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ORIGINAL ARTICLE

Wound Healing Potential of Low Temperature Plasma in Human Primary Epidermal Keratinocytes

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Abstract

BACKGROUND: Low temperature plasma (LTP) was recently shown to be potentially useful for biomedical applications such as bleeding cessation, cancer treatment, and wound healing, among others. Keratinocytes are a major cell type that migrates directionally into the wound bed, and their proliferation leads to complete wound closure during the cutaneous repair/regeneration process. However, the beneficial effects of LTP on human keratinocytes have not been well studied. Therefore, we investigated migration, growth factor production, and cytokine secretion in primary human keratinocytes after LTP treatment.

METHODS: Primary cultured keratinocytes were obtained from human skin biopsies. Cell viability was measured with the EZ-Cytox cell viability assay, cell migration was evaluated by an *in vitro* wound healing assay, gene expression was analyzed by quantitative real-time polymerase chain reaction, and protein expression was measured by enzyme-linked immunosorbent assays and western blotting after LTP treatment.

RESULTS: Cell migration, the secretion of several cytokines, and gene and protein levels of angiogenic growth factors increased in LTP-treated human keratinocytes without associated cell toxicity. LTP treatment also significantly induced the expression of hypoxia inducible factor-1α (HIF-1α), an upstream regulator of angiogenesis. Further, the inhibition of HIF-1α expression blocked the production of angiogenic growth factors induced by LTP in human keratinocytes.

CONCLUSION: Our results suggest that LTP treatment is an effective approach to modulate wound healing-related molecules in epidermal keratinocytes and might promote angiogenesis, leading to improved wound healing.

Keywords Low temperature plasma · Keratinocyte · Cytokine · Growth factor · Wound healing

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1 Introduction

Low temperature plasma (LTP) is formed by electric discharge drilling for argon or helium under atmospheric pressure at room temperature, and consists of electric fields, a variety of reactive species, and UV emissions that are responsible for multiple biological effects [1–4]. Studies have demonstrated the beneficial effects of LTP in biomedical fields, including antimicrobial, antitumor, and wound repair activities [1, 4].

One previous study investigated the safety of an LTP device by treating human dermal fibroblasts, and the results indicated that it can be safely used for therapeutic treatment [5]. LTP treatment at low doses does not induce toxicity, and instead it was found to induce porcine aortic endothelial cell proliferation, whereas longer exposures have been shown to lead to cell death [6]. Moreover, its therapeutic potential for wound healing was systematically investigated using human dermal fibroblasts, and it was demonstrated that LTP treatment modulates cell parameters relevant to wound healing, such as the promotion of cell motility in an *in vitro* wound healing assay and the induction of both gene and protein expression of MCP-1, IL-6, and TGF-β1, which are important cytokines for wound repair [7]. In addition, several animal studies have demonstrated accelerated wound healing after LTP treatment [7–9], based on one such mechanism, specifically the stimulation of inflammatory responses [7]. Furthermore, in clinical studies, daily treatment with plasma significantly reduced bacterial counts in chronically-infected wounds [10] and enhanced re-epithelialization, wherein fewer blood scabs and fibrin layers were observed, without any inflammation in the wound of the donor site, in a placebo-controlled randomized clinical study [11]. Whereas the development of LTP has approached clinical applications, the mechanisms underlying its effects on mammalian cells and tissues during wound repair are not entirely known.

Keratinocytes comprise the major cellular component of the skin epidermis and have important roles in the complex mechanisms underlying the initiation, maintenance, and completion of the cutaneous wound healing process. At the early stages after injury, keratinocytes begin to migrate, close to the wound, and release a number of pro-inflammatory cytokines, chemokines, and growth factors that can stimulate not only keratinocytes themselves, but also other inflammatory cells and fibroblasts [12]. In particular, the interactions between keratinocytes and fibroblasts, which occur in a paracrine manner via growth factors, play an extremely important role in the completion of wound closure during wound healing [13, 14].

Keratinocytes are also an important source of angiogenic growth factors such as platelet-derived growth factor (PDGF) [12], heparin-binding EGF-like growth factor (HB-EGF) [15], and vascular endothelial growth factor (VEGF) [16], all of which stimulate the migration of endothelial cells to the wound site and contribute to angiogenesis. HB-EGF and PDGF also stimulate fibroblast proliferation, differentiation, and extracellular matrix (ECM) production [13]. During normal wound healing, VEGF is abundantly expressed in keratinocytes at the edge of the wound [17]. In contrast, defective the VEGF mRNA expression, might be associated with delayed wound repair in diabetic db/db mice [16].

Until now, there have been few reports on the effects of LTP on primary human epidermal keratinocytes. Therefore, in this study, we investigated migration, growth factor production, and cytokine secretion in keratinocytes after LTP treatment. Furthermore, we evaluated the expression of angiogenic growth factors induced by LTP via HIF-1α upregulation.

2 Materials and methods

2.1 Primary human epidermal keratinocyte culture

Skin biopsies of volunteer were collected from Hangang Sacred Heart Hospital. Informed written consent was obtained from all patients who participated in this study, and the study protocol was approved by the institutional review board of Hallym University Hangang Sacred Heart Hospital (2018-018). Tissues were repeatedly washed five times with cold phosphate buffered saline (PBS) and 70% alcohol for medical disinfection. The tissues were then cut to a width of approximately 2–4 mm and digested with dispase II (1.0 unit/ml, 30 ml; Gibco, Life Technologies, Carlsbad, CA, USA) solution and agitated at 4 °C for 16–21 h. The next day, the epidermis was separated from tissues using sterile forceps, and then digested with 0.125% trypsin (Gibco, Life Technologies, Carlsbad, CA, USA) for 15 min at 37 °C. The samples were treated with KGM-Gold™ keratinocyte growth medium containing supplements (Lonza Bioscience, Basel, Switzerland) to inactivate the trypsin. After filtering and centrifugation at 1200×*g* for 3 min, the pellet was suspended in keratinocyte culture media and cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Cells from the second passage were applied to subsequent experiments.

2.2 LTP device

The LTP system was as used in our previous study [18]. The LTP was a developed dielectric barrier discharge device, and applied 13 kHz of frequency, 5.9 kV of voltage, and 42 W of electric power. The working gas for LTP

generation was a mixture of 5000 ccm of helium and 50 ccm of air at 28 ± 2 °C.

2.3 LTP treatment

Keratinocytes were seeded in 35-mm cell culture dishes at a density of 5×10^4 cells, and cultured for 2 days. Culture dishes were washed once with Dulbecco's phosphate buffered saline (DPBS) and added to 1.2 ml of DPBS before LTP treatment. Untreated dishes were subjected to the same process. The distance between the LTP torch and culture dish was 3 cm and the treatment diameter was 2 cm. The cells were treated with LTP for 30 s, 1 min, or 3 min, depending on the experiment. Analyses were performed 6 and/or 24 h after LTP treatment.

2.4 Cell viability assay

Keratinocyte viability was measured by an improved MTT assay method (EZ-Cytox, Dogen, Seoul, Korea) according to the manufacturer's instructions. The final value was calculated according to the following formulae: sample absorbance − background absorbance = original absorbance; original absorbance/control absorbance × 100 = % viability.

2.5 Cell migration assay

Keratinocyte migration was measured by wound healing assays in 35 mm μ-dishes with 2-well culture inserts (Ibidi GmbH, Planegg, Germany) according to the manufacturer's instructions. Keratinocytes were plated in the culture insert dish at a density of 2×10^4 cells per well and cultured for 24 h. The culture insert was then withdrawn, which produced a defined cell-free wound of 500 ± 50 μm. Mitomycin C (Sigma, St. Louis, MO, USA) was added at 5 μg/ml to the cell culture medium to inhibit cell proliferation during migration. The images of cell migration in the wound area were captured 6 and 24 h after exposure to LTP for 30 s or 3 min using a light microscope (IX 70, Olympus, Tokyo, Japan). The migration was normalized to that of untreated keratinocytes as a control, which was set to 100%, and expressed as a fold-change.

2.6 Cytokine array

The supernatants of keratinocyte cultures were collected 24 h after exposure to LTP for 1 or 3 min. The supernatants from untreated cells were used as controls. Cytokine levels were measured by cytokine arrays (R&D, Minneapolis, MN, USA), which included nine cytokine targets, specifically GM-CSF, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17, IL-12, and IL-13.

2.7 Enzyme-linked immunosorbent assay

The supernatants of keratinocyte cultures were collected 24 h after exposure to LTP for 30 s or 3 min, or from untreated control cells, and were analyzed by enzyme-linked immunosorbent assay (ELISA) (Cusabio Technology, Wuhan, China) for eight selected molecular targets as follows: PDGF-A, PDGF-B, VEGF-A, HB-EGF, vascular angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), fibroblast growth factor 2 (FGF-2), and fibroblast growth factor 10 (FGF-10). For the inhibitor study, after exposure to LTP for 3 min and then immediate treatment with a HIF1α inhibitor [19], 30 μM of CAY10585 (Cayman Chemical, Ann Arbor, MI, USA) was added for 24 h. Unexposed cells were treated with dimethyl sulfoxide (DMSO).

2.8 Quantitative real-time PCR

The keratinocytes were harvested 24 h after LTP treatment for 30 s or 3 min and lysed with Trizol (Promega, Madison, WI, USA). Total RNA was extracted with a column-based total RNA extraction kit (MG-Med Geumchun-gu, Seoul, Korea) according to the manufacturer's instructions. RNA concentration measurements, cDNA synthesis, and quantitative real-time PCR (qPCR) were conducted as previously described [18]. Sequences of primers are listed in Table 1. The mRNA expression of target genes was normalized based on the $2 - \Delta\Delta Ct$ ratio [20].

2.9 Western blotting analysis

Keratinocytes were exposed to LTP for 3 min, immediately incubated with 30 μM CAY10585, and harvested 24 h after LTP treatment. Cells were lysed in ice-cold RIPA buffer (Biosesang, Seongnam, Gyeonggi-do, Korea) with a protease inhibitor cocktail and complete phosphatase inhibitor (Sigma, St. Louis, MO, USA) pre-added. The samples were incubated for 30 min at 4 °C with constant agitation, and centrifuged for 30 min (30,000×*g*, 4 °C). Protein concentration was measured with a Quick start Bradford protein assay kit (Bio-Rad, Irvine, CA, USA). Lysates were mixed with 5 × reducing sample buffer (Biosesang, Seongnam, Gyeonggi-do, Korea) and heated for 3 min at 95 °C. The samples were then loaded for gel electrophoresis, electro-transferred onto polyvinylidene difluoride (PVDF) membranes, and then blocked with 5% (w/v) skim milk in tris-buffered saline containing 0.1% tween-20 (TBST) at room temperature (RT). Membranes were then incubated with polyclonal rabbit anti-HIF1α primary antibody (1:500, Santa Cruz, CA, USA) or polyclonal rabbit anti-β-actin antibody (1:2000, Cell Signaling, Danvers, MA, USA) for 18 h at 4 °C. Samples were then incubated with horseradish peroxidase (HRP)-conjugated

Table 1 Real-time PCR primer sequences

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)
ANG-1	CAACCTTGTCAATCTTTGCACT	TCTGCACAGTCTCTAAATGGT
ANG-2	TAAGGACCCCACTGTTGCTA	TAGATGCCATTCGTGGTGTG
VEGF A	CGGTGCTGGAATTTGATATTCATTG	CGATTCAAGTGGGGAATGGC
HB-EGF	ACAGCGTGGGAACTCACTTT	TCTCGGTAGCAATTGGCAGG
PDGF-A	GAAGGCCTAGGGAGTCAGGT	TCACATCTGGTTGGCTGCTT
PDGF-B	GCCAGCGCCCATTTTTCAT	GTGTGTGCGCGCAAAGTATC
FGF-2	GAGAAGAGCGACCCTCACATCA	TCCTTCATAGCCAGGTAACGGT
FGF-7	GGCAAGTTTCCCTCCCTTTT	CAAGTGCTGTGTGCTAGACT
HPRT	GGACCCCACGAAGTGTTGGATAT	TCTCATCTTAGGCTTTGTATTTTGCT

goat anti-rabbit IgG antibody, as the secondary antibody (1:3000, Merck Millipore, Billerica, MA, USA), for 2 h at RT. The membranes were developed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Carlsbad, CA, USA), and imaged on a chemiluminescence imaging system (WAE-6100, Atto, Tokyo, Japan). The intensity of band images was analyzed with CS analyzer 4 software and then normalized to β-actin levels.

2.10 Statistical analysis

Results are expressed as the mean ± standard error (S.E.). Comparisons between groups were performed using PASW statistics 24 (SPSS Inc., Chicago, IL, USA), applying the Mann–Whitney *U* test. Differences were considered statistically significant at $p < 0.05$.

3 Results

3.1 LTP maintains cell viability and enhances *in vitro* wound healing

To investigate cell viability after LTP treatment, the EZ-Cytox cell viability assays were conducted to assess the cytotoxic effects of this treatment towards keratinocytes. According to the results, keratinocyte viability was not affected by LTP exposure (Fig. 1A). Moreover, we performed an *in vitro* wound healing assay in an insert μ-dish culture system 6 h and 24 h after LTP exposure for 30 s or 3 min. To inhibit the effects of cell proliferation during migration, mitomycin C (5 mg/ml) was added to the culture medium for 24 h after LTP treatment. Keratinocyte migration significantly increased 24 h after LTP exposure for 3 min compared to that in the untreated control group ($p < 0.05$; Fig. 1B, C).

3.2 LTP increases cytokine production in keratinocytes

To determine whether LTP treatment modulates cytokine secretion, we performed a cytokine array with the supernatant of keratinocytes after LTP treatment. According to our data, the level of GM-CSF significantly decreased at 24 h in supernatants of keratinocyte cultures after exposure to LTP for 1 or 3 min. However, the levels of IL-6, IL-10, and IL-17 significantly increased in the keratinocyte culture medium 24 h after exposure to LTP for 3 min (Table 2). Although the levels of IL-1β and IL-8 were slightly reduced, there was no significant difference. At the same time, IL-4, IL-12, and IL-13 were not detected (Table 2).

3.3 LTP increases the expressions of angiogenic genes and proteins in keratinocytes

Next, we measured the mRNA expression of angiogenic growth factors by real-time PCR. Ang-1, Ang-2, VEGF-A, HB-EGF, FGF-2, and FGF-10 mRNA levels were significantly increased 24 h after exposure to LTP for 30 s, compared to those in untreated controls ($p < 0.05$; Fig. 2A–F). Furthermore, LTP treatment for 3 min more robustly induced the expression of these markers compared to that after exposure for 30 s ($p < 0.05$; Fig. 2A–F). In addition, PDGF-AA and PDGF-BB were highly induced 24 h after exposure to LTP for 3 min, compared to that in untreated cells ($p < 0.05$; Fig. 2G, H).

We then determined the protein expression of angiogenic factors in the cell culture medium, 24 h after LTP treatment, by ELISA. The levels of Ang-1, Ang-2, VEGF-A, HB-EGF, FGF-2, and FGF-10 were significantly increased 24 h after exposure to LTP for 3 min, compared to those in the untreated controls ($p < 0.05$; Fig. 3A–F). Furthermore, the levels of PDGF-A and PDGF-B were

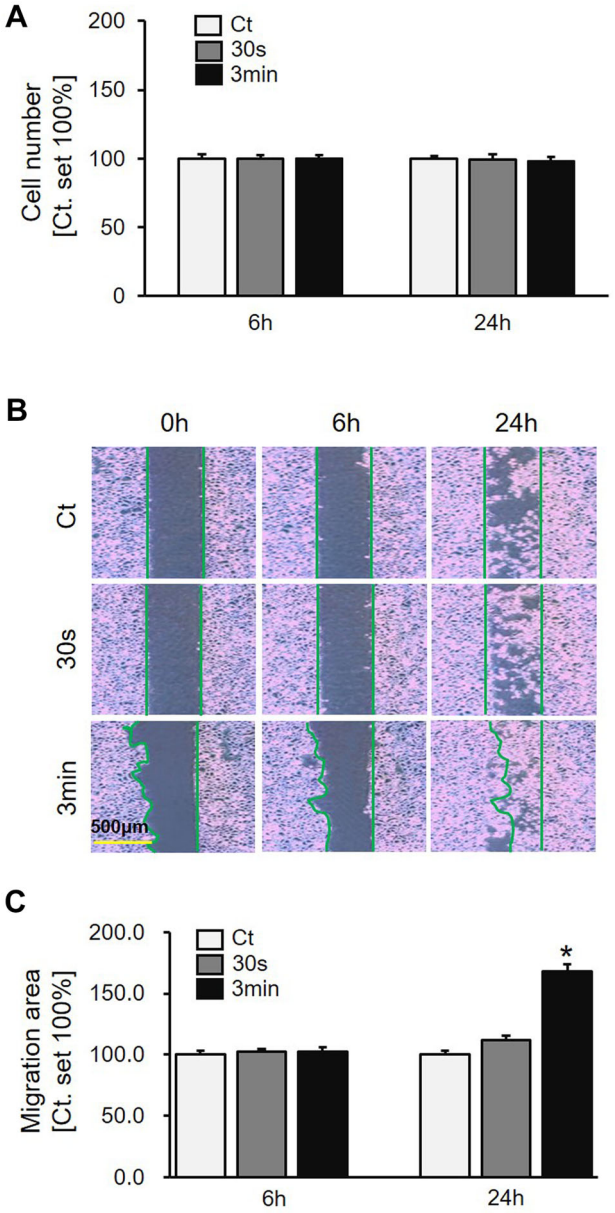

Fig. 1 Cell viability and migration of primary human skin keratinocytes after LTP treatment. LTP treatment maintains viability and induces migration of keratinocyte. **A** Keratinocyte viability was expressed as a percentage value of untreated cells. The untreated control are regarded as 100%. Values are expressed as mean ± SE from three independent experiments. *$p < 0.05$ versus the corresponding untreated control. **B** Photographs of keratinocyte migration at 6 and 24 h after exposure to LTP for 30 s or 3 min. Scale bar, 500 μm. **C** Quantification of keratinocyte migrations was expressed as a fold change, normalized to untreated cell (set to 100%). Data are expressed as mean ± S.E. from three independent experiments. *$p < 0.05$ versus the corresponding untreated control

significantly increased 24 h after exposure to LTP for 30 s and 3 min, compared to those in the untreated control group ($p < 0.05$; Fig. 3G, H).

Table 2 LTP treatment induces cytokine expression

Cytokine	Multiplex cytokine assay		
		1 min	3 min
GM-CSF	Fold change (LTP/control)	0.69 ± 0.134	0.76 ± 0.101
	P value	0.0303*	0.0306*
IL-1β	Fold change (LTP/control)	0.94 ± 0.063	0.86 ± 0.163
	P value	0.257	0.187
IL-6	Fold change (LTP/control)	0.90 ± 0.010	1.11 ± 0.024
	P value	0.055	0.026*
IL-8	Fold change (LTP/control)	0.91 ± 0.151	0.92 ± 0.162
	P value	0.216	0.262
IL-10	Fold change (LTP/control)	1.09 ± 0.013	1.15 ± 0.016
	P value	0.028*	0.022*
IL-17	Fold change (LTP/control)	1.03 ± 0.104	1.21 ± 0.012
	P value	0.383	0.013*
IL-4	Not determined		
IL-12			
IL-13			

Multiplex cytokine array analysis was performed using cell culture media from 24 h after LTP treatment for 1 min or 3 min or form untreated cells. The results are expressed as a fold change, untreated samples are marked as value 1. Each sample was assayed in duplicate, and the experiments were performed in three times independently. Data are expressed as mean ± SE *$p < 0.05$ versus the control group

3.4 LTP increases the production of angiogenic cytokines in a HIF-1α-dependent manner in keratinocytes

To confirm whether the expression of angiogenic growth factors is controlled by HIF-1α, keratinocytes were treated with CAY10585 for 24 h, which is an inhibitor of HIF-1α accumulation and transcriptional activity, after exposure to LTP for 3 min. Subsequently, the protein expression of HIF-1α was evaluated by western blot analysis. HIF-1α expression in the cell lysate was significantly induced 24 h after exposure to LTP for 3 min, compared to that in untreated keratinocytes ($p < 0.05$; Fig. 4A). However, CAY10585 treatment suppressed LTP-induced HIF-1α expression, compared to that in the untreated controls ($p < 0.05$; Fig. 4A). Similarly, levels of VEGF-A, Ang-1, and Ang-2 were significantly induced 24 h after exposure to LTP for 3 min, compared to those in the untreated keratinocytes ($p < 0.05$; Fig. 4B–D). Moreover,

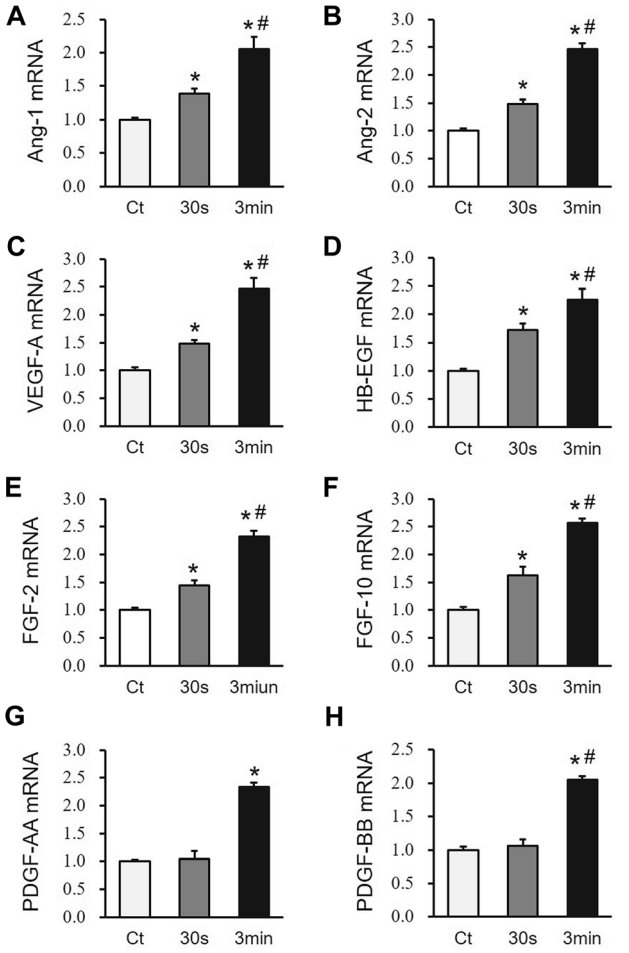

Fig. 2 mRNA expression of angiogenic growth factors after LTP treatment. Cell culture dishes were rinsed once with DPBS before exposure to LTP, then added 1.2 ml DPBS. Untreated dishes also undergone the above process. **A–H** The mRNA expression of Ang-1, Ang-2, VEGF-A, HB-EGF, FGF-2, FGF-10, PDGF-AA and PDGF-BB was measured 6 and 24 h after LTP treatment for 30 s or 3 min. The mRNA expression was calculated as ratio = $2^{-\triangle\triangle Ct}$, untreated cells are marked as value 1, and data are the mean ± SE from three independent experiments. $^{*}p < 0.05$ versus the corresponding untreated control group

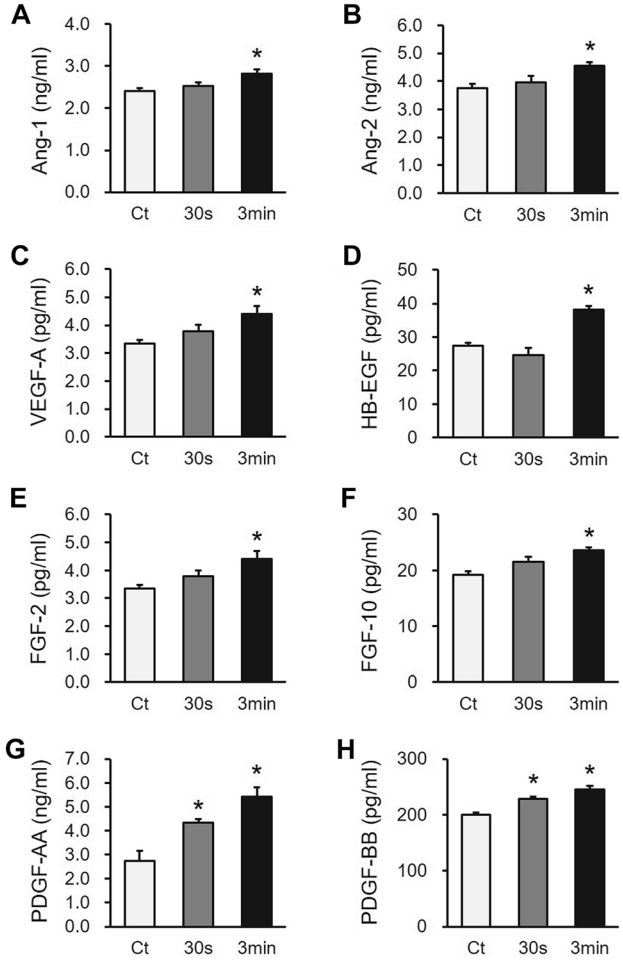

Fig. 3 Protein expressions of angiogenic growth factors after LTP treatment. **A–H** The concentration of Ang-1, Ang-2, VEGF-A, HB-EGF, FGF-2, FGF-10, PDGF-AA and PDGF-BB were measured by ELISA in keratinocytes cell culture supernatants 6 and 24 h after LTP treatment for 30 s or 3 min. Each sample assessed in duplicate, and the analysis performed three times independently. Data are the mean ± SE $^{*}p < 0.05$ versus the untreated control group

CAY10585 treatment significantly blocked the LTP-mediated increase in VEGF-A, Ang-1, and Ang-2 in the keratinocyte culture medium ($p < 0.05$; Fig. 4B–D).

4 Discussion

Over the past 10 years, plasma applications have been gradually developed for the field of medicine. LTP has been shown to be anti-bacterial, induce the stimulation of dermal cell migration and proliferation, and modulate redox signaling during wound regeneration [21, 22]. However, before plasma becomes a medical device, its efficacy and safety should be further evaluated at both the cellular and organism levels. In the present study, we aimed to identify whether cell migration, the expression of different cytokines, and growth factor production are induced by LTP without cytotoxicity in human primary keratinocytes.

We found that LTP treatment for 3 min did not affect keratinocyte viability (Fig. 1A), indicating that it is a safe dose for mammalian cells or for future *in vivo* studies. Moreover, we observed that LTP influences keratinocyte migration, as determined by a scratch wound healing assay, where cells were treated with mitomycin C to eliminate the effect of cell proliferation (Fig. 1B, C). These results are in consistent with a study by Schmidt et al. [9] which showed enhanced HaCaT cell migration with an indirect plasma treatment and indicated that these phenomena were related

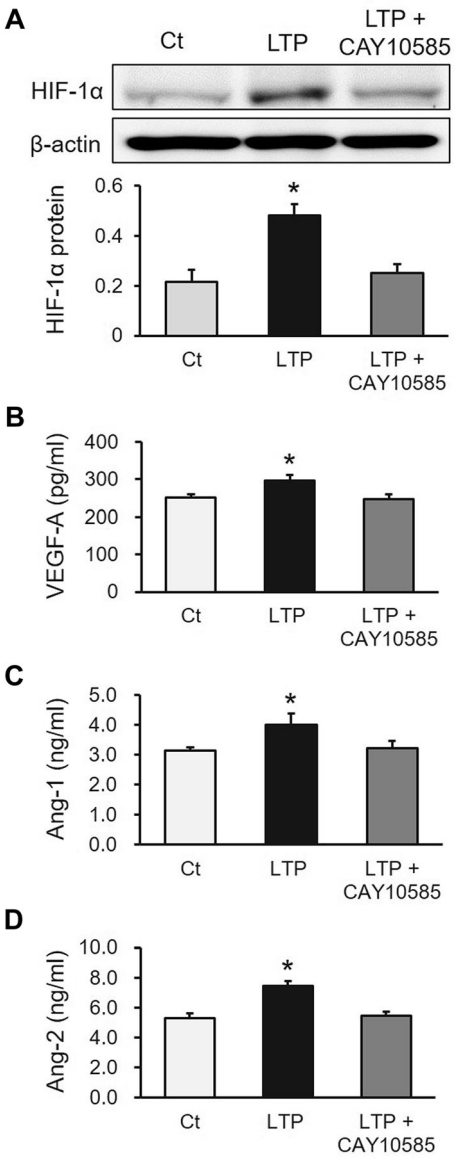

Fig. 4 HIF-1α inhibitor blocked the LTP-induced production of angiogenic growth factor in keratinocytes. **A** Expression of HIF-1α in keratinocytes after exposure to LTP was evaluated by western blotting. CAY10585 was administrated with 30 μM for 24 h immediately after LTP treatment for 3 min. The intensity of each protein band measured and HIF-1α expression was normalized to the ratio of β-actin. *$p < 0.05$ versus the untreated control group or CAY10585 treated group. **B–D** The levels of VEGF-A, Ang-1, and Ang-2 measured by ELISA in keratinocyte cell culture supernatant 24 h after LTP treatment for 3 min. All data are expressed as mean ± SE from three independent experiments. Data are expressed as mean ± SE *$p < 0.05$ versus the untreated control group or CAY10585 treated group

to changes in junction proteins and adhesion molecules induced by LTP. Moreover, there is evidence that ERK activation also contributes to the cell migration induced by LTP [23]. In addition, animal experiments showed that pro-inflammatory cytokines and growth factors are abundant at the wound site, suggesting that they play an important role in keratinocyte migration [24, 25]. We found that LTP induced the secretion of IL-6 (Table 2), VEGF-A, HB-EGF, FGF2, and FGF-10 (Figs. 2C–F, 3C–F) in keratinocytes. These factors can exert multiple effects on keratinocytes individually or in combination, particularly with respect to cell proliferation and migration [25]. Thus, we suggest that enhanced keratinocyte migration is partially a response to the production of pro-inflammatory cytokines and growth factors induced by LTP.

LTP also treatment induced the expression of pro-inflammatory cytokines including IL-6 and IL-17 and the anti-inflammatory cytokine IL-10 (Table 2). In an *in vitro* study, IL-17 induced the expression of antimicrobial peptides in keratinocytes [26]. In addition, IL-17 administration promoted scar formation by increasing the number of macrophages in a cutaneous excisional mouse model [27]. Conversely, blocking or the genetic deletion of IL-17 resulted in delayed wound closure in animals [28]. The cytokine IL-6 induces keratinocyte proliferation *in vitro*. IL-6 knockout mice were shown to exhibit a delay in re-epithelialization and impaired granulation tissue formation; in contrast, excessive IL-6 leads to cutaneous scarring [29, 30]. IL-10 inhibits the overexpression of chemokines and pro-inflammatory cytokines including IL-6 and TNF-α *in vivo*. Lenti-IL-10 injection to a wound was found to result in reduced inflammation, scar-less healing, and the restoration of normal dermal architecture [31, 32]. Therefore, we suggest the LTP treatment might have an important effect in regulating the coordinated expression of multiple cytokines for the purpose of maintaining normal wound repair. Moreover, the expression of pro-inflammatory cytokines was found to be prominently decreased in wound healing-impaired mice [33] and exaggerated inflammation is a necessary prerequisite for scar formation. For example, the enhanced activity of pro-inflammatory cytokines increases the concentration of profibrotic cytokines such as TGF-β, which might induce hypertrophic scars in burn or infected wounds [34].

It is well known that angiogenesis plays an important role in wound healing. Newly formed blood vessels contribute to the formation of granulation tissue and provides nutrition and oxygen to support growing tissues [35]. HIF-1α is widely recognized as a controller of angiogenesis, as it regulates the expression various pro-angiogenic factors including VEGF and FGF [36]. In our study, we observed the increased expression of intracellular HIF-1α following LTP treatment in keratinocytes (Fig. 4A). Moreover, LTP treatment also significantly induced both the mRNA and protein expression of angiogenic growth factors including Ang-1, Ang-2, VEGF-A, HB-EGF, PDGF-AA, PDGF-BB, FGF-2, and FGF-7, as measured by qPCR and ELISA (Figs. 2, 3). However, treatment with a HIF-1α inhibitor,

CAY10585, blocked the LTP-induced upregulation of angiogenic growth factors (Fig. 4). A recent study showed that LTP treatment increases angiogenesis in an animal pressure ulcer model [8]. Several studies also suggested specific role for HIF-1α in cell migration. In one study, th HIF-1α inhibitor vitexin significantly inhibited the migration of rat pheochromocytoma PC12 cells [1, 37]. The migration of embryonic fibroblasts cultured from HIF-1α-knockout mice was also found to be significantly reduced when compared with that of wild-type cells. However, this phenomenon was partially rescued by HIF-1α gene transfer [2, 38]. Furthermore, HIF-1α knock-down by siRNA transfection in HaCaT keratinocytes inhibited their migration [3, 39]. This evidence clearly shows that HIF-1α is an upstream regulator of cell migration. Our results showed that LTP treatment upregulates HIF-1α expression in keratinocytes, thereby increasing their migration.

In summary, this study demonstrated that LTP improves wound healing in human primary keratinocytes by inducing inflammation-relevant cytokines, cell migration, and the production of angiogenic factors, which are mediated HIF-1α upregulation in response to LTP. Impaired angiogenesis has been shown by many studies to be associated with pathological wound repair seen in delayed and impaired wound healing animal models or chronic, non-healing wound repair in patients. Keratinocyte-derived angiogenic growth factors are critical for impaired angiogenesis. Therefore, we believe that LTP might improve angiogenesis during delayed wound repair. Future research will confirm the results of the current *in vitro* experiments using an animal study and will evaluate other beneficial effects of LTP treatment *in vivo*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The study protocol was approved by Institutional Review Board of Hallym University Hangang Sacred Heart Hospital (2018-018). This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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