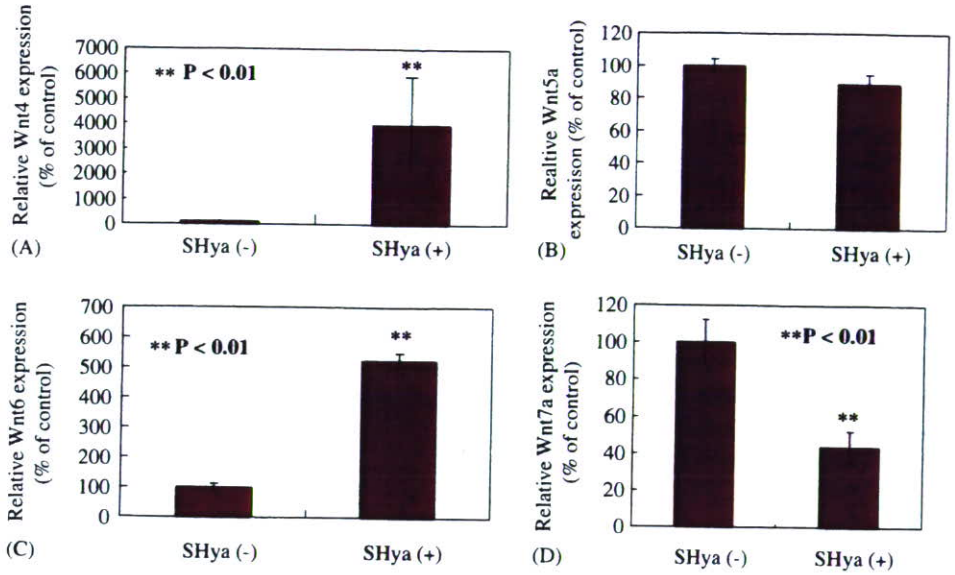

Fig. 5. The expression level of Wnt mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya-coated 60 mm dishes (0 and 0.8 mg/cm^2) and cultured in medium with 0.20 mM calcium for 20 h. Then RNA was extracted, and real-time RT-PCR was performed to determine the expression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

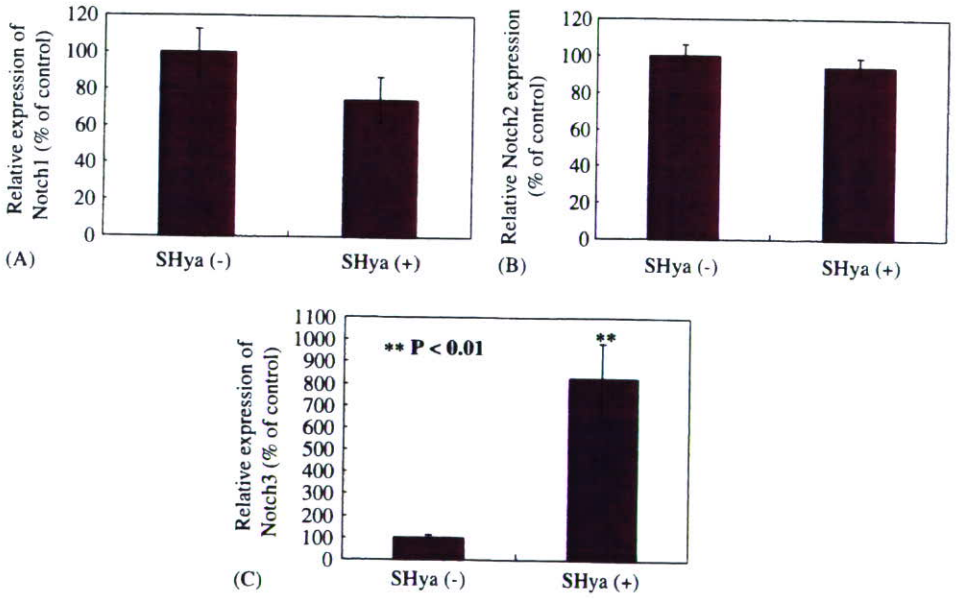

Fig. 6. The expression level of Notch mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm^2) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed to measure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

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

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

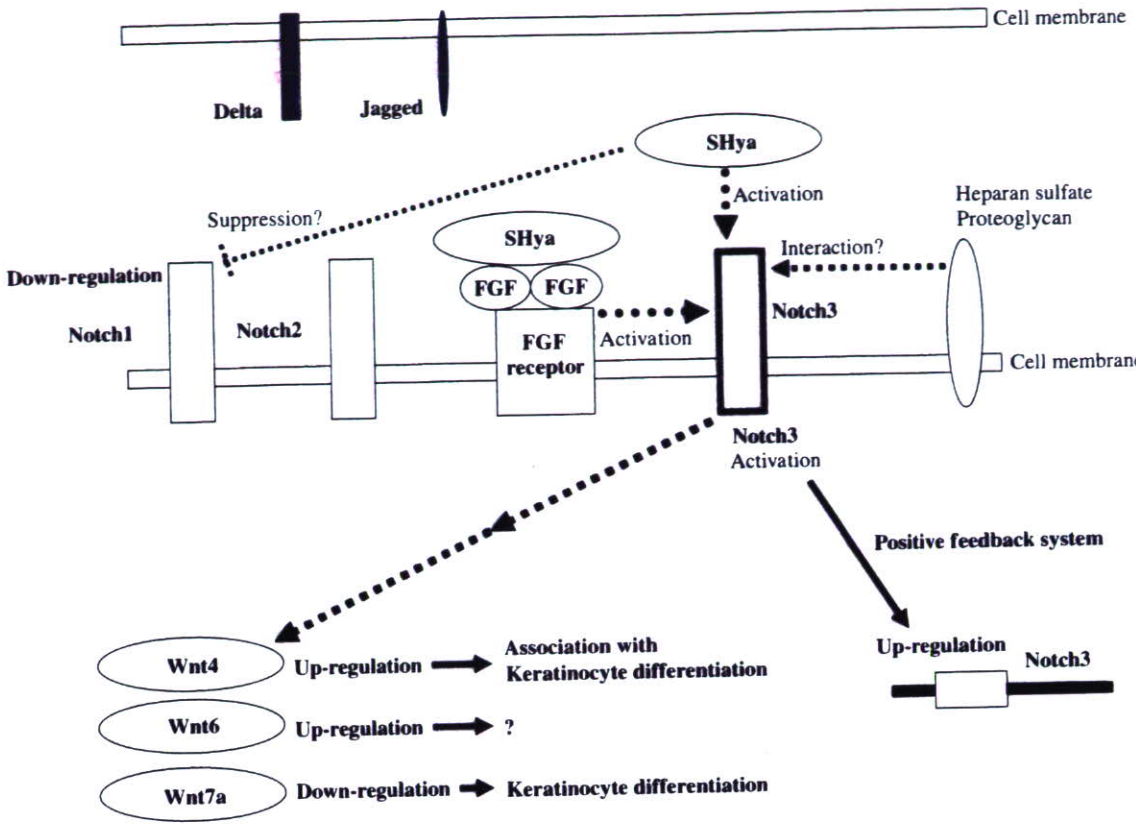

Fig. 7. Diagram of the effect of SHya on Wnt expression via Notch3.

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

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

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

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

5. Conclusion

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

Acknowledgments

Special thanks to Dr. Misao Nagahata-Ishiguro for providing sulfated hyaluronan. This work was supported by Health and Labour Sciences Research Grants on Advanced Medical Technology (H14-001) and the Health Sciences focusing on Drug Innovation (KH61059) and on

Human Genome, Tissue Engineering (H17-022), from the Japanese Ministry of Health, Labour and Welfare.

References

[1] Blacker KL, William ML, Goldyne M. Mitomycin C-treated 3T3 fibroblasts used as feeder layers for human keratinocyte culture retain the capacity to generate eicosanoids. J Invest Dermatol 1987;89: 536–9.

[2] Lam PK, Chan ES, Yen RS, Lau HC, King WW. A new system for the cultivation of keratinocytes on acellular human dermis with the use of fibrin glue and 3T3 feeder cells. J Burn Care Rehabil 2000;21:1–4.

[3] Meana A, Iglesias J, Del RM, Larcher F, Madrigal B, Fresno MF, et al. Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels. Burns 1998;24:621–30.

[4] Hollander D, Stein M, Bernd A, Windolf J, Wagner R, Pannike A. Autologous keratinocyte culture on hyaluronic acid ester membrane: an alternative in complicated wound management? Unfallchirurgie 1996;22:268–72.

[5] Boyce S, Michel S, Reichert U, Shroot B, Schmidt R. Reconstructed skin from cultured human keratinocytes and fibroblasts on a collagen–glycosaminoglycan biopolymer substrate. Skin Pharmacol 1990;3:136–43.

[6] Lapcik L Jr and L, Lapcik L, De Smedt S, Demeester J, Chabrecek P. Hyaluronan: preparation, structure, properties, and applications. Chem Rev 1998;98:2663–84.

[7] Laurent TC, Fraser JE. Hyaluronan. FASEB J 1992;6:2397–404.

[8] Kimata K, Honma Y, Okayama M, Oguri K, Hozumi M, Suzuki S. Increased synthesis of hyaluronic acid by mouse mammary carcinoma cell variants with high metastatic potential. Cancer Res 1983;43: 1347–54.

[9] Knudson CB, Knudson W. Hyaluronan-binding proteins in development, tissue homeostasis, and disease. FASEB J 1983;7:1233–41.

[10] Park JU, Tsuchiya T. Increase in gap junctional intercellular communication by high molecular weight hyaluronic acid associated with fibroblast growth factor 2 and keratinocyte growth factor production in normal human dermal fibroblasts. Tissue Eng 2002;8: 419–27.

[11] Park JU, Tsuchiya T. Increase in gap-junctional intercellular communications (GJIC) of normal human dermal fibroblasts (NHDF) on surfaces coated with high-molecular-weight hyaluronic acid (HMW HA). J Biomed Mater Res 2002;60:541–7.

[12] Takada T, Katagiri T, Ifuku M, Morimura N, Kobayashi M, Hasegawa K, et al. Sulfated polysaccharides enhance the biological activities of bone morphogenetic proteins. J Biol Chem 2003;278: 43229–35.

[13] Chamow SM, Schwall RH, Stack RJ. Sulphated oligosaccharides promote hepatocyte growth factor association and govern its activity. J Biol Chem 1995;14:16871–8.

[14] Nagahata M, Tsuchiya T, Ishiguro T, Matsuda N, Nakatsuchi Y, Teramoto A, et al. A novel function of *N*-cadherin and connexin43: marked enhancement of alkaline phosphatase activity in rat calvarial osteoblast exposed to sulfated hyaluronan. Biochem Biophys Res Commun 2004;315:603–11.

[15] Kavalkovich KW, Boynton RE, Murphy JM, Barry F. Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system. In Vitro Cell Dev Biol Anim 2002;38:457–66.

[16] Yang R, Yan Z, Chen F, Hansson GK, Kiessling R. Hyaluronic acid and chondroitin sulphate A rapidly promote differentiation of immature DC with upregulation of costimulatory and antigen-presenting molecules, and enhancement of NF-κB and protein kinase activity. Scand J Immunol 2002;55:2–13.

[17] Matsuda M, Shikata K, Shimizu F, Suzuki Y, Miyasaka M, Kawachi H, et al. Therapeutic effect of sulphated hyaluronic acid, a potential selectin-blocking agent, on experimental progressive mesangial proliferative glomerulonephritis. J Pathol 2002;198:407–14.

[18] Saitoh A, Laura A, Vogel HJC, Udey MC. Characterization of *Wnt* gene expression in murine skin: possible involvement of epidermis-derived Wnt-4 in cutaneous epithelial–mesenchymal interactions. Exp Cell Res 1998;243:150–60.

[19] Schmidit C, Stoeckelhuber M, Mckinnell I, Putz R, Christ B, Patel K. Wnt6 regulates the epithelialisation process of the segmental plate mesoderm leading somite formation. Dev Biol 2004;271: 198–209.

[20] Jungmook L, Choun-Ki J. Wnt-7a up-regulates matrix metalloproteinase-12 expression and promotes cell proliferation in corneal epithelial cells during wound healing. J Biol Chem 2005;280: 21653–60.

[21] Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, et al. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J 2001;20: 3427–36.

[22] Devgan V, Mammucari C, Millar SE, Brisken C, Dotto GP. p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation. Genes Dev 2005;19: 1485–95.

[23] Domenga V, Fardoux P, Lacombe P, Monet M, Maciazek J, Krebs LT, et al. *Notch3* is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev 2004;18:2730–5.

[24] Vacca A, Felli MP, Palermo R, Di Mario G, Calce A, Di Giovine M, et al. Notch3 and pre-TCR interaction unveils distinct NF-kappaB pathways in T-cell development and leukemia. EMBO J 2006;25: 1000–8.

[25] Li Y, Nagira T, Tsuchiya T. The effect of hyaluronic acid on insulin secretion in HIT-T15 cells through the enhancement of gap-junctional intercellular communications. Biomaterials 2006;27: 1437–43.

[26] Anderegg G, Flaschka H, Sallmann R, Schwarzenbach G. Metallindikatoren VII. Ein auf Erdalkaliionen ansprechendes Phatalein und sein analytische Verwendung. Helv Chim Acta 1954;37: 113–20.

[27] Yang L, Yamasaki K, Shirakata Y, Dai X, Tokumaru S, Yahata Y, et al. Bone morphogenetic protein-2 modulates Wnt and frizzled expression and enhances the canonical pathway of Wnt signaling in normal keratinocytes. J Dermatol Sci 2006;42:111–9.

[28] Kispert A, Vainio S, McMahon AP. Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. Development 1998;125:4225–34.

[29] Kispert A, Vainio S, Shen L, Rowitch DH, McMahon AP. Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. Development 1996;122:3627–37.

[30] Nery KYS, Rutlin ML, Radtke F, Fishell G, Gaiano N. Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. J Neurosci 2004;24:9497–506.

[31] Akai J, Halley PA, Storey KG. FGF-dependent Notch signaling maintains the spinal cord stem zone. Genes Dev 2005;19:2877–87.

Rheumatology 2007;46:1652–1656
Advance Access publication 12 September 2007

doi:10.1093/rheumatology/kem220

Serum keratan sulfate is a promising marker of early articular cartilage breakdown

S. Wakitani[1], M. Nawata[2], A. Kawaguchi[1], T. Okabe[3], K. Takaoka[1], T. Tsuchiya[4], R. Nakaoka[4], H. Masuda[5] and K. Miyazaki[5]

Objectives. To find serum markers that may serve as indices for an early diagnosis of degeneration or damage of the articular cartilage.
Methods. Twenty-four healthy volunteers, 19 individuals with knee trauma (KT) and 31 with knee osteoarthritis (OA) were evaluated. KT patients were divided into a group ($n=5$) with an injury <2 months old (recent KT) and a group ($n=14$) with that >2 months old (old KT). Articular cartilage damage was assessed using either arthroscopy or direct observation. Serum concentrations of hyaluronic acid (HA), cartilage proteoglycan aggrecan turnover epitope (CS846) and cartilage oligomeric protein (COMP) were measured using enzyme-linked immunosorbent assay kits and those of keratan sulfate (KS) and chondroitin-6-sulfate (C6S) using high-performance liquid chromatography.
Results. Serum KS in the recent KT group (2095 ± 594 ng/ml) was significantly higher than that in the old KT group (1373 ± 418 ng/ml; $P=0.021$), and serum COMP in the recent KT group (1572 ± 182 ng/ml) showed a tendency that was higher than that in the old KT group (1350 ± 250 ng/ml; $P=0.079$).
Serum KS in OA patients with Kellgren and Lawrence (KL) grades 0 and I (1456 ± 334 ng/ml) showed a tendency that was higher than that in OA patients with KL grades II, III and IV (1248 ± 220 ng/ml; $P=0.084$).
Conclusions. The serum concentration of KS correlated with the damage of the articular cartilage and it was significantly increased even at an early stage after the injury.

KEY WORDS: Keratan sulfate, Glycosaminoglycan, Cartilage oligomeric protein, Cartilage injury, Osteoarthritis, Serum marker.

Introduction

The prevalence of patients with articular cartilage defects among patients with symptomatic knees requiring arthroscopy has been reported as 5–20% [1–3]; when left untreated, osteoarthritic changes are observed on X-rays taken after 10–20 yrs [4, 5]. Thus, articular cartilage injury is considered a cause of osteoarthritis (OA). Even if there is no articular cartilage injury, degeneration of the articular cartilage is considered to begin in humans at a young age, and articular cartilage changes, such as changes in colour and fibrillation, can occur. Injury or early-stage alterations of the articular cartilage in OA cannot be detected using X-ray examination. Magnetic resonance imaging (MRI) can detect articular cartilage defects and cartilaginous quality changes to some extent, but this technique is not sensitive enough to detect early OA changes and is expensive to be used as a routine examination. Serum markers, on the other hand, are suitable as screening tests, and only patients with high values of serum markers should be subjected to MRI or arthroscopy to detect articular cartilage degeneration. If it were possible to detect OA or articular cartilage damage at an early stage, patients could be educated to prevent the progression of OA. Moreover, it would be useful to monitor the natural course of articular cartilage damage or repair after, for instance, autologous chondrocytes implantation, whose effectiveness is still controversial because there is no method to effectively evaluate cartilage repair.

In 1985, Thonar *et al.* [6] measured serum keratan sulfate (KS) using an enzyme-linked immunosorbent assay (ELISA) by anti-KS antibody (1/20/5-D-4), and suggested its usefulness as a marker of OA. However, the correlation was weak and it did not correlate with X-ray grading [7]. Many researchers have tried to detect the metabolic products of articular cartilage components (proteoglycan, type II collagen and non-collagenous proteins) in joint fluid or blood and thereby a marker of OA [8–11]. As reported by Okumura *et al.* [12], early OA articular cartilage destruction begins with a loss of glycosaminoglycans (GAGs) from articular cartilage surfaces, followed by collagenolysis. Thus, the first event in OA or articular cartilage damage is the release of GAGs, which play an important role in maintaining articular cartilage function. Consequently, early markers of articular cartilage damage or OA change might be among GAG metabolic products. We selected KS, chondroitin 6 sulfate (C6S), cartilage proteoglycan aggrecan turnover epitope (CS846) and hyaluronan (HA) as candidate markers, and cartilage oligomeric protein (COMP), which is not a component of GAGs but has been reported to be a marker of OA [9]. These components have been reported to correlate with OA, to some extent, but not with cartilage damage caused by degradation and/or injury. These metabolic products can be measured in joint fluid, serum and urine, but we measured them in serum because it is easy to collect.

We measured KS using high-performance liquid chromatography (HPLC), which has been reported to be more accurate than ELISA [13]; C6S using HPLC, and CS846, HA and COMP using ELISA. We measured these markers in healthy volunteers and in patients with knee trauma (KT) or OA, who were subjected to knee surgery and whose articular cartilage was optically assessed (by arthroscopy or direct observation). We examined the correlation of these markers with the articular cartilage assessment to evaluate their usefulness as markers of early articular cartilage breakdown caused by degeneration and/or injury but that showed no change by X-ray examination.

Patients and methods

This study was approved by the institutional Review Board of Marunouchi Hospital and was conducted in accordance with the Helsinki Declaration of 1975, revised in 1983. Written informed consent was obtained from the healthy volunteers and patients prior to their participation in the study.

[1]Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, [2]Department of Orthopaedic Surgery, Marunouchi Hospital, [3]Department of Orthopaedic Surgery, Shinshu University School of Medicine, [4]Division of Medical Devices, National Institute of Health Sciences and [5]Department of Pharmaceuticals Information, Seikagaku Corporation.

Submitted 11 April 2007; revised version accepted 19 July 2007.

Correspondence to: Shigeyuki Wakitani, MD, Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, 1-4-3 Asahi, Abeno-ku, Osaka 545-8585, Japan. E-mail: wakitani@med.osaka-cu.ac.jp

Blood collection from healthy volunteers

Ten men and 14 women (23–52 yrs old) volunteered to participate in the study. The volunteers were healthy with no gross obesity, inferior limb malalignment, history of knee injury or knee disorders. Sera were collected and stored at −80°C.

Patients with KT or knee OA

Nineteen KT patients (11 men and 8 women; 20–54 yrs old) and 31 patients with knee OA (11 men and 20 women; 40–80 yrs old) who were diagnosed to undergo knee surgery participated in the study. X-rays of knee and lumbar spine were available for all the patients. Sera samples were collected before surgery and stored at −80°C. The condition of the knee articular cartilage was observed at the time of surgery either arthroscopically or by direct observation. Among the 19 KT patients, two had meniscal injuries, 12 had ligament injuries and five had both meniscal and ligament injuries. KT patients were divided into a group of 5 patients with injuries <2 months old (recent KT) and a group of 14 patients with injuries >2 months old (old KT). Among the 31 OA patients, eight underwent total knee replacement, one underwent a high tibial osteotomy and 22 underwent arthroscopic debridement.

Assessment of articular cartilage surfaces by X-ray and visual inspection

X-ray images were assessed using the Kellgren and Lawrence (KL) grading scale [14]. All the KT patients were KL grade 0. Seven of the OA patients were KL grade 0, seven were KL grade I, four were KL grade II, six were KL grade III and seven were KL grade IV. Articular cartilage damage was assessed using the Societé Francaise d' Arthroscopie (SFA) scaling system [15]. In brief, the degree of articular cartilage damage was estimated from 0 to IV according to the SFA grading scale. The width of the damaged area was evaluated as a percentage of the damaged area in the medial and medial femoro-tibial and patello-femoral areas, separately. The SFA score was then calculated using a coefficient. The SFA score represents not only the degree of articular cartilage surface damage, but also the width of the damaged area.

Determination of the serum markers

Keratan sulfate was determined by HPLC after digestion with keratanase II (Seikagaku Corporation, Tokyo, Japan) according to the method of Tomatsu *et al.* [13] Each serum sample (0.2 ml) was treated with a protease (actinase E: Kaken Pharmaceutical Co. Ltd., Tokyo) and the negatively charged substance containing KS was fractionated by Q sepharose and digested by keratanase II. The KS-derived β-galactosyl-(1-4)-6-*O*-sulfo-*N*-acetylglucosamine (m-ks) and β-6-*O*-sulfo-galactosyl-(1-4)-6-*O*-sulfo-*N*-acetylglucosamine (d-ks) were contained in the solution that was digested by the enzyme and were measured using HPLC. Standard KS derived from bovine cornea (Seikagaku) was used to measure KS under identical conditions; and the quantity of KS in each serum sample was calculated as the sum of m-ks and d-ks. To determine C6S concentration, 0.2 ml of each serum sample was first treated with chondroitinase ABC (Seikagaku). The quantity of unsaturated disaccharide contained in the digested fluid was determined and C6S was detected by HPLC [16]. For the determination of CS846, COMP and HA, the Aggrecan Chondroitin Sulfate 846 Epitope ELISA Kit (IBEX Technologies, Inc., Montreal, Quebec, Canada), Human COMP ELISA Kit (Kamiya Biomedical Company, Seattle, WA, USA) and Hyaluronan Assay Kit (Seikagaku Corporation) were used respectively.

Statistical analysis

To determine the statistical significance of inter-group differences, Steel's multiple comparison test for patient group *vs* control and Wilcoxon rank-sum test for inter-patient group were conducted, and the *P*-level was set at <0.05.

Results

Arthroscopic findings in KT patients and serum concentrations of KS, C6S, CS846, HA and COMP

All KT patients had articular cartilage damage. Their cartilaginous damage scores (SFA) for the recent KT group and the old KT group were 1.2 ± 0.7 and 3.8 ± 3.9, respectively. On X-ray examination, no changes were noted in the knee or intervertebral joints (Fig. 1).

The serum concentrations of KS, C6S, CS846, HA and COMP in KT patients are shown in Table 1. KS, C6S, CS846 and COMP were significantly higher in the recent KT group ($P=0.001$, $P=0.047$, $P=0.022$ and $P=0.001$, respectively), and KS and COMP higher in the old KT group (both $P<0.001$) than in controls.

X-ray and arthroscopic examination of OA patients and serum concentrations of KS, C6S, CS846, HA and COMP

The SFA scores of OA patients distributed by their KL grade are presented in Table 2. The SFA score increased in relation with

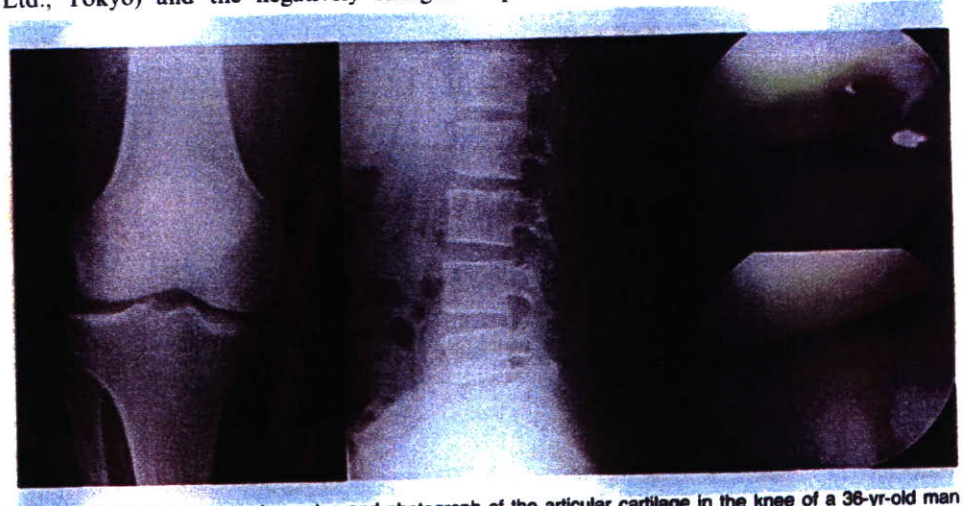

FIG. 1. Representative radiographs of the knee and lumbar spine and photograph of the articular cartilage in the knee of a 36-yr-old man with traumatic arthropathy. The X-ray grade, SFA score and serum KS concentration were normal, 0.7 and 2537 ng/ml, respectively. The serum KS concentration was high, although damage of the cartilage was minimal.

increased KL grade. Even in patients with KL grade 0 OA, degeneration or damage of the articular cartilage surface was observed by direct optical methods. In such patients, no X-ray findings were detected in the knee nor in the intervertebral discs (Fig. 2).

Serum concentrations of KS were significantly higher in most OA stages (KL grade 0: $P=0.004$, I: $P<0.001$, III: $P=0.004$ and IV: $P=0.008$) and serum COMP were significantly higher in all OA stages (KL grade 0: $P=0.004$, I: $P=0.002$, II: $P=0.008$, III: $P=0.002$ and IV: $P<0.001$) than in controls. C6S and HA in patients with KL grade IV were significantly higher than in controls (both $P<0.001$).

TABLE 1. Serum concentration of markers of cartilage degeneration or damage in patients with knee trauma

	Healthy subjects	KT patients	
		Recent trauma[a]	Old trauma[b]
n	24	5	14
SFA		1.2 ± 0.7	3.8 ± 3.9
KS (ng/ml)	910 ± 145	2095 ± 594*	1373 ± 418*
C6S (ng/ml)	97 ± 28	122 ± 10*	104 ± 22
CS846 (ng/ml)	137 ± 24	214 ± 77*	142 ± 46
COMP (ng/ml)	1030 ± 150	1572 ± 182*	1350 ± 250*
HA (ng/ml)	41 ± 15	44 ± 19	39 ± 12

The values are the mean ± S.D.
[a]Patients evaluated within 2 months after the injury.
[b]Patients evaluated >2 months after the injury.
*$P<0.05$ *vs* Healthy subjects (Steel's multiple comparison test).
SFA, Societé Francaise d' Arthroscopie score; KS, keratan sulfate; C6S, Chondroitin-6-sulfate; CS846, cartilage proteoglycan aggrecan turnover epitope; COMP, cartilage oligomeric protein and HA, hyaluronic acid.

Comparison of the serum markers between patient groups

Since the serum concentrations of KS and COMP were higher in most stages of KT and OA than in controls, those differences between stages were compared in Fig. 3. The serum KS in the recent KT group (2095 ± 594 ng/ml) was significantly higher than that in the old KT group (1373 ± 418 ng/ml; $P=0.021$) and those in OA patients with KL grades 0 and I (1456 ± 334 ng/ml) showed a tendency that was higher than that in patients with KL grades II, III and IV (1248 ± 220 ng/ml; $P=0.084$). The serum concentrations of COMP in the recent KT group (1572 ± 182 ng/ml) showed a tendency that was higher than that in the old KT group (1350 ± 250 ng/ml; $P=0.079$), but those in OA patients showed no difference between the patient group with KL grades 0 and I (1639 ± 434 ng/ml) and the patient group with KL grades II, III and IV (1731 ± 355 ng/ml).

Discussion

This study showed that the serum concentration of KS was high in patients with early-stage damage of the articular cartilage undetectable by X-ray imaging. Serum KS may be suitable as a screening test for articular cartilage damage and to monitor the natural course of articular damage or repair.

In the KT patients with recent injuries, KS was significantly higher than in those with old injuries, suggesting that serum

TABLE 2. Serum concentration of markers of cartilage degeneration or damage in OA patients

X-ray grade	Healthy subjects	OA patients				
		0	I	II	III	IV
n	24	7	7	4	6	7
SFA		2.4 ± 2.1	5.1 ± 3.5	28.8 ± 47.5	>100	>200
KS (ng/ml)	910 ± 145	1501 ± 360*	1411 ± 326*	1253 ± 241	1352 ± 242*	1155 ± 176*
C6S (ng/ml)	97 ± 28	116 ± 18	115 ± 15	102 ± 22	131 ± 22	157 ± 53*
CS846 (ng/ml)	137 ± 24	147 ± 67	151 ± 108	140 ± 70	112 ± 16	211 ± 184
COMP (ng/ml)	1030 ± 150	1710 ± 550*	1570 ± 310*	1580 ± 200*	1630 ± 470*	1907 ± 268*
HA (ng/ml)	41 ± 15	80 ± 65	72 ± 21*	76 ± 68	116 ± 76	258 ± 230*

The patients were grouped by their X-ray grade.
The values are the mean ± S.D.
*$P<0.05$ *vs* healthy subjects (Steel's multiple comparison test).
SFA, Societé Francaise d' Arthroscopie score; KS, keratan sulfate; C6S, chondroitin-6-sulfate; CS846, cartilage proteoglycan aggrecan turnover epitope; COMP, cartilage oligomeric protein and HA, hyaluronic acid.

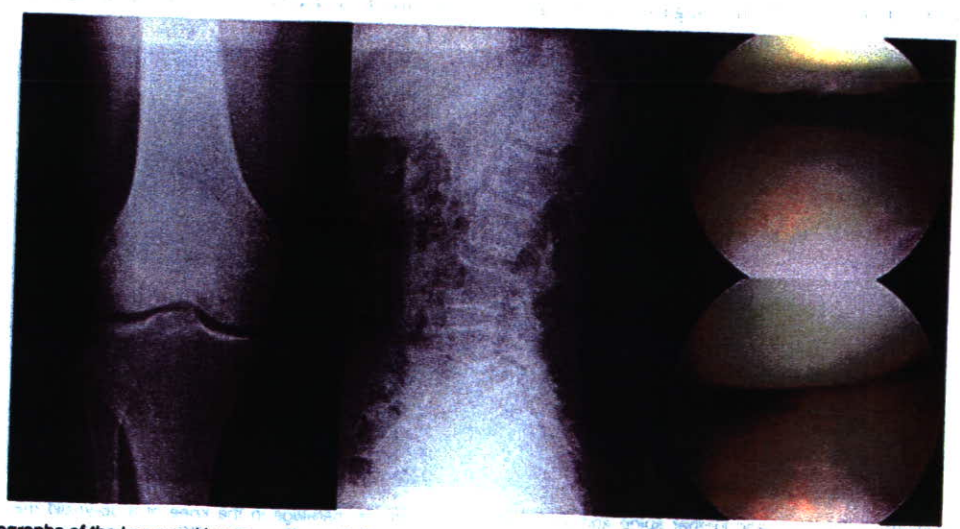

FIG. 2. Representative radiographs of the knee and lumbar spine and photograph of the articular cartilage in the knee of a 66-yr-old woman with KL grade 0 OA. The SFA score and serum KS concentration were 1.7 and 2145 ng/ml, respectively. The serum KS concentration was high, although the patient had early-stage OA.

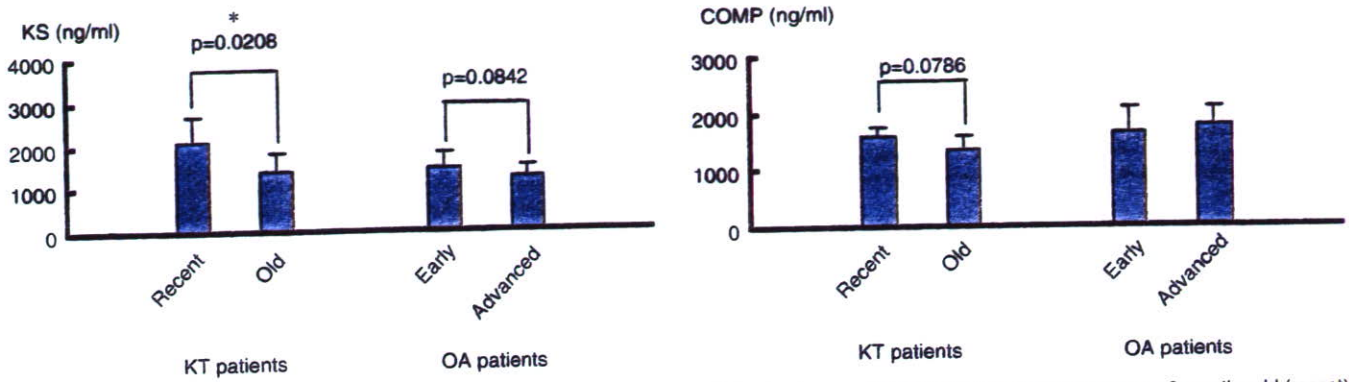

FIG. 3. Comparison of the serum markers between stage groups in KT or OA patients. KT patients were divided into a group with an injury <2 months old (recent) and a group with an injury >2 months old (old). OA patients were divided into a group with KL grades 0 and I (early) and a group with KL grades II, III and IV (advanced). Data are the mean ± S.D. *$P < 0.05$ (Wilcoxon rank-sum test).

KS might indicate the release of cartilaginous GAG in the early stage after injury in spite of moderate cartilaginous damage.

In OA patients, the serum concentrations of KS, C6S, HA and COMP were significantly higher than in healthy controls as reported previously [6, 17–19]. Among these parameters, KS was high in patients with KL grades 0 and I, indicating that KS might serve as a marker of early-stage OA. The KS concentrations in OA patients tended to decrease as the KL grade increased from 0 to IV, which reflects disappearance of the joint space. It may mean that in OA, a greater quantity of the cartilage matrix is released when the joint space has not yet narrowed. On the contrary, COMP was high in KL grade IV. This can be explained by the fact that COMP is a non-collagen protein that exists in the synovial membrane, meniscus and tendon, as well as in the cartilage and its increase is most likely related to the inflammation of various intra-articular tissues. The changes in C6S and HA were marked in KL grade IV, indicating that these are not markers of early-stage cartilage destruction.

KS is a component of proteoglycans found in the articular cartilage, intervertebral discs and corneas. Because corneas are relatively small tissues, serum KS mainly originates from articular cartilage and intervertebral discs. Thus, the serum concentration of KS is not only a marker of knee articular cartilage, but also of other joints and intervertebral discs. Therefore, before concluding that the elevated serum concentration of KS originated from damage to the knee joint articular cartilage, the possibility of spondyloarthropathy and OA in other joints must be examined. We verified that there were no X-ray changes in the lumbar spine nor symptoms caused by lumbar spinal abnormalities in KT patients (Fig. 1), although spondyloarthrotic changes existed in OA patients because most of these patients were of advanced age (Fig. 2). We verified that no OA symptoms were observed in joints other than the knee in these patients. We are planning to investigate serum KS in patients with spondyloarthropathy or intervertebral disk herniation in the future.

Serum KS is considered to reflect the normal metabolism of cartilage, and KS increases in case of mechanical injuries within a few months after injury. Budsberg *et al.* [20] found that serum KS increased 1–3 months after resection of the anterior cruciate ligaments of dog knees. In our report, KT patients who were evaluated within 2 months after the injury exhibited an acute release of KS. Although the SFA score of KT patients was very small, indicating that damage was confined, serum KS was high (Table 1). After the rapid release of KS ends, release from the injured surfaces continues at a relatively high rate. This phase is considered to continue for a few years to a couple of decades as in KT evaluated >2 months after the injury and in early-stage OA patients (KL grades 0 and I). The persistence of this condition leads to OA in a few decades. This phase corresponds to advanced OA (KL grades II, III, IV).

This report is the first study to show that serum KS increases early after an injury causing small articular damage and in patients with early-stage OA undetectable by X-ray imaging. As only a small volume of blood is required for the measurement of serum KS, this parameter may serve as a screening test to detect articular cartilage injury and it is expected to contribute greatly to the decision on a therapeutic strategy for the management of OA or cartilage injury.

Rheumatology key messages

- Serum keratan sulfate correlates with damage of the articular cartilage.
- It may serve as a screening and monitoring test of the natural course of articular cartilage damage or repair.

Disclosure statement: The authors have declared no conflicts of interest.

References

1 Aroen A, Loken S, Heir S *et al.* Articular cartilage lesions in 993 consecutive knee arthroscopies. Am J Sports Med 2004;32:211–5.
2 Curl WW, Krome J, Gordon ES, Rushing J, Smith BP, Poehling GG. Cartilage injuries: a review of 31,516 knee arthroscopies. Arthroscopy 1997;13:456–60.
3 Hjelle K, Solheim E, Strand T, Muri R, Brittberg M. Articular cartilage defects in 1,000 knee arthroscopies. Arthroscopy 2002;18:730–4.
4 Messner K, Maletius W. The long-term prognosis for severe damage to weight-bearing cartilage in the knee: a 14-year clinical and radiographic follow-up in 28 young athletes. Acta Orthop Scand 1996;67:165–8.
5 Shelbourne KD, Jari S, Gray T. Outcome of untreated traumatic articular cartilage defects of the knee: a natural history study. J Bone Joint Surg Am 2003;85–A (Suppl 2):8–16.
6 Thonar EJ, Lenz ME, Klintworth GK *et al.* Quantification of keratan sulfate in blood as a marker of cartilage catabolism. Arthritis Rheum 1985;28:1367–76.
7 Campion GV, McCrae F, Schnitzer TJ, Lenz ME, Dieppe PA, Thonar EJ. Levels of keratan sulfate in the serum and synovial fluid of patients with osteoarthritis of the knee. Arthritis Rheum 1991;34:1254–9.
8 Belcher C, Yaqub R, Fawthrop F, Bayliss M, Doherty M. Synovial fluid chondroitin and keratan sulphate epitopes, glycosaminoglycans, and hyaluronan in arthritic and normal knees. Ann Rheum Dis 1997;56:299–307.
9 Mundermann A, Dyrby CO, Andriacchi TP, King KB. Serum concentration of cartilage oligomeric matrix protein (COMP) is sensitive to physiological cyclic loading in healthy adults. Osteoarthr Cartilage 2005;13:34–8.
10 Bruyere O, Collette J, Kothari M *et al.* Osteoarthritis, magnetic resonance imaging, and biochemical markers: a one year prospective study. Ann Rheum Dis 2006;65:1050–4.
11 Mazzuca SA, Poole AR, Brandt KD *et al.* Associations between joint space narrowing and molecular markers of collagen and proteoglycan turnover in patients with knee osteoarthritis. J Rheumatol 2006;33:1147–51.
12 Okumura M, Tagami M, Fujinaga T. Measurement of serum and synovial fluid keratan sulphate and antibody to collagen type II in equine osteoarthritis. J Vet Med A 1998;45:513–6.
13 Tomatsu S, Okamura K, Maeda H *et al.* Keratan sulphate levels in mucopolysaccharidoses and mucolipidoses. J Inherit Metab Dis 2005;28:187–202.

14 Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494–502.
15 Ayral X, Altman RD. Arthroscopic evaluation of knee articular cartilage. In: Brandt KD, Doherty M, Lohmander LS, eds. Osteoarthritis. Oxford: Oxford University Press, 1998;494–505.
16 Shinmei M, Miyauchi S, Machida A, Miyazaki K. Quantitation of chondroitin 4-sulfate and chondroitin 6-sulfate in pathologic joint fluid. Arthritis Rheum 1992;35:1304–8.
17 Uesaka S, Nakayama Y, Shirai Y, Yoshihara K. Serum and synovial fluid levels of chondroitin sulfate in patients with osteoarthritis of the knee joint. J Nippon Med Sch 2001;68:165–70.
18 Salisbury C, Sharif M. Relations between synovial fluid and serum concentrations of osteocalcin and other markers of joint tissue turnover in the knee joint compared with peripheral blood. Ann Rheum Dis 1997;56:558–61.
19 Gemero P, Pipernno M, Gineyts E, Christgau S, Delmas PD, Vignon E. Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. Ann Rheum Dis 2001;60:619–26.
20 Budsberg SC, Lenz ME, Thonar EJ. Serum and synovial fluid concentrations of keratan sulfate and hyaluronan in dogs with induced stifle joint osteoarthritis following cranial cruciate ligament transection. Am J Vet Res 2006;67:429–32.

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

Received 20 September 2005; revised 19 April 2006; accepted 22 May 2006
Published online 29 August 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30931

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
Contract grant sponsor: Health and Labour Sciences Research
Contract grant sponsor: Research on Advanced Medical Technology, Ministry of Health, Labour and Welfare and Japan Health Sciences Foundation

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

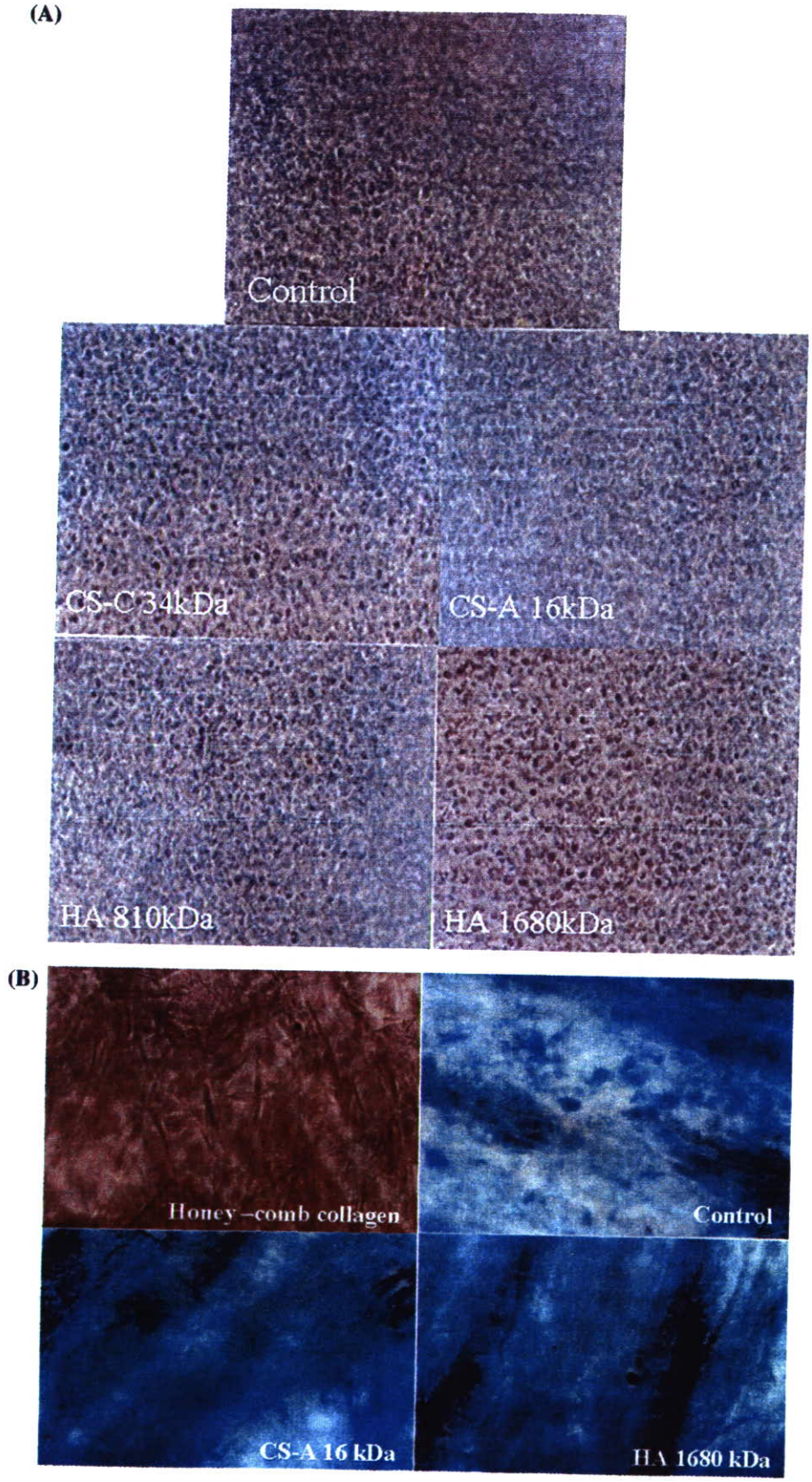

Figure 1. Light microscopic appearance of cultured HC in (A) micromass and (B) 3-D honeycomb rotation conditions spotted as high density cultures treated with different molecular weights and molecular structures of HA and CS for 4 weeks (after alcian blue staining, original magnification ×200).

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).

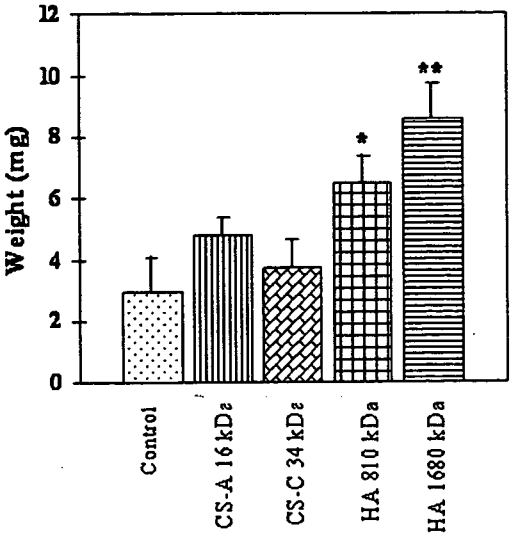

Figure 2. Wet weight of tissue constructs obtained by culturing HC on collagen honeycomb in rotation condition and treated with different molecular weights and structures of HA and CS for a period of 4 weeks. HA 810 kDa (*$p < 0.05$), and HA 1680 kDa (**$p < 0.01$) are significantly different compared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

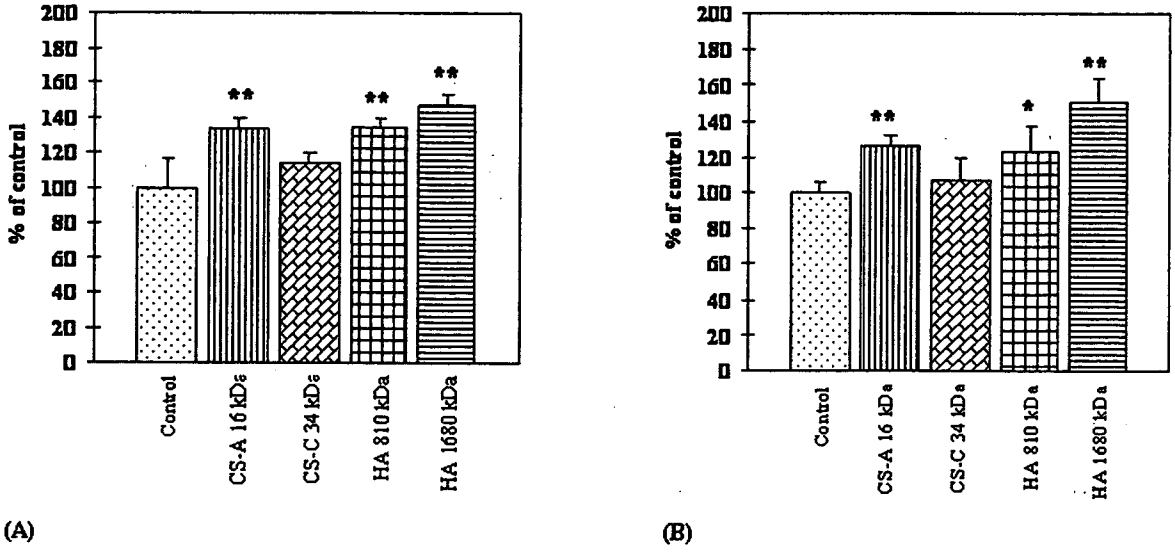

Figure 3. Cell proliferation of HC estimated by alamar blue method. (A) Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. CS-A 16 kDa, HA 810 kDa, and HA 1680 kDa are significantly different compared with control (**$p < 0.01$). (B) In rotation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

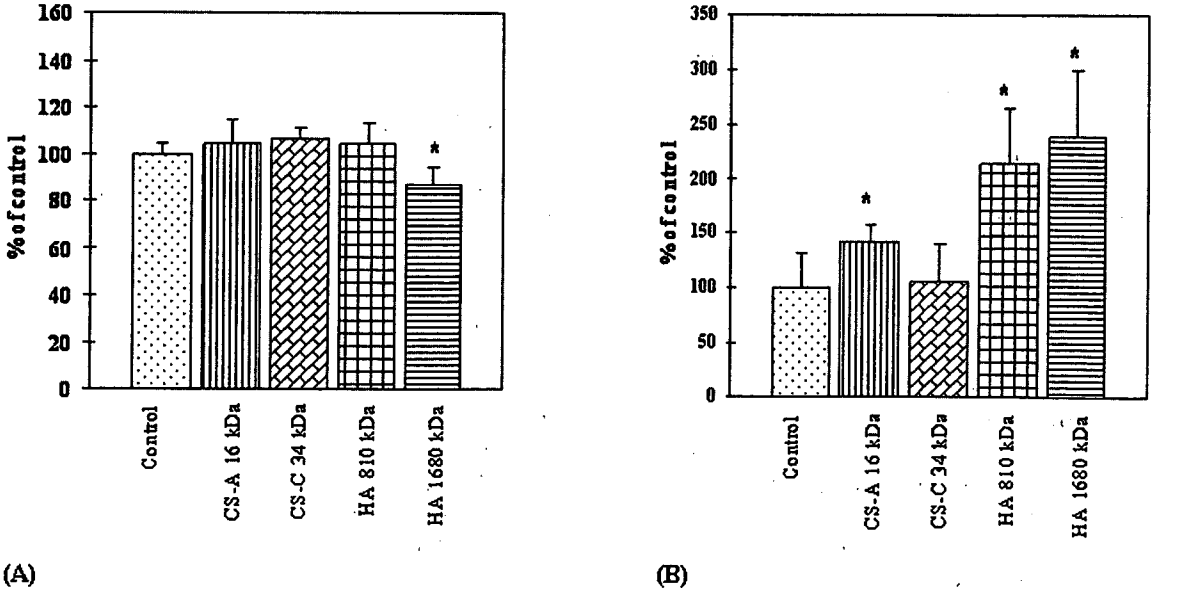

Figure 4. Cell differentiation of HC estimated by alcian blue method. (A) Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. HA 1680 kDa is significantly different compared with control (*$p < 0.05$). (B) In rotation conditions, cultures were treated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

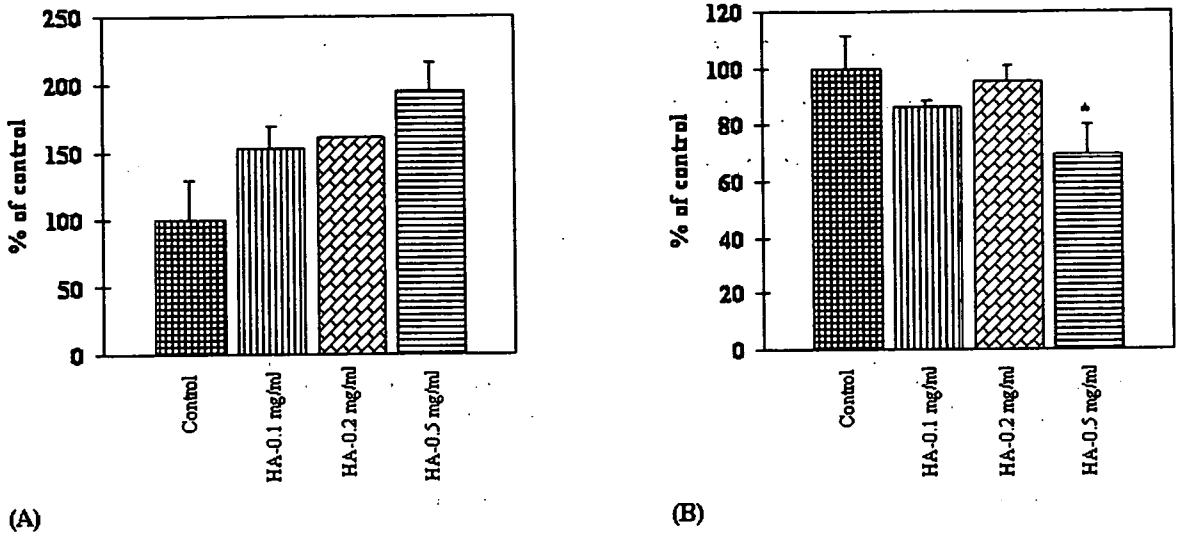

Figure 5. Chondrogenic effect of HA (1680 kDa) with different concentrations under micromass conditions for 4 weeks. (A) As demonstrated by crystal violet staining, cell proliferation was increased with 0.1, 0.2, and 0.5 mg/mL of HA 1680 kDa respectively, compared with the control culture. (B) Cell differentiation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

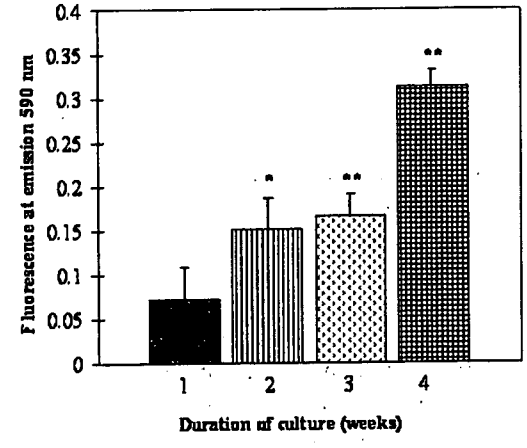

Figure 6. Time course of cell proliferation under micromass conditions for 4 weeks, estimated by crystal violet staining. Cell proliferation was significantly increased after 2 weeks (*$p < 0.05$), 3 weeks (**$p < 0.01$), and 4 weeks (**$p < 0.01$) of culture, compared with the chondrocytes cultured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

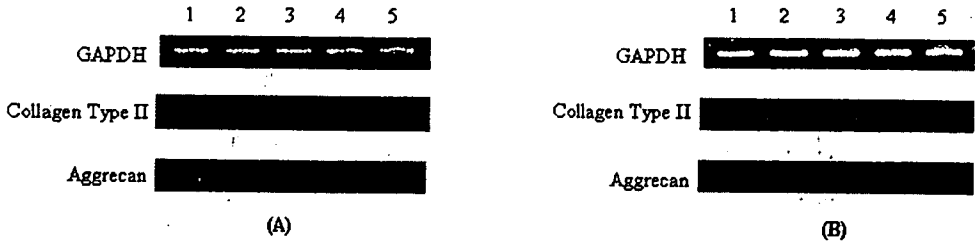

Figure 7. (A) Reverse transcription-polymerase chain reaction amplification of GAPDH, collagen type II, and aggrecan in cultures of HC with four different types of CS and HA for 4 weeks in micromass conditions. Lane 1, control; lane 2, CS-A 16 kDa; lane 3, CS-C 34 kDa; lane 4, HA 810 kDa; and lane 5,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

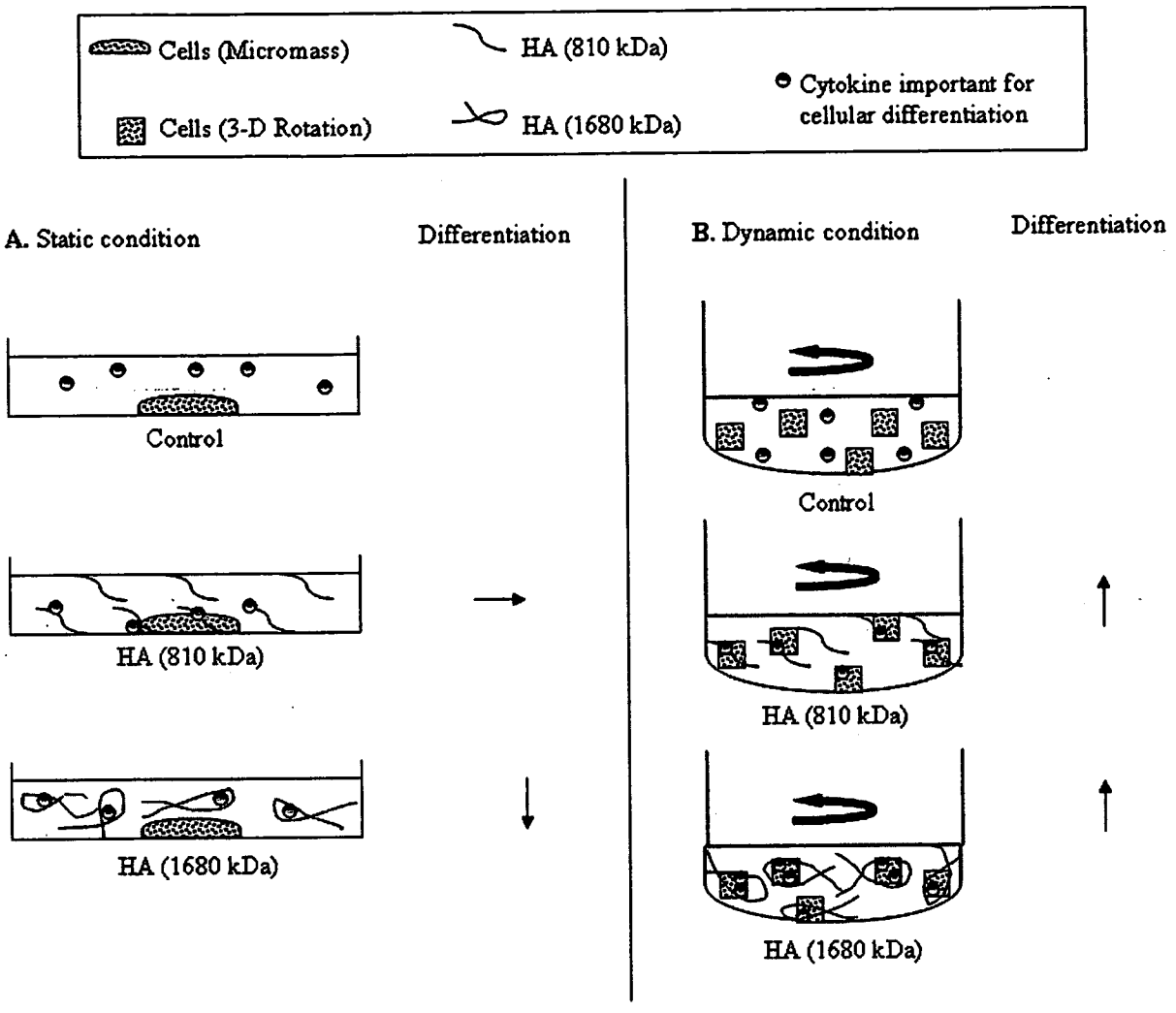

Figure 8. Schematic representation of the effects of different molecular weights of HA on the differentiation of HC. (A) Static condition (micromass). (B) Dynamic condition (rotation culture).

(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.

References

1. Mayhew TA, Williams GR, Senica MA, Kuniholm G, Du Moulin GC. Validation of a quality assurance program for autologous cultured chondrocyte implantation. Tissue Eng 1998; 4:325–334.

2. Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin Orthop 2000;374:212–234.
3. Freed LE, Hollander AP, Martin I, Barry JR, Langer R, Vunjak-Novakovic G. Chondrogenesis in a cell-polymer-bioreactor system. Exp Cell Res 1998;240:58–65.
4. Vacanti CA, Langer R, Schloo B, Vacanti JP. Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. Plast Reconstr Surg 1991;88:753–759.
5. Nehrer S, Breinan HA, Ramappa A, Shortkroff S, Young G, Minas T, Sledge CB, Yannas IV, Spector M. Canine chondrocytes seeded in type I and type II collagen implants investigated in vitro. J Biomed Mater Res 1997;38:95–104.
6. Messner K, Gillquist J. Synthetic implants for the repair of osteochondral defects of the medial femoral condyle: a biomechanical and histological evaluation in the rabbit knee. Biomaterials 1993;14:513–521.
7. Sommerlath K, Gillquist J. The effects of an artificial meniscus substitute in a knee joint with a resected anterior cruciate ligament. An experimental study in rabbits. Clin Orthop 1993;289: 276–284.
8. Messner K. Durability of artificial implants for repair of osteochondral defects of the medial femoral condyle in rabbits. Biomaterials 1994;15:657–664.
9. Klompmaker J, Jansen HWB, Veth RP, Nielsen HKL, de Groot JH, Pennings AJ. Porous polymer implants for repair of full-thickness defects of articular cartilage: an experimental study in rabbit and dog. Biomaterials 1992;13:625–634.
10. Kon M, de Visser AC. A poly (HEMA) sponge for restoration of articular cartilage defects. Plast Reconstr Surg 1981;67:288–294.
11. Cobey MC. Arthroplasties using compressed ivalon sponge ('intra-medic sponge') long-term follow-up studies in 109 cases. Clin Orthop 1967;54:139–144.
12. Corkhill PH, Trevett AS, Tighe BJ. The potential of hydrogels as synthetic articular cartilage. Proc Inst Mech Eng (H) 1990; 204:147–155.
13. Corkhill PH, Fitton JH, Tighe BJ. Towards a synthetic articular cartilage. J Biomater Sci Polym Ed 1993;4:615–630.
14. Yasui N, Osawa S, Ochi T, Nakashima H, Ono K. Primary culture of chondrocytes embedded in collagen gels. Exp Cell Biol 1982;50:92–100.
15. Kimura T, Yasui N, Ohsawa S, Ono K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. Clin Orthop 1984;186:231–239.
16. Grandolfo M, D' Andrea P, Paoletti S, Martina M, Silvestrini G, Bonu Vitter F. Culture and differentiation of chondrocytes entrapped in alginate gels. Calcif Tissue Int 1993;52:42–48.
17. Loty S, Sautier JM, Loty C, Boulekbache H, Kokubo T, Forest N. Cartilage formation by fetal rat chondrocytes cultured in alginate beads: a proposed model for investigating tissue-biomaterial interactions. J Biomed Mater Res 1998;42:213–222.
18. Perka C, Spitzer RS, Lindenhayn K, Sittinger M, Schultz O. Matrix-mixed culture: new methodology for chondrocyte culture and preparation of cartilage transplants. J Biomed Mater Res 2000;49:305–311.
19. Meinhart J, Fussenegger M, Hobling W. Stabilization of fibrin-chondrocyte constructs for cartilage reconstruction. Ann Plast Surg 1999;42:673–678.
20. Perka C, Schultz O, Lindenhayn K, Spitzer RS, Muschik M, Sittinger M, Burmester GR. Joint cartilage repair with transplantation of embryonic chondrocytes embedded in collagen-fibrin matrices. Clin Exp Rheumatol 2000;18:13–22.
21. Perka C, Schultz O, Spitzer RS, Lindenhayn K, Burmester GR, Sittinger M. Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials 2000;21:1145–1153.
22. George-Weinstein M, Gerhart JV, Foti GJ, Lash JW. Maturation of myogenic and chondrogenic cells in the presomitic mesoderm of the chick embryo. Exp Cell Res 1994;211:263–274.
23. Nelson JF, Standford HG, Cutright DE. Evaluation and comparisons of biodegradable substances as osteogenic agents. Oral Surg Oral Med Oral Pathol 1977;43:836–843.
24. Hollinger JO. Preliminary report on the osteogenic potential of a biodegradable copolymer of polylactide (PLA) and polyglycolide (PGA). J Biomed Mater Res 1983;17:71–82.
25. Freed LE, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer F. Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. J Biomed Mater Res 1993;27:11–23.
26. Lohmann CH, Schwartz Z, Niederauer GG, Carnes DL Jr, Dean DD, Boyan BD. Pretreatment with platelet derived growth factor-BB modulates the ability of costochondral resting zone chondrocytes incorporated into PLA/PGA scaffolds to form new cartilage in vivo. Biomaterials 2000;21:49–61.
27. Lu L, Peter SJ, Lyman MD, Lai HL, Leite SM, Tamada JA, Uyama S, Vacanti JP, Langer R, Mikos AG. In vitro and in vivo degradation of porous poly(DL-lactic-*co*-glycolic acid) foams. Biomaterials 2000;21:1837–1845.
28. Cowman MK, Li M, Balazs EA. Tapping mode atomic force microscopy of hyaluronan: Extended and intramolecularly interacting chains. Biophys J 1998;75:2030–2037.
29. Greco RM, Iocono JA, Ehrlich HP. Hyaluronic acid stimulates human fibroblast proliferation within a collagen matrix. J Cell Physiol 1998;177:465–473.
30. Culty M, Nguyen HA, Underhill CB. The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. J Cell Biol 1992;116:1055–1062.
31. Heldin P, Laurent TC, Heldin CH. Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts. Biochem J 1989;258:919–922.
32. Ehlers E-M, Behrens P, Wünsch L, Kühnel W, Russlies M. Effects of hyaluronic acid on the morphology and proliferation of human chondrocytes in primary cell culture. Ann Anat 2000;183:13–17.
33. Kawasaki K, Ochi M, Uchio Y, Adachi N, Matsusaki M. Hyaluronic acid enhances proliferation and chondroitin sulphate synthesis in cultured chondrocytes embedded in collagen gels. J Cell Physiol 1999;179:142–148.
34. Buckwalter JA, Hunziker EB. Orthopaedics. Healing of bones, cartilages, tendons, ligaments: A new era. Lancet 1996;348:S1118.
35. van Susante Job LC, Pieper J, Buma P, van Kuppevelt TH, van Beuningen H, van der Kraan PM, Veerkamp JH, van den Berg WB, veth Rene PH. Linkage of chondroitin-sulfate to type I collagen scaffolds stimulates the bioactivity of seeded chondrocytes in vitro. Biomaterials 2001;22:2359–2369.
36. Bassleer CT, Combal JPA, Bougaret S, Malaise M. Effects of chondroitin sulphate and interleukin-1 on human articular chondrocytes cultivated in clusters. Osteoarthr Cartil 1998;6: 196–204.
37. Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, Freed LE. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. J Orthop Res 1999;17:130–138.
38. Rahman MS, Tsuchiya T. Enhancement of chondrogenic differentiation of human articular chondrocytes by biodegradable materials. Tissue Eng 2001;7:781–790.
39. Tsuchiya T, Ikarashi Y, Arai T, Ohhashi J, Nakamura A. Improved sensitivity and decreased sample size in a cytotoxicity test for biomaterials: a modified colony microassay using a microplate and crystal violet staining. J Appl Biomater 1994;5: 361–367.
40. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–895.
41. Fukuda K, Dan H, Takayama M, Kumano F, Saitoh M, Tanaka S. Hyaluronic acid increases proteoglycan synthesis in bovine

articular cartilage in the presence of interleukin-1. J Pharmacol Exp Ther 1996;277:1672–1677.
42. Vunjak-Novakovic G, Obaradovic B, Martin I, Freed LE. Bioreactor studies of native and tissue engineered cartilage. Biorheology 2002;39:259–268.
43. Loty S, Foll C, Forest N, Sautier JM. Association of enhanced expression of gap junctions with in vitro chondrogenic differentiation of rat nasal septal cartilage-released cells following their dedifferentiation and redifferentiation. Arch Oral Biol 2000;45: 843–856.
44. Schwab W, Hofer A, Kasper M. Immunohistochemical distribution of connexin 43 in the cartilage of rats and mice. Histochem J 1998;30:413–419.
45. Donahue HJ, Guilak F, Vander Molen MA, McLeod KJ, Rubin CT, Grande DA, Brink PR. Chondrocytes isolated from mature articular cartilage retain the capacity to form functional gap junctions. J Bone Miner Res 1995;10:1359–1364.
46. Neumann A, Schinzel R, Palm D, Riederer P, Münch G. High molecular weight hyaluronic acid inhibits advanced glycation endproduct-induced NF-κB activation and cytokine expression. FEBS Lett 1999;453:283–287.
47. Park JU, Tsuchiya T. Increase in gap-junctional intercellular communications (GJIC) of normal human dermal fibroblasts (NHDF) on surfaces coated with high-molecular-weight hyaluronic acid (HMW HA). J Biomed Mater Res 2002;60:541–547.
48. Park JU, Tsuchiya T. Increase in gap junctional intracellular communication by high molecular weight hyaluronic acid associated with fibroblast growth factor 2 and keratinocyte growth factor production in normal human dermal fibroblasts. Tissue Eng 2002;8:419–427.
49. Nevo Z, Dorfman A. Stimulation of chondromucoprotein synthesis in chondrocytes by extracellular chondromucoprotein. Proc Natl Acad Sci USA 1972;69:2069–2072.
50. Saldanha V, Grande DA. Extracellular matrix protein gene expression of bovine chondrocytes cultured on resorbable scaffolds. Biomaterials 2000;21:2427–2431.
51. Freed LE, Martin I, Vunjak-Novakovic G. Frontiers in tissue engineering. In vitro modulation of chondrogenesis. Clin Orthop Relat Res 1999;367:S46–S58.
52. Gooch KJ, Kwon JH, Blunk T, Langer R, Freed LE, Vunjak-Novakovic G. Effects of mixing intensity on tissue-engineered cartilage. Biotechnol Bioeng 2001;72:402–407.

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

Received 21 January 2007; revised 27 September 2007; accepted 11 December 2007
Published online 00 Month 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.32018

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 oliogodendrocytes.[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, 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

Contract grant sponsors: Japanese Ministry of Health, Labour and Welfare, The Japan Health Sciences Foundation