Compound K Activates Hyaluronan Synthase 2 in transformed human Keratinocytes and Fibroblasts and Increases hyaluronan in hairless mouse skin

Sujong Kim, Byung Yong Kang, Si Yong Cho, Dae Suk Sung, Eiu Suk Shin, Hui Kyung Chang, Myung Hun Yeom, Kwang Sik Woo, Duk Hee Kim, Young Chul Sim and Yong Sung Lee#

From the R & D Center, Amore-Pacific Corporation, 314-1 Borari, Kiheung-eup, Yongin-si, Kyounggi-do 449-729, Korea and #Department of Biochemistry, College of Medicine, Hanyang University, Seoul, Korea

Key Words: cDNA microarray, compound K, ginsenosides, hyaluronan (HA), hyaluronan synthase 2 (HAS2)

Summary

Ginsenosides, the major active ingredients of ginseng, show a variety of biomedical efficacies such as anti-aging, anti oxidation and anti-inflammatory activities. To understand the effects of compound K (20-O-•-D-glucopyranosyl-20 (S)-protopanaxadiol), one of the major metabolite of ginsenosides on the skin, we assessed the expression level of ~ 100 transcripts in compound K-treated HaCaT cells using cDNA microarray analysis. Compound K treatment induced differential expression of 21 genes, which have been reported to be involved in the organization of ECM structure as well as defense responses in human skin cells. One of the most interesting findings is 2-fold increase in hyaluronan synthase2 (HAS2) gene expression by compound K. We found that change in expression of HAS2 gene represents a specific response of HaCaT cells to compound K because hyaluronan synthase 1, 3 was not changed by treatment with compound K. We also demonstrated that the compound K effectively induced hyaluronan synthesis in human skin cells and hairless mouse skin. The human clinical study indicates that topical application of compound K-containing oil-in-water emulsion showed improvement of xerosis, wrinkle and fine lines in the aged skin. We concluded that compound K has anti-aging effects by the induction of HAS2 gene expression and following hyaluronan synthase.

Introduction

Panax ginseng C. A. Meyer has been reported to possess a wide range of biological and pharmacological activities such as anti-aging, anti-inflammation, and anti-oxidation in the central nerve system, cardiovascular system, endocrine system, and immune function, which are mainly, attributed to its triterpenoid saponin components [1,2]. A number of saponins called ginsenosides have so far been isolated from ginseng [3], and have extensively investigated with regard to their antiapoptotic, antitumoric and anti-inflammatory activity in diverse biological system [4-10]. For example, protopanaxadiol saponins such as ginsenoside Rh2 and Rg3 have been reported to inhibit tumor cell proliferation [4,5], induce differentiation and apoptosis [6,7], and inhibit metastasis [8], whereas ginsenoside Rg1 prevents rat cortical neurons from apoptosis [9]. Recently, the ginseng saponin metabolites formed by intestinal bacteria were identified after oral administration of ginseng extract in human and rats [10]. One of the major metabolite, the Compound K (20-O-• •D-glucopyranosyl-20 (S)-protopanaxadiol) (Figure 1) has been receiving increasing attention because *in vivo* antimetastatic and antiallergic activities by the ginsenosides is mediated by this metabolite [11, 12]

Although total extract from ginseng have so far been included in the cosmetic formulation and several benefits of ginseng in the human skin have been reported [13-18], the action mechanism and potential role of ginsenosides in skin are not completely understood yet. In the present study, we prepared the compound K by enzymatic-modifying ginsenoside R(c). To gain a more broad understanding of the effects of compound K on the skin at the molecular level, we assessed the expression level of ~100 transcripts in compound K-treated HaCaT using cDNA microarray analysis. Comparisons of compound K-treated HaCaT and vehicle-treated control revealed that apparent difference in mRNA expression of 21 genes. One of the most interesting findings is upregulation of hyaluronan synthase2 (HAS2) gene expression by compound K. Human hyaluronan synthase (HAS1, HAS2 and HAS3) use sugar substrates from UDP donors to form disaccharides consisting of D-glucuronic acid (• 1,3-linked) and N-acetyl-D-glucosamine (• 1,4-linked), which is an essential component of hyaluronan, the major extracellular matrix molecules in the skin. Generally, hyaluronan has biological functions of water retention, the maintenance of intercellular space, and regulation of cellular proliferation and differentiation in the skin. The age-dependent decrease in the hyaluronan content of human skin has been accepted to be one of the major causes for several deteriorations of intrinsic skin aging. We demonstrated that compound K effectively induced hyaluronan synthesis in human skin cells and hairless mouse skin. We also showed that emulsion containing compound K reduced dryness and wrinkle in aged skin, as the result of the human clinical test.

•

Materials and Methods

•

Compound K preparation

10 g of total ginseng extracts (red ginseng, white ginseng, tiny ginseng roots, and ginseng leaves) were dissolved in 2 L of citrate buffer (pH 4.0) and incubated with 10 g of naringinase (Sigma Co. Ltd.) or 10 g of pectinase (Novozyme Co. Ltd.) for 48 h at 38• ◦ When the enzymatic hydrolysis reaction was complete, the reaction mixture was extracted with 2 L of ethyl acetate. After evaporation in vacuum, 2.8 g of the residue was obtained. To obtain pure compound K, the resulting products were separated through silicagel column chromatography and eluted with chloroform:methanol (9:1). Further elution with chroloform:methanol (6:1) afforded the pure compound K (0.28 g). The identification was done by FAB-MS, 1H-, and 13C-NMR spectra and the purity was assessed by HPLC and determined to be about 99%. Compound K was dissolved in 100% ethanol to make a stock solution. Ethanol concentration was kept below 0.001% in all the cell culture and did not exert any detectable change in cell behavior.

Cell culture

The spontaneously transformed human keratinocyte cell line HaCaT was kindly provided by Dr. N.E. Fusenig (Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany) [19]. Human diploid fibroblasts (HDF) was kindly provided by Dr. S. C. Park [Seoul National University, Seoul, Korea]. The cells were grown under proper culture conditions (37°C, 5% CO_2, 95% air) in Dulbecco's modified Eagle's media (Life Technologies, Inc.) containing 10% fetal bovine serum (HyClone), 3.6 g sodium bicarbonate per liter, and antibiotics (100 μg streptomycin per ml, 100 IU penicillin per ml) (Life Technologies, Inc.). The cells received fresh medium every 3 d and were subcultured at a split ratio of 1:5 as soon as they reached confluence. The cells were treated with 0.1% trypsin/EDTA, and plated at 1×10^5 cells per 75 cm^2 tissue culture flask and cultivated to confluence in medium containing 10% FCS. They were then cultured in serum-free medium for 24 h and incubated in fresh serum-free medium in the presence or absence of 1–5 μM compound K for 3, 6, 12, 24 or 48 h.

RNA preparation

HaCaT cells and HDF cells were washed twice with phosphate buffered saline (Life Technologies), and total cellular RNA was isolated using Trizol reagent (GibcoBRL Life Technologies) according to the manufacturer's instructions.••

•

cDNA microarray analysis

Fabrication 100 cDNA transcripts were spotted onto silylated slides(CEL Associates) with a Pixsys 5500 arrayer (Cartesian Technologies), as previously described [20]. These 100 transcripts were selected based on their functions. Housekeeping genes and yeast DNA also were spotted as negative controls.

Probe preparation and Hybridization Fluorescence-labeled cDNA probes were made from 100 μg of total RNA by oligo (dT)-primed polymerization using SuperScript II reverse transcriptase (Gibco BRL Life Technologies), as previously described [20]. The Cy5 and Cy3 probes were mixed, and 8 μg of poly (dA) (Pharmacia), 4 μg of *Escherichia coli* tRNA (Sigma Chemical Co.), 10 μg of Human Cot1 DNA (Life Technologies, Inc.), 0.3 μl of 10% SDS, 3 μl of 20 x SSC (final concentration, 3 x SSC), and water were added to a final volume of 20 μl. The probe was incubated at 98•C for 2 min, chilled on ice for 10 sec and placed onto the glass slide with a coverslip. Hybridization was performed at 55•C for 16 h. The slides were washed in 2 x SSC, 0.2% SDS at room temperature for 5 min, 0.1 x SSC, 0.2% SDS at 55•C for 5 min, after a quick rinse in 0.1 x SSC. The slides were dried at 500 r.p.m. for 5 min.

Scanning and analysis of DNA microarrays Two fluorescent images (Cy3 and Cy5) were scanned separately from a GMS 418 Array Scanner (Affymetrix). The images were analyzed using ImaGene 4.2 and GeneSight (Biodiscovery). Values at least twofold of the background intensity were considered significant. Hierarchical clustering was applied to genes using the weighted pair-group method with centroid average as implemented in the program Cluster and the results were analyzed with Tree View (M. Eisen; http//www.microarrays.org/software).

Reverse transcription coupled to polymerase chain reaction

Total RNA was subjected to reverse transcription and subsequent PCR to show the presence of HAS 1,2, 3 mRNA in analyzed cells. 4 μg of total RNA were reverse-transcribed for single-strand cDNAs with Superscript II reverse transcriptase (Life Technologies, Inc.). Subsequently, 10 μl of the reaction mixture were taken and used in PCR reaction. Each PCR was carried out in a 50 μl volume of 1x PCR buffer for 5 min at 95•C for initial denaturing, followed by 25 –35 cycles of 95•C for 45 sec, 56•C for 45 sec, and 72•C for 1 min, in the Perkin-Elmer Cycler 9600 (Perkin-Elmer Applied Biosystems). Table 1 shows the primer sequences for the cDNAs. PCR products were transferred to an agarose gel, and visualized by ethidium bromide staining.

Immunocytochemistry

HaCaT and HDF cells were washed with phosphate-buffered saline and fixed at room temperature for 20 min in a fixative with 2% paraformaldehyde (v/v) and 0.5% glutaraldehyde (v/v). After fixation, the cells were washed three times for 2 min each with 0.1 M sodium phosphate buffer, pH 7.4, and then blocked in 1% bovine serum albumin (w/v) containing 0.1%

Triton X-100 (v/v) in the same buffer for 30 min at room temperature. Hyaluronan staining was done with a specific probe, biotinylated hyaluronan binding protein (bHABP)(Seikagaku). The bHABP probe, diluted to 5 μg/ml in 3% bovine serum albumin (w/v), was added to the fixed cells and was incubated overnight at 4 °C. After washing, avidin- fluorescein-isothocyanate (FITC) was added. The images were analyzed under a fluorescence microscope.

•

Animals study

Topical Application Hos: hr-1 albino male hairless mice, purchased from Biogenomics (Seoul, Korea) at 30 weeks of age, were fed standard rodent chow and water ad libitum. After adaptation to a environment of 24 ± 2°C and 55 ± 10% relative humidity for 1 week, 200 μl of compound K solution (1 % wt/vol) in vehicle (1,3 BG : ethanol = 7 : 3) was topically applied to the back of the mice twice during 2 days. At 24 h after the final application, each skin sample was collected.

Histochemical Detection of Hyaluronan Hyaluronan staining was performed using a biotinylated HABP (Seikagaku). Each skin sample was fixed with 2% formaldehyde and 0.5% glutaraldehyde in PBS by irradiation in a microwave oven at 45° C for 1 min, washed, embedded and sectioned with a microtome. After deparaffinization, the section was treated with 0.3% H_2O_2 in methanol at room temperature for 30 min, washed in PBS and blocked with 1% BSA. Thereafter, the section was stored with biotinylated HABP (5 mg/ml) in PBS at 4°C overnight, washed and incubated with streptoavidin-peroxidase (1/300 diluted in PBS) at room temperature for 30 min. After further washing, each slide was reacted with 3,3)-diaminobenzidine tetrahydrochloride reagent at room temperature for 5 min, washed in water and subjected to Mayer•s hematoxylin staining.

Image and Data Analyses Immunostained slides were characterized quantitatively via digital image analysis using ImagePro-Plus (Media Cybernetics). Images were captured through an Olympus BH-2 microscope fitted with a MicroImage video camera (Boyertown, PA). A series of 10 random images on several slides were taken for each immunostained parameter to obtain a mean value for statistical comparison. Staining was defined via color intensity, and a color mask was made. The mask was then applied equally to all images and measurements were determined. Immunohistochemical measurement parameters included total tissue area, total stained area, and intensity of stain. Student's *t* test was performed using SigmaStat (SPSS, Inc.) Significance was considered at P , 0.05 and data are presented as mean ± SEM.

Human Clinical Test

Topical Application A clinical study for evaluating the efficacies of compound K-containing cosmetic formula was carried out. Two formula of oil-in-water emulsion with and without 0.03%

of compound K were applied to 49 healthy Korean women aged from 31 to 47 years, having a normal, dry, mixed or dry tendency skin face typology and presenting wrinkles and fine lines on the face. Before the test, the degree of wrinkle and fine lines on the face of all the volunteers were classified by detecting global photodamage score [25]. All of the measurements were performed before the first application and after 4, 8, and 12 weeks of use. The testing samples were used twice a day (in the morning and evening) by the volunteers themselves, at home, to the facial skin, especially on the crows' feet and the eye contour.

Evaluation of skin efficacies Assessment of wrinkles, fine lines, moisturization, firmness, smoothness, roughness, and radiance of facial skin was measured by the volunteers and by the dermatologists. Photometric evaluation using Camscope® (model DCS-105), image analysis by making silicone replica and using Skin-Visiometer SV 600 (Courage & Khazaka, Germany) were used for instrumental analysis of the difference and improvement of before and after the application of the cosmetic sample.

Tolerance Assessment Erythema, oedema, dryness, scaling and burning are taken into account for clinical rating. Itching, stinging, tightness, dryness and prickling are taken into account for the volunteer's rating.

Results and Discussion

Identification of compound K- responsive genes

To identify genes differentially regulated by compound K, gene expression level in compound K- treated cells was compared with that of vehicle-treated cells as control among 100 transcripts(Figure 2). Three replicated cDNA array analyses revealed 20 known genes (20%) of 100 genes to be expressed with more than a 2-fold difference in mRNA levels. Among these 20 genes, 14 genes were up-regulated and 6 genes down-regulated (Table 2). Genes related to cell adhesion and ECM, such as laminin, integrin, collagen and HAS2, were increased by treatment of compound K. In other hand, genes of matrix metalloproteinase family involved in ECM modification were decreased in mRNA expression after compound K treatment. Glutathione S-transferases, which have been reported to be involved in defense responses were expressed at an average of 2-fold higher levels in compound K-treated cells. All together, our findings demonstrated that compound K globally affects expression of genes involved in organization of ECM structure as well as defense responses.

Compound K induced an increase of Hyaluronan via upregulation of the HAS 2 mRNA expression in HaCaT and HDF cell culture

HAS2 was selected for further functional studies. HASs have attracted increasing scientific attention, as they synthesize HA, one of the major extracellular matrix molecules which has biological functions of water retention, the maintenance of intercellular space and regulation of cellular proliferation and differentiation in the skin [21]. The age- dependent decrease in the HA content of human skin has been accepted to be one of the major causes for several deteriorations of intrinsically aged skin [22-24].

At first, to verify a change of HAS2 expression by compound K, mRNA level of HAS2 was measured by semiquantitative RT-PCR. This comparative analysis showed that mRNA of HAS2 was detected in basal level and increased up to 3-fold and 2.5-fold by compound K in HaCaT and HDF cells, respectively (Figure 3). In contrast, HAS1 and HAS3 mRNA were not detected in HaCaT cell (Figure 4). The distribution of hyaluronan was shown by staining the HaCaT and HDF cell cultures with the hyaluronan-specific probe (bHABP) (Figure 5). Compound K increased the amount of hyaluronan associated with the cell layer. This visual impression was confirmed by determination of the total fluorescence densities, resulting in higher values for compound K-treated culture. A good correlation was found between increase in the HAS2 mRNA levels and increase in HA staining.

Compound K increased Hyaluronan synthesis and skin thickness in Hairless mouse

skin

HA, widely deposited in epidermis and dermis, was significantly increased in compound K- treated hairless mouse skin (Figure 6). In compound K- treated skin, the amount of HA predominantly increased in the extracellular papillary dermis and in all the viable epidermis including basal, spinous and granular layers. (Figure 6A and B). Quantitative image analysis showed the amount of HA increased 3 folds in epidermis and dermis of compound K-treated skin, respectively as compared with untreated skin. (Figure 6C). The general histological features of compound K-treated skin were compared with those of untreated skin in H&E-stained sections.

Because HA has known to have biological functions of water retention and the maintenance of intercellular space, epidermal thickness was measured in the same skin sections that had HA staining measurement taken. As shown in Figure 6D, compound K caused a thickening of skin after two daily treatment. The decrease in skin thickness and water content, which is frequently observed in intrinsically aged human skin has been reported to be caused, in part, by age- dependent decrease in the HA content [22-24]. The upregulation of HAS2 gene expression and the following increase in HA synthesis by compound K treatment might improve several deteriorations of intrinsically aged skin such as skin ••••••• and wrinkles.

Compound K-containing oil-in-water emulsion clearly improved skin facial wrinkles and fine lines

As a result of the assessment by the dermatologists based on global photodamage score, Compound K-containing emulsion has shown statistically significant reduction of wrinkles and fine lines of the facial skin in comparison to the initial measurements and to the values obtained on the control emulsion after 12 weeks of use (Figure 7A). Actually, 76% of volunteers have shown positive and affirmative judgment for anti-wrinkle effect after 8 weeks of use and 92% after 12 weeks of use (Figure 7B). Based on the result of skin replica analysis, total number of wrinkles was reduced after 8 weeks with statistical significance. Skin smoothness increased for 92% of panelists, skin radiance was improved for 68%, skin firmness for 68%, and skin roughness for 94%. Skin moisturization was enhanced for over 88% of them.

Conclusion

Compound K, the major active metabolite of ginseng saponins globally affects expression of several genes involved in organization of ECM structure as well as defense responses in human skin cells. Specially, Compound K increased the HAS2 mRNA expression in HaCaT and HDF cell culture and induced an increase of HA synthesis in human skin cells and hairless mouse skin. Compound K-containing oil-in-water emulsion has shown clear anti-wrinkle effect

with statistical significance as the result of human clinical efficacy test.

References

1. Lee FC, Facts about Ginseng, the Elixir of Life. Hollyn International Corp., Elizabeth, NJ, 1992
2. Huang KC, The pharmacology of Chinese Herbs. CRC Press, Boca Raton, FL, 1999
3. Gillis CN: Panax ginseng pharmacology: A nitric oxide link? *Biochem Pharmacol* **54**:1-8, 1997
4. Kikuchi Y, Sasa H, Kita T, Hirata J, Tode T, Nagata I. Inhibition of human ovarian cancer cell proliferation in vitro by ginsenoside Rh2 and adjuvant effects to cisplatin in vivo. *Anticancer Drugs*. **2**(1):63-7, 1991
5. Tode T, Kikuchi Y, Kita T, Hirata J, Imaizumi E, Nagata I. Inhibitory effects by oral administration of ginsenoside Rh2 on the growth of human ovarian cancer cells in nude mice. *J Cancer Res Clin Oncol.* **120**(1-2):24-6, 1993
6. Lee YN, Lee HY, Chung HY, Kim SI, Lee SK, Park BC, Kim KW. In vitro induction of differentiation by ginsenoides in F9 teratocarcinoma cells. *Eur J Cancer.* **32A**(8):1420-8, 1996
7. Park JA, Lee KY, Oh YJ, Kim KW, Lee SK. Activation of caspase-3 protease via a Bcl-2-insensitive pathway during the process of ginsenoside Rh2-induced apoptosis. *Cancer Lett.* **121**(1):73-81, 1997
8. Shinkai K, Akedo H, Mukai M, Imamura F, Isoai A, Kobayashi M, Kitagawa I. Inhibition of in vitro tumor cell invasion by ginsenoside Rg3. *Jpn J Cancer Res.* **87**(4):357-62, 1996
9. Yu SC, Li XY: Effect of ginsenoside on IL-1•• and IL-6 mRNA expression in hipopcampal neurons in chronic inflammation model of aged rats. *Acta Pharmaco Sin* **21**: 915-918, 2000
10. Akao T, Kida H, Kanaoka M, Hattori M, Kobashi K. Intestinal bacterial hydrolysis is required for the appearance of compound K in rat plasma after oral administration of ginsenoside Rb1 from Panax ginseng. *J Pharm Pharmacol.* **50**(10):1155-60, 1998
11. Hasegawa H, Sung JH and Hur JD, Ginseng intestinal bacterial metabolite IH901 as a new antimetastatic agent. *Arch Pharm Res* **20**: 539-544, 1997
12. Bae EA, Choo MK, Park EK, Park SY, Shin HY, Kim DH., Metabolism of ginsenoside R(c) by human intestinal bacteria and its related antiallergic activity. *Biol Pharm Bull* **25**(6):743-7, 2002
13. Choi S. Epidermis proliferative effect of the Panax ginseng ginsenoside Rb2. *Arch Pharm Res.* **25**(1):71-6, 2002

14. Surh YJ, Na HK, Lee JY, Keum YS. Molecular mechanisms underlying anti-tumor promoting activities of heat-processed Panax ginseng C.A. Meyer. *J Korean Med Sci.* **16** Suppl:S38-41. Review, 2001
15. Chang LK, Whitaker DC. The impact of herbal medicines on dermatologic surgery. *Dermatol Surg.* **27**(8):759-63. Review, 2001
16. Pribitkin ED, Boger G. Herbal therapy: what every facial plastic surgeon must know. *Arch Facial Plast Surg.* **3**(2):127-32. Review, 2001
17. Keum YS, Park KK, Lee JM, Chun KS, Park JH, Lee SK, Kwon H, Surh YJ. Antioxidant and anti-tumor promoting activities of the methanol extract of heat-processed ginseng. *Cancer Lett.* **13**;150(1):41-8, 2000
18. Kim SH, Jeong KS, Ryu SY, Kim TH. Panax ginseng prevents apoptosis in hair follicles and accelerates recovery of hair medullary cells in irradiated mice. *In Vivo.* **12**(2):219-22, 1998
19. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol.* **106**(3):761-71, 1988
20. Lee, K.H., Chang, M.Y, Ahn J.I., Yu, D.H., Jung, S.S., Choi, J.H., Noh, Y.H., Lee, Y.S., Ahn, M.J. (2000). Differential gene expression in retinoic acid-induced differentiation of acute promyelocytic leukemia cells, NB4 and HL-60 cells, *Biochem. Biophys. Res. Commun.* **296**, 1125-1133.
21. Laurent TC, Fraser JR. Hyaluronan. *FASEB J.* **6**(7):2397-404. Review, 1992
22. Fleischmajer R, Perlish JS, Bashey RI. Human dermal glycosaminoglycans and aging. *Biochim Biophys Acta.* **279**(2):265-75,1972
23. Longas MO, Russell CS, He XY. Evidence for structural changes in dermatan sulfate and hyaluronic acid with aging. *Carbohydr Res.***159**(1):127-36, 1987
24. Ghersetich I, Lotti T, Campanile G, Grappone C, Dini G. Hyaluronic acid in cutaneous intrinsic aging. *Int J Dermatol.* **33**(2):119-22,1994
25. Jin Ho Chung, Seong Hun Lee, Choon Shik Youn, Byung Joo Park, Kyu Han Kim, Kyung Chang Park, Kwang Hyun Cho, Hee Chul Eun. Cutaneous photodamage in Koreans: Influence of Sex, Sun Exposure, Smoking, and Skin Color. *Arch Dermatol* **137**(8): 1043 – 1051, 2001

Table 1. HAS primer sequences for semiquantitative RT-PCR

Table 2. The list of genes altered by more than 2-fold by Compound K treatment[a] and the log values of expression ratio[b].

Gene name	3h	6h	24h	48h
collagen, type I, alpha 1 (COL1A1)	0.48		-0.04	-0.21
glutathione S-transferase A3 (GSTA3)	0.28		0.40	0.60
glutathione S-transferase A4 (GSTA4)	0.46			
heat shock protein, DNAJ-like 2 (HSJ2)	-0.19		0.13	-0.17
heat shock transcription factor 4 (HSF4)	0.33		0.16	0.28
heat shock 70kD protein 9B (mortalin-2)	0.57	0.60	0.14	
heat shock protein 75 (TRAP1)	0.60	0.31		
hyaluronan synthase 2 (HAS2)	0.37	0.40	-0.38	
integrin alpha 6 subunit	-0.32		0.49	0.90
integrin, beta 4 (ITGB4)	0.02			
integrin, beta 5 (ITGB5)	-0.48		0.26	0.52
matrix metalloproteinase 1 (interstitial collagenase) (MMP1)	-0.20	-0.14		-0.06
matrix metalloproteinase 10 (stromelysin 2) (MMP10)	-0.80	-0.92		
matrix metalloproteinase 12 (macrophage elastase) (MMP12)		-0.34	-0.97	0.37
matrix metalloproteinase 13 (collagenase 3) (MMP13)		-0.75		
matrix metalloproteinase 2 (72kD gelatinase, 72kD type IV collagenase)	-0.45	-0.90	-0.38	
matrix metalloproteinase 7 (matrilysin, uterine) (MMP7)				
matrix metalloproteinase 16 (membrane-inserted) (MMP16)	0.39		0.42	
matrix metalloproteinase like 1 (MMPL1)	0.00		-0.60	0.03
laminin, gamma 2, transcript variant 1	-0.03	0.95		

[a] HaCaT cells were treated with compound K for the indicated times.

[b] Data are medians of $\log_2$ (Cy5/Cy3 ratio); positive 1 means 2-fold increase, whereas negative 1 means 2-fold decrease in compound K-treated cells.

FIGURE LEGENDS

Figure 1. Structure of compound K(20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol)

Figure 2. Cluster analyses of genes encoding ECM components or genes involved in ECM modification and defense responses. These 100 genes were clustered hierarchically on the basis of the similarity of their expression profiles. The expression pattern of each gene is displayed as a horizontal strip. Grey indicates missing data.

Figure 3. Further confirmations of microarray data by RT-PCR. Semiquantative RT-PCR was performed to verify the relative mRNA levels of genes measured by microarray analysis. The hyaluronan synthase 2 mRNA expression in HaCaT cells(A) and HDF cells (B) was increased by treatment with compound K at the doses indicated. Total RNA isolated were reversely transcribed and amplified with 25-35 PCR cycles for the HAS2 and GAPDH, an internal control. Data were normalized relative to the expression of GAPDH and denoted as a relative amount in mRNA expression in graphs(C and D).

Figure 4. The different regulation of HAS1, HAS2 and HAS3 transcripts in HaCaT cells by compound K. HaCaT cells were cultured in 0 or 1 μM of compound K for 24 or 48 h. Total RNA isolated were reversely transcribed and amplified with 30 PCR cycles for the different HAS isotypes and GAPDH, an internal control. Although HAS2 transcript level was increased about 3 - and 5 - fold in HaCaT cells treated with compound K for 24h and 48 h respectively, no significant difference was found in HAS1 and HAS3 transcript expression. Data were normalized relative to the expression of GAPDH and denoted as a relative amount in mRNA expression.

Figure 5. Effects of compound K on the hyaluronan distribution in HaCaT and HDF cell cultures. Confluent HaCaT cells (A, B) and HDF cells (C, D) were incubated in the absence (A, C) or presence of 1μM compound K (B, D). All cultures were grown on chamber slides, fixed, and probed for hyaluronan by bHABP as described under "Materials and Methods."

Figure 6. Effects of compound K on hyaluronan distribution and epidermal thickness in hairless mouse skin. The hairless mice were topically treated with vehicle (ethanol:propylene glycol=7:3) (A), and compound K at 1 % (wt/vol) (B) as described in "Materials and Methods". Hairless mouse skin sections stained with HABP and Mayer's hematoxylin. The staining with HABP showed the wide distribution of HA in epidermis and dermis. Especially, the staining was

abundantly localized in intercellular space of epidermal basal cells and the upper dermal region. The staining density and intensity (C), and epidermal thickness (D) in the hairless skin were quantitatively analyzed using computer-assisted digital morphometric analyzer, ImagePro-Plus software as described in "Materials and Methods". * $p < 0.01$: a significant difference from control.

Figure 7. Evaluation of anti-wrinkle effect of oil-in-water emulsion with or without compound K. Skin morphology improvement was observed in comparison between measurements before (A) and 12 weeks after treatments (B). Silicone replica were analyzed at the initial time point and 8 or 12 weeks after applications (C). (ΔR1:depth difference of wrinkles, ΔR2: depth difference of 5 fragmented skin wrinkles, ΔR3: difference of 5 fragmented skin roughness, ΔR4: difference of smoothness depth, ΔR5: difference of arithmetic average roughness.)

Figure 1

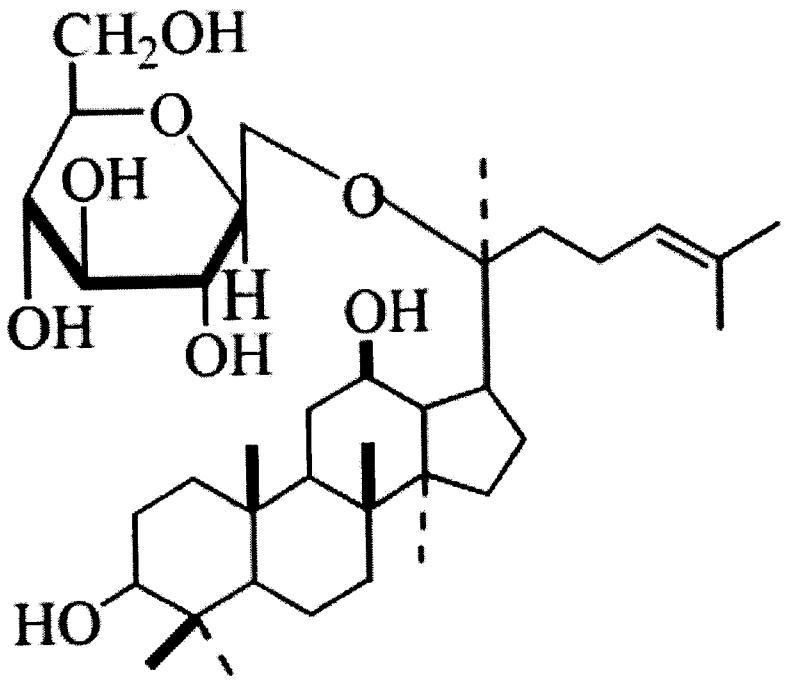
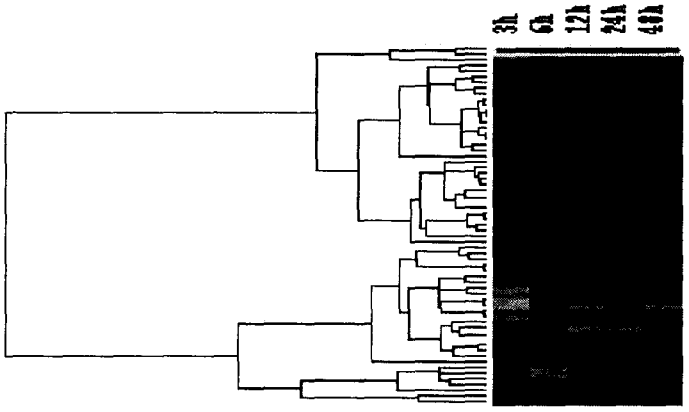


Figure 2

A



B

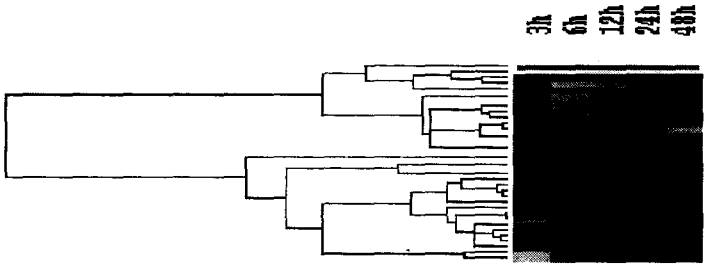


Figure 3

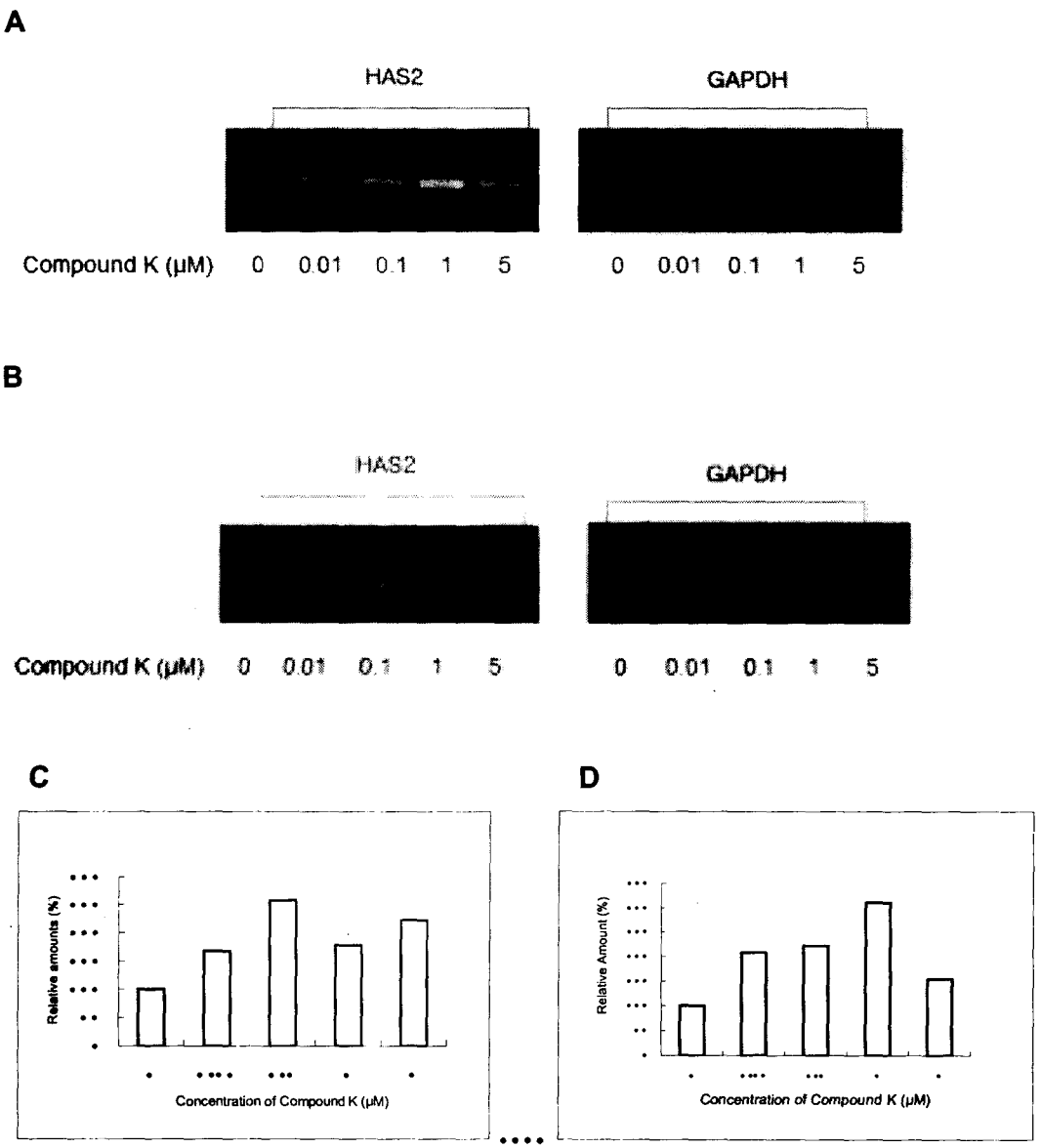
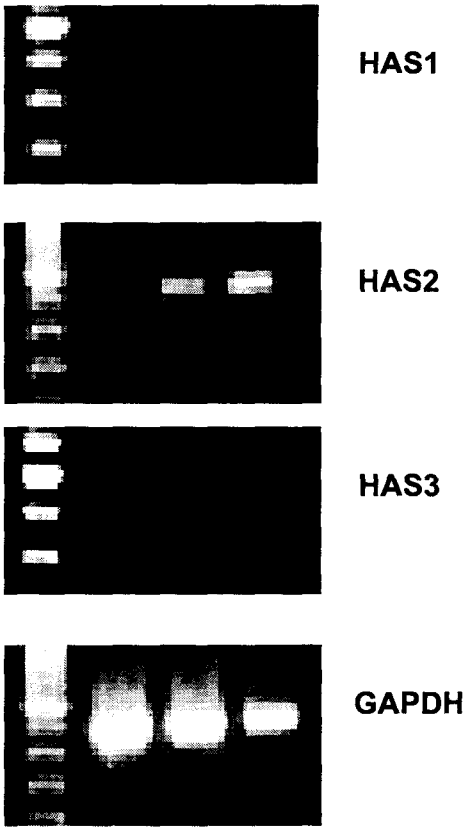

A
HAS2
GAPDH
Compound K (μM) 0 0.01 0.1 1 5
0 0.01 0.1 1 5
B
HAS2
GAPDH
Compound K (μM) 0 0.01 0.1 1 5
0 0.01 0.1 1 5
C
Relative amounts (%)
Concentration of Compound K (μM)
D
Relative Amount (%)
Concentration of Compound K (μM)

Figure 4

A


HAS1
HAS2
HAS3
GAPDH
SM 0h 24h 48 h

B

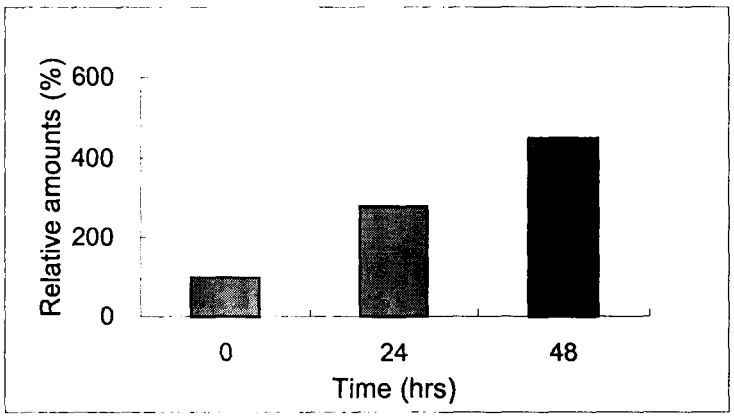

Relative amounts (%)
600
400
200
0
0
24
48
Time (hrs)

Figure 5.

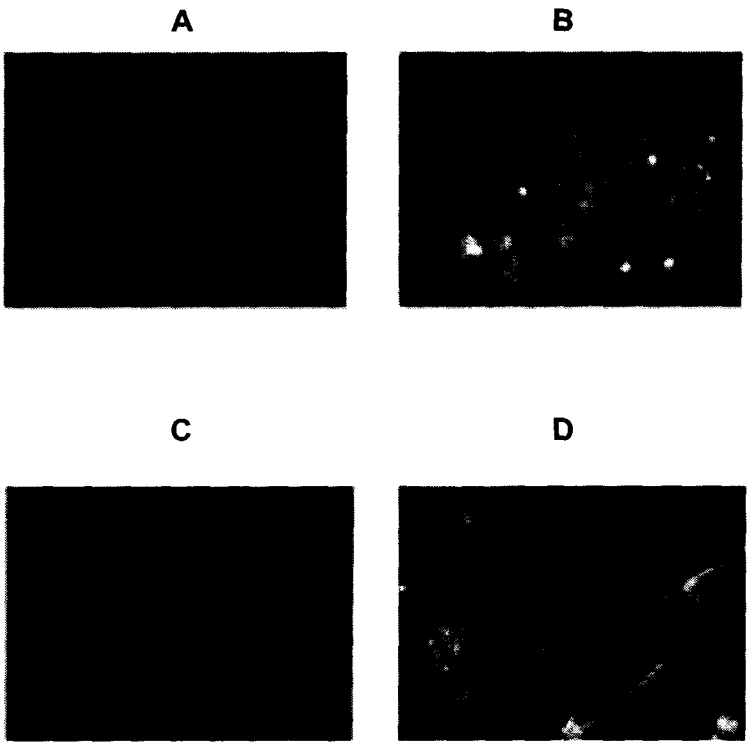
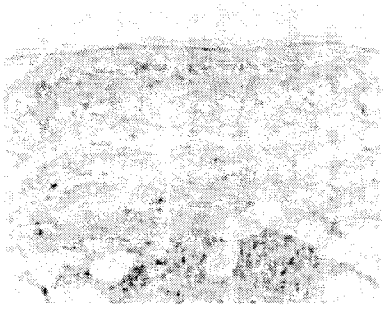
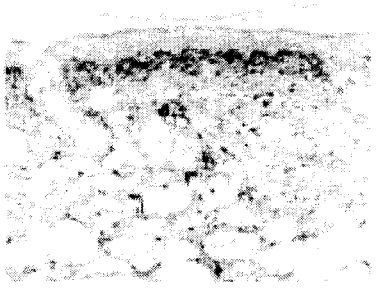


Figure 6

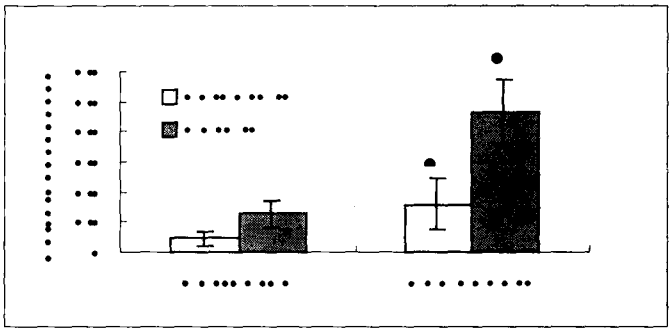
A



B



C



D

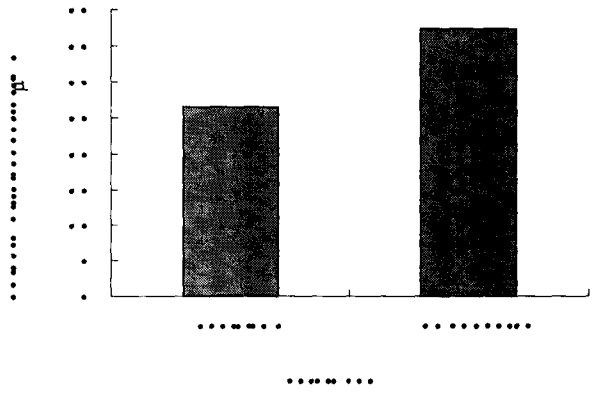
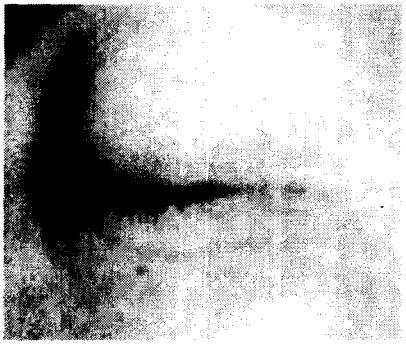
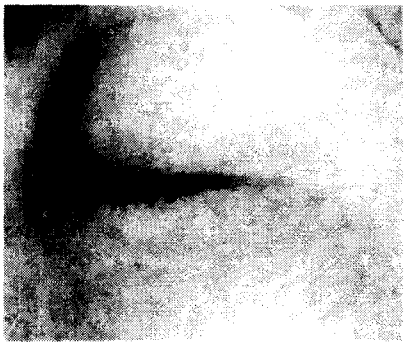


Figure 7

A



B



C