Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay

Menno J. Oudhoff,*[,1] Jan G. M. Bolscher,* Kamran Nazmi,* Hakan Kalay,[†] Wim van 't Hof,* Arie V. Nieuw Amerongen,* and Enno C. I. Veerman*

*Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, Amsterdam, The Netherlands; and [†]Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands

ABSTRACT **Wounds in the oral cavity heal much faster than skin lesions. Among other factors, saliva is generally assumed to be of relevance to this feature. Rodent saliva contains large amounts of growth factors such as epidermal growth factor (EGF) and nerve growth factor (NGF). In humans, however, the identity of the involved compounds has remained elusive, especially since EGF and NGF concentrations are ~100,000 times lower than those in rodent saliva. Using an *in vitro* model for wound closure, we examined the properties of human saliva and the fractions that were obtained from saliva by high-performance liquid chromotography (HPLC) separation. We identified histatin 1 (Hst1) and histatin 2 (Hst2) as major wound-closing factors in human saliva. In contrast, the D-enantiomer of Hst2 did not induce wound closure, indicating stereospecific activation. Furthermore, histatins were actively internalized by epithelial cells and specifically used the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway, thereby enhancing epithelial migration. This study demonstrates that members of the histatin family, which up to now were implicated in the antifungal weaponry of saliva, exert a novel function that likely is relevant for oral wound healing.—Oudhoff, M. J., Bolscher, J. G. M., Nazmi, K., Kalay, H., van 't Hof, W., Nieuw Amerongen, A. V., Veerman, E. C. I. Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. *FASEB J.* 22, 3805–3812 (2008)**

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WOUNDS IN THE ORAL CAVITY HEAL much faster than skin lesions, with similar wounds healing in 7 days in the oral cavity compared with several weeks on the skin (1, 2). Accelerated healing in the oral cavity has been attributed to various factors, including better microcirculation in oral tissue, a higher turnover rate for oral epithelium, and the presence of saliva. The role of saliva in accelerating wound healing has been investigated predominantly in animal models: desalivated rats exhibit delayed oral wound healing (3), and accelerated healing takes place in mice that apply saliva to their skin by licking (4). In addition, gastric ulcer healing is delayed in desalivated rats. This delay can be reversed by the addition of epidermal growth factor (EGF; ref. 5). The discovery of growth-stimulating compounds in saliva dates back to 1962, when Stanley Cohen isolated EGF from mouse submandibular gland tissue. Isolated EGF accelerated both incisor eruption and neonatal eyelid opening (6). Subsequent studies (7, 8) revealed that EGF plays a crucial role in several cellular processes that take place during wound healing, including cell proliferation, cell differentiation, and cell migration. A number of other growth factors, such as nerve growth factor (NGF) and fibroblast growth factor, have also been found in saliva. Taken together, these studies indicate that growth factors, especially EGF, are responsible for saliva-enhanced wound healing in rodents.

Although it is tempting to extrapolate the rodent results to humans, there is little direct experimental evidence that EGF is a key determinant of saliva-promoted wound healing in humans. Furthermore, since EGF and NGF concentrations in human saliva are ~100,000 times lower than in rodent saliva (9–12), it seems unlikely that these factors play the same prominent role in human oral wound healing as in mice and rats.

Human saliva contains a myriad of proteins and peptides that protect against microbial, mechanical, and chemical injuries (13). In the present study, we addressed the question of which factors in human saliva contribute to its wound-healing properties. Saliva and saliva protein fractions were tested in an established *in*

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

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vitro model for wound closure using an epithelial cell line. This revealed that histatins, rather than EGF, were the major wound-closing factors in human saliva. Further characterization indicated that the activation by histatins has several features in common with that by "classic" growth factors. These include stereospecific and active uptake by the cell and the requirement of a specific intracellular signaling pathway [extracellular signal-regulated kinases 1/2 (ERK1/2)]. This study demonstrates that members of the histatin family, which up to now were implicated in the antifungal weaponry of saliva, exert a novel function that likely is relevant for the maintenance of the integrity of the oral soft tissues.

MATERIALS AND METHODS

Cell culture

The human buccal epithelial cell lines TR146 and HO-1-N-1 were provided by Cancer Research UK (London, UK) and the Japanese Collection of Research Bioresources (Osaka, Japan), respectively. Cells were cultured in prescribed growth media: TR146 in Dulbecco modified Eagle medium (DMEM) with 4.5 g/L glucose and HO-1-N-1 in DMEM-F12 medium (Invitrogen, Carlsbad, CA, USA), both appended with 10% fetal calf serum (HyClone, South Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (antibiotic antimycotic solution, Sigma-Aldrich, St. Louis, MO, USA), at 37°C, 95% humidity and 5% CO_2. Cells were maintained until near confluence, detached with 0.25% trypsin-EDTA (Invitrogen), counted in a hepacytometer, and seeded into new flasks or multiwell plates at the required cell densities.

Saliva collection and fractionation of saliva

Parotid saliva was used in all experiments and collected as described previously (14). Before use, saliva samples were sterilized by filtration through a 0.45 μm pore filter (Schleicher and Schuell Biosciences, Keene, NH, USA).

Parotid saliva (2 ml) was fractionated by reverse phase (RP) -HPLC using a C8 column (10×120 mm). Elution was performed with a linear gradient, from 5–45% acetonitrile containing 0.1% trifluoroacetic acid (TFA) for 45 min at a flow rate of 4 ml/min. Eluted proteins were pooled in three fractions and tested for wound-closing activity. The active fraction was lyophilized, reconstituted in 2 ml HPLC-grade water, and further fractionated over the same column, eluted with a gradient from 10–40% acetonitrile containing 0.1% TFA in 30 min, at a flow rate of 4 ml/min. Again, fractions with wound-closing activities were lyophilized and reconstituted to the initial volume and applied on a Vydac C18 column (218 TP, 10×250 mm, and 10 μm particles; Grace, Deerfield, IL, USA), eluted with a gradient from 10–35% acetonitrile containing 0.1% TFA in 45 min, at a flow rate of 4 ml/min. The peak fraction containing wound-closure activity was identified by ion-trap mass spectrometry with an LCQ Deca XP (Thermo Finnigan, Waltham, MA, USA), as described previously (15, 16).

Peptide synthesis

Peptides and fluorescein isothiocyanate (FITC) -labeled peptides (F-peptides) were synthesized by solid phase peptide synthesis using Fmoc chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Biosearch, Bedford, MA, USA). Purification by RP-HPLC and confirmation of authenticity by mass spectrometry were conducted as described previously (16). For the FITC labeling, peptides were extended with the linker Fmoc-L-γ-aminobutyric acid, and after the detachment of the Fmoc group, labeled overnight at room temperature with 30-fold excess FITC in DIPEA/DMF before removal of the side chain protecting groups and simultaneous detachment of the resin support. F-Hst1 accelerated wound closure similar to unlabeled Hst1.

EGF determination

EGF concentrations in saliva were determined by ELISA using a human EGF Cytoset kit (Invitrogen), following the manufacturer's instructions.

In vitro wound-closure assay

Wound-closure experiments were performed as described previously (17). In brief, TR146 cells were grown in 12-well plates until confluence, and serum deprived for 24 h in keratinocyte serum-free medium (SFM; Invitrogen). In each well a scratch was made using a sterile tip, and cellular debris was removed by washing with SFM. The width of the scratch was determined microscopically immediately after creation and 16 h later. The effects of the following conditions on wound closure were analyzed: *1*) parotid saliva, diluted 3:10 in SFM with saliva buffer used as a control (30 mM Na_2CO_3, 10 mM KCl, 6 mM K_2HPO_4, 3 mM KSCN, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 7.3) diluted 3:10 in SFM; *2*) human epidermal growth factor (rhEGF; Invitrogen), dissolved in SFM; *3*) RP-HPLC fractions containing salivary proteins, dissolved in SFM; and *4*) synthetic peptides dissolved in SFM, at final concentrations of 30 μg/ml Hst1, 10 μg/ml Hst2, 10 μg/ml D-Hst2, 30 μg/ml Hst3, and 30 μg/ml Hst5 (**Table 1**). For conditions *2*, *3*, and *4*, SFM was used as a negative control.

For the inhibitor studies, mechanically wounded cells were exposed to inhibitors of ERK1/2 (U0126, 5 μM; LC Laboratories, Woburn, MA, USA) of the EGF receptor (EGFR) (AG1478, 1 μM, Calbiochem), or of p38MAPK (SB203580, 5 μM; LC Laboratories) during the experiment. The inhibitor in SFM was used as negative control.

Relative closure was calculated as $(X_0 - X_{16h})/(C_0 - C_{16h})$, where X_0 = width of the scratch at time 0, X_{16h} = width of the scratch after 16 h exposure to a condition, C_0 = width of the scratch at time 0, and C_{16h} = width of the scratch after 16 h exposure to the control (saliva buffer or SFM).

Boyden chamber assay

On an 8 μm pore-size Thincert for 24-well plates (Greiner Bio-One, Frickenhausen, Germany), 2×10^4 HO-1-N-1 cells were seeded. After attachment overnight and serum deprivation for 6 h, Hst2, D-Hst2 (10 μg/ml), rhEGF (10 ng/ml), or SFM only was added to the lower compartment. After 16 h, cells at the top side of the Thincert membrane were removed with a cotton swab. The remaining cells at the bottom side were washed with PBS, fixed with 70% ethanol, and stained with 10 μM propidium iodide (PI; Invitrogen) to visualize the nuclei. These nuclei were counted in three representative high-power fields (HPFs; ×40) per well, using a fluorescence microscope (Leica DM IL PLAN, ×40–400; Leica Microsystems, Wetzlar, Germahy).

TABLE 1. *Amino acid sequence, wound closure, and candidacidal properties of synthetic histatins*

Peptide	Amino acid sequence	Wound closure (relative closure)	Candidacidal activity (LC_{50}) 1 mM PPB	Candidacidal activity (LC_{50}) SFM
Hst1	D<u>S</u>HEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN	1.22 ± 0.12*	6.0 ± 0.3	>100
Hst2	RKFHEKHHSHREFPFYGDYGSNYLYDN	1.36 ± 0.13*	13.8 ± 1.9	>100
D-Hst2	rkfhekhhshrefpfygdygsnylydn	0.99 ± 0.14	10.7 ± 1.2	>100
Hst3	DSHAKRHHGYKRKFHEKHHSHR.....G.YRSNYLYDN	1.21 ± 0.12*	1.1 ± 0.1	>100
Hst5	DSHAKRHHGYKRKFHEKHHSHR.....G.Y	1.01 ± 0.11	2.3 ± 0.1	>100
rhEGF		1.41 ± 0.12*	ND	ND

Lowercase letters indicate D-amino acid residues; <u>S</u> = phosphoserine. Values are means ± SD. rhEGF concentration (10 ng/ml) is 10× higher than normally occurring in human saliva. Wound-closure assays, $n = 4$; candidacidal assay, $n = 3$. LC_{50}, concentration (μM) at which 50% of the *C. albicans* cells were killed; ND, not determined. *$P<0.01$.

Determination of the candidacidal activity

Candidacidal activity was determined by measuring the fluorescence enhancement of PI (Invitrogen), a membrane-impermeable probe that on binding to DNA becomes 20–30 times more fluorescent, essentially as described previously (18). In short, a *Candida albicans* (ATCC 10231; American Type Culture Collection, Manassas, VA, USA) midlog phase culture of 10^7 yeast cells/ml was supplemented with PI (final concentration of 10 μM) and subsequently added to serial dilutions of peptides. PI fluorescence was measured after 1 h incubation, at excitation and emission wavelengths of 544 and 620 nm, respectively, in a Fluostar Galaxy microplate fluorimeter (BMG Labtechnologies, Offenburg, Germany). LC_{50} was defined as the peptide concentration at which 50% of *C. albicans* cells were killed.

Depletion from supernatant and localization of histatins

Depletion of peptides from the supernatant was analyzed as follows. Epithelial cells were grown until confluence, washed with PBS, and incubated with 100 μg/ml of the peptide of interest in SFM at 37°C, unless otherwise noted. Directly after admission of the peptide and after 20 h of incubation, aliquots from the supernatant were taken for quantification of the remaining peptides by RP-HPLC. Data were compared with peptide admission in wells lacking cells.

For the localization of F-Hst1, cells were grown until near confluence and incubated with 50 μg/ml F-Hst1 for 24 h at 4°C or for 2 h at 37°C. To explore the effect of energy depletion on internalization, cells were treated with sodium azide (10 mM), an inhibitor of the oxidative phosphorylation, for 1 h before and during incubation with F-Hst1 for 2 h at 37°C. To test the necessity of membrane proteins to be present for internalization, cells were treated with trypsin for ~3 min, washed with PBS, and then incubated with F-Hst1 for 2 h at 37°C. Subsequently to all conditions described, cells were washed vigorously with PBS 3 times to remove nonspecific binding of F-Hst1. Cells were examined by fluorescence microscopy (Leica DM IL PLAN, ×40–400).

Statistical analysis

Each experiment was conducted at least 3 times and minimally in triplicate. Data were analyzed using 1-way ANOVA with an additional least significance difference test to determine significance between samples. Values of $P < 0.05$ were considered significant.

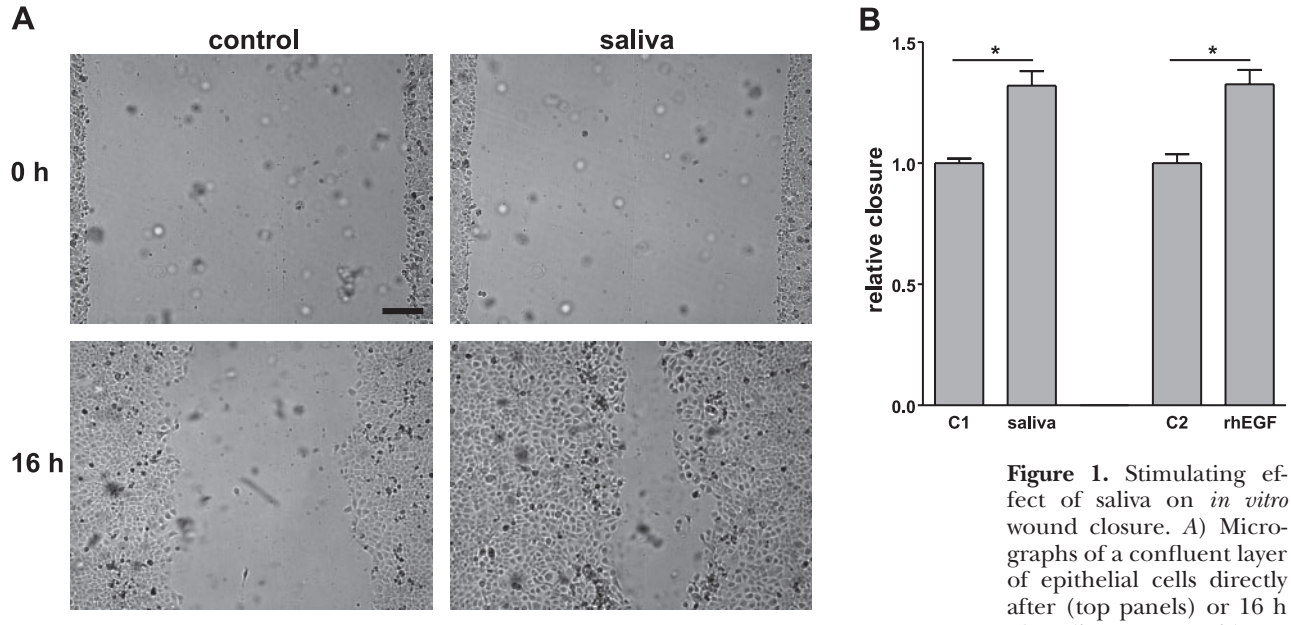
RESULTS

Human saliva accelerates *in vitro* wound closure

Experimental evidence that saliva contains wound-healing constituents comes largely from animal studies. Therefore, we wanted to verify that human saliva also accelerates wound healing by studying the effect of saliva in an established wound-closure assay. Incubation of epithelial cells with human saliva strongly enhanced wound closure *in vitro* (**Fig. 1*A, B***). The accelerated closure reached levels comparable with those of rhEGF at a concentration of 10 ng/ml, which is much higher than the concentration naturally occurring in human saliva (Fig. 1*B*). In rodents, EGF is the main factor responsible for saliva-enhanced wound healing. To investigate the role EGF plays in human saliva-induced wound closure, we tested saliva from 6 individuals on their wound-closure ability and in parallel determined the EGF concentration. All but one of the saliva samples enhanced wound closing significantly (Fig. 1*C*). The EGF concentrations ranged from 374 to 1151 pg/ml (Fig. 1*C*), all well below the minimal rhEGF concentration needed to accelerate wound closure in our system (~5 ng/ml, data not shown). In accordance, we found no correlation between EGF concentration and wound-closure activity. Next, we supplemented saliva with the EGFR inhibitor AG1478. This had no effect on the saliva-enhanced wound closure, while the activity of the control (rhEGF) was strongly reduced. AG1478 diminished the basal wound-closure rate in the buffer-treated cells also, indicating that the epithelial cell line TR146 exhibits a basal level of endogenous EGFR activation (Fig. 1*D*), which is relatively normal in such assays (19). Altogether, these experiments indicate that EGF evidently does not play a prominent role in wound-closure activities of human saliva.

Histatins are the wound-closing factors in saliva

Having excluded that EGF was responsible for saliva-enhanced wound closure, we aimed to identify the main factors contributing to the wound-closure effect.



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Person	rel. clos. ± SD	EGF (pg/ml)
I	1.43 ± 0.12*	374
II	1.33 ± 0.21*	852
III	1.30 ± 0.12*	1151
IV	1.20 ± 0.18*	946
V	1.19 ± 0.13*	865
VI	1.16 ± 0.22	699
Control	1.00 ± 0.07	0

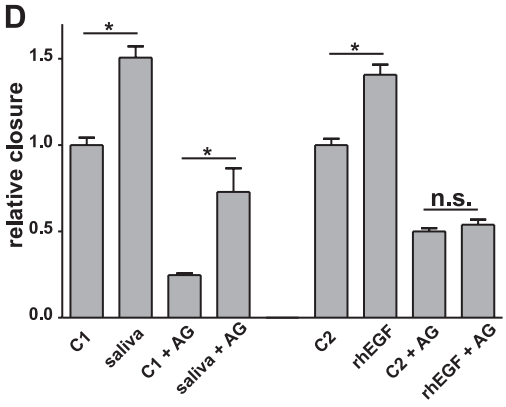


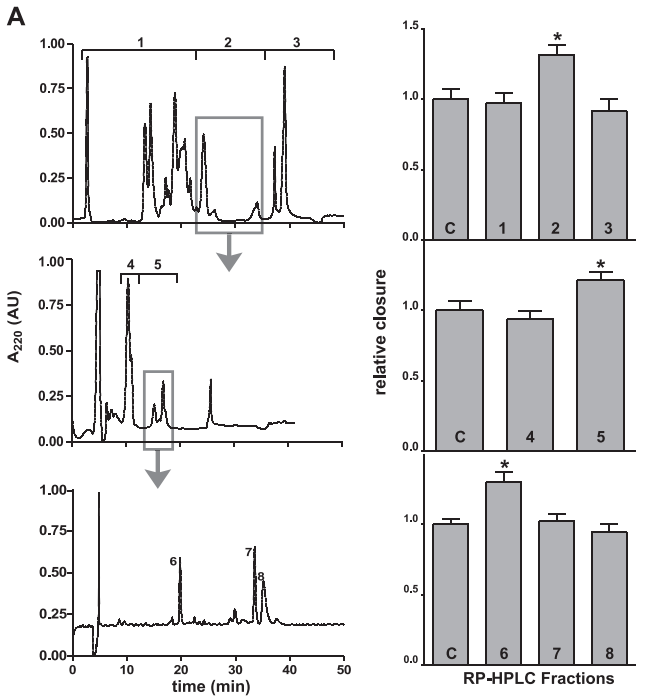
Figure 1. Stimulating effect of saliva on *in vitro* wound closure. *A*) Micrographs of a confluent layer of epithelial cells directly after (top panels) or 16 h after (bottom panels) application of a scratch in the absence (left panels) or the presence (right panels) of saliva (30%, v/v in SFM). Scale bar = 200 μm. *B*) Relative wound closure after 16 h of incubation, calculated as described in Materials and Methods from micrographs similar to those shown in *A*. C1, control for saliva (saliva buffer, 3:10 diluted in SFM); C2, control for rhEGF (SFM). Saliva (n=7) and 10 ng/ml rhEGF (n=4) induced wound closure (*P<0.01). *C*) Wound-closure activity and EGF levels in saliva from different people (*P<0.05). *D*) Effect of the EGFR inhibitor AG (AG1478) on saliva-induced and rhEGF-induced wound closure. Both in the presence and absence of AG, saliva accelerated wound closure (*P<0.01; n=4). In contrast, AG almost completely suppressed rhEGF-induced wound closure.

To do so, we fractionated saliva by RP-HPLC and tested the biological activity of the collected fractions. The left panel of **Fig. 2*A*** shows the RP-HPLC profiles of the 3-step saliva fractionation. The corresponding activities of the fractions in the wound-closure assay are shown in the right panel of Fig. 2*A*. The enhanced wound-closure activity of saliva could be assigned to one specific fraction (Fig. 2*A*, peak 6). Subsequent identification of this fraction by ion-trap mass spectrometry revealed the presence of an *Htn1* gene product (Fig. 2*B*). The *Htn1* gene gives rise to two proteins, Hst1 and Hst2.

To verify that the biological activity found in the HPLC fraction can indeed be attributed to histatins, we synthesized the histatins that are most commonly present in saliva. In Table 1, the amino acid sequences of Hst1, Hst2, D-Hst2 (the D-enantiomer of Hst2), Hst3, and Hst5 are shown, as are their activities in the *in vitro* wound-closure assay. Both candidates indicated by the mass spectrometric analysis, Hst1 and Hst2, accelerated wound closure (Table 1) in the concentration range from 5 to 100 μg/ml (data not shown). In addition, Hst3, one of the *Htn2* gene products, induced wound closure. Remarkably, Hst5, the *Htn2* gene product that lacks the 8 C-terminal amino acid residues of Hst3, was completely inactive (Table 1). This implies that the C terminus of Hst3 holds a key domain for activating epithelial cells. Interestingly, the D-enantiomer of Hst2 (D-Hst2) did not enhance wound closure. This indicates the involvement of a stereospecific interaction in histatin-enhanced wound closure (Table 1). Essentially the same data were obtained when another buccal epithelial cell line (HO-1-N-1), which had a very low level of basal wound closure, was used (data not shown).

Hst2 induces cell migration

We next investigated the effects of histatins on the migration of epithelial cells, which is an important



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peak	sequence found	XC	gene	proteins
6	FHEKHHSHREFPFYGDYGSNYLYDN	4.531	*Htn1*	Hst1, Hst2

Figure 2. Isolation and identification of wound-closure-inducing factors in saliva by RP-HPLC. *A*) Fractionation of parotid saliva. Top panel: parotid saliva (2 ml) was loaded on a C8 column. Elution was performed with a linear gradient of 5–45% acetonitrile. Fractions were pooled as indicated to obtain pools 1–3 and were tested for wound-closure activity. Middle panel: fraction 2 was loaded on the same C8 column and eluted with a linear gradient of 10–45% acetonitrile. Fractions were pooled as indicated to obtain fractions 4 and 5 and tested for wound-closure activity. Bottom panel: fraction 4 was loaded on a C18 column and eluted with a linear gradient of 10–35% acetonitrile. Fractions were pooled as indicated to obtain fractions 6–8 and tested for wound-closure activity (*$P<0.01$). *B*) Identification of the protein in fraction 6 by ion-trap mass spectrometry, as an *Htn1* gene product.

element of wound closure, in a chemotaxis assay. Hst2 induced cell migration with comparable values to those of rhEGF (10 ng/ml), whereas D-Hst2 did not (**Fig. 3*A, B***). Thus Hst2, at concentrations commonly present in saliva, can induce cell migration at levels that are likely relevant for oral wound healing.

Antifungal mechanism of histatins is different from their wound-closure mechanism

Histatins, in particular Hst3 and Hst5, have generally been recognized as antimicrobial peptides that play a role in the protection of the oral cavity against microbial invasion due to their membrane disrupting activity (18). To determine whether the molecular mechanisms underlying the antimicrobial activity of histatins are related to those involved in inducing wound closure, we tested the candidacidal activities of the synthesized histatins. At low ionic strength (1 mM potassium phosphate buffer), all histatin variants were candidacidal, including D-Hst2 and Hst5 (Table 1). The finding that D-Hst2 was as fungicidal as L-Hst2 illustrates that the histatin-mediated killing of *C. albicans* is nonchiral in nature, contrary to its wound-closure activities. In addition, Hst5 is one of the most potent antifungal histatins agents, which is completely opposed to its lack of wound-closure properties. In SFM (150 mM), the medium used in the wound-closure assay, no candidacidal activity was detected for any of the histatin species tested (Table 1). In saliva buffer (50 mM), the candidacidal effects of histatins were also completely abolished (data not shown). Taken together, these data indicate that the antifungal and cell-stimulating activities of histatins require completely different physicochemical and structural features.

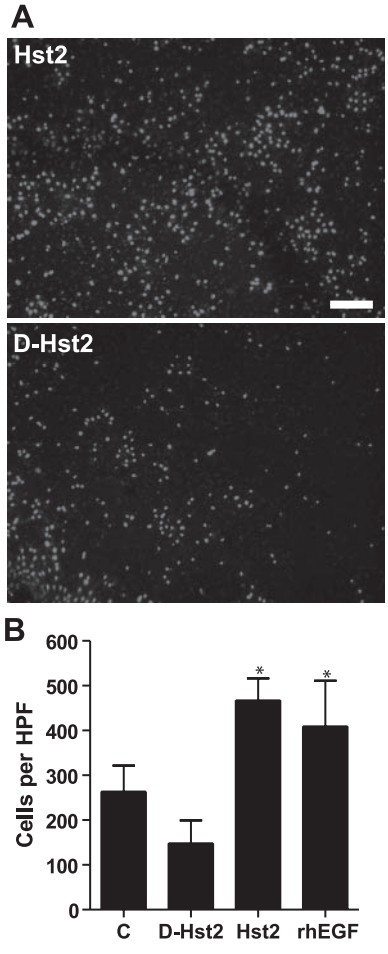

Figure 3. Hst2-induced cell migration. Cell migration-inducing activities of Hst2, D-Hst2 (both 10 μg/ml), and rhEGF (10 ng/ml) were analyzed with a Boyden chamber assay. *A*) PI staining of the nuclei of cells at the bottom side of the Thincert membrane after removal of the cells on the top side. Scale bar = 200 μm. *B*) Cell migration-inducing activities of SFM (C), Hst2, D-Hst2, and rhEGF as expressed in cells counted per HPF after 16 h (×40; $n=3$; *$P<0.01$).

Cells internalize Hst1, Hst2, and Hst3, but not D-Hst2, *via* an active energy-dependent mechanism

The lack of epithelial cell-inducing activity of the D-enantiomer of Hst2 (Table 1; Fig. 3), suggested a stereospecific interaction between histatins and epithelial cells. We further examined the interaction of Hst1, Hst2, Hst3, and D-Hst2 with epithelial cells by monitoring the depletion of these peptides from the supernatant during incubation with epithelial cells (**Fig. 4*A***). Hst1, Hst2, and Hst3 were depleted from the medium after incubation for 20 h at 37°C (Fig. 4*A*). In contrast, no depletion of D-Hst2 was observed. Also, at 4°C hardly any depletion of Hst2 occurred. This further indicates the involvement of a stereospecific interaction, suggesting that the activation is receptor mediated.

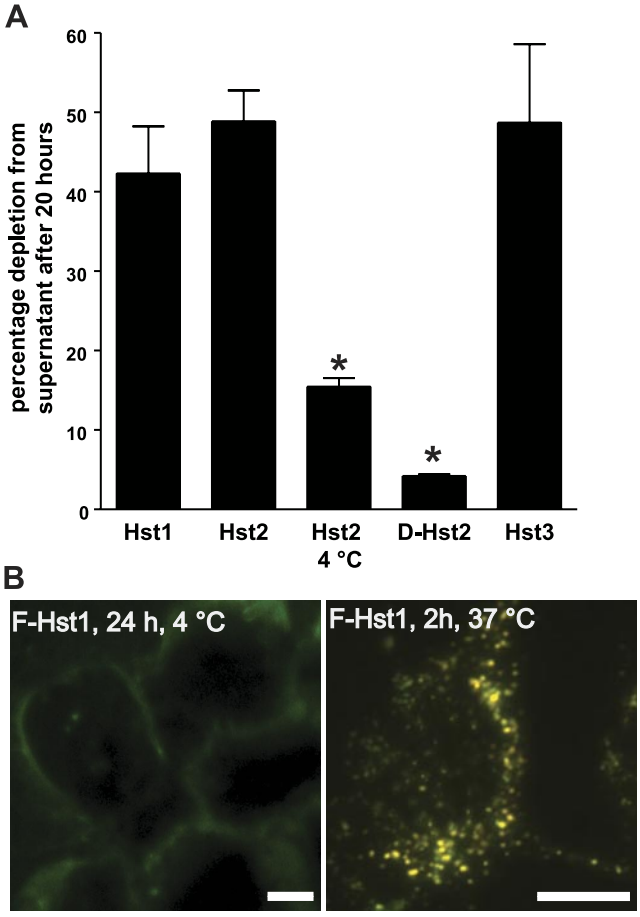

Figure 4. Interaction of histatins with epithelial cells. *A*) Depletion of synthetic histatins from the culture medium supernatant by epithelial cells. Synthetic Hst1, Hst2, Hst3, and D-Hst2 (100 μg/ml) were incubated with a monolayer of cells. After 20 h, the concentration of the peptide remaining in the culture medium was determined by RP-HPLC and presented as percentage of total amount of peptide. Hst1 and Hst3 were depleted from the cell supernatants, but D-Hst2 was not (n=3; *P<0.01). In the absence of cells, no depletion of peptides occurred (not shown). *B*) Fluorescence microscopy of epithelial cells after incubation with F-Hst1. Confluent layers of epithelial cells were incubated with F-Hst1 (50 μg/ml) at 37°C for 2 h (right panel) or at 4°C for 24 h (left panel). Scale bars = 20 μm.

Receptor-mediated activation of processes such as cell migration is often accompanied by internalization of the receptor and its ligand, which commonly is an active process. We therefore examined whether epithelial cells are able to take up F-Hst1. When cells were incubated with F-Hst1 at 4°C, mainly at the perimeter of the cells a weak, diffuse labeling pattern was visible, whereas the cytoplasm was virtually negative (Fig. 4*B*). In contrast, after incubation at 37°C, an intracellular bright, granular labeling pattern was observed, indicating uptake of the peptide into the cell (Fig. 4*B*). Pretreatment of cells with trypsin completely abolished fluorescent labeling of the cells. Depletion of the energy charge of the cells by treatment with sodium azide also abolished internalization of F-Hst1 (data not shown). Taken together, these results suggest that the wound-closure effects of histatins involve a receptor on the membrane of epithelial cells that is internalized (together with bound histatin) by the cell in an energy-dependent manner.

ERK1/2 signal transduction pathway mediates histatin-induced wound closure

Two studies (20, 21) have shown that, within the wound-closure model, EGF-induced cell migration is regulated by p38MAPK, whereas proliferation involves activation of the ERK1/2 pathway. To identify the intracellular pathways involved in histatin-induced wound closure, we examined the involvement of the two MAPK cascades that have been implicated in wound closure *in vitro*. We tested the effects in the wound-closure assay of the inhibitors U0126, which inhibits ERK1/2; SB203580, which inhibits p38MAPK; and AG1478, which inhibits the EGFR. Hst2-induced wound closure was abolished by U0126 but not by SB203580 or AG1478 (**Fig. 5**). The EGFR inhibitor had no effect on the Hst2-mediated wound closure, which is in line with our finding that EGF plays no prominent role in saliva-mediated wound closure (Fig. 1*D*). On the other hand, rhEGF activity was inhibited by SB203580 and by AG1478 but not by U0126 (Fig. 5), these results concur with previous studies (20, 21). Further experiments are required to fully elucidate the intracellular pathways and activation processes; however, these experiments already reveal that Hst2-activated and EGF-activated cell migration are controlled by different intracellular mechanisms.

DISCUSSION

Histatin is a major wound-closure factor in human saliva

In this study, we aimed to identify the components involved in the wound-healing properties of human saliva. The most surprising finding was that salivary

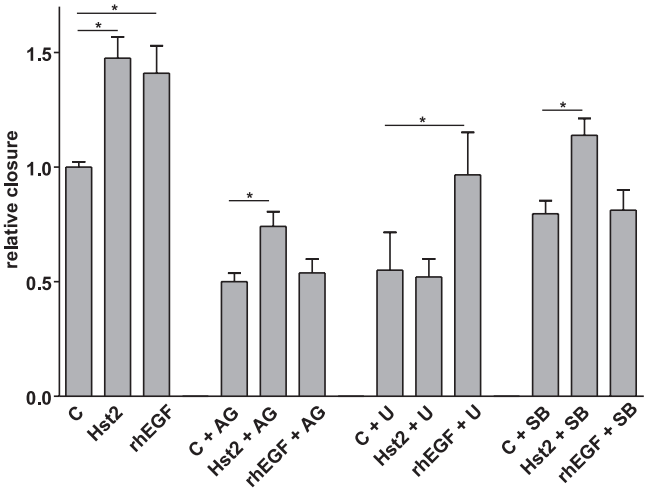

Figure 5. Hst2-induced and rhEGF-induced wound closure in the presence of inhibitors of EGFR, ERK1/2, and p38MAPK. Wound-closure activities of rhEGF (10 ng/ml) and Hst2 (10 μg/ml) were determined in the presence of 1 μM AG1478 (AG), 5 μM U0126 (U), and 5 μM SB203580 (SB) inhibitors of EGFR, ERK1/2, and p38MAPK, respectively. The *y* axis shows relative wound closure compared with the control (C; SFM). Only U0126 inhibited the wound-closure activity of Hst2 significantly, demonstrating the involvement of the ERK1/2 signal transduction pathway and exclusion of the EGFR/p38MAPK signaling pathway ($n=4$; $^*P<0.01$ *vs.* control).

histatins, which up to now had been acknowledged primarily as antimicrobial peptides in the oral defense against microorganisms, exhibited potent *in vitro* effects on epithelial cells. Histatins are a family of at least 12 histidine-rich cationic peptides encoded by the *Htn1* and *Htn2* genes that are specifically expressed in human salivary glands (22). Hst1 is the primary gene product of *Htn1*, and Hst2 is a shorter variant, which probably originated from Hst1 by intracellular processing before secretion. The other histatins are products of *Htn2* of which Hst3 and Hst5 are most abundant in saliva. Together, Hst1, Hst3, and Hst5 comprise ~85% of the total of histatin proteins.

Although a major role in oral wound healing has generally been attributed to salivary EGF, we did not obtain data corroborating this view. First, the EGF concentrations in human saliva (Fig. 1*C*) are orders of magnitude lower than in rodent saliva (9, 11, 12) and well below the concentration needed for detectable activation of epithelial cells *in vitro*. Second, the inhibition of the salivary-EGF activity did not diminish the stimulatory effects of saliva (Fig. 1*D*). In the relatively few studies (23–27) showing that human saliva has wound-healing-related activity, often isolated salivary proteins were used. This makes it difficult to evaluate the physiological relevance of these findings and the exact function these proteins have in saliva, especially since other growth factors, such as NGF, fibroblast growth factor, and trefoil peptide-3 are present in saliva at relatively low concentrations (10, 28, 29). In contrast, for histatins we found enhanced wound closure at least within the range from 5 to 100 μg/ml, which is the physiological concentration of histatins in human saliva (30).

Differences in mode of action between histatin and other antimicrobial peptides

Histatins are members of the large family of cationic antimicrobial peptides that are ubiquitously present among all living organisms (31). It has become clear that besides their antimicrobial functions, a number of these peptides also have effects on the tissue of the host. Our newly found function for histatins thus seems in line with findings that other antimicrobial peptides present in human saliva (such as defensins and LL-37) have growth stimulating properties (24, 26). It was found that LL-37 acts *via* transactivatition of the EGFR by activation of a metalloproteinase (32). For defensins, it was also found that the EGFR is essential for activation (24). In contrast, we show that histatins activate the cells independently from the EGFR (Fig. 5). Furthermore, it is shown that both LL-37 and defensins activate cells in a narrow concentration range, whereas at somewhat higher concentrations cell death occurs (24, 26, 33). We found that histatins induce wound closure within a range from 5 to 100 μg/ml without causing cell death. The concentrations used are those naturally occurring in saliva (34). Furthermore, the D-enantiomer of LL-37 is as active as LL-37 (26), while in the present study it was found that D-Hst2 was completely inactive (Table 1; Fig. 3). This corroborates that the mechanism of action of histatin is essentially different from that of LL-37. The interaction of histatin with its target cells displays characteristics that resemble those of regular growth factors, such as EGF, which on binding are taken up by endocytosis (35). Histatins are also actively taken up by epithelial cells, but not at 4°C or in the presence of the energy poison sodium azide. The active uptake likely occurs *via* a stereospecific receptor since D-Hst2 is not taken up (Fig. 4). Furthermore, the cell migration activity of Hst2 is diminished in the presence of a specific ERK1/2 inhibitor (Fig. 5). The involvement of the ERK1/2 pathway in growth factor-enhanced cell migration is not uncommon; for review, see Huang *et al.* (36).

CONCLUSIONS

These data demonstrate that the wound-closure properties of human saliva can be attributed to histatins and not to EGF. Histatins thus exert a function that may be relevant for oral wound healing. Compared with growth factors such as EGF, histatins are relatively stable molecules and accessible for cheap and large-scale production, which make them attractive candidates for development as therapeutics for promoting wound healing. FJ

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