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

Nasreen Banu, Toshie Tsuchiya
Division of Medical Devices, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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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 80A: 257–267, 2007

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 (3-D) hyaline cartilage, although production of hyaline cartilage remains a challenge. Different non-biodegradable 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 extra-cellular ground substance. By interacting with other matrix molecules, HA provides stability and elasticity to the extra-cellular matrix (ECM).[28–31] Among many biochemical regulators of articular cartilage, HA of animal origin plays an important role in maintaining the articular chondrocyte morphology and prolifera-

Correspondence to: T. Tsuchiya Ph.D.; e-mail: tsuchiya@nihs.go.jp
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tion[32] and it enhanced proliferation, as well as chondroitin sulfate (CS) synthesis, of rabbit articular cartilage.[33]

CS is an important ECM 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 3-D 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 3-D 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 3-D 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 3-D 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 3-D 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

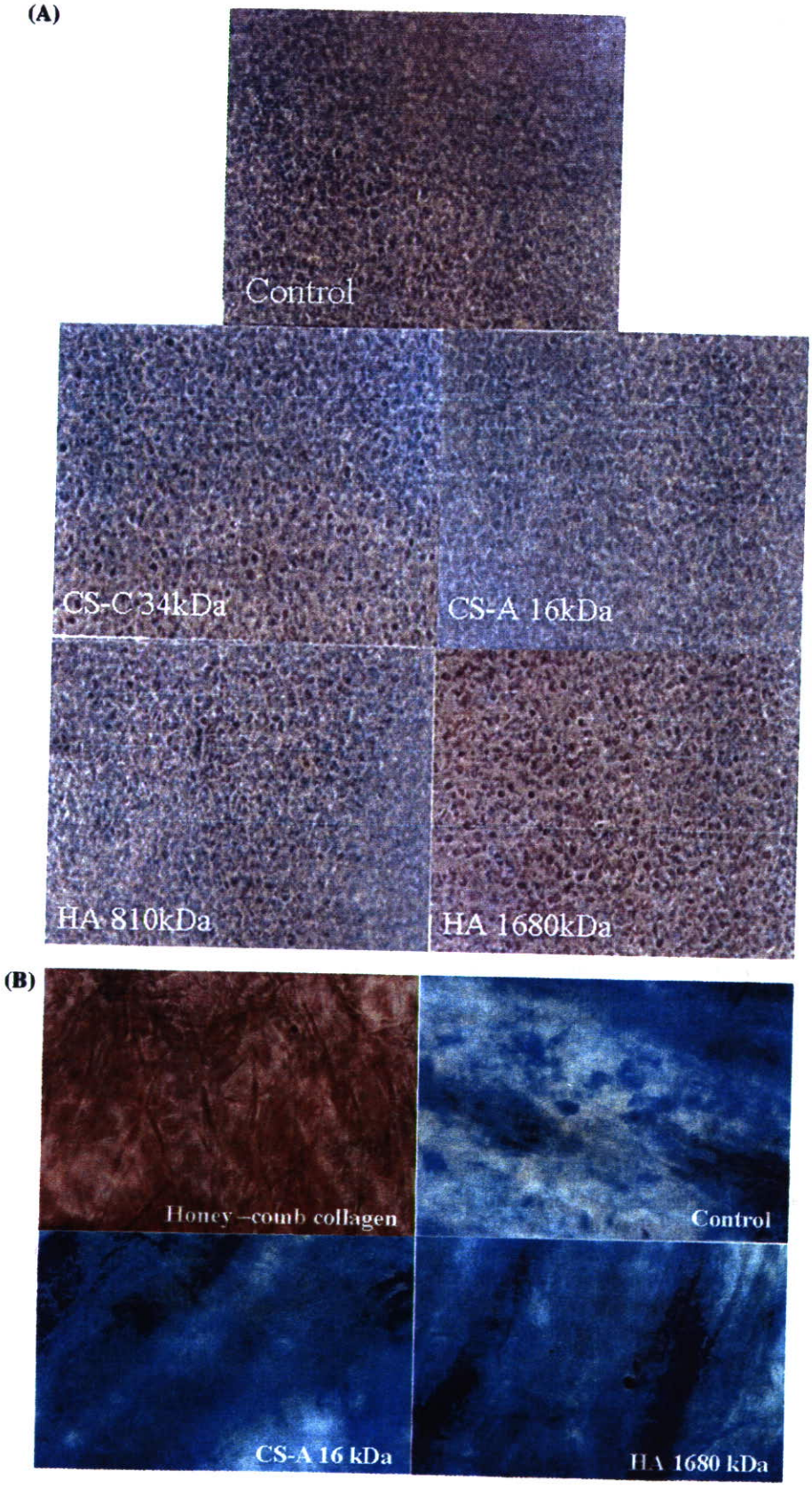

Figure 1. Light microscopic appearance of cultured HC in (A) micromass and (B) 3-D honeycomb rotation conditions spotted as high density cultures treated with different molecular weights and molecular structures of HA and CS for 4 weeks (after alcian blue staining, original magnification ×200).

samples were statistically 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. 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 3-D 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).

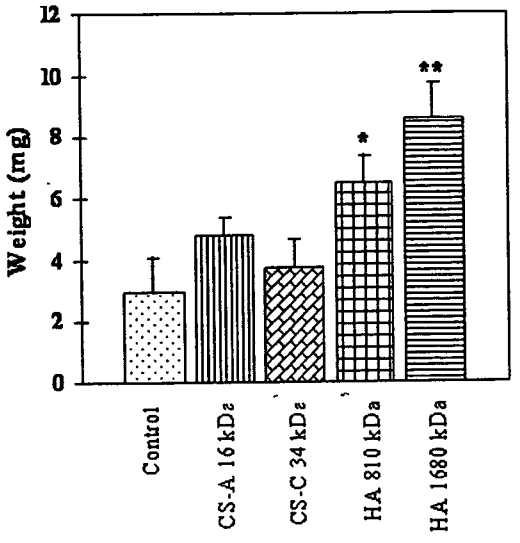

Figure 2. Wet weight of tissue constructs obtained by culturing HC on collagen honeycomb in rotation condition and treated with different molecular weights and structures of HA and CS for a period of 4 weeks. HA 810 kDa (*$p < 0.05$), and HA 1680 kDa (**$p < 0.01$) are significantly different compared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

Cell 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)].

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)].

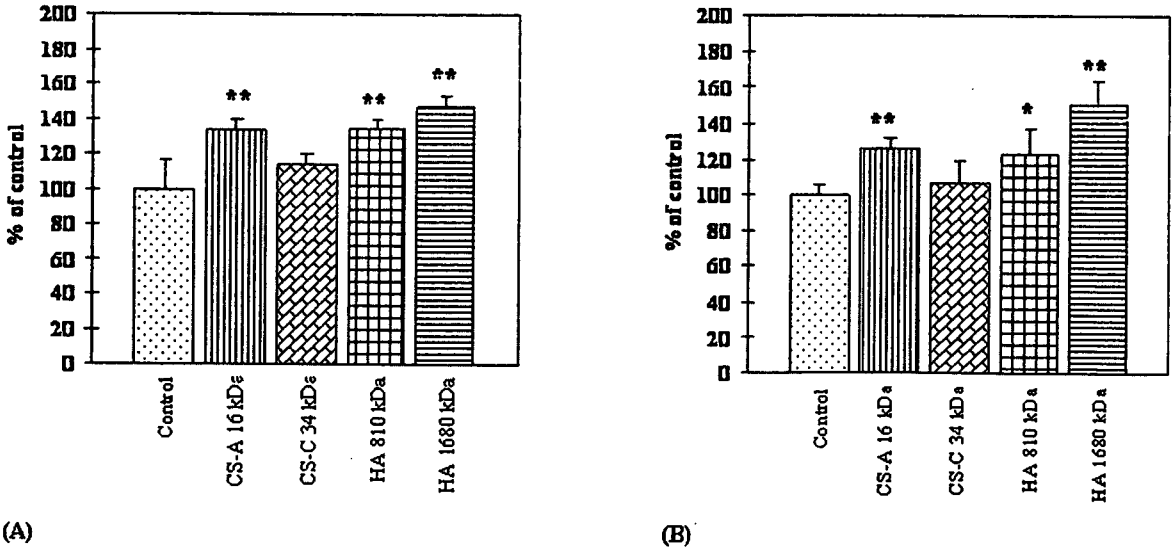

Figure 3. Cell proliferation of HC estimated by alamar blue method. (A) Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. CS-A 16 kDa, HA 810 kDa, and HA 1680 kDa are significantly different compared with control (**$p < 0.01$). (B) In rotation 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.

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)].

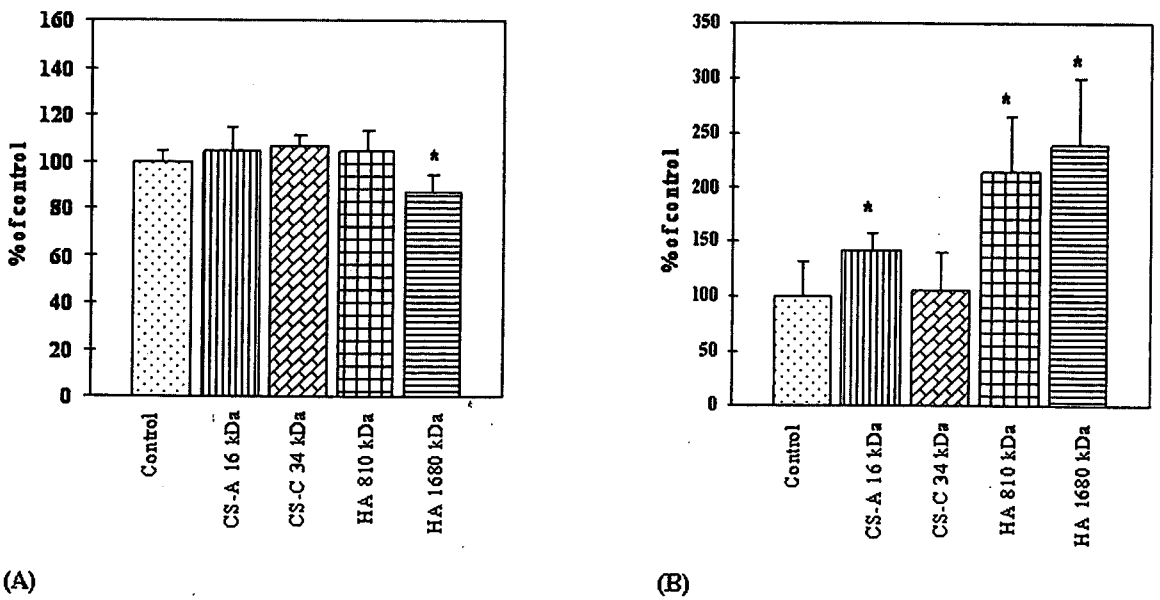

Figure 4. Cell differentiation of HC estimated by alcian blue method. (A) Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. HA 1680 kDa is significantly different compared with control (*$p < 0.05$). (B) In rotation conditions, cultures were treated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

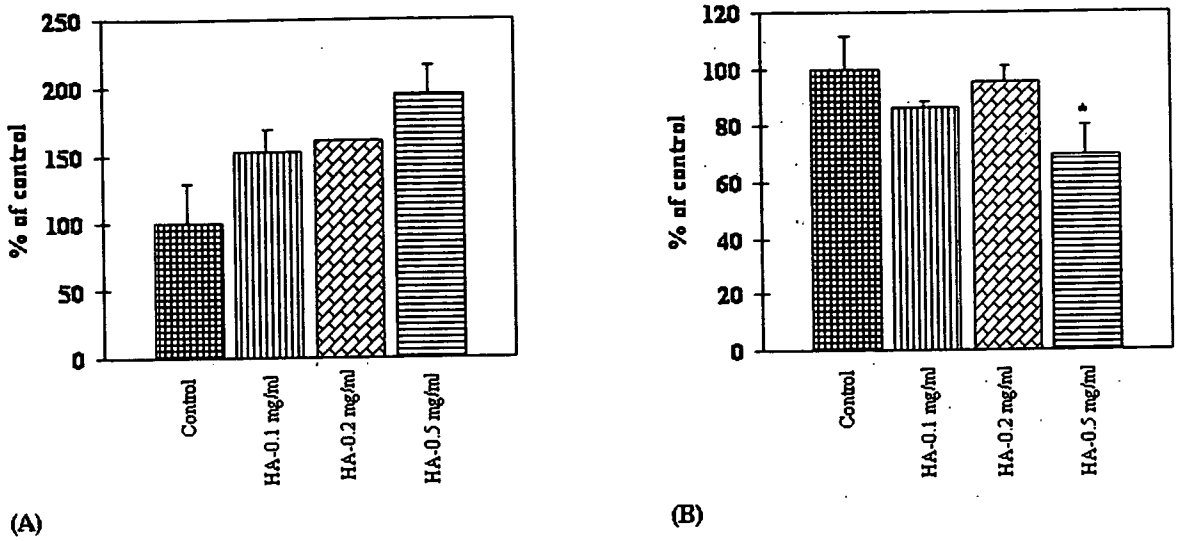

Figure 5. Chondrogenic effect of HA (1680 kDa) with different concentrations under micromass conditions for 4 weeks. (A) As demonstrated by crystal violet staining, cell proliferation was increased with 0.1, 0.2, and 0.5 mg/mL of HA 1680 kDa respectively, compared with the control culture. (B) Cell differentiation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

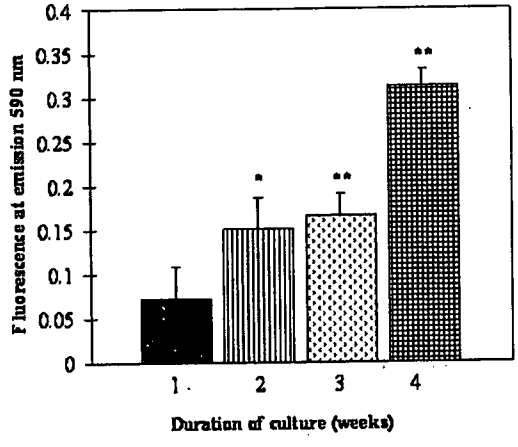

Figure 6. Time course of cell proliferation under micromass conditions for 4 weeks, estimated by crystal violet staining. Cell proliferation was significantly increased after 2 weeks (*$p < 0.05$), 3 weeks (**$p < 0.01$), and 4 weeks (**$p < 0.01$) of culture, compared with the chondrocytes cultured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

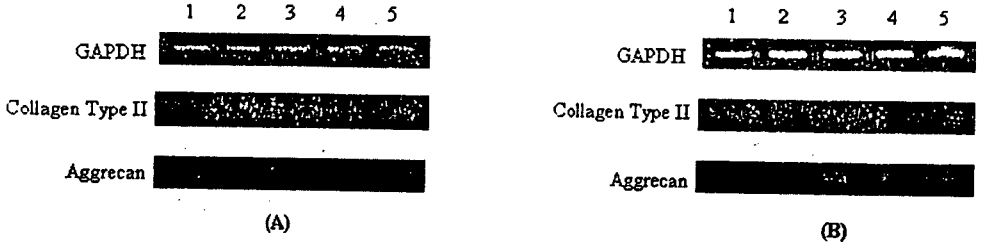

Figure 7. (A) Reverse transcription-polymerase chain reaction amplification of GAPDH, collagen type II, and aggrecan in cultures of HC with four different types of CS and HA for 4 weeks in micromass conditions. Lane 1, control; lane 2, CS-A 16 kDa; lane 3, CS-C 34 kDa; lane 4, HA 810 kDa; and lane 5, 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 3-D 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

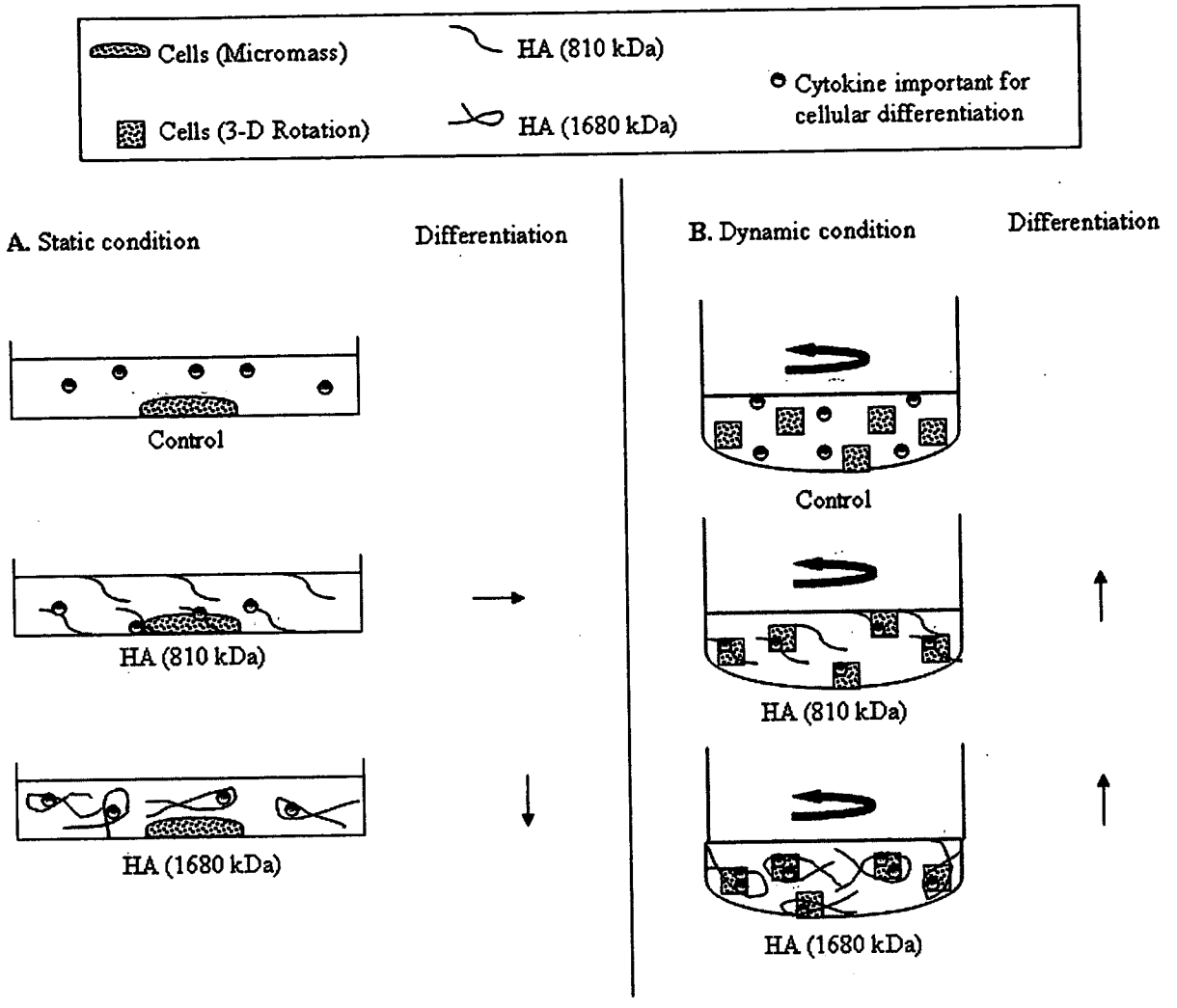

Figure 8. Schematic representation of the effects of different molecular weights of HA on the differentiation of HC. (A) Static condition (micromass). (B) Dynamic condition (rotation culture).

(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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AQ2

Enhancing action by sulfated hyaluronan on connexin-26, -32, and -43 gene expressions during the culture of normal human astrocytes

Saifuddin Ahmed, Toshie Tsuchiya, Misao Nagahata-Ishiguro, Rumi Sawada, Nasreen Banu, Tsutomu Nagira
Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-Ku, Tokyo 158-8501, Japan

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Abstract: Astrocyte proliferation is strictly controlled during development and in the adult nervous system. In this study, we examined the role of sulfated hyaluronan (SHya) in the proliferation and differentiation of normal human astrocytes (NHA). Cells were cultured with different concentrations of SHya for 7 days, and the number of viable cells and the presence of neural cell-specific genes were determined to assess their proliferation and development, respectively. With SHya, cell proliferation increased nonsignificantly. Furthermore, remarkable enhancing action by SHya on connexin-26, -32, and -43 gene expressions were observed during the culture of NHA. It has been suggested that a part of NHA have neural precursor activity that gives rise to astrocytes itself, oligodendrocytes and neurons. Our results clearly demonstrated that the expression of specific genes for neural precursor cells, astrocytes, neurons, and oligodendrocytes was significantly increased to 50 μg/mL in SHya-treated cultures when compared with that of the control culture. These findings suggest that SHya plays an important role in the proliferation and differentiation of NHA and in the production of a novel material for tissue engineering. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 00A: 000–000, 2008

Key words: astrocyte; sulfated hyaluronan; cell proliferation; real-time PCR; gene expression

INTRODUCTION

Telencephalic neuroepithelial cells contain neural precursors that give rise to the neuronal lineage and the glial lineage, which includes astrocytes and oligodendrocytes.[1,2] Astrocyte proliferation is strictly controlled during development and in the adult nervous system. In all regions of the central nervous system (CNS), astrocytes are comprehensively coupled by gap junctions.[3] Gap-junctional intercellular communication (GJIC) occurs through clusters of intercellular channels that directly connect the cytoplasm of adjacent cells, allowing selective passage of ions and small molecules between coupled cells.[4,5] GJIC involves two hemichannels or connexons,[6] and each connexon is composed of six basic protein subunits named connexin (Cx) that allow the cell–cell transfer of small molecules. Approximately 20 connexins are known, and they are expressed in a cell- and development-specific manner.[7,8] Among them, Cx26, Cx30, and Cx43 are expressed in astrocytes, and Cx32 and Cx47 are expressed in oligodendrocytes.[9]

Because of its important biological activities, hyaluronan (Hya) has been widely used in medical practice. Hya is a negatively charged glycosaminoglycan (GAG) that facilitates cell migration, adhesion, proliferation, and tissue repair. By interacting with other matrix molecules, Hya contributes stability and elasticity to the extra-cellular matrix (ECM). It also plays the main structural role in the formation of the brain ECM.[10] Recently, hyaluronan synthesized with varying degrees of sulfation was reported to affect cell aggregation, proliferation, and differentiation of rat calvarial osteoblasts.[11] In this study, we investigated the effect of sulfated hyaluronan (SHya) on the cell function of normal human astrocytes (NHA).

Correspondence to: T. Tsuchiya; e-mail: tsuchiya@nihs.go.jp
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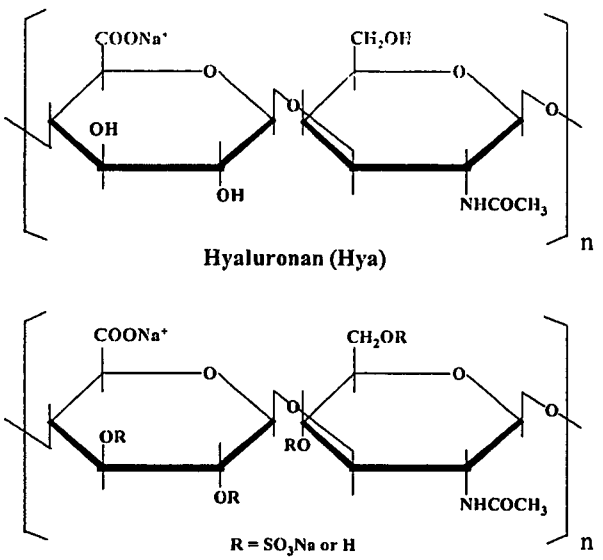

Figure 1. Structure of Hya and SHya. SHya is composed of Hya and a sulfate group. The molecular weight of SHya is 2.0×10^4, and the degree of substitution of SHya is 0.6.

MATERIALS AND METHODS

SHya preparation

SHya was synthesized by the method reported earlier (Fig. 1).[11] The molecular weight of SHya was 2.0×10^4, and the degree of substitution (D.S.) of SHya was 0.6, as determined by the chelate titration method.[12] Briefly, 2% Hya120 (molecular weight, 1.2×10^6) solution in *N,N*-dimethylformamide (DMF) (Wako Pure Chemical Industries, Osaka, Japan) was mixed with trimethylamine (TMA)-SO_3 complex (Aldrich Chemical, Milwaukee, WI) and stirred at 60°C for 24 h. The reaction mixture was then diluted, neutralized, and precipitated by adding acetone (Wako Pure Chemical Industries). The precipitate was dissolved in distilled water and dialyzed against distilled water. Moreover, the effectiveness of sulfation was also demonstrated by FTIR 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. Stock solutions of SHya were made directly in ABM medium (Cambrex Bio Science, Walkersville, MD) supplemented with 5% FCS, and recombinant human epidermal growth factor.

Astrocyte cell culture

NHA (Cambrex Bio Science) was maintained in ABM medium supplemented with 5% FCS, and recombinant human epidermal growth factor, and cultured in a humidified atmosphere of 5% CO_2 under 95% air at 37°C.

Giemsa staining

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

MTT assay for cell proliferation

NHA was seeded into 24-well plates for MTT assay at a density of 1×10^4/well in ABM medium supplemented with 5% FCS, recombinant human epidermal growth factor, and cultured in a humidified atmosphere of 5% CO_2 under 95% air at 37°C. After 1-week culture with different concentrations of SHya (10 or 50 μg/mL), the viability of NHA cells was determined by MTT assay. TetraColor ONE (Seikagaku Kogyo, Tokyo, Japan) was used to measure changes in cell numbers. This assay measures the activity of the enzyme in mitochondria for counting living cells. The medium was replaced with 300 μL of fresh medium containing 6 μL TetraColor ONE reagents. After 2 h, samples were measured in a microplate reader.

Scrape loading and dye transfer assay

The scrape loading and dye transfer (SLDT) technique was performed by the method of El-Fouly et al.[13] Confluent monolayer cells in 35-mm culture dishes were rinsed with Ca^{2+}, Mg^{2+} phosphate-buffered saline [PBS(+)], and the cell dishes were loaded with 0.1% Lucifer Yellow (Molecular Probes, Eugene, OR) in PBS(+) solution and immediately scraped with a sharp blade. After incubation for 5 min at 37°C, cells were washed four 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).

Expression of gap junctional and neural cell marker genes

For quantitative RT-PCR, NHA was seeded into 12-well plates at a density of 2×10^4 cells/well in ABM medium (Cambrex Bio Science) supplemented with 5% FCS, and recombinant human epidermal growth factor, and cultured in a humidified atmosphere of 5% CO_2 under 95% air at 37°C. After 1-week cell culture with 10 or 50 μg/mL SHya, 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). Aliquots of the cDNA (1/20) 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 mixture including 1 μL of RT reaction mixture, 2 μL Light Cycler-Fast Start Reaction Mix SYBR Green 1 (Roche), 0.5 μ*M* each primer, and 3 m*M* $MgCl_2$. The PCR program consisted of 40 cycles of 8 s at 94°C, 5 s at 65°C, and 10 s at 72°C. Primer sequences for amplification were

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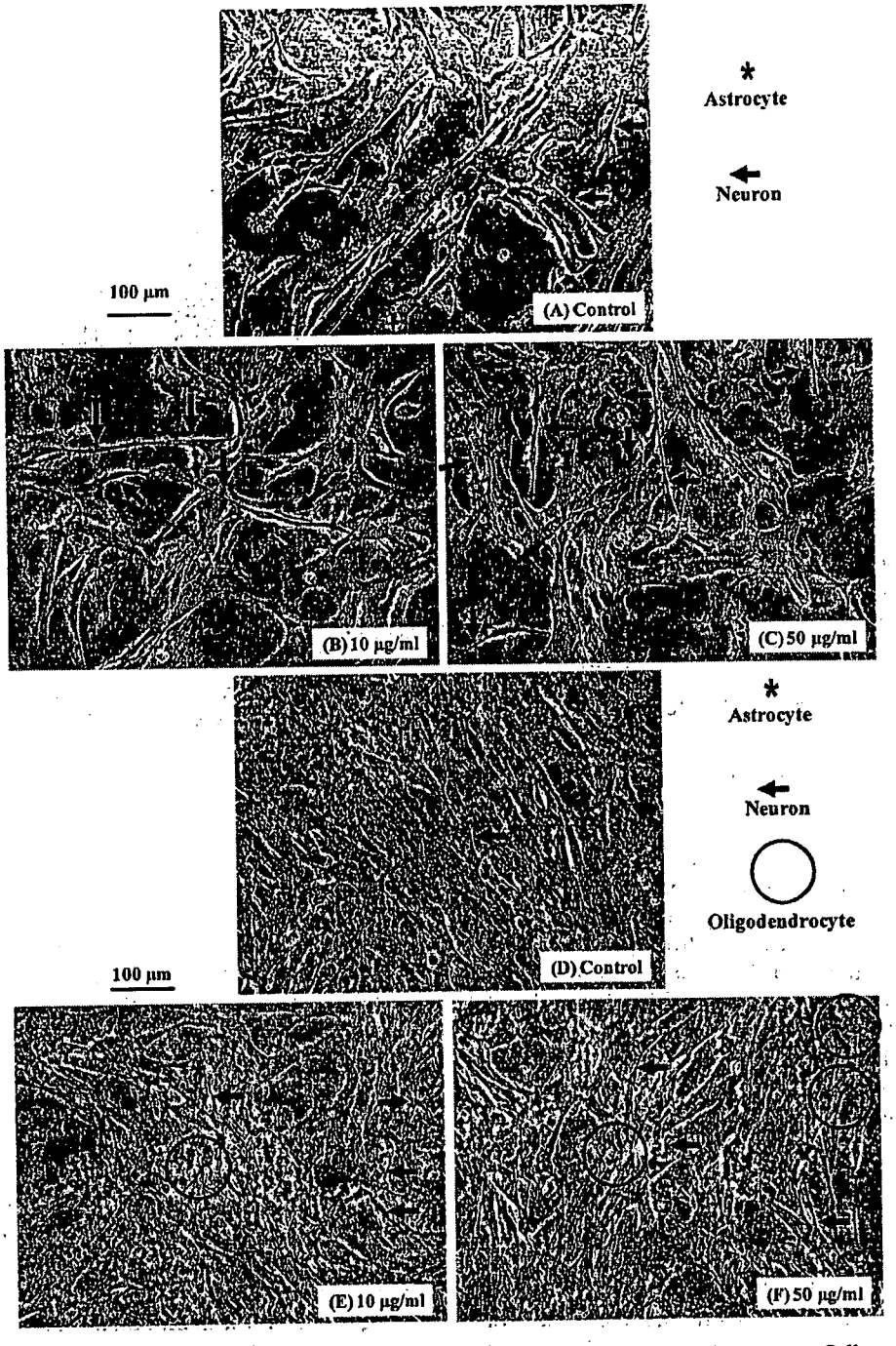

Figure 2. NHA cell morphology. Cultures were cultured with SHya in different concentrations. Cells were observed by inverted light microscopy observation and Giemsa staining. After 3 days of culture, some cells looked like the branches or spikes extending out from the cell body (neuron) in control (A) and 10 μg/mL (B) of SHya-treated cultures, and the neurons were elongated and increased in number in 50 μg/mL (C) of SHya-treated cultures. After 1 week, in addition to neuron, some cells appeared like the sparse branches of a tree (oligodendrocyte) with 10 μg/mL (E) and 50 μg/mL (F) of SHya. The number of oligodendrocytes was increased at 50 μg/mL of SHya-treated cultures, compared with the control (D) culture.

5′-GGGCTAATTACAGTGCAG-3′ and 5′-CATGTCCAG CAGCTAGTT-3′ for Cx43, 5′-ATAGACAGCATGAGAGG GAT-3′ and 5′-AGACAGGCATAGAATTAGGC-3′ for Cx26, 5′-CTTCCTTCCCTGGCTACTTC-3′ and 5′-CATCCC ATCTCTTGTACCCA-3′ for Cx32, 5′-GAGATCAGAGCCC AGGATGCT-3′ and 5′-CTGAGGGGTGGTGCCAAGGAG-3′

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for nestin, 5′-TCCGCTGCTCGCCGCTCCTAC-3′ and 5′-TCAT CTCTGCCCGCTCACTGG-3′ for glial fibrillary acidic protein (GFAP), 5′-CACTTCCTCCTCCTCCACGAC-3′ and 5′-GTCCATGGCCAGGTTCAGGTC-3′ for oligodendrocyte transcription factor 1 (OLIG1), 5′-CTAAGGAGGA GATTGGACAGG-3′ and 5′-AGTGGTGGCAGTGATTTCA GT-3′ for Nurr-1, and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for GAPDH. The RNA preparation and RT-PCR in this study were performed in triplicate.

Statistical analysis

Student's *t*-test was used to assess whether differences observed between the SHya-supplemented 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 comparison was 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 mean ± SD.

RESULTS AND DISCUSSION

NHA was cultured with different concentrations (10 and 50 μg/mL) of SHya. After 3 days and 1-week culture, cells were observed by inverted light microscopy observation and Giemsa staining. After 3-day culture, some cells looked like the branches or spikes extending out from the cell body (neuron) in control and 10 μg/mL of SHya-treated cultures, and the neurons were elongated and increased in number in
F2 50 μg/mL of SHya-treated cultures [Fig. 2(A–C)]. After 1 week, in addition to neuron, some cells appeared like the sparse branches of a tree (oligodendrocyte) with 10 μg and 50 μg/mL of SHya. The number of oligodendrocytes was increased at 50 μg/mL of SHya-treated cultures, compared with the control culture [Fig. 2(D–F)]. Cell proliferation was nonsignificantly increased about 1.2-fold with 50 μg/mL SHya com-
F3 pared with control (Fig. 3) but was almost similar to control when treated with 10 μg/mL of SHya.

Astrocytes are coupled to a cellular network via gap junction channels predominantly composed of Cx43. Astrocytes are believed to play an important role in neuroprotection by providing energy substrates to neurons and by regulating the concentrations of K^+ and neurotransmitters via gap junctions. Therefore, we measured the GJIC function by SLDT assay. GJIC was significantly increased in cells cul-
F4 tured with SHya [Fig. 4(A,B)].

The expression of the Cx43 gene was also significantly increased in 50 μg/mL SHya-treated cultures
F5 [Fig. 5(A)]. It was reported that Cx26, Cx30, and Cx43 are expressed in astrocytes,[9] and Cx47 and

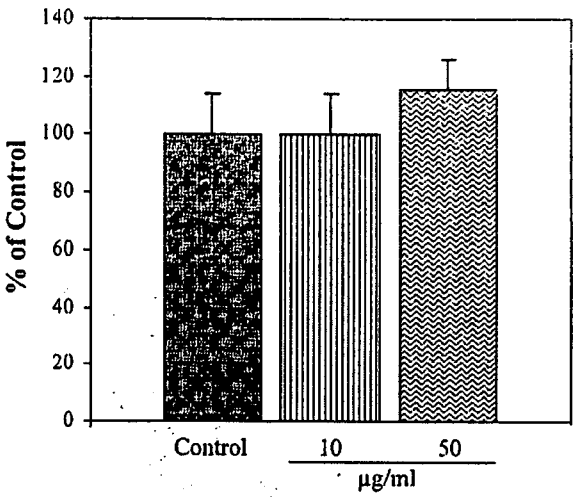

Figure 3. MTT assay for cell proliferation. Cell proliferation was nonsignificantly increased about 1.2-fold with 50 μg/mL SHya-treated cultures compared with control.

Cx32 are expressed in oligodendrocytes; Cx32 is also expressed by neurons in mouse.[9,14] Astrocyte Cx26 is associated mainly with oligodendrocyte Cx32, whereas astrocyte Cx43 and Cx30 are associated with oligodendrocyte Cx47.[15] To evaluate their presence, we further estimated the expression levels of these specific genes. Expressions of Cx26 and Cx32 genes were increased in 50 μg/mL SHya-treated cultures about 4- and 3.5-fold, respectively, compared with the control culture [Fig. 5(B,C)]. In contrast, no expression of Cx30 and Cx47 genes was detected (data not shown). As mention earlier, mouse astrocyte and oligodendrocyte were capable to express Cx30 and Cx47 gene, respectively. But, in our experiment, we used human cell that failed to express these genes. We postulated that the difference in the species may play a vital role for the expression of these genes. Our data suggest that SHya promotes a strong association of astrocyte Cx26 with oligodendrocyte Cx32 and neuron Cx32.

The majority of cells in the CNS are generated during the embryonic and early postnatal period. Brain has long been regarded as incapable of regeneration. Therefore, discovery of new neurons in certain regions of adult mammalian brain has generated intense interest. Neural stem cells were reported to have the ability for expansion and differentiation into astrocytes, oligodendrocytes, and neurons *in vitro*.[16,17] It was suggested that a part of NHA have neural precursor activity that gives rise to astrocytes itself, oligodendrocytes and neurons, that express their original specific markers (Fig. 6).[18] F6
Neural precursor cells express nestin, a class IV intermediate filament protein. Astrocytes express GFAP, a glial filamentous acidic protein. Oligodendrocytes express OLIG1, and differentiated neurons

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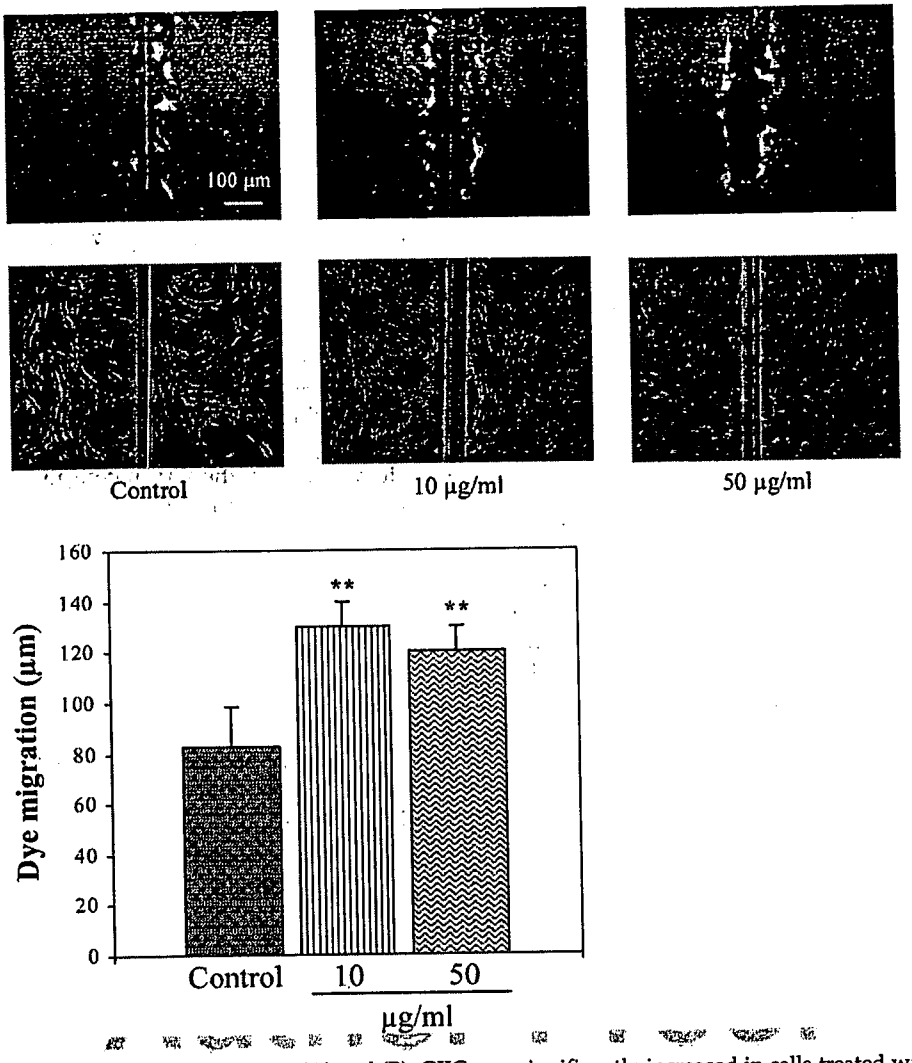

Figure 4. Statistical analysis of SLDT assay. (A) and (B), GJIC was significantly increased in cells treated with 10 and 50 μg/mL of SHya. $^{**}p < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.] AQ3

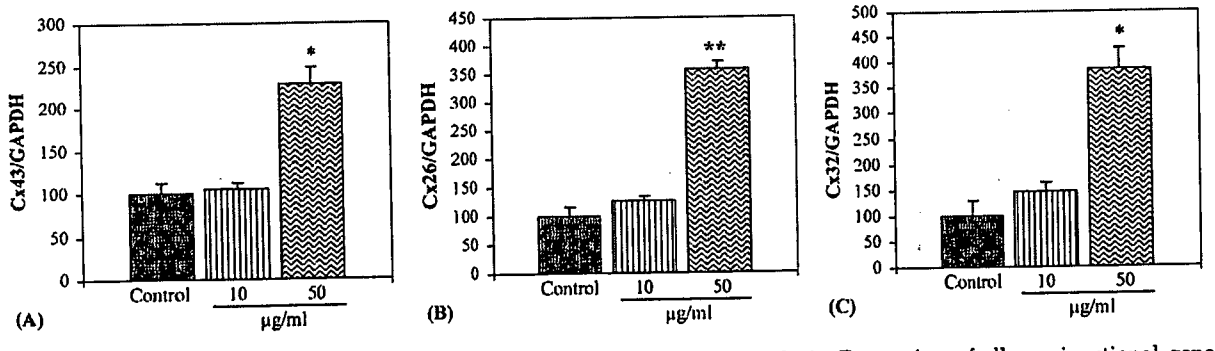

Figure 5. mRNA expression of Cx43, Cx26, and Cx32 by real-time PCR analysis. Expression of all gap junctional genes was significantly increased in 50 μg/mL SHya-treated cultures compared with control. (A) Expression of Cx43 was increased about 2.2-fold, (B) expression of Cx26 was increased about 4-fold, and (C) expression of Cx32 gene was increased about 3.5-fold. $^{*}p < 0.05$, $^{**}p < 0.01$.

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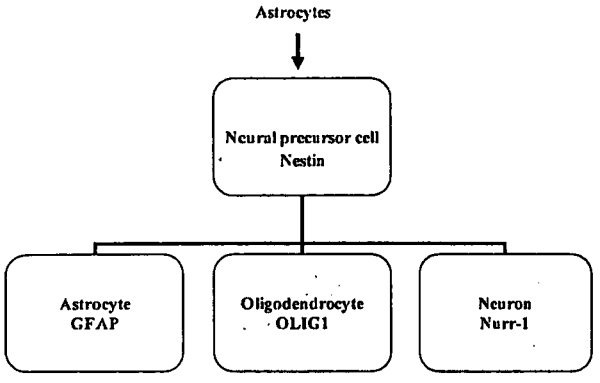

Figure 6. Schematic representation of astrocytes differentiation *in vitro*.

express Nurr-1, a transcription factor. In this study, we also determined the role of SHya in differentiation and expression of specific neural genes. When NHA was cultured with SHya, the expressions of nestin, GFAP, OLIG1, and Nurr-1 were significantly increased in 50 μg/mL SHya-treated cultures [Fig. F7 7(A–D)]. In all cases, the increases (about 2-, 3-, 1.8-, and 1.7-fold, respectively) were statistically significant at the concentration of 50 μg/mL SHya compared to the control. From this finding, it was suggested that astrocytes may be differentiated into neurons and/or oligodendrocytes with SHya-treated cultures.

Sulfated polysaccharides, such as heparan sulfate and heparin, are reported to mediate the activity of basic fibroblast growth factor in the ECM.[19] SHya, a semisynthetic material, composed of Hya and a sulfate group,[11] was synthesized by using Hya extracted from microorganisms; therefore, it has a lower infectivity and a lower risk of containing virus-induced carcinogens. Interaction of SHya with cells was already reported in several studies,[20] but the effect of SHya on cell proliferation, differentiation, and intercellular signaling was not clear. As reported earlier, SHya affected the osteoblasts responding to serum components supplied by FBS in the culture medium.[11] In our experiment, FBS was also added to the culture medium suggesting that SHya may affect cell function by interacting with the serum components. Moreover, Abatangelo et al.[21] reported SHya as a better nutrient for cells than Hya. Also, the SHya do not stimulate damage to the erythrocyte membrane. Therefore, we hypothesized a positive role of SHya on NHA.

CONCLUSION

In this study, we identified several distinct roles for SHya in NHA: increasing cell proliferation and facilitating GJIC function in a dose-dependent manner. Expressions of the specific markers of certain

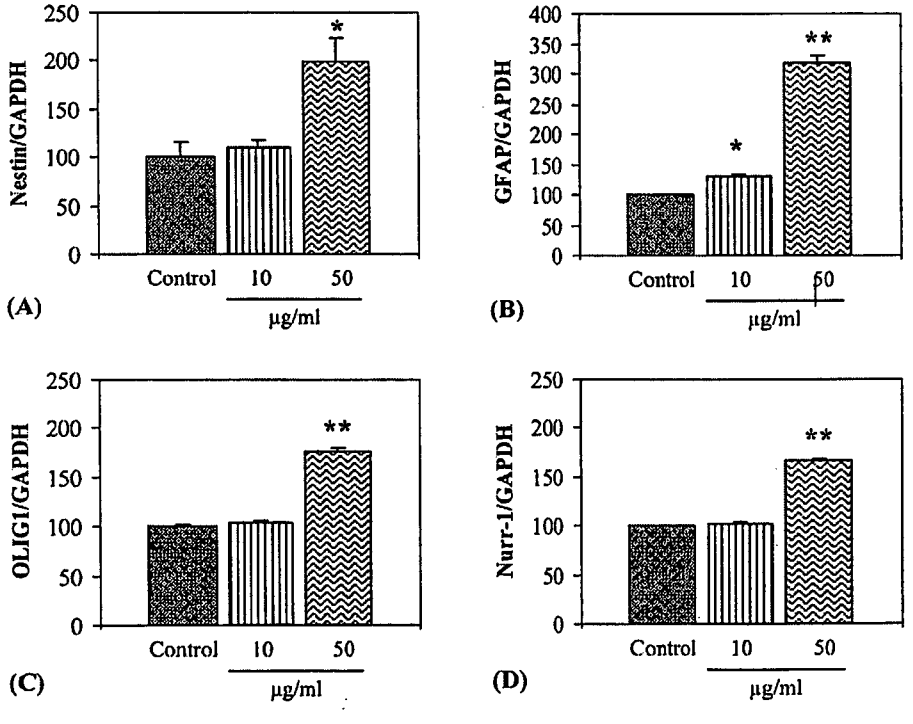

Figure 7. mRNA expression of neural cell marker genes by real-time PCR. The expressions of (A) Nestin, (B) GFAP, (C) OLIG1, and (D) Nurr-1 were increased. In all cases, at the concentration of 50 μg/mL, the increase was significant (about 2-, 3-, 1.8-, and 1.7-fold, respectively), compared to control. $^{*}p < 0.05$, $^{**}p < 0.01$.

ID: sangeethag Date: 15/3/08 Time: 13:52 Path: J:/Production/JBMM/VOL00000/080132/3B2/C2JBMM080132

genes were also enhanced with SHya treatment. Therefore, we postulate that SHya can bind with NHA and facilitate cell migration, adhesion, proliferation, and differentiation. Thus, SHya seems to play an important role in NHA and thus could help provide a novel material for the advancement of the field of tissue engineering.

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