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Glucagon-like peptide-1 analogue liraglutide facilitates wound healing by activating PI3K/Akt pathway in keratinocytes

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ABSTRACT

Aims: Diabetes induces various skin troubles including foot ulcer. This type of skin ulcer is refractory but the pathogenesis is not so certain. Recent study show that glucagon-like peptide-1 (GLP-1) analogues reduce foot complications with diabetes (Pérez et al., 2015), however, the role of GLP-1/GLP-1R axis is not fully understood, and clear evidence of GLP-1 to facilitate wound closure is still lacking. In this study, we investigated whether a potent GLP-1R agonist liraglutide affects wound healing process.

Methods: The expression of GLP-1R in HaCaT cells were indentified by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and immunoblotting analysis. To assess the effect on wound closure in keratinocytes, we performed in vitro scratch assay using the IncuCyte system (Essen BioSciences, Ann Arborm MI). We applied ointment containing liraglutide on full-thickness wounds in the dorsum of female balb/c mice (n = 6) until healing. To investigate the effect on PI3K/Akt pathway, we used IncuCyte system in HaCaT treated with PI3K inhibitor and Akt inhibitor.

Results: Keratinocytes expressed GLP-1R and liraglutide induced their migration. Liraglutide facilitated the wound healing in mice. Liraglutide upregulated keratinocyte migration via PI3K/Akt activation.

Conclusions: Our study suggests that liraglutide may be a potential target drug to improve skin ulcer with diabetes through its specific receptor GLP-1.

1. Introduction

Skin is one of the important target organs affected by diabetes mellitus, therefore, diabetes induces multifactorial complications in skin including intractable foot ulcer [2,3]. Although both micro- and macroangiopathy are believed to contribute to the development and delayed healing of diabetic wounds, the pathogenesis of diabetic skin ulcer is complex and still incompletely understood [4,5].

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Recently, glucagon-like peptide-1 (GLP-1) analogues and inhibitors of dipeptidyl peptidase-4 (DPP4) receive much attention in diabetic treatment because these drugs significantly reduce glycemic condition as well as cardiovascular and metabolic comorbidities [6,7]. Given that DPP4 is a potent degrading enzyme for GLP-1, the DPP4 inhibitors augment the GLP-1 levels [8]. Of note, DPP4 inhibitors promote wound healing in diabetic mouse [9]. A GLP-1 analogue liraglutide is also estimated to reduce foot complications with diabetes [1], however, clear evidence of GLP-1 to facilitate wound closure is still lacking.

GLP-1 exerts its biological effects through binding to specific GLP-1 receptor (GLP-1R) of the G-protein coupled receptor family [8,10]. Upon binding to GLP-1R, GLP-1 increases the intracellular levels of cyclic adenosine monophosphate in pancreatic β cells and thereby stimulates insulin secretion in a glucose-dependent manner [8,10]. Moreover, GLP-1R is expressed in various extrapancreatic tissues including intestine, lung and innate immune system [8,11–13] and the GLP-1/GLP-1R signaling plays a distinct biological role in each tissue [8,11–13].

The GLP-1R is also expressed in skin [14–16], however, the role of GLP-1/GLP-1R axis is not fully understood in the skin. In this study, we investigated whether a potent GLP-1R agonist liraglutide upregulates migration and/or proliferation of keratinocytes and affects wound healing process. We found that liraglutide did upregulate the keratinocyte migration via PI3K signal activation and promoted the wound healing in mice.

2. Material and methods

2.1. Reagents and antibodies

Liraglutide was provided from NOVO (Nordisk Co., Denmark), dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) at a concentration of 100 μM, and further diluted in medium. mitomycin C was obtained from Sigma-Aldrich; Akt inhibitor(ab142088) and LY 294002(ab120243) were purchased from abcam (Cambridge, UK). The antibodies used in this study were rabbit anti-β-actin, rabbit anti-GLP-1R, rabbit anti-Akt, rabbit anti-phosphorylated Akt and horseradish peroxidase-conjugated secondary antibody from Cell Signaling Technology (Danvers, MA).

2.2. Cell culture

The human immortalized keratinocyte cell line, HaCaT, were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% antibiotics (10,000 μg/ml streptomycin and 10,000 units/ml penicillin) at 37 °C with 5% CO_2 incubator.

2.3. In vitro scratch assay

HaCaT cells (3×10^4 cells/well) were seeded onto a collagen-1-coated 96-well ImageLock tissue culture plate (Essen BioScience) and incubated at 37 °C with 5% CO_2 incubator for 24 h. Wounds were made by the 96-well WoundMaker (Essen BioScience). The wounded cells were washed twice with culture medium to remove the detached cells and then treated with 100 μl of medium containing several concentrations of test materials (Fig. 1. liraglutide 1 nM, 10 nM, 100 nM and Fif 2. liraglutide 100 nM and Fig. 4. Liraglutide 100 nM, LY 294002 0.5 μM). Image of the wounds were automatically acquired within the CO_2 incubator by IncuCyte zoom software (Essen BioScience). The wound image updates were taken at 2 h intervals for the duration of the experiment. The data were analyzed with respect to wound confluence and calculated by using the IncuCyte software package (Essen BioScience). And to inhibit cell proliferation HaCaT cells were pretreated with 5 μg/ml mitomycin C for 2 h.

2.4. CCK-8 assay

HaCaT cells were incubated in 96-well plates at 37 °C with 5% CO_2. When the cell confluence reached 60% after cell inoculation, cell culture medium of each wells were changed to several concentration Liraglutide (1 nM, 10 nM and 100 nM), and incubated further 24 h. CCK-8 solution was added and the optical density values were detected at 450 nm using a quantitative automatic microplate reader (BIORAD).

2.5. Real-time quantitative reverse transcriptase-PCR

Total RNA was isolated from HaCaT cells using the RNeasy Mini kit

(Qiagen). Quantitative real-time reverse transcriptase-PCR was preformed with PrimeScript RT reagent and SYBR Premic Ex Taq II (Takara Bio, Ohtsu, Japan) in accordance with the manufacturer's instructions. PCR amplifications were performed with the following cycling conditions: 95 °C for 30 s initially, followed by 40 cycles of 95 °C for 5 s (denaturation step) and 60 °C for 20 s (annealing/extension steps). The cycle threshold for each amplification was normalized using β-actin (internal control). Normalized gene expression is shown as the quantity of gene-specific mRNA relative to that of control mRNA (fold induction). Oligonucleotide primers used in this study are listed below.

Sequences of nucleotides used as primer for PCR amplification:

human GLP-1R: sense, 5′-CAGCGCTCCCTGACTGAG-3′
antisense, 5′-CAGGCGTATTCATCGAAGGT-3′
human β-actin: sense, 5′-CTACAGGTTCAGATGATGTC-3′
antisense, 5′-CAGCTTCTCCTTCTCCATTG-3′

2.6. Animals

Female Balb/c normal mice (from Charles River Laboratories, Kanagawa, Japan) housed in vivarium in accordance with the guidelines of the animal facility center of Kyushu University. The mice were caged individually and maintained on food and water ad libitum.

2.7. Wound healing in vivo

Their skins were prepped for surgery by shaving the fur with electric clipper followed by a depilatory agent. The mice were anesthetized with sevoflurane, after which full-thickness wounds were made in the dorsal skin using a biopsy punch with a diameter of 6 mm (Kai Industries, Gifu, Japan).

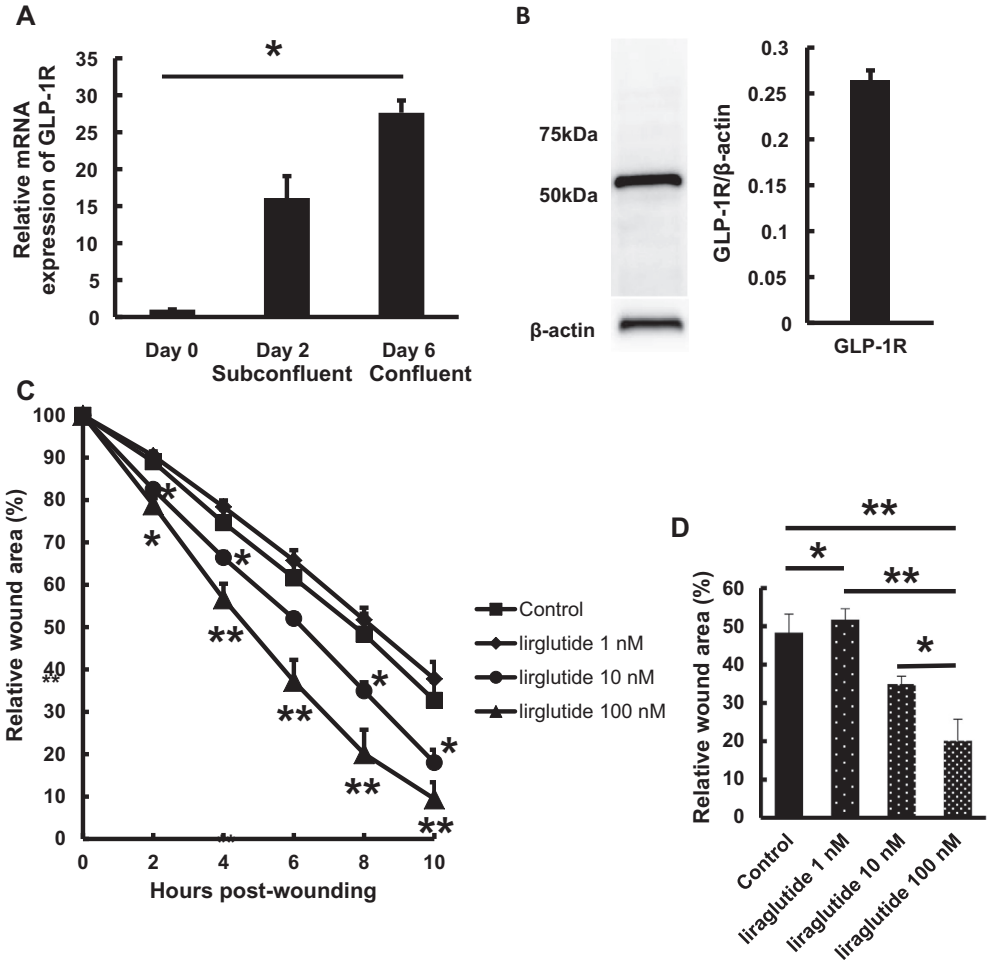

Fig. 1 – A: HaCaT cells were cultured in DMEM for 2 days (50% confluent), and 6 days (100% confluent) (n = 6). B: HaCaT cells were cultured in DMEM for 5 days (90% confluent) (n = 3). C, D: HaCaT cells were scratched and incubated with liraglutide 1 nM, 10 nM, 100 nM or DMSO (n = 6).

To inhibit the contraction of wound, toric silicon rubber (diameter of the inner circle was 8 mm) was affixed with 8 interrupted sutures on each wound. On the day of wound creation and every 2 days thereafter, ointment (vaseline containing liraglutide [liraglutide: 10 pm/g vaseline, DMSO: 100 μl/g vaseline] or vehicle [DMSO: 100 μl/g vaseline]) was occlusively applied to each wound. Wound was dressed with aluminum plate (Finn Chambers, smart practice Japan) and taped after applied each agents. Digital photographs were taken under sevoflurane anesthesia until healing. The wound area was calculated from the photographs using the ImageJ software (NIH, Bethesda, MD).

2.8. *Immunoblotting*

HaCaT cells were seeded in six-well plated, and at full confluence, cell monolayers were scratched with a blue pipette tip. Scratched cells were then treated with liraglutide (100 nM) or DMSO supplemented in DMEM for 3 h and protein lysates from cells were isolated with lysis buffer (25 mM HEPES, 10 mM $Na_4P_2O_7{\cdot}10H_2O$, 100 mM NaF, 5 mM EDTA, 2 mM Na_3VO_4, 1% TriTon X-100) and analyzed by SDS-PAGE on a 10% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membraned (Millipore, Bedford, MA) and probed with specific antibodies. Immunological bands were identified with a horseradish peroxidase-conjugated secondary antibody followed by visualization with SuperSignal west pico chemiluminescence substrate (Pierce, Rockford, IL).

2.9. *Statistics*

Data are presented as mean ± standard error. The significance of differences between groups was assessed using Student's unpaired two-tailed *t* test (when two groups were analyzed) or one-way analysis of variance (for three or more groups). A P-value of <0.05 was considered statistically significant.

3. Result

3.1. *Keratinocytes expressed GLP-1R and liraglutide induced their migration*

We first examined whether human keratinocytes express GLP-1R. As shown in Fig. 1A, keratinocytes express GLP-1R

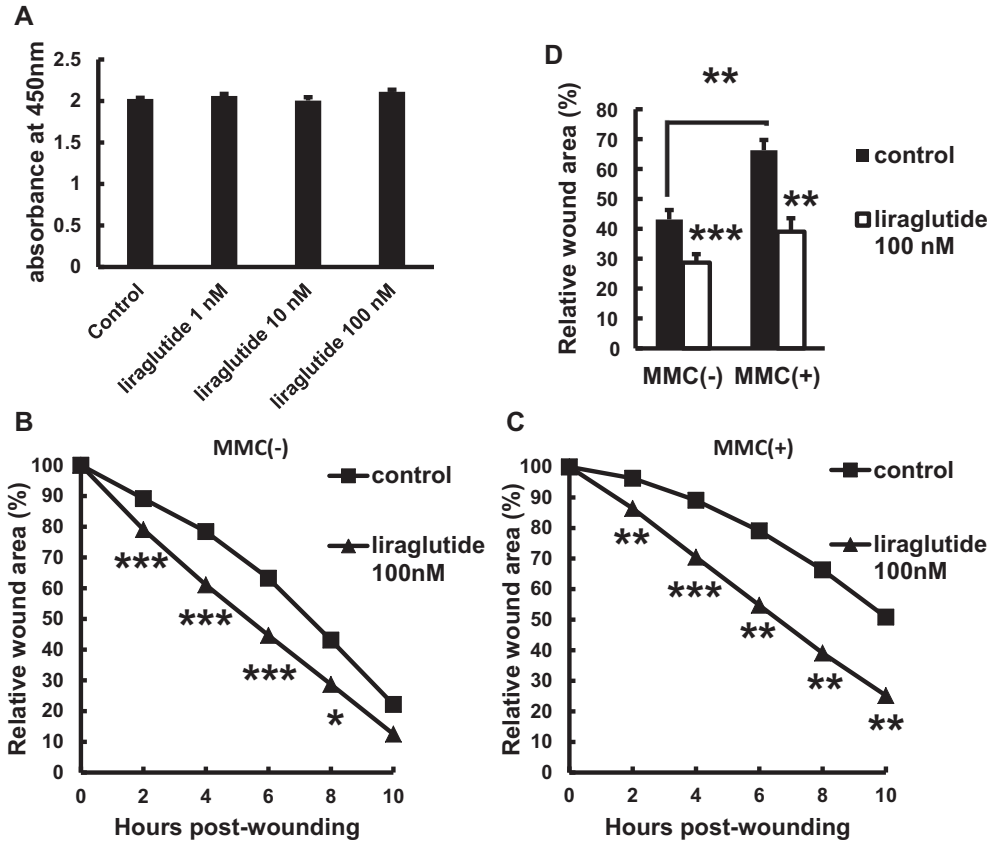

Fig. 2 – A: HaCaT cells were treated with liraglutide (1 nM, 10 nM, 100 nM) or DMSO for 24 h and the reaction products of CCK-8 assay were quantified (n = 6). B, C, D: HaCaT cells were treated (B) without or (C) with 5 μg/ml mitomycin C for 2 h. Cells were scratched and incubated with 100 nM liraglutide or DMSO (n = 6). (D) The relative wound area at 8 h is shown.

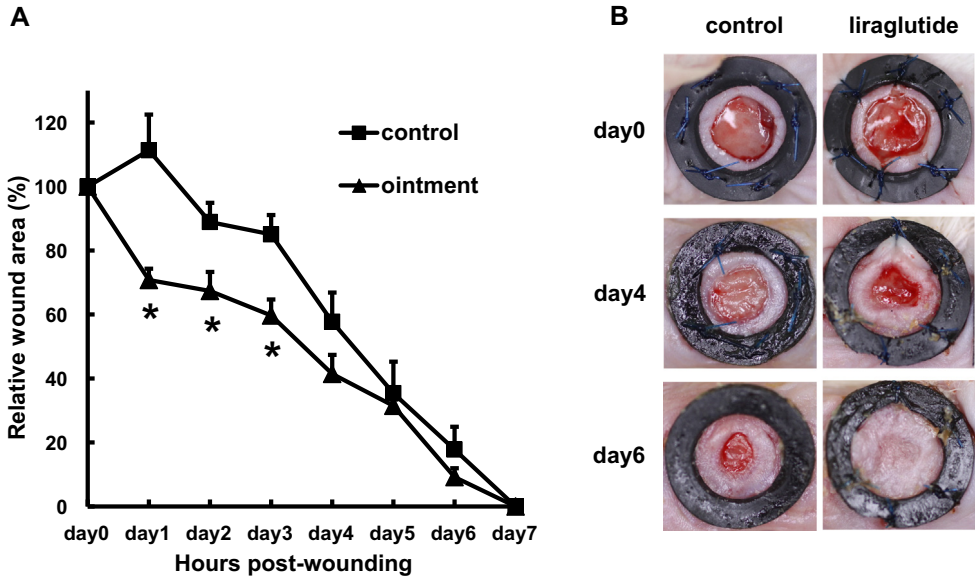

Fig. 3 – A, B: Representative photographs and the time courses of the relative wound area after wound creation in balb/c mice are shown (n = 5).

mRNA. The mRNA level of GLP-1R was low at the initiation of culture, however, it was upregulated when keratinocyte culture became confluent. The protein expression of GLP-1R was also confirmed by Western blot analysis (Fig. 1B).

We then examined whether liraglutide affects migratory capacity of keratinocytes. Confluent keratinocyte cultures were scratched by WoundMaker and wound closure was measured. In graded concentrations of liraglutide (1–100 nM), 10 nM and 100 nM of liraglutide significantly enhanced the keratinocyte migration at each time points until 10 h post-wounding (Fig. 1C). At 8 h post-wounding, significant dose-dependency was evident between 10 nM and 100 nM liraglutide (Fig. 1D)

The augmented wound closure was not due to the enhanced proliferation of keratinocytes by liraglutide, because liraglutide did not enhance the keratinocyte proliferation (Fig. 2A). Moreover, the liraglutide-induced upregulation of migration was demonstrated in keratinocytes treated with or without mitomycin C (Fig. 2B, C and D). As mitomycin C inhibited the proliferation of keratinocytes, these results suggested that liraglutide-induced upregulation of migration was independent of keratinocyte proliferation.

3.2. *Liraglutide facilitated the wound healing in mice*

As these in vitro studies suggested a migration-promoting effects of liraglutide in keratinocytes, we applied liraglutide to the experimental wounds made by 6-mm size punch excision in mice. Topical application of liraglutide (7.5 ng per 6-mm wound) significantly accelerated the wound closure at day 1 to day3 (Fig. 3A and B), indicating the promoting capacity of liraglutide in wound healing in vivo. Topical administration of liraglutide did not affect blood glucose level in mice (data not shown).

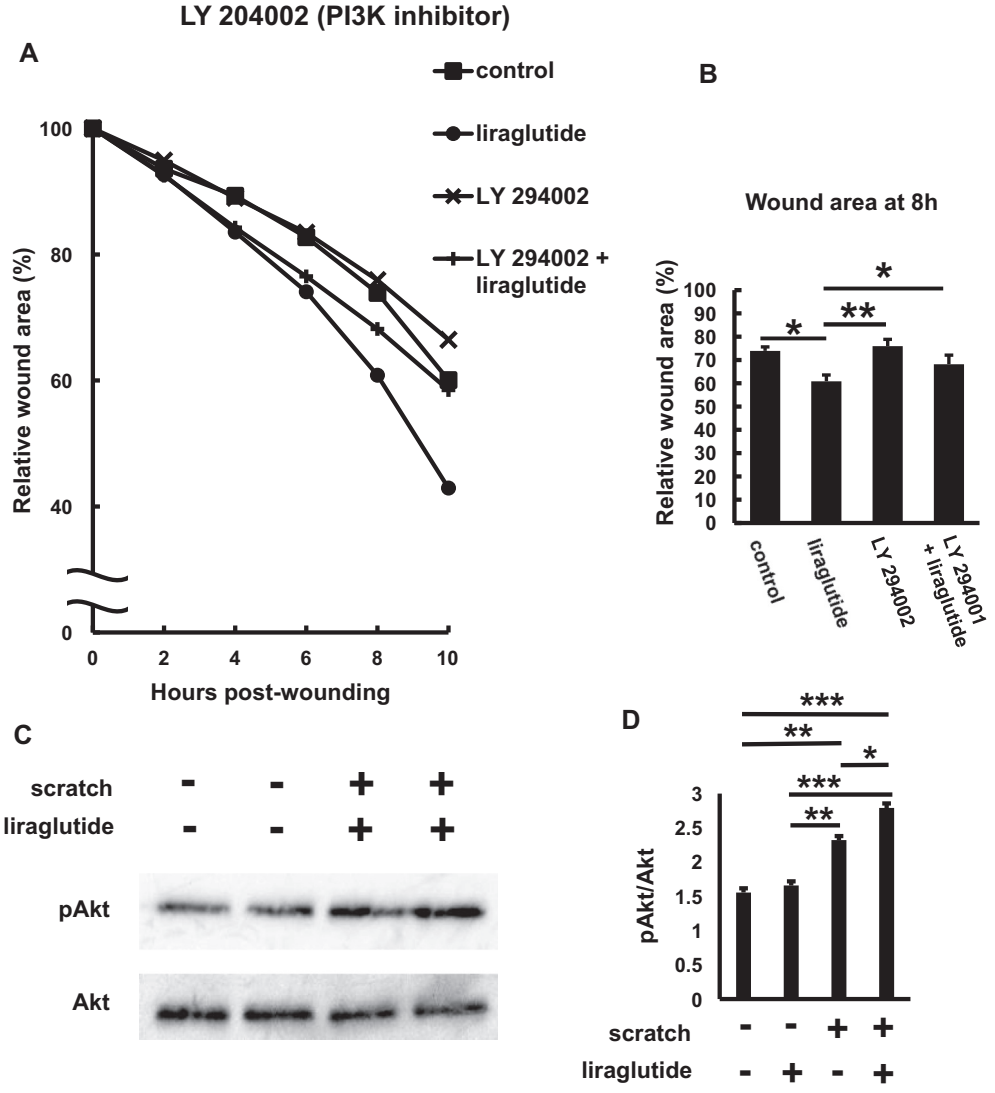

Fig. 4 – A, B: (A) HaCaT cells were scratched and incubated with DMSO, 100 nM liraglutide and/or LY 294002 (PI3K inhibitor). (B) The relative wound area at 8 h is shown. C, D: HaCaT cells were scratched and treated with 100 nM liraglutide or DMSO for 2 h (n = 3).

3.3. *Liraglutide upregulated keratinocyte migration via PI3K/Akt activation*

It has been reported that phosphorylation of Akt is essentially involved in the keratinocyte migratory capacity [17,18]. As liraglutide/GLP-1R signaling has been demonstrated to activate PI3K/Akt pathway [19,20], we then examined the effects of PI3K inhibitor LY294002. In the presence of LY294002, the migration-facilitating effect of liraglutide was canceled in 10 h incubation period (Fig. 4A). The inhibitory action of LY294002 was evident even at 8 h incubation period (Fig. 4B). We next examined the ratio of phosphorylated Akt (pAkt) and total Akt. Scratch stimulation per se upregulated the pAkt/Akt ratio in the confluent keratinocyte culture as has been reported previously [18]. Consistent to this notion, scratch injury enhanced phosphorylation of Akt, but liraglutide further augmented the scratch-induced phosphorylation of Akt (Fig. 4C and D).

4. Discussion

GLP-1 is a gut-derived incretin hormone that stimulates insulin and suppresses glucagon secretion, inhibits gastric emptying, and reduces appetite and food intake [21]. Systemic administration of GLP-1 mimetics such as liraglutide is beneficial not only in treating type II diabetes but also in reducing comorbid cardiovascular diseases [22]. GLP-1 is metabolically unstable and is rapidly degraded by DPP-4. Therefore, DPP-4 inhibitors increase the GLP-1 levels and eventually improve diabetic condition [8]. In addition, DPP-4 inhibitors are capable of accelerating wound healing in diabetic mouse model [9]. However, no studies have challenged a question whether GLP-1 mimetic liraglutide exhibits such a wound healing property.

In the present study, we first demonstrated (1) that keratinocytes expressed functional GLP-1R at mRNA and protein level, (2) that exogenous liraglutide promoted keratinocyte migration in vitro and facilitated wound closure in mice, and (3) that the upregulation of migratory capacity was mediated, at least in part, via PI3K/Akt pathway. In addition, the gene expression of GLP-1R was culture-time-, namely, confluency-dependent manner. Faurschou et al. first reported that mRNA expression of GLP-1R was detected in normal skin as well as uninvolved and involved skin in psoriasis patients [15]. However, they did not mention about cell types. We showed that liraglutide did activate PI3K/Akt pathway via functional GLP-1R.

The present results corroborated the previous findings that DPP-4 inhibitor linagliptin strongly reduced DPP-4 activity, stabilized active GLP-1 in chronic wounds, and improved healing in *ob/ob* mice [9]. Increase of endogenous GLP-1 by DPP-4 inhibitor linagliptin or administration of GLP-1 mimetic liraglutide facilitate wound closure. Among various signaling pathways, recent studies have stressed an importance of PI3K/Akt pathway in keratinocyte migration and wound closure [23,24]. In parallel, our study revealed that the PI3K/Akt pathway was involved in the liraglutide/GLP-1R-induced keratinocyte migration.

Besides wound healing acceleration, liraglutide has been expected to be beneficial in psoriasis frequently comorbid with diabetes and cardiovascular diseases [16,25], a randomized placebo-controlled study failed to prove this expectation [26]. Given that major pathogenetic pipeline in psoriasis is TNF/IL-23/IL-17A axis, the activation of GLP-1R/PI3K/Akt pathway is not influential. However, the cardinal signaling for wound healing is mediated via PI3K/Akt pathway [24] so that liraglutide may be a potential target drug to improve diabetic ulcer through its specific receptor GLP-1R.

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