In addition, we made microscopic images of the wells that were used for DNA quantification by measuring fluorescence (**Fig. 2*A***). The results clearly show that in contrast to rhEGF, Hst1(12–38) does not enhance cell proliferation (Fig. 2*B*).

In addition to cell migration and proliferation, cell spreading, which precedes migration, is very important for reepithelialization. To test the effect of histatin on cell spreading, low densities of epithelial cells were seeded in the presence or absence of Hst1(12–38). After 16 h, cells were fixed and permeabilized and subsequently stained for F-actin and nuclei. Representative fluorescent microscopic images are shown in **Fig. 3*A***. The surface of 35 individual cells was calculated using ImageJ software.

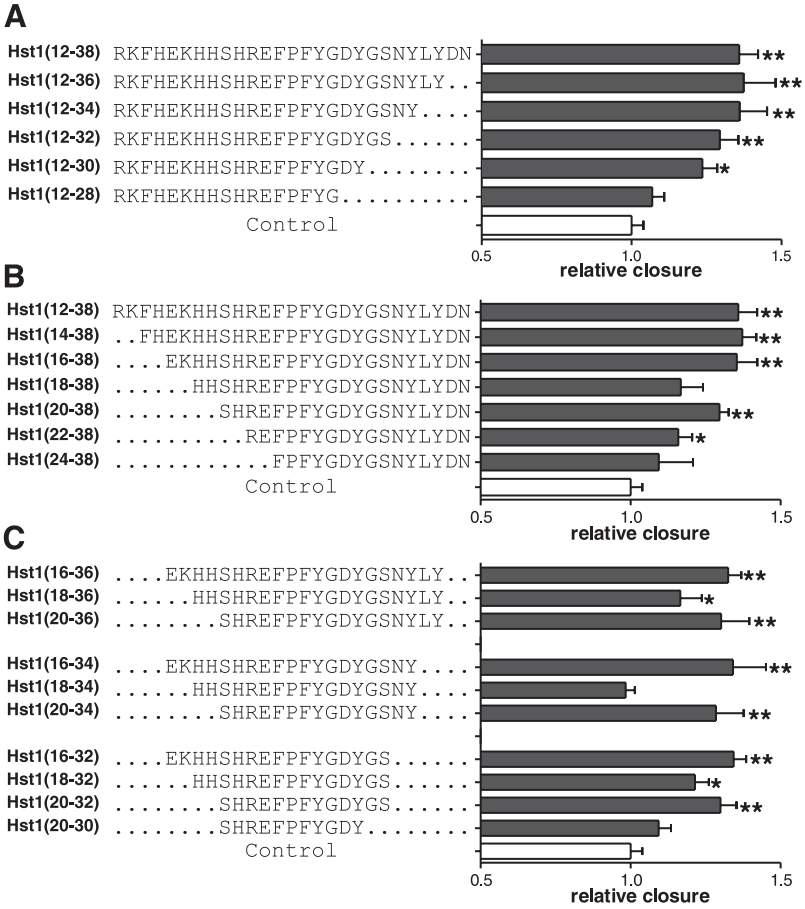

Figure 4. Hst1(20–32) is the minimal active domain of Hst1 in the *in vitro* wound-scratch assay. Wound-scratch experiments and relative-closure measurements of confluent HO-1-N-1 wells were done as described and shown in Fig. 1*D, E*. *A*) C-terminal stepwise truncation of Hst1(12–38) and subsequent measuring of relative-wound-closure characteristic for each peptide. *B*) N-terminal stepwise truncation of Hst1(12–38) and subsequent measuring of relative-wound-closure characteristic for each peptide. *C*) N- and C-terminal truncation of Hst1(12–38); peptides were made based on the results in *A* and *B*. Error bars represent means ± SD; $n = 12$. $^*P < 0.05$, $^{**}P < 0.01$ *vs.* control.

The area per cell was larger in the Hst1(12–38)-treated cells as compared with the control (Fig. 3*B*) or D-Hst1(12–38)-treated cells (data not shown), indicating that Hst1(12–38) promotes cell spreading. To further study histatin-stimulated cell spreading, we examined the effect of Hst1(12–38) on spreading with the aid of ECIS (22). This technique provides quantitative information, in real time, on cell attachment and spreading on coated golden electrodes. Serum-starved cells (2×10^5) were added to the chambers with or without Hst1(12–38), and impedance was measured online. Hst1(12–38)-treated cells already showed higher impedance after 2.5 h, as well as for the remainder of the experiment (Fig. 3*C*). These results show that histatin does enhance cell spreading.

Minimal active domain of Hst1 is SHREFPFYGDYGS

Our next step was to identify the minimal domain that mediates the biological activity of histatin. Since the *in vitro* wound-scratch properties of Hst1(12–38) are comparable to those of the parent peptide Hst1 (10), this peptide was used as a starting point for mapping the minimal active domain. We compared the activities of fragments that differ by 2-residue-stepwise truncation in the C terminus, the N terminus, or both (**Fig. 4**). Removal of the first 6 C-terminal residues had no significant effect on the cell-stimulating properties in the *in vitro* wound-scratch assay (Fig. 4*A*). Removal of the next 2 residues resulted in a fragment Hst1(12–30) with a decreased activity. Further truncation resulted in a complete loss of activity (Fig. 4*A*). Stepwise truncation of the first 4 residues at the N terminus did not affect the activity (Fig. 4*B*). Removal of aa 16 and 17, generating the fragment Hst1(18–38) starting with a double histidin at positions 18 and 19, resulted in a response that was not statistically different from the control ($P=0.07$). Strikingly, on further truncation, the activity was completely restored (Fig. 4*B*). Similarly, when variants were prepared in which residues had been removed from both termini, the peptides starting with $His^{18}His^{19}$ also showed decreased activity compared with the shorter, N-terminally truncated fragments. This decreased activity was statistically significant for Hst1(18–34) (Fig. 4*C*). Our hypothesis is that these variants are less or not able to activate the putative receptor because of the presence of clustered positive charges at the N terminus. Overall, on the basis of these results we conclude that the region between residues 20 and 32 is critical for the wound healing properties of Hst1.

Cyclization of Hst1 potentiates its activity 1,000-fold

The structure-activity relationship of histatins, particularly the anomalous behavior in the N-truncated series (Fig. 4), suggests that, in addition to a specific amino acid sequence, additional structural requirements govern the activity of histatin. This prompted us to prepare a variant in which the conformational freedom was constrained by covalently linking the C terminus to the N terminus. The activity of this cyclic Hst1 (cHst1) was compared with that of linear Hst1 in the *in vitro* wound-scratch assay. We found a dose-dependent enhancement of wound closure with both peptides (**Fig. 5*A***). However, the minimal concentration needed to enhance wound closure differed greatly, 0.001 μM for cHst1 *vs.* 1 μM for Hst1 (Fig. 5*A*). Thus, constraining the conformation of Hst1 by cyclization resulted in a >1000-fold stimulation of the molar activity, likely because the affinity for the receptor was increased.

Similar to high concentrations of linear Hst1(12–38), low concentrations of cHst1 also promoted wound healing in a PTx-sensitive and ERK1/2-dependent fashion (Fig. 5*B*). This indicates that the 2 histatin variants signal through the same receptor.

DISCUSSION

The superior wound healing in the oral cavity compared with cutaneous wound healing has been an

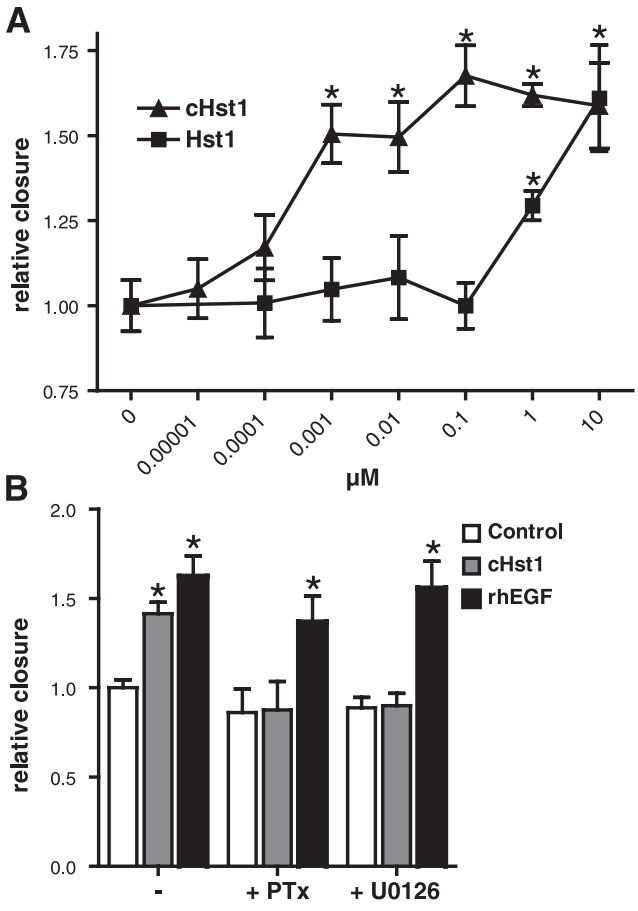

Figure 5. Cyclization of Hst1 potentiates its effect 1000-fold. Wound-scratch experiments and relative closure measurements of confluent HO-1-N-1 wells were done as described in Materials and Methods and shown in Fig. 1*D, E. A*) Relative wound closure of Hst1 (■) and cHst1 (▲) compared with control (0 μM in graph). cHst1 remains active until 0.001 μM, whereas Hst1 is active until 1 μM. *B*) Relative closure by cHst1 (0.1 μM) and rhEGF (10 ng/ml) with and without the addition of the pharmaceutical inhibitors PTx or U0126 (inhibitor of MEK, the activator of ERK1/2). *A, B)* Error bars and symbols represent means ± SD; $n = 12$. $^*P < 0.01$ *vs.* appropriate control.

intriguing yet unexplained phenomenon. Although several mechanisms have been proposed, saliva has always been considered to be of significant beneficial relevance to the outcome of oral healing. It is also possible that the healing effects of saliva are not only limited to the oral cavity since licking of a skin wound by animals and humans is a readily acknowledged event that may also promote skin wound healing. Recently, we isolated histatin from human saliva and identified histatin as the component that was responsible for the *in vitro* epithelial-cell-migration-inducing properties of saliva (10). In the current study, we examined the effect of histatin on reepithelialization of a wound created in a tissue-engineered 3-dimensional skin model. Reepithelialization of full thickness wounds is enhanced by histatin, with rates comparable to those of the gold standard rhEGF. Histatin does not enhance proliferation but stimulates reepithelialization by stimulating cell migration and cell spreading. We also showed that the activation by histatins is not only sequence, but also structure dependent, by using a cyclic version of Hst1. This cyclization of the peptide increased its concentration-dependent effect on wound healing 1000-fold.

Chemokines and growth factors are generally small proteins (5–10 kDa) that have a stable conformation by sulfur bridges of their cysteine residues. Nevertheless, linear peptides that were derived from chemokines or growth factors can activate cells but at 1000 times higher concentrations than the original ligand (23). The binding affinities of these linear peptides are greatly enhanced on cyclization (23–25). This seems similar to our finding that cyclization increases the molar activity of histatin. However, most studies aim to find antagonists of receptors to inhibit pathological processes such as cancer (23) or HIV-1 entry *via* CXCR4 (26). In contrast, to our knowledge this is the first study to show that cyclization results in improved cell-stimulating activity. The only known cyclic protein motif expressed in mammals is presented by theta defensins, which thus far have been considered as broad-spectrum antimicrobial peptides (27, 28). In view of the strong potentiating effect of cyclization on histatin activity, it is tempting to speculate that theta defensins may play also a role in the activation of host cells.

Our current data show that a GPCR is involved in the activation of cells by histatin. This is in line with our previous study in which we excluded the EGF receptor (10). The EGF receptor is commonly utilized by indirect activation of cells by various factors including ultraviolet light, lysophosphatic acid, or other antimicrobial peptides (29–33). Thus, histatin appears to signal through a specific receptor, albeit that this as yet unidentified receptor may be shared with other ligands, similar to the situation for the chemokine receptor subgroup of GPCRs that also signal through $G\alpha_i$ (34).

At first sight, our findings have some similarities with other proclaimed antimicrobial peptides that can affect host tissues, such as LL-37 and defensins. At a closer look, however, there are substantial differences. First, contrary to D-Hst1(12–38), the D-enantiomer of LL-37 is as active as the natural-occurring peptide (35). Second, both LL-37 and defensins have been shown to act *via* the EGF receptor (30, 31, 33), whereas we have previously shown that histatin does not (10). Third, LL-37 and defensins enhance cell proliferation (30, 35), whereas in this study we show that cell proliferation is unaffected by histatins. Finally, LL-37 and defensins enhance several cellular processes, but at somewhat higher concentrations they are cytotoxic (36–38). This is in contrast to histatins, which are not cytotoxic at least up to 100 μg/ml.

As a concluding remark, we note that unlike manufactured recombinant growth factors currently in clinical trials (39, 40), histatins are stable molecules that can be produced easily and on a large scale. They therefore have a high potential for use as novel therapeutics suited for the treatment of wounds. For clinical implementation of histatins in the treatment of wounds, it is of key interest to minimize the production costs. Also, in general, the smaller the compound, the better it is able to penetrate the skin. Our identification of the minimal domain might thus be very important for the development of histatin as a new therapeutic agent for wound healing. Our finding of potentiation of the molar activity by cyclization is obviously clinically relevant but also gives valuable insight into the mode of action utilized by histatin. We think that a specific spatial conformation of histatin is required to bind and activate the receptor, which in turn triggers signal transduction and cell activation. FJ

We thank Hakan Kalay (VU Medical Center, Amsterdam) for determining the mass of the peptides by mass spectrometry. E.C.I.V. gratefully acknowledges the support from the Skeletal Tissue Engineering Group Amsterdam (STEGA).

REFERENCES

1. Vissink, A., Jansma, J., Spijkervet, F. K., Burlage, F. R., and Coppes, R. P. (2003) Oral sequelae of head and neck radiotherapy. *Crit. Rev. Oral Biol. Med.* **14,** 199–212
2. Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G. S., Boontheung, P., Cociorva, D., Delahunty, C. M., Denny, T., Dunsmore, J., Faull, K. F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S. C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J. A., Ogorzalek Loo, R. R., Malamud, D., Melvin, J. E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S. K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M. A., Takashima, J., Than, S., Wang, J., Whitelegge, J. P., Witkowska, H. E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D. T., Yates, J. R., 3rd, and Fisher, S. J. (2008) The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *J. Proteome Res.* **7,** 1994–2006
3. Engeland, C. G., Bosch, J. A., Cacioppo, J. T., and Marucha, P. T. (2006) Mucosal wound healing: the roles of age and sex. *Arch. Surg.* 141, 1193–1197; discussion 1198
4. Kiecolt-Glaser, J. K., Marucha, P. T., Malarkey, W. B., Mercado, A. M., and Glaser, R. (1995) Slowing of wound healing by psychological stress. *Lancet* **346,** 1194–1196
5. Szpaderska, A. M., Zuckerman, J. D., and DiPietro, L. A. (2003) Differential injury responses in oral mucosal and cutaneous wounds. *J. Dent. Res.* **82,** 621–626
6. Hutson, J. M., Niall, M., Evans, D., and Fowler, R. (1979) Effect of salivary glands on wound contraction in mice. *Nature* **279,** 793–795
7. Konturek, S. J., Dembinski, A., Warzecha, Z., Brzozowski, T., and Gregory, H. (1988) Role of epidermal growth factor in healing of chronic gastroduodenal ulcers in rats. *Gastroenterology* **94,** 1300–1307
8. Fisher, D. A., and Lakshmanan, J. (1990) Metabolism and effects of epidermal growth factor and related growth factors in mammals. *Endocr. Rev.* **11,** 418–442

9. Murphy, R. A., Watson, A. Y., Metz, J., and Forssmann, W. G. (1980) The mouse submandibular gland: an exocrine organ for growth factors. *J. Histochem. Cytochem.* **28,** 890–902
10. Oudhoff, M. J., Bolscher, J. G., Nazmi, K., Kalay, H., van 't Hof, W., Amerongen, A. V., and Veerman, E. C. (2008) Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. *FASEB J.* **22,** 3805–3812
11. Sun, X., Salih, E., Oppenheim, F. G., and Helmerhorst, E. J. (2009) Kinetics of histatin proteolysis in whole saliva and the effect on bioactive domains with metal-binding, antifungal, and wound-healing properties. [E-pub ahead of print] *FASEB J.* doi: 10/1096/fj.09-131045
12. Baum, B. J., Bird, J. L., Millar, D. B., and Longton, R. W. (1976) Studies on histidine-rich polypeptides from human parotid saliva. *Arch. Biochem. Biophys.* **177,** 427–436
13. Oppenheim, F. G., Xu, T., McMillian, F. M., Levitz, S. M., Diamond, R. D., Offner, G. D., and Troxler, R. F. (1988) Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. *J. Biol. Chem.* **263,** 7472–7477
14. Ruissen, A. L., Groenink, J., Van't Hof, W., Walgreen-Weterings, E., van Marle, J., van Veen, H. A., Voorhout, W. F., Veerman, E. C., and Nieuw Amerongen, A. V. (2002) Histatin 5 and derivatives. Their localization and effects on the ultra-structural level. *Peptides* **23,** 1391–1399
15. Veerman, E. C., Valentijn-Benz, M., Nazmi, K., Ruissen, A. L., Walgreen-Weterings, E., van Marle, J., Doust, A. B., van't Hof, W., Bolscher, J. G., and Nieuw Amerongen, A. V. (2007) Energy depletion protects *Candida albicans* against antimicrobial peptides by rigidifying its cell membrane. *J. Biol. Chem.* **282,** 18831–18841
16. Mochon, A. B., and Liu, H. (2008) The antimicrobial peptide histatin-5 causes a spatially restricted disruption on the *Candida albicans* surface, allowing rapid entry of the peptide into the cytoplasm. *PLoS Pathog* **4,** e1000190
17. Gibbs, S., van den Hoogenband, H. M., Kirtschig, G., Richters, C. D., Spiekstra, S. W., Breetveld, M., Scheper, R. J., and de Boer, E. M. (2006) Autologous full-thickness skin substitute for healing chronic wounds. *Br. J. Dermatol.* **155,** 267–274
18. Stawikowski, M., and Cudic, P. (2006) A novel strategy for the solid-phase synthesis of cyclic lipodepsipeptides. *Tetrahedron. Lett.* **47,** 8587–8590
19. Ponec, M., Kempenaar, J. A., and De Kloet, E. R. (1981) Corticoids and cultured human epidermal keratinocytes: specific intracellular binding and clinical efficacy. *J. Invest. Dermatol.* **76,** 211–214
20. Breetveld, M., Richters, C. D., Rustemeyer, T., Scheper, R. J., and Gibbs, S. (2006) Comparison of wound closure after burn and cold injury in human skin equivalents. *J. Invest. Dermatol.* **126,** 1918–1921
21. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) Image processing with ImageJ. *Biophotonics International* **11,** 26–42
22. Lorenowicz, M. J., Fernandez-Borja, M., van Stalborch, A. M., van Sterkenburg, M. A., Hiemstra, P. S., and Hordijk, P. L. (2007) Microtubule dynamics and Rac-1 signaling independently regulate barrier function in lung epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **293,** L1321–L1331
23. Brennand, D. M., Dennehy, U., Ellis, V., Scully, M. F., Tripathi, P., Kakkar, V. V., and Patel, G. (1997) Identification of a cyclic peptide inhibitor of platelet-derived growth factor-BB receptor-binding and mitogen-induced DNA synthesis in human fibroblasts. *FEBS Lett.* **413,** 70–74
24. Yokoyama, F., Suzuki, N., Haruki, M., Nishi, N., Oishi, S., Fujii, N., Utani, A., Kleinman, H. K., and Nomizu, M. (2004) Cyclic peptides from the loop region of the laminin alpha 4 chain LG4 module show enhanced biological activity over linear peptides. *Biochemistry* **43,** 13590–13597
25. Jackson, S., DeGrado, W., Dwivedi, A., Parthasarathy, A., Higley, A., Krywko, J., Rockwell, A., Markwalder, J., and Wells, G. (1994) Template-constrained cyclic peptides: design of high-affinity ligands for GPIIb/IIIa. *J. Amer. Chem. Soc.* **116,** 3220–3230
26. Zhou, N., Luo, Z., Luo, J., Fan, X., Cayabyab, M., Hiraoka, M., Liu, D., Han, X., Pesavento, J., Dong, C. Z., Wang, Y., An, J., Kaji, H., Sodroski, J. G., and Huang, Z. (2002) Exploring the stereochemistry of CXCR4-peptide recognition and inhibiting HIV-1 entry with D-peptides derived from chemokines. *J. Biol. Chem.* **277,** 17476–17485
27. Tang, Y. Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C. J., Ouellette, A. J., and Selsted, M. E. (1999) A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* **286,** 498–502
28. Garcia, A. E., Osapay, G., Tran, P. A., Yuan, J., and Selsted, M. E. (2008) Isolation, synthesis, and antimicrobial activities of naturally occurring theta-defensin isoforms from baboon leukocytes. *Infect. Immun.* **76,** 5883–5891
29. Huang, R. P., Wu, J. X., Fan, Y., and Adamson, E. D. (1996) UV activates growth factor receptors via reactive oxygen intermediates. *J. Cell Biol.* **133,** 211–220
30. Niyonsaba, F., Ushio, H., Nakano, N., Ng, W., Sayama, K., Hashimoto, K., Nagaoka, I., Okumura, K., and Ogawa, H. (2007) Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J. Invest. Dermatol.* **127,** 594–604
31. Tjabringa, G. S., Aarbiou, J., Ninaber, D. K., Drijfhout, J. W., Sorensen, O. E., Borregaard, N., Rabe, K. F., and Hiemstra, P. S. (2003) The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J. Immunol.* **171,** 6690–6696
32. Xu, K. P., Yin, J., and Yu, F. S. (2007) Lysophosphatidic acid promoting corneal epithelial wound healing by transactivation of epidermal growth factor receptor. *Invest. Ophthalmol. Vis. Sci.* **48,** 636–643
33. Tokumaru, S., Sayama, K., Shirakata, Y., Komatsuzawa, H., Ouhara, K., Hanakawa, Y., Yahata, Y., Dai, X., Tohyama, M., Nagai, H., Yang, L., Higashiyama, S., Yoshimura, A., Sugai, M., and Hashimoto, K. (2005) Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J. Immunol.* **175,** 4662–4668
34. Zlotnik, A., Yoshie, O., and Nomiyama, H. (2006) The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol.* **7,** 243
35. Von Haussen, J., Koczulla, R., Shaykhiev, R., Herr, C., Pinkenburg, O., Reimer, D., Wiewrodt, R., Biesterfeld, S., Aigner, A., Czubayko, F., and Bals, R. (2008) The host defence peptide LL-37/hCAP-18 is a growth factor for lung cancer cells. *Lung Cancer* **59,** 12–23
36. Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D., and Agerberth, B. (1998) Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J. Biol. Chem.* **273,** 3718–3724
37. Aarbiou, J., Tjabringa, G. S., Verhoosel, R. M., Ninaber, D. K., White, S. R., Peltenburg, L. T., Rabe, K. F., and Hiemstra, P. S. (2006) Mechanisms of cell death induced by the neutrophil antimicrobial peptides alpha-defensins and LL-37. *Inflamm. Res.* **55,** 119–127
38. Van Wetering, S., Mannesse-Lazeroms, S. P., Dijkman, J. H., and Hiemstra, P. S. (1997) Effect of neutrophil serine proteinases and defensins on lung epithelial cells: modulation of cytotoxicity and IL-8 production. *J. Leukoc. Biol.* **62,** 217–226
39. Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H., and Tomic-Canic, M. (2008) Growth factors and cytokines in wound healing. *Wound Repair Regen.* **16,** 585–601
40. Occleston, N. L., O'Kane, S., Goldspink, N., and Ferguson, M. W. (2008) New therapeutics for the prevention and reduction of scarring. *Drug Discov. Today* **13,** 973–981

Received for publication May 20, 2009.
Accepted for publication July 9, 2009.