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Chitosan Increases α6 Integrin^{high}/CD71^{high} Human Keratinocyte Transit-Amplifying Cell Population

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Abstract − Glycosaminoglycans (GAGs) and chitosan have been used as matrix materials to support the dermal part of skin equivalent which is used for both pharmacological and toxicological evaluations of drugs potentially used for dermatological diseases. However, their biological roles of GAGs and chitosan in the skin equivalent are still unknown. In the present study, we evaluated whether GAGs and chitosan directly affect keratinocyte stem cells (KSCs) and their transit-amplifying cells (TA cells). Among supporting matrix materials, chitosan significantly increased the number of α6 integrin^{high}/CD71^{high} human keratinocyte TA cells by 48.5%. In quantitative real-time RT-PCR analysis, chitosan significantly increased CD71 and CD200 gene transcription whereas not α6 integrin. In addition, the level of the gene transcription of both keratin 1 (K1) and K10 in the chitosan-treated human keratinocytes was significantly lower than those of control, suggesting that chitosan inhibit keratinocyte differentiation. We also found that N-acetyl-D-glucosamine (NAG) and β-(1-4)-linked D-glucosamine (D-glc), two components of chitosan, have no effect on the expression of CD71, K1, and K10, suggesting that each monomer component of chitosan is not enough to regulate the number of epidermal keratinocyte lineage. Conclusively, chitosan increases keratinocyte TA cell population which may contribute to the cellular mass expansion of the epidermal part of a skin equivalent system.

Keywords: Chitosan, Keratinocyte stem cells, Transit-amplifying cells, CD71, K1, K10

INTRODUCTION

Human keratinocytes in the basal layer of epidermis is heterogeneously composed of proliferative and differentiating cells; keratinocyte stem cells (KSCs), transit-amplifying cells (TA cells), and postmitotic differentiating keratinocytes (Fuchs, 2008). The characteristics of KSCs have been reported to be relatively slow-cycling or quiescent cells. However, KSCs have high *in vitro* proliferating potential in colony forming assay (Morris *et al*., 1985). The TA cells, the first progeny of KSCs, can undergo limited number of cell divisions to expand keratinocyte population to generate postmitotic differentiating keratinocytes. Especially, these KSCs and TA cells are thought to be indispensible as a source of cells for skin regeneration (Alonso and Fuchs, 2003; Li *et al*., 2004; Blanpain and Fuchs, 2006; Fuchs, 2007; Fuchs, 2008).

Cell surface markers have been widely used to isolate and purify stem cells and TA cells by exploiting fluorescence activated cell sorting (FACS)-based techniques (Li *et al*., 1998; Kaur and Li, 2000). However, the cell surface markers for KSCs and their TA cells remain relatively poorly defined. Currently, α6 integrin and CD71/transferin receptor are widely used to define and detect KSCs and their TA cells by FACS analysis (Li *et al*., 1998; Kaur and Li, 2000; Tani *et al*., 2000). KSC population shows the high level of α6 integrin and the low level of CD71 (designated α6^{high} CD71^{low}), whereas TA cell population has the high level of both α6 integrin and CD71(α6^{high} CD71^{high}). In addition, keratinocytes in the early stage of epidermal differentiation show the low level of α6 integrin (α6^{low}).

The skin equivalent, a three-dimensional model system,

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has been widely used for examining many aspects of skin biology, pharmacological evaluation of new drugs, and toxicological assessments of potential skin irritants (Shahabeddin *et al*., 1990). The skin equivalent can be constructed by seeding human keratinocytes on the top of the dermal equivalent, and subsequently exposed to the air-liquid interface to induce keratinocyte stratification and differentiation (Augustin *et al*., 1997; Black *et al*., 2005). The dermal equivalent is made of fibroblasts with substrates such as collagen, glycosaminoglycans (GAGs) and chitosan (Augustin *et al*., 1997; Black *et al*., 2005).GAGs and chitosan have been extensively studied as suitable substrates for cultured human epidermal keratinocytes, and their mixtures with the with the cells yield a composite material that is histologically similar to skin. However, their biological roles of GAGs and chitosan in the regulation of keratinocytes during generation of skin equivalent have not been elucidated.

Here we examined whether GAGs and chitosan have effects on the population of keratinocyte lineages including KSCs, TA cells and early differentiating cells. As results of the evaluation of GAGs and chitosan, we found that chitosan may contribute to the formation of reconstructed skin by increasing keratinocyte TA cells.

MATERIALS AND METHODS

Materials

Normal human epidermal keratinocytes (NHEK), keratinocyte growth medium-2 (KGM-2) and fetal bovine serum (FBS) were purchased from Lonza, Co (Basel, Switzerland). Bovine serum albumin (BSA), GAGs (chondroitn sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), hyaluronic acid (HA)), chitosan, and its components (N-acetyl-glucosamine and D-glucosamine) were purchased from Sigma (St. Louis, MO, USA). FITC-conjugated anti-α6 integrin antibody, PE-Cy5 conjugated anti-CD71 antibody and FITC-, PE-Cy5 isotype control antibody were purchased from BD Biosciences (San Jose, CA, USA).

Human epidermal keratinocytes in monolayer culture

NHEK were cultured in KGM-2 medium. The culture dishes were maintained in an atmosphere of 5% CO_2 and 37^{o}C. NHEKs were used for the experiments within second passage. After starvation for 24 hr with incomplete KGM-2 medium, the cells were incubated for 24 hr after treated with or without GAGs, chitosan, N-acetyl-glucosamine (NAG), and D-glucosamine (D-glc) at the concentration of 30 μg/ml.

Fluorescence activated cell sorting

NHEKs were treated with GAGs and chitosan were used for flow cytometric analysis, respectively. Dual staining was conducted using FITC-conjugated anti-α6 integrin antibody and PE-Cy5 conjugated anti-CD71 antibody at 10 μg/ml. Cells were incubated with reaction buffer (KGM-2 medium with 2% FBS and 2% BSA) containing both antibodies for 1hr at 4^{o}C. Cells were washed in blocking buffer (KGM-2 medium with 2% BSA) two times, and were resuspended in reaction buffer. Each cell subpopulation (KSCs, $\alpha6^{high}CD71^{low}$; TA cells, $\alpha6^{high}$ $CD71^{high}$; early differentiating cells, $\alpha6^{low}$) was analyzed by FACSCalibur (BD Biosciences).

RNA isolation and quantitative real-time PCR

Total RNA was isolated by using the Trizol method (Invitrogen) and quantitated by using NanoDrop. For reverse transcription, aliquots of 4 μg RNA were transcribed into cDNA with a SuperScript reverse transcriptase III Kit (Invitrogen). The RT reaction was stopped by adding Tris-EDTA buffer (pH 8.0) to a total of 200 μl of cDNA solution. TaqMan® Gene Expression Assays were purchased from Applied Biosystems. And RT-PCR was done following the supplier`s instruction. Briefly, 20 μl of PCR mixture contained 10 μl of 2X Taqman universal PCR Master Mix, 1 μl of 20X of TaqMan® expression assay mix, and 50 ng cDNA. Realtime PCRs were done in a 7500 Fast Real-Time PCR system (Applied Biosystems). cDNA samples were analyzed for α6 integrin, Hs01041011_m1; CD71, Hs00174609_ m1; CD200, Hs00245978_m1; FST, Hs00246260_m1; K1, Hs00196158_m1; K10, Hs00166289_m1; TGM1, Hs00165929_ m1; FLG, Hs00856927_g1 and LOR, Hs01894962_s1. Human GAPDH (43333764F) was used for normalize variation in cDNA quantities from different samples. Quantification of relative differences among various conditions was calculated by using equation from mathematical model developed by Pfaffl (Pfaffl *et al*., 2002).

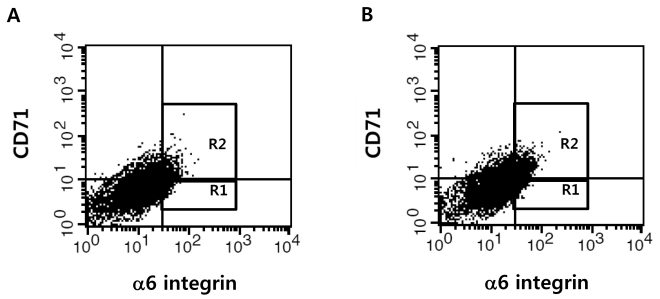
Statistical analysis

The data were representative of three or more independent experiments *in vitro*. Statistical analyses were performed using the Student's two-sample t-test. Data are expressed as means ± SEM or representative data. $p < 0.05$ is considered to be significant.

RESULTS

Chitosan increases keratinocyte TA cell population

We first evaluated whether supporting matrix materials such as CS, DS, HA, HS, and chitosan change the epi-



C

10,000 cells (N=3)

	Con	CS	DS	HA	HS	Chitosan
R1 region (KSCs)	492	561 (+14%)	383 (-22%)	305* (-38%)	266* (-46%)	513 (+4%)
R2 region (TA cells)	565	616 (+9%)	483 (-15%)	377* (-33%)	479 (-15%)	839* (+48.5%)

Fig. 1. Flow cytometric analyses for expression of α6 integrin and CD71 in early passage human keratinocytes. The R1 and R2 squares represent $\alpha6^{high}/CD71^{low}$ as KSCs and $\alpha6^{high}/CD71^{high}$ as TA cells, respectively. FACS analysis data are shown in Control (A), chitosan (B). Each KSCs and TA cell population was changed by GAGs and chitosan treatment, respectively (C). Representative data are shown, n=3. *$p<0.05$.

dermal cell population in NHEK culture. FACS analysis revealed that chitosan significantly increased 48.5% of $\alpha6^{high}CD71^{high}$ keratinoctye TA cell population but had no effect on $\alpha6^{high}CD71^{low}$ KSC population compared to control (Fig. 1). In contrast, HA reduced 38% of $\alpha6^{high}CD71^{low}$ KSC population and also 34% in TA cells. HS also decreased 46% of KSC population compared to control (Fig. 1).

Chitosan favors the expansion of keratinocyte TA cells

Next, we evaluated the effects of chitosan on the gene transcription of specific markers for KSCs/TA cells and epidermal differentiation by quantitative real-time PCR. Both GAGs and chitosan had no effect on the expression level of α6 integrin mRNA (Fig. 2A). However, unlike GAGs, chitosan significantly increased the CD71 gene transcription over 3 fold, suggesting that chitosan promote keratinocyte TA cell expansion (Fig. 2B). In addition, the expression level of CD200 mRNA, which is considered as another specific marker of KSCs/TA cells, was also significantly enhanced by chitosan treatment (Fig. 2C)

When we tested the effect of chitosan on epidermal differentiation markers, we found chitosan significantly downregulated the gene transcription of K1 and K10, which is known to be expressed during the early stage of epidermal differentiation (Fig. 2D, E). On the contrary, HA significantly increased the expression level of K1, K10 by about 3 fold, 8 fold, respectively (Fig. 2D, E). DS also enhanced the K1 and K10 gene transcription. However, GAGs and chitosan did not change the mRNA levels of transglutaminase 1 (TGM1), filaggrin, and loricrin, the late stage marker for epidermal differentiation (Fig. 2F-H).

NAG and D-glc, two components of chitosan, are not related to the effect of chitosan on KSCs/TA cells

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (D-glc, deacetylated unit) and N-acetyl-D-glucosamine (NAG, acetylated unit). Next, we evaluated whether NAG and/or D-glc contribute to the effects of chitosan on KSCs/TA cells. Both NAG and D-glc had no effect on the mRNA levels of α6 integrin, CD71, and CD200 (Fig. 3A-C), and also had no effects on the expression level of K1, K10, TGM1, filaggrin, and loricrin (Fig. 3D-H). Taken together, these results suggested that both NAG and D-glc, the monomer components of chitosan, are not related to the effect of chitosan on the expression of keratinocyte lineage specific markers.

DISCUSSION

KSCs and their TA cells are both critical for the continued regeneration of the epidermis in human skin (Fuchs., 2007; Houben *et al*., 2007; Fuchs., 2008). KSCs undergo a cell division to generate two daughter cells, KSC itself and its differentiating progeny TA cell (Fuchs., 2007; Houben *et al*., 2007; Fuchs., 2008). Keratinocyte TA cells exist in the basal layer of epidermis and can perform a limited number of cell division to increase the pool of keratinocytes in the suprabasal layer. After several rounds of a cell division, TA cells turn into the terminally differentiated non-mitotic keratinocytes that proceed through complex processes of morphological and biochemical changes to generate the cornified layer of skin (Alonso *et al*., 2003; Candi *et al*., 2005; Blanpain and Fuchs, 2006; Kaur, 2006; Larderet *et al*., 2006). Differentiation of keratinocytes causes a loss of growth potential and results in the serial expression of differentiation markers; K1 and K10, which are specific early markers expressed in suprabasal layers (Eichner *et al*., 1986; Drozdoff *et al*., 1993; Houben *et al*., 2007) followed by late differentiation markers, such as filaggrin (Harding and Scott, 1983) and epidermal TGM1 (Rice and Green, 1979). In the present study, we systematically investigated the effects of various supporting materials used for the manufacture of the dermal compartment of skin equivalent on KSCs/TA cells. We found that chitosan significantly in-

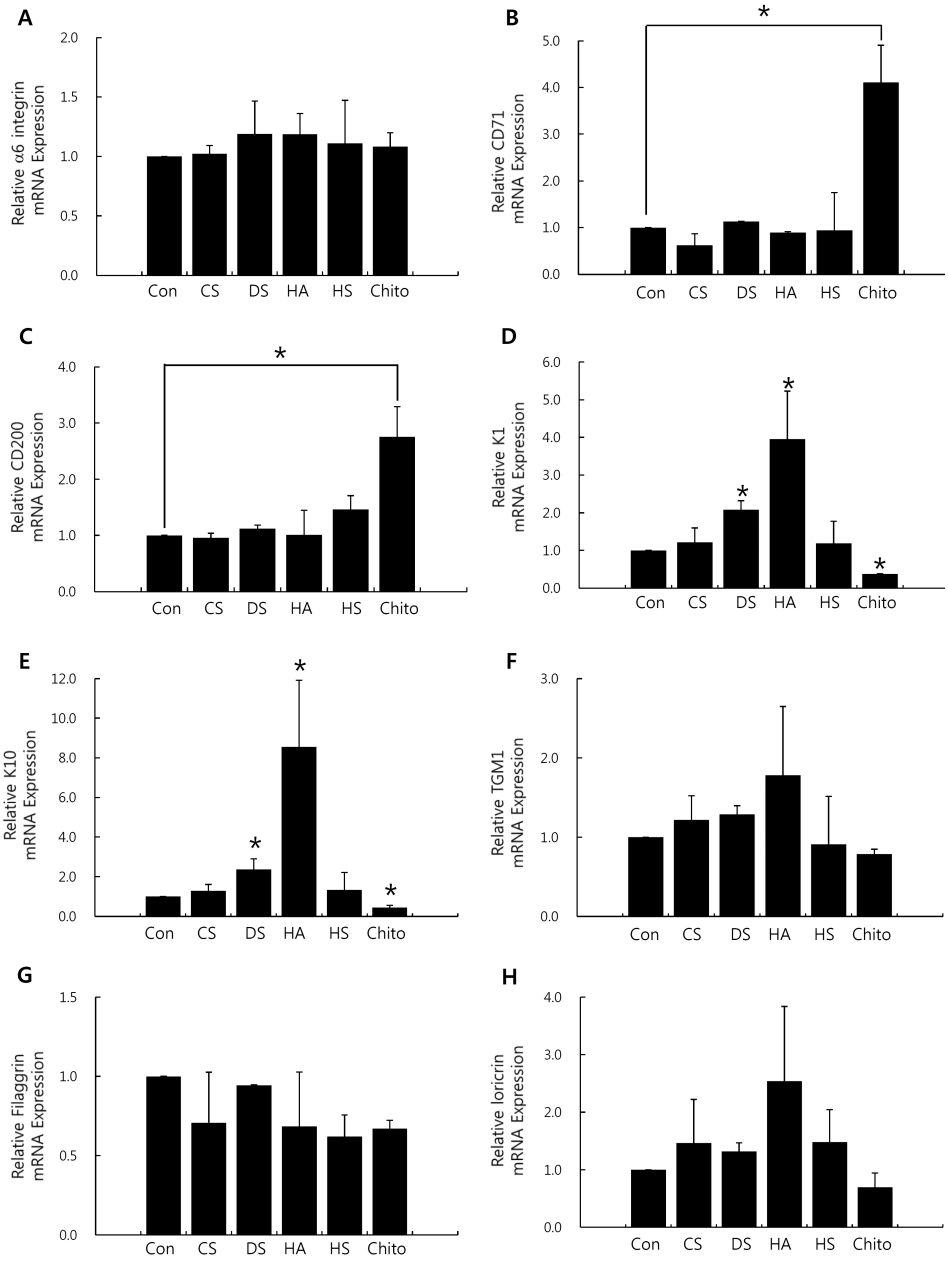


Fig. 2. The effects of GAGs and chitosan on specific expression markers of KSCs, TA cells and differentiated keratinocytes. Each condition was subjected to quantitative real-time PCR for KSCs/TA cell markers, α6 integrin (A), CD71 (B) and CD200 (C). The expression of genes associated with early and late differentiation stages of keratinocyte as K1 (D), K10 (E), TGM1 (F), filaggrin (G) and loricrin (H). The relative mRNA expression was normalized to that of GAPDH in each sample. Data are means ± SEM, n=4, $*p < 0.05$.

creased the number of α6 integrin^{high}/ CD71^{high} human keratinocyte TA cell population (Fig. 1) and confirmed the up-regulation of CD71 gene transcription (Fig. 2). However, chitosan did not change the mRNA level of α6 integrin (Fig. 2). Chitosan has been conventionally used as the supporting matrix material for the reconstitution of skin equivalent (Duplan-Perrat *et al*., 2000; Noblesse *et al*., 2004). These results first demonstrated that chitosan promoted the epidermal TA cell expansion to provide the enough number of keratinocytes to generate the epidermal compartment of skin equivalent.

Interestingly, HA increased the level of early differentiation markers, K1 and K10, whereas it has no effect on the KSC/TA cell marker gene expression (Fig. 2). It was reported that the NHEK proliferation was down-regulated when they were cultured on the sulfated HA coated surface (Nagira *et al*., 2007). In addition, the increase in K1 gene expression was found in the same study, which is consistent with our observation. These results suggest that HA favor the epidermal differentiation of keratinocyte rather than the expansion of KSCs and their TA cells. During epidermal development, the balance between proliferation and differentiation must be tightly controlled. Both HA and chitosan have been exploited to produce skin equivalent.

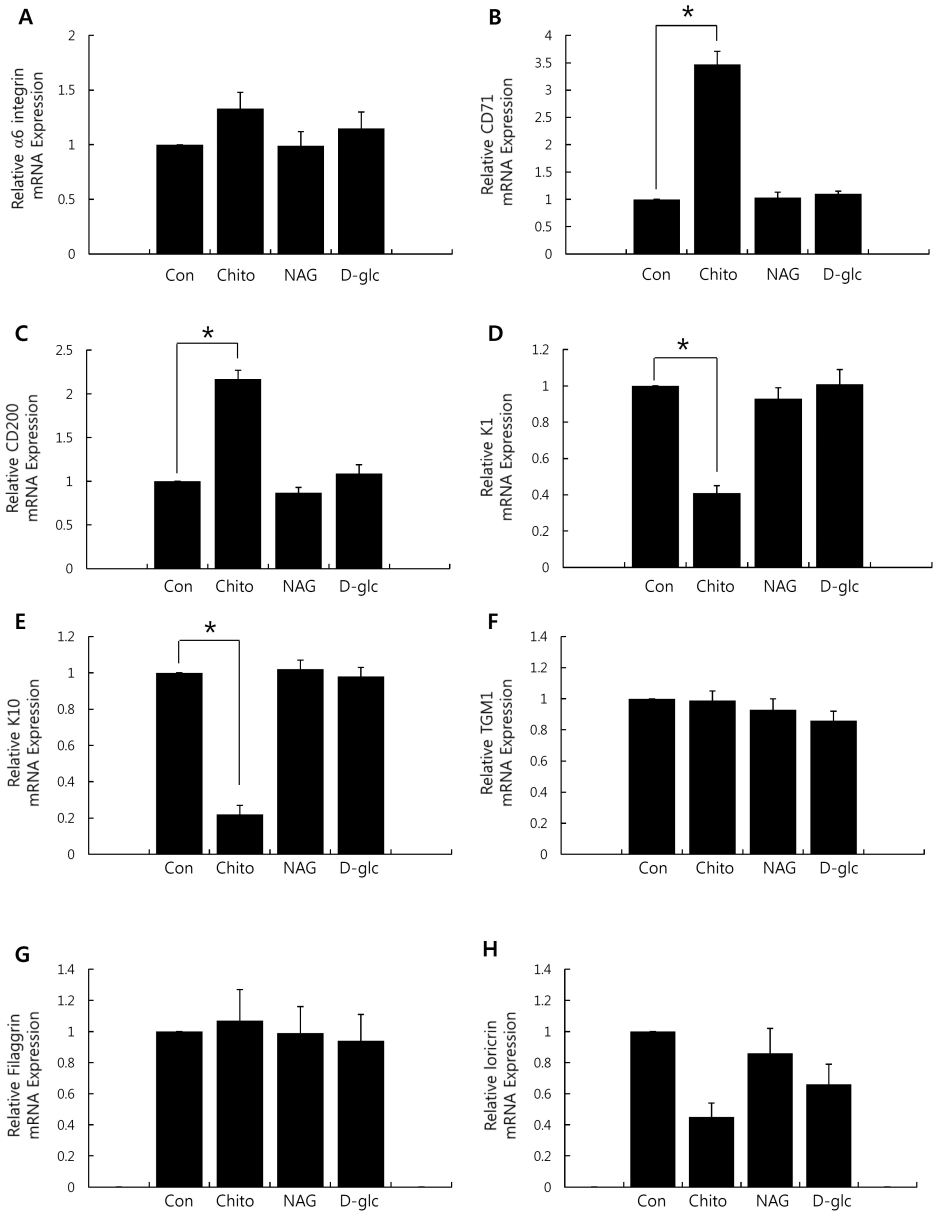


Fig. 3. The effects of chitosan and its monomers on specific expression markers of KSCs, TA cells and differentiated keratinocytes. Each condition was subjected to quantitative real-time PCR for KSCs/TA cell markers, α6 integrin (A), CD71 (B) and CD200 (C). Keratinocyte differentiation marker analyses were performed for K1 (D), K10 (E), TGM1 (F), filaggin (G) and loricrin (h). Data were calculated using GAPDH as an internal standard and were normalized by control as 1. Data are means ± SEM, n=4, $*p < 0.05$.

However, HA and chitosan play an opposite role in the generation of skin equivalent. Because skin equivalent is used in various purposes such as pharmacological evaluation of dermatological drugs, penetration studies for drugs and cosmetic ingredients, and toxicological testing for potential skin irritants, further studies will be required which supporting matrix material is appropriate the purpose of study.

Taken together, our results suggest that chitosan and GAGs play a role in providing appropriate biological signals to regulate both KSC/TA cell expansion and stage-specific keratinocytes differentiation processes.

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CONFLICT OF INTEREST

Authors Dong Wook Shin, Joong Hyun Shim, Yoonkyung Kim, Eui Dong Son, Han Kon Kim, Seung Ha Yang, Hye-Jin Jeong, Minsoo Noh are employees of Amore-Pacific Co.

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