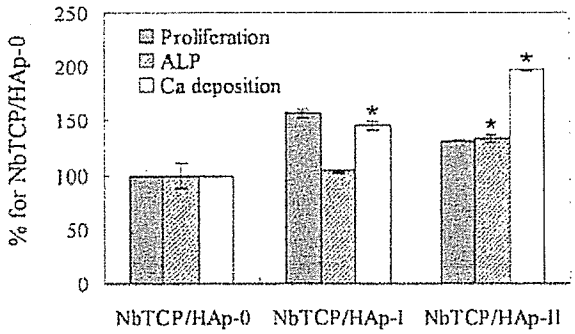

Fig. 6. Osteogenic properties (proliferation, ALP activity, and Ca deposition) of NHOst cultured in extracts from various NbTCP/HAp samples for 14 days. *P < 0.01 against NbTCP/HAp-0 (without Nb ions)

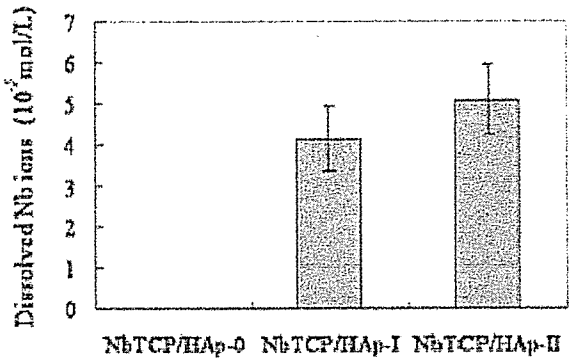

Fig. 7. Concentrations of Nb ions in extracts from various NbTCP/HAp samples. The concentration of Nb ions in cell culture medium was measured by inductively coupled plasma analysis

NHOst adhered to and spread on NbTCP/HAp-I and -II, while little spreading of NHOst was observed on HAp. In addition, as shown in Fig. 4, NHOst cultured on the NbTCP/HAp-II pellets expressed high ALP activity, compared with those cultured on NbTCP/HAp-0. Figure 6 shows the proliferation, ALP activity, and Ca deposition of NHOst cultured in extracts from various NbTCP/HAp samples for 14 days. Like the NHOst cultured on pellets, NHOst cultured in the extract from NbTCP/HAp-II expressed higher ALP activity than those in the extract from NbTCP/HAp-0. Furthermore, the amount of deposited calcium from NHOst increased with increasing Nb ion concentration in NbTCP/HAp, and the calcium deposition in the extract from NbTCP/HAp-II was twice that in the extract from NbTCP/HAp-0.

Figure 7 shows the concentration of Nb ions in extracts from NbTCP/HAp samples. It was found that Nb ions were released into the cell culture medium at concentrations of the order of 1×10^{-5} mol/l. To investigate the effect of Nb ions on NHOst function, NHOst were cultured in a medium containing Nb ions. The dependence of osteogenesis by NHOst on Nb ion concentration is shown in Fig. 8. Nb ions did not affect the proliferation of NHOst, but the ALP activity and Ca deposition of NHOst proceeded proportionally when the concentration of Nb ions was more than 1×10^{-5} mol/L.

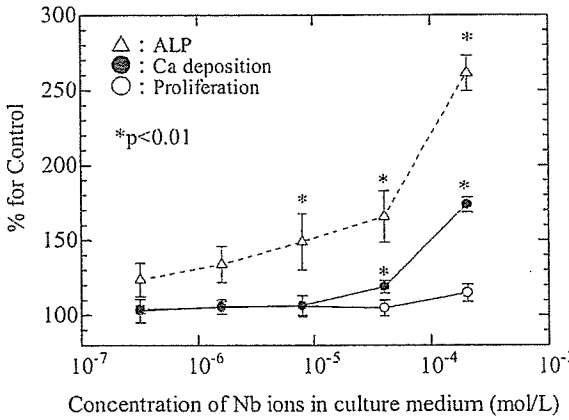

Fig. 8. Relationship between concentration of Nb ions in culture medium and osteogenic properties of NHOst. *P < 0.01 against cell culture medium without Nb ions

Discussion

Characterization of NbTCP/HAp biphasic calcium phosphate ceramics

As summarized in Table 1, before annealing the precipitates, the NbHAp samples were hydroxyapatite with low levels of crystallite. The hydroxyapatite structure is known to be very tolerant of ionic substitution.[12] Ca^{2+} ions, PO_4^{3-} ions, and OH^- ions can be replaced, partly or completely, by various cationic or anionic ions. Notably, as shown in Table 1, the lattice parameter of HAp increased when the Nb content in NbTCP/HAp was high. This fact suggests that Nb ions are taken into the apatite lattice. If a substitution of an Nb^{5+} ion for a Ca^{2+} ion in HAp occurred, the lattice parameter should decrease, since the ionic radius of Ca^{2+} and Nb^{5+} are 0.1 nm and 0.064 nm, respectively. Therefore, the possibility of substitution of Nb ions for Ca ions is low. On the other hand, although the structure of Nb ions in aqueous solution is not fully understood at present, it has been reported that Nb ions in solution are not present as Nb^{5+} but as niobiumate acid, $H_xNb_6O_{19}^{(8-x)-}$ ions (x = 0,1,2) for basic conditions,[14,15] and the niobiumate acid cluster ($H_xNb_6O_{19}^{(8-x)-}$) was polymerized or dissociated depending on the pH and ion concentration.[15] According to these reports, $H_4NbO_6^{3-}$ anionic monomer can exist in basal and low Nb concentrations (<0.08 M). Since the Nb concentration in this study was 0.01 M, Nb ions would exist as $H_4NbO_6^{3-}$ anionic monomers. $H_4NbO_6^{3-}$ may be substituted at the PO_4 site since the PO_4 site in HAp can be replaced by anionic

atomic groups. In addition, the ionic radius of the $H_4NbO_6^{3-}$ monomer and PO_4 are approximately 0.30 nm and 0.23 nm, respectively, suggesting that an increase in lattice parameter of NbTCP/HAp is ascribed to the substitution of PO_4 sites by this monomer in HAp. Furthermore, the fact that both the Ca/(Nb + P) and Nb/(P + Nb) molar ratios of the precipitates, as measured by ICP, approximately agreed with their theoretical values may support this hypothesis. Despite the theoretical Nb/(Nb + P) ratio being 0.1667, the Nb/(Nb + P) molar ratio in NbTCP/HAp-II was about 0.07, which suggests that the maximum amount of substituted Nb ions at the PO_4 site is around 0.07.

The Ca/(P + Nb) molar ratio in the NbHAp obtained in this study was lower than that of the stoichiometric value of 1.67 for HAp. Hydroxyapatite having a lower Ca/P molar ratio is known as calcium-deficient hydroxyapatite [Ca-def HAp, $Ca_{10-Z}(HPO_4)_Z(PO_4)_{6-Z}(OH)_{2-Z}$, $Z = 0$–1]. Therefore, NbHAp can be regarded as a Ca-def HAp in which the PO_4 sites are partly occupied by Nb ions.

Ca-def HAp decomposes to stoichiometric HAp and β-TCP at temperatures above 600°C according to the following reaction:[16,17]

$$Ca_{10-Z}(HPO_4)_Z(PO_4)_{6-Z}(OH)_{2-Z}\cdot nH_2O \rightarrow (1-Z)Ca_{10}(PO_4)_6(OH)_2 + 3Z\cdot\beta\text{-}Ca_3(PO_4)_2 + Z\cdot nH_2O$$

The above thermal decomposition reaction occurred during the annealing of NbHAp, resulting in a lower Ca/P molar ratio than the stoichiometric value of HAp because of partial β-TCP formation. In addition, the homogenously distributed Nb ions in NbTCP/HAp may result from thermal diffusion of Nb ions during the thermal decomposition process.

Osteogenesis of NHOst cultured on NbTCP/HAp

In this study, NbTCP/HAp showed potential to promote calcification of NHOst. This study indicated that osteogenic behavior of NHOst cultured on NbTCP/HAp pellets was consistent with that of NHOst cultured in extracts from the pellets, suggesting that dissolved ions from the NbTCP/HAp pellets affect calcification of NHOst. As shown in Fig. 7, Nb ions were apparently released from NbTCP/HAps and dissolved in the medium at concentrations of the order of 1×10^{-5} mol/l. When 4×10^{-5} mol/l of $NbCl_5$ was added to the culture medium, Ca deposition clearly increased (Fig. 8). Therefore, the enhancement of Ca deposition is considered to be due to the dissolved Nb ions. One possible mechanism for enhancement of calcification is discussed below.

ALP is known to play an important role in the calcification of bone.[18–20] Generally, the calcification of bone mineral occurs in the matrix vesicles budding from the surface of osteoblasts.[21] The nucleation of biological apatite, which is the initial stage of calcification, occurs due to the reaction between inorganic PO_4^{3-} ions produced by the ALP and calcium ions in matrix vesicles.

NHOst cultured on the NbTCP/HAp pellets containing Nb ions expressed high ALP activity compared with those cultured on HAp without Nb ion. Similarly, it was found that NHOst cultured in an extract from NbTCP/HAp containing Nb ions expressed higher ALP activity than those in the extract from HAp without Nb ions. These results suggest that Nb ions affect the enhancement of ALP activity. Based on the above calcification mechanism in matrix vesicles, the enhancement of calcification might result from the enhancement of ALP activity due to dissolved Nb ions from NbTCP/HAp. The enhancement of ALP activity increases the production of inorganic PO_4^{3-} ions, and then the inorganic PO_4^{3-} ions produced may be taken into the matrix vesicles. The subsequent nucleation of biological hydroxyapatite occurs due to a reaction of Ca ions and inorganic PO_4^{3-} ions, followed by calcification. Although we cannot deny that Nb ions directly promote calcification by NHOst unrelated with ALP expression, the essence of the calcification enhancement by NbTCP/HAp may be the enhancement of ALP activity by Nb ions dissolved from NbTCP/HAp. The biological effect of Nb ions on NHOst is under investigation. Although further studies are necessary to clarify the mechanism of enhanced calcification by Nb ions, this study strongly suggests that NbTCP/HAp is a more promising material for use as a bone tissue engineering scaffold than HAp.

Conclusion

In order to promote the osteogenicity of osteoblasts, we synthesized a combination of HAp and β-TCP biphasic calcium phosphate containing Nb ions (NbTCP/HAp). The NbTCP/HAp samples were prepared by annealing precipitates obtained by coprecipitation of an aqueous solution of $Ca(NO_3)_2$ with a mixture of $(NH_4)_2HPO_4$ and aqueous Nb solution. The precipitates obtained by the coprecipitation process can be identified as Ca-def HAp, the PO_4 sites of which are partly occupied by Nb ions. NbTCP/HAp samples were successfully obtained by thermal decomposition of the precipitates.

NbTCP/HAp enhanced calcification of NHOst. The enhancement of calcification of NbTCP/HAp was ascribed to the enhancement of ALP activity due to the dissolved Nb ions from NbTCP/HAp.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research on Advanced Medical Technology from the Ministry of Labour, Health and Welfare of Japan, and a Grant-in-Aid from the Japan Health Sciences Foundation.

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Markedly different effects of hyaluronic acid and chondroitin sulfate-A on the differentiation of human articular chondrocytes in micromass and 3D honeycomb rotation cultures

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Abstract: A source of morphologically and functionally available human cartilagenous tissue for implantation is required in the field of tissue engineering. To achieve this goal, we evaluated the effects of hyaluronic acid (HA-810 and 1680 kDa), and chondroitin sulfate (CS-A 16 and C-34 kDa) on human articular chondrocytes (HC) in micromass and rotation culture conditions. Cell proliferation was increased by CS-A 16 kDa under micromass and rotation cultures, while cell differentiation was increased under rotation but not micromass conditions. Proliferation and differentiation due to CS-C 34 kDa were very similar to the control under both culture conditions. With HA, cell proliferation was increased depending on the molecular weight under micromass and rotation conditions. In contrast, chondrocyte differentiation was enhanced under rotation conditions, but decreased under micromass conditions depending on the molecular weight of HA. In both culture conditions, aggrecan gene was continuously expressed. However, the collagen type II gene was more weakly expressed in rotation than the micromass culture conditions. Thus, the chemical structures of polysaccharides, and the culture condition, rotation or micromass, caused differences in chondrogenesis. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 000–000, 2006

Key words: human articular cartilage; hyaluronic acid; chondroitin sulfate; chondrogenesis; *in vitro* culture

INTRODUCTION

The limited potential of human hyaline cartilage for self-renewal has encouraged research in autologous chondrocyte transplantation for the regeneration of hyaline cartilage following traumatic cartilage damage.[1,2] The development of bioengineered cartilaginous implants is being studied in the field of tissue engineering. A primary approach in tissue engineering involves the regeneration of tissue by growing isolated chondrocytes on polymorphic scaffolds to produce a three-dimensional articular cartilage tissue suitable for implantation.[3–5] Cell seeded scaffolds were tested in the *in vitro* engineering of three-dimensional (3D) hyaline cartilage, although production of hyaline cartilage remains a challenge. Different nonbiodegradable materials tested for cartilage tissue repair in different experimental animals include polytetrafluoroethylene (PTFE),[6] polyethylene terephthalate (Dacron),[7,8] polyurethanes,[9] polyhydroxyethyl methacrylate (PHEMA),[10] polyvinyl alcohol (PVA, Ivalon™),[11] and a variety of other hydrogels.[12,13] Many studies have evaluated the potential of various natural bioabsorbable polymers such as collagen,[14,15] alginates,[16–18] fibrin,[19–21] and gelatin.[22] In recent years, extensive experiments have been performed that support the growth of chondrocytes by using various synthetic bioabsorbable materials in animal models to facilitate the regeneration of cartilage tissue.[23–27]

Hyaluronic acid (HA) is a negatively charged glycosaminoglycan (GAG) composed of repeated disaccharides of D-glucuronic acid and *N*-acetyl-D-glucosamine monomers that is considered the "backbone" of the extracellular ground substance. By interacting with other matrix molecules, HA provides stability and elasticity to the extracellular matrix[28–31] Among many biochemical regulators of articular cartilage, HA of animal origin plays an important role in maintaining the articular chondrocyte morphology and proliferation[32] and

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it enhanced proliferation, as well as chondroitin sulfate (CS) synthesis, of rabbit articular cartilage.[33]

CS is an important ~~extracellular matrix~~ component of native cartilage tissue.[34] Two types of CS were used in this experiment, CS-A [sulphated on the C6 position of the *N*-acetylglucosamine (GlcNAc)] and CS-C (sulphated on the C4 position of the GlcNAc). Recently, CS was shown to stimulate the bioactivity of seeded chondrocytes *in vitro*[35] and to increase matrix component production by human articular chondrocytes (HC) cultivated in clusters *in vitro*.[36] A recent study suggested that the hydrodynamic conditions in tissue culture bioreactors could modulate the composition, morphology, mechanical properties, and electromechanical function of engineered cartilage.[37] Although comprehensive studies have been done with animal cells using bioabsorbable materials, little information is available on the chondrogenic effects of HA and CS on HC. We know of no studies that have assessed the effects of different molecular weights of HA obtained from bacteria and CS using HC in both micromass and 3D honeycomb rotation culture conditions. The aim of the present *in vitro* study was to investigate the effects of HA and CS on HC in the generation of a 3D human hyaline cartilage that imitates native cartilage. For this purpose, bacterially produced HA of different molecular weights, and CS with different molecular structures were used in culturing HC under micromass and rotation conditions.

MATERIALS AND METHODS

Chondrocytes and medium

Human articular chondrocytes (HC) of the knee joint and chondrocyte growth medium were commercially obtained from Cambrex Bio Science Walkersville (Walkersville, MD). The chondrocyte growth medium contained basal medium (CC-3217) and growth supplement (CC-4409) which includes 25 mL of fetal bovine serum, 1.0 mL of R3-insulin-like growth factor-1, 2.5 mL of basic fibroblast growth factor, 1.0 mL of insulin, 0.5 mL of transferrin, and 0.5 mL of gentamicin/amphotericin-B.

Preparation of materials

The CS of two different molecular weights and structures (CS-A 16 kDa, CS-C 34 kDa) and HA of two different molecular weights (HA 810 kDa, HA 1680 kDa) used in this experiment were obtained from Lifecore Biomedical, (Minneapolis, MN). HA was of bacterial origin. The collagen honeycomb used in this experiment as 3D scaffolds was obtained from Koken, Japan.

Both kinds of CS and HA (810 kDa) were dissolved in chondrocyte growth medium at a final concentration of 0.5 mg/mL. HA (1680 kDa) was dissolved in chondrocyte growth medium at concentrations of 0.1, 0.2, and 0.5 mg/mL.

Preparation of siliconized vessel

One-hundred-milliliter glass bottles for use as culture vessels were siliconized using AquaSil™ siliconizing agent (Pierce, Illinois) according to the manufacturer's protocol with slight modification. Briefly, the bottles were thoroughly washed with soap and water, rinsed with distilled water to remove all residues, and then dried in an oven at 100°C for at least 1 h. A 0.5% solution of AquaSil™ siliconizing agent was made with Milli-Q water, and all glass bottles were completely filled with freshly prepared siliconizing solution and agitated for 1 min to coat the inner surface with a thin film of silicon. The bottles were then rinsed with 100% methanol to remove excess siliconizing fluid, dried at 100°C for 1 h, rinsed with distilled water, dried again at 100°C for 1 h, and autoclaved.

Cell culture

The HC were seeded in monolayers at a density of 2×10^4 cells/cm^2 in Corning 75-cm^2 cell culture flasks (Corning, type 430720, Corning, NY). When subconfluent, the cells were trypsinized (trypsin-EDTA [Gibco, Grand Island, NY] in phosphate-buffered saline [PBS]) and again subcultured in monolayers. After adequate growth, chondrocytes from passage three (P3) were collected by trypsinization and prepared for micromass and rotation cultures. In one set of the micromass cultures, 4×10^5 cells in 20 µL of medium were spotted onto Costar 24-well microplates for tissue culture (Costar type 3526, Corning), and media was added after 2 h of cell attachment at 37°C in a CO_2 incubator. Medium was supplemented with four different kinds of CS and HA (0.5 mg/mL); control cultures were grown with medium only. In another set of cultured chondrocytes, media was supplemented with HA (1680 kDa) of different concentrations (0.1, 0.2, and 0.5 mg/mL). The medium was changed twice a week. For 3D honeycomb rotation culture, chondrocytes (4×10^5 cells/20 µL) were spotted on each scaffold inside the siliconized glass bottles. To allow the cells to settle and attach to the scaffolds, culture vessels were incubated in a CO_2 incubator for 2 h at 37°C before 6 mL of medium with HA or CS (0.5 mg/mL) was added to each bottle. Cell-free scaffolds that were similarly cultured and rotated served as blanks. Half of the medium was changed every 3 days, and fresh CS and HA were added each time. Both cultures were incubated in 5% CO_2 and 95% air at 37°C for 4 weeks. In the rotation culture, all bottles rested on the platform of a shaker (Shaker SRR-3, Iuchi, Tokyo, Japan), which was placed inside a 37°C cell culture incubator and rotated electrically to develop a flow condition in the media. The clockwise rotation maintained equal intensity of turbulence for the cells and scaffolds, and all constructs were dynamically suspended in a laminar flow. Gas exchange was allowed by surface aeration by loosely capping the bottles.

Cell morphology assay

Cell morphology was determined by inverted light microscopy. Twice weekly observations were done, and photographs were taken with Fuji film.

Measurement of wet weight

After 4 weeks of 3D honeycomb rotation culture, scaffolds were taken out of the siliconized bottle, and extra medium attached with them was wiped with sterile gauge. Then wet weights were measured using a scale.

Proliferation assay

Alamar blue method

Cell proliferation was quantitatively measured by alamar blue (Biosource International, Camarillo, CA) assay after 4 weeks of culture, as previously described.[38] The assay reveals the metabolic activity of cells by detecting mitochondrial activity. Alamar blue used as an indicator dye is incorporated into the cells, reduced, and excreted as a fluorescent product. In the micromass culture, medium was discarded from all wells after 4 weeks of culture, and each well was filled with 1 mL of a 20-fold dilution of alamer blue solution with the fresh medium. For the rotation culture, the newly formed cartilaginous constructs were placed in the wells (a single construct per well, at least four samples in each group) of 24-well tissue culture plates. The wells were filled with a 20-fold dilution of alamer blue solution, similar to the micromass condition. The culture plates were incubated at 37°C for 4 h.

After the incubation period, two 100-μL aliquots of the solution in each well were transferred to wells of a Costar 96-well tissue culture microplate (Costar type 3595, Corning). An equal volume of fresh medium per well (total four wells) served as blanks. The extent of cell proliferation was quantitated using a Cytofluor II fluorescence multiwell cell reader (PerSeptive Biosystems, Framingham, MA) at 535-nm excitation and 590-nm emission. The intensity of the blue color obtained was directly proportional to the metabolic activity of the cell populations. Blank values were subtracted from the experimental values to exclude background activity.

Crystal violet staining

Cell proliferation was quantitatively estimated by crystal violet (Wako Pure Chemical Industries, Osaka, Japan) staining as previously described.[39] Briefly, medium from all wells in the micromass culture was discarded after the culture period, and cells were fixed with 100% methanol at room temperature. After fixation, cells were stained with 0.1% crystal violet in methanol for 20 min. After a proper wash, methanol was again applied and incubated for 10 min. Hundred microliters from each well was transferred to a new 96-well plate, and the absorbance was measured at a wavelength of 590 nm using an ELISA reader (Bio-Tek Instruments, Winooski, VT). Blank values were subtracted from experimental values to exclude background activity.

Differentiation assay

Proteoglycans are known components of the cartilage matrix. The degree of chondrogenesis was determined by staining the cartilage specific proteoglycan with alcian blue solution (Wako Pure Chemical Industries, Osaka, Japan) as described earlier.[38] Following the alamar blue assay, the medium was discarded from the 24 well plates containing the newly formed cartilaginous constructs and from the micromass culture plates. The plates were then washed once with 0.5 mL/well of PBS at room temperature and stained in 0.5 mL/well with 1% (v/v) alcian blue, pH 1.0, overnight at 4°C. The alcian blue solution was then removed, and wells were rinsed with 3% (v/v) acetic acid and distilled water to completely remove the free dye. The cartilage proteoglycan was extracted using 4*M* guanidine hydrochloride, and the absorbance was measured at a wavelength of 600 nm using an ELISA reader (Bio-Tek Instruments). A 100-μL sample of fresh 4*M* guanidine hydrochloride per well in a total of four wells served as blanks. Blank values were subtracted from experimental values to exclude background activity.

Reverse transcription and polymerase chain reaction

The matrix molecules were confirmed, as part of this study, to be collagen type II and aggrecan. For detection of the presence of these proteoglycans, single stranded cDNA was prepared from 1 μg of total RNA by reverse transcription (RT) using a commercially available First-Strand cDNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Subsequent PCR was performed with 1 μL of cDNA in a 24.75 μL of reaction mixture (10× PCR buffer 2.5 μL, dNTP 2 μL, $MgCl_2$ 2 μL, forward and reverse, each primer 0.5 μL, Taq DNA polymerase 0.25 μL, and distilled water 17 μL). The codon sequence used for the primer sets was as follows:

Collagen type II: forward 5′-GGCAATAGCAGCAGGTTCACGTACA-3′
reverse 5′-CGATAACAGTCTTGCCCCACTT-3′
Aggrecan: forward 5′-TCGAGGACAGCGAGGCC-3′
reverse 5′-TCGAGGGTGTAGCGTGTAGAGA-3′.

An initial denaturation step at 94°C for 5 min, followed by 25 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s), and a final extension at 72°C for 5 min for collagen type II, and an initial denaturation at 95°C for 5 min, followed by 33 cycles (95°C for 5 min, 60°C for 1 min, 72°C for 1 min), and a final extension at 72°C for 5 min for aggrecan were carried out. Electrophoresis of PCR products was performed on 3% agarose gel and visualized with SYBR Green I (Bio Whittaker Molecular Applications, Rockland, ME). The relative intensity of signals from each lane was analyzed using a computerized scanner. For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH):

forward 5′-CCCATCACCATCTTCCAGGAGCGAGA-3′
reverse 5′-TGGCCAAGGTCATCCATGACAACTTTGG-3′.

Statistical analysis

Student's *t* tests were used to assess whether differences observed between the polymers treated and the control samples were statistically significant. For comparison of groups of means, one-way analysis of variance was carried

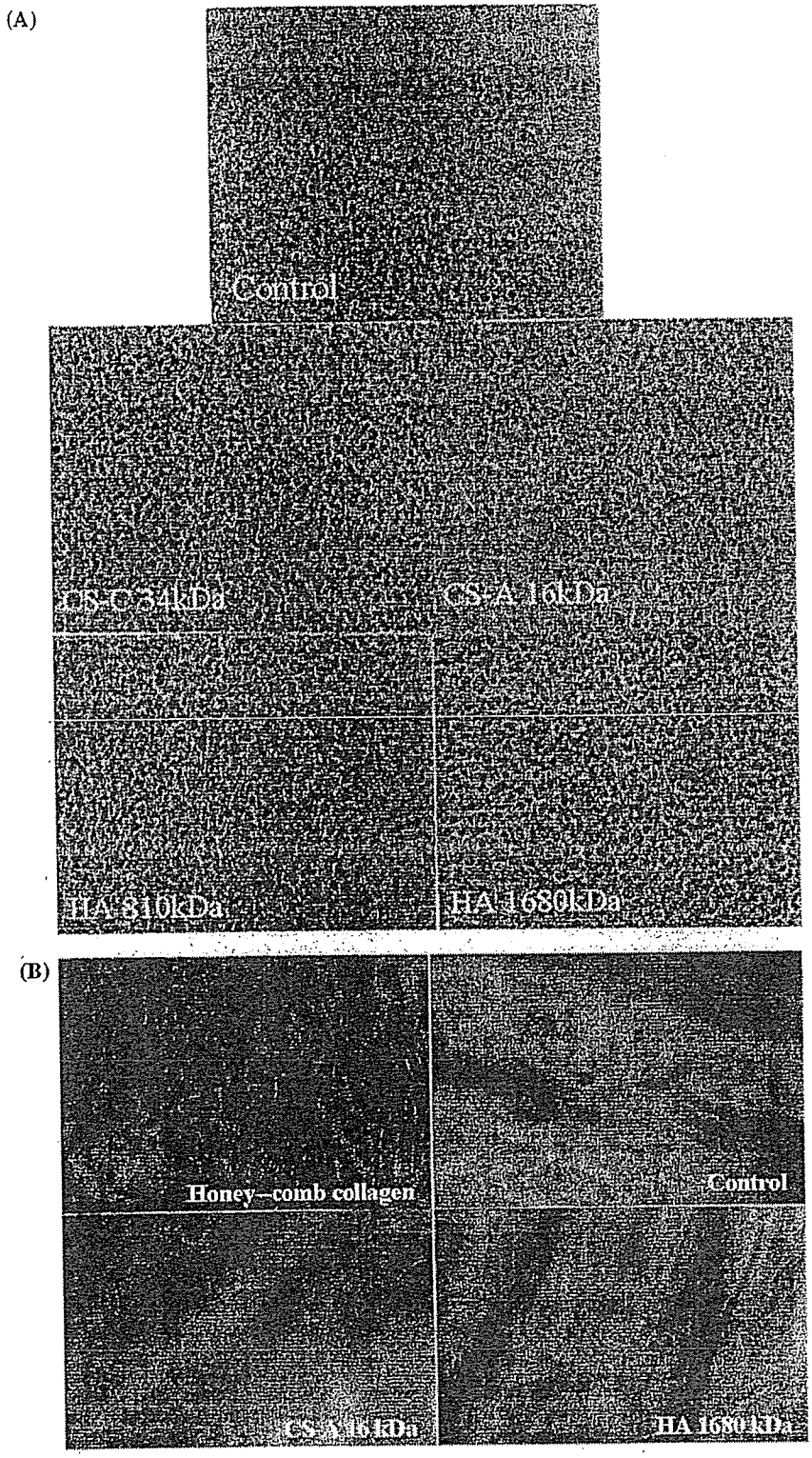

Figure 1. Light microscopic appearance of cultured HC in (A) micromass and (B) 3D honeycomb rotation conditions spotted as high density cultures treated with different molecular weights and molecular structures of HA and CS for 4 weeks (after alcian blue staining, original magnification ×200). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.] AQ1

out. When significant differences were found, Tukey's pairwise comparisons were used to investigate the nature of the difference. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± SD (standard deviation). Four samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

RESULTS

Cell morphology

The morphology of chondrocytes in micromass culture and chondrocyte constructs grown on a 3D collagen honeycomb under rotation was determined by inverted light microscopy after alcian blue staining and is shown in Figure 1. In micromass culture, the cartilage-specific proteoglycans were comparatively less stained with alcian blue in cultures treated with HA 1680 kDa than in the other culture conditions [Fig. 1(A)]. In contrast, in the rotation culture, the cartilage-specific proteoglycans were more prominently stained with alcian blue in cultures treated with HA 1680 kDa than in the other cultures, and the intensity of the blue color obtained was directly proportional to the amount of specific proteoglycans present in the cartilage constructs [Fig. 1(B)].

Wet weight

To obtain the actual weight of the newly formed cartilaginous constructs, the wet weights of 4-week-cultured cell-free scaffolds were subtracted from the wet weights of all cells-seeded constructs. After 4 weeks of culture, the wet weight of the constructs grown with CS-A 16, CS-C 34, HA 810, and HA 1680 kDa were increased 1.6, 1.2, 2.2 ($p < 0.05$), and 2.9 ($p < 0.01$) times compared with control (Fig. 2).

Cell proliferation assay

The proliferation rates of all cultures done both in micromass [Fig. 3(A)] and rotation conditions [Fig. 3(B)] are shown in Figure 3, with error bars representing the SD of the mean. All values of the samples exposed to the factors are expressed as a percentage of the average control value, which was calculated as 100%. Under micromass condition, cell proliferation of the cultures treated with CS-A 16, HA 810, and HA 1680 kDa at 0.5 mg/mL was increased 1.3 ($p < 0.01$), 1.34 ($p < 0.01$), and 1.5 ($p < 0.01$) times, respectively, compared with the control culture [Fig. 3(A)]. The same figure shows that CS-C 34 kDa treatment caused a 1.1-fold increase of cell proliferation, but this was not significant compared with the control culture. Under the rotation condition, cell proliferation of the cultures treated with CS-A 16, HA 810, and HA 1680 kDa at 0.5 mg/mL was increased to 1.3 ($p < 0.01$), 1.2 ($p < 0.05$), and 1.5 ($p < 0.01$) times, respectively, compared with the control culture. CS-C 34 kDa treatment also caused a 1.1-fold increase in cell proliferation, which was not statistically significant compared with the control culture [Fig. 3(B)].

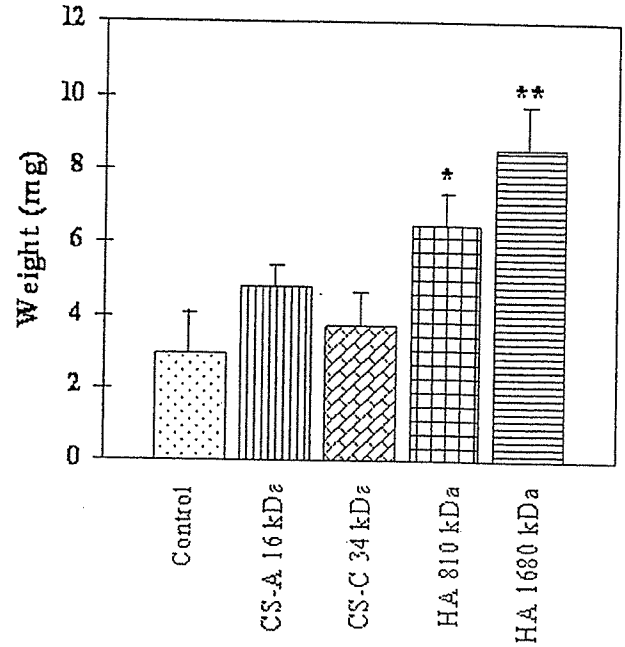

Figure 2. Wet weight of tissue constructs obtained by culturing HC on collagen honeycomb in rotation condition and treated with different molecular weights and structures of HA and CS for a period of 4 weeks. HA 810 kDa (*$p < 0.05$), and HA 1680 kDa (**$p < 0.01$) are significantly different compared with controls. All experiments were run in quadruplicate for two separate times.

Cell differentiation

The form of proteoglycan bound with alcian blue was extracted with 4*M* guanidine hydrochloride. The amounts were expressed as a percentage of the average control value, which was calculated as 100%. Under micromass culture, differentiation of chondrocytes treated with HA 1680 kDa was 87% ($p < 0.05$) of that of the control culture. At the same time, cultures treated with CS-A 16, CS-C 34, and HA 810 kDa showed a slight but not significant increase in cell differentiation [Fig. 4(A)]. The intensity of alcian blue staining was found to be higher in all cultures under the rotation condition than that found with the control culture. Here, cultures treated with CS-A 16, HA 810, and HA 1680 kDa were increased to 1.4 ($p < 0.05$), 2.1 ($p < 0.05$), and 2.4 ($p < 0.05$) times compared with the control culture. CS-C 34 kDa treatment caused a 1.1-fold nonsignificant increase in cell differentiation compared with control culture [Fig. 4(B)].

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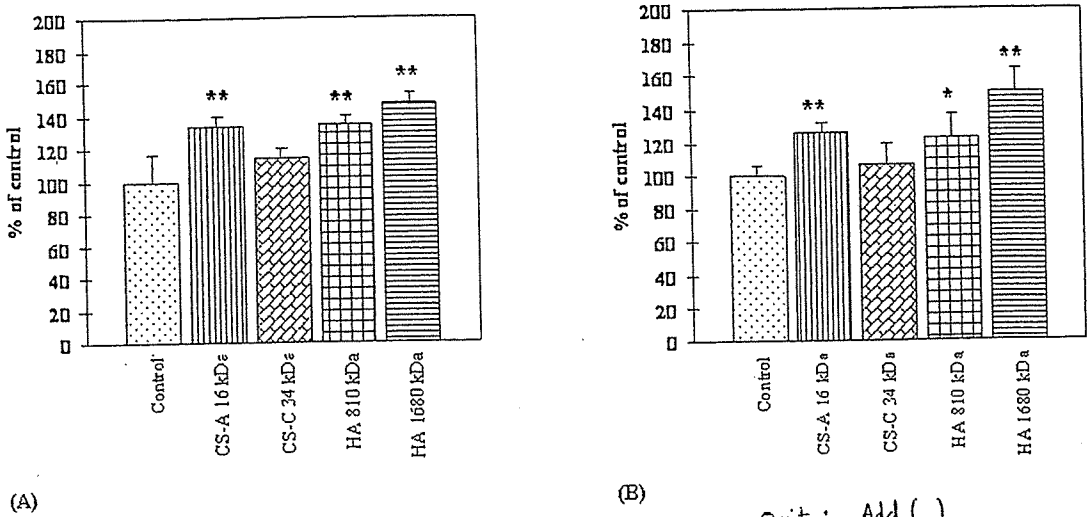

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Figure 3. Cell proliferation of HC estimated by alamar blue method. A: Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. CS-A 16 kDa, HA 810 kDa, and HA 1680 kDa are significantly different compared with control ($^{**}p < 0.01$). B: In rotation conditions, cultures were treated with four different types of CS and HA for 4 weeks. CS-A 16 kDa ($^{**}p < 0.01$), HA 810 kDa ($^{*}p < 0.05$), HA 1680 kDa ($^{**}p < 0.01$) are significantly different compared with controls. All experiments were run in quadruplicate for two separate times.

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Chondrogenic effect of HA (1680 kDa) in different concentrations

To examine the chondrogenic effect of HA (1680 kDa) in different concentrations, we again performed proliferation and differentiation assays under the micromass culture condition. Cell proliferation was increased about 1.5-, 1.6-, and 2-fold when treated with 0.1, 0.2, and 0.5 mg/mL of HA 1680 kDa, respectively, compared with the control culture [Fig. 5(A)]. F5 On the other hand, cell differentiation was significantly decreased to about 70% ($p < 0.05$) when treated with 0.5 mg/mL compared with control culture. However, cultures treated with 0.1 and 0.2 mg/mL of HA 1680 kDa also showed slight but nonsignificant decreases in cell differentiation [Fig. 5(B)].

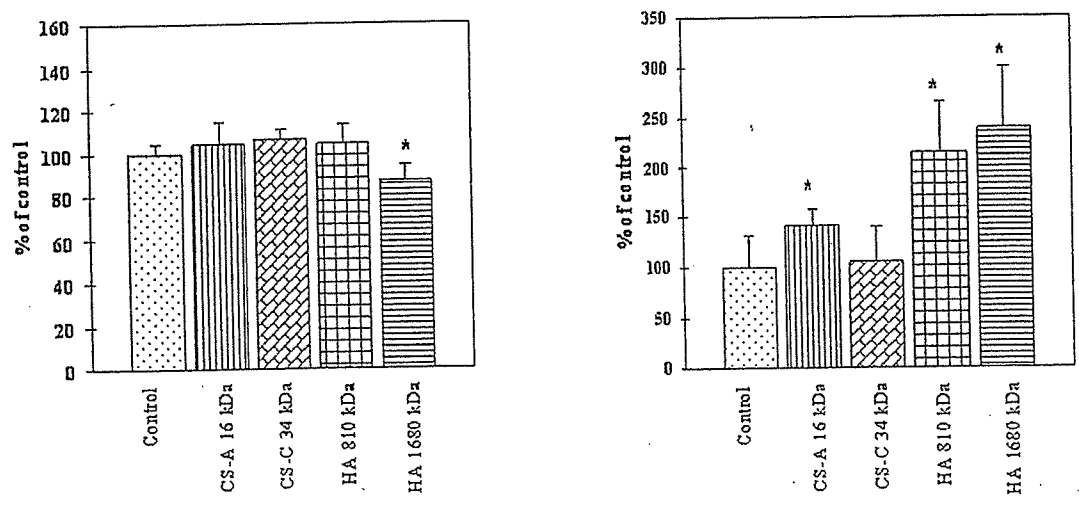

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Figure 4. Cell differentiation of HC estimated by alcian blue method. A: Under micromass conditions in control, CS-A 16 kDa; CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. HA 1680 kDa is significantly different compared with control ($^{*}p < 0.05$). B: In rotation conditions, cultures were treated with four different types of CS and HA for 4 weeks. CS-A 16 kDa, HA 810 kDa, and HA 1680 kDa are significantly different compared with controls ($^{*}p < 0.05$). All experiments were run in quadruplicate for two separate times.

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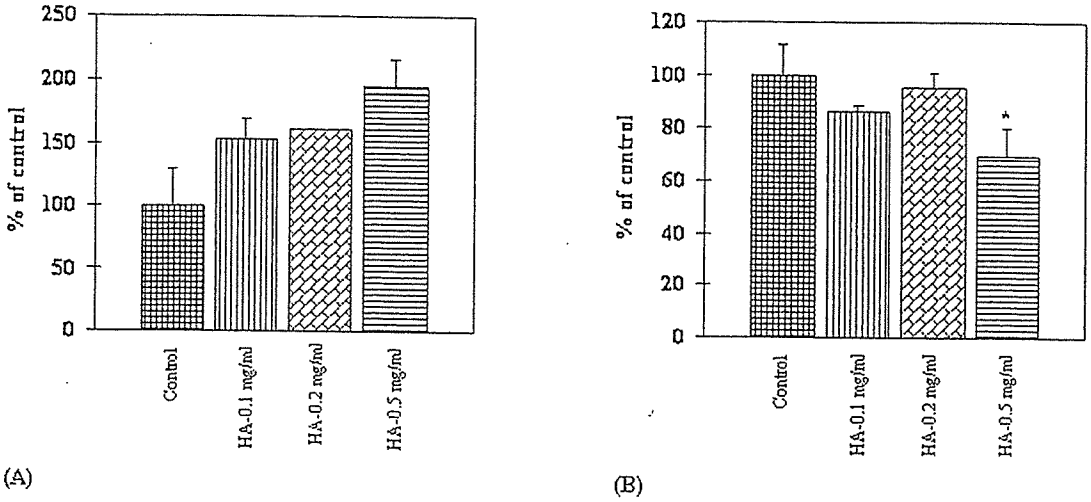

Figure 5. Chondrogenic effect of HA (1680 kDa) with different concentrations under micromass conditions for 4 weeks. A: As demonstrated by crystal violet staining, cell proliferation was increased with 0.1, 0.2, and 0.5 mg/mL of HA 1680 kDa respectively, compared with the control culture. B: Cell differentiation estimated by alcian blue staining was significantly decreased with 0.5 mg/mL ($*p < 0.05$) and slightly but non-significantly decreased with 0.1 and 0.2 mg/mL of HA 1680 kDa compared with control culture. All experiments were run in quadruplicate for two separate times.

Time course of cell proliferation

Under the micromass condition, cell proliferation was increased about 2.2 ($p < 0.05$)-, 2.3 ($p < 0.01$)-, and 4.3 ($p < 0.01$)-fold after 2, 3, and 4 weeks of culture, respectively, compared with the chondrocytes cultured for 1 week (Fig. 6).

Extracellular matrix gene expression

Under the micromass condition, RT-PCR analysis showed that chondrocytes treated with CS-A 16, CS-C 34, HA 810, and HA 1680 kDa at 0.5 mg/mL all consistently expressed collagen type II and aggrecan genes in culture [Fig. 7(A)]. On the other hand, under rotation, all cultures expressed the aggrecan gene and the intensity of expression was similar in all cultures, but there was a gradual decrease in the expression of collagen type II gene in all culture conditions. In fact, the level of expression was even less than in the control culture [Fig. 7(B)]. Therefore, this qualitative detection assay demonstrates that the cartilage extracellular matrix (ECM) expressed both collagen type II and aggrecan gene molecules in all samples under both culture conditions.

DISCUSSION

Clarification of the basic mechanisms of chondrocyte proliferation and differentiation is essential to develop new biological therapies for better treatment of patients suffering from joint diseases. Recently, autologous chondrocyte transplantation (ACT) has been introduced as a novel biological treatment.[40] Thorough understanding of experimental methods that produce adequate cell proliferation and differentiation is required for the clinical application of ACT. Here, we examined the effects of HA and CS of different molecular weights and structures by culturing HC in both micromass and rotation culture conditions. HC were

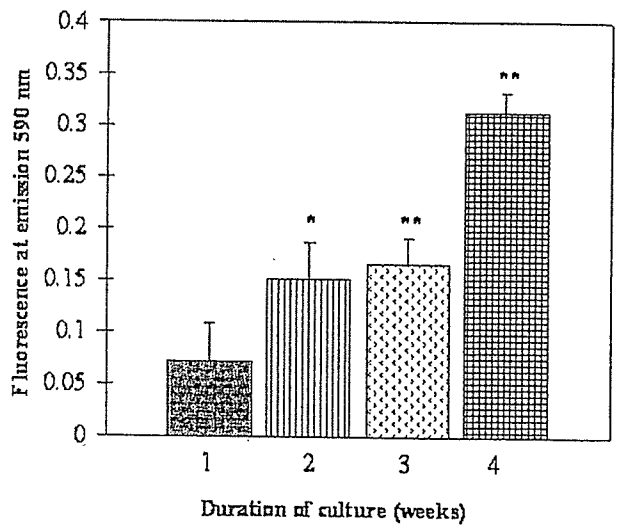

Figure 6. Time course of cell proliferation under micromass conditions for 4 weeks, estimated by crystal violet staining. Cell proliferation was significantly increased after 2 weeks ($*p < 0.05$), 3 weeks ($**p < 0.01$), and 4 weeks ($**p < 0.01$) of culture, compared with the chondrocytes cultured for 1 week. All experiments were run in quadruplicate for two separate times.

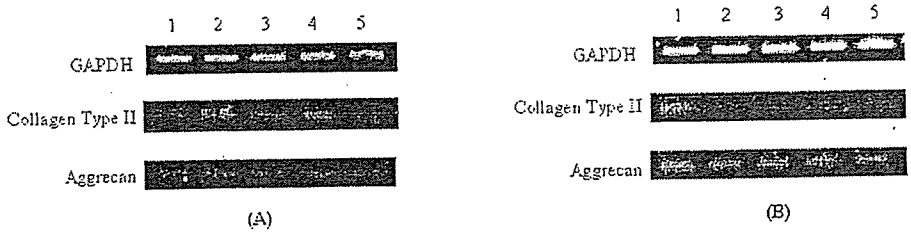

Figure 7. A: Reverse transcription-polymerase chain reaction amplification of GAPDH, collagen type II, and aggrecan in cultures of HC with four different types of CS and HA for 4 weeks in micromass conditions. Lane 1, control; lane 2, CS-A 16 kDa; lane 3, CS-C 34 kDa; lane 4, HA 810 kDa; and lane 5, HA 1680 kDa. B: Reverse transcription-polymerase chain reaction amplification of GAPDH, collagen type II, and aggrecan in cultures of HC with four different types of CS and HA for 4 weeks in rotation conditions. Lane 1, control; lane 2, CS-A 16 kDa; lane 3, CS-C 34 kDa; lane 4, HA 810 kDa; and lane 5, HA 1680 kDa. All experiments were run in quadruplicate for two separate times.

cultured on a collagen honeycomb under rotation to generate a 3D human hyaline cartilage that has the capacity to mimic native cartilage. After 4 weeks culture in this condition, the wet weight of the constructs grown with HA and CS of different molecular weights and structures were markedly increased compared with controls (Fig. 2). HA obtained from an animal source was reported to show stimulatory effects on cell proliferation and differentiation, respectively, of chondrocytes of rabbit and bovine origin cultured *in vitro* in a static condition.[33,41] We used HA of bacterial origin with different molecular weights for the *in vitro* study of HC and showed a similar tendency toward a marked increase in chondrocyte proliferation both in micromass and rotation culture conditions compared with the control cultures (Fig. 3). Under rotation, cell differentiation was significantly increased in cultures treated with HA of different molecular weights, especially with HA 1680 kDa. In contrast, in micromass culture, the HC treated with HA 1680 kDa showed a significant decrease in cell differentiation compared with controls, while a slight increase was observed in the HA 810 kDa treated cells (Fig. 4). The results obtained by morphological examination of cultured chondrocytes as well as cartilage constructs after alcian blue staining under micromass and rotation conditions (Fig. 1) also correlated with the findings presented in Figure 4.

To find the appropriate concentration of HA 1680 kDa, we again cultured HC under the micromass condition. Figure 5(A,B) revealed that the cell proliferation was increased and the differentiation was decreased in a dose-dependent manner. These findings also corroborated the results shown in Figures 3(A) and 4(A). It is supposed that small amounts of growth factors are bound to HA of animal origin. In contrast, HA of bacterial origin, such as that used in this study, is free from such constituents and is highly pure. Thus, we hypothesized that HA of animal and bacteria origins might have different effects on HC proliferation and differentiation. In micromass culture, chondrocyte differentiation was inhibited by HA of different molecular weights, and this inhibitory effect may have been overcome by the flow of turbulence occurring during rotation culture (Fig. 8). A recent study suggested that hydrodynamic conditions for culturing bovine articular cartilage provided efficient mass transfer essential for cell proliferation and synthesis of matrix components and that dynamic laminar flow patterns promoted cell differentiation, retention of newly synthesized macromolecules, and maintenance of cartilaginous tissue.[42] It was also suggested that the rotation condition is more effective than the micromass condition when HC is cultured with or without HA (data not shown). It was recently ascertained by immunofluorescence assay that the gap-junction protein connexin 43 was localized in the cultured cartilage *in vitro*, further indicating that functional gap junctions[43] and gap junctional intracellular communication (GJIC) coupling by connexin 43 play important roles in the cartilage development,[44] and it was demonstrated that chondrocytes isolated from adult articular cartilage expressed functional gap junctions.[45] Neumann et al. reported that HA can both promote and inhibit cytokine expression depending on its molecular size.[46] Recent studies suggested that the function of GJIC,[47] as well as biosynthesis of growth factors, was inhibited by the addition of HMW HA 800 kDa.[48] The molecular weight of HA used in the present study was 1680 kDa. This higher molecular weight HA apparently inhibited GJIC under the micromass condition and caused an inhibitory effect on cell differentiation.

CS-A was reported to increase proteoglycan production by human chondrocytes in culture media and in clusters.[36] Other experimental models have shown that CS-A increases GAG synthesis by chondrocytes *in vitro*.[49] It was also reported that CS linked to type I collagen scaffolds stimulates the bioactivity of seeded bovine chondrocytes *in vitro*.[35] To the best of our knowledge, no study has investigated HC proliferation and differentiation using CS of different chemical structures in micromass and rotation culture conditions. Under both micromass and rotation conditions, CS-A 16 kDa-treated cultures showed a significant increase in HC proliferation (Fig. 3) and differentiation

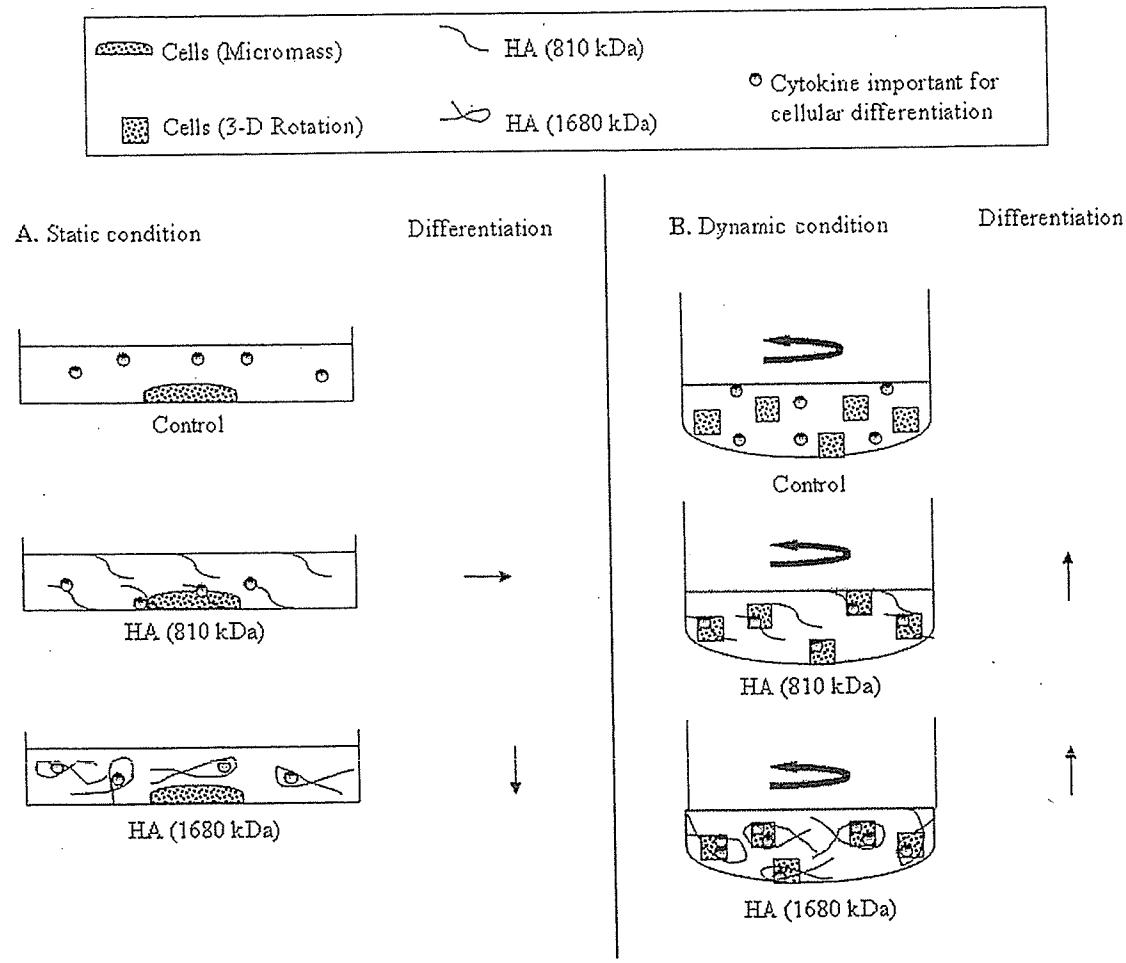

Figure 8. Schematic representation of the effects of different molecular weights of HA on the differentiation of HC. A: Static condition (micromass). B: Dynamic condition (rotation culture).

(Fig. 4) over CS-C 34 kDa. The substituted position of sulfate is different between CS-A and CS-C, and their different chemical structure played a vital role in chondrogenesis of HC rather than their different molecular weights.

A recent study reported consistent expression of the aggrecan gene in cultures of bovine articular chondrocytes with different scaffold materials cultures or as a monolayer, but scaffolds were weaker in inducing collagen type II gene expression compared with the monolayer culture.[50] In the present study, qualitative RT-PCR analysis demonstrated that the ECM of HC treated with HA and CS of different molecular weights and structures also expressed the aggrecan gene in both culture conditions. It was found that under the presence of CS-A, CS-C, and HA, expressions of collagen type II gene were lower than that of controls even in the rotation culture, although most relating researches have reported that the culture under fluid flow, mixing, or physical stimulation increased collagen contents.[51,52] Therefore, CS-A, CS-C, and HA may suppress the expression of collagen type II gene but enhance the expression of aggrecan gene under the rotation culture [Fig. 7(B)].

CONCLUSIONS

In the present *in vitro* study, considering the chemical structure and culture condition in the case of CS and the source of origin, molecular weight, and culture condition in the case of HA, it can be postulated that both the hyaluronic acids and CS-A 16 kDa will probably be effective in the field of HC repair under the *in vivo* mechanical stimulation.

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Effects of sulfated hyaluronan on keratinocyte differentiation and Wnt and Notch gene expression

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Abstract

Sulfated hyaluronan (SHya), which is composed of a sulfated group and hyaluronan (Hya), has high activity on and biocompatibility with cells. When normal human epidermal keratinocytes (NHEKs) were incubated in dishes coated with SHya, cell proliferation was suppressed in a dose-dependent manner. The expression levels of keratin 1 and loricrin mRNAs, as detected by real-time RT-PCR, were increased significantly. The expressions of Wnt mRNAs, which play important roles in cell proliferation and differentiation, were modulated. Wnt4 and Wnt6 mRNA expressions were increased compared to controls, while expression of Wnt5a was similar to the control and that of Wnt7a mRNA was decreased. In addition, the expression of Notch mRNAs, which play a critical role in keratinocyte differentiation, were affected. Notch3 mRNA was increased significantly, while Notch1 mRNA was decreased compared to controls, and expression of Notch2 was similar to that of control. These results suggested that a SHya-coated scaffold might be useful for regulating cell activity in tissue engineering.

Keywords: Sulfated hyaluronan; Normal human epidermal keratinocyte; Differentiation; Wnt; Notch

1. Introduction

Normal human epidermal keratinocytes (NHEKs) are usually cultured on 3T3 mouse feeder-layer cells [1]. Several biomaterials have been developed from other animals or humans, but they carry the risk of infection from prions and viruses [2,3]. In contrast, biomaterials from microorganisms carry lower risks of infection, and the development of a semi-synthetic material promoting cell activity will enable safer cell culture.

Several types of polysaccharides for culturing NHEKs have been studied [4,5]. Hyaluronan (Hya) is a negatively charged glycosaminoglycan that is a major component of the extracellular matrix (ECM) [6,7]. Hya plays important roles in cell adhesion, migration, proliferation, and differentiation [7–9]. Park and Tsuchiya reported that a Hya-coated surface is capable of enhancing gap junctional intercellular communication (GJIC) and differentiation or cell growth [10,11]. Sulfated polysaccharides, such as heparin or heparin sulfate, stabilize some growth factors, resulting in enhancement of their effects [12], and promote their mitogenic activity [13]. Sulfated hyaluronan (SHya), a semi-synthetic material composed of Hya and a sulfate group [14] can be synthesized using Hya extracted from microorganisms; therefore, it has a lower infectivity and a lower risk of containing virus-induced carcinogens.

It has been reported that Hya and chondroitin sulfate A enhanced chondrogenesis of human mesenchymal stem cells [15,16]. We hypothesized that SHya has the potential to function as a biomaterial promoting keratinocyte differentiation because it has been reported that SHya stimulates cell activities [14,17]. Analysis of the effects of SHya on cell differentiation and intercellular signaling will provide the information allowing construction of biomaterials of greater usefulness for tissue engineering.

Wnts are secreted glycoproteins that bind Frizzled receptors and play a critical role in the process of cell differentiation in the canonical pathway. In the canonical

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pathway, beta-catenin stabilized by the binding of Wnt proteins to Frizzled receptors stimulates TCF/LEF transcription. A mouse keratinocyte cell line with decreased Wnt4 expression showed a more malignant morphology and was less differentiated [18], Wnt6 was required for epithelialization of the segmental plate mesoderm [19], and Wnt7a promoted cell proliferation by activation of Rac-GTPase and beta-catenin [20].

Notch receptors play a crucial role in determination of cell fate. Notch1 signaling plays an essential role in regulation of mouse keratinocyte differentiation [21]. In mouse keratinocytes, Notch1 activation suppressed Wnt4 activity mediated by the cyclin/CDK inhibitor p21 (WAF1/Cip1) [22]. Notch3 was required for the differentiation of vascular smooth muscle cells and T-cells [23,24].

In a previous study, we demonstrated that a Hya coating promoted several cell functions better than a Hya-supplemented one [25]. Therefore, in this study, we investigated the effects of a SHya coating on keratinocyte differentiation.

2. Materials and methods

2.1. Sulfated hyaluronan

SHya was prepared by the method reported previously [4]. A solution of 2% Hya120 (molecular weight, 1.2×10^6) solution in *N*,*N*-dimethylformamide (DMF) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was mixed with trimethylamine (TMA)–SO_3 complex (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) and stirred for 24 h at 60 °C. The reaction mixture was then diluted, neutralized, and precipitated by adding a large quantity of acetone (Wako Pure Chemical Industries). The precipitate was dissolved in distilled water and dialyzed against distilled water. The molecular weight of SHya was 2.0×10^5, and the degree of substitution (D.S.) of SHya was 1.0, as determined by the chelate titration method [26] (Fig. 1). Moreover, the effectiveness of sulfation was also demonstrated by FT-IR analysis. The IR spectrum of SHya exhibited two absorption bands at 1240 and 820 cm^{-1} due to S=O and SO_3^- stretching, respectively.

2.2. Cell culture

NHEKs isolated from neonatal human foreskins (Cambrex Bioscience, Walkersville, MD, USA) were cultured with K-110 Type II medium (Kyokuto, Tokyo, Japan) supplemented with 2% whole bovine pituitary extract, 50 IU penicillin G, 50 μg/ml streptomycin, and 0.03 mM $CaCl_2$ (low-calcium condition) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Keratinocyte differentiation was induced in 0.20 mM $CaCl_2$ medium (high-calcium condition). The medium was exchanged for a fresh one every 2 days.

2.3. Preparation of SHya-coated culture dishes and well plates

Both 35 and 100 mm polystyrene dishes (Iwaki, Funabashi, Japan) and 24-well plates (Corning, Corning, NY, USA) were coated with SHya dissolved in distilled water at a final density of 0.4 or 0.8 mg/cm^2. The SHya-coated dishes and plates were dried under a sterile airflow at room temperature for 8 h.

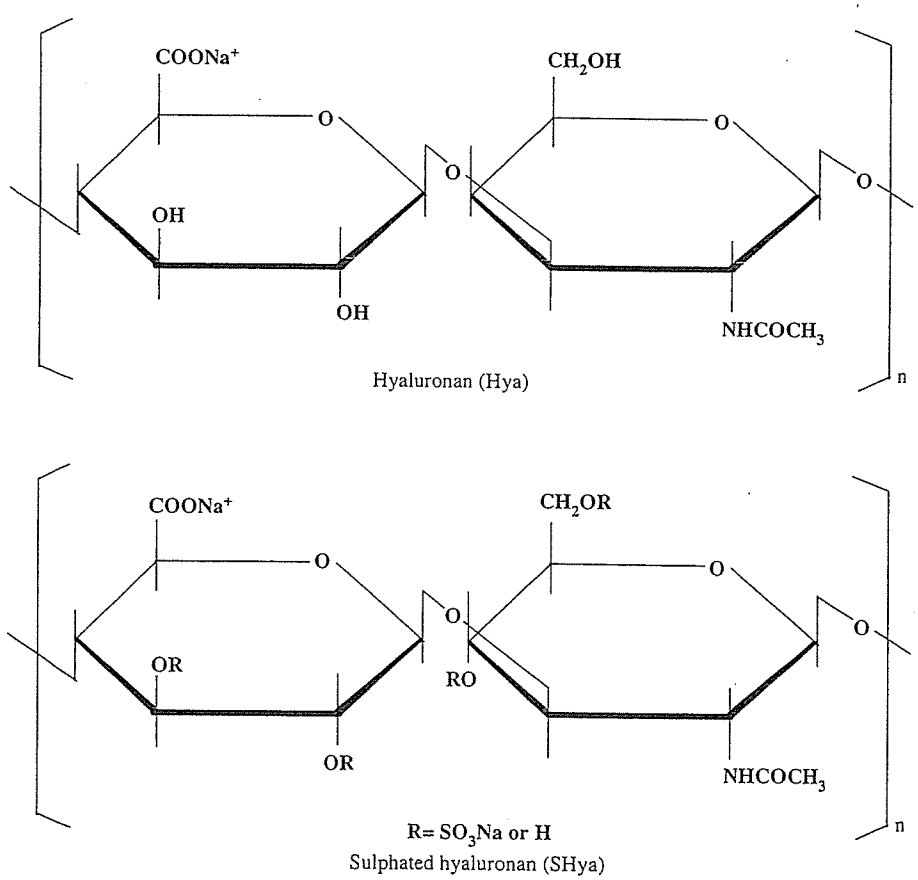

Fig. 1. The structures of hyaluronan and sulfated hyaluronan. Sulfated hyaluronan (SHya) is composed of Hya and a sulfate group. The molecular weight of SHya is 2.0×10^5, and the degree of substitution of SHya was 1.0.

2.4. Determination of cell numbers by crystal violet assay

NHEKs were seeded at 1.0×10^4 cells/cm^2 in wells of 24-well plates coated with various concentrations of SHya (0, 0.4, and 0.8 mg/cm^2) and incubated in the high-calcium condition (0.20 mM) for 5 days. After washing with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline [PBS(−)], cells were exposed to 0.4% crystal violet (Wako) in methanol for 15 min. NHEKs were washed with PBS(−) three times and destained with 500 μl of methanol for 20 min. Then, absorbance was read at 590 nm using a plate reader.

2.5. Quantitative real time RT-PCR

NHEKs were seeded at 1.0×10^4 cells/cm^2 in various concentrations on SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm^2) and incubated in the high-calcium condition (0.20 mM) for 5 days. Cells were washed with PBS(−) three times, and total RNA was extracted from NHEKs using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed into 10 μl DNA using an ExScript RT reagent kit (Takara Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Aliquots of the cDNA were used as templates for PCR analysis using a Lightcycler system (Roche, Mannheim, Germany). PCR amplification was performed in a total volume of 20 μl including 1 μl of RT reaction, 10 μl of SYBR *Premix Ex Taq* (Takara), and 0.4 μM/l of each primer. The PCR reaction was performed as follows: 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 65 °C for 15 s. The PCR primers were purchased from Takara Co., Ltd. The PCR primer sequences for amplification of keratin 1 were forward primer 5′-AGATCACTGCTGGCAGACATGG-3′, and reverse primer 5′-TGATGGACTGCTGCAAGTTGG-3′. The PCR primer sequences for amplification of loricrin were forward primer 5′-TCATGATGC-TACCCGAGGTTTG-3′, and reverse primer 5′-CAGAACTAGATG CAGCCGGAGA-3′. The PCR primer sequences for amplification of Wnt4 were forward primer 5′-CCAGCAGAGCCCTCATGAAC-3′, and reverse primer 5′-TCCACCTCAGTGGCACCATC-3′. The PCR primer sequences for amplification of Wnt6 were forward primer 5′-CTG GAATTGCTCCAGCCACA-3′, and reverse primer 5′-GCAGTGAT GGCGAACACGA-3′. The PCR primer sequences for amplification of Wnt7a were forward primer 5′-GCCCGGACTCTCATGAACTTG-3′, and reverse primer 5′-CCTCGTTGTACTTGTCCTTGAGCA-3′. The PCR primer sequences for amplification of Notch1 were forward primer 5′-TGCGAGGTCAACACAGACGAG-3′, and reverse primer 5′-GTG TAAGTGTTGGGTCCGTCCAG-3′. The PCR primer sequences for amplification of Notch2 were forward primer 5′-TGAACACTGGGTC GATGATGAAG-3′, and reverse primer 5′-AGCGATGGTGTCC TACGGATG-3′. The PCR primer sequences for amplification of Notch3 were forward primer 5′-TGATGGCATGGATGTCAATGTG-3′, and reverse primer 5′-CAGTTGGCATTGGCTCCAGA-3′. The PCR primer sequences for amplification of GAPDH were forward primer 5′-GCACCGTCAAGGCTGAGAAC-3′, and reverse primer 5′-ATGG TGGTGAAGACGCCAGT-3′. Each sample was tested in triplicate.

2.6. Statistical analysis

Significant differences between groups were evaluated with Student's *t*-test. Mean differences were considered significant when $^{**}p < 0.01$. Three samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

3. Results

3.1. Adhesiveness of NHEKs to SHya

Normal human dermal fibroblasts showed low adhesion to Hya-coated surfaces [9,10]. In order to assess the adhesion of NHEKs to SHya-coated surfaces, NHEKs were seeded on SHya- and Hya-coated surfaces (0, 0.4, and 0.8 mg/cm^2). Fig. 2 shows that NHEKs adhered to SHya-coated surfaces as well as to an uncoated dish, but they did not adhere to the Hya-coated dishes.

3.2. Effect of SHya coating on keratinocyte proliferation

NHEKs were seeded on SHya-coated dishes (0.4 or 0.8 mg/cm^2) and incubated in a high-calcium condition

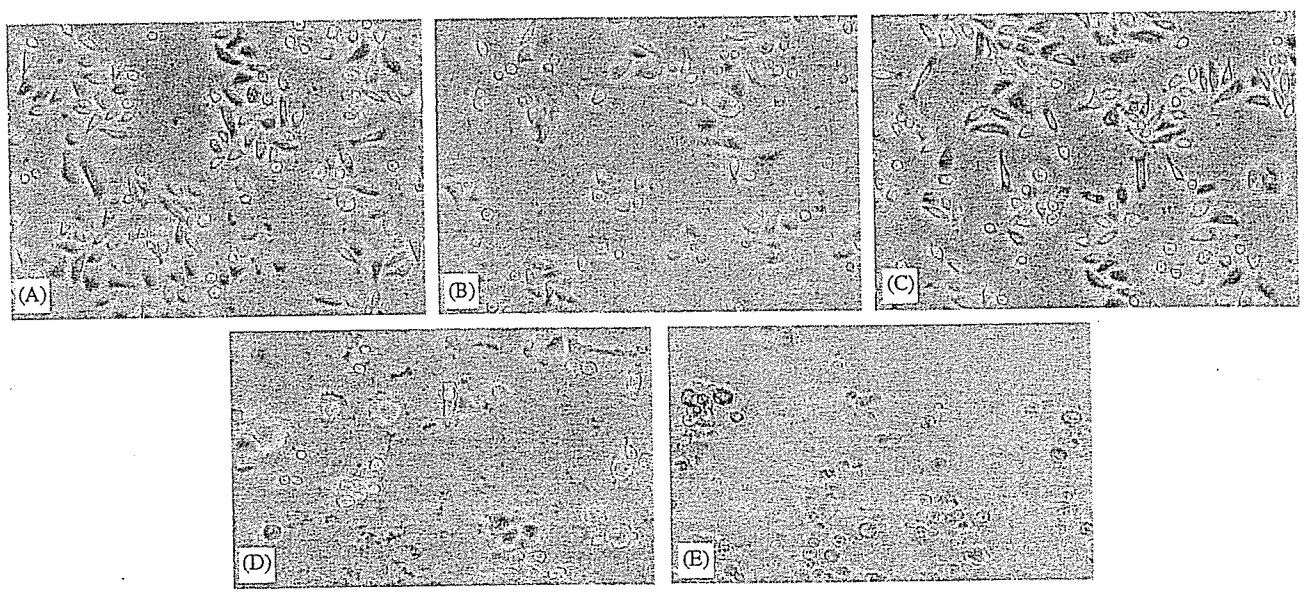

Fig. 2. The adhesion of NHEKs to SHya-coated surfaces. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya- and Hya-coated 24-well plates. Then NHEKs were incubated with non-coated (A), 0.4 mg/cm^2 SHya-coated (B), 0.8 mg/cm^2 SHya-coated (C), 0.4 mg/cm^2 Hya-coated (D), and 0.8 mg/cm^2 Hya-coated (E) wells for 16 h.

(0.20 mM) for 5 days. The cell proliferation assay showed that the SHya coating suppressed keratinocyte proliferation remarkably in a dose-dependent manner (Fig. 3).

3.3. Effect of SHya coating on keratin1 and loricrin expression

To ensure that the SHya coating promoted keratinocyte differentiation, we detected the relative expression levels of differential marker mRNA by real-time RT-PCR. After NHEKs were incubated with SHya coating in a high-calcium condition (0.20 mM) for 5 days, the expression level of keratin1 mRNA on NHEKs was increased more than eight-fold compared to the control (Fig. 4A) and that of loricrin mRNA was increased in a dose-dependent manner (Fig. 4B).

3.4. Effect of SHya coating on Wnts expressions

The expression levels of Wnt4 and Wnt6 mRNA on NHEKs incubated with SHya coating in the low-calcium condition were measured (Fig. 5A and B); the expression level of Wnt5a of NHEKs incubated with SHya coating was decreased to 95% of the control (Fig. 5C) and that of Wnt7a mRNA to about 40% of the control (Fig. 5D).

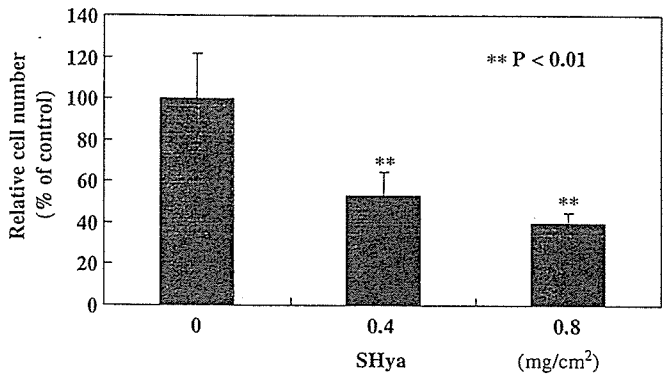

Fig. 3. Suppressive effect of SHya on keratinocyte proliferation. NHEKs were seeded at 1.0×10^4 cells/cm^2 densities onto non-coated (A), 0.4 mg/cm^2 SHya-coated (B), and 0.8 mg/cm^2 SHya-coated (C) 24-well plates and cultured for 5 days. Then numbers of NHEKs were determined by crystal violet assay. Each value is expressed as the mean ± SD. $^{**}P < 0.01$ compared to control.

3.5. Effect of SHya coating on Notch expressions

The expression level of Notch1 mRNA on NHEKs incubated with SHya coating was decreased about 75% compared to the control, and that of Notch2 mRNA was similar to the control (Fig. 6A and B). However, the expression level of Notch3 mRNA was increased about eight-fold compared to the control (Fig. 6C).

4. Discussion

Several studies have suggested that SHya interacts with cells [4], but the effect of SHya on cell differentiation and intercellular signaling was not clear. We demonstrated that a SHya coating promoted keratinocyte differentiation and modulated the expression levels of Notch and Wnt mRNAs.

In this study, the expression levels of Wnt4 and Wnt6 on NHEKs incubated with SHya were increased. A mouse keratinocyte cell line with a deficit of Wnt4 expression showed less differentiation [18]. Wnt6 regulated epithelization [19], suggesting that the SHya-induced upregulation of Wnt4 and Wnt6 is associated with the regulation of keratinocyte differentiation. Wnt7a promoted cell proliferation in corneal epithelial cells during wound healing [20], suggesting that SHya down-regulated Wnt7a expression, resulting in the enhancement of keratinocyte differentiation. The activation of beta-catenin, a downstream factor of Wnt signaling, contributes to keratinocyte differentiation [27]. A sulfated proteoglycan-induced Wnt-11 expression in mouse kidney cells, and sulfated polysaccharides were required in Wnt signaling in mouse kidney cells [28,29]. Therefore, it was suggested

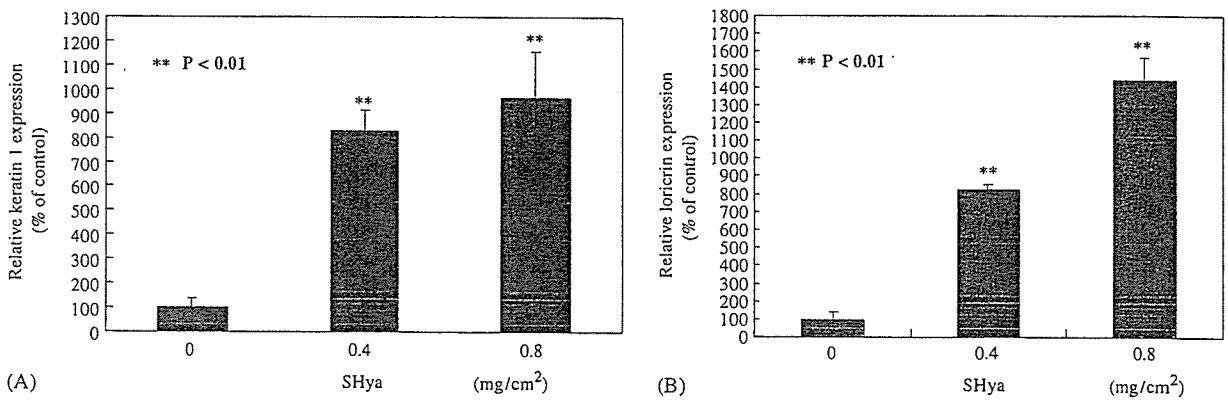

Fig. 4. The expression levels of keratin1 and loricrin mRNA of NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm^2) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed to determine the expression levels of keratin1 and loricrin mRNA. Effect of SHya on the expression level of (A) keratin1 mRNA, and (B) loricrin mRNA. Each value is expressed as the mean ± SD. $^{**}P < 0.01$ compared to control.

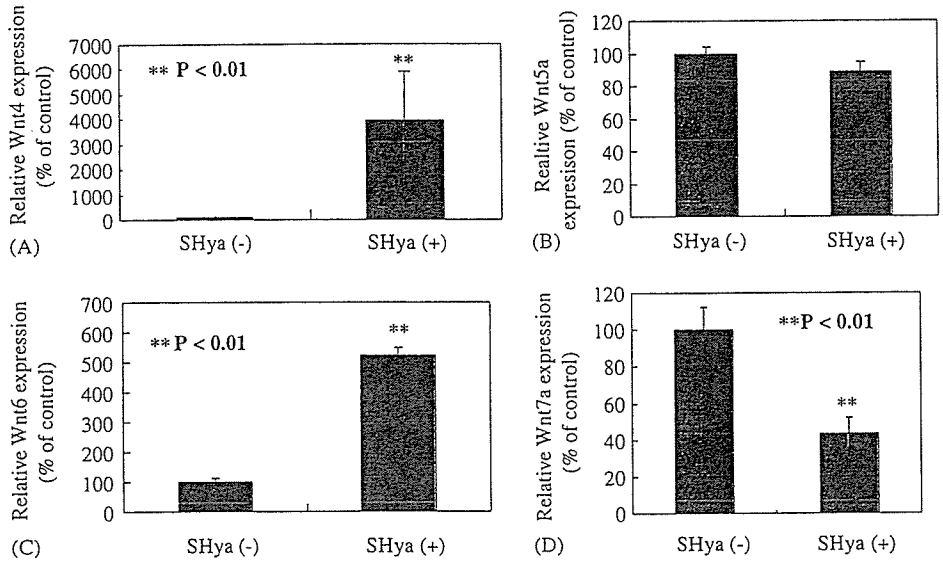

Fig. 5. The expression level of Wnt mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya-coated 60 mm dishes (0 and 0.8 mg/cm^2) and cultured in medium with 0.20 mM calcium for 20 h. Then RNA was extracted, and real-time RT-PCR was performed to determine the expression level of Wnt mRNA. Effect of SHya on the expression level of (A) Wnt4 mRNA, (B) Wnt5a mRNA, (C) Wnt6 mRNA, and (D) Wnt7a mRNA. Each value is expressed as the mean ± SD. $^{**}P<0.01$ compared to control.

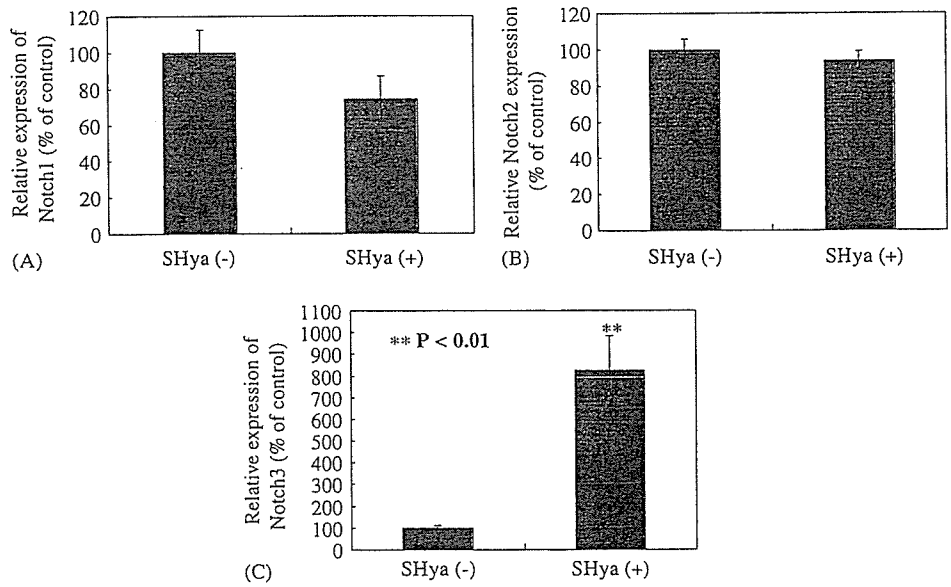

Fig. 6. The expression level of Notch mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm^2) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed to measure the expression level of notch mRNA. The effect of SHya on the expression level of (A) Notch1 mRNA, (B) Notch2 mRNA, and (C) Notch3 mRNA. Each value is expressed as the mean ± SD. $^{**}P<0.01$ compared to control.

that SHya modulated Wnt signaling leading to beta-catenin activation.

In mouse keratinocytes, Notch1 is associated with the regulation of cell differentiation via p21, and Notch1 activation down-regulates Wnt4 expression [21]. Notch1 is required in keratinocyte differentiation and in the regulation of Wnt expression [24]. Activation of Notch receptors induces an increase in its own expression level by a positive feedback mechanism [24]. The expression of Notch1 in NHEKs incubated in SHya-coated dishes was decreased compared to that of the control. It was suggested that the decrease in Notch1 expression triggered by SHya induced the increase of Wnt4. The expression level of Notch3 mRNA in NHEKs incubated with SHya coating was increased, suggesting that SHya interacted with Notch3, particularly resulting in the modulation of Wnt expression (Fig. 7). Notch3 is required for the differentiation of vascular smooth muscle cells or T cells [23,24]. The role of Notch3 in keratinocyte differentiation triggered by elevation of the extracellular calcium-ion concentration may not

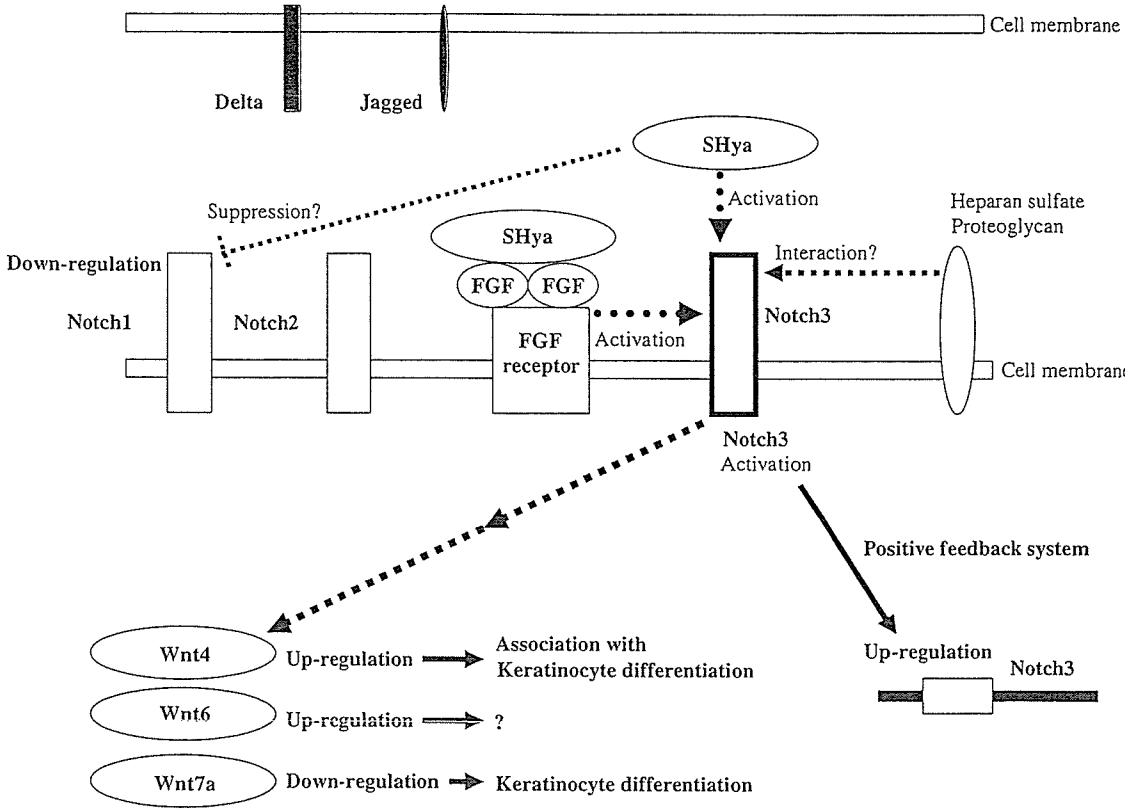

Fig. 7. Diagram of the effect of SHya on Wnt expression via Notch3.

be important, but Notch3 may be activated by SHya, leading to a modulation of intracellular signaling and enhancement of keratinocyte differentiation. Notch3 may be required for interaction with the sulfate groups of sulfated polysaccharides.

Notch1 activation stimulates p21 via the RBP-J kappa transcription factor, resulting in growth arrest, keratinocyte differentiation, or a decrease of Wnt4 expression [25]. Therefore, SHya might activate p21 via Notch3 activation, leading to the modulation of Wnt expression.

The differentiation of NHEKs incubated in SHya was better than that in Hya (data not shown). This suggests that the introduction of sulfate groups into Hya may be a key factor in the enhancement of keratinocyte differentiation.

Normal human dermal fibroblasts showed very low adhesiveness to Hya-coated surfaces because of the anionic surface of Hya [10,11]. However, NHEKs showed very high adhesiveness to SHya-coated surfaces. This suggested that the introduction of sulfate groups into Hya may change the property of its surface, resulting in a high adhesiveness. Sulfated polysaccharides such as heparin and heparan sulfate enhance the stabilities of some growth factors or adsorption of them by the cell membrane, resulting in the enhancement of cell differentiation [13]. The sulfate groups of SHya may bind to and stabilize cationic growth factors, and stabilized growth factors may neutralize the negative charge of the SHya surface, resulting in high cell attachment. Further, it was reported that fibroblast growth factors (FGF) receptors interact with the Notch signaling pathway [30,31]. Therefore, it was suggested that SHya binds and stabilizes FGF to activate FGF receptors leading to stimulation of Notch3 and intercellular signaling.

5. Conclusion

This study demonstrated that a SHya coating promoted keratinocyte differentiation triggered by an elevated extracellular calcium ion concentration. Furthermore, SHya modulated Wnt expressions and increased the expression level of Notch3 mRNA. These results suggest that Notch3 may be an important target for the regulation of cell differentiation. SHya may be a useful biomaterial to regulate Wnt signaling in tissue engineering. This study provides new information that clarifies the interaction between sulfate groups and Notch families. Studies are in progress to clarify the roles of these modulations of *Notch* and *Wnt* genes in keratinocyte differentiation.

Acknowledgments

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