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Stem cell recovering effect of copper-free GHK in skin

Hye-Ryung Choi,[a] Youn-A Kang,[a] Sun-Jong Ryoo,[c] Jung-Won Shin,[a,b] Jung-Im Na,[a,b] Chang-Hun Huh[a,b] and Kyoung-Chan Park[a,b]*

The peptide Gly-His-Lys (GHK) is a naturally occurring copper(II)-chelating motifs in human serum and cerebrospinal fluid. In industry, GHK (with or without copper) is used to make hair and skin care products. Copper-GHK plays a physiological role in the process of wound healing and tissue repair by stimulating collagen synthesis in fibroblasts. We also reported that copper-GHK promotes the survival of basal stem cells in the skin. However, the effects of copper-free GHK (GHK) have not been investigated well. In this study, the effects of GHK were studied using cultured normal human keratinocytes and skin equivalent (SE) models. In monolayer cultured keratinocytes, GHK increased the proliferation of keratinocytes. When GHK was added during the culture of SE models, the basal cells became more cuboidal than control model. In addition, there was linear and intense staining of α6 and β1 integrin along the basement membrane. The number of p63 and proliferating cell nuclear antigen positive cells was also significantly increased in GHK-treated SEs than in control SEs. Western blot and slide culture experiment showed that GHK increased the expression of integrin by keratinocytes. All these results showed that GHK increased the stemness and proliferative potential of epidermal basal cells, which is associated with increased expression of integrin. In conclusion, copper-free GHK showed similar effects with copper-GHK. Thus, it can be said that copper-free GHK can be used in industry to obtain the effects of copper-GHK *in vivo*. Further study is necessary to explore the relationship between copper-free GHK and copper-GHK. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: GHK; skin equivalents; stem cells

Introduction

Peptides have the potential as promising pharmacological agents [1]. Furthermore, peptides are a rapidly expanding category of cosmeceuticals because they can affect many processes such as cell proliferation, inflammation, and melanogenesis [2].

The peptide Gly-His-Lys (GHK) is a naturally occurring copper (II)-chelating motifs in human serum and cerebrospinal fluid [3] and possesses a high affinity for copper(II) ions [4]. Copper-GHK, which was first described as a growth factor for a variety of differentiated cells, modulates the formation of new connective tissue [5]. In addition, recent studies have shown that copper-GHK plays a physiological role in the process of wound healing and tissue repair by stimulating collagen synthesis in fibroblasts [6]. We also reported that copper-GHK may increase the proliferative potential of keratinocytes by regulating extracellular matrix proteins such as α6 and β1 integrins [7]. Interestingly, it is reported that copper can alter the keratinocyte integrin expression during the re-epithelization and remodeling phases of wound healing [8]. In industry, GHK (with or without copper) is used to make hair and skin care products. Then, it will be important to investigate the effects of copper-free GHK (GHK). However, the effects of GHK are not studied well. In the skin, keratinocytes are the major cell type of the epidermis, and integrins are a family of cell surface receptors present on keratinocytes that enable keratinocyte signaling and interaction with other cells and the extracellular matrix [8]. Because of the complexity of skin biology, skin equivalent (SE) models are considered as an attractive model. In the present study, the effects of GHK were studied using cultured normal human keratinocytes and SE models.

Materials and Methods

Chemicals

Gly-His-Lys was obtained from Beadtech Co. (Seoul, Korea). Antibodies against integrin α6 (#sc-6597), integrin β1 (#sc-9970), p63 (4A4, #sc-8431), and actin (#sc-1616) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody against proliferating cell nuclear antigen (PCNA; #M0879) was obtained from Dako (Glostrup, Denmark).

* *Correspondence to: Kyoung-Chan Park, Department of Dermatology, Seoul National University Bundang Hospital, Gumi-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-707, Korea. E-mail: gcpark@snu.ac.kr*

a *Department of Dermatology, Seoul National University Bundang Hospital, Seongnam-si, Gyeonggi-do 463-707, Korea*

b *Department of Dermatology, Seoul National University College of Medicine, Seoul 110-744, Korea*

c *Beadtech Inc, Suite 312, 135-dong, Seoul National University, Gwanak-gu, Seoul 151-744, Korea*

Cell Culture

Normal human keratinocytes and fibroblasts were isolated from human foreskins obtained during circumcision. All samples were obtained with informed consent. Skin specimens were processed according to the method described [9], as modified in our laboratory using thermolysin (Sigma, St. Louis, MO, USA). Keratinocytes were cultured in keratinocyte growth medium (KGM; Clonetics, San Diego, CA, USA), and fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; LM001-05, WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT, USA).

MTT Assay

To test the cytotoxicity of GHK, cells (4×10^4 cells/well) were seeded into 24-well plates. After 24 h serum starvation, cells were then incubated with GHK (0.1, 1, and 10 μM) for 24 h at 37 °C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT solution; 100 μl of 5 mg/ml) was then added, after which the plates were incubated for an additional 4 h. The supernatant was then removed, and the formazan crystals were solubilized in dimethylsulfoxide (1 ml). Next, the optical density was determined at 540 nm using an ELISA reader (TECAN, Salzburg, Austria).

Keratinocytes Proliferation Assay

To test the proliferative effects of GHK on keratinocytes, cells were incubated with 0.1–100 μM of GHK for 72 h at 37 °C. In addition, long-term proliferation assay was performed. Second passage keratinocytes were cultured in KGM (Clonetics), and GHK was added at a concentration of 1 μM. When the cells reached approximately 80% confluence, they were detached and cultured into new dishes. To prevent GHK from having an effect on the attachment of cells or trypsin treatment, GHK was added 1 day after splitting the cells. Each time the samples were split, the total cell numbers were determined using a hemocytometer.

5-Bromo-2′-deoxyuridine Incorporation Assay and Propidium Iodide Staining

Keratinocytes (1×10^6 cells) were plated onto 100 mm culture dish. After 24 h, cells were starved for another 24 h. Cells were treated with or without GHK (1 μM) for 4 h prior to 5-bromo-2′-deoxyuridine (BrdU; 1 μM, 203806, Merck Millipore Calbiochem, Darmstadt, Germany) treatment. After 72 h, trypsinized cells were fixed in cold 70% ethanol at 4 °C for 1 h. For BrdU detection, cells were incubated with fluorescein isothiocyanate (FITC) mouse anti-BrdU or FITC mouse IgG1 isotype control (FITC mouse anti-BrdU set, 556028, BD Biosciences Pharmingen, San Diego, CA, USA) at RT for 30 min. Then, for apoptosis detection, cells were incubated at RT for 30 min in the buffer containing 5 μg/ml propidium iodide (Sigma), 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7), and 25 μg/ml RNase A. Stained cells were analyzed by flow cytometry (FACSCalibur System, BD Biosciences).

Construction of Three-dimensional Skin Models

Skin equivalents were constructed following our previous method [10]. Briefly, dermal substitutes were prepared according to the method described with some modification [11]. Type I collagen was extracted from the tendons of rat tails. Dermal substitutes were then made by mixing eight volumes of type I collagen with one volume of 10× concentrated DMEM and one volume of neutralization buffer (0.05N NaOH, 0.26 mM $NaHCO_3$, and 200 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)) and then adding 5×10^5 fibroblasts. After gelling in a 30 mm polycarbonate filter chamber (3.0 μm Millicell; Millipore, Bedford, MA, USA), human keratinocytes (1×10^6 cells) were then seeded onto the dermal substitute. After 1 day in a submerged state, they were cultured at the air–liquid interface for 12 days. The growth medium consisted of DMEM and Ham's nutrient mixture F12 at a ratio of 3 : 1, supplemented with 5% fetal bovine serum, 0.4 μg/ml hydrocortisone, 1 μM isoproterenol, 25 μg/ml ascorbic acid, and 5 μg/ml insulin. A low concentration of Epidermal Growth Factor (EGF) (1 ng/ml, Invitrogen Co., Carlsbad, CA, USA) was also added during the submerged culture, and a higher concentration of EGF (10 ng/ml) was added during the air–liquid interface culture. The medium was changed three times per week, and all experiments were repeated at least twice under the same conditions. In addition, GHK (1 μM) was added 1 day after air exposure, and fresh peptides were added each time when the medium was changed.

Histology and Immunohistochemistry

After 13 days, SEs were fixed in Carnoy's solution (ethanol/chloroform/acetic acid, 6 : 3 : 1) for 30 min and then processed for conventional paraffin embedment. Briefly, 4- to 6-μm-thick sections were prepared and stained with hematoxylin and eosin. For immunohistochemical analysis, the sections were processed using the avidin–biotin–peroxidase complex technique (Dako).

Western Blot Analysis

Second passage keratinocytes were detached and cultured into new dishes. One day after splitting, GHK (1 μM) was added, and the cells were then incubated for an additional 3 days. Next, the cells were lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete™, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Some 10 μg of protein per lane were then separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes, which were saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were then incubated with the appropriate primary antibodies at a dilution of 1 : 1000, followed by additional incubation with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK).

Immunocytochemistry

Keratinocytes (3.5×10^4 cells) were plated onto two-well chamber slides (154852, Thermo Scientific Nunc, Rochester, NY, USA) and cultured for 24 h. Cells were treated with or without GHK (1 μM) for 48 h. Cultured cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 10 min and were blocked with peroxidase block solution (K4005, Dako, Carpinteria, CA, USA) for 5 min. Slides were then incubated with an integrin β1 antibody (sc-9970, Santa Cruz Biotechnology) at a dilution of 1 : 100 for 30 min. Integrin β1 was detected with a peroxidase-labeled polymer conjugated antibody and 3-Amino-9-ethylcarbazole (AEC) + substrate-chromogen solution (K4005, Dako) according to the manufacturer's protocol. Slides were counterstained with Mayer's hematoxylin solution.

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Statistics

Data were compared using a student's *t*-test, and $p < 0.05$ was considered to be statistically significant.

Results

The Effects of GHK on Cultured Keratinocytes

Cultured cells were treated with GHK (0.1, 1, and 10 μM) for 24 h and then analyzed using an MTT assay. The results showed that GHK was not cytotoxic to keratinocytes or fibroblasts in these range of concentration (data not shown).

Keratinocyte Proliferation Assay

The proliferative effects were tested by adding 0.1–100 μM of GHK (Figure 1). The results revealed dose-dependent stimulatory effects in response to treatment with GHK. Then, long-term proliferation assays were conducted using a concentration of 1 μM GHK. Treatment with GHK increased the number of keratinocytes when compared with control cells. Specifically, the total number of keratinocytes after six passages was 260 times that of the initial seeding in the control group but 300 times greater than the initial seeding in the GHK group (Figure 2)

5-Bromo-2′-deoxyuridine Incorporation Assay

Results showed that the treatment of GHK significantly increased the stainability of BrdU (Figure 3(B, D)).

Reconstruction of SEs and the Effect of GHK

For the treatment of SE models, 1 μM of GHK was used. Histologic findings showed characteristic multilayering and stratification of the epidermis in all SE models. There was no significant difference in the thickness of the epidermis (Figure 4). However, the basal cells were more cuboidal when GHK was added (Figure 4(C), inset). Integrin $\alpha 6$ is a marker of extracellular adhesion receptors that is distributed along the dermal epidermal junction and indicates the existence of hemidesmosomes [12]. Integrin $\beta 1$, which is expressed throughout the basal cell membrane, plays a role in cell–matrix and cell–cell interactions [13].

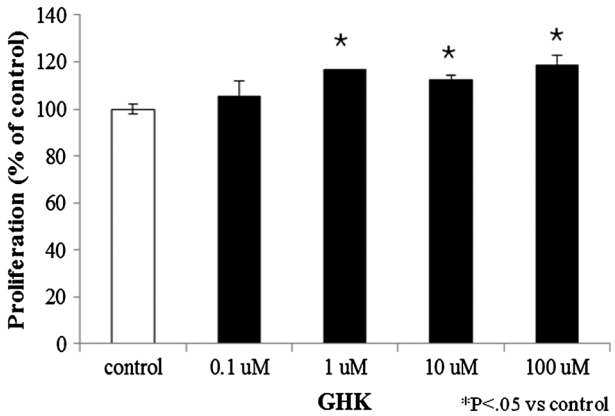


Figure 1. The effect of GHK on keratinocytes. Cells were treated with GHK (0.1–100 μM) for 72 h at 37 °C under 5% CO_2, as described in the Materials and Methods Section. The results are shown as the optical density value or absorbance value (O.D.) following MTT assay. The values shown are the means ± SD of triplicate wells. *$p < 0.05$, **$p < 0.01$ compared with control.

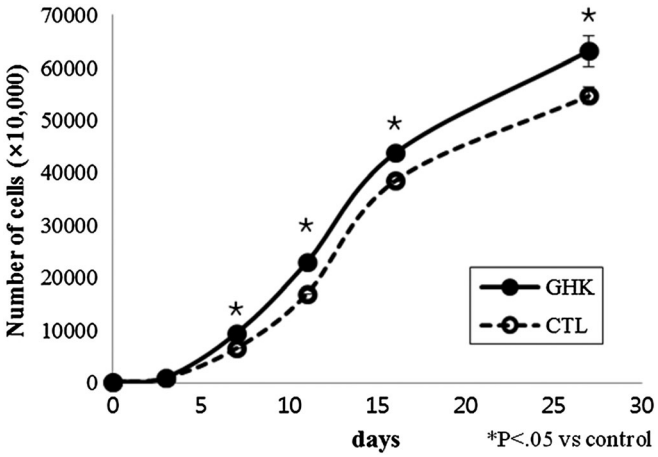
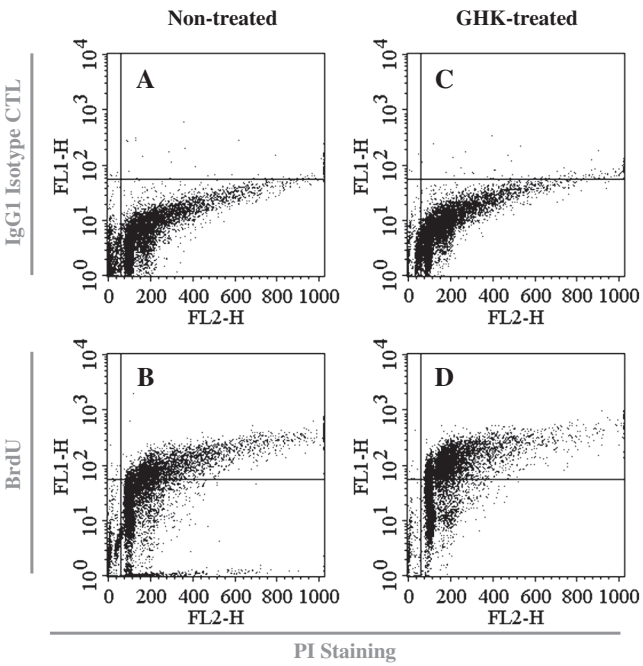


Figure 2. The long-term effect of GHK on the proliferation of keratinocytes. Cells were treated with GHK, as described in the Materials and Methods Section. The results are shown as the number of cells that were counted at each passage. Initially, 2.1×10^6 cells were split for each treatment. At the final harvest, the number of cells in the group that was treated with GHK was significantly higher than the number of cells in the control group (GHK, 6.30×10^8; control, 5.46×10^8). All experiments were repeated twice, and the values shown are from a representative experiment.



%	UL	UR	LL	LR
B	0.09	**30.77**	8.74	60.40
D	0.07	**55.17**	2.43	42.33

Figure 3. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay. Cells were treated with or without GHK (1 μM) for 4 h prior to BrdU, as described in the Materials and Methods Section. Results (B and D, lower table) showed that the treatment of GHK significantly increased the stainability of BrdU [(A) IgG1 Isotype CTL/PI stained non-treated cells, (B) BrdU/PI stained non-treated cells, (C) IgG1 Isotype CTL/PI stained GHK-treated cells, and (D) BrdU/PI stained GHK-treated cells]. The results shown are representative of experiments that were conducted in duplicate.

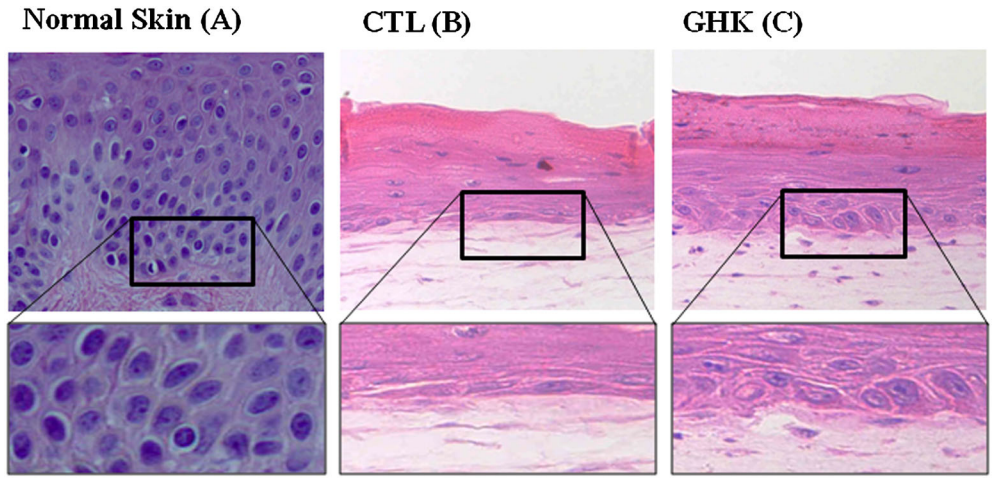


Figure 4. Histology of normal skin (A) and SEs [(B) control SE, (C) SE with GHK]. SE were treated with GHK (1 μM) as described in the Materials and Methods Section. Following hematoxylin and eosin staining, epidermal thickness and the shapes of basal cells were observed by microscopic examination. There was no significant difference in epidermal thickness, but the basal cells appeared to be more cuboidal in GHK-treated models (×200).

Immunohistochemical staining also revealed linear and intense staining of α6 and β1 integrin along the basement membrane in GHK-treated models (Figure 5).

p63 is a potential stem cell marker that belongs to a family that includes two structurally related proteins, p53 and p73 [14]. It is also known that PCNA is present throughout the cell cycle in

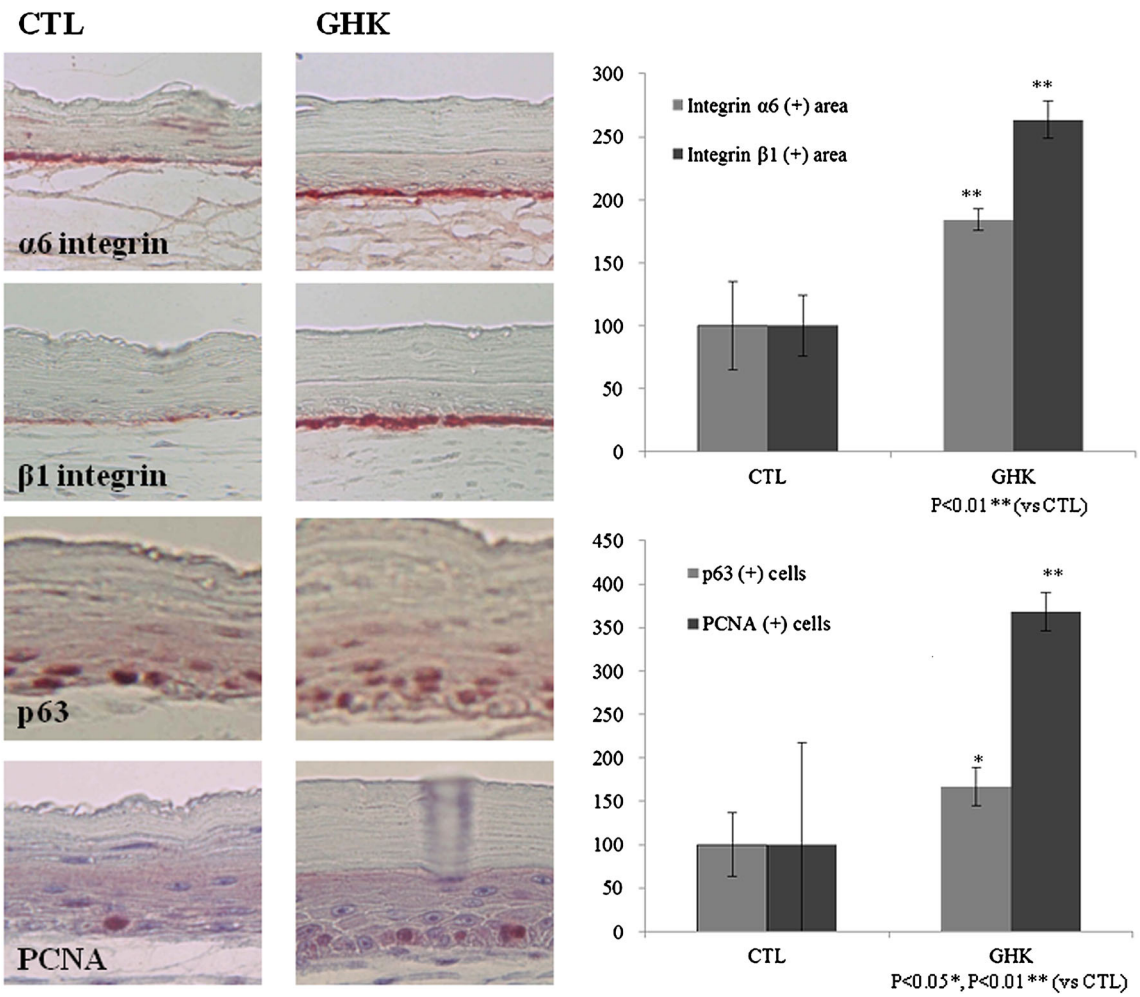


Figure 5. Immunohistochemical findings of GHK-treated three-dimensional SE models as described in the Materials and Methods Section. The expression of α6 and β1 integrin was significantly increased by the addition of GHK (×200). The number of p63 and PCNA positive cells was also significantly increased in response to treatment with GHK. Experiments were repeated at least twice, and the results shown are a representative data.

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proliferating cells and a marker of proliferating cells [15]. Immunohistochemical staining for p63 and PCNA was performed, and the number of p63 and PCNA positive cells was counted in randomly selected five different high power fields. Results showed that the number of p63 and PCNA positive cells was significantly higher in GHK-treated SEs than in control SEs (Figure 5). All experiments were repeated at least twice.

GHK Increased the Integrin Expression

Western blot analysis revealed that GHK led to increase expression of both integrin $\alpha 6$ and $\beta 1$ at passage 3 (Figure 6(A)). Furthermore, third passage keratinocytes, cultured on a chamber slide, express more $\beta 1$ integrin when GHK was added (Figure 6(C)).

Discussion

Gly-His-Lys is a tripeptide with affinity for copper(II) ions, and copper-GHK stimulates collagen synthesis by fibroblasts [4]. Therefore, copper-GHK is frequently used to make skin and hair care formulations. However, to the best of our knowledge, the effects of copper-free GHK have not been studied well. The epidermis is the outermost layer of the skin. Thus, it is essential for the epidermis to be able to continually self-renew and regenerate following injury. These characteristics are critically dependent on the ability of the principal epidermal cell type, the keratinocyte, to proliferate and to respond to differentiation cues [16]. In this study, the effect of GHK was tested using cultured normal human keratinocytes and cultured SEs. We investigated the effects of GHK on keratinocytes and found that it increased the proliferation and the number of keratinocytes after repeated passages of culture (Figures 1 and 2). These results suggest that GHK can act as a growth factor for keratinocytes. Then, three-dimensional skin models were constructed to study the effects of GHK. When GHK was added to the SEs, the basal cells became cuboidal (Figure 4 (C), inset). In normal skin, basal cells are cuboidal in shape, with their long axes aligned perpendicular to the dermoepidermal junction (Figure 4(A), inset). These cuboidal keratinocytes become flattened as differentiation progresses [17]. Thus, it can be said that cuboid basal cells have greater potential of proliferation than flattened cells. Accordingly, our findings showed that treatment with GHK increased the proliferative potential of basal cells. Furthermore, our results showed an increased expression of $\alpha 6$ and $\beta 1$ integrin along the basement membrane when SEs were treated with GHK (Figure 5). It is known that integrin expression is reduced in standard SE models compared with normal skin [17]. Then, our findings showed that GHK improved the microenvironment of epidermal basal cells, which is associated with the modulation of integrin expression. As already mentioned, p63 is known as a putative stem cell marker of skin. In normal skin, expression of p63 was noticeable in the nuclei of cells in the basal and suprabasal layers of the epidermis [18]. Our results revealed that GHK increased the number of p63 positive cells (Figure 5). These findings suggest that GHK promotes the survival of basal stem cells in SEs. Then, PCNA positive cells were counted and found to increase by the addition of GHK (Figure 5). These results showed that the number of proliferating cells is also increased by the addition of GHK (Figure 5). All these findings are exactly consistent with results that were obtained by long-term culture experiment (Figure 2) or flow cytometry analysis using BrdU staining (Figure 3). It means that addition of GHK increased the stemness and the proliferative potential of epidermal basal cells.

Integrin $\alpha 6\beta 4$ plays a positive role in the maintenance of basal keratinocyte properties and epidermal homeostasis [12]. Then, cells were treated with GHK, and western blot analysis was performed to study the effects of GHK on integrin expression. Results showed that the integrin $\alpha 6$ and integrin $\beta 1$ levels were increased in response to treatment with GHK (Figure 6(A)). Slide culture experiment also showed that GHK increased the expression of integrin $\beta 1$, which is also known as a putative stem cell marker (Figure 6(C)).

In summary, our results showed that addition of copper-free GHK increased the expression of both $\alpha 6$ and $\beta 1$ integrins. In addition, the increased number of p63 and PCNA positive cells was observed in response to treatment with GHK. Therefore, it can be said that copper-free GHK increased the stemness and the proliferative potential of epidermal basal cells.

Previously, we reported that copper-GHK increases integrin expression and p63 positivity in three-dimensional SE models [7]. However, the present study showed that copper-free GHK also has beneficial effects on keratinocytes even without copper ions. Although the exact composition of KGM (Clonetics) is not known, the presence of copper in culture medium is suspected. Then, trace

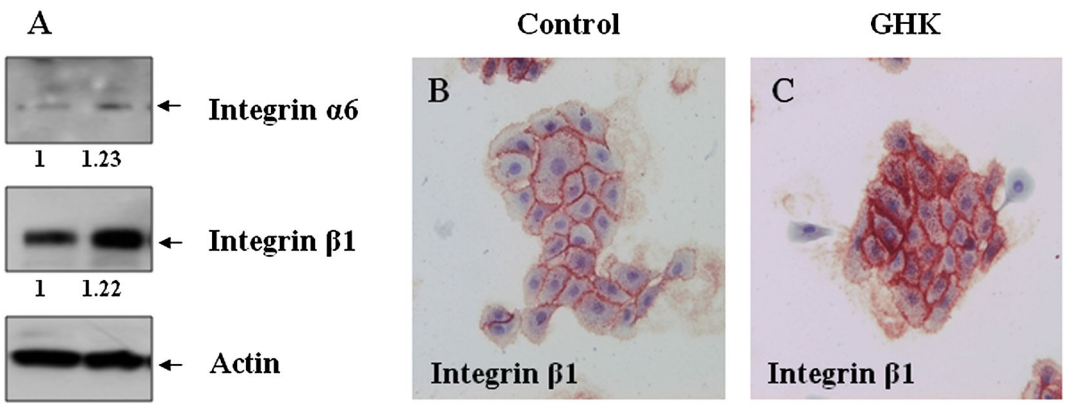

Figure 6. (A) Cultured keratinocytes were treated with or without 1 μM GHK for 3 days. The cells were then lysed, and western blot analyses were conducted as described in the Materials and Methods Section. Equal protein loadings were confirmed using anti-actin antibody. Fold increases over the control were determined by densitometric analysis. The result shown is representative of experiments that were conducted in triplicate. (B) Cells were also cultured for 1 day on chamber slides. Then, cells were treated with or without GHK (1 μM) for 2 days as described in the Materials and Methods Section. GHK-treated cells express more $\beta 1$ integrin. Experiments were repeated twice, and the results shown are a representative data.

amount of copper in the medium may bind GHK and can show these effects. This study provided evidences that copper-free GHK can be beneficial in various biological conditions, but further study is necessary to explore the relationship between copper-free GHK and copper-GHK.

Acknowledgements

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