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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

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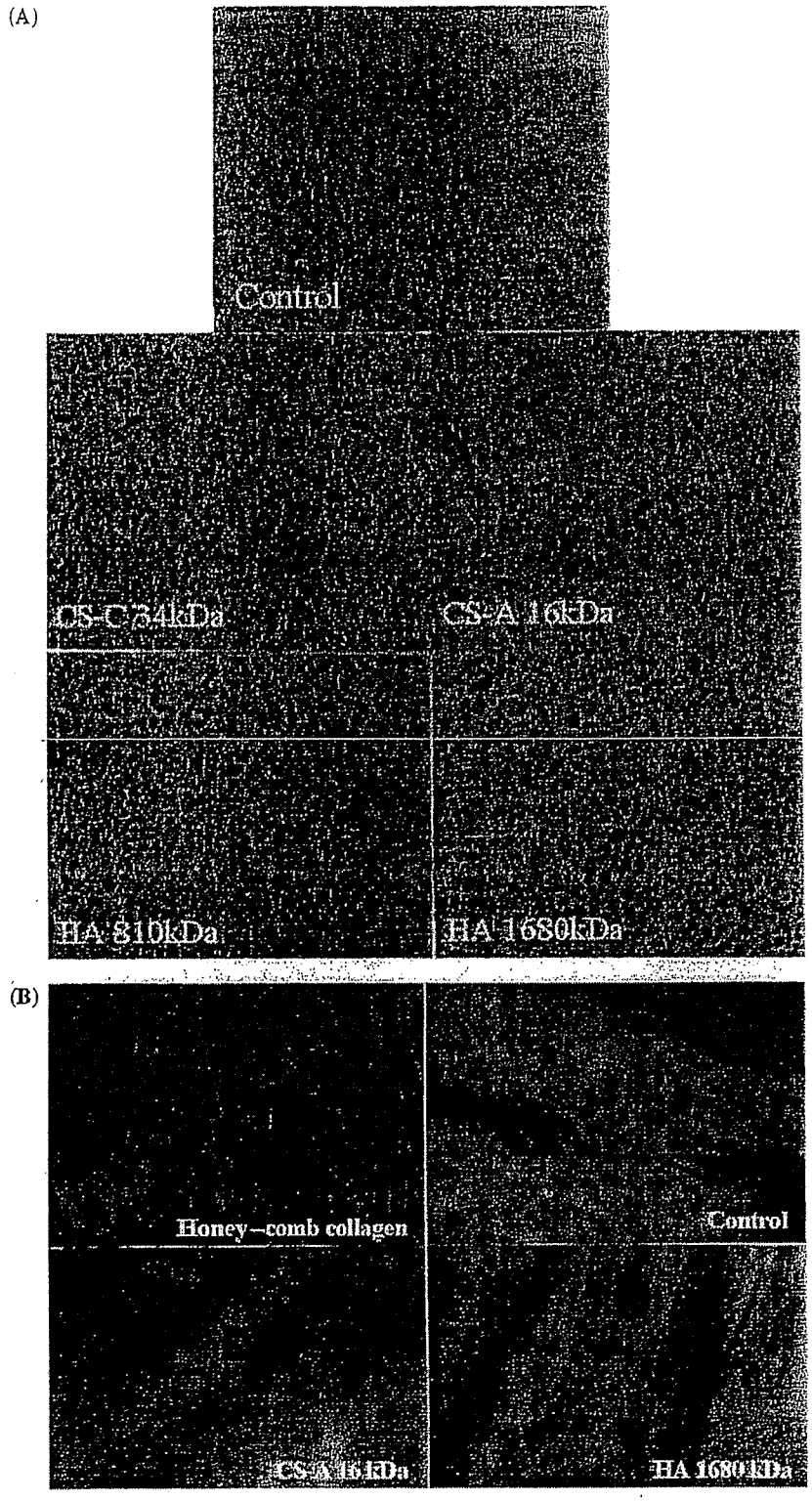

Figure 1. Light microscopic appearance of cultured HC in (A) micromass and (B) 3D honeycomb rotation conditions spotted as high density cultures treated with different molecular weights and molecular structures of HA and CS for 4 weeks (after alcian blue staining, original magnification ×200). [Color 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)].

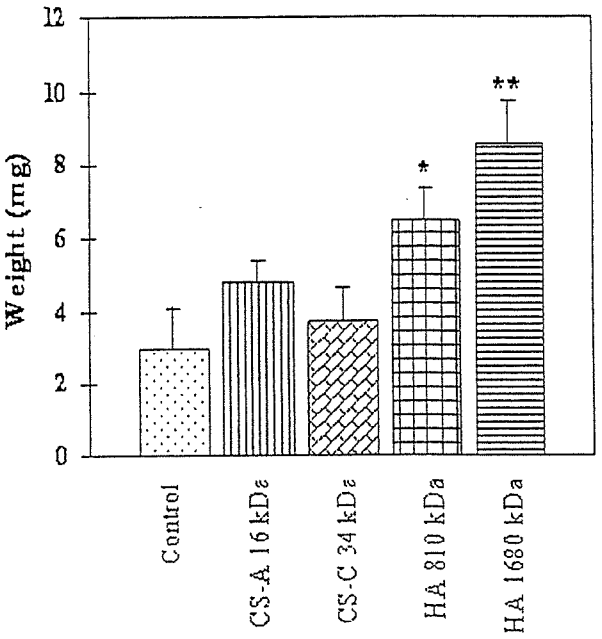

Figure 2. Wet weight of tissue constructs obtained by culturing HC on collagen honeycomb in rotation condition and treated with different molecular weights and structures of HA and CS for a period of 4 weeks. HA 810 kDa (*$p < 0.05$), and HA 1680 kDa (**$p < 0.01$) are significantly different compared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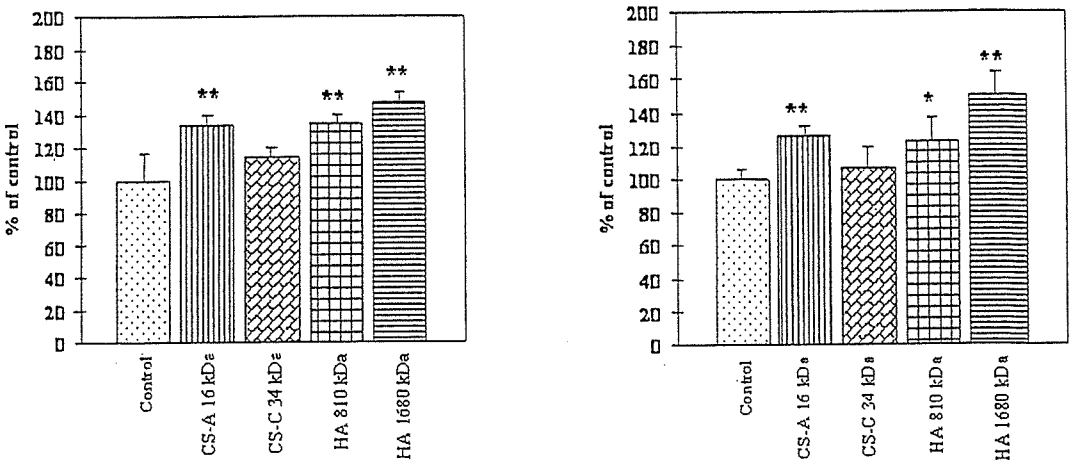

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Figure 3. Cell proliferation of HC estimated by alamar blue method. A: Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. CS-A 16 kDa, HA 810 kDa, and HA 1680 kDa are significantly different compared 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)]. 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)]. F5

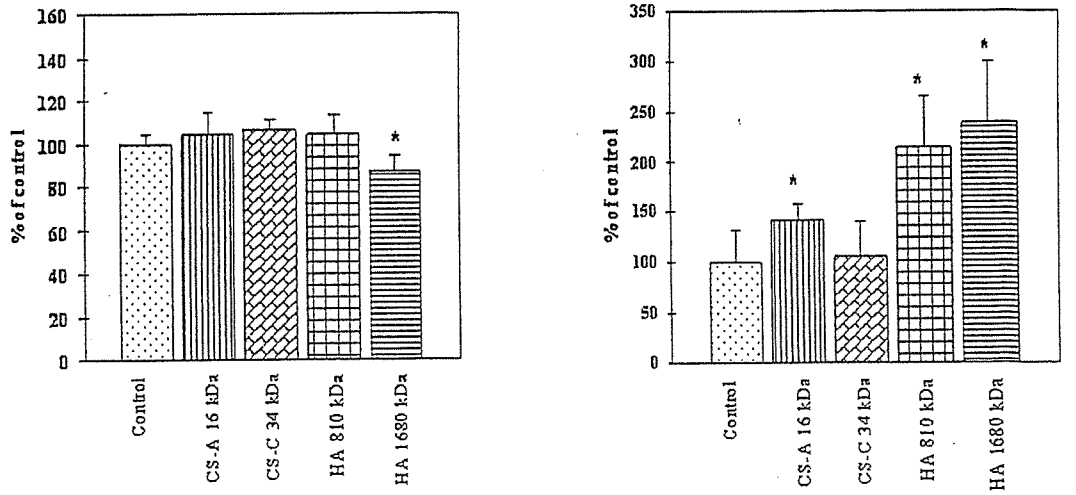

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Figure 4. Cell differentiation of HC estimated by alcian blue method. A: Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. HA 1680 kDa is significantly different compared with control (*$p < 0.05$). 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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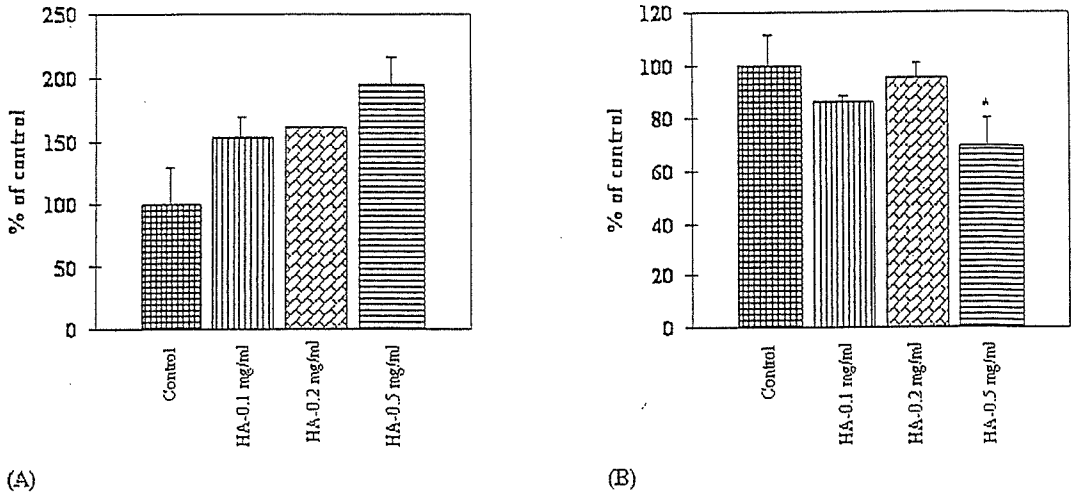

Figure 5. Chondrogenic effect of HA (1680 kDa) with different concentrations under micromass conditions for 4 weeks. A: As demonstrated by crystal violet staining, cell proliferation was increased with 0.1, 0.2, and 0.5 mg/mL of HA 1680 kDa respectively, compared with the control culture. B: Cell differentiation 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

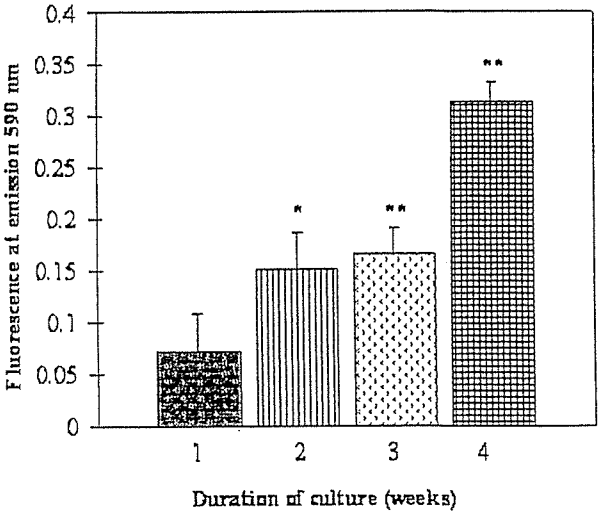

Figure 6. Time course of cell proliferation under micromass conditions for 4 weeks, estimated by crystal violet staining. Cell proliferation was significantly increased after 2 weeks ($*p < 0.05$), 3 weeks ($**p < 0.01$), and 4 weeks ($**p < 0.01$) of culture, compared with the chondrocytes cultured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

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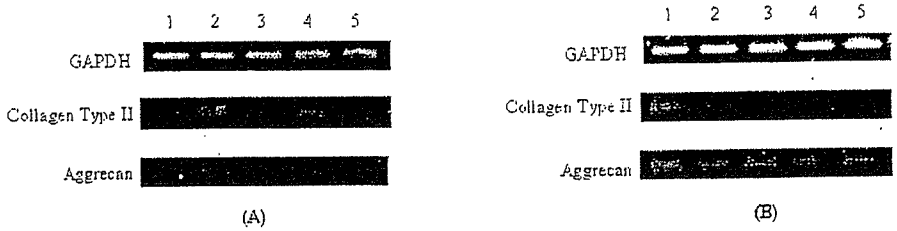

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Figure 7. A: Reverse transcription-polymerase chain reaction amplification of GAPDH, collagen type II, and aggrecan in cultures of HC with four different types of CS and HA for 4 weeks in micromass conditions. Lane 1, control; lane 2, CS-A 16 kDa; 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.

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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. F8

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

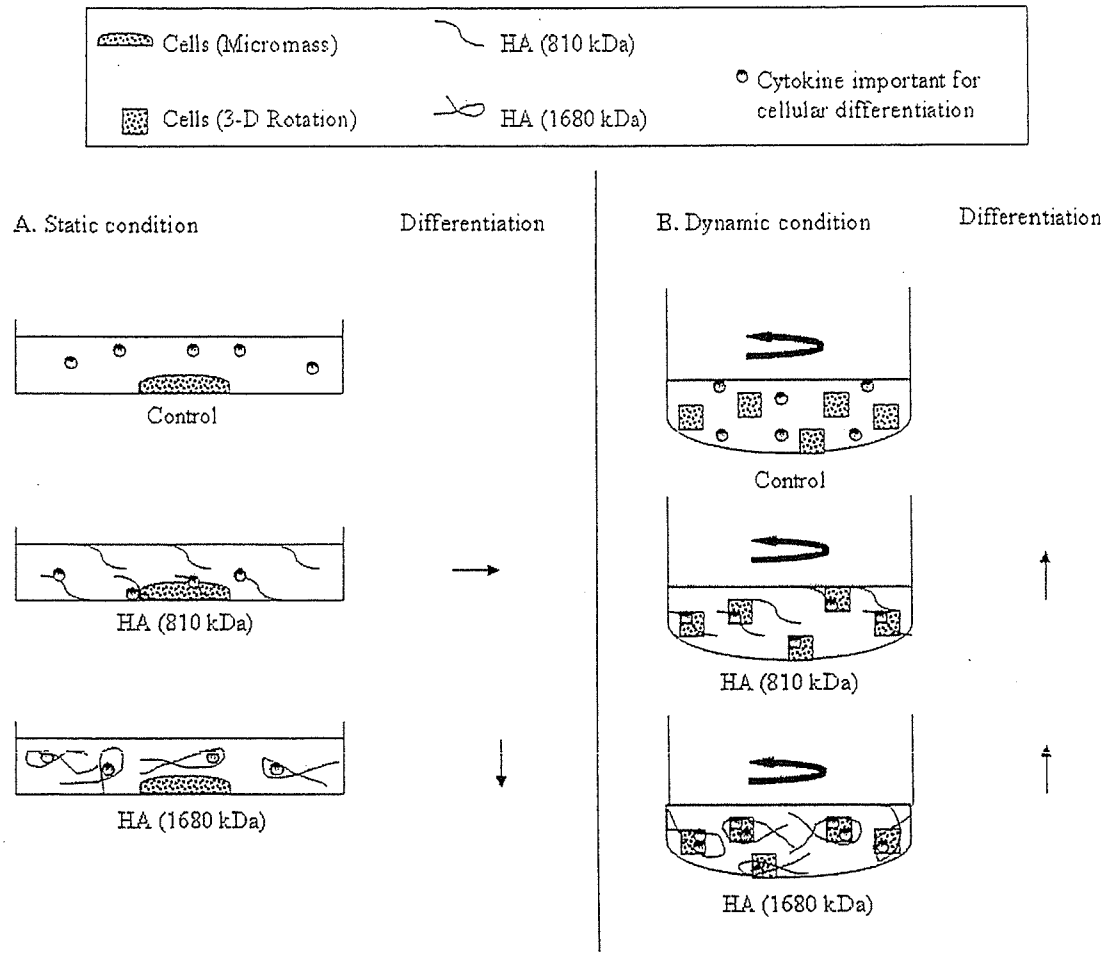

Figure 8. Schematic representation of the effects of different molecular weights of HA on the differentiation of HC. A: Static condition (micromass). B: Dynamic condition (rotation culture). → Omit ; Add ( )

(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

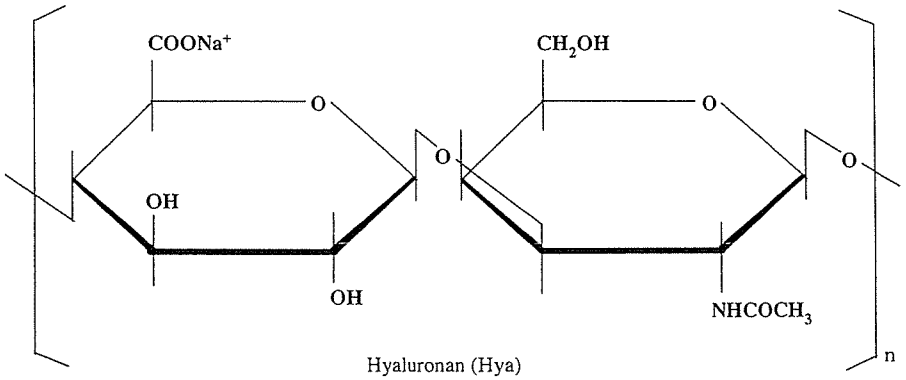
SHya was prepared by the method reported previously [4]. A solution of 2% Hya120 (molecular weight, 1.2×10^6) solution in *N*,*N*-dimethylformamide (DMF) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was mixed with trimethylamine (TMA)–SO_3 complex (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) 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

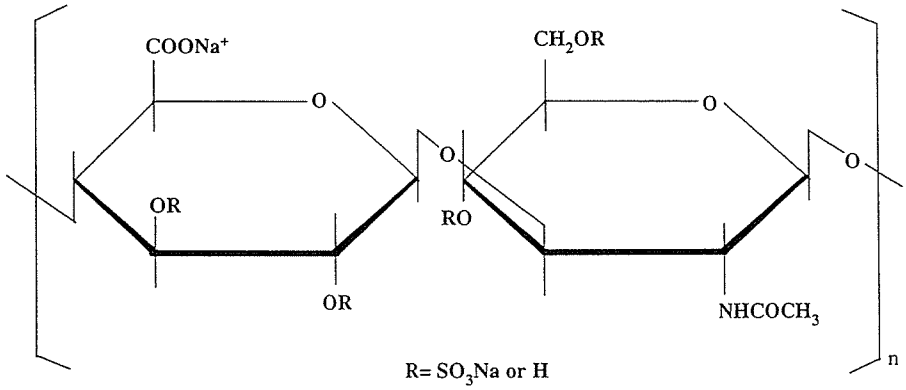
NHEKs isolated from neonatal human foreskins (Cambrex Bioscience, Walkersville, MD, USA) were cultured with K-110 Type II medium (Kyokuto, Tokyo, Japan) supplemented with 2% whole bovine pituitary extract, 50 IU penicillin G, 50 μg/ml streptomycin, and 0.03 mM $CaCl_2$ (low-calcium condition) at 37 °C 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.



Hyaluronan (Hya)



R= SO_3Na or H

Sulphated hyaluronan (SHya)

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

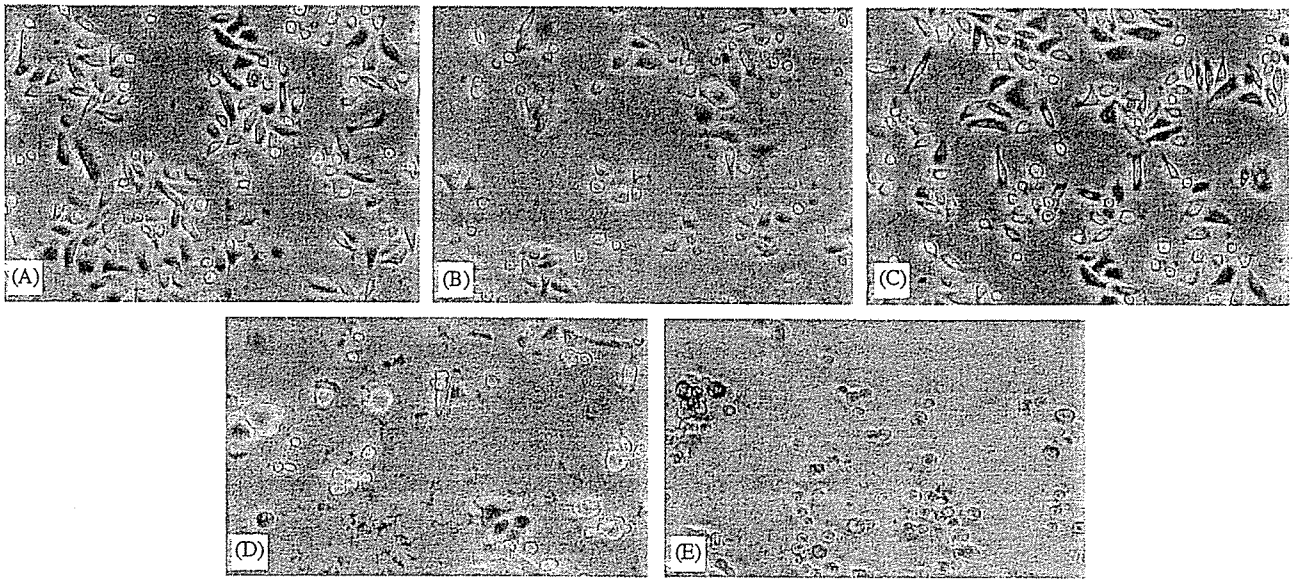


Fig. 2. The adhesion of NHEKs to SHya-coated surfaces. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya- and Hya-coated 24-well plates. Then NHEKs were incubated with non-coated (A), 0.4 mg/cm^2 SHya-coated (B), 0.8 mg/cm^2 SHya-coated (C), 0.4 mg/cm^2 Hya-coated (D), and 0.8 mg/cm^2 Hya-coated (E)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

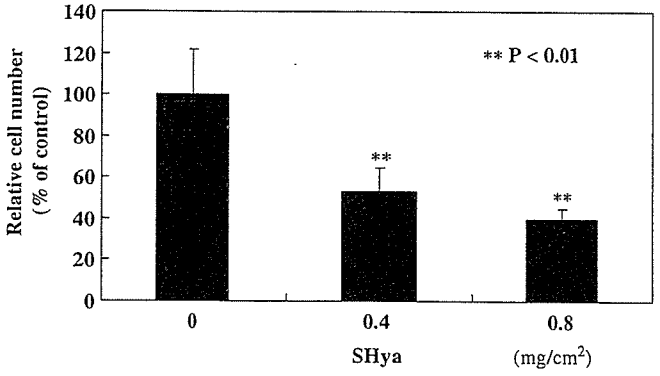


Fig. 3. Suppressive effect of SHya on keratinocyte proliferation. NHEKs were seeded at 1.0×10^4 cells/cm^2 densities onto non-coated (A), 0.4 mg/cm^2 SHya-coated (B), and 0.8 mg/cm^2 SHya-coated (C) 24-well plates and cultured for 5 days. Then numbers of NHEKs were determined by crystal violet assay. 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

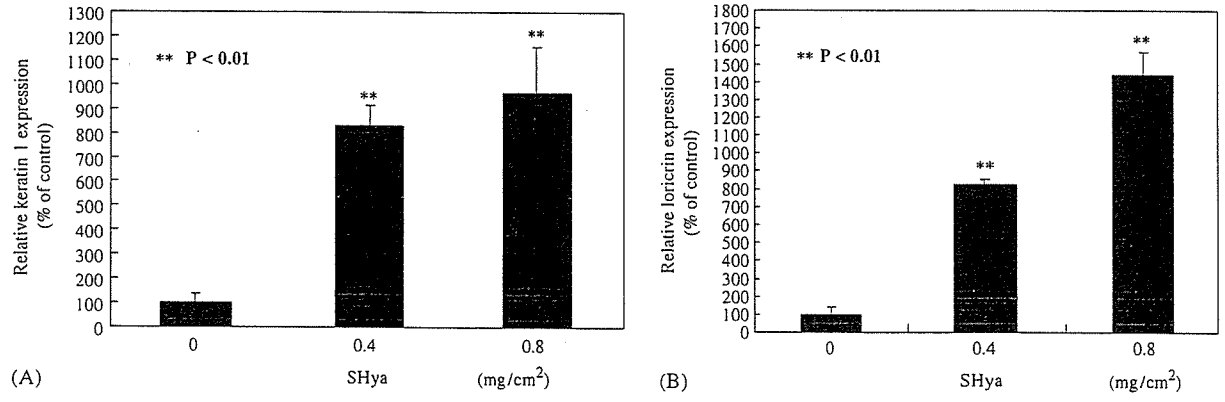


Fig. 4. The expression levels of keratin1 and loricrin mRNA of NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm^2) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed 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.

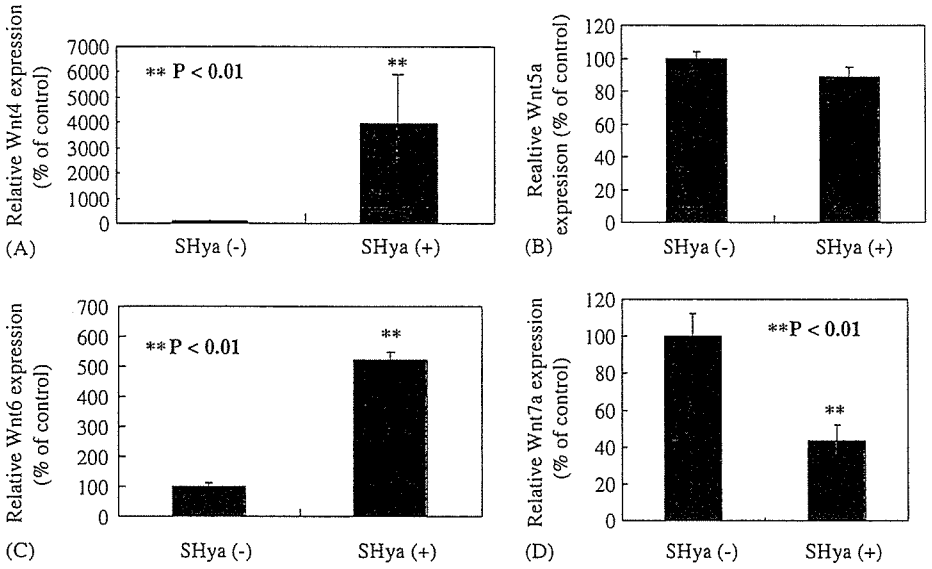


Fig. 5. The expression level of Wnt mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya-coated 60 mm dishes (0 and 0.8 mg/cm^2) and cultured in medium with 0.20 mM calcium for 20 h. Then RNA was extracted, and real-time RT-PCR was performed to determine the expression 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.

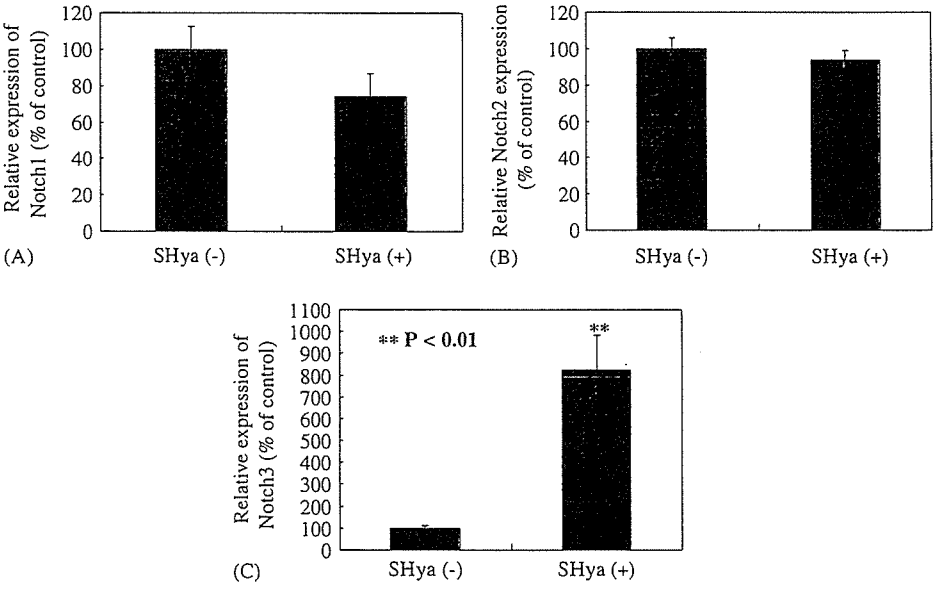


Fig. 6. The expression level of Notch mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm^2) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed to measure 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

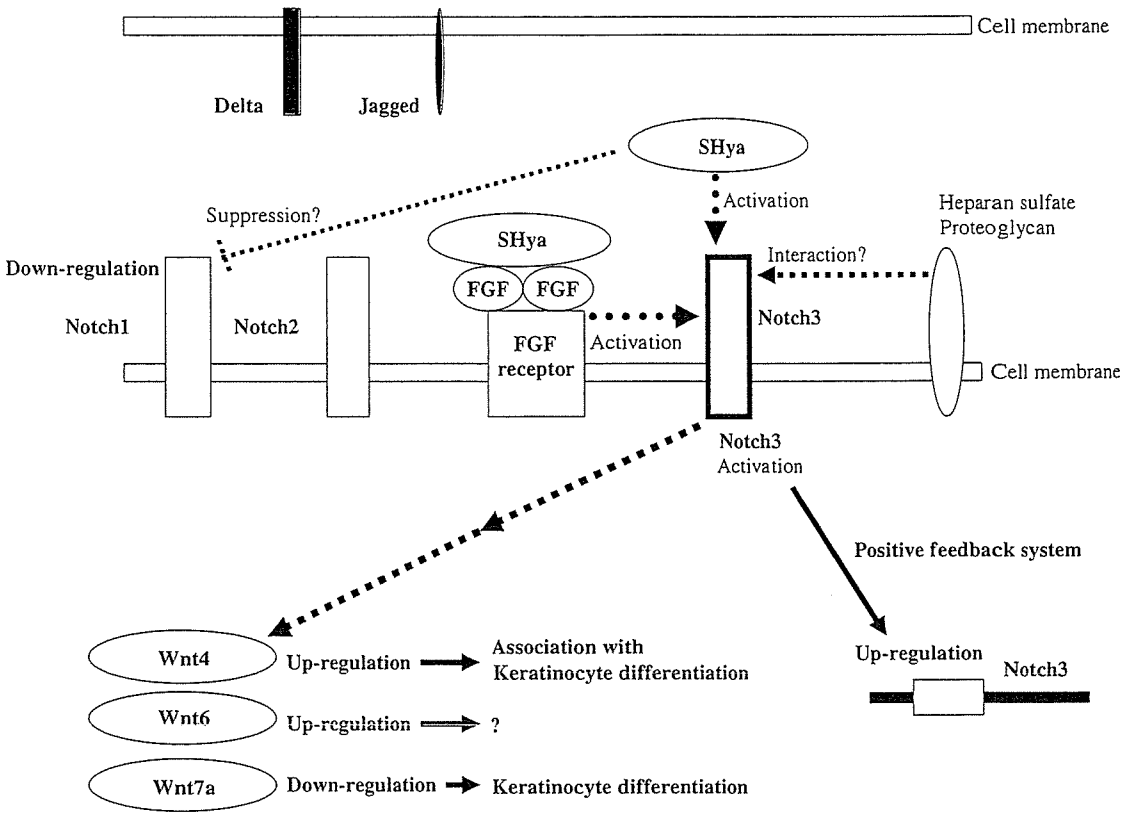


Fig. 7. Diagram of the effect of SHya on Wnt expression via Notch3.

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.

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A mouse strain difference in tumorigenesis induced by biodegradable polymers

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Abstract: The use of poly-L-lactic acid (PLLA) surgical implants for repair of bone fractures has gained popularity in the past decade. The aim of this study was to evaluate the *in vivo* effect of PLLA plates on subcutaneous tissue in two mouse strains, BALB/cJ and SJL/J, which have higher and lower tumorigenicity, respectively. Gap-junctional intercellular communication and protein expression of connexin 43 were significantly suppressed, whereas secretion of transforming growth factor-β1 and expression of extracellular matrix, insulin-like growth factor binding protein 3, and cysteine-rich intestinal protein 2 were significantly increased in PLLA-implanted BALB/cJ mice when compared with BALB/cJ controls. Finally, tumors were formed after implantation of cultured cells from the more-tumorigenic BALB/cJ, but not SJL/J, mice into nude mice. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 409–417, 2006

Key words: poly-L-lactic acid; gap-junctional intercellular communication; transforming growth factor-β1; connexin 43; nude mice

INTRODUCTION

The morphologic, chemical, and surface electrical characteristics of a biomaterial can influence the extent of the cellular response to an implant,[1,2] but host factors also contribute, so that an identical material implanted in different species[3,4] or at different anatomical locations[5,6] may elicit different degrees of response. Poly-L-lactic acid (PLLA) is a synthetic degradable polymer with good biocompatibility that is widely used clinically for surgical implants and as a bioabsorbable suture material.[7,8] Long-term implants of PLLA produced tumors in rats,[9] and adverse effects were also reported in other animal experiments.[10] All tumors are generally viewed as the result of disruption of the homeostatic regulation of the cell's ability to respond to extracellular signals, which triggers intracellular signal transduction abnormalities.[11] During the transition from the single-cell organism to the multicellular organism, many genes evolved to regulate these cellular functions. One of these genes is the gene coding for a membrane-associated protein channel (the gap junction).[12] Gap-junctional intercellular communication (GJIC) involves two hemichannels or connexons,[13] and each connexon is composed of six basic protein subunits named connexin (Cx), which allow the cell–cell transfer of small molecules. Approximately 20 connexins are known, and they are expressed in a cell- and development-specific manner.[14,15] GJIC also plays an important role in the maintenance of cell homeostasis and in the control of cell growth.[16] Thus, disruption of GJIC has been shown to contribute to the multi-step, multi-mechanism process of carcinogenesis.[17–19] Several tumor-promoting agents have been shown to restrict GJIC by phosphorylation of connexin proteins, such as connexin 43, which is essential in forming the gap junction channel.[20,21] Our previous study revealed that PLLA increased the secretion of transforming growth factor-β1 (TGF-β1), suppressed the mRNA expression of Cx 43, and inhibited GJIC in the early stage after implantation, thus promoting tumorigenesis in BALB/cJ mice.[22] We have hypothesized that the difference in tumorigenic potentials of PLLA is caused mainly by the different tumor-promoting activities of these biomaterials and that TGF-β1 might have an important role in PLLA-implanted BALB/cJ mice. Therefore, in our present experimental approach, we aimed to determine the novel effects of PLLA plates in two mouse strains, BALB/cJ and SJL/J, after long-term implantation. Among mouse strains, the former is a more tumorigenic strain when compared with the later.[23]

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Immune-deficient nude mice, which are highly susceptible to tumorigenicity, were also used in this experiment.

MATERIALS AND METHODS

Animals

Five-week-old female BALB/cJ and SJL/J, and five-week-old male BALB/cAnCrj-nu mice were purchased from Charles River (Japan) and maintained in the animal center according to the NIHS animal welfare guidelines. All mice were fed standard pellet diets and water *ad libitum* before and after PLLA implantation.

Implantation of PLLA

PLLA was obtained from Shimadzu Co. Ltd. as uniform sheets. The implants (size, 20 × 10 × 1 mm^3; Mw, 200,000) were sterilized using ethylene oxide gas prior to use. Sodium pentobarbital (4 mg/kg) was intraperitoneally administered to the mice. The dorsal skin was shaved and scrubbed with 70% alcohol. Using an aseptic technique, an incision of about 2 cm was made; a subcutaneous pocket was formed by blunt dissection away from the incision, and one piece of PLLA was placed in the pocket. The incision was closed with silk sutures. In both strains, controls were obtained by sham operation and subsequent subcutaneous pocket formation. Following surgery, the mice were housed in individual cages. After 10 months, mice from the implanted group were killed, implanted materials were excised, and subcutaneous tissues from the adjacent sites were collected for culture. At the same time, subcutaneous tissues were removed from the sites in the sham-operated controls that correlated with the implant sites. Similar experiments were also performed 1 month after PLLA implantation.[22]

Cell culture of subcutaneous tissues

The subcutaneous tissues were maintained in minimum essential medium (MEM) supplemented with 10% FBS in a 5% CO_2 atmosphere at 37°C.

Giemsa staining

When cells reached confluence in tissue culture dishes, they were fixed and stained with Giemsa solution. Cell morphology was determined under an inverted light microscope.

Western blot analysis

When cells had grown confluent in 60-mm tissue culture dishes, all cells were lysed directly in 100 μL 2% sodium dodecyl sulfate (SDS) gel loading buffer (50 m*M* Tris-HCl, pH 6.8, 100 m*M* 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). The protein concentration of the cleared lysate was measured using a micro-plate BCA protein assay (Pierce, Rockford, IL). Equivalent protein samples were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech UK, Buckinghamshire, UK), and Cx 43 protein was detected by anti-Cx 43 polyclonal antibodies (ZYMED Laboratories, San Francisco, CA). The membrane was soaked with Block Ace (Yukijirushi Nyugyo, Sapporo, Japan), reacted with the anti-Cx 43 polyclonal antibodies for 1 h, and after washes with phosphate-buffered saline (PBS) containing 0.1% Tween20, reacted with the secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase for 1 h. After several washes with PBS-Tween20, the membrane was detected with the ECL detection system (Amersham Pharmacia Biotech UK).

Scrape-loading and dye transfer assay

The scrape-loading and dye transfer (SLDT) technique was performed by the method of El-Fouly et al.[24] Confluent monolayer cells in 35-mm culture dishes were used. After rinsing with Ca^{2+}, Mg^{2+} PBS(+), cell dishes were loaded with 0.1% Lucifer Yellow (Molecular Probes, Eugene, OR) in PBS(+) solution and were scraped immediately with a sharp blade. After incubation for 5 min at 37°C, cells were washed three times with PBS(+), and the extent of dye transfer was monitored using a fluorescence microscope equipped with a type UFX-DXII CCD camera and a super high-pressure mercury lamp power supply (Nikon, Tokyo, Japan).

Enzyme-linked immunosorbent assay

Cells were seeded onto 60-mm dishes. The conditioned medium was collected after centrifugation at 1000 rpm for 2 min. The TGF-β1 levels of the media were measured with commercially available enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN).

DNA microarray analysis

At least 10^7 cells were harvested and frozen in liquid nitrogen. Total RNA was extracted, purified, and assessed for yield and purity, and cDNA probes were synthesized with the Atlas™ Pure Total RNA Labeling System (Clontech) according to the manufacturer's instructions. Hybridization of the ^{33}P-labeled probes to the Atlas Array of Mouse Cancer 1.2 k Array (Clontec 7858–1), on which 1176 cDNAs

of cancer-related genes were spotted, was performed with Atlas™ cDNA Expression Arrays according to the manufacturer's instructions. The phosphor images of hybridized arrays were analyzed with AtlasImage™ (Clontech). Genes that were up- or downregulated more than fivefold relative to the negative controls are discussed.

Determination of tumorigenicity in nude mice

Cultured cells were harvested by trypsinization, and 2×10^6 washed cells suspended in 0.2 mL of PBS were inoculated at a single subcutaneous site into 6–8-week-old nude mice. All mice were examined regularly for the development of tumor.

Soft agar assay

Approximately 100,000 cells per well from each clone were seeded in 2 mL of 0.3% soft agar in culture medium on a solidified basal layer in 6-well tissue culture plates. The plates were cultured for 4 weeks and then stained with *p*-iodotetrazolium violet for 48 h before counting.

Statistical analysis

Student *t* tests were used to assess whether differences observed between the implanted and control samples were statically significant. For comparison of groups of means, one-way analysis of variance was carried out. When significant differences were found, Tukey's pairwise comparisons were used to investigate the nature of the difference. The confidence level was set at 95% for all tests. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± SD.

RESULTS

Giemsa staining

Cells with different morphologies formed a slightly crisscrossed pattern in the BALB/cJ control group, whereas cells in the implanted groups of BALB/cJ showed a markedly crisscrossed pattern. The cells were extensively piled up, which decreased contact inhibition, under inverted light microscopy observation and Giemsa staining [Fig. 1(A,B)]. In contrast, the cells of the SJL/J group formed a parallel, flat, confluent monolayer that maintained contact inhibition [Fig. 1(C,D)].

Western blot analysis

We examined the protein expression of the connexin 43 gene and found that the total protein level was significantly decreased in PLLA-implanted BALB/cJ mice when compared with that in BALB/cJ controls (Fig. 2). However, protein expression was decreased in both control and PLLA-implanted groups in SJL/J mice (Fig. 2).

SLDT assay

The SLDT assay was used to assess functional GJIC. GJIC was significantly inhibited in PLLA-implanted BALB/cJ mice when compared with that in BALB/cJ controls (Fig. 3). A significant difference was also observed between the two strains of mice in that the GJIC was lower in SJL/J than in BALB/cJ group (Fig. 3).

ELISA

The secretion of TGF-β1 was significantly increased in PLLA-implanted BALB/cJ subcutaneous tissues in comparison with that from BALB/cJ control mice. On the contrary, secretion of TGF-β1 tended to decrease in the SJL/J implanted mice when compared with that in SJL/J control mice (Fig. 4).

DNA microarray analysis of the four kinds of cells

Expression of the major ECM [fibronectin 1, procollagen VIIIα 1, and osteopontin precursor (OPN)] proteins [Fig. 5(A–C)], insulin-like growth factor binding protein (IGFBP) 3 [Fig. 5(D)], and cysteine-rich intestinal protein 2 (CRIP 2) [Fig. 5(E)] were increased in the PLLA-implanted BALB/cJ mouse cells when compared with that in BALB/cJ control mouse cells. No such difference was observed between SJL/J implanted and control mouse cells.

Tumorigenicity in nude mice

No tumor was formed in PBS(−) injected nude mice [Fig. 6(A)]. Rapid growth of large tumors was observed in nude mice within 2 weeks of injection of cultured cells from PLLA-implanted BALB/cJ mice [Fig. 6(B,C,E,F)]. Nude mice injected with HeLa cells, which served as positive controls, showed slower growth of tumor 4 weeks after cell injection [Fig. 6(D,G)].