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Novel cholinergic peptides SLURP-1 and -2 regulate epithelialization of cutaneous and oral wounds

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Abstract

It is well established that auto/paracrine acetylcholine (ACh) is essential for wound epithelialization, and that the mechanisms include regulation of keratinocyte motility and adhesion *via* nicotinic ACh receptors (nAChRs). Keratinocyte nAChRs can be also activated by non-canonical ligands, such as secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein (SLURP)-1 and -2. In this study, we determined effects of recombinant (r)SLURP-1 and -2 on migration of human epidermal and oral keratinocytes under agarose and epithelialization of cutaneous and mucosal excisional wounds in mice, and also identified nAChRs mediating SLURP signals. Both *in vitro* and *in vivo*, rSLURP-1 decreased and SLURP-2 increased epithelialization rate. The mixture of both peptides accelerated epithelialization even further, indicating that their simultaneous signaling renders an additive physiologic response. The specificity of rSLURP actions was illustrated by similar effects on cutaneous and oral wounds, which feature distinct responses to injury, and also by abrogation of rSLURP effects with neutralizing antibodies. rSLURP-1 acted predominantly *via* the α_7 nAChR-coupled upregulation of the sedentary integrins α_2 and α_3 , whereas SLURP-2—through α_3 , and α_9 nAChRs upregulating migratory integrins α_5 and α_V . The biologic effects of rSLURPs required the presence of endogenous ACh, indicating that auto/paracrine SLURPs provide for a fine tuning of the physiologic regulation of crawling locomotion *via* the keratinocyte ACh axis. Since nAChRs have been shown to regulate SLURP production, cholinergic regulation of keratinocyte migration appears to be mediated by a reciprocally arranged network. The cholinergic peptides, therefore, may become prototype drugs for the treatment of wounds that fail to heal.

Keywords

nicotinic acetylcholine receptors; skin and oral wounds; epithelialization; keratinocytes; lateral migration

Introduction

Non-healing wounds represent a significant burden to patients, health care professionals, and the health care system. Although many different treatments have been used, successful repair of wounds remains a major healthcare and biomedical challenge in the 21st century (reviewed in (1, 2)). More efficient treatment of wounds that fail to heal are desperately needed, and this problems constitutes a subject of intense research. Lateral migration of epidermal and oral keratinocytes (**EKC** and **OKC**) is central to epithelialization of cutaneous (3) and mucosal (4) wounds, respectively. In the intact epithelium, keratinocytes

express $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ integrin receptors (5). Crawling cells enlarge their integrin repertoire by expressing $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$ integrins (6–8). Keratinocyte expression of $\alpha_2\beta_1$ integrin in wound is decreased (9). When the wound is closed, the expression of the "sedentary" integrins is renewed. Thus, based on their differential expression in wounded vs. intact epithelium and relation to the keratinocyte migratory function, the integrins can be tentatively divided into two groups: sedentary (α_2 and α_3) and migratory (α_5 , α_v , β_5 and β_6).

Although functional components of wound epithelialization are well-characterized, much less is known about the signaling mechanisms that initiate, sustain and terminate migration of keratinocytes. Recent studies have demonstrated that keratinocyte cholinergic system represents a previously overlooked and potentially important subject in the wound healing area. The mucocutaneous cytotransmitter acetylcholine (**ACh**) produced by both EKC (10) and OKC (11) modulates random cell migration (chemokinesis) and stimulates directional migration (chemotaxis) (12). Simultaneous stimulation of nicotinic and muscarinic receptors by ACh may be required to synchronize and balance ionic and metabolic events in a moving keratinocyte. Binding of ACh to its receptors on the cell membrane simultaneously elicits several diverse biochemical events the "biologic sum" of which, taken together with cumulative effects of other hormonal and environmental stimuli, determines a distinct change in cell behavior during epidermal turnover (reviewed in (13, 14)).

The mechanisms of cholinergic auto/paracrine control of keratinocyte motility involves regulation of expression of integrin genes *via* the nicotinic class of ACh receptors (**nAChRs**) (15). The nAChRs are classic representatives of the superfamily of ligand-gated ion channel proteins, or ionotropic receptors, mediating the influx of Na^+ and Ca^{2+} and efflux of K^+ (16). The homomeric nAChRs expressed in EKC and OKC can be comprised by α_7 or α_9 subunits, whereas the heteromeric nAChRs—by the α_3 , α_5 , α_9 , α_{10} , β_1 , β_2 and β_4 subunits, e.g., $\alpha_3(\beta_2/\beta_4)\pm\alpha_5$ and $\alpha_9\alpha_{10}$ (reviewed in (13)). While the nAChRs containing α_3 or α_9 subunits stimulate chemokinesis, activation of the nAChRs comprised by α_7 subunits exhibit reciprocal effects on cell motility by stimulating chemotaxis and inhibiting chemokinesis (12, 17).

Recent research clearly indicates that in addition to ACh and cholinergic drugs, the keratinocyte nAChRs can be activated by non-canonical ligands, including members of the Ly-6 protein family termed SLURP (secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein)-1 and -2 (reviewed in (18)). SLURP-1 has been identified in the studies of the autosomal recessive keratotic palmoplantar skin disorder Mal de Meleda that features inactivation of SLURP-1 through point mutation. SLURP-1 has been detected in neurons, respiratory and digestive epithelia, cornea, fibroblasts, lymphocytes, uterus and bone (reviewed in (19)). SLURP-2 was discovered during microarray analysis of novel genes whose expression in skin samples of patients with psoriasis was predominantly increased (20). Thus, classic studies showed that both SLURP-1 and -2 play important roles in regulating keratinocyte vital functions (20–22), suggesting that these cholinergic peptides may be useful for wound healing.

We have cloned SLURP-1 and -2, produced recombinant proteins and generated monoclonal antibodies that visualized each endogenous SLURP protein in human epidermis and oral mucosa (23, 24). Radioligand binding inhibition studies showed that SLURP-1 and -2 preferentially ligate α_7 and non- α_7 nAChRs, respectively, which allows SLURP-1 and -2 to induce physiologic responses *via* different pathways. SLURP-1 has been shown to alter expression of cell cycle regulators, differentiation markers and activate caspases (23). In marked contrast, SLURP-2 delayed keratinocyte differentiation and prevented apoptosis (24). Different biologic effects observed for SLURP-1 and -2 apparently resulted from

differential regulation of keratinocyte gene expression downstream of different nAChR subtypes preferentially ligated by each SLURP molecule.

In this study, we measured effects of recombinant (r)SLURP-1 and -2 on lateral migration of EKC and OKC under agarose, and epithelialization of mucocutaneous wounds in mice. We observed that each cholinergic peptide exhibited differential regulation of epithelialization and that both required the presence of auto/paracrine ACh. rSLURP-1 inhibited crawling locomotion of both EKC and OKC *in vitro* and delayed wound epithelialization *via* the $\alpha 7$ nAChR-coupled pathway that upregulates expression of the sedentary integrins α_2 and α_3 . rSLURP-2 produced stimulatory effects on cell migration and wound epithelialization mediated by both $\alpha 3$ -, and $\alpha 9$ -made nAChRs upregulating expression of the migratory integrins α_5 and α_V . Combining rSLURP-1 with -2 augmented epithelialization rate, indicating that SLURP-1 and -2 exhibit synergistic regulation of cell activities comprising the keratinocyte migratory function. Therefore, these cholinergic peptides may become prototype drugs for the treatment of wounds that fail to heal.

Materials and Methods

Chemicals and tissue culture reagents

The full length rSLURP-1 and rSLURP-2 were manufactured at Virusys Corporation (Sykesville, MD), as detailed by us before (25). Agarose type A was obtained from Accurate Chemical & Scientific Corporation (Westbury, NY). The predesigned and tested small hairpin RNA (**shRNA**) targeting human *CHRNA3* (NM_000743), *CHRNA7* (NM_001190455), *CHRNA9* (NM_017581), SLURP-1 (NM_020427.2), and SLURP-2 (NM_023946) and scrambled shRNA (negative control) were purchased from OriGene Technologies (Rockville, MD). The metabolic inhibitor of ACh synthesis hemicholinium-3 (**HC-3**), heat-inactivated newborn calf serum and all secondary antibodies were purchased from Sigma-Aldrich Corporation, Inc. (St. Louis, MO). The anti-SLURP-1 and -2 monoclonal antibodies 336H12-1A3 and 341F10-1F12, respectively, characterized by us in previous studies (23, 24), and the blocking peptides were from Research and Diagnostic Antibodies (North Las Vegas, NV). The antibodies to nAChRs used to evaluate the efficacy of inhibition of receptor protein expression in experiments with shRNA were from Research and Diagnostic Antibodies (anti- $\alpha 3$ and anti- $\alpha 7$) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; anti- $\alpha 9$).

Keratinocyte migration assay

Human EKC were purchased from Invitrogen Life Technologies (Carlsbad, CA) and grown in the serum-free keratinocyte growth medium containing 5 ng/ml EGF and 50 μ g/ml bovine pituitary extract (GIBCO-BRL, Cambridge, MA) in accordance the protocol provided by the vendor. Human OKC were purchased from ScienCell Research Laboratories (Carlsbad, CA) and grown in the oral keratinocyte medium (Cat. No: 2611; ScienCell Research Laboratories). Each type of keratinocytes was used in experiments between passages 2 and 4 grown in the respective growth medium (**GM**) to approximately 80% confluence. The pH of GM containing test compounds was maintained within the range 7.2–7.4, and the osmolarity was 290–310 mOsm/kg under all experimental conditions. The rate of epithelialization was measured by the random migration distance in agarose gel keratinocyte outgrowth system (**AGKOS**), as detailed by us elsewhere (12, 26). Briefly, a confluent keratinocyte monolayer was formed by loading EKC or OKC at a high density (4×10^4 cells/10 μ l) into 3 mm well in an agarose gel and incubating in GM to allow the cells to adhere to the dish bottom and form intercellular junctions. The cultures were incubated for 10 d in a humid CO₂ incubator with daily changes of GM containing test chemicals *vs.* GM without any additions (control). Some cells were first transfected with shRNA. To standardize results obtained in different

experiments, the mean values of the migration distances were converted into the percentage of control. The control value in each experiment was determined by measuring the baseline migration distance (in mm) and taken as 100%.

***In vivo* wounding and morphometric assay of epithelialization rate**

This study was approved by the University California-Irvine Animal Care and Use Committee. The assay of the skin wound epithelialization rate was performed in accordance to our previously published protocols (17, 27). Briefly, using a uniform 1 × 1 cm square template, full thickness excisions through the panniculus carnosus were made on the anesthetized skin of 6–7 weeks old BALB/c mice (The Jackson Laboratory, Sacramento, CA), in whom the hair cycle had been synchronized by the anagen induction technique (28). Each animal received 2 wounds at the symmetric sites of the central back, 0.5 cm off the vertebral line. Wounds were left undressed, and wounded animals were individually housed under aseptic conditions till the end of experiment, after which the mice were euthanized, and the wound border was harvested by shaving a narrow strip along the perimeter of the wound. The bilateral round excisional wounds of buccal mucosa were inflicted by a 2 mm in diameter biopsy puncher in accordance to the established procedure (29) in mice intraperitoneally anesthetized with ketamine/xylazine (80/10 mg/kg). Starting 1 h after wounding, we initiated daily treatments of experimental wounds by microinjections of 100 µL saline containing 1 µg/ml of rSLURP-1 and/or -2 with or without 1 µg of anti-SLURP-1 and/or -2 antibody to block SLURP effects. The control wound in each animal was similarly treated with plain saline solution. The effective dose of rSLURPs was identified in pilot studies, which also demonstrated that the majority of skin and oral wounds fully epithelialized by the 10th and 4th, respectively, day after wounding. Therefore, mice with skin wounds were euthanized on days 2, 5 and 8 and mice with oral wounds on days 1, 2 and 3 after injury. At least 3 animals per time point were used in each experiment. The epithelialization rate of both cutaneous and oral wounds was assayed in hematoxylin and eosin stained cryostat sections of the wounds by measuring the lengths of the tongues of new epithelium extending from either side of the wound. The immunofluorescence analysis of freshly frozen specimens of wounded skin and oral mucosa was performed as detailed previously (11).

shRNA transfection experiments

For transfection of EKC and OKC with the HuSH-29TM predesigned shRNA plasmids specific for human SLURP-1 and -2 molecules, and $\alpha 3$, $\alpha 7$ and $\alpha 9$ nAChR subunits, we followed a standard protocol described by us in detail elsewhere (30). Briefly, one day before transfection EKC and OKC were seeded at a density of 3×10^5 cells per well of a 6-well plate for real-time quantitative polymerase chain reaction (qPCR) experiments or 1×10^4 cells per well of a 96-well plate for the in-cell western (ICW) experiments, and exposed to experimental, i.e., nAChR or each SLURP gene-specific shRNA, or normal control shRNA (shRNA-NC) plasmids in GIBCOTM Opti-MEM I Reduced-Serum Medium (Invitrogen, Carlsbad, CA) with the TransIT[®]-Keratinocyte Transfection Reagent (Mirus Bio LLC, Madison, WI). The transfection was continued for 24 h at 37°C in a humid, 5% CO₂ incubator. On the next day, the transfection medium was replaced by GM, and the cells were incubated for additional periods of time to determine by immunoblotting and immunofluorescence relative protein levels of each SLURP peptide and nAChR subunit under consideration. The maximum inhibition was achieved at 72 h after transfection (Fig. 1A), at which point the shRNA-transfected cells were used in experiments.

qPCR experiments

Total RNA was extracted from EKC and OKC at the end of exposure experiments with the RNeasy Mini Kit (Qiagen, Valencia, CA) and used in the qPCR assays of integrin $\alpha 2$, $\alpha 3$, $\alpha 5$

and α_V gene expression, as detailed by us elsewhere (31). The qPCR primers were designed with the assistance of the Primer Express software version 2.0 computer program (Applied Biosystems, Foster City, CA) and the service Assays-on-Demand provided by Applied Biosystems. The qPCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) and the TaqMan Universal Master Mix reagent (Applied Biosystems) in accordance to the manufacturer's protocol, as described by us in detail elsewhere (24). To correct for minor variations in mRNA extraction and reverse transcription, the gene expression values were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The data from triplicate samples were analyzed with a sequence detector software (Applied Biosystems) and expressed as mean $\pm$ SD of integrin mRNA relative to that of control.

ICW experiments

The ICW assays were performed as described by us in detail elsewhere (23), using the reagents and equipment from LI-COR Biotechnology (Lincoln, NE). After exposure of 4×10^4 cells/well of a 96-well plate to test agents, the experimental and control keratinocytes were fixed *in situ*, washed, permeabilized with Triton solution, incubated with the LI-COR Odyssey Blocking Buffer for 1.5 h and then treated overnight at 4°C with a primary rabbit polyclonal antibody to integrins α_2 , α_3 , α_5 or α_V (Millipore, Temecula, CA). After that, the cells were washed, and stained for 1 h at room temperature with a secondary LI-COR IRDye 800CW goat anti-rabbit diluted 1:800, and Sapphire700 (1:10000) to normalize for cell number/well. The protein expression was quantitated using the LI-COR Odyssey Imaging System.

Statistical analysis

All experiments were performed in triplicates and repeated at least three times. Statistical significance was determined using the Student's *t*-test. The differences were deemed as significant when the calculated *p* value was <0.05 .

Results

Differential regulation of keratinocyte migration under agarose by rSLURP proteins

Using the chemokinesis AGKOS assay, we measured direct effects of rSLURP-1 and -2 on the migration distance of normal human EKC and OKC. We found that responses of both types of keratinocytes were similar. rSLURP-1 significantly ($p<0.05$) decreased and rSLURP-2 increased migration distances (Fig. 1 B, D). Surprisingly, when keratinocytes were exposed to a mixture of rSLURP-1 and -2 peptides, they migrated farther than the cells exposed to rSLURP-2 given alone ($p<0.05$).

These findings suggested that SLURP-1 and -2 exhibit synergistic regulation of cellular activities comprising the keratinocyte migratory function.

Requirement of endogenous ACh for SLURP-1 and -2 regulation of keratinocyte migration

Next, we evaluated the requirement of auto/paracrine ACh for SLURP effects on EKC and OKC. The cells seeded in the AGKOS plates were fed with the medium containing HC-3, which inhibits ACh synthesis and arrests keratinocyte migration (12, 15), and then exposed to rSLURP proteins. As expected, the migration distance was significantly ($p<0.05$) diminished in the presence of HC-3 (Fig. 1 B, D). Addition of rSLURP-1, -2 or a mixture of both peptides to the cells deprived of endogenous ACh had no effect on their arrested migration (Fig. 1 B, D).

These results were consistent with the previous report that SLURP-1 exhibits its biologic action on keratinocytes *via* an allosteric agonistic action at $\alpha 7$ nAChR requiring the presence of the endogenous agonist ACh (21).

The role of endogenous SLURPs in auto/paracrine regulation of keratinocyte migration by ACh

To identify the role of endogenous SLURP proteins produced and secreted by keratinocytes in the physiologic control of crawling locomotion, we deprived EKC and OKC of endogenous SLURP-1 or -2 and then measured migration distances. The >90% inhibition of SLURP protein expression in keratinocytes transfected with shRNA-SLURP-1 or shRNA-SLURP-2, compared to that in control cells transfected with shRNA-NC (Fig. 1 A). Silencing of the SLURP-1 or -2 genes did not produce significant changes in the keratinocyte migration distance ($p>0.05$; Fig. 1 C, E). However, when a rSLURP molecule was added to keratinocytes deprived of corresponding endogenous SLURP protein, the cells responded in the same way as did the intact cells, i.e., rSLURP-1 downregulated whereas rSLURP-2 upregulated migration ($p<0.05$; Fig. 1 C, E).

These results suggested the endogenously produced and secreted SLURPs provide for a fine tuning of the physiologic control of EKC and OKC by auto/paracrine ACh.

Differential regulation of epithelialization of murine mucocutaneous wounds by rSLURP-1 and -2

To be able to extrapolate the *in vitro* findings to the *in vivo* situation, we measured the effects of each rSLURP protein on the rate of epithelialization of the full thickness excisional skin and oral wounds in BALB/c mice. Similarly to keratinocyte migration under agarose, the epithelialization rate of mucocutaneous wounds was decreased by rSLURP-1 and increased by rSLURP-2, and even more so by a mixture of both peptides (Fig. 2). The changes became significant ($p<0.05$) on day 5 after skin wounding and on day 2 after mucosal wounding. The fact that human rSLURPs produced biologic effects on murine keratinocytes was not surprising because of considerable homology, >70%, between human and murine SLURP molecules.

To confirm specificity of rSLURP effects, we tested the ability to abolish significant changes in the epithelialization rate of wounds treated with rSLURPs in the presence of corresponding anti-SLURP antibodies. As expected, co-administration of SLURPs and neutralizing antibodies abolished effects of both rSLURP peptides (Fig. 2). In the presence of antibodies to both SLURP-1 and -2, the epithelialization of both skin and oral wounds decreased below the normal levels, with the differences reaching significance ($p<0.05$) in mucosal, but not skin, wounds. Normal mouse IgG, used as a control for murine monoclonal anti-SLURP antibodies, did not affect the epithelialization rate of mucocutaneous wounds (Fig 2).

As expected based on homology between human and murine SLURP molecules, the antibodies raised against human SLURP-1 and -2 visualized their murine counterparts in the epithelializing murine mucocutaneous wounds. Interestingly, keratinocytes comprising epithelial tongues both in the skin (Fig. 3 A, B) and oral mucosa (not shown) abundantly expressed SLURP-2, but very little amounts of SLURP-1. The opposite was observed in perilesional epithelium, consistent with our previous findings that SLURP-2 is important for keratinocyte survival (24), whereas SLURP-1—for keratinocyte maturation and terminal differentiation (23).

These results indicated that in contrast to keratinocyte cultures, endogenous SLURP-1 and -2 are involved in normal epithelialization of mucocutaneous wounds *in vivo*.

Differential effects of rSLURP-1 and -2 on epithelial morphology in mucocutaneous wounds

The distinct biologic effects of each rSLURP protein on wound epithelialization were also illustrated by considerable differences in the morphology of epithelialization tongues in wounds treated with rSLURP-1 vs. rSLURP-2. In the skin, rSLURP-1 facilitated vertical enlargement, producing a thick but short epithelialization tongue (Fig. 3 C), whereas rSLURP-2 caused horizontal extension, resulting in a thin but long tongue (Fig. 3 D). In buccal mucosa treated with rSLURP-1, thickening of short epithelial tongues was chiefly due piling up of terminally differentiated anucleated OKC (Fig. 3 E). In contrast, treatment of mucosa with rSLURP-2 produced elongated tongues comprised by several layers of nucleated OKC (Fig. 3 F). These phenotypes were observed if the vast majority of mice in each subgroup (Fig. 3 C–F, legend). The diverse phenotypes of epithelial tongues in response to treatments with rSLURP-1 vs. rSLURP-2 were consistent with reciprocal effects of each SLURP protein on keratinocyte development, with SLURP-1 promoting and SLURP-2 impeding terminal differentiation and apoptosis of keratinocytes (23, 24).

Taken together, these findings suggested that simultaneous activation of signaling by both SLURP-1 and -2 in keratinocytes provides for an additive physiologic effect stimulating their cellular functions mediating wound epithelialization.

Molecular mechanisms of reciprocal effects of SLURP-1 and -2 on keratinocyte migration

To elucidate molecular mechanisms mediating effects of SLURPs on keratinocyte migration, we performed quantitative analysis of the effects of rSLURP-1 and -2 on integrin gene expression at the mRNA and the protein levels using qPCR (Fig. 4 A) and ICW (Fig. 4 B), respectively. Both assays brought consistent results in experiments with EKC and OKC. rSLURP-1 significantly ($p < 0.05$) upregulated by several fold expression of the sedentary integrins α_2 and α_3 without altering the expression of the migratory integrins α_5 and α_V . In marked contrast, rSLURP-2 significantly ($p < 0.05$) upregulated the expression of α_5 and α_V integrins without affecting integrins α_2 and α_3 . As expected, simultaneous exposure to both rSLURPs upregulated both sedentary and migratory integrins in both EKC and OKC (Fig. 4 A, B).

These results indicated that downstream signaling emanating from the nAChRs ligated by SLURP-1 in both EKC and OKC is coupled to upregulation of the sedentary integrin gene expression, whereas that by SLURP-2—to upregulation of the migratory integrins. Furthermore, since one rSLURP protein did not abolish the effect of the other when both were administered together, it appeared that each rSLURP molecule acted through a distinct nAChR-coupled pathway.

Identification of the keratinocyte nAChR subtypes mediating regulatory effects of SLURP-1 and -2 on integrin expression in EKC and OKC

To identify the nAChR subtypes predominantly involved in mediating the regulatory effects of rSLURP-1 and -2 molecules on the expression of sedentary and migratory integrins in EKC and OKC, we silenced each of the major keratinocyte nAChRs using shRNA against α_3 , α_7 or α_9 subunits, and then measured the ability of rSLURP-1 and -2 to alter the proteins levels of integrins under consideration. Functional inactivation of α_7 in the keratinocytes transfected with shRNA- α_7 abolished the effects of rSLURP-1 on α_2 and α_3 integrins ($p < 0.05$), whereas transfection with shRNA- α_3 or shRNA- α_9 had no significant effects on these sedentary integrins ($p > 0.05$; Fig. 5 A, C). The opposite was observed in experiments with rSLURP-2. While transfection of either EKC or OKC with shRNA- α_7 did not alter the stimulatory effect of rSLURP-2 on the expression of the migratory integrins α_5 and α_V ($p > 0.05$), functional inactivation of α_3 or α_9 nAChR in both cases produced significant

($p < 0.05$) reduction of the protein levels of both migratory integrins, compared to the effect of rSLURP-2 on keratinocytes transfected with shRNA-NC (Fig. 5 B, D).

These findings indicated that the inhibitory action of SLURP-1 on keratinocyte migration is predominantly mediated by the $\alpha 7$ nAChR that upregulates expression of sedentary integrins $\alpha 2$ and $\alpha 3$, and that the stimulatory effect of SLURP-2 is mediated by both $\alpha 3$ - and $\alpha 9$ -made nAChRs coupled to upregulation of the migratory integrins $\alpha 5$ and αv .

Discussion

This study describes novel paradigm of regulation of keratinocyte migration by cholinergic peptides. Both *in vitro* and *in vivo*, SLURP-1 and -2 exhibited differential regulation of mucocutaneous epithelialization, with SLURP-1 decreasing and SLURP-2 increasing the epithelialization rate. When combined, SLURP-1 and -2 further accelerated epithelialization, indicating that their simultaneous signaling renders an additive physiologic response stimulating migration. The action of SLURP-1 was mediated predominantly by the $\alpha 7$ nAChR coupled to upregulation of sedentary integrins, whereas that of SLURP-2—by both the $\alpha 3$ -, and $\alpha 9$ -made nAChRs coupled to upregulation of migratory integrins. The biologic effects of rSLURPs required the presence of endogenous ACh, indicating that auto/paracrine SLURPs provide for a fine tuning of the physiologic regulation of crawling locomotion *via* the keratinocyte ACh axis. Since nAChRs have been previously shown to regulate the production and secretion of SLURPs (30, 32), cholinergic regulation of keratinocyte migration appears to be mediated by a reciprocally arranged network.

Many factors have been implicated in the mechanism of wound epithelialization, and there is no consensus as to which factors are critical for keratinocyte migration. The epithelium and several other types of non-neuronal cells synthesize, degrade and respond to ACh that functions outside the nervous system as an auto/paracrine hormone, or a cytotransmitter, activating the nicotinic and muscarinic classes of cholinergic receptors expressed by non-neuronal cells (reviewed in (33)). Studies of the role of keratinocyte ACh axis in wound epithelialization extend beyond the central paradigm of growth factor-mediated migration. Arrest of random and directional migration of EKC due to inhibition of ACh synthesis by HC-3 reported earlier (12, 15), which was reproduced in this study using both EKC and OKC, provides direct evidence that ACh is essential for normal epithelialization and serve as a pacemaker of keratinocyte motility. Both cutaneous and mucosal epithelia showed similar response to rSLURPs, despite the fact that epidermis is the stratified squamous epithelium comprised by "keratinizing" keratinocytes, whereas buccal mucosa is a non-keratinizing epithelium comprised by "non-keratinizing" keratinocytes. Furthermore, although healing of both cutaneous and oral wounds proceeds through the same stages (34), the morphologic and molecules differences in response to injury between skin and oral mucosa are well documented (35, 36). Therefore, the common effects of SLURPs on epithelialization of cutaneous and oral wounds observed in this study could be mediated by the same type of nAChRs coupled to regulation of the migratory function of both EKC and OKC. Indeed, we and others have demonstrated that the signaling pathways downstream of keratinocyte nAChRs regulate related cellular functions such as adhesion and migration, thus facilitation healing of cutaneous wounds (reviewed in (13, 37)). In a mouse model of cutaneous wound epithelialization, the healing effect of micromolar concentrations of nicotine is comparable to that of basic fibroblast growth factor (38).

In this study, we used novel rSLURPs that, in contrast to our previously used peptides (23, 24), do not contain SUMO protein and exhibit biologic effects at lower concentrations (25). Abrogation of the effects of rSLURPs in the presence of anti-SLURP antibodies demonstrated that changes in the epithelialization rate were causally related to the

pharmacologic action of these cholinergic peptides. Since endogenous SLURP-1 protein is detectable in plasma and urine at the concentration ranging from 6 to 35 ng/ml (22), the dose of rSLURPs required to alter epithelialization rate in our experiments exceeded the physiologic levels. This was not surprising, because the therapeutic doses of various hormones and growth factors used in medicine usually exceed their physiologic levels maintaining homeostasis. Lack of significant changes of keratinocyte migration due to functional inactivation of SLURP-1 and -2 gene expression in cultured EKC and OKC is consistent with this paradigm, because hormones and growth factors present in GM can compensate for abrogated stimulation of keratinocytes with auto/paracrine SLURPs. On the other hand, the inhibitory effect of anti-SLURP-1/2 antibodies on epithelialization of mucocutaneous wounds indicates that endogenous SLURP-1 and -2 play important roles in the biological machinery of wound epithelialization *in vivo*.

By activating nAChRs, SLURPs augment the physiologic control of keratinocyte migration by ACh. Abrogation of SLURP effects due to inhibition of ACh synthesis with HC-3 is in keeping with the notion that SLURPs exhibit their biologic effects on keratinocytes *via* an allosteric agonistic action requiring the presence of ACh (21). Experiments with shRNA against nAChR subunits demonstrated that SLURP-1 and -2 exhibited their effects on integrin expression by activating distinct nAChR subtypes. The obtained results are consistent with previously reported inverse effects of $\alpha 3$ - and $\alpha 9$ -made nAChRs *vs.* $\alpha 7$ nAChR on keratinocyte chemokinesis and selective coupling of distinct keratinocyte nAChR subtypes to intracellular effector molecules (12, 17). However, shRNA against neither nAChR subtype could completely abolish the stimulatory effect of rSLURP-1 and -2 on the expression of sedentary and migratory, integrins, respectively, indicating that other subtypes of keratinocyte nAChRs were also involved, albeit to a lesser degree.

The nAChR-mediated mechanism of action should allow SLURPs to synchronize the ionic events and metabolic signaling cascades that drive crawling locomotion of keratinocytes during wound epithelialization. A recent study has demonstrated that SLURP-1 activates the Raf-1/MEK1/ERK1/2 cascade in keratinocytes *via* two complementary signaling pathways: 1) Ca^{2+} -entry dependent CaMKII/PKC activation and 2) Ca^{2+} -independent involvement of Jak2 (39). Hence, in addition to upregulation of sedentary integrins, the inhibitory effect of SLURP-1 on keratinocyte migration observed in this study could be mediated by other biological processes elicited due to increase of intracellular Ca^{2+} , such as launching the terminal differentiation program and upregulation of cell adherence to the substrate, both of which can slow locomotion.

One of the most intriguing observations of this study, however, is an enhanced epithelialization rate due to simultaneous stimulation of keratinocytes with both rSLURP peptides. This additive physiological effect may be explained by activation of the reciprocal signaling pathways downstream of the nAChRs mediating biologic effects of each SLURP molecule. In the intact epidermis, an expression pattern of endogenous SLURP-1 and -2 appears to be somewhat different, with SLURP-1 being predominant in the upper epithelial layers and SLURP-2—in the lower layers (23, 24). This pattern coincides with that of $\alpha 7$ and $\alpha 3$ nAChRs preferentially mediating effects of SLURP-1 and -2, respectively (reviewed in (13, 18)). Upon wounding, keratinocytes dedifferentiate and crawl over the denuded dermis to re-epithelialize the wound. Since EKC and OKC growing in cell cultures express both the $\alpha 7$ and non- $\alpha 7$, such as $\alpha 3$, nAChR subtypes (11, 40), the immature keratinocytes should be able to simultaneously respond to both SLURP proteins. This ability may be essential for normal epithelialization, but it apparently decreases when the cells lose their "migratory" non- $\alpha 7$ nAChRs during their progression through the differentiation process rebuilding the epidermal barrier.

In conclusion, we demonstrated that SLURP-1 and -2 exhibit reciprocal regulation of keratinocyte migration by modulating the nicotinic signaling of auto/paracrine ACh, and that simultaneous stimulation of keratinocytes with both SLURPs augments their migratory function. These findings reveal a novel paradigm of the physiologic regulation of mucocutaneous epithelialization by cholinergic peptides. Therefore, rSLURPs may be a powerful tool for therapeutic modulation of keratinocyte migration. The advantage of using rSLURPs to accelerate wound epithelialization is their higher stability (half-life >24 h; data not shown) and affinity at keratinocyte nAChRs, compared to conventional agonists. Future studies of the SLURP effects on cellular and biochemical events comprising the epithelialization program should further justify their therapeutic use in wounds that fail to heal.

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Figure 1. Regulation of keratinocyte migration under agarose by rSLURP-1 and -2

A, Western blot of EKC lysates probed with anti-SLURP-1 or -2 antibodies at specified time points after transfection with a corresponding shRNA. A MW ladder is shown on the right. Similar results were obtained with OKC transfected with anti-SLURP-1 and -2 shRNA.

B, C. Effects of rSLURPs on random keratinocyte migration. Second passage human EKC (**A**) and OKC (**C**) were loaded into the chemokinesis AGKOS plates and incubated overnight to allow cells to settle. Migration distances were measured as described in the Materials and Methods after incubation for 10 d with 0.1 μ g/ml rSLURP-1 (**rS-1**) and/or rSLURP-2 (**rS-2**) in the presence or absence of 20 μ M of HC-3 to abolish ACh synthesis. Each experiment was performed in triplicate and repeated at least 3 times ($n = 3$), and the

combined results were averaged. The results are expressed as means $\pm$ SD% of untreated control, taken as 100%.

D, E. Effects of SLURP-1 or -2 gene silencing on keratinocytes migration in AGKOS plates. Second passage human EKC (**B**) or OKC (**D**) were loaded into AGKOS plates, incubated overnight to allow cells to settle, and transfected with shRNA-NC, anti-SLURP-1 or -2 shRNA, as detailed in Materials and Methods. After that, the cells transfected with shRNA-SLURP-1 (**shRNA-S-1**) were incubated for 10 d in GM containing 0.1 μ g/ml of rSLURP-1 (**rS-1**), whereas the cells transfected with shRNA-SLURP-2 (**shRNA-S-2**) were exposed to 0.1 μ g/ml of rSLURP-2 (**rS-2**), after which the migration distances were measure as detailed in the Materials and Methods.

Asterisks are p<0.05 compared to intact keratinocytes.

Figure 2. Effects of rSLURPs on epithelialization of murine cutaneous and oral wounds

Changes in the rate of epithelialization of excisional cutaneous (A) or oral (B) wounds in BALB/c mice treated daily by microinjections of 100 μ l saline containing 1 μ g/ml of each or both rSLURP-1 and -2 in the presence or absence of 1 μ g of anti-SLURP-1 and/or -2 monoclonal antibodies (S-1ab or S-2ab, respectively), or normal mouse IgGs (NlgG). The epithelialization rate was calculated, as detailed in Materials and Methods.

Asterisks are $p < 0.05$ compared to control mice treated with saline alone.

Figure 3. Microscopic studies of epithelialization tongues

A, B. Immunolocalization of endogenous SLURP-1 (**A**) and SLURP-2 (**B**) in the epithelial tongues of murine epidermis on the 2nd post wounding. The edge of the full thickness wound extending from the epidermis down to the dermis is depicted by an arrow. Bars = 30 (**A**) and 100 (**B**) μm.

C, D. Typical appearances of cutaneous epithelial tongues after 5 days of treatment with rSLURP-1 (**C**; observed in 7 out of 8 mice in the subgroup) or rSLURP-2 (**D**; 8 out of 8 mice). Bar = 150 μm.

E, F. Typical appearances of mucosal epithelial tongues after 2 days of treatment with rSLURP-1 (**E**; 8 out of 8 mice) or rSLURP-2 (**F**; 7 out of 8 mice). Bar = 30 μm.

Figure 4. Quantitative analysis of alterations in the integrin gene expression in EKC and OKC treated with rSLURP-1 or -2

Cultures of EKC and OKC were stimulated for 24 h at 37°C and 5% CO₂ with 0.1 µg/ml of each rSLURP peptide or their mixture added to GM. After incubation, the relative amounts of mRNA and proteins of α_2 , α_3 , α_5 and α_V integrins were quantified by qPCR (A) and ICW (B), respectively, as detailed in Materials and Methods.

Asterisks are $p < 0.05$ compared to intact keratinocytes.

Figure 5. Keratinocyte nAChRs mediating differential regulation of integrin gene expression by rSLURP-1 and -2

The EKC (A, B) and OKC (C, D) transfected with either shRNA-NC (control) or anti- α_3 , α_7 or α_9 nAChR subunit shRNA were incubated with 0.1 $\mu\text{g/ml}$ of either rSLURP-1 (A, C) or rSLURP-2 (B, D) for 24 h at 37°C and 5% CO_2 , after which the relative protein amounts of α_2 , α_3 , α_5 and α_V integrins were quantified by ICW, as detailed in Materials and Methods. Asterisks are $p < 0.05$ compared to control cells transfected with shRNA-NC; pound signs are $p < 0.05$ compared to control cells transfected with shRNA-NC and treated with respective rSLURP molecule.