JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Enhancements of Skin Cell Proliferations and Migrations via 6-Dehydrogingerdione

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ABSTRACT: Human skin protects the body from mechanical and chemical damages, and skin wound healing is a costly procedure and worldwide issue. A *Zingiber officinale* compound, 6-dehydrogingerdione (6-DG), is presented as a novel biofunctional healing agent for human skin wound repair. The effectiveness on cell growth/migration, growth factor, collagen amount, and enzymatic activity was assessed. 6-DG treatment accelerated cellular proliferation and migration dose-dependently. Enzyme-linked immunosorbent assay (ELISA) showed that 6-DG brought about higher growth factor productions on transforming growth factor-β (TGF-β), platelet-derived growth factor-$\alpha\beta$ (PDGF-$\alpha\beta$), and vascular endothelial growth factors (VEGF). Under phorbol 12-myristate 13-acetate (PMA) incubation, 6-DG increased fibroblast collagen yield obviously, reduced matrix metalloproteinase-1 (MMP-1) protein expression, and recovered tissue inhibitor of metalloproteinase-1 (TIMP-1) secretion. 6-DG also blocked the mitogen-activated protein kinase (MAPK) pathway by suppressing c-Jun protein levels and extracellular signal-regulated kinases (ERK) phosphorylation in fibroblasts. From all of the above, 6-DG has potential to be a novel agent for human skin repair.

KEYWORDS: *6-dehydrogingerdione, skin cells, growth factor, migration, collagen*

INTRODUCTION

Ginger, the powdered rhizomes of the herb *Zingiber officinale* Roscoe (Zingiberaceae, dietary ginger), is used widely as a spice throughout the world. In Chinese medicine, ginger has been used traditionally as a treatment for allergy, asthma, constipation, diabetes, gingivitis, nervous diseases, rheumatism, stroke, toothache, and antimicroorganism infection.[1−3] Ginger species have been documented for the treatment of chemotherapy-associated nausea, the suppression of platelet aggregation, and the inhibition of tyrosinase, cyclooxygenase, or nitric oxide synthase.[4−7]

The skin is the largest organ in the body of vertebrates and is composed of the epidermis, dermis, and hypodermis.[8] Keratinocytes in the epidermis and fibroblasts in the dermis are responsible for protecting the body from many mechanical and chemical damages and for wound repair. Cutaneous wound healing, a complicated and highly orchestrated process, is a worldwide concern and a costly procedure for all age ranges. At the present time, there is only one agent for skin wound healing with the approval from the U.S. Food and Drug Administration: *all-trans*-retinoic acid. Retinoic acid elicits collagen production and reduces the matrix metalloproteinase (MMP) amount. However, this agent has several side effects, such as skin irritation, skin peeling, and teratogenicity.[9] The counteractive side effects offset the benefits on skin repair and may quench the use of retinoic acid. Steroids are another kind of well-known pharmaceutical used to regenerate skin injuries; however, their prolonged use shows side reactions of collagen degradation and inhibition of wound repair.[9] In the early stage of injury repair, it is believed that fibroblast migration around the wound margins initiates the wound closure. With the migratory force, the wound begins to close and the newly generated tissue begins to produce a resistance, which results in fibroblast differentiation.[10] These processes are characterized by local expression profiles of the skin cells, several growth factors, and extracellular matrix (ECM) changes. High cell growth and migration of keratinocytes and fibroblasts prompt the wound-healing progressions. Transforming growth factor-β (TGF-β),[11,12] vascular endothelial growth factor (VEGF),[13,14] and platelet-derived growth factor-$\alpha\beta$ (PDGF-$\alpha\beta$)[15] are three factors participating in the facilitation of skin cell proliferation. Skin cells are settled in the ECM, which belongs to the connective tissue, provides structural support, and regulates cell behaviors and communications. Collagen is the main component of ECM and is the most bountiful protein in connective tissue.

TGF-β is one of the growth factors that is related to cell differentiation, adhesion, proliferation, migration, and ECM precipitation. In wound repair procedures, TGF-β, produced by fibroblasts, keratinocytes, macrophages, and platelets, is essential in angiogenesis, connective tissue regeneration, inflammation, and re-epithelialization.[11] TGF-β, known to be the most potent growth factor, also regulates wound contraction, cell migration, and fibrotic scar formation throughout the body. Additionally, TGF-β boosts the proliferation of the myofibroblast phenotype and thus promotes scarring commonly related to cell migratory moments.[12] VEGF is generated, expressed, and secreted by fibroblasts, keratinocytes, macrophages, neutrophils, platelets, and endothelial and smooth muscle cells.[13] Also illustrated as the vascular permeability factor, VEGF is a key

Received: October 10, 2012
Revised: December 25, 2012
Accepted: December 26, 2012
Published: December 26, 2012

dx.doi.org/10.1021/jf304340q | J. Agric. Food Chem. 2013, 61, 1349−1356

moderator of endothelial cell migration, proliferation, and permeability in physiological and pathological angiogenesis.[14] PDGF-$\alpha\beta$ plays a vital role in human skin cell proliferations in all stages. Upon injury to the skin, PDGF is excreted from related cells and can be found in wound fluid.[15] PDGF and TGF-β allow the penetration of fibroblasts and the conversion of fibroblasts to myofibroblasts, which form constrictive forces to assist wound closures in ECM developments. PDGF stimulates immune macrophages to secrete TGF-β. PDGF also cooperates synergistically to increase the VEGF expression of hypoxia ischemic damage in tissues.[16] All of the growth factors mentioned have interactions with each other during skin cell proliferations and wound-healing processes.

MMPs comprise over 20 different human zinc peptidases of the metzincin superfamily.[17] This series of enzymes is responsible for the maintenance and turnover of macromolecules of ECM, such as collagen. MMP-1 (collagenase) is a vital initiator of ECM deterioration and collaborates with other MMPs in collagen degradation. MMP-1 expression from dermal fibroblasts is induced by some physiological factors, including growth factors, inflammatory cytokines, tumor promoters, and ultraviolet radiation. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, is recognized with the contribution to MMP-1 production through the activation of c-Jun and extracellular signal-regulated kinases (ERK) and for inhibiting the tissue inhibitor of metalloproteinases-1 (TIMP-1).[18] Remodeling and synthesis of ECM collagen in human skin dermal fibroblasts are important to cell proliferation and wound repair.

The biofunctions of dietary ginger extracts on skin wound healing have been recognized.[19,20] 6-Dehydrogingerdione (6-DG) is our target compound and a major phenolic alkanone, which is constituted from ginger and has strong antioxidative ability.[21] On the basis of our findings, this work is the first study to determine the effects of 6-DG on human normal skin cell regeneration according to in vitro cellular signaling pathway examinations.

MATERIALS AND METHODS

Chemicals and Reagents. Coomassie Brilliant Blue R250, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and PMA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO BRL (Gaithersburg, MD, USA). All other chemical buffers and reagents were purchased at the highest commercial purity and quality possible.

Plant Materials. Dried and chipped rhizomes of *Z. officinale* were extracted repeatedly with different mixtures of chloroform–methanol at room temperature, on the basis of previous papers.[2] The extraction yielded pure 6-DG from different fractions by silica gel column chromatography with gradients of *n*-hexane/$CHCl_3$ (Figure 1). Using

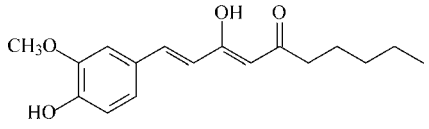

Figure 1. Structure of 6-DG.

spectroscopy, the compound was analyzed, identified, and compared with literature values.[22]

Human Dermal Fibroblasts and Epidermal Keratinocytes Cultures. Both human skin cell culture procedures followed the processes of the previous study.[23] Briefly, the primary cultures of human skin fibroblasts, derived from Chung-Ho Memorial Hospital, Kaohsiung Medical University, Taiwan, ROC (Institutional Review Board-II, KMUH-IRB-990269), were incubated in DMEM with 10% fetal calf serum, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. Human keratinocytes were isolated from foreskin primary culture and cultured in keratinocyte-SFM, supplemented with bovine pituitary extract (BPE) and EGF. The medium and growth supplements for keratinocytes contain γ-epidermal growth factor, BPE, insulin, fibroblast growth factor, and calcium (0.09 mM). All cell types were incubated at 37 °C in a humidified incubator with a 5% CO_2 atmosphere.

Cell Growth Assay. MTT assay was used to measure cell growth to test whether the compound used in this study could induce cell proliferation.[24] The cells were preincubated for 30 min in the absence or presence of 20 ng/mL PMA followed by incubation in testing samples. Briefly, skin cells were seeded in 96-well plates and treated with different concentrations of ginger compound or were untreated (as positive control) for 24 h. Stock MTT solution (5 mg/mL, dissolved in phosphate-buffered saline, PBS) was diluted 1:10 in culture medium, added to a culture dish, and then incubated at 37 °C for 2 h. At the end of the incubation period, the medium was removed and replaced with 0.05 mL of DMSO to dissolve the formazan crystals. The culture dishes were gently shaken for 20 min in the dark and added to a 96-well plate. The cell growth was calculated by the percentage of the control OD value (595 nm) on a multiwall scanning spectrophotometer (UV–vis, BioTek, Winooski, VT, USA). In consideration of the possible antiproliferative effects of DMSO, cultures were added with maximal 1% DMSO and used as a control, which was not found to affect skin cell growth or other assays. The standard deviations (SDs) of data derived from triplicate measurements.

Enzyme-Linked Immunosorbent Assay (ELISA). We performed ELISA to determine the amounts of TGF-β, VEGF, and PDGF produced after skin cells had been exposed to the testing compound. The skin cells were cultured in 6-well plates under conditioned medium, and the supernatant was collected at 24 h for analysis. Amounts of TGF-β, VEGF, and PDGF-$\alpha\beta$ secreted in the culture medium were determined from DuoSet ELISA development kits (R&D Systems, Minneapolis, MN, USA). The assay was performed according to the manufacturer's instructions, and protein amounts were measured and given as picograms per milliliter.

In Vitro Cell Culture Wound-Healing Assay. The potential of cellular migration was determined by cell culture wound-healing migration assays, which were performed according to the methods reported by ref 25. In brief, 5×10^5 cells were cultured onto 12-well plates and cultivated to complete confluence. A yellow 200 μL plastic pipet tip was used to create a clean 1 mm wide cellular wound area on a confluent culture of skin cells and washed three times to remove floating cells. Then either vehicle (medium containing 0.5% DMSO) or various concentrations of testing samples were added for the indicated time periods. After the indicated incubation time, the cellular wound gaps were photographed using an inverted phase-contrast microscope (TE2000-U; Nikon, Tokyo, Japan) equipped with NIS-Elements (Nikon) software. The migration and cell movement throughout the wound area were examined and calculated by the free software "TScratch" (www.cse-lab.ethz.ch/software.html).[26] Magnification used was 100×, bars indicate the SD.

Western Blot Analysis. A total of 1×10^6 cells were treated with 6-DG or vehicle control for 24 h. The cells were washed twice with ice-cold phosphate-buffered saline, harvested, and disrupted in lysis buffer (50 mM Tris-HCl, pH 7.5, 137 mM sodium chloride, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM phenylmethanesulfonyl fluoride, 2 μM leupeptin, and 2 μg/mL aprotinin).[27] The cell lysate was centrifuged at 10000*g* for 30 min at 4 °C, and the protein concentration in the supernatant was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Briefly, equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto a polyvinylidene fluoride (PVDF, PALL Life Science, Ann Arbor, MI, USA). The membrane was blocked for 1 h with 5% nonfat milk in PBS-T buffer (phosphate-buffered saline

containing 0.1% Tween 20). The membranes were incubated with corresponding primary antibodies, washed twice with PBS, and then incubated with secondary antibodies against the corresponding primary antibodies. The signals were visualized using enhanced chemiluminescence substrate (PerkinElmer, Boston, MA, USA). The signal intensities of proteins were analyzed using the software Gel-Pro v 3.0 (Media Cybernetics, Carlsbad, CA, USA).

Collagen Amount Measurement. For measurement of the total collagen amount synthesized by fibroblasts, Sirius Red dye (Direct Red; Sigma-Aldrich, St. Louis, MO, USA) was used to stain total collagen.[28] We compared the collagen secreted by fibroblasts incubated on 24-well plates with the testing sample. After the indicated time interval, media were removed and cells washed with PBS twice. One hundred microliters of 0.1% Sirius Red stain (0.05 g powder per 50 mL picric acid) was added to each well and kept at room temperature for 1 h. The unattached stain was removed and washed at least five times with 200 μL of 0.1 N HCl. The attached stain was extracted with 100 μL of 0.1 N NaOH and mixed well. The stain was placed into a 96-well plate to read the absorbance at 540 nm using a spectrometer (UV−vis, BioTek, Winooski, VT, USA). The specific cell collagen production was defined as the collagen amount produced per fibroblast cell.

Collagen Zymography. Collagen zymography was performed using 10% polyacrylamide gels containing 0.1% collagen.[29] After electrophoresis, the SDS present was removed by treatment with Triton X-100 (2.5%), followed by incubation in a Tris-based buffer containing NaCl, $CaCl_2$, and $ZnCl_2$ at 37 °C overnight. The gel was then stained with Coomassie Brilliant Blue R250, and the MMP-1 (interstitial collagenase) activity was detected (52 kDa) as unstained collagen degradation zones within the gel. The signals were analyzed using Gel Pro v.4.0 software (Media Cybernetics, Silver Spring, MD, USA).

Statistical Analysis. All data values are presented as the mean values (±SD) of at least three independent experiments. When appropriate, data were analyzed by the Student's t test. Statistical significance was assumed of a p value of <0.05.

RESULTS

Cell Growth of 6-DG Treated in Human Fibroblasts and Keratinocytes. Ten natural pure compounds, including gingerols and shogaols, isolated from *Z. officinale* were used to screen their impacts on skin cell proliferations. The MTT assay was used to investigate the cell growth of human skin dermal fibroblasts and epidermal keratinocytes treated for 24 h. Using 100 μM concentrations of 10 compounds for preliminary screenings, 6-DG was identified as the target compound for further research (Figure 1). Human normal skin fibroblasts and keratinocytes were treated with various concentrations from 0 to 100 μM 6-DG (Figure 2). We found the compound at low doses (2 and 10 μM) yielded high cell growths in these two cells. With the increased concentration of 100 μM, the growth rate of both cells decreased. With 6-DG, fibroblast growth was boosted within 50 μM (Figure 2A), and on keratinocytes, this compound showed a similar effective tendency under each equivalent condition (Figure 2B).

Cell Migrations of Fibroblasts and Keratinocytes Were Activated by 6-DG. The activations of fibroblast and keratinocyte migration affected by 6-DG were examined by the in vitro cell culture wound-healing assay, and the results are shown in Figure 3. The migratory capacity was evaluated by monitoring how effectively the monolayer cell invaded the wound region at both 2 and 10 μM for treatment periods of 12 and 24 h. Photographs in Figure 3A were the migration semblance of fibroblasts to show the repairing effects of 6-DG. Obvious enhancing effects were observed at treatment conditions, and the quantification analysis is shown in Figure 3B.

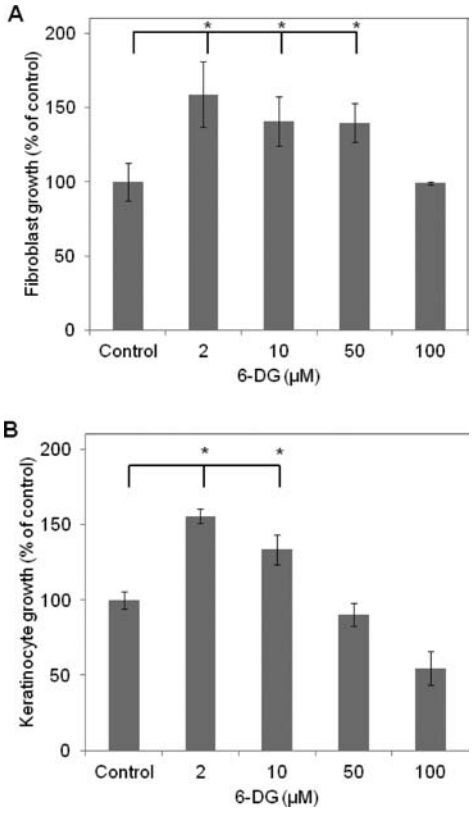

Figure 2. Human fibroblast (A) and keratinocyte (B) cell growth with 6-DG at various concentrations for 24 h. The data are shown as the mean value ± SD of three independent experiments. Significance for three different time-point groups was accepted at $p < 0.05$ (*) versus their corresponding controls.

6-DG at 2 μM, 24 h, repaired the clearing area more quickly (266%) than at 10 μM and the vehicle control, and the migratory cells almost covered the vacant area. Keratinocyte migration was also enhanced due to 6-DG at the same testing conditions (Figure 3C,D). With 2 and 10 μM 6-DG, the migration of keratinocytes was enhanced 230 and 175% at 12 h and 355 and 273% at 24 h, respectively.

Increase of Growth Factors (TGF-β, PDGF-$\alpha\beta$, and VEGF) from Human Fibroblasts and Keratinocytes by 6-DG Treatment. Growth factors, such as TGF-β, PDGF-$\alpha\beta$, and VEGF, previously well-known cell proliferation enhancers, have been selected for scrutinizing by ELISA (Table 1). Because the compound was dissolved in DMSO, we added 1% of DMSO to the vehicle control to be certain that it did not affect these assays. Our results showed that there was no considerable difference with 1% DMSO or not. We found that TGF-β in fibroblasts increased from 778 to 985 pg/mL (26% enhancement) and to 1003 pg/mL (29% enhancement) after we incubated 6-DG at 2 and 10 μM for 24 h, respectively. In keratinocytes, with both concentrations of this compound, TGF-β increased from 665 to 823 pg/mL (24% increment) and to 768 pg/mL (15% increment), respectively.

With the treatments of 6-DG at 2 and 10 μM on fibroblasts, the secretions of PDGF-$\alpha\beta$ increased from 2.5 to 7.1 pg/mL (184% enhancement) and to 5.3 pg/mL (112% enhancement), respectively, compared to the control group. In keratinocytes, 2 and 10 μM 6-DG treatments induced the increase from 2.2 to 5.8 pg/mL (165% increment) and to 4.6 pg/mL (107% increment) of PDGF-$\alpha\beta$ expression in contrast with the vehicle.

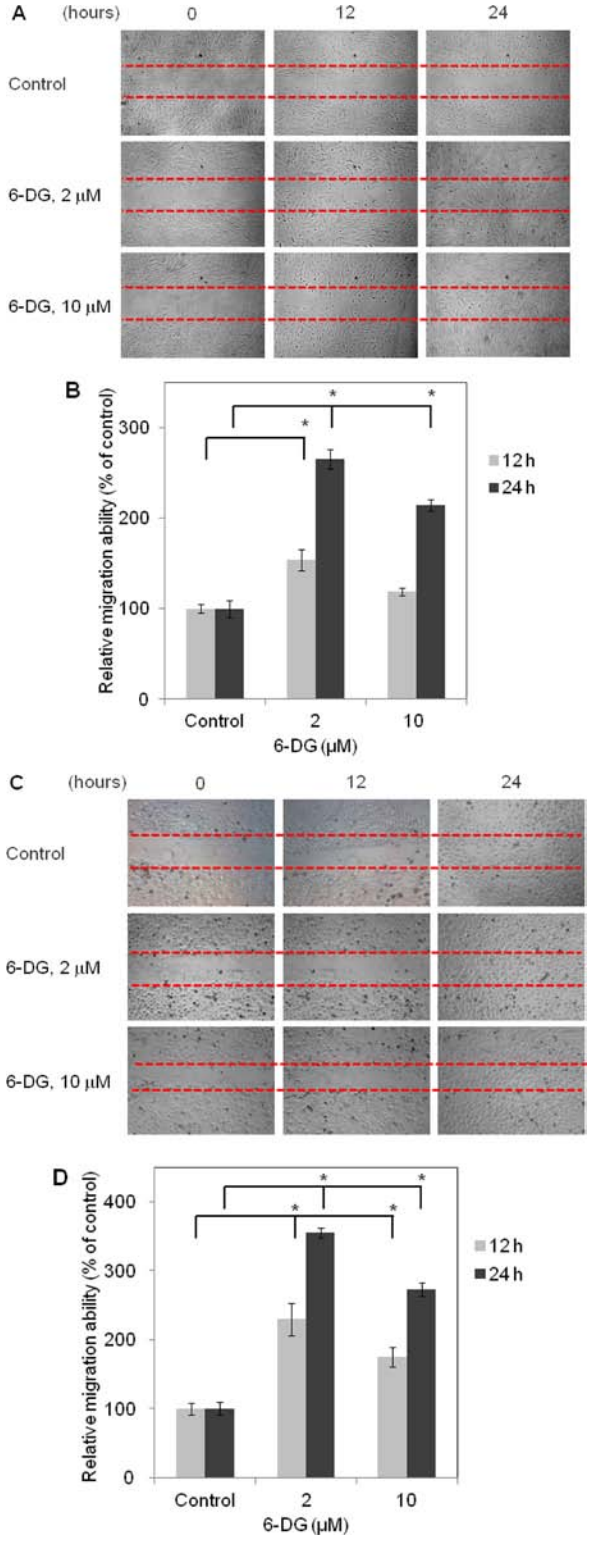

Figure 3. In vitro wound-healing assay on 6-DG-treated human skin cells: (A) migratory photographs of human fibroblasts; (B) quantification of the migration potential of 6-DG or PBS-treated cells; (C) photographs of keratinocytes; (D) keratinocytes migration quantifications. Fibroblasts or keratinocytes were cultured to monolayer confluence on an uncoated 6-well culture dish followed by applying a scratch with a sterile pipet tip. After extensive washing with PBS, cells were treated with either vehicle or various concentrations of 6-DG for indicated time periods.

VEGF, induced in fibroblasts by 6-DG at 2 μM, increased from 287 to 432 pg/mL (51% higher), and at 10 μM, no significant difference compared to the control was observed. In keratinocytes, with 2 and 10 μM 6-DG, the amount of VEGF presented an increase from 251 to 344 pg/mL (37% enhancement) and to 293 pg/mL (17% enhancement), respectively. The 6-DG treatment effects on the three growth factor expressions in both skin cells were found to be similar.

6-DG Reversed the Effect of PMA on Fibroblast Growth. Because PMA (collagen synthesis inhibitor) enhances the expression of MMP-1 (collagen digester) and harms fibroblasts,[30] we applied this agent to induce cell damage as a negative control. Only 37% of fibroblasts survived treatment with 20 ng/mL PMA for 24 h (Figure 4A). With 6-DG only, fibroblast growth was higher than with the vehicle control. At 2, 10, and 25 μM 6-DG, fibroblast growth demonstrated increases of 35, 20, and 17%, respectively, which revealed that this compound, from 2 to 25 μM, did no harm to fibroblasts (consistent with Figure 2). After treatment with 2, 10, and 25 μM 6-DG, the cell amount recovered from 37 to 75, 75, and 67%, respectively, which showed the enhancements to reverse fibroblast cell growth damage after PMA incubation for 30 min.

Collagen Contents of Human Fibroblasts Promoted by 6-DG. There are many patients with injuries, burns, and metabolic disease suffering from various dermatologic damages, such as skin cell death and collagen loss.[28] Figure 4B shows the collagen content of human fibroblasts in the absence or presence of 20 ng/mL PMA prestimulation for 30 min followed with various doses of 6-DG for 24 h. As expected, PMA provoked collagen digestion and decreased the collagen amount to 32% compared to the control group. Treated with 6-DG, the collagen content dramatically presented enhancements to 87, 75, and 40% (at 2, 10, and 25 μM), respectively. With the same treatments of 6-DG after PMA management, we observed that the collagen amounts recovered to 100, 92, and 73%, respectively. In Figure 4C, we discovered that the specific cell collagen production was higher with 6-DG treatments from 2 to 25 μM. No matter with or without 20 ng/mL PMA, the specific cell collagen production ranged from 115 to 138% due to 6-DG enhancements compared to the control group.

Effects of 6-DG on PMA-Stimulated MMP-1 and TIMP-1 Production in Human Fibroblasts. MMP-1 was responsible for collagen destruction and was related to cell growth and migration. The following data were gained from Western blot analysis to explore PMA-activated MMP-1 and TIMP-1 expressions in normal human dermal fibroblasts (Figure 5). The cells in the absence or presence of 20 ng/mL PMA were preincubated for 30 min and then cultured with 6-DG at 2 and 25 μM. The presence of PMA increased the level of MMP-1 production. After treatment with 6-DG at 2 μM only, the MMP-1 amount showed no significant difference compared to the control (Figure 5A). When 6-DG was added after the PMA preincubation, we found that 6-DG decreased the MMP-1 amount. After the addition of PMA, the TIMP-1 amount became less, and when treated with 6-DG, TIMP-1 recovered. 6-DG improved the TIMP-1 expression, which subsequently blocked the activation of MMP-1 and inhibited collagen degradation. With 25 μM 6-DG, the Western blot data showed similar results to 2 μM (Figure 5B).

Inhibitory Effect of 6-DG on Mitogen-Activated Protein Kinase (MAPK) Activation. We attempted to determine whether 6-DG inhibited collagen degradation in PMA-treated

dx.doi.org/10.1021/jf304340q | J. Agric. Food Chem. 2013, 61, 1349−1356

Table 1. Growth Factor Secretive Productions Effected by 6-DG on Human Skin Fibroblasts and Keratinocytes

	control	DMSO (1%)	6-DG	
			2 μM	10 μM
		Fibroblasts		
TGF-β (pg/mL)	778.3 ± 15.6	778.3 ± 3.3	984.5 ± 21.6	1003.3 ± 24.0
PDGF-$\alpha\beta$ (pg/mL)	2.5 ± 0.1	1.7 ± 0.2	7.1 ± 0.1	5.3 ± 0.1
VEGF (pg/mL)	287.0 ± 11.1	279.5 ± 12.9	432.0 ± 14.6	294.5 ± 14.6
		Keratinocytes		
TGF-β (pg/mL)	665.4 ± 8.2	642.3 ± 20.1	822.8 ± 15.4	768.0 ± 9.6
PDGF-$\alpha\beta$ (pg/mL)	2.20 ± 0.1	1.46 ± 0.2	5.84 ± 0.2	4.55 ± 0.1
VEGF (pg/mL)	250.7 ± 6.3	234.5 ± 11.7	344.3 ± 9.5	292.8 ± 7.3

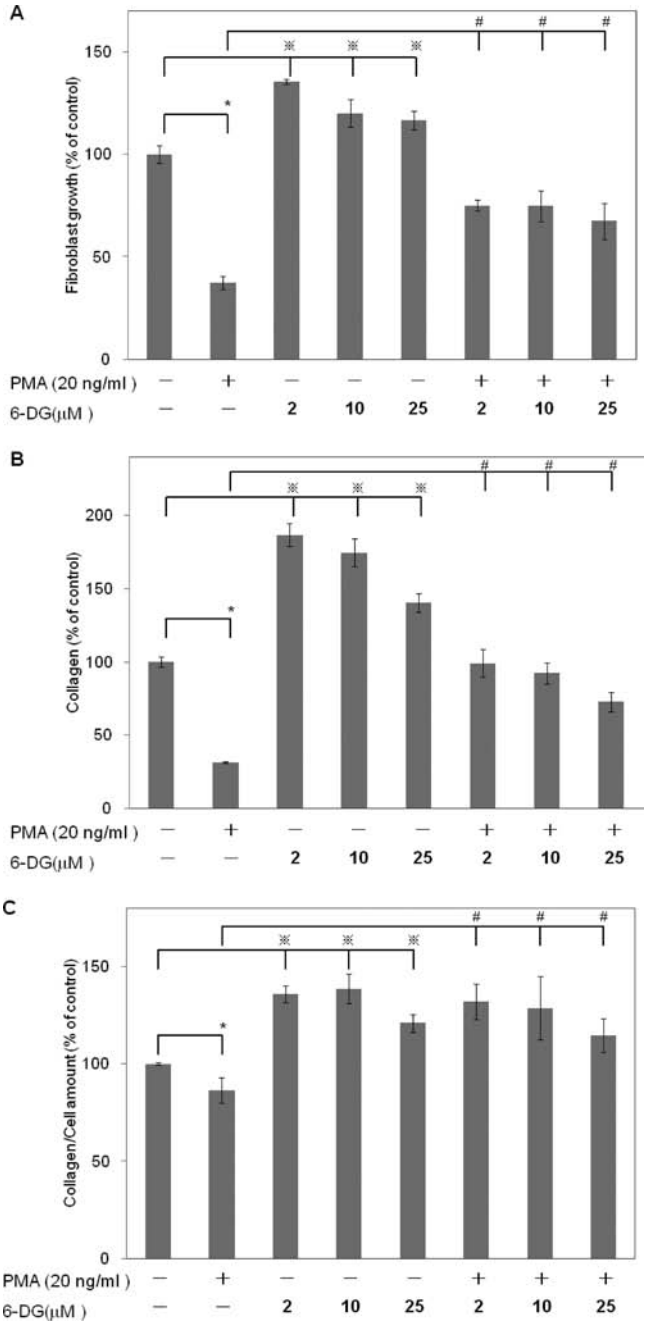

Figure 4. (A) Cell growth of fibroblasts treated with or without PMA in advance of and following 6-DG treatments from 2 to 25 μM; (B) collagen contents; (C) specific cell collagen production.

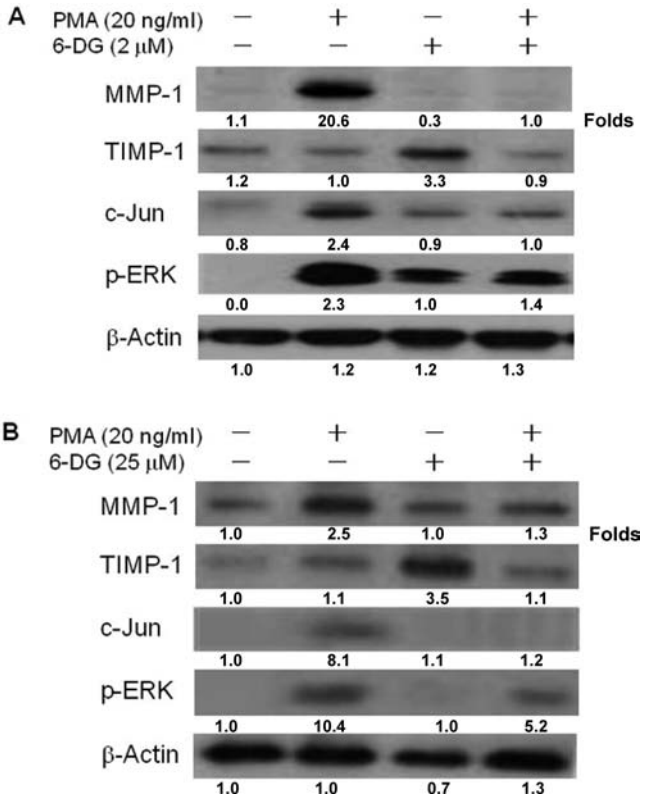

Figure 5. Effects of 6-DG on cell migration related proteins. PMA was used as collagen inhibitor. Cells were pretreated with PMA for 30 min and then treated with (A) 2 μM or (B) 25 μM 6-DG for 48 h to reveal by Western blot. β-Actin was used as an internal control.

dermal fibroblasts through blocking the MAPK signaling cascade. The 20 ng/mL PMA treatment on fibroblasts induced c-Jun and ERK phosphorylation (Figure 5). 6-DG, at both 2 and 25 μM, blocked c-Jun and ERK activation induced by PMA. PMA also triggered ERK phosphorylation, which was decreased by the following 6-DG treatment.

Inhibitory Effect of 6-DG on MMP-1 Activity. To examine the effect of 6-DG on the activity of collagenase, MMP-1, collagen zymography was performed. We exhibit the consequence in Figure 6A; the treatment of 20 ng/mL PMA increased the activity of MMP-1, and Figure 6B shows the quantitative analysis. As expected, the PMA-induced activation of MMP-1 was dramatically inhibited by the dose of 25 μM 6-DG, indicating the specific inhibition of 6-DG on MMP-1 activity in dermal fibroblast.

dx.doi.org/10.1021/jf304340q | J. Agric. Food Chem. 2013, 61, 1349−1356

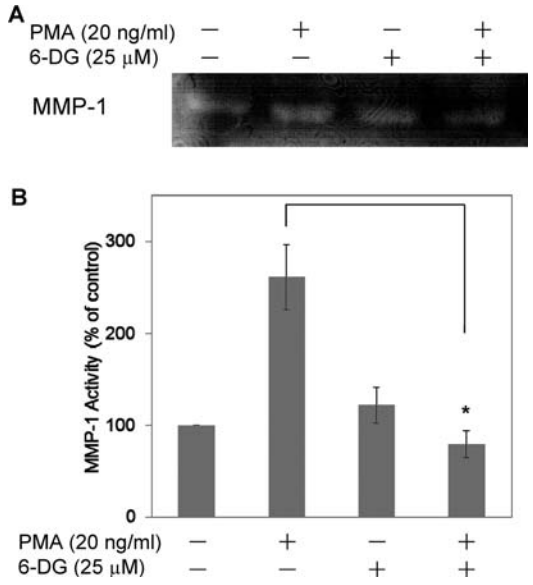

Figure 6. Effects of 6-DG on MMP-1 activities. Cells were pretreated with PMA for 30 min and then treated with (A) 25 μM 6-DG for 24 h. (B) Quantitative analysis. *, $p < 0.05$ against PMA treatment alone.

DISCUSSION

Our target plant, ginger, is a natural food ingredient that has been used for thousands of years and was proved to be without biosafety concerns.[31,32] We screened dozens of natural pure compounds including ginger species, and suitable concentrations were according to our preliminary experimental results. We discovered that 6-DG proliferated and accelerated migrations of human fibroblasts and keratinocytes effectively (Figures 2 and 3). We have identified 6-DG as a strong inhibitor in human hepatoblastoma and breast cancer cells.[21,33] There is a discrepancy between the present study and our previous data, where ginger compounds were discovered for their anticancer properties in inhibiting cell growth and migration.[21,33] It is assumed that 6-DG dosage amounts, treatment time courses, or cell types in the different experimental designs resulted in various cell proliferations. To distinguish this issue, we confirmed the related growth factor secretions and protein expressions to vary our hypothesis.

TGF-β assists in protecting the healthy surrounding tissues around injured areas by forming granulations. With the assistances of TGF-β, monocytes are converted to macrophages, which are inflammatory response cells that produce growth factors on the tissue debridement and granulations.[34] PDGF-$\alpha\beta$ is generated by fibroblasts, keratinocytes, vascular endothelium, macrophages, and platelets. During the wound-healing process, PDGF-$\alpha\beta$ serves predominantly as the main key in blood vessel maturations by recruiting pericytes and increasing its structural integrities.[35] VEGF is a noteworthy growth factor on skin wound healing as it helps angiogenesis, cell proliferations, and migrations.[36] Around the wound tissue area, ECM reformations are vital and synthesized, deposited, and organized with the help of VEGF from these related cells. As mentioned in the Introduction, PDGF stimulates immune macrophages to secrete TGF-β, and it also helps increase VEGF expression in some injury situations. In this study, we found that TGF-β and VEGF significantly increased after 6-DG application in addition to PDGF. We suggested that the increase of TGF-β and VEGF secretions might be due to PDGF increases.

Bhagavathula et al. reported in vitro and in vivo wound repair induced by ginger compounds with curcumin and ginger extracts in hairless rat skin.[19] Our assay system was cultured from normal human skin, and the higher cell growths were observed at a low concentration (2 μM) in both fibroblasts and keratinocytes. With the in vitro cell culture wound-healing assay, the treated cells were permitted to migrate over the clearing area more rapidly than the control group. Twenty-four hours after 6-DG treatment, fibroblast and keratinocyte migratory cells almost entirely covered the wound region (Figure 3). Our study suggested that skin cell growth was enhanced by the growth factor activations in the presence of 6-DG (Table 1). This was the first time that the ginger constituent was revealed to enhance growth factor production in normal human skin cells. We considered that the overexpression of the three growth factors used in this study might have contributed to increased cell migrations evident in the cell culture wound-healing assay.

We discovered that the specific cell collagen productions promoted by 6-DG were 25−50% higher than the control group (Figure 4). Fibroblast cell growth was also enhanced due to 6-DG treatments. Both aspects caused an increase in collagen amount and reverted the collagen content reduced by PMA. Previous studies found that TGF-β-treated adipose-derived stem cells CM induced higher expression of type I collagen along with cell cycle regulatory proteins and migration of fibroblasts.[37] After treatment of the skin cells with 6-DG, the expression of TGF-β and collagen increased (Table 1 and Figure 5). The increase in collagen amount might be due to greater TGF-β expression.

The downstream factors of dermal fibroblast migration were next examined. MMP-1 serves as a key enzyme in the degradation of collagen and stimulates the degradation of major dermal components, which sequentially causes aging and wrinkling. Other MMP members, such as MMP-3, MMP-9, and MMP-10, also contribute to keratinocyte migration during epithelialization. Recently, Muller et al. indicated that the ratio of MMP-1 and TIMP-1 is critical to the prediction of wound healing.[38] Therefore, our research focused on the relationships of TIMP-1, growth factors, ERK MAPK, collagen production, and MMP-1. Additionally, the transcription factor, PMA, regulates directly or indirectly via intracellular kinase phosphorylation, such as MAPK to stimulate MMP-1 production in dermal fibroblasts.[39] Considering the master cytokine leading to fibrosis, TGF-β up-regulated collagen and TIMP-1 while down-regulating the transcription of the MMP-1 gene.[40] 6-DG stimulated the overexpression of TGF-β, which was the major reason for increased collagen production. Besides the PMA-induced c-Jun, ERK and MAPK activation was attenuated by 6-DG, which suggested that 6-DG may also down-regulate the MMP-1 protein level through c-Jun and ERK (Figure 5). One important factor that regulates MMP-1 secretion is the MAPK signaling pathway, which plays an essential role in ruling various cellular functions. The present study discovered that, in PMA-treated dermal fibroblasts, MAPK activation, upstream of MMP, was rapidly down-regulated by 6-DG.

The results showed that 6-DG inhibited the MMP-1 amount and that its activity returned to the basal level (Figure 6). Consistent with our result, there was a report that adipose-derived stem cells facilitated wound healing by enlarging the expression of collagen I/III and down-regulating MMP-1 expression in human dermal fibroblasts.[41] Therefore, MMP-1 activity down-regulation seems to promote the progression of wound healing.

Taken together, our present work demonstrates the wound-healing effect of 6-DG on human epidermal keratinocytes and dermal fibroblasts via up-regulation of TGF-β, VEGF-$\alpha\beta$, and PDGF. 6-DG also decreased c-Jun and ERK and enhanced TIMP-1, which subsequently reduced the MMP-1 protein, increasing the amount of collagen (Figure 7). Accordingly,

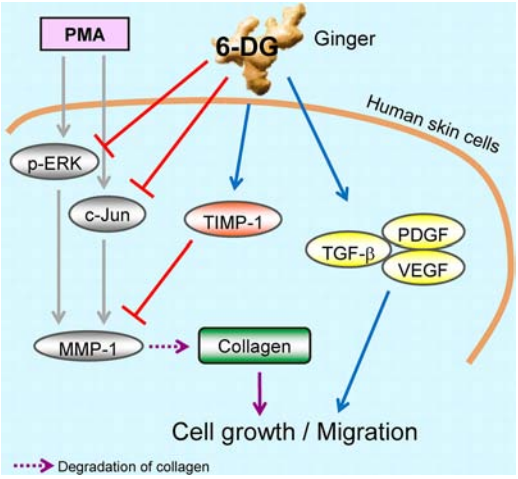

Figure 7. Proposed schematic diagram of 6-DG biofunctions on human fibroblasts and keratinocytes, including cell growth, migration, growth factor expression, and collagen secretion.

6-DG could be a human skin cell growth and migration enhancer, and our present work sheds light on the molecular mechanism for wound repair.

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Funding

This work was financially supported by the National Science Council of the Republic of China under Grants NSC-97-2320-B-242-002-MY3 and NSC-99-2221-E-037-006-MY3 as well as by the Taiwan Tech Trek program (2010), the academic community about industry program.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Mei-Yin Chen for assistance and Katherine Chang for English editing.

ABBREVIATIONS USED

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 6-DG, 6-dehydrogingerdione; BPE, bovine pituitary extract; COSY, correlation spectroscopy; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; ECM, extracellular matrix; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum coherence; MMP-1, matrix metalloproteinase-1; MAPK, mitogen-activated protein kinase; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectrometry; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PDGF-$\alpha\beta$, platelet derived growth factor-$\alpha\beta$; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SD, standard deviation; TIMP-1, tissue inhibitor of metalloproteinase-1; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factors

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