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〈Regular Article〉

Expression of Hyaluronan Synthase and Collagen Type I mRNA by Hyaluronan Tetrasaccharides in Normal Human Dermal Fibroblasts

Madoka KAGE[1], Yoshihiro TOKUDOME[1], Yukiko MATSUNAGA[2], Takeshi HARIYA[2], Fumie HASHIMOTO *, [1]

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Abstract

The major compounds in the dermis are hyaluronan and type I collagen, which decrease with aging, UV and various other factors. The loss of hyaluronan and collagen with aging is associated with increased dehydration and wrinkling of the skin. We aimed to investigate the influence of hyaluronan tetrasaccharide (HA4) on regulation of high molecular weight hyaluronan (HA) and collagen synthesis in normal human dermal fibroblasts (NHDFs). Expression of hyaluronan synthase (HAS) 1−3 and collagen (COL) 1A1 mRNA were evaluated by quantitative real-time PCR. HAS1 mRNA expression was found to be increased in NHDFs treated with HA and HA4. In addition, it was observed that NHDFs co-cultured with normal human epidermal keratinocytes (NHEKs) showed up-regulation of HAS1 mRNA expression, as compared with NHDFs single cell culture treated with HA4. Treatment of NHDFs with HA4 + derivatized vitamin C (VC-PMg) significantly increased COL1A1 mRNA expression. In this study, we confirmed that HA4 affected HAS1 and COL1A1 mRNA expression; thus, HA4 application may show various action in skin.

Key words: hyaluronan tetrasaccharide, hyaluronan synthase, collagen, mRNA.

1. Introduction

Hyaluronan, which is composed of repeated *β*-1,4-glucuronic acid-*β*-1,3-*N*-acetylglucosamine disaccharide units, is a non-sulfated glycosaminoglycan with a molecular weight of over 1,000 kDa. Hyaluronan is abundant in the extracellular matrix (ECM) of the skin, and is involved in many biological processes such as tissue homeostasis, cell proliferation, cell migration, cell differentiation, angiogenesis, tumor biology and repair processes by interacting with various proteins[1)]. In addition, because of physical properties such as viscoelasticity, hydrophilicity and extensibility, hyaluronan seems to play an important role as a space filler, osmotic buffer, plasma protein sieve and lubricant[2)]. In the skin, hyaluronan synthases (HAS) are synthesized by dermal fibroblasts and epidermal keratinocytes. Synthesis of hyaluronan is accomplished by three HAS (HAS1, HAS2 and HAS3), which produce different sizes of polysaccharide chains with average molecular weights of about 3×10^5 to 2×10^6 Da for HAS1 and HAS2, and about 2×10^5 to 3×10^5 Da for HAS3[3)].

Collagen is a major component of ECM, and type I collagen accounts for approximately 80% of the total collagen in adult human dermis[4)]. Type I collagen is a product of two genes, *α*1 (I) (COL1A1) and *α*2 (I) (COL1A2), which are coordinately regulated[5)]. The major structural protein in the dermis is type I collagen, which comprises 90% of the dry weight of skin and diminishes with normal aging[6)−8)]. Collagen plays a role in cell adhesion, and is important for maintaining normal tissue architecture and function.

Stimulation of living tissue by hyaluronan appears to depend on various factors, including hyaluronan chain length. For example, high molecular weight hyaluronan was demonstrated to suppress angiogenesis[9)] and inflammation[10)], whereas low molecular weight hyaluronan stimulated induction of angiogenesis[11)], inflammation[12)] and expression heat shock protein 72[13)]. In addition, injection of cross-linked hyaluronan stimulates collagen synthesis, partially restoring dermal matrix components that are lost in photo-damaged skin[14)]. In this way, hyaluronan possesses numerous functions, but little is known about the effects of hyaluronan oligosaccharides on skin.

In this study, we aimed to investigating the influence of high molecular weight hyaluronan (HA) and hyaluronan tetrasaccharide (HA4) on regulation of HASs and COL1A1 mRNA expression in normal human dermal fibroblasts (NHDFs).

2. Materials and Methods

2−1. Materials

HA4 (99.14%) (776.3 Da) was provided by Glycoscience Laboratories Inc. (Tokyo, Japan). HA (>1,200 kDa) produced by a *Streptococcus zooepidemicus* was used. All other chemicals and solvents were of analytical grade. Abdominal skin origin normal human dermal fibroblasts (NHDFs) were purchased from Kurabo (Osaka, Japan). Abdominal skin origin normal human epidermal keratinocytes (NHEKs) were

*, [1] Faculty of Pharmaceutical Sciences, Josai University (1−1 Keyakidai, Sakado, Saitama 350−0295, Japan)
[2] SHISEIDO Co., Ltd., Research Center
連絡先　E-mail: hasimoto@josai.ac.jp

purchased from Biopredic (Rennes, France). HuMedia-KG2 was purchased from Kurabo (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Nichirei Bioscience (Tokyo, Japan), and Dulbecco's modified Eagle's medium (DMEM) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Magnesium ascorbyl phosphate (VC-PMg) was obtained from Nikko Chemicals (Tokyo, Japan). RNAiso Plus, PrimeScript® RT reagent Kit and SYBR® *Premix Ex Taq*™ were purchased from TaKaRa Bio (Shiga, Japan). Primers were purchased from Invitrogen (CA, U.S.A.).

2−2. Cell culture

NHDFs were seeded into 100-mm dishes (1.0×10^5 cells per dish), and were cultured in DMEM supplemented with 10 % FBS. NHEKs were seeded into 100-mm dishes (2.0×10^5 cells per dish), and were cultured in HuMedia-KB2 supplemented with 10 μg/m*l* insulin, 0.1 ng/m*l* human epidermal growth factor, 0.5 μg/m*l* hydrocortisone, 50 μg/m*l* gentamicin, 50 ng/m*l* amphotericin B, and 0.4% (v/v) bovine pituitary extract. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37ºC, and culture medium was changed every day. After cells had become subconfluent, they were subcultured in 60-mm dishes ($0.3-0.5\times10^5$ cells per dish) in the same medium. After culture for 1 day, cells were treated at doses of 0.1 μg/m*l* HA or HA4, followed by further culture.

2−3. Keratinocyte-fibroblast co-culture

NHDFs were plated into six-well plates at 0.2×10^5 cells per well. At the same time, NHEKs were plated into BD Falcon Cell Culture Inserts at 0.2×10^5 cells per insert. These inserts consist of a porous translucent low-protein binding polyethylene terephthalate (PET) membrane with a pore size of 0.4 mm and pore density of 1×10^8 pores per cm^2. After culture for 1 day, NHEKs were treated at doses of 0.1 μg/m*l* HA or HA4, followed by further culture.

2−4. 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/m*l* MTT solution and were incubated for a further 3 h. Medium was removed and the resulting formazan crystal was solubilized in 1 m*l* of 0.04 M hydrochloric acid/isopropyl alcohol. Optical density at 570 nm was determined using a microplate reader (SpectraMax M2^e; Molecular Devices, Tokyo, Japan).

2−5. RNA extraction and quantitative real-time PCR

Total RNA was isolated from cells following standard procedures using RNAiso Plus. First-strand cDNA was synthesized from 0.5 μg total RNA from cells sample using PrimeScript® RT reagent Kit and thermal cycler (Veriti; Applied Biosystems, CA, USA). The cDNA samples generated were diluted and used for real-time PCR analysis. Briefly, 2 μ*l* of diluted cDNA was mixed with both forward and reverse primers (Table 1) and SYBR® *Premix Ex Taq*™ in 20 μ*l* final volumes. Amplification was performed using the Real-Time PCR System (ABI PRISM® 7500; Applied Biosystems). 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 normalized with the GAPDH mRNA. The results were expressed as an *n*-fold difference relative to normal controls (relative expression levels).

2−6. Data analysis

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

3. Results

3−1. Cytotoxicity of HA and HA4 on NHDFs

In order to investigate the cytotoxicity of HA and HA4 on NHDFs, we treated NHDFs with HA and HA4. After treatment for 24 h, cell viability was examined by MTT assay. HA and HA4 showed no cytotoxicity up to a dose of 0.1 μg/m*l* (Fig. 1).

3−2. HAS mRNA expression in NHDFs and NHEKs by HA and HA4 treatment

mRNA expression levels of HAS, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were assessed by quantitative real-time PCR in NHDFs. NHDFs were cultured with DMEM (control), HA or HA4 for 24 h. HA and HA4 was applied at doses of 0.1 μg/m*l*. After 24 h of treatment, quantitative real-time PCR was used to determine the levels of HAS1, HAS2 and HAS3 mRNA relative to the control gene. Treatment of NHDFs with HA and HA4 significantly increased HAS1 mRNA expression, but HAS2 and

Table 1. Primers.

Primer		Sequence
GAPDH	Forward	5′-GAAGGTGAAGGTCGGAGT-3′
	Reverse	5′-GAAGATGGTGATGGGATTTC-3′
HAS1	Forward	5′-ACGTGCGGATCCTTAACCCT-3′
	Reverse	5′-AGGCCTAGAGGACCGCTGAT-3′
HAS2	Forward	5′-GTCATGGGCAGAGACAAATCAG-3′
	Reverse	5′-CGTTACGTGTTGCGAGCTTTC-3′
HAS3	Forward	5′-GGGCATTATCAAGGCCACCTA-3′
	Reverse	5′-CAGATTTGTTGATGGTAGCAATGG-3′
COL1A1	Forward	5′-CCGCCGCTTCACCTACAGC-3′
	Reverse	5′-TTTTGTATTCAATCACTGTCTTGCC-3′

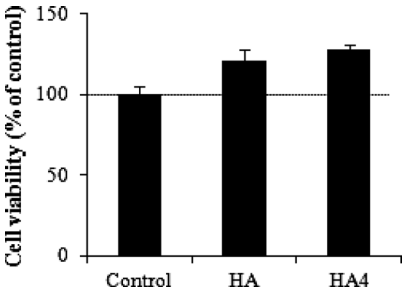

Fig. 1. Effects of HA and HA4 treatment on cell viability of NHDFs. Cell viability was determined by MTT assay. NHDFs were treated for 24 h with 0.1 μg/m*l* HA and HA4. Values are means±S.D. of three experiments.

HAS3 mRNA expression were unchanged. The increases were 1.7-fold by HA treatment, and 1.6-fold by HA4 treatment (Fig. 2A). Furthermore, after 48 h of treatment, HA and HA4 further increased HAS1 mRNA expression (Fig. 2B). Next, to determine whether HA and HA4 treatment affects the expression of HAS mRNA, cultured NHEKs were incubated with 0.1 μg/m*l* HA or HA4 for 24 h. No significant differences in HAS mRNA expression were detected between HA and HA4 treated samples and untreated controls (Fig. 3).

3−3. Effects of HA and HA4 treatment HAS mRNA expression in co-culture

mRNA expression of HAS was assessed by quantitative real-time PCR in NHEK-NHDF co-culture. Treatment of NHEKs with HA and HA4 increased HAS1 mRNA expression in NHDFs (6.6-fold increase with HA, and 5.0-fold increase with HA4; Fig. 4).

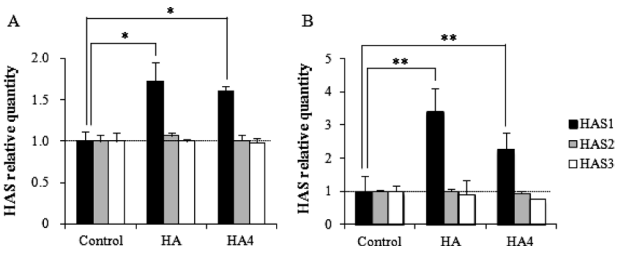

Fig. 2. Effects of HA and HA4 treatment for 24 and 48 h on HAS1, HAS2 and HAS3 mRNA expression in NHDFs. HAS1, HAS2 and HAS3 mRNA expression was determined by quantitative real-time PCR. NHDFs were treated for 24 h (a) and 48 h (b) with 0.1 μg/m*l* HA and HA4. Values were normalized against GAPDH housekeeping gene levels and were compared with untreated controls. Values are means ± S.D. of three experiments.
$^{*}p<0.05$, $^{**}p<0.01$, Tukey's *post-hoc* multiple comparison test.

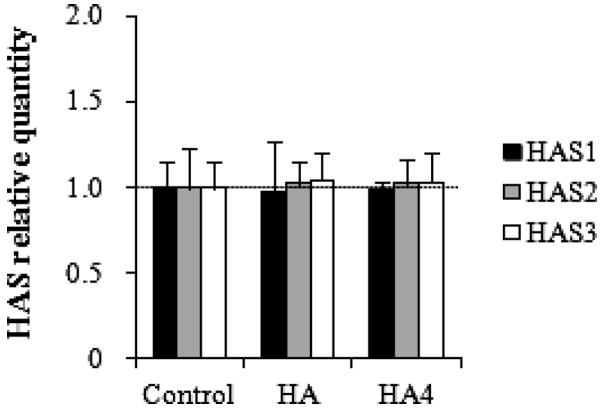

Fig. 3. Effects of HA and HA4 treatment for 24 h on HAS1, HAS2 and HAS3 mRNA expression in NHEKs. HAS1, HAS2 and HAS3 mRNA expression was determined by quantitative real-time PCR. NHEKs were treated for 24 h with 0.1 μg/m*l* HA and HA4. Values were normalized against GAPDH housekeeping gene levels and were compared with untreated controls. Values are means ± S.D. of three experiments.

3−4. COL1A1 mRNA expression in NHDFs by HA and HA4 treatment

mRNA expression of COL1A1 was assessed by quantitative real-time PCR in NHDFs. Treatment of NHDFs with HA and HA4 were small increased (not significant) COL1A1 mRNA expression (Fig. 5A). Furthermore, combinations of HA4 and VC-PMg were accompanied by additional increases in COL1A1 mRNA expression (Fig. 5B).

4. Discussion

In this study, we examined the effects of HA and HA4 on HAS and COL1A1 expression in an *in vitro* study. The results showed that treatment of NHDFs with HA and HA4 increased HAS1 and COL1A1 mRNA expression.

Although hyaluronan is a simple, unbranched polysaccharide chain composed of repeating disaccharide units, it exerts

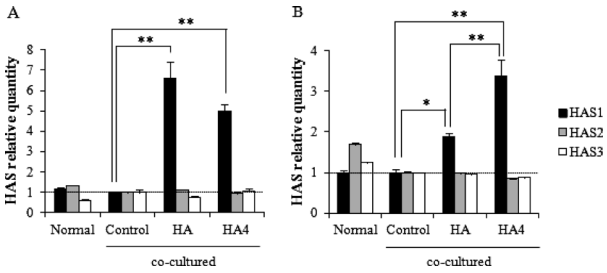

Fig. 4. Effects of HA and HA4 treatment for 24 h on HAS1, HAS2 and HAS3 mRNA expression in co-culture of NHDFs and NHEKs. HAS1, HAS2 and HAS3 mRNA expression in NHDFs was determined by quantitative real-time PCR. Normal was monolayer culture of NHDFs. NHEKs were treated for 24 h with 0.1 μg/m*l* HA and HA4. Values were normalized against GAPDH housekeeping gene levels and were compared with untreated controls. Values are means ± S.D. of three experiments.
$^{*}p < 0.05$, $^{**}p < 0.01$, Tukey's *post-hoc* multiple comparison test.

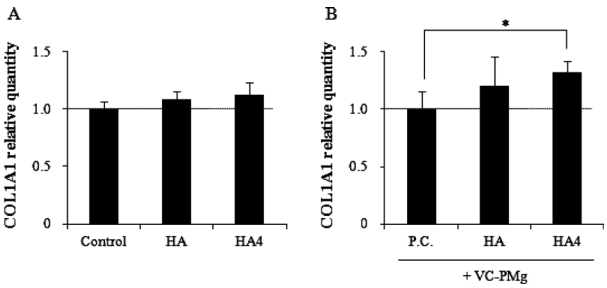

Fig. 5. Effects of HA and HA4 treatment for 24 h on COL1A1 mRNA expression in NHDFs. COL1A1 mRNA expression was determined by quantitative real-time PCR. (a) NHDFs were treated for 24 h with 0.1 μg/m*l* HA and HA4. Values were compared with untreated controls. (b) NHDFs were treated with HA + VC-PMg and HA4 + VC-PMg. Values were compared with VC-PMg treatment control (positive control; PC). Values were normalized against GAPDH housekeeping gene levels. Values are means ± S.D. of three experiments.
$^{*}p < 0.05$, Dunnett's *post-hoc* multiple comparison test.

a broad spectrum of physiological functions depending on location, concentration and chain length, by binding to hyaluronan -binding proteins. HAS1, HAS2 and HAS3 are the enzymes responsible for hyaluronan production. While the sizes of the hyaluronan synthesized *in vitro* by HAS1 and HAS2 were 3×10^5 to 2×10^6 Da, HAS3 expression leads to the synthesis of a substantially smaller hyaluronan (2×10^5 to 3×10^5 Da) [3]. In addition, these enzymes differ in catalytic activity (HAS3＞HAS2＞HAS1), as well as the sizes of their final products[15]. Depending on hyaluronan concentration changes in the cell environment, NHDFs regulate hyaluronan production itself. In our study, HAS1 mRNA expression was increased in NHDFs treated with HA and HA4 (Figs. 2A, 2B). HAS mRNA expression in NHDFs treated with HA4 was similar to that after treatment with HA. In contrast, HAS mRNA expression was unchanged in NHEKs treated with HA and HA4 (Fig. 3). The major function of the epidermis is to provide a barrier between the external environment and the organism. Thus, NHEKs showed no change in HAS mRNA expression, as hyaluronan production is not a major function. We have new experimental data that HA4 enhanced keratinocyte differentiation. Thus, the function of HA4 in NHEKs is not hyaluronan synthesis, but rather, is related to keratinocyte differentiation. HAS1 mRNA expression was increased in NHEK-NHDF co-cultures treated with HA and HA4 (Fig. 4). Treatment of fibroblasts with growth factors (epidermal growth factor, platelet derived growth factor BB, and transforming growth factor 1β) up-regulated HAS gene expression and increased HAS enzymes and hyaluronan production[16]. Therefore, HAS1 mRNA expression was increased in NHEKs-NHDFs, as compared with NHDFs, as a result of growth factor signaling.

Injectable dermal fillers are becoming increasingly popular for improving skin contour defects related to aging (wrinkles and lines), depressed acne scars, and other traumatic or congenital conditions. After cross-linked hyaluronan injections, collagen deposition around the filler was found to be higher than in controls[14]. In addition, VC-PMg was demonstrated to stimulate collagen synthesis[17)–20)]. In our study, treatment of NHDFs with HA4 tended result in increased COL1A1 mRNA expression (Fig. 5A). Furthermore, COL1A1 mRNA expression was significantly higher in NHDFs treated with HA4 + VC-PMg when compared with single VC-PMg treatment (Fig. 5B). This suggests that HA4 treatment may get involved in collagen synthesis.

In this study, HA4 was suggested to affect hyaluronan and collagen synthesis. In aged skin, particularly photo-aged skin, HA content is significantly lower. On the other hand, by injecting hyaluronan into the skin, symptoms of photo-aging, such as wrinkles, diminish. Therefore, hyaluronan has been recognized as a compound that can suppress aging. We previously reported that HA4 permeates through skin by passive diffusion. In addition, we show that treatment of HA4 improved skin functional recovery after UVA irradiation[21]. In this study, HAS and COL1A1 mRNA expression of HA4 was similar to HA. Hyaluronan can not penetrate into the skin because of high water solubility and a high molecular weight. For these reason, HA4 application may show various action in skin.

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