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The *Pseudomonas aeruginosa* quorum sensing signal molecule *N*-(3-oxododecanoyl) homoserine lactone enhances keratinocyte migration and induces *Mmp13* gene expression *in vitro*

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ABSTRACT

Re-epithelialization is an essential step of wound healing involving three overlapping keratinocyte functions: migration, proliferation and differentiation. While quorum sensing (QS) is a cell density-dependent signaling system that enables bacteria to regulate the expression of certain genes, the QS molecule *N*-(3-oxododecanoyl) homoserine lactone (AHL) exerts effects also on mammalian cells in a process called inter-kingdom signaling. Recent studies have shown that AHL improves epithelialization in *in vivo* wound healing models but detailed understanding of the molecular and cellular mechanisms are needed. The present study focused on the AHL as a candidate reagent to improve wound healing through direct modulation of keratinocyte's activity in the re-epithelialization process. Results indicated that AHL enhances the keratinocyte's ability to migrate in an *in vitro* scratch wound healing model probably due to the high *Mmp13* gene expression analysis after AHL treatment that was revealed by real-time RT-PCR. Inhibition of activator protein 1 (AP-1) signaling pathway completely prevented the migration of keratinocytes, and also resulted in a diminished *Mmp13* gene expression, suggesting that AP-1 might be essential in the AHL-induced migration. Taken together, these results imply that AHL is a promising candidate molecule to improve re-epithelialization through the induction of migration of keratinocytes. Further investigation is needed to clarify the mechanism of action and molecular pathway of AHL on the keratinocyte migration process.

1. Introduction

In today's society, chronic wounds represent a major health care burden. Many factors can delay wound healing such as chronic disease, vascular insufficiency, diabetes, nutritional deficiencies, advanced age and others [1]. Wound healing disorders, especially impaired re-epithelialization, are a therapeutic problem of extensive clinical importance [2]. Recently, several approaches are being evaluated for treating patients with chronic ulcers [3]. However, still no advancement of wound closure is observed in some cases. Thus, it is reasonable to search for factors regulating wound healing processes to establish novel therapeutic approaches.

Although wound healing is a complex, multi-step process involving many cell types, an essential feature of a healed wound is the restoration of an intact epidermal barrier through wound epithelialization, also known as re-epithelialization. It can be conceptually viewed as the result of three overlapping keratinocyte functions: migration, proliferation and differentiation [4]. Defects in these functions are associated with the clinical phenomenon of chronic non-healing wounds.

During re-epithelialization, keratinocytes both migrate to cover the wound and proliferate to form a dense hyperproliferative epithelium feeding the epithelial tongue. To reconstitute full thickness skin and tissue integrity, migration and proliferation are sustained by growth factors, assisted by integrins and matrix metalloproteinases (MMPs) [5]. Integrins induce leading keratinocytes to migrate over the provisional matrix at the wound edges and the expression of this protein play a key role in the mechanism of migration [6]. Moreover, accumulated evidence has demonstrated that MMPs also play a crucial role in this process and are secreted by various cells in the wound in order to degrade and change matrix proteins at the wound site [7].

Several studies have shown that quorum sensing (QS) molecules produced by bacteria can influence the behavior of eukaryotic cells. QS is a cell density-dependent signaling system that

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enables bacteria to regulate the expression of certain virulent genes. The molecule *N*-(3-oxododecanoyl) homoserine lactone (AHL) is a QS-related signal molecule in *Pseudomonas aeruginosa* and it is reported to exert effects on mammalian cells, in a process called inter-kingdom signaling [8]. Huang [9] found that topical administration of AHL enhanced epithelialization of wounds in diabetic rats by improving impairments in the basement membrane, evidencing a clear organization of cell migration. Furthermore, Kanno et al. [10] reported that rat wounds inoculated with low doses of wild type *P. aeruginosa*, the strain which produces AHL, had a paradoxal acceleration of the re-epithelialization process. Although these results indicated that AHL improves re-epithelialization *in vivo*, it is still unknown which keratinocyte function is being modulated by AHL.

Based on those previous findings, we hypothesized that AHL directly modulates the molecular and cellular mechanisms that regulate keratinocyte migration at wound sites. Therefore, this research is focused on providing evidence of the potential effects of AHL on the modulation of keratinocyte migration process in an *in vitro* model.

2. Methods

2.1. Cell culture and reagents

Fetal rat skin keratinocyte cell line (FRSK, JCRB005) was from Health Science Research Resources Bank (Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle Medium (DMEM, Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, BioWest, Nuaillé, France) and 1% antibiotics (100 U/mL penicillin, 100 U/mL streptomycin; Nacalai Tesque) at 37 °C under 5% CO_2. The *P. aeruginosa* QS signal, *N*-(3-oxododecanoyl) homoserine lactone, was from Sigma (St. Louis, MO) and a stock solution of 10 mM was prepared by dissolving it in ethanol (EtOH) and subsequently stored in a −20 °C freezer. Cell proliferation reagent WST-1 (tetrazolium salt) was from Roche Diagnostics (Indianapolis, IN). RNeasy® Plus Mini Kit was from Qiagen (Valencia, CA). High Capacity complimentary DNA Reverse Transcription Kit and SYBR Green PCR Master Mix were from Applied Biosystems (Carlsbad, CA). Tanshinone IIA was from Enzo Life Sciences (Playmouth, PA).

2.2. In vitro wound healing assay

FRSK cell line was seeded at a density of 2×10^5 cells/well in non-coated 12-well plates with DMEM supplemented with 10% FBS and 1% antibiotics. After cell attachment, cells were serum starved for 24 h, scratch-wounded with a sterile 200 μL pipette tip and washed two times with serum free medium (SFM). Then, medium was subsequently changed to one of the two types of treatment: SFM supplemented with 1% antibiotics and 0.1% EtOH (control group) or 1 μM of AHL (AHL group). The wound area was photographed with phase contrast microscopy at marked positions. Cells were allowed to migrate for 24 and 48 h and the same fields were photographed again. Scratched areas of 3 randomly chosen fields per well in quadruplicate were measured with ImageJ software (ver. 1.42q, NIH, Bethesda, USA) and recovered surface area over all time points was calculated. For each condition, results were expressed as a percent wound closure relative to baseline and values were compared between the two groups at each time point.

In a different experiment, FRSK cells were seeded at a density of 5×10^5 cells/well in type I collagen-coated 6-well plates (BD Biosciences) on DMEM supplemented with 10% FBS and 1% antibiotics. After cell attachment, cells were serum starved for 24 h, scratch-wounded with a sterile 200 μL pipette tip and washed two times with SFM. Then, medium was subsequently changed to one of the two types of treatment: SFM supplemented with 1% antibiotics and 0.1% EtOH (control group) or 1 μM of AHL (AHL group). The wound area was photographed with phase contrast microscopy at marked positions. Cells were allowed to migrate for 24, 48 and 96 h and the same fields were photographed. Measurements of scratched areas of 3 randomly chosen fields per well in triplicate were performed as described above.

2.3. Proliferation assay

Proliferative activity of FRSK cells was analyzed by incubating cells in the WST-1 proliferation reagent after treatment with AHL. Briefly, 5×10^3 cells/well were seeded into type I collagen-coated and non-coated 96-well plates (Iwaki, Japan) on DMEM supplemented with 10% FBS and 1% antibiotics, and incubated overnight at 37 °C under 5% CO^2. Subsequently, cells ($n = 6$) were treated SFM supplemented with 1% antibiotics and 0.1% EtOH (control group) or 1 μM of AHL (AHL group) for 24 h and incubated with WST-1 reagent for 4 h. Absorbance was measured using a microplate reader and proliferative activity of the AHL group was shown relative to control group.

2.4. Total RNA isolation and real-time reverse transcription polymerase chain reaction (RT-PCR)

FRSK Cells were seeded at 3.6×10^5 cells/well on type I collagen-coated 6-well plates cultured in DMEM supplemented with 10% FBS and 1% antibiotics at 37 °C under 5% CO_2. After cell attachment, cells were serum starved for 24 h. Subsequently, medium was replaced in quadruplicate ($n = 4$) with the two types of medium supplemented with 0.1% EtOH (control group) or 1 μM AHL (AHL group). Six hours after treatment, total RNA was extracted using RNeasy® Plus Mini Kit according to the manufacturer's recommendation and first-strand cDNA was synthesized using a High Capacity cDNA reverse transcription kit. The expression of genes encoding all integrin subunits expressed in keratinocyte cells and MMPs involved in the re-epithelialization process, in which type I collagen is the substrate [11,12] were analyzed by real-time RT-PCR (Stratagene MX 3000P, Agilent Technologies, Japan) using SYBR Green PCR Master Mix. A gene encoding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as internal control. Primer sequences are described in Table 1. The expression levels of each target gene were calculated using comparative CT method.

2.5. Activator protein 1 (AP-1) inhibition assay

FRSK cells were prepared for *in vitro* wound healing assay in type I collagen-coated 6-well plates as described above. Tanshinone IIA, which suppresses AP-1 DNA complex formation, was applied to the cells in 100 μM diluted in SFM for 1 h prior to the AHL treatment and cells were allowed to migrate for 24 h. The marked areas of the wound were photographed and 6 fields of each treatment group were randomly chosen. Scratched areas were measured with ImageJ software and recovered surface over all time points were calculated. For each condition, results were expressed as a percent wound closure compared to baseline. Additionally, the effect of AP-1 inhibition on *Mmp13* expression after AHL treatment was investigated. FRSK Cells were seeded at 3.6×10^5 cells/well on type I collagen-coated and treated with tanshinone IIA for 1 h prior to the AHL treatment as described above. Cells were treated with control or 1 μM of AHL ($n = 4$) for 6 h, total RNA was extracted, first-strand cDNA was synthesized as described above and the expression of *Mmp-13* was analyzed by real-time RT-PCR.

Table 1
Primer sequences and product sizes for real time RT-PCR.

Gene symbol	Accession no.	Forward	Reverse	Product size (bp)
Gapdh	NM_017008.3	TGGTGAAGGTCGGTGTGAAC	GACTGTGCCGTTGAACTTGC	173
Itga1	NM_030994.2	GCATCAAAGACATCAGCATCG	GGTTGAAGCCTGGACAGCAT	158
Itga2	XM_345156	CTAGCACTCCAACGGAGAGG	CACTGCACCTAGCATCAGGA	200
Itga3	NM_001108292.1	CCCTTCAAACGGAACCAGAG	TCCACCTGCAAAGTCAGGAA	152
Itga5	NM_001108118.1	AGGTGACGGGACTCAACAAC	GGGCATTTCAGGACTTGTGT	151
Itga6	XM_002729169.1	GGGTACAACGCCTTTCTTCG	TTCTGGCGGAGGTCAATTCT	180
Itgb1	NM_017022.2	ACCCACTGCAAGGAGAAGGA	CAAATCAGCAGCAAGGCAAG	188
Itgb3	NM_153720.1	GTCATCCCAGGCCTCAAGTC	AAAGGCCTGGCAGTCACAGT	186
Itgb4	NM_013180.1	TGTCCTTTGAGCAGCCTGAA	CCCCTTCCACAGGGACATAA	159
Itgb5	NM_147139.2	CAGATGCAGTGGGAATGGAA	CAAAGCAGGAGCACTGGTTG	188
Itgb6	NM_001004263.1	ACTGAACCGAATGGGGATTG	AAATTCTCCTGGGAGCACCA	156
Mmp2	NM_031054.1	ACAGGACCCTGGAGCTTTGA	CTTGCAGATCTCGGGAGTGA	175
Mmp3	NM_133523.1	AAGATGCTGGCATGGAGGTT	TTCGAGTCCAGCTTCCCTGT	166
Mmp9	NM_031055.1	GCGCTGGGCTTAGATCATTC	TGGGACACATAGTGGGAGGA	199
Mmp10	NM_133514.1	CTGGCCCTGGATTTTATGGA	GAAAGGCGAATGTTGGCTTG	196
Mmp11	NM_012980.1	CAGAACCCAACGAGTGGACA	GACCTATGGGTCGAGGGAAA	185
Mmp13	NM_133530.1	ATGTGGAGTGCCTGATGTGG	GCCATCATGGATCCTGGTAAA	193
Mmp14	NM_031056.1	CCCAAAGCAGCAACTTCAGC	GGCCTTGCCTGTCACTTGTA	158
Mmp19	NM_001107159.1	AACCCCTGCAGCAGTGAAGT	GCTGTGTCCGGGGAGAGTAG	277

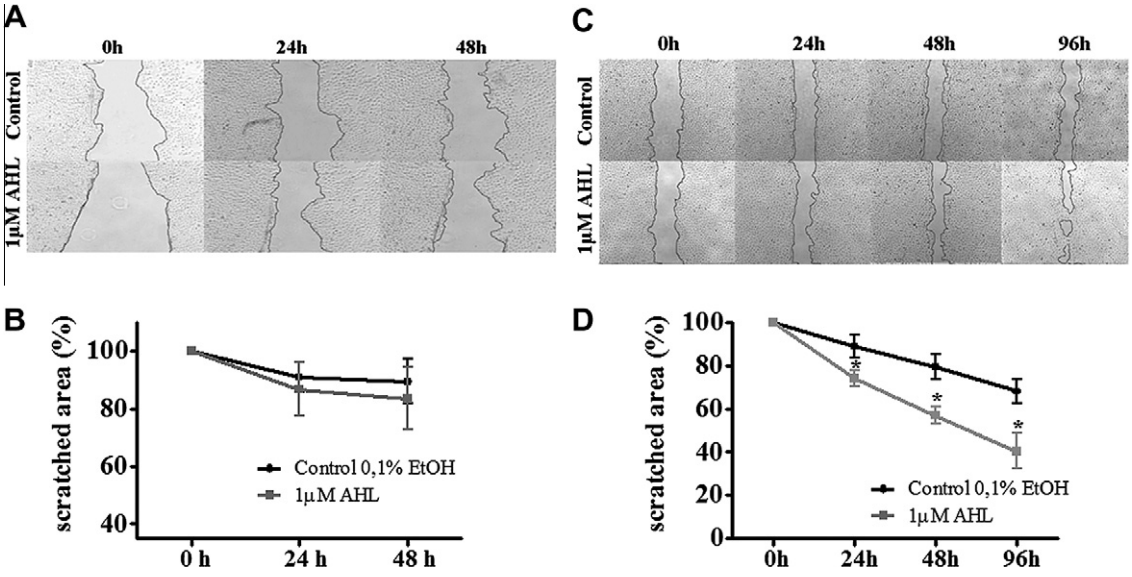

Fig. 1. *In vitro* wound healing assay: representative observation of FRSK migration in non-coated plate (A). Migration rate of FRSK cultured in non-coated plate, treated with 1 μM AHL or control 0.1% EtOH (B). Representative observation of FRSK migration in type I collagen-coated plate (C). Migration rate of FRSK cultured in type I collagen-coated plate, treated with 1 μM AHL or control 0.1% EtOH (D). $^{*}p < 0.001$ two-way ANOVA, Bonferroni post-hoc test.

2.6. Statistical analysis

Data are shown as means ± standard deviation. Differences among groups were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Student's *t* test was used when comparing two groups at one time point. Values of $p < 0.05$ were considered statistically significant. The software Graphpad Prism 5.0 (GraphPad Software Inc., CA) was used for all statistical analysis.

3. Results

3.1. AHL improves migration of FRSK cultured on type I collagen-coated surface

Initially, we investigated whether AHL induces keratinocyte migration. After treatment with 1 μM of AHL, FRSK cultured in non-coated plate, cell migration in the AHL-treated group was not significantly different compared to control when observed by phase contrast microscopy (Fig. 1A and B). Interestingly, after treatment with 1 μM of AHL, keratinocytes cultured in type I collagen-coated plate, an enhanced keratinocyte migration was observed and it was statistically significant at 24, 48 and 96 h compared to control (Fig. 1C and D).

3.2. AHL does not induce proliferation of FRSK

To determine whether the apparent induction of migration was associated with the proliferation of keratinocytes in response to AHL treatment, cells were subjected to WST-1 assay. As showed in Fig. 2, AHL did not induce proliferation of keratinocytes at the tested concentration (i.e., associated with migration) on both coated and non-coated surfaces.

3.3. AHL does not modulate the gene expression of integrins but enhances the Mmp-13 expression after 6 hours

After confirming that AHL enhances migration of keratinocytes, we next analyzed the expression changes of genes involved in the

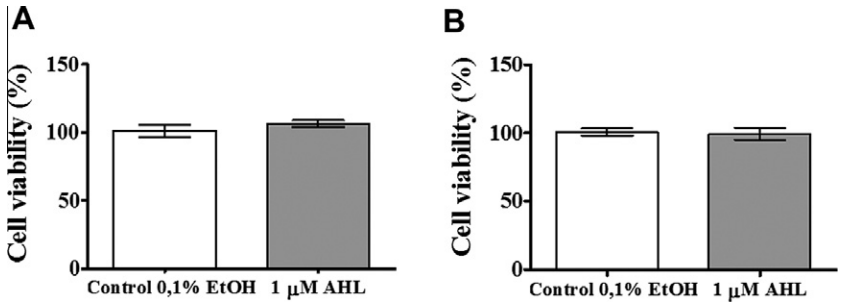

Fig. 2. Proliferation assay: proliferative activity of 1 μM AHL on FRSK cultured on non-coated plate (A) and type I collagen-coated plate (B).

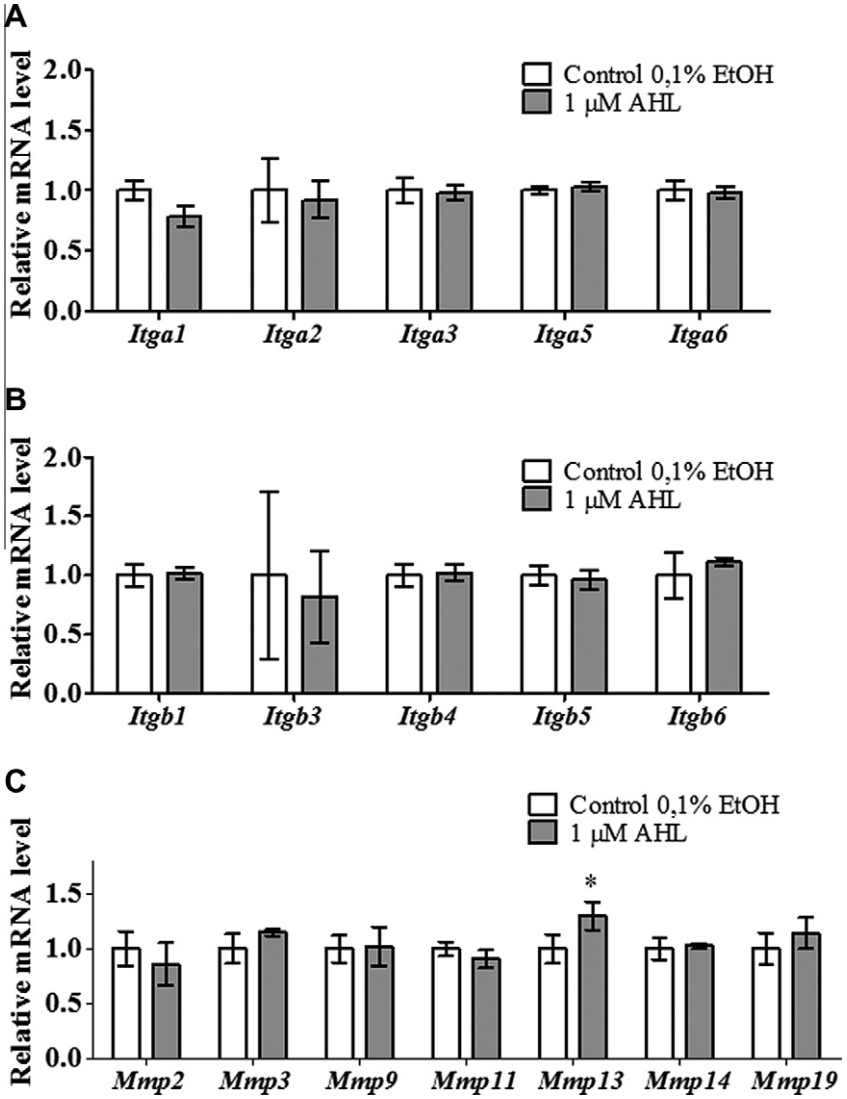

Fig. 3. Gene expression analysis: relative mRNA level of subunits of alpha (A) and beta integrins (B) expressed in FRSK cultured in type I collagen-coated plate and treated with AHL. Relative mRNA level of MMPs expressed in FRSK cultured in type I collagen-coated plate and treated with AHL (C). $^{*}p < 0.05$ (Student's *t* test).

mechanism of cell migration. The effect of AHL on gene expression of integrin subunits and MMPs were determined by real-time RT-PCR. Results show that AHL treatment did not significantly induce or suppress gene expression of all analyzed integrin subunits (Fig. 3A and B). Interestingly, AHL treatment significantly induced the overexpression of *Mmp13* as showed in Fig. 3C. None of the other analyzed MMPs had a significant modulation of gene expression.

3.4. AP-1 signaling pathway is essential for the AHL-induced migration

In this study we further focused on a possible involvement of AP-1 transcription factor, which is known to play an important role in the induction of keratinocyte migration during wound healing [13], to be a mediator of MMP expression [14] and a downstream effector of AHL signaling in rat dermal fibroblasts [15].

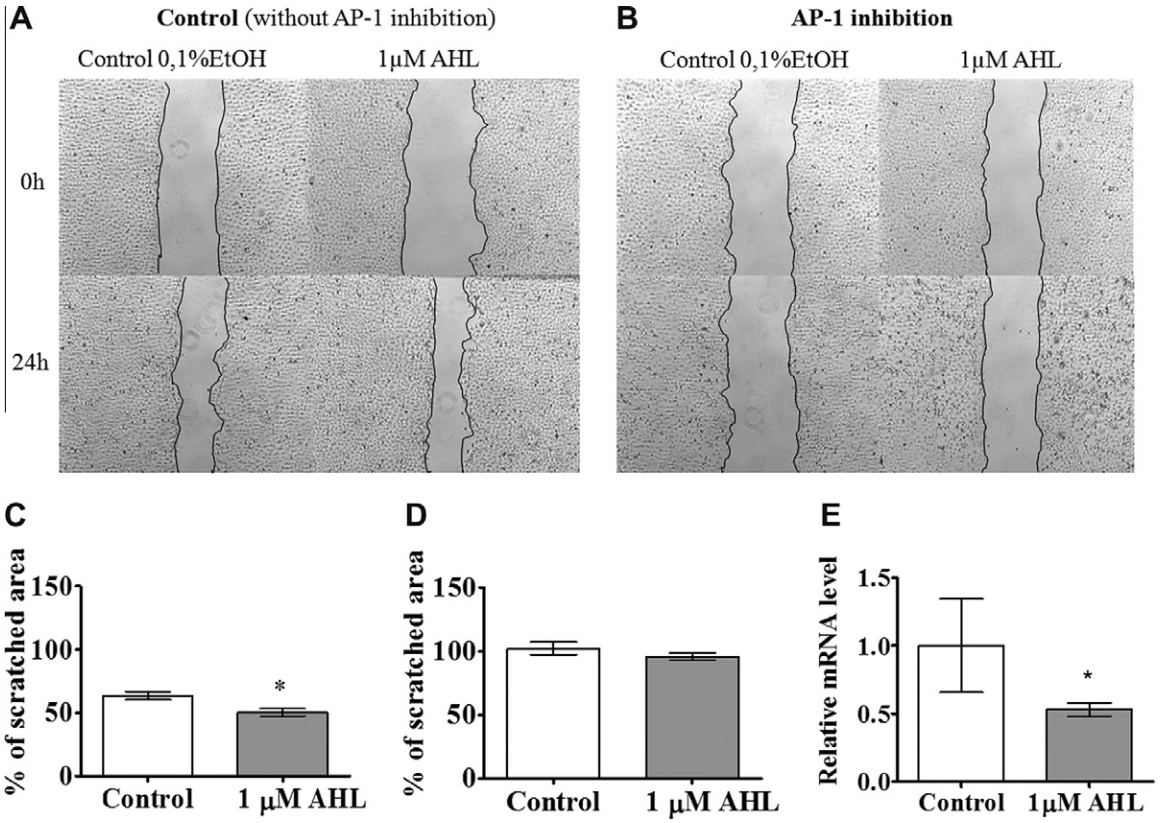

Fig. 4. AP-1 inhibition assay: representative observation of the involvement of AP-1 transcription factor in the migration of FRSK treated with AHL. Migration in the group cultured without AP-1 inhibition (A). Migration in the group cultured with AP-1 inhibition (B). Migration rates at 24 h of FRSK treated with control 0.1% EtOH or 1 μM AHL without AP-1 inhibition (C) or with AP-1 inhibition (D). Expression change of *Mmp13* after AP-1 inhibiton under AHL treatment (E). $^{*}p < 0.05$ (Student's *t* test).

In order to determine if AP-1 pathway is involved in the AHL induced migration, the *in vitro* wound healing assay was performed and cells treated with an inhibitor of AP-1 DNA complex formation prior to the AHL treatment. In the group cultured without AP-1 inhibition, similarly to the previous results, the treatment with 1 μM AHL enhanced the migration of keratinocytes compared to control after 24 h (Fig. 4A and C). In contrast, the treatment with AP-1 inhibitor completely inhibited overall migration ability of keratinocytes cultured in type I collagen-coated plate in both groups at the same time point (Fig. 4B and D).

The effect of AP-1 inhibition on *Mmp13* gene expression after AHL treatment was further analyzed by real-time RT-PCR. Results showed that the treatment with the inhibitor of AP-1 transcriptor factor diminished the elevation of *Mmp13* gene expression induced by AHL compared to the control.

4. Discussion

The effects of AHL, the *P. aeruginosa* QS signal molecule, on mammalian cells have been recently investigated in several types of cells [16]. In the present study, we have provided the first evidence on the effect of AHL on the migration of keratinocyte cells.

Re-epithelialization, which is necessary for wound closure and restoration of barrier function after skin injury, requires directional keratinocyte migration from the wound edges as well as cell proliferation. Both, proliferation and migration of keratinocytes are controlled by extracellular hormones, providing attractive opportunities for therapeutic intervention [17].

Our research group is currently focused on investigating the potential effects of AHL for wound healing improvement. Huang [9] showed that when AHL is administered to full-thickness diabetic wounds in rats, the re-epithelialization was enhanced through improved organization of the basement membrane. Likely, Nakagami et al. [18] affirmed that AHL accelerates wound healing through myofibroblast differentiation in an *in vivo* wound healing model in rats. An enhanced ability of keratinocytes to migrate over the *in vitro* wound found in our study, further support the hypothesis suggested from that author's results. Taken those findings together, we were able to reveal multiple effects of AHL on the wound healing process.

In this study, cells treated with 1 μM of AHL had a greater and significant migration rate compared to cells of the control group. Interestingly, this enhanced migration was noticed only in cells cultured on type I collagen-coated surface. One of the stimuli that induces keratinocyte migration is the contact with type I collagen from damaged cell matrix of the wound [19]. Therefore we suggest that type I collagen matrix is needed for mediating the AHL effects on keratinocyte migration.

While both proliferation and migration of keratinocytes contribute to re-epithelialization, these events appear to be two separate phenomenon [20]. Herein, we verified the modulation of keratinocyte proliferation as a possible mechanism by which AHL may influence re-epithelialization. As there was no change in the proliferation profile of keratinocytes after treatment with 1 μM of AHL, we confirmed that AHL does not affect proliferation of these cells, suggesting that the re-epithelialization occurs due to migration rather than proliferation.

Exposure to 1 μM of AHL for 6 h significantly increased the expression of *Mmp13* in keratinocytes. The duration of AHL treatment was based on a previous finding from our laboratory, in

which the fibroblastic cell line Rat-1, showed the upregulation of specific MMP gene expression after 6 h of AHL treatment [15]. Although the migration continuously occurred for a period more than 96 h after the scratch, the increased expression of *Mmp13* at 6 h is consistent with a previous study in which reports the increase of MMP levels in the early stages of wound healing [4]. Nevertheless, the study by Hattori et al. [21] emphasizes that *Mmp13* plays a key role in keratinocyte migration, angiogenesis, and contraction in wound healing. They also reported that *Mmp13* is expressed in keratinocytes at the migrating border and their study on *Mmp13* KO mice showed significant delay in wound healing. MMP-13 has wide substrate specificity, degrading collagen type I, collagen type III and collagen type IV [22]. Previous study says that keratinocyte migration over the wound bed is known to be dependent on the attachment of keratinocytes to type I collagen [23]. Therefore we could say that MMP-13 might contribute to the enhanced migration of keratinocytes due to its effect on collagen type I degradation, causing detachment of basal keratinocytes from the basal membrane.

Integrins play a key role in keratinocyte migration since its interaction to the extra cellular matrix is crucial at the wound edges during migration [24]. Therefore, we hypothesized that they might be involved in the AHL mediated migration. In this study, the exposure to AHL did not affect mRNA expression of integrins. Since keratinocyte migration assays were allowed to occur over a long period, it is possible that the migratory factors are modulated at a later time point. In keratinocytes, as in other cell types, the function of integrins can also be regulated independently of gene expression levels [11]. Therefore, further investigation would be needed to elucidate the effects of AHL on integrins.

Some of the key transcription-binding sites involved in the regulation of various *Mmp* genes are: the AP-1 and -2 sites, the polyomavirus enhancer-A binding protein-3 (PEA3) site, the nuclear factor kappa B (NF-κB) site, and the signal transducer and activator of transcription protein (STAT) site. The AP-1 transcription factor which is a heterodimeric complex appears to be a major mediator of the regulation of *Mmp* genes, including *Mmp13* [16]. Thus, some authors report that many signaling pathways including some that control cell proliferation and migration act modulating AP-1 activity [25]. AP-1 also regulates genes encoding cell adhesion molecules such as integrins and laminins that allow migrating cells to attach over the provisional matrix [26]. As *Mmp13* is known to be regulated by AP-1 transcription factor, we hypothesized that it could be possibly the key mechanism involved in the modulation of AHL-induced keratinocyte migration. Interestingly, the results showed that no migration was observed in the AP-1 inhibited cells, indicating that AP-1 might be essential for the AHL-induced keratinocyte migration.

Since we speculated that AP-1 transcription factor is involved in the AHL-induced upregulation of *Mmp13*, which is an early response factor, we also investigated the change of gene expression after 6 h of AHL treatment. The decreased expression of *Mmp13* after treatment with AP-1 inhibitor under the presence of AHL provided more evidence in the direct relationship among AP-1, AHL and *Mmp13*. From this result we could say that AP-1 transcription factor might be located downstream of AHL signaling, as suggested in the abovementioned hypothesis.

Based on these results, it is possible to propose a model where AHL enhances the keratinocyte's ability to migrate in an *in vitro* scratch wound model, which might be mediated by *Mmp13* upregulation through involvement of AP-1 transcription factor. Herein we have provided an *in vitro* evidence of the potential effect of AHL on the modulation of the migration process, suggesting further researches to support the topical AHL administration as a possible treatment to improve epithelialization of chronic wounds.

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