# Effect of hyaluronan tetrasaccharides on epidermal differentiation in normal human epidermal keratinocytes

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**Abstract**

Hyaluronan plays a role in keratinocyte proliferation and differentiation. In addition, hyaluronan has been shown to have different biological activities depending on its molecular weight. It has been reported that hyaluronan-mediated CD44 activation regulates keratinocyte differentiation. Therefore, the aim of the present study was to investigate the influence of hyaluronan tetrasaccharides (HA4) on the regulation of keratinocyte differentiation, CD44 gene expression, and CD44-phosphorylated protein in human keratinocytes, and compare HA4 with high molecular weight hyaluronan (HA). Normal human epidermal keratinocytes (NHEKs) were treated at doses of 1 μg/mL HA or HA4. After treatment, cell viability was checked using an MTT assay. Each differentiation marker and CD44 mRNA expression was detected by real-time PCR. Each differentiation marker and CD44-phosphorylated protein was assessed by western blotting. HA and HA4 showed no cytotoxicity up to a dose of 1 μg/mL. On day 3 after HA4 treatment, each differentiation marker mRNA and K10 protein level was more than that of the control. On day 9, late differentiation marker mRNA and protein levels were increased with HA and HA4 treatment. In addition, HA4 treatment increased the expression of CD44 mRNA, CD44-phosphorylated protein, and intracellular calcium concentrations. HA4 enhanced keratinocyte differentiation and increased CD44-phosphorylated protein levels. HA4 may induce epidermal differentiation through phosphorylation of CD44.

***Keywords:*** hyaluronan oligosaccharide / keratinocyte differentiation / CD44 phosphorylation

## Introduction

The major function of the epidermis is to provide a barrier between the external environment and the organism. The main cell in the epidermis is the keratinocyte. In the epidermis, dividing stem cells give rise to progeny cells destined to migrate upward through the epidermal layer until they die and are lost from the surface. This process is called differentiation. In the normal mammalian epidermis, cell division and expression of keratins K5/K14 in the basal layer are followed by K1/K10, the keratin bundling protein filaggrin, and cornified envelope proteins such as involucrin, loricrin, and transglutaminase (TGase) [1, 2]. Terminal differentiation of mammalian epidermal keratinocytes results in the formation of the stratum corneum, which consists of dead cells filled with cross-linked keratin protein surrounded by a toughened cornified cell envelope.

Hyaluronan, which is composed of repeated β-1,4-glucuronic acid-1,3-*N*-acetylglucosamine disaccharide units, is a non-sulfated glycosaminoglycan with a molecular weight of over 1,000 kDa. Hyaluronan is one of the major extracellular matrix components in the skin. Hyaluronan exists freely in extracellular matrix spaces, but is also involved in many biological processes such as tissue homeostasis, cell proliferation, cell migration, cell differentiation, angiogenesis, tumor biology, and repair processes by the interface of each protein [3].

CD44 is the major receptor of hyaluronan [4]. The interaction between CD44 and hyaluronan can mediate both cell-cell and cell-extracellular matrix interactions and has been shown to be important in a variety of physiological and pathophysiological processes, including tumour metastasis, wound healing, and leukocyte extravasation at sites of inflammation [5]. The ability of CD44 to bind hyaluronan is tightly regulated. CD44 can exist in inactive non-binding forms or an active ligand binding form.

Stimulation by hyaluronan of the living body appears to depend on many factors including hyaluronan chain length and hyaluronan receptors such as CD44. For example, high molecular weight hyaluronan was demonstrated to induce anti-inflammatory and anti-angiogenic responses [6, 7]. In contrast, low molecular weight hyaluronan has been implicated in several biological processes including angiogenesis, cell proliferation, maturation, migration, activation of protein tyrosine kinase cascades, and inflammatory gene expression [8-14] Hyaluronan oligosaccharides, which are much smaller than low molecular weight hyaluronan, up-regulates heat shock protein 72 expression [15]. It has been reported that hyaluronan-mediated

CD44 activation regulates keratinocyte differentiation [16]. In this way, hyaluronan was reported to have many functions, but little is known about the effect of low molecular hyaluronan on keratinocyte differentiation. In this study, we aimed at investigating the influence of hyaluronan oligosaccharides (HA4) with two repeats of the disaccharide unit on the regulation of keratinocyte differentiation in human keratinocytes, and compared HA4 with high molecular weight hyaluronan. In addition, CD44 gene expression and CD44-phosphorylated protein amounts were measured and assessed.

## Materials and methods

### Materials

Abdominal skin origin normal human epidermal keratinocytes (NHEKs) were purchased from Biopredic (Rennes, France). HuMedia-KG2 was purchased from Kurabo (Osaka, Japan). RNAiso Plus, the PrimeScript® RT reagent Kit, and SYBR® *Premix Ex Taq*™ were purchased from Takara Bio (Shiga, Japan). Primers were purchased from Invitrogen (CA USA). Polyvinylidene difluoride membranes and western blotting detection reagents were purchased from GE Healthcare (Buckinghamshire, UK). HA4 (99.14%) (776.3 Da) were provided by Glycoscience Laboratories Inc. (Tokyo, Japan). High-molecular weight hyaluronan (HA) (>1200 kDa) produced by a *Streptococcus zooepidemicus* was used. All other chemicals and solvents used were analytical grade.

### Cell culture

Cells were seeded in 100 mm dishes ($2.0 \times 10^5$ cells per dish). NHEKs were grown in HuMedia-KB2 supplemented with 10 μg/mL insulin, 0.1 ng/mL human epidermal growth factor, 0.5 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphotericin B, and 0.4% (v/v) bovine pituitary extract. NHEKs were incubated in a humidified atmosphere of 5% $CO_2$ at 37ºC. The culture medium was changed every day. Once cells had become subconfluent, they were subcultured in 60 mm dishes ($1.5\text{-}2.0 \times 10^5$ cells per dish) in the same medium. After culturing for 1 day, cells were treated at doses of 1 μg/mL HA or HA4 and cultured.

### MTT assay

NHEKs were seeded on 24-well culture plates and incubated for 1 day. After treatment with HA and HA4 for 24 h, cells received 0.5 mg/mL MTT solution and were incubated for a further 3 h. The medium was removed and the resulting formazan crystal was solubilized in 1 mL of 0.04 M hydrochloric acid/isopropyl alcohol. The optical density at 570 nm was determined using a microplate reader (SpectraMax M2$^e$, Molecular Devices, Tokyo, Japan).

**RNA extraction and quantitative real-time PCR**

Total RNA was isolated from cells following standard procedures using RNAiso Plus. The first strand of cDNA was synthesized from 0.5 μg total RNA from cell samples using the PrimeScript® RT reagent Kit and a thermal cycler (Veriti, Applied Biosystems, CA, USA). The cDNA samples generated were diluted and used for real-time PCR analysis. Briefly, 2 μL of diluted cDNA was mixed with both forward and reverse primers (Table 1) and SYBR® *Premix Ex Taq*™ in 20 μL final volumes. Amplification was performed using the Real-Time PCR System (ABI PRISM® 7500, Applied Biosystems, CA, USA). Amplified PCR products were quantified by measuring each gene and GAPDH mRNA calculated cycle thresholds ($C_T$). The amount of specific mRNA in samples was calculated from the standard curve and was normalized with GAPDH mRNA. Results were expressed as an *n*-fold difference relative to normal controls (relative expression levels).

**Western blotting assay**

NHEKs were washed twice in PBS and subsequently dissolved in lysis buffer solution (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol), 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. Proteins were heated in sample buffer (0.1 M Tris-HCl, pH 6.8, 25% glycerol, 5% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol) for 4 min at 95ºC and subjected to SDS-PAGE. Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes using a tank apparatus (Bio-Rad, CA, USA). Subsequently, blots were transferred to a blocking buffer (Tris-buffered saline (TBS), pH 7.6, 0.1% Tween 20, 5% w/v powdered-skim milk) and incubated for 1 h at room temperature. After being washed in three stages in wash buffer (TBS, pH 7.6, 0.1% Tween 20), blots were incubated with primary antibody in 5% powdered-skim milk at room temperature. After 1 h of gentle shaking, blots were washed three times in wash buffer. Antibodies were detected with peroxidase-conjugated anti-rabbit IgG and peroxidase-positive bands were detected by LAS-1000 (Fujifilm, Tokyo, Japan).

**Data analysis**

Analysis was performed using Statistical Analysis SAS statistical software ver. 9.2 (SAS Institute, Cary, NC). Indicated *P*-values were derived from the Tukey's *post-hoc* multiple comparison test.

## Results

### The cytotoxicity of HA and HA4 on NHEKs

To investigate the cytotoxicity of HA and HA4 on cells, we treated NHEKs with HA and HA4. After treatment for 24 h, cell viability was checked by the MTT assay. As a result, HA and HA4 showed no cytotoxicity up to a dose of 1 μg/mL (Fig. 1).

Fig. 1

### Differentiation marker mRNA expressions and protein evaluation

The mRNA expression levels of differentiation marker mRNA were assessed by quantitative real-time PCR in NHEKs relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). NHEKs were cultured with medium as controls and HA and HA4 (1 μg/mL) for 4, 8, 24, 72, and 216 h. After each hour of treatment, quantitative real-time PCR was used to determine the levels of K5, K10, involucrin, profilaggrin, and TGase mRNA relative to the control gene. As a result, treatment of NHEKs with HA and HA4 increased K10, involucrin, profilaggrin and TGase mRNA expression in a time dependent manner. HA and HA4 treatment for 4, 8, and 24 h enhanced little change in the expressions of all differentiation marker mRNA (data not shown). The expressions of involucrin, profilaggrin, and TGase mRNA with HA4 treatment for 72 h (3 days) were 1.3-, 1.3-, and 1.5-fold higher than control (Fig. 2). The expressions of all differentiation marker mRNA were increased with HA and HA4 treatment for 216 h (9 days). Specifically, the expressions of involucrin, profilaggrin, and TGase mRNA of late differentiation markers were notably increased with HA and HA4 treatment. The expressions of profilaggrin and TGase mRNA with HA treatment were 1.6- and 1.6-fold higher than control, increases which were considered to be significant. In addition, the expressions of profilaggrin and TGase mRNA with HA4 treatment were increased 1.5- and 1.6-fold over that of control (Fig. 3), increases which were also considered to be significant.

Fig. 2

Fig. 3

Next, the protein levels of K10, involucrin, and filaggrin were assessed by western blotting in NHEKs relative to β–actin. NHEKs were cultured with medium as controls and HA and HA4 for 3 and 9 days. As a result, K10 protein levels with HA4 treatment for 3 days was 2.2-fold higher than control, but the cultured did not express involucrin and filaggrin proteins of late differentiation markers (Fig. 4). Involucrin protein levels with HA and HA4 treatment for 9 days were 1.4- and 2.0-fold higher, and filaggrin protein levels with HA and HA4 treatment were 1.4- and 1.2-fold higher than control (Fig. 5).

Fig. 4

Fig. 5

**CD44 mRNA expression and CD44-phosphorylated protein levels**

The expression of CD44 mRNA was investigated by real-time PCR. NHEKs were cultured with medium as controls and HA and HA4 for 4, 8, 24, and 216 h. As a result, HA4 treatment increased the expression of CD44 mRNA. Specifically, CD44 mRNA expression with HA4 treatment was increased 1.5-fold over on 216 h (9 days) that of control (Fig. 6), an increase that was considered to be significant. The CD44-phosphorylated protein was assessed by western blotting. NHEKs were cultured with medium as controls and HA and HA4 for 8 and 24 h. As a result, CD44-phosphorylated protein levels were significantly increased with HA4 than that of the control and HA after HA and HA4 treatment for 8 h (Fig. 7a). HA and HA4 treatment for 24 h enhanced little change in CD44-phosphorylated protein levels (Fig. 7b).

Fig. 6

Fig. 7

**Discussion**

Our group previously reported that HA4 permeated through skin by passive diffusion and treatment with HA4 improved skin functional recovery after UVA irradiation [17]. Next, to elucidate the healing mechanisms of HA4, we examined the effects of HA4 on epidermal differentiation in NHEKs. Our studies have suggested that keratinocyte differentiation was enhanced by HA4.

Involucrin and filaggrin were late markers of differentiation that were specifically expressed in suprabasal (granular) epidermal layers [18]. Involucrin protein and mRNA levels were regulated by agents that regulate keratinocyte differentiation, calcium, hydrocortisone, retinoids, and vitamin D [19-21]. Hydrocortisone, which inhibits epidermal proliferation and enhances terminal differentiation, is accompanied by a decrease in epidermal hyaluronan [22]. In *Streptomyces* hyaluronidase-treated cultures, profilaggrin levels were increased relative to controls [23]. In addition, it has been shown using CD4 knockout mice that epidermal differentiation is stimulated by hyaluronan [16]. In this way, hyaluronan and keratinocyte differentiation were related closely. In this study, it was revealed that HA4 enhanced keratinocyte differentiation in the same manner as HA (Fig. 2-5). Moreover, the keratinocyte differentiation induction by HA4 was faster than HA (Fig. 2, 4). The expression of each differentiation marker mRNA with HA4 treatment for 3 days was increased than that of the control (Fig. 2). In addition, K10 protein levels with HA4 treatment for 3 days were significantly increased 2.2-fold higher than control (Fig. 4). The expressions of late differentiation marker mRNA with HA4 and HA treatment for 9 days were significantly increased than that of the control (Fig. 3). In addition, involucrin and filaggrin protein levels with HA4 and HA treatment for 9 days were increased 2.0- and 1.2-fold over that of the control (Fig. 5). HA4 permeated through skin by passive diffusion [17]. However, hyaluronan can't penetrate into the skin membrane because of high water solubility and a high molecular weight (100 kDa). Consequently, HA4 may induce differentiation of the skin by only application.

Next, we examined the mechanism of keratinocyte differentiation. CD44 was widely distributed, which makes it the major hyaluronan receptor on most cell types. The major receptor for hyaluronan on the surface of epidermal keratinocytes was CD44. Activation of CD44 by hyaluronan increased signal transduction and several downstream biological activities. For example, hyaluronan and CD44 interaction induced intracellular signal cascades that regulate cell-cell adhesion, cell migration, and proliferation, which are required for morphogenesis and

wound healing [24-30]. In addition, the interaction between hyaluronan and CD44 is related to keratinocyte differentiation, cholesterol synthesis, and lamellar body formation, which are required for maintenance of normal stratum corneum structure and epidermal barrier function [16, 28]. In cultured cells, CD44 was constitutively phosphorylated at serine in the cytoplasmic tail [31-33]. In addition, hyaluronan-CD44 interaction with a small GTPase (Rac1)-dependent protein kinase N-γ (PKNγ) kinase activation promotes phosphorylation of phospholipase C (PLC) γ1 phosphorylation and cortactin, leading to the onset of PLC γ1-mediated $IP_3$ production/$Ca^{2+}$ signaling and cortactin-mediated cytoskeleton function required for keratinocyte function (e.g. cell-cell adhesion and differentiation) [28]. In this way, various signaling pathways were promoted by the hyaluronan-CD44 interaction. In our study, CD44 mRNA expression and CD44-phosphorylated protein levels with HA4 treatment were increased that of the control (Fig. 6 and 7). Altogether, HA4 enhanced keratinocyte differentiation and increased CD44-phosphorylated protein levels. However, CD44 mRNA expression and CD44-phosphorylated protein level have a time lag. Therefore, we thought that these two have not directly-involved. The CD44 mRNA expression was increased by HA4 may involve in secondary action. From these results, it was suggested that HA4 enhanced keratinocyte differentiation through phosphorylation of CD44. However, we don't examine other factors which increases and decreases by epidermal differentiation through the phosphorylation of the CD44. Accordingly, it is necessary to clarify the detailed mechanism of epidermal differentiation by HA4. Our group previously reported that HA4 improved skin functional recovery after UVA irradiation [17]. That epidermal differentiation was enhanced by HA4 may be one of the factors for skin functional recovery after UVA irradiation.

**Acknowledgements**

The authors thank Mr. Yuichiro Mori and Ms. Akiko Miyake (Shiseido Research Center) for insightful comments and suggestions.

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**Figure 1** Effect of HA and HA4 treatment on the cell viability of NHEKs. Cell viability was determined by the MTT assay. NHEKs were treated for 24 h with 1 μg/mL HA and HA4. Values are the mean ± S.D. of three experiments.

**Figure 2** Effect of HA and HA4 treatment for 3 days on each differentiation marker mRNA expression in NHEKs. K5 (A), K10 (B), involucrin (C), profilaggrin (D), and TGase (E) mRNA expressions were determined by quantitative real-time PCR. NHEKs were treated for 3 days with 1 μg/mL HA and HA4. Values were normalized to GAPDH housekeeping gene levels and compared with untreated controls. Values are the mean ± S.D. of three experiments. $*p < 0.05$, Tukey's *post-hoc* multiple comparison test.

**Figure 3** Effect of HA and HA4 treatment for 9 days on each differentiation marker mRNA expression in NHEKs. K5 (A), K10 (B), involucrin (C), profilaggrin (D), and TGase (E) mRNA expressions were determined by quantitative real-time PCR. NHEKs were treated for 9 days with 1 μg/mL HA and HA4. Values were normalized to GAPDH housekeeping gene levels and compared with untreated controls. Values are the mean ± S.D. of three experiments. $*p < 0.05$, $**p < 0.01$, Tukey's *post-hoc* multiple comparison test.

**Figure 4** Effect of HA and HA4 treatment for 3 days on each differentiation marker protein evaluation in NHEKs. K10 (A), involucrin (B), and filaggrin (C) proteins were determined by western blotting. NHEKs were treated for 3 days with 1 μg/mL HA and HA4. Values were normalized to β-actin housekeeping protein levels and compared with untreated controls. Values are the mean ± S.D. of three experiments. N.D., not determined. $*p < 0.05$, Tukey's *post-hoc* multiple comparison test.

**Figure 5** Effect of HA and HA4 treatment for 9 days on each differentiation markers protein evaluation in NHEKs. K10 (A), involucrin (B) and filaggrin (C) protein were determined by western blotting. NHEKs were treated for 9 days with 1 μg/mL HA and HA4. Values were normalized to the β-actin housekeeping protein levels and compared with untreated control. Values are the mean ± S.D. of three experiments. $*p < 0.05$, Tukey's *post-hoc* multiple comparison test.

**Figure 6** Effect of HA and HA4 on CD44 mRNA expression in NHEKs. CD44 mRNA expression was determined by quantitative real-time PCR. NHEKs were treated for 4 h, 8 h, 24 h,

and 216 h (9 days) with 1 μg/mL HA and HA4. Values were normalized to GAPDH housekeeping gene levels and compared with untreated controls. Values are the mean ± S.D. of three experiments. $^{**}p < 0.01$, Tukey's *post-hoc* multiple comparison test.

**Figure 7** Effect of HA and HA4 on CD44-phosphorylated protein evaluation in NHEKs. CD44-phosphorylated protein was determined by western blotting. NHEKs were treated for 8 h (A) and 24 h (B) with 1 μg/mL HA and HA4. Values were normalized to β-actin housekeeping protein levels and compared with untreated controls. Values are the mean ± S.D. of three experiments. $^{**}p < 0.01$, Tukey's *post-hoc* multiple comparison test.

Fig. 1

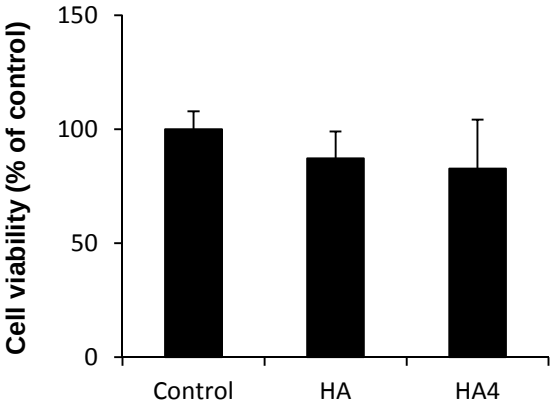

Fig. 2

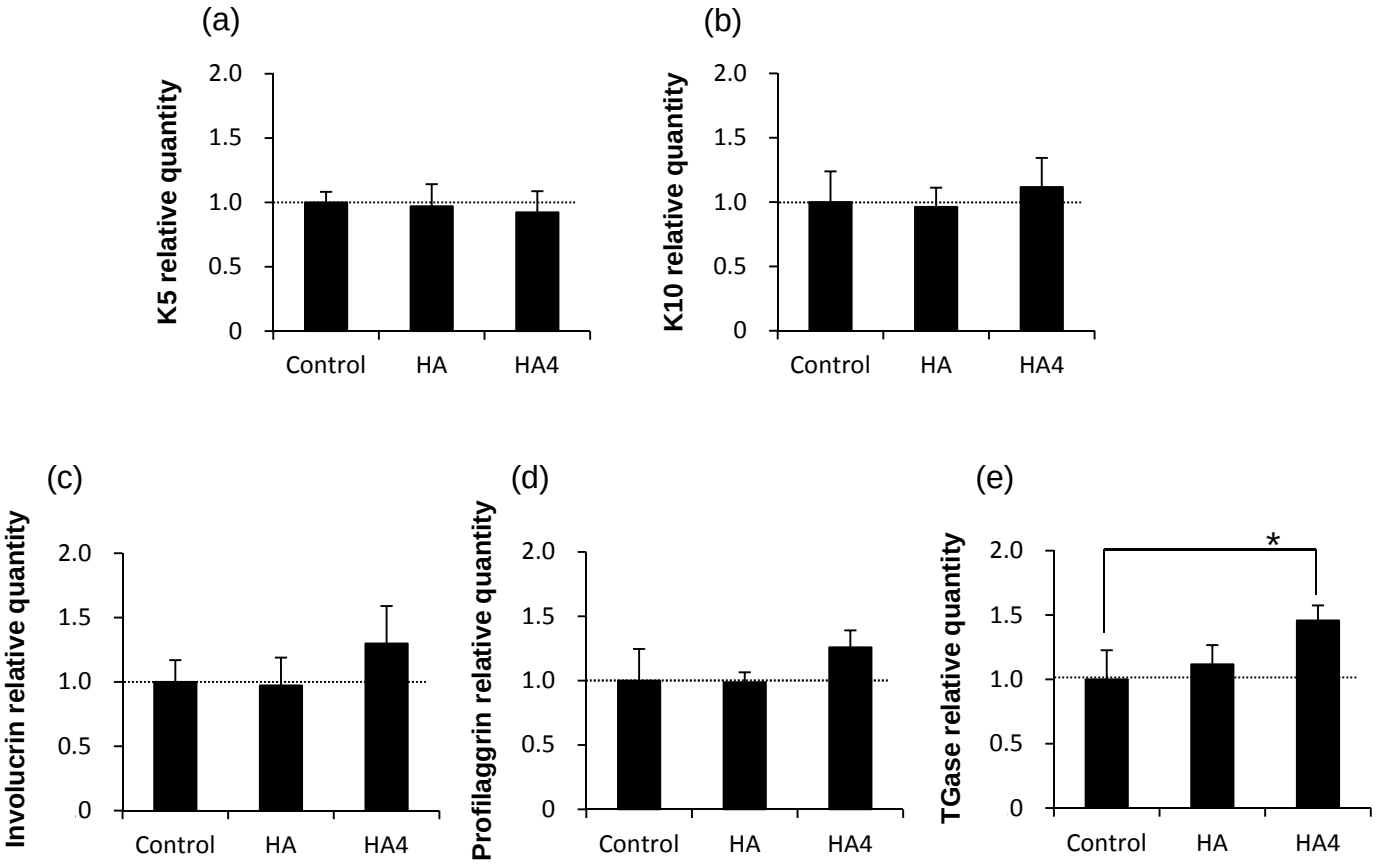
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Fig. 3

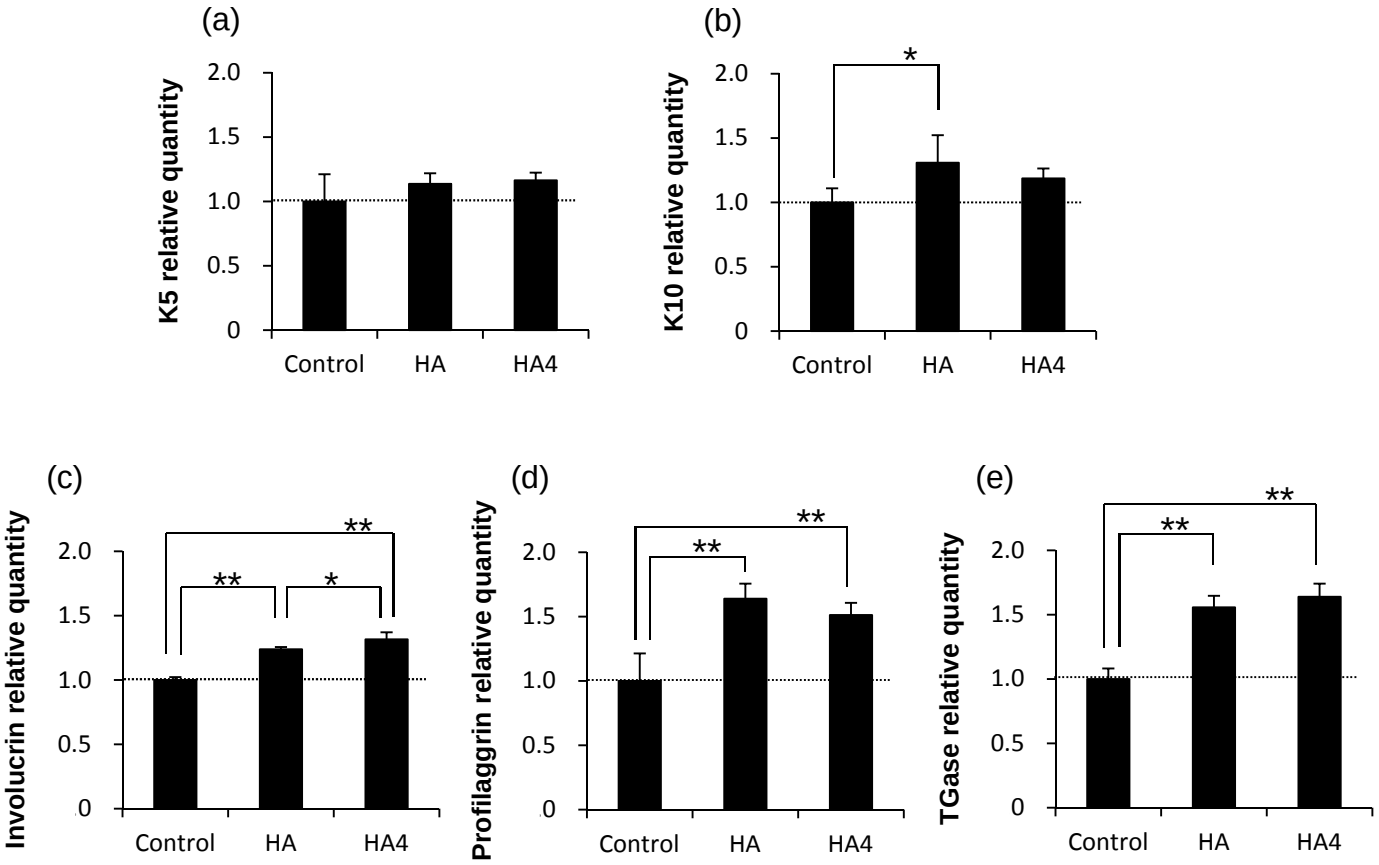
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Fig. 4

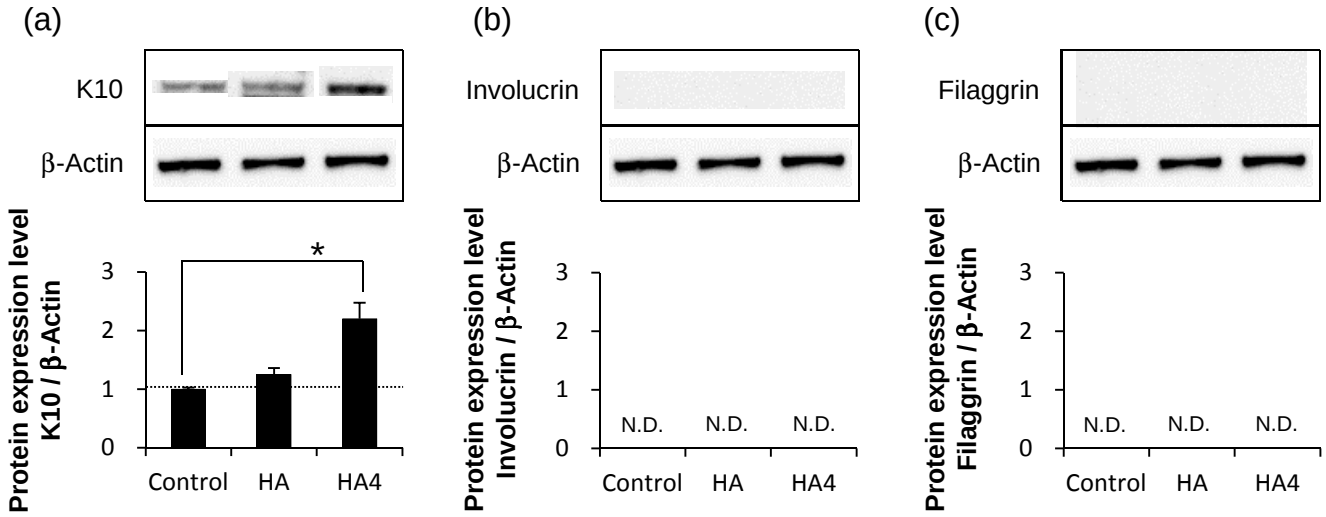
(a)
K10
β-Actin
Protein expression level
K10 / β-Actin
3
2
1
0
*
Control
HA
HA4
(b)
Involucrin
β-Actin
Protein expression level
Involucrin / β-Actin
3
2
1
0
N.D.
N.D.
N.D.
Control
HA
HA4
(c)
Filaggrin
β-Actin
Protein expression level
Filaggrin / β-Actin
3
2
1
0
N.D.
N.D.
N.D.
Control
HA
HA4

Fig. 5

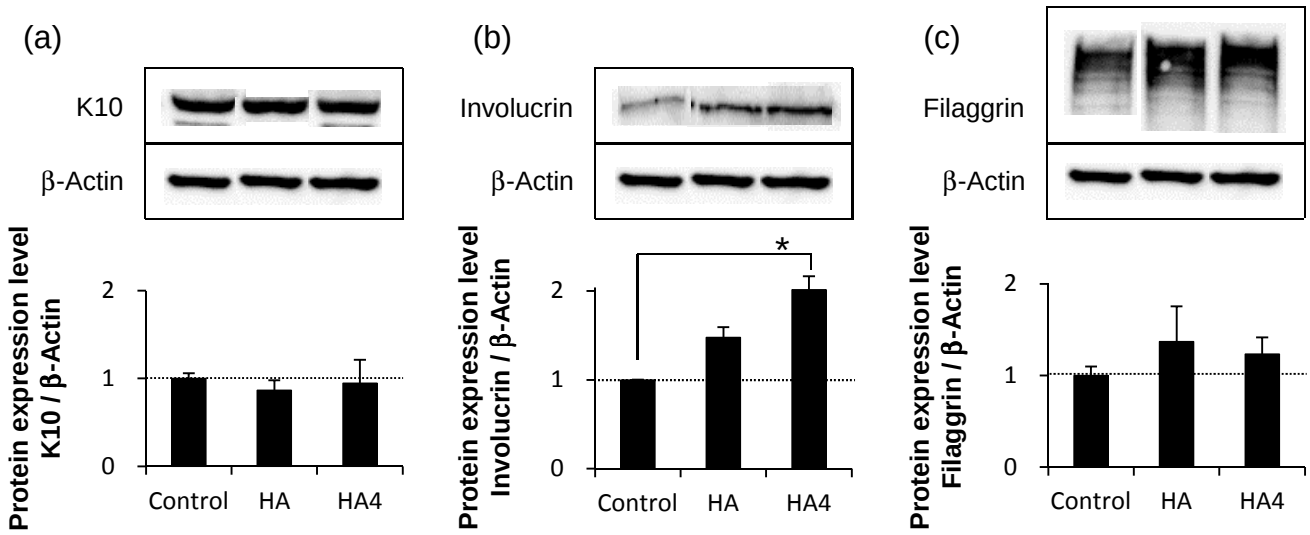
(a)
K10
β-Actin
Protein expression level K10 / β-Actin
2
1
0
Control
HA
HA4
(b)
Involucrin
β-Actin
Protein expression level Involucrin / β-Actin
*
2
1
0
Control
HA
HA4
(c)
Filaggrin
β-Actin
Protein expression level Filaggrin / β-Actin
2
1
0
Control
HA
HA4

Fig. 6

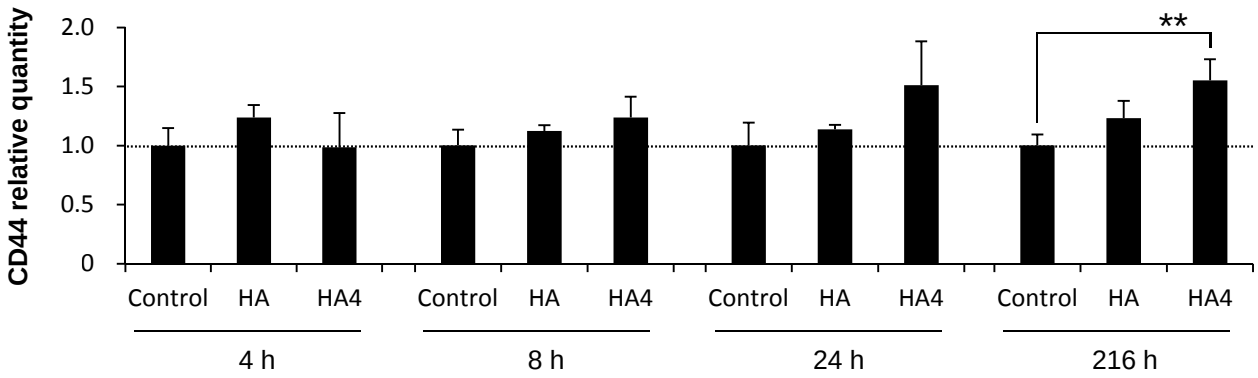
CD44 relative quantity
2.0
1.5
1.0
0.5
0
**
Control
HA
HA4
Control
HA
HA4
Control
HA
HA4
Control
HA
HA4
4 h
8 h
24 h
216 h

Fig. 7

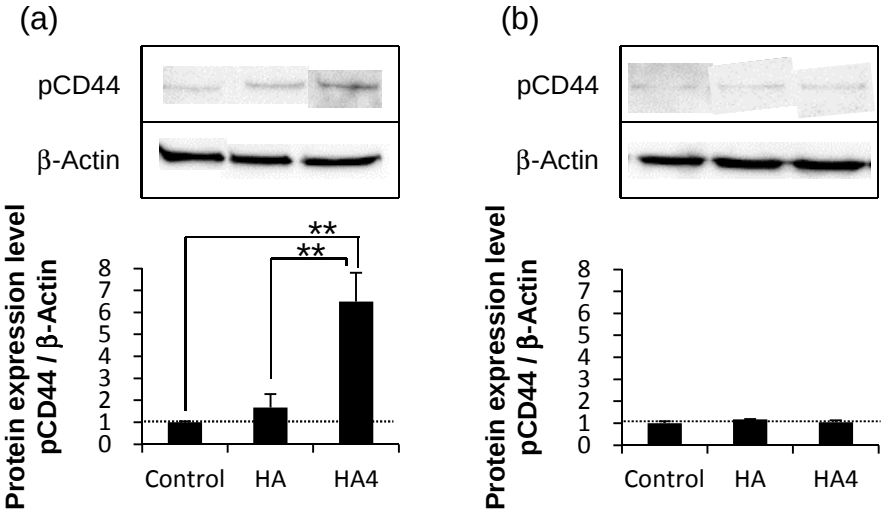
(a)
pCD44
β-Actin
Protein expression level
pCD44 / β-Actin
**
**
0
1
2
3
4
5
6
7
8
Control
HA
HA4
(b)
pCD44
β-Actin
Protein expression level
pCD44 / β-Actin
0
1
2
3
4
5
6
7
8
Control
HA
HA4

**Table I** Primer sequences

| Primer | | Sequence |
|---|---|---|
| GAPDH | Forward | 5’-GAAGGTGAAGGTCGGAGT-3’ |
| | Reverse | 5’-GAAGATGGTGATGGGATTTC-3’ |
| Keratin 5 | Forward | 5’-GAGCTGAGAAACATGCAGGA-3’ |
| | Reverse | 5’-TCTCAGCAGTGGTACGCTTG-3’ |
| Keratin 10 | Forward | 5’-CCATCGATGACCTTAAAAATCAG-3’ |
| | Reverse | 5’-GCAGAGCTACCTCATTCTCATACTT-3’ |
| Involucrin | Forward | 5’-TGCCTGAGCAAGAATGTGAG-3’ |
| | Reverse | 5’-TTCCTCATGCTGTTCCCAGT-3’ |
| Profilaggrin | Forward | 5’-CCATCATGGATCTGCGTGG-3’ |
| | Reverse | 5’-CACGAGAGGAAGTCTCTGCGT-3’ |
| Transglutaminase | Forward | 5’-TCTTCAAGAACCCCCTTCCC-3’ |
| | Reverse | 5’-TCTGTAACCCAGAGCCTTCGA-3’ |
| CD44 | Forward | 5’-GACACCATGGACAAGTTTTGG-3’ |
| | Reverse | 5’-CGGCAGGTTATATTCAAATCG-3’ |