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Black Rice (*Oryza sativa* L. var. *japonica*) Hydrolyzed Peptides Induce Expression of Hyaluronan Synthase 2 Gene in HaCaT Keratinocytes

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Abstract Black rice (*Oryza sativa* L. var. *japonica*) has been used in folk medicine in Asia. To understand the effects of black rice hydrolyzed peptides (BRP) from germinated black rice, we assessed the expression levels of about 20,000 transcripts in BRP-treated HaCaT keratinocytes using human 1A oligo microarray analysis. As a result, the BRP treatment showed a differential expression ratio of more than 2-fold: 745 were activated and 1,011 were repressed. One of the most interesting findings was a 2-fold increase in hyaluronan synthase 2 (HAS2) gene expression by BRP. Semiquantitative RT-PCR showed that BRP increased HAS2 mRNA in dose-dependent manners. ELISA showed that BRP effectively increased hyaluronan (HA) production in HaCaT keratinocytes.

Key words: *Oryza sativa*, germinated black rice, microarray, peptides, hyaluronan synthase

Protein active substances for personal care preparations are obtained from natural sources of both animal and vegetable origin, such as wheat and almonds, as well as soybean and milk proteins. Rice is the primary grain in Asia and is a staple food for many Asian countries. However, more people are familiar with soy and whey protein products. The enzymatic hydrolysis of these plant proteins leads to hydrolysates that possess improved functional properties, as well as having the potential to work as active substances for personal care preparations. Furthermore, germinated plant seeds are receiving increasing attention owing to their possible improvement of nutritional qualities and their biological effects. During germination, some of the seed storage materials are degraded and used for respiration and partly for synthesis of new cell constituents of the developing embryo, including several enzymes and growth factors.

Hyaluronan (HA) is well known to hold water, maintain the extracellular space, and facilitate the transport of ion solutes and nutrients [16, 20]. HA has also been suggested to modulate cell adhesiveness, to alter the structure of the extracellular matrix, and to influence cell migration, differentiation, growth, angiogenesis, and immune regulation [30]. The HA content in the skin is among the highest in the body's organs (0.5–1.0 mg HA per gram wet tissue weight), comprising about 50% of the total HA in a given organism [16]. The HA content is reported to decline with age [19, 9], which may contribute to wrinkle formation and the decrease in elasticity of the skin. Chemicals that modulate the synthesis and degradation of HA are thought to hold the potential for new anti-inflammatories and anti-wrinkle drugs.

HA is synthesized at the inner surface of the plasma membrane by hyaluronan synthase (HAS) and is extruded through the plasma membrane into the extracellular space simultaneously with the ongoing synthesis [35]. Currently, three different HAS genes have been identified in mammalian cells: HAS1, HAS2, and HAS3. The three HAS genes are highly homologous but appear to differ from each other in kinetic properties and product size [2, 11]. Limited data are available on the factors that regulate the expression level and enzymatic activity of the different HAS enzymes in various cells and tissues [8, 37, 13, 6, 22], but a number of studies have suggested that the overall synthesis rate of HA is stimulated by some growth factors and cytokines [10, 27, 32, 33].

In skin epidermis, the narrow extracellular space surrounding keratinocytes contains a high concentration of HA, but it is found mainly between the basal and spinous cell layers of normal human epidermis and much less in terminally differentiated layers [24]. Both in normal and diseased

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epidermis, keratinocyte growth and differentiation are regulated by paracrine and endocrine signaling molecules, such as epidermal growth factor (EGF) and the nuclear hormone all-*trans*-retinoic acid (RA). Interestingly, the HA synthesis rate is stimulated by EGF in epidermal keratinocytes in monolayer [25] and organotypic [24] cultures and by all-*trans*-RA in human skin organ cultures [31]. Direct evidence for the biological role of HA in epidermal keratinocytes emerged from the finding that HAS2-mediated HA synthesis controls the migration rate of keratinocytes in scratch-wounded monolayer cultures [26]. HA concentration is closely correlated with the proliferative activity and volume of the vital part of the epidermis and inversely related with the markers of differentiation, suggesting that HA synthesis regulated by HAS2 and HAS3 is an important component in the proliferative reactions of the epidermis and is also involved in the epidermal differentiation process [24].

Recently, a cDNA microarray-based method was introduced for the high throughput monitoring of gene expressions. This technology has revolutionized gene expression studies by providing the means to measure mRNA levels in thousands of genes simultaneously in simple and complex biological samples [3, 18, 4, 17, 14, 23].

The *Oryza sativa* L. var. *japonica* [Family: Poaceae (syn: Gramineae)], which is commonly known as "Heuk-Mi" (black rice) in South Korea, is a dark purple-colored seed and a major rice crop in South Asia and China. It is broadly known as enriched rice and has been used in traditional medicine for treating various allergic disorders, such as dermatitis and bronchitis [15].

In this study, to develop new active biomaterials through enzyme-treated biotechnology, we obtained novel peptides from the germinated black rice by using microarray technology, and investigated transcriptional responses of HaCaT keratinocytes upon treatment with black rice hydrolyzed peptides (BRP). RT-PCR and ELISA showed that BRP increased HAS2 mRNA expression and HA production in HaCaT keratinocytes.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Cambrex/BioWhittaker Inc. (Walkersville, MD, U.S.A.). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was purchased from Sigma (St. Louis, MO, U.S.A.). RNeasy Mini Kit was purchased from Qiagen (MD, U.S.A.). Human 1A Oligo Microarray Kit (V2), *in situ* Hybridization Kit Plus, and stabilization and drying solution were purchased from Agilent Technologies (Palo Alto, CA, U.S.A.). Albumin (Bovine serum, M.W. 66,000), Carbonic anhydrase (M.W. 29,000), Aprotinin (M.W. 6,500), Angiotensin II acetate (M.W. 1,046), and GLY-TYR (M.W. 238.2) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and used as molecular weight standards for the molecular weight determination by HPLC. All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of Black Rice Hydrolyzed Peptides

Seeds of black rice (*Oryza sativa* L. var. *japonica*) were soaked in distilled water for 12 h at room temperature (~25°C). The soaked seeds were kept between thick layers of cotton cloth and allowed to germinate in the dark for 2 days at room temperature until the length of the sprout ranged between 5–15 mm. The germinated seeds were rinsed with distilled water, ground, and freeze-dried. The germinated rice flour (100 g) was suspended in deionized water (1,000 ml) and then heated to 90°C for 20 min and cooled down to room temperature. After the addition of papain (100 mg) and incubation with shaking for 6 h, the suspensions were heated to 90°C for 20 min and cooled down to room temperature. The enzymatic hydrolysis conditions for papain were pH 5.0 at a temperature of 45°C. The remaining insoluble portion was removed by centrifugation (12,000 rpm, 15 min), and the supernatant was lyophilized and stored at −20°C. Separation and fractionation of the hydrolyzed rice peptide were conducted by gel filtration chromatography. The peptide solution (500 mg/ml) was applied to a Sephadex G-25 column (2.5×50 cm) and eluted with distilled water at a flow rate of 10 ml/h. The eluate was monitored for peptides by measuring absorbance at 210 nm. Fraction tubes that formed a peak the estimated molecular weight below 3,000 daltons were pooled, lyophilized, and stored at −20°C (Fig. 1).

Molecular Weight Determination by HPLC

The molecular weight of BRP was estimated on the basis of the calibration curve made by the gel permeation chromatography (GPC) with a Shodex OHpak KB-804 column (300×0.8 mm, Showa Denko K.K., Tokyo, Japan) eluted with the isocratic mode of 50 mM phosphate buffer, 100 mM NaCl, pH 7.0, at a flow rate of 0.8 ml/min. The eluate was monitored for peptides by measuring absorbance at 210 nm.

Cell Culture

The spontaneously transformed human keratinocyte cell line HaCaT was kindly provided by Dr. N. E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany) [1]. The cells were grown under proper culture conditions (at 37°C in humidified atmosphere containing 5% CO_2) in Dulbecco's modified Eagle's media containing 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin

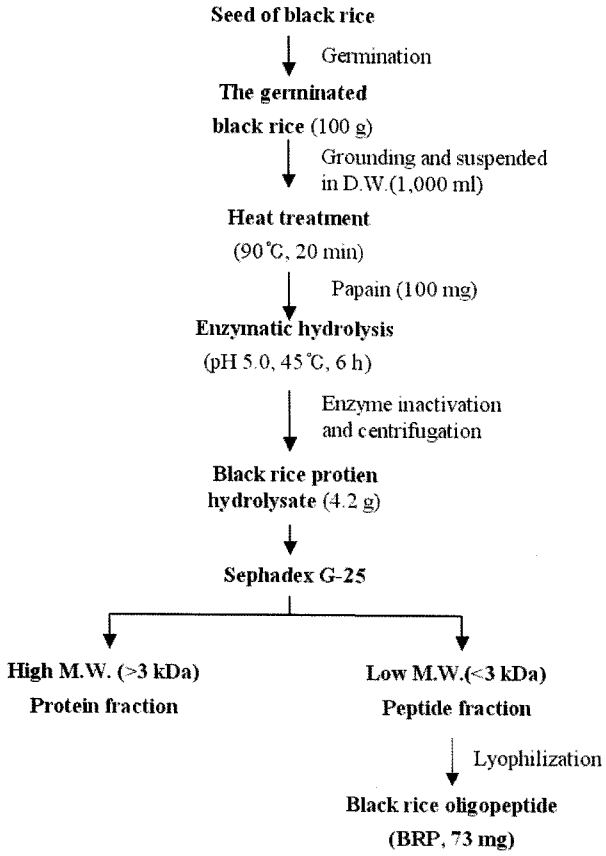

Fig. 1. Preparation procedure of black rice hydrolyzed peptides.

(100 mg/ml). The cells were plated at 1×10^5 cells per 75 cm^2 tissue culture flask and cultivated in medium containing 10% FBS for 24 h. BRP was added and incubated for the indicated time periods.

RNA Preparation

Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen, MD, U.S.A.) according to the manufacturer's instructions. The integrity of the RNA was assessed by electrophoresis with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.A.).

Microarray and Data Analysis

To analyze BRP-responsive genes in the HaCaT keratinocytes, the total cells RNAs were extracted 24 h and 48 h after the treatment of BRP to HaCaT keratinocytes. Antisense amino allyl RNA (aRNA) targets from 2 μg total RNA were prepared using the Amino Allyl MessageAmp aRNA Kit (Ambion, Austin, TX, U.S.A.) according to the manufacturer's protocol. Human 1A Oligo Microarray (V2), which contains over 20,000 60-mer probes corresponding to over 18,000 human genes, was purchased from Agilent. Microarray hybridizations were carried out on Agilent Human oligonucleotide microarrays using a 2 μg Cy3-labeled "reference" sample and 2 μg Cy5-labeled "experimental" sample. Hybridizations were carried out using the Agilent hybridization kit and a Robbins Scientific "22k chamber" hybridization oven (Robbins Scientific, Sunnyvale, CA, U.S.A.). After incubating overnight at 65°C, the slide was washed twice with 2×SSC containing 0.1% SDS for 5 min at 42°C, once with 0.1×SSC containing 0.1% SDS for 10 min at room temperature, and finally with 0.1×SSC for 1 min at room temperature. The slide was dried by centrifugation at 700 rpm for 5 min. Hybridization images on the slide were scanned by a GenePix 4000B scanner (Axon Instruments, Union City, CA, U.S.A.) and analyzed by GenePix Pro 3.0 software (Axon Instruments, CA, U.S.A.) to obtain gene expression ratios (non-treated *vs.* BRP treated). Logged gene expression ratios were normalized by LOWESS (locally weighted scatterplot smoother) regression [36]. The fluorescent intensity of each spot was calculated by local median background subtraction. We used the robust scatterplot smoother LOWESS function to perform intensity-dependent normalization for gene expression. A scatterplot analysis was made by Microsoft Excel 2000 (Microsoft, Redmond, WA, U.S.A.). Significance analysis of microarray (SAM) was performed for the selection of the genes with significant expression changes [34].

Semiquantitative RT-PCR Analysis

Total RNA was subjected to reverse transcription and subsequent PCR to confirm the change of HAS2 mRNA in analyzed cells. A volume of 1 μg of total RNA from each sample was subjected to reverse transcription using an Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification was performed in a reaction volume of 25 μl of cDNA product and HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) using an automatic heat-block DNA thermal cycler (ASTEC PC801, ASTEC Inc., Tokyo, Japan). Amplification of the constitutively expressed GAPDH was used as an internal control to assess the reverse transcription efficiency. The oligonucleotide primers used were 5'- GCT ACC AGT TTA TCC AAA CG-3' (sense) and 5'-GTG ACT CAT CTG TCT CAC CG-3' (antisense) for HAS2; and 5'-ATT GTT GCC ATC AAT GAC CC-3' (sense) and 5'-AGT AGA GGC AGG GAT GAT GT-3' (antisense) for GAPDH. The temperature cycling condition of amplification was as follows for HAS2 and GAPDH: 15 min at 94°C, then 26–28 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 10 min. The amplification products were electrophoresed on 1.5% agarose gel, visualized by ethidium bromide staining, and photographed. Gel images were scanned using an image analysis system (BIS303PC, DNR Imaging Systems Ltd., U.K.). The intensities of specific PCR bands were quantitated in relation to GAPDH bands amplified from the same cDNA using a densitometric program (NIH Image Software, MD, U.S.A.).

Hyaluronan Measurement

HaCaT keratinocytes were grown to high density in 24-well plates. Immediately before the experiments, cells were washed two times with serum-free medium to completely remove HA accumulated during cell growth. Subsequently, HaCaT keratinocytes were cultured with or without BRP in 0.5 ml serum-free medium for 24 h. At the indicated time, aliquots of medium were removed, centrifuged at 15,000 ×*g* for 5 min, and supernatants were analyzed for HA using an enzyme-linked immunosorbent assay (ELISA) kit (Echelon Bioscience, Salt Lake, U.S.A.) according to the manufacturer's instructions. Finally, optical density was monitored at 405 nm on a microplate reader (ELX 800, Bio-Tek Instruments, VT, U.S.A.).

Statistical Analysis

All data except for the microarray results were expressed as the mean±SEM of independent experiments. Statistical significance was compared between the BRP treatment group and the control by the Student's *t*-test. Results with $p<0.05$ were considered statistically significant.

Results

Preparation of Black Rice Hydrolyzed Peptides

We confirmed the differences of protein contents, the molecular weight distribution of proteins by SDS-PAGE, and the composition of amino acids through the black rice germination. During germination, the total soluble protein of black rice was increased by 35% through the germination process. However, the protein contents analysis by SDS-PAGE indicated that the content of low molecular weight proteins was significantly increased, whereas the protein contents of high molecular weight proteins were decreased by germination (data not shown). Furthermore, we could produce novel peptides that had low molecular weight by the combination of the germination and the treatment with papain. The hydrolysates were fractionated on the basis of their molecular weight by gel filtration chromatography. Additionally, the black rice hydrolyzed peptides (BRP) were prepared from the fraction tubes that formed a peak estimated molecular

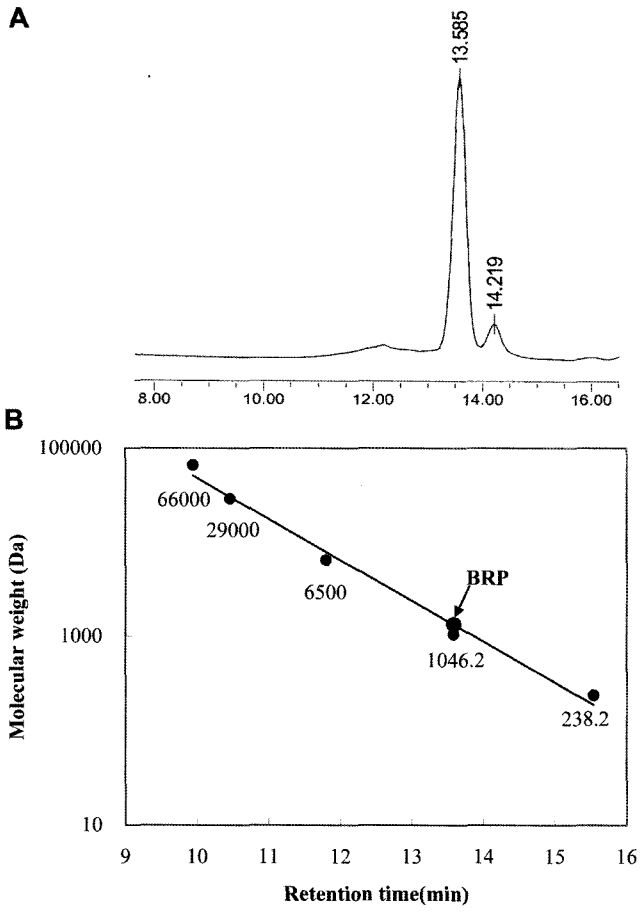

Fig. 2. Typical elution chromatogram in a gel permeation chromatography by HPLC (**A**) and determination of the molecular weight of black rice hydrolyzed peptides (**B**).

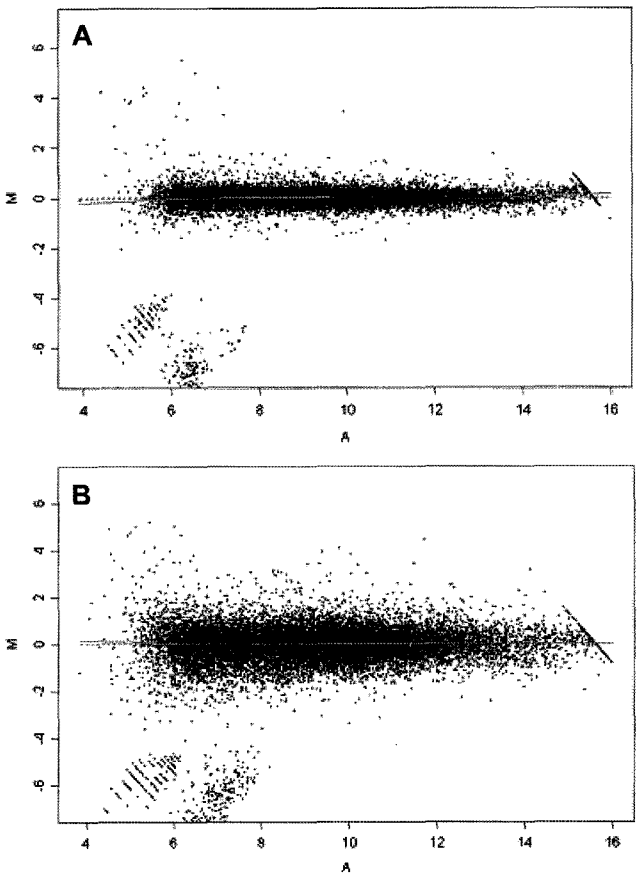

Fig. 3. MA-plots (M, expression ratio; A, signal intensity) represent genes activated and repressed by black rice hydrolyzed peptides in HaCaT keratinocytes.

The MA-plot is used to represent the (R, G) data (R, red for Cy5; G, green for Cy3) where M=log2R/G and A=log2(RxG). **A**. Black rice hydrolyzed peptides treatment/24 h; **B**. Black rice hydrolyzed peptides treatment/48 h.

weight below 3,000 daltons and were lyophilized. The molecular size distribution of BRP was measured by HPLC on a GPC column. The chromatograms obtained from the gel permeation column showed that the major peak of BRP was located at approximately 1,300 daltons. (Fig. 2).

Microarray Analysis of Differential Gene Expression in HaCaT Keratinocytes after BRP Treatment

We used HaCaT keratinocytes as a model system for investigating BRP responsive gene expression. Total RNA was isolated at two different times (24 and 48 h) after BRP treatment and fluorescently labeled probes were prepared. Hybridization was performed with a human 1A oligonucleotide microarray slide. After hybridization, the microarray slide was scanned and analyzed using commercial software. To confirm their reproducibility, experiments were repeated independently three times and relative changes were calculated. The mean values of the intensities of each spot in the three experiments were calculated and are plotted in Fig. 3. Of the 20,173 genes examined in the BRP treatment/24 h protocol, changes in mRNA expression

Table 1. Genes upregulated by black rice hydrolyzed peptides in HaCaT keratinocytes.

Gene name	GenBank no.	Fold change	
		24 h	48 h
Cell cycle			
Mdm2, transformed 3T3 cell double minute 2, p53	NM_002392	2.21	3.39
Histone deacetylase 5	NM_005474	1.45	2.89
Lymphocyte-specific protein tyrosine kinase	NM_005356	2.14	2.11
Cellular physiological process			
ATPase, H+ transporting, lysosomal 38 kDa, V0 subunit d isoform 2	NM_152565	1.99	8.17
Arachidonate 12-lipoxygenase, 12R type	NM_001139	2.00	6.96
Superoxide dismutase 2, mitochondrial	NM_000636	1.91	4.14
Cytochrome P450, family 1, subfamily A, polypeptide 1	NM_000499	1.54	4.14
Transient receptor potential cation channel, subfamily V, member 2	NM_016113	1.75	3.41
Activating transcription factor 3	NM_004024	1.54	3.25
Serine (or cysteine) proteinase inhibitor, clade B, member 4	NM_002974	2.57	2.75
Arachidonate lipoxygenase 3	NM_021628	1.53	2.75
Ferritin, heavy polypeptide 1	NM_002032	1.85	2.57
Cytochrome P450, family 4, subfamily F, polypeptide 8	NM_007253	1.58	2.11
Immune response			
Guanylate-binding protein 2, interferon-inducible	NM_004120	1.42	3.41
Immunoglobulin lambda constant 1 (Mcg marker)	BC015833	1.87	3.12
Lymphocyte antigen 96	NM_015364	2.03	3.07
Signal transduction			
Growth differentiation factor 15	NM_004864	1.34	22.16
Defensin, beta 4	NM_004942	3.39	4.56
G protein-coupled receptor 45	NM_007227	1.57	2.48
Growth factor receptor-bound protein 10	AF000018	1.61	2.23
Interleukin 1 receptor-like 1	NM_016232	3.12	1.97
Melatonin receptor 1B	NM_005959	18.51	1.85
Others			
Thioredoxin interacting protein	NM_006472	10.78	17.15
Hypothetical protein BC012317	NM_138397	1.82	10.70
Interleukin 23, alpha subunit p19	NM_016584	1.78	10.41
Stress 70 protein chaperone, microsome-associated, 60 kDa	NM_006948	1.30	7.73
S100 calcium-binding protein P	NM_005980	2.08	5.90
Small proline-rich protein 1A	NM_005987	3.39	3.16
Brain-enriched guanylate kinase-associated protein	NM_020836	4.41	3.32
Interferon-stimulated gene 20 kDa	NM_002201	1.93	3.23
Hyaluronan synthase 2	NM_005328	3.45	3.17
Insulin growth factor-like family member 1	NM_198541	1.45	2.51
Keratinocyte-associated protein 3	AY358993	1.44	2.03
Interleukin 13 receptor	NM_000640	3.36	1.58

Table 2. Genes downregulated by black rice hydrolyzed peptides in HaCaT keratinocytes.

Gene name	GenBank no.	Fold change	
		24 h	48 h
Cell cycle			
Asp (abnormal spindle)-like, microcephaly-associated	NM_018136	-1.45	-4.53
M-phase phosphoprotein 1	NM_016195	-1.41	-3.61
PMS1 postmeiotic segregation increased 1	NM_000534	-1.48	-3.39
Chromosome condensation protein G	NM_022346	-1.42	-3.32
Cellular physiological process			
Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_001511	-1.47	-9.00
Clathrin, heavy polypeptide-like 1	NM_001835	-1.60	-5.31
Splicing factor, arginine/serine-rich 5	NM_006925	-1.44	-4.59
Hypothetical zinc finger protein FLJ14011	NM_022103	-1.49	-4.50
Zinc finger and SCAN domain-containing 5	NM_024303	-1.71	-4.38
Solute carrier organic anion transporter family, member 3A1	AK130644	-1.54	-3.68
Ubiquitin-specific protease 13 (isopeptidase T-3)	NM_003940	-1.73	-3.53
Fanconi anemia, complementation group A	NM_000135	-1.40	-3.34
Pyruvate dehydrogenase (lipoamide) alpha 2	NM_005390	-1.43	-3.32
3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	NM_002130	-3.20	-3.25
Hypothetical protein dJ122O8.2	NM_020466	-1.51	-3.20
Distal-less homeobox 3	NM_005220	-2.11	-2.31
Immune response			
Chemokine (C-X-C motif) ligand 2	NM_002089	-2.16	-8.17
Chemokine (C-X-C motif) ligand 3	NM_002090	-2.10	-3.07
Signal transduction			
Phosphoinositide-3-kinase, class 2, gamma polypeptide	NM_004570	-1.65	-2.01
Others			
Hypothetical protein FLJ14397	NM_032779	-1.73	-12.47
Fusion [involved in t(12;16) in malignant liposarcoma]	NM_004960	-1.46	-5.46
SLIT and NTRK-like family, member 6	NM_032229	-1.45	-5.13
Serum deprivation response (phosphatidylserine-binding protein)	NM_004657	-1.42	-3.61
Hypothetical protein FLJ25414	NM_152343	-1.60	-3.23
Epsin 3	NM_017957	-1.59	-3.05
DNA glycosylase hFPG2	NM_018248	-1.56	-2.95
FLJ38822 protein	NM_207398	-1.48	-2.91
Kinesin family member 20A	NM_005733	-1.35	-2.91

were detected in 170 genes: 121 were activated and 49 were repressed (Tables 1 and 2). Only 0.8% of all genes were activated or repressed more than 2-fold, with BRP causing no significant differences in the expression of the remaining 99.2% of the genes. However, in the BRP treatment/48 h protocol, 2,105 genes showed a differential expression ratio of more than 2-fold, or less than 50%: 1,019 were activated and 1,086 were repressed. About 10.4% of all genes were activated or repressed (Tables 1 and 2). Out of the 2,105 activated or repressed genes, 1,979 genes were listed as known genes in GenBank, and 126 genes were unknown. These genes were classified into nine functions: signal transduction-related, cell cycle-related, cellular physical process-related, cell death-related, response to stress-related, transcription-related, immune response-related, apoptosis-related, and others (including expressed sequence tags and unknown genes).

BRP Increased HA Synthase via Upregulation of HAS2 Gene in HaCaT Keratinocytes

HAS2 was selected for further functional studies. First, to confirm a change of HAS2 expression by BRP, the mRNA level of HAS2 was measured by semiquantitative RT-PCR. This comparative analysis showed that HAS2 mRNA was hardly detectable or was detected at a low level in the controls; however, HAS2 mRNA dramatically increased by BRP in dose-dependent manners (Fig. 4). Next, competitive ELISA experiments were performed in order to determine whether the observed elevated levels of HAS2 mRNA in BRP-treated HaCaT keratinocytes also resulted in increased HA production. As shown in Fig. 5, BRP-treated cells produced higher levels of detectable HA than untreated cells. HA levels in the media of untreated cells were 352.1 ng/ml and HA levels in the media of cells treated with 0.5, 1, or 5 mg/ml BRP were

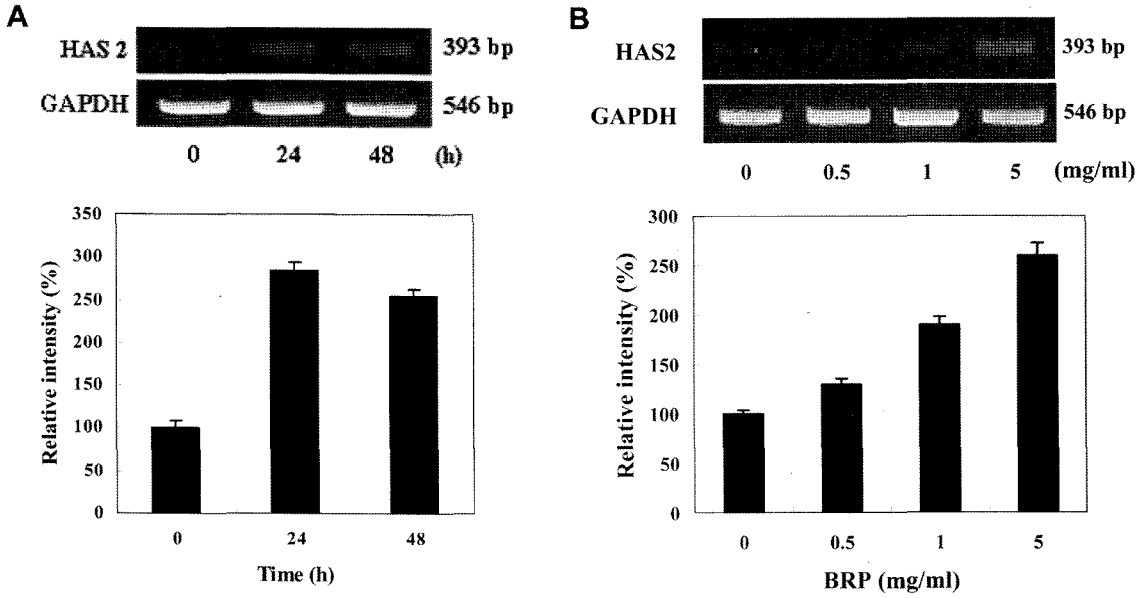

Fig. 4. Further confirmation of microarray data by semiquantitative RT-PCR.
HaCaT keratinocytes were treated with the 5 mg/ml black rice hydrolyzed peptides for indicated time periods (**A**) or the indicated concentrations of black rice hydrolyzed peptides for 24 h (**B**). At the end of incubation, total RNA was isolated and reversely transcribed. The cDNA of HAS2 and GAPDH, an internal control, were amplified with 26–28 PCR cycles.

428.7 ng/ml, 494.9 ng/ml, and 647.5 ng/ml, respectively. These results seemed to be closely correlated with the changes in mRNA observed in the semiquantitative RT-PCR experiments.

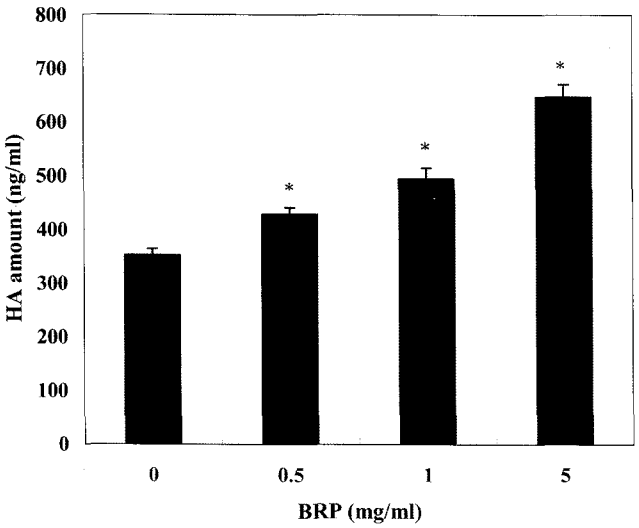

Fig. 5. Effects of black rice hydrolyzed peptides on the amount of hyaluronan released by HaCaT keratinocytes.
Cells were grown to high density and washed with serum-free medium to completely remove HA accumulated during cell growth. Subsequently, cells were treated with the indicated concentrations of black rice hydrolyzed peptides for 24 h. At the end of incubation, aliquots of culture media were removed, centrifuged at 15,000 ×*g* for 5 min, and supernatants were analyzed for the presence of hyaluronan using an enzyme-linked immunosorbent assay kit. All values are means±SD of 3 independent experiments. *$p<0.05$ compared with control.

DISCUSSION

In this study, we adopted microarray analysis to understand the effects of BRP, black rice hydrolyzed peptides, on the HaCaT keratinocytes. When the expression levels of about 20,173 transcripts in BRP-treated HaCaT keratinocytes were assessed, marked alterations in the expressions of 2,105 genes, which have been reported to be involved in the organization of ECM structure as well as defense responses in human skin cells, were observed. HAS2 was one of the most significantly regulated genes with respect to its transcriptional responses to BRP.

Human HASs use sugar substrates from UDP donors to form disaccharides, consisting of D-glucuronic acid and N-acetyl-D-glucosamine, which is an essential component of HA, the major extracellular matrix molecule in the skin. Generally, HA has biological functions, such as water retention and maintenance of intercellular space. The proposed roles of HA in the skin include providing moisture and elasticity, maintaining the dermal structure, and facilitating the transport of ion solutes and nutrients [16, 21]. Since HA has a short half-life of only 0.5 day and HAS has an extremely short half-life of 2–4 h [16], the regulation of HAS gene expression is proposed to play an important role in the control process of HA status [21].

To confirm a change of HAS2 gene expression by BRP, we conducted semiquantitative RT-PCR. Our results showed that BRP at as low as 0.5 mg/ml significantly upregulated HAS2 gene expression in cultured HaCaT keratinocytes. The increase in HAS2 mRNA after BRP treatment was

dose-dependent. This observation was in good accordance with the result from the microarray analysis. Consistent with mRNA levels of the HAS2 gene, a pronounced increase in the HA content was observed in BRP-treated HaCaT keratinocytes when analyzed by ELISA.

Previously, changes in the content or metabolism of HA in the skin have been implicated in many pathophysiological conditions or pharmacological treatments [12]. In particular, a linear, age-dependent decrease in the content of HA has been showed in aged human skin [7, 19]. Determining if the age-dependent decrease in the HA content is caused by the activation of the system for degrading HA or by the decreased HAS activities remains unclear; however, pharmacological agents, such as estrogen or retinoic acid, are known to enhance HA synthesis and to prevent skin atrophy, dryness, and wrinkles in human skin [29, 28, 5].

In conclusion, our study demonstrated that BRP induced the expression of the HAS gene and enhanced HA synthesis in HaCaT keratinocytes. Although further clinical studies with BRP are needed to investigate whether or not it is truly applicable to human skin, BRP holds great promise for new therapeutic applications to xerosis of aged persons.

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