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図2●次世代医療機器評価指標検討会の5分野

ェック項目を明らかにしていこうと考えています．

ただ，この分野はまだ確立した評価法がありませんし，未知のリスクや有効性が出てきて，確認の手技手法も新しく出てくるのではないかと思います．そこで，現時点でどこまで確認できるのかを研究者の皆さまにもお考えいただいて，あまり硬直した指針にならないように，その時点での最新の科学の目で評価していただけるようにもっていきたいと思います．あまり画一的な方法をガイドラインとして示す必要はないと思っています．むしろ技術の進歩の方が早いので，その時々で最適な評価をしていくように心がけなくてはいけないと思います．

2005年11月に，EUは再生医療に対して新しい規制を提唱しました．まだファイナルにはなっていませんが，これまではEU全域としての規制がなかったため，産業化を推進するためにも明確な安全性評価の体制をとるべきだという議論が出て今回の提唱に繋がったとされています．米国でもFDAが1990年代後半から枠組みを作り始めて安全性の評価を進めています．きちんとした安全性の評価体制があってこそ世の中に認知されるし，社会の役に立っていくのだと思います．

土屋　もう1つのトピックは，ISO150という外科用インプラントの国際標準化技術委員会があるのですが，そこでは整形外科から循環器までの色々な用具に対して，個別にどういったスタンダードを作るかと議論しています．ここにWG11という組織工学製品のワーキンググループ（WG）がありますが，現在日本より，WGからサブコミッティ（SC）への格上げの提案をしています．このSCを作ればその下にいくつもWGが作れますし，また日本が幹事国になれる．そういった国際的な場に日本の標準文書をたくさん持っていけば，世界との調和が取れて，製品化を進めやすくなると思います*2．

次世代医療機器の展望

俵木　平成17年から厚生労働省は経済産業省と連携して次世代医療機器評価指標検討会／医療機器開発ガイドライン評価検討委員会を設置しました．5つの分野（図2）について開発の推進と審査の迅速化を目指して新たなガイドラインをつくり，それを通知していくという試みで，審査WGを土屋先生に推進していただいています．ただ，やはり次世代医療機器もまだ評価指標が固まっているわけではありませんので，評価をしてくださる先生方も方法論が難しい分野だと思います．その内容が硬直したものになってしまうと，逆に開発を阻害するようなことにもなるので，緩やかな評価指標を世の中にお示しして，開発や審査の参考になるようなものを示していく必要があるのだと思います．

―あまり自由度があり過ぎると，現場が不安に思うことはないのでしょうか？

俵木　実際の審査を行っている医薬品医療機器総合機構では，相談制度を設けています．例えば，治験の前のプロトコールの内容についての相談であるとか，申請に当たって必要となる試験の相談などです．医療機器の相談制度自体は平成16年からスタートしていますが，まだまだ活用しにくいところがあるので，色々な相談のコースをつくって，開発の各段階で活用いただきたいと思います．事前に審査サイドとディスカッションしてから申請に持ってきていただければ，審査が非常にスムーズにいくのではないかと思います．FDAでもやはり承認申請の前の段階，あるいは治験の前の段階でかなり企業とコンサルテーションを行っていて，申請から承認までの期間を短くすることができたと言われています．

土屋　心筋シートの次世代評価指標作成事業審査WGであった話なのですが，細胞の生存率が例えば80％以上でないといけないという限定的な指標であった場合，それならば70％はだめなのか？　という問題が出てきます．何かが多少混ざっていたとしても，それが別の作用をしているかもしれません．器官は色々な細胞のミクスチャ

＊2：追加情報
インタビュー後，日本の提案が可決され，TC150WG11はSC7に格上げとなった．

ーで構成されています．フィーダー細胞の役割をしたり，サイトカインを出していたり，複合化しているのです．そういう意味で，明確な数値は避け，今の知見に基づいて科学的・合理的な説明ができればいいということにしたいと思います．実際 FDA では，具体的な数値が設定されていたためにある製品の開発が止まっているという話も聞いています．

それから材料開発という意味では，従来の医療機器は工業製品を使っていました．ステンレスやチタンなどです．しかし今は，色々な生体成分を作れるようになった．そしてそれがどういうふうに反応するかのメカニズムがわかる時代になってきました．だからこそ，今までのように，製品を作ってから厚生労働省に申請するために安全性試験をするという流れではなく，最初に材料をセレクションする過程で安全性評価を踏まえることが大切になるのです．なぜ安全性が低いのかがメカニスティックにわかれば，今度はそれを有効性に置き換えるにはどうすれば良いかと次のステップが考えられるようになります．我々はその過程を経て新しいセラミクスを開発し，国際特許にも出しています．

新たな医療機器の可能性

ー次世代医療機器検討会では５つのテーマが進められていますが，今後さらに新しい６つ目のテーマが出てくる可能性はあるのでしょうか？

俵木　検討すべきテーマがあれば５つで終わりということにはならないと思います．

土屋　この間すごいと思ったのは，国立循環器病センターの杉町　勝先生のバイオニック臓器のお話でした．杉町先生の発想は，先端を行っておられるという感じがしましたね．あのような研究を我々が協力して上手く軟着陸させることができればと思っています．

それから先ほども言いましたように，ペースメーカーなどは日本も非常に技術があるので，日本の企業が日本人に合ったより良いものを出していただきたいと思います．企業間で連携して進めないと，これからの医療機器開発は進まないと思います．プラスチックだけを入れるのではなく，その治療部位に薬を投与した方がさらに効果が上がるのは，どなたでも理解しやすいですよね．逆に，薬を体内に大量に投与しても目的の部位に行き着く量は少ないですが，医療機器だとまさにその部位に効率良く入れることができる．そう言った意味で，これからはコンビネーションの医療機器が増えてくると思いますし，薬の業界がもっと参入する必要があると感じています．じつは世界的にもコンビネーション医療機器は進められていて，ISO の中にもコンビネーション WG ができつつあります．また，先日のバイオマテリアル学会の会場で，Johnson&Johnson 社の方に未来志向バイオマテリアルの世界戦略を発表していただきましたが，あらゆるものに薬と細胞のコンビネーションを使うと講演されていました．彼らは現在，心筋梗塞時のステント治療で遅発性に生じる血栓を溶かすために，さらに抗血栓剤を２段構えで入れることを考えているそうです．また，合金を使うのであればノンアレルギーの金属をコーティングすれば良いのではないかなど，様々な改良や開発品ができてくると思いますし，今まさにその転換期を迎えていると感じています．

俵木　５分野だけではないさらに新たな次世代の医療機器開発に向けて，今後も評価指標を作る必要があると思います．何を評価すればいいのかという指標を作っていくことが，患者さんの手元に有用な医療機器を早くお届けすることに繋がるのではないでしょうか．

土屋　次世代医療機器の会議を始めてから，非常に医療機器の研究開発が進んでいるように感じます．コミュニケーションは非常に大切ですね．

俵木　やはり情報や認識の共有は重要だと思います．開発する方々も，相手は何を考えているのかな，どこまでやればいいのかな，と手探りの状態で進めるのは相当大変なことですし．皆がこういうことを心配しているのだ，という共通認識を持って進めていっていけたらと思います．

土屋　いま，半年に１回くらい新しい技術が出ています．最近も，間葉系幹細胞が無血清培地で培養できるという厚生労働科学研究費の再生医療研究班の成果が報告されました．これを製品化するには，製造工程から様々な手段に従ってやらなくてはいけませんので，小さい会社だけではなく大手の製薬企業にも協力して進めてもらう必要があります．産業と行政と研究の連携が，これからさらに大きく変わっていくと思います．

ー貴重なお話をありがとうございました．

（インタビュー実施　2007 年 1 月）

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

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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-

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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

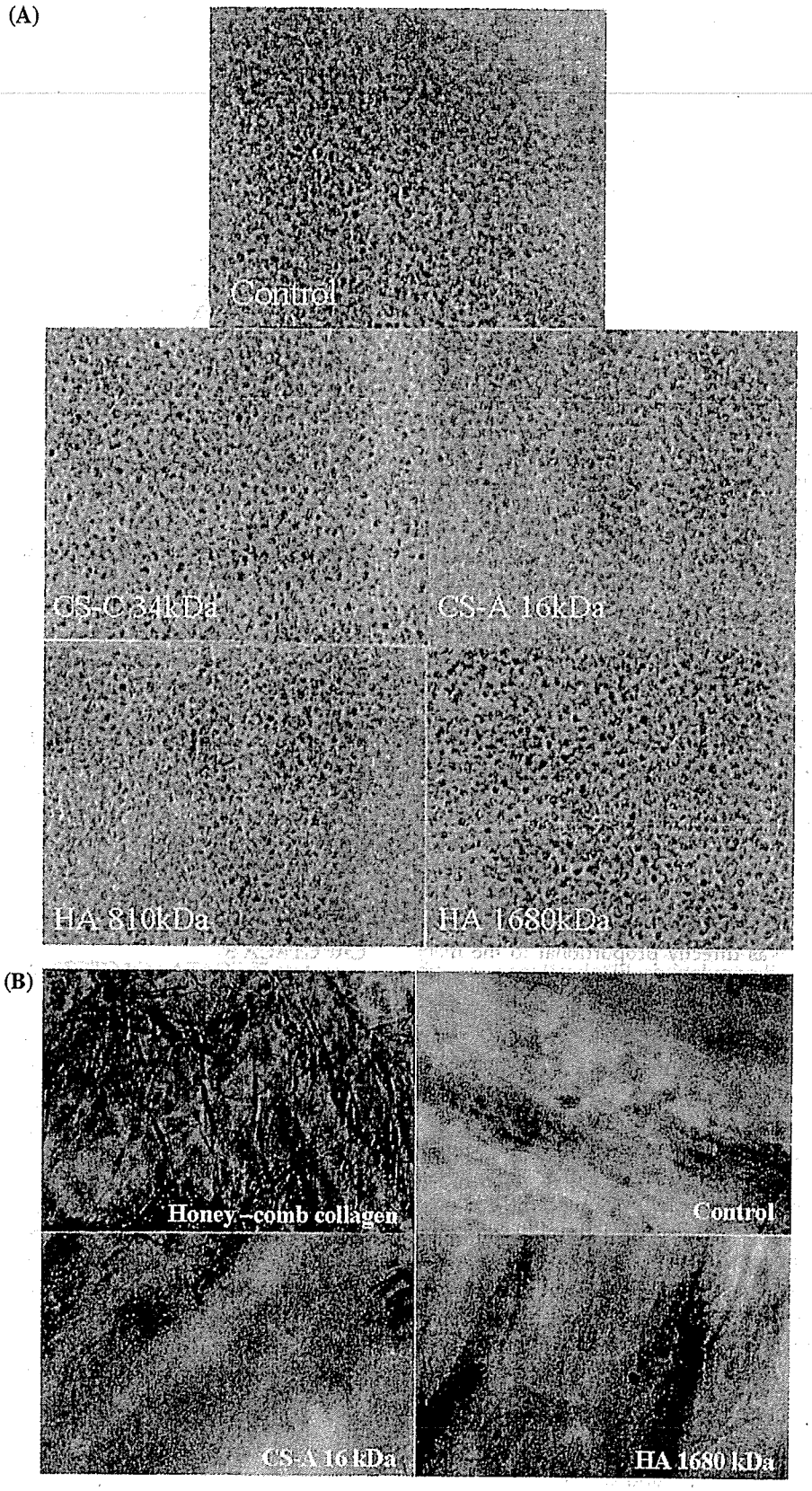

Figure 1. Light microscopic appearance of cultured HC in (A) micromass and (B) 3-D honeycomb rotation conditions spotted as high density cultures treated with different molecular weights and molecular structures of HA and CS for 4 weeks (after alcian blue staining, original magnification ×200).

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).

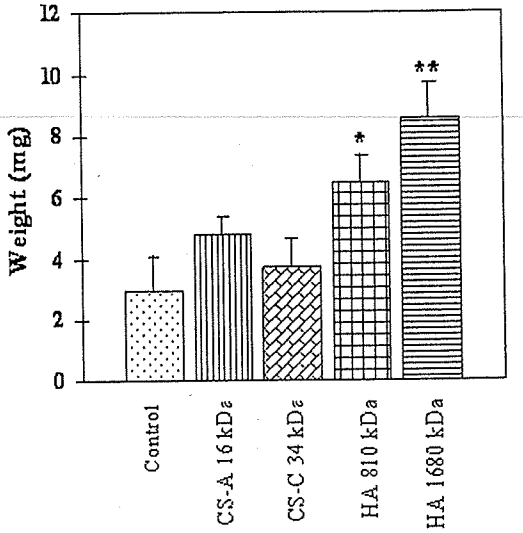

Figure 2. Wet weight of tissue constructs obtained by culturing HC on collagen honeycomb in rotation condition and treated with different molecular weights and structures of HA and CS for a period of 4 weeks. HA 810 kDa (*$p < 0.05$), and HA 1680 kDa (**$p < 0.01$) are significantly different compared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

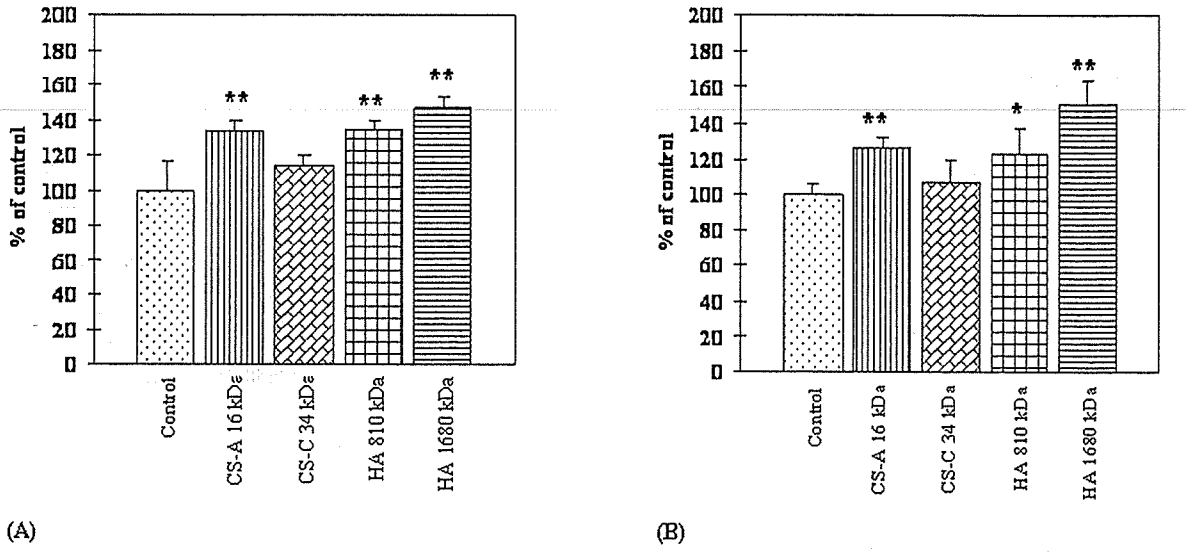

Figure 3. Cell proliferation of HC estimated by alamar blue method. (A) Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. CS-A 16 kDa, HA 810 kDa, and HA 1680 kDa are significantly different compared with control (**$p < 0.01$). (B) In rotation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

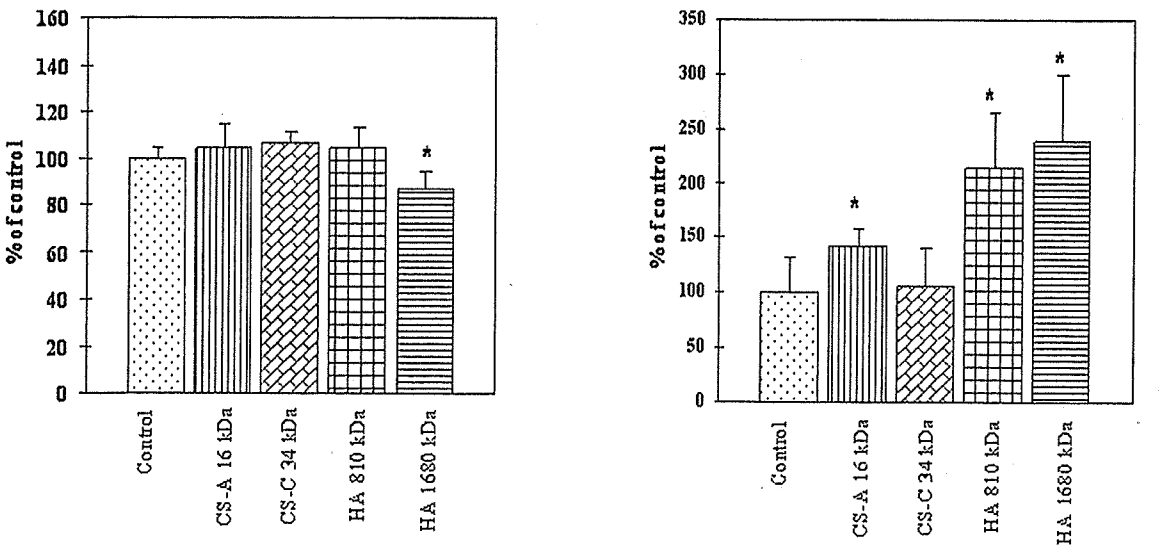

Figure 4. Cell differentiation of HC estimated by alcian blue method. (A) Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. HA 1680 kDa is significantly different compared with control (*$p < 0.05$). (B) In rotation conditions, cultures were treated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

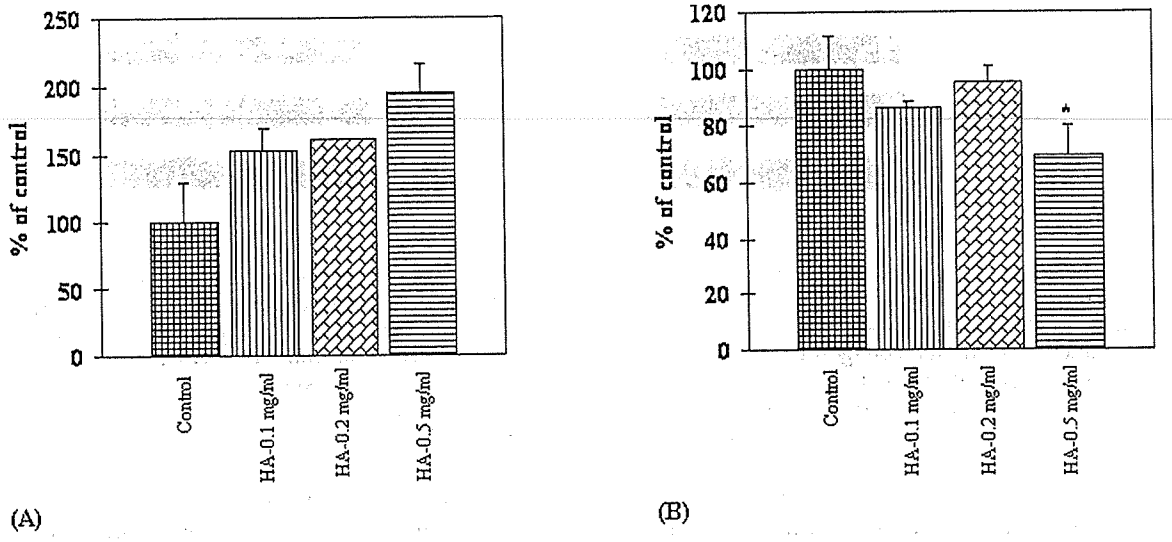

Figure 5. Chondrogenic effect of HA (1680 kDa) with different concentrations under micromass conditions for 4 weeks. (A) As demonstrated by crystal violet staining, cell proliferation was increased with 0.1, 0.2, and 0.5 mg/mL of HA 1680 kDa respectively, compared with the control culture. (B) Cell differentiation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

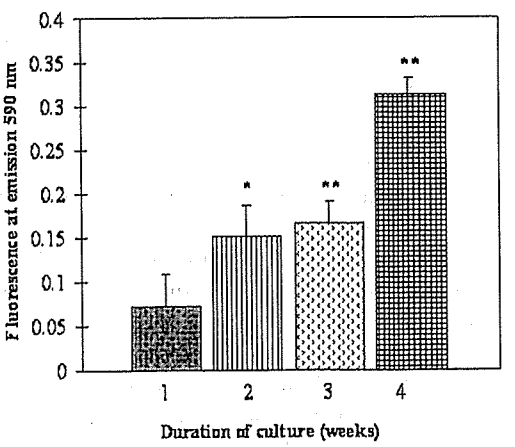

Figure 6. Time course of cell proliferation under micromass conditions for 4 weeks, estimated by crystal violet staining. Cell proliferation was significantly increased after 2 weeks (*$p < 0.05$), 3 weeks (**$p < 0.01$), and 4 weeks (**$p < 0.01$) of culture, compared with the chondrocytes cultured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

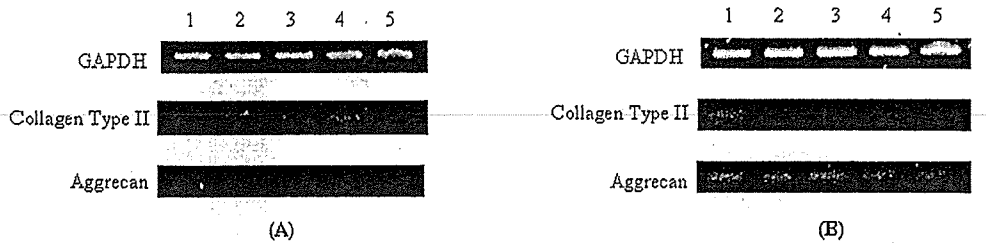

Figure 7. (A) Reverse transcription-polymerase chain reaction amplification of GAPDH, collagen type II, and aggrecan in cultures of HC with four different types of CS and HA for 4 weeks in micromass conditions. Lane 1, control; lane 2, CS-A 16 kDa; lane 3, CS-C 34 kDa; lane 4, HA 810 kDa; and lane 5,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

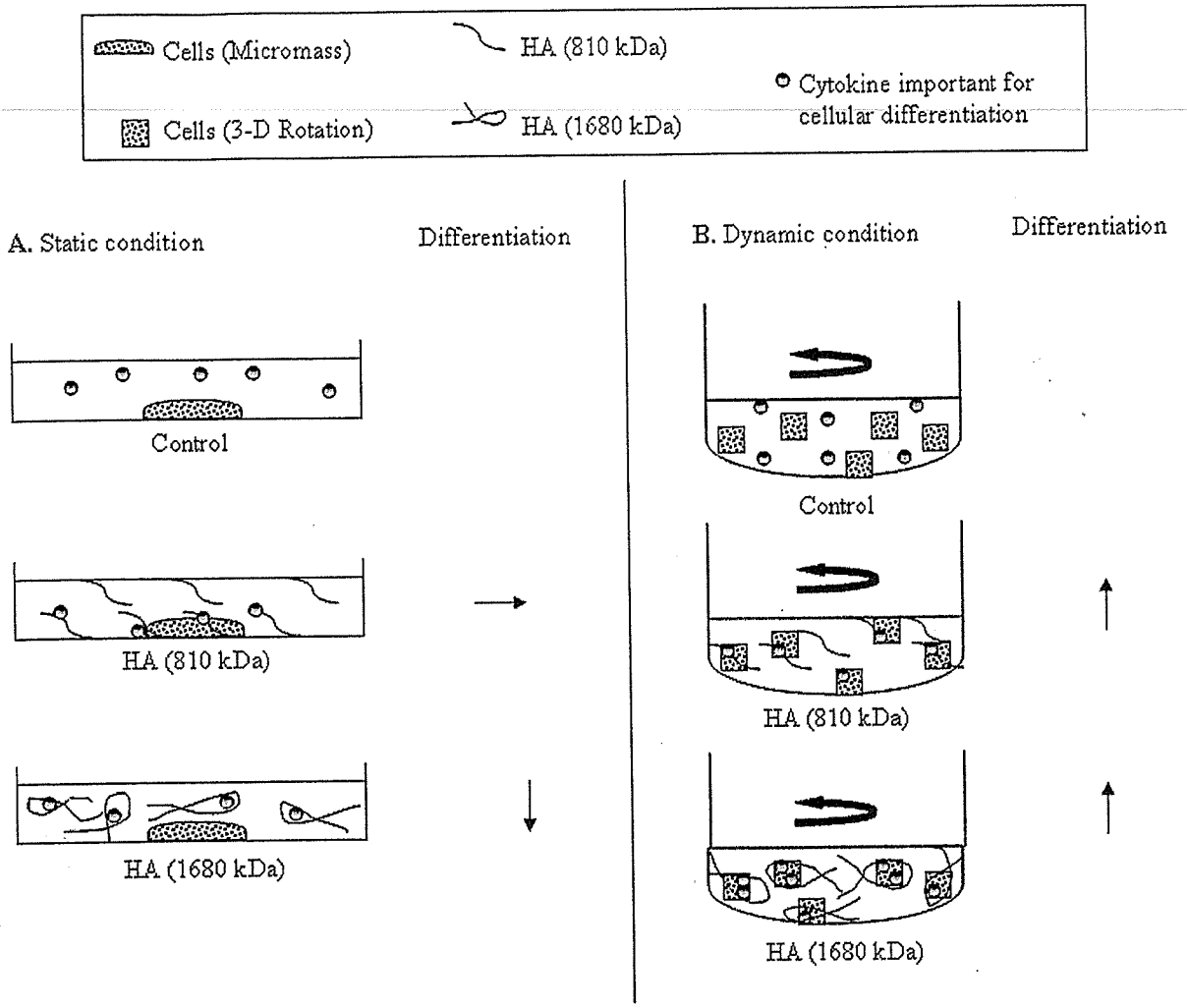

Figure 8. Schematic representation of the effects of different molecular weights of HA on the differentiation of HC. (A) Static condition (micromass). (B) Dynamic condition (rotation culture).

(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.

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Key Engineering Materials Vols. 342-343 (2007) pp. 853-856
online at http://www.scientific.net

A Novel Non-Destructive Method for Measuring Elastic Moduli of Cultivated Cartilage Tissues

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Keywords: Mechanical Property, Bulk Modulus, Volume, Pressure, Human Articular Chondrocyte, Cultivated Cartilage

Abstract. Accurate measurement of the mechanical properties of artificial or cultivated cartilage is a major factor for determining successive regeneration of defective soft tissues. In this study, we developed a novel method that enabled the bulk modulus (k-modulus) to be measured nondestructively using the relationship between volume and pressure of living soft tissues. In order to validate this method we estimated the bulk modulus of soft silicone rubbers using our new method and a conventional method. The results showed a 5 ~ 10% difference between the results obtained with the two methods. Our method was used subsequently to measure the mechanical properties of cultivated cartilage samples (collagen gel type), that had been incubated for four weeks in the presence or absence of human articular chondrocytes (HACs). Our experiments showed that cultivated cartilage tissues grown in the presence of HACs had a higher bulk modulus (120 ± 20 kPa) than samples grown without HACs (90 ± 15 kPa). The results indicated that our novel method offered an effective method for measurement of volume changes in minute living soft tissues, with the measurements having a high degree of accuracy and precision. Furthermore, this method has significant advantages over conventional approaches as it can be used to rapidly and accurately evaluate the strength of soft tissues during cultivation without causing damage to the specimen.

Introduction

Recently, artificial cartilage, obtained by cultivation with human articular chondrocyte (HACs) and mesenchymal stem cell (MSCs) on collagen scaffolds, has provided a method with the potential to regenerate damaged articular cartilage [1, 2]. In order to achieve successfully regeneration of damaged soft tissues, it is well established that biomechanical function and the biological construct of the artificial tissues play an important role in the tissue engineering [3-5]. For this reason, many experimental approaches have been used to assess the mechanical properties of factitious soft tissues. Some researches recently proposed a method for measuring mechanical properties with micro-needles or other specially designed devices [6-8]. However, using these approaches it has proved very difficult to accurately assess the mechanical properties of biological materials, such as skeletal muscles, cartilage, or other soft tissues including artificial tissues. This is due to the technical difficulties associated with non-constructive measurements, in addition to the irregular geometries of living tissues. In spite of these practical limitations, mechanical assessments are crucial when determining the maturity of cultivated soft tissues for transplantation and to ensure the success of regenerative medicine. We therefore developed a novel method that enabled the bulk modulus of elasticity (modulus of volume elasticity) to be measured rapidly, using the relationship

between volume and pressure of living soft tissues. This method was then applied to measure the elastic modulus of cartilage cultivated for four weeks on collagen scaffolds with and without human articular chondrocyte (HACs).

Materials & Methods

Theory and Experiment. Figure 1 shows a diagrammatic illustration of our device, which was used in a pressure pot under different air pressures. The system was composed of Chambers 1 and 2, a pressure sensor, an A/D converter and an air compressor. The sample was placed in Chamber 1, with Chamber 2 acting as a reference for the device. The pressure sensor detected the difference in pressure (ΔP) produced when a pressure change occurred in Chamber 1. The volume change (ΔV) was expressed as an electronic signal using a FFT (Fast Fourier Transform) analyzer as shown in Fig. 1-(A). In order to calculate the relationship between volume (V) and pressure (P), we used the classical equation of the relationship between volume and pressure [6]. If the volume of the sample changed (V_x) in chamber 1, the equation (1) can be written as:

$$\Delta P_1 - \Delta P_2 = \frac{nP_1\Delta V_1}{V_1 - V_X} - \frac{nP_2\Delta V_2}{V_2} \quad . \qquad (1)$$

where P_1, V_1 are the pressure and volume in chamber 1, respectively, and P_2, V_2 are the pressure and volume in chamber 2, respectively. n represents the number of the polytropic index. When the pressure increases, ΔP is inserted into the equation (1). The realtionship between pressure and volume in this system can be rewritten as equestion (2).

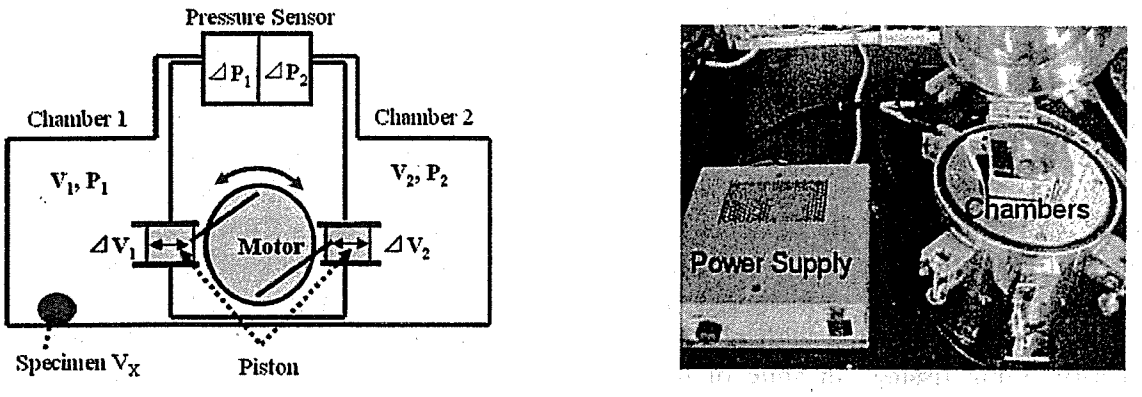
$$\Delta P_1 - \Delta P_2 = \frac{n(P+\Delta P)\Delta V}{V_1 - V_X} - \frac{n(P+\Delta P)\Delta V}{V_2} = n(P+\Delta P)\Delta V\left(\frac{1}{V_1 - V_X} - \frac{1}{V_2}\right) \qquad (2)$$

The bulk modulus is calculated and converted by the following equation (3), (4).

$$k = P \ / \varepsilon_v \qquad (3)$$

$$k = E / 3\,(1 - 2\nu) \qquad (4)$$

where k, P, ε_v, E, and ν are the bulk modus, pressure, volume strain, elastic modulus, and possion ratio, respectively.



(A) (B)

Fig. 1 Schematic diagram (A) and photograph (B) of the novel testing device using the principle of the relationship between pressure and volume changes

Validation of New Testing Method. Before calculating the bulk modulus of the samples, the relationship curves between the known volumes and pressure changes between 0 ~ 120 kPa in this system were first defined using stainless steel balls. Soft silicone rubber, which had similar mechanical properties to human soft tissues, was then used to validate our specially-designed device. The elastic modulus of the silicone rubbers was measured by two methods: 1) the conventional dynamic elastic modulus (E') test and 2) our method. In the dynamic test, the silicone samples were loaded with a cyclic strain of amplitude 0.5% at a frequency of 0.5 ~ 100 Hz by Rheogel-E4000 (UBM Co., Japan). The dynamic elastic moduli at 1 Hz were converted to bulk moduli using equation (4). The two bulk moduli calculated using the conventional dynamic test and our method were then compared using the statistical t-test ($p < 0.05$).

HACs and Culture Methods. Two types of the cultivated cartilage grown with HACs (+) and without HACs (−) on collagen scaffolds were used to measure the bulk modulus. The artificial cartilages were prepared by the following process [2]. HACs of the knee joint were commercially obtained from BioWhittaker (Walkersville, USA) and cultured in chondrocyte growth medium (Walkersville, USA). After the collagen scaffold (BD Science, USA) was placed in a 24-well tissue culture micro-plate (Coring, USA), high-density micromass cultures were started by seeding 4×10^4 HACs in 20 μL of medium onto the collagen scaffold. After a 2 h attachment period in a 5% CO_2 incubator at 37°C, 1 mL of culture medium was added to each well. The cultures were then incubated for a further 4 weeks with the medium being changed twice weekly. Figure 3-(A) shows samples of the cultivated cartilages on the collagen scaffolds grown with and without HACs.

Results & Discussions

In order to validate this method, we mesured the bulk modulus of soft silicone rubber using our proposed method and the conventional method, The results were then compared. The results obtained using the novel method (409 ± 14 kPa) compared favorably with those obtained using the conventional biomechanical measurements (417 ± 22 kPa). As shown in Table 1, this result represented a mere 5 ~ 10% difference between the two methods, thereby confirming the accuracy of our new method. We found no significant difference between the two measuring methods in two types of silicone ($p > 0.05$).

We then applied our method to measure the bulk moduli of cultivated cartilage samples incubated for four weeks in the presence or absence of HACs. Figure 2 shows the relationship between volume and pressure changes of the cultivated cartilages. The volume of the cultivated cartilages was decreased with increasing pressure. As shown in Fig. 3-(B), cultivated cartilage tissues grown in HACs had a higher bulk modulus (120 ± 20 kPa) than samples grown without HCAs (90 ± 15 kPa). This differenence between the two samples of cultivated cartilage was statistically significant ($p < 0.05$). These findings indicated that growing cells such as HACs could increase the mechanical property of cultivated cartilages. Although the results showed a lower elastic modulus than that reported for normal human articular cartilage of 0.3 ~ 1.5 MPa, the lower mechanical property of an initial artifical cartilage would be suitable for assimilation around normal living cartilage [8,9].

Table 1 Comparison of the elastic moduli calculated using elastic modulus and our novel method

Samples	Bulk Modulus by Conventional Method	Bulk Modulus by Our Novel Method
Silicone gel (n=3)	176 ± 34 [MPa]	120 ± 20 [MPa]*
Silicone rubber (n=3)	417 ± 22 [kPa]	409 ± 14 [kPa]**

*, **: were converted with $\nu = 0.48$ and $\nu = 0.42$

Conclusions

On the basis of these results, it can be concluded that our novel method offers an effective method for measurement of the biomechanical properties of artificial or cultivated soft tissues as well as living soft tissues. The method has a high degree of accuracy and precision. In addition, the method can be used for rapid and accurate evaluation of changes in strength of soft tissues during cultivation without causing damage to the specimen.

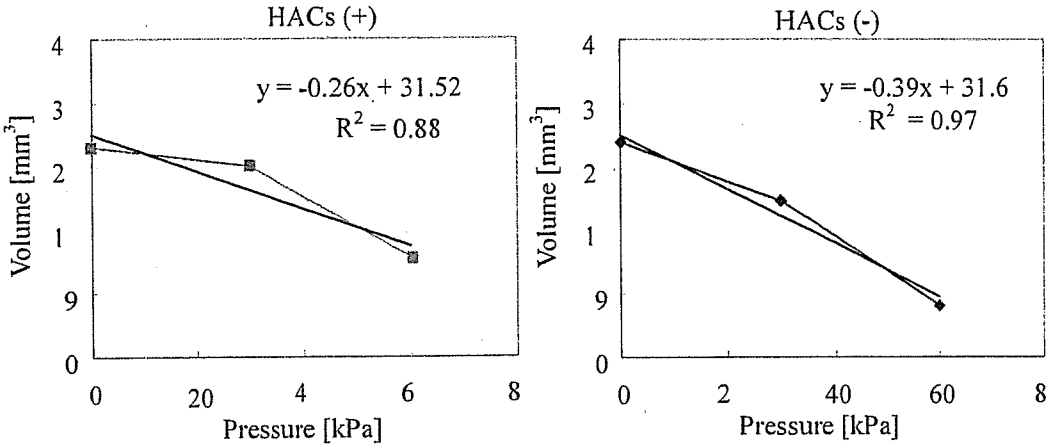


Fig. 2 Comparison of volume changes in relationship to changes in pressure in two types of cultivated cartilages grown with HACs (+) and without HACs (-)

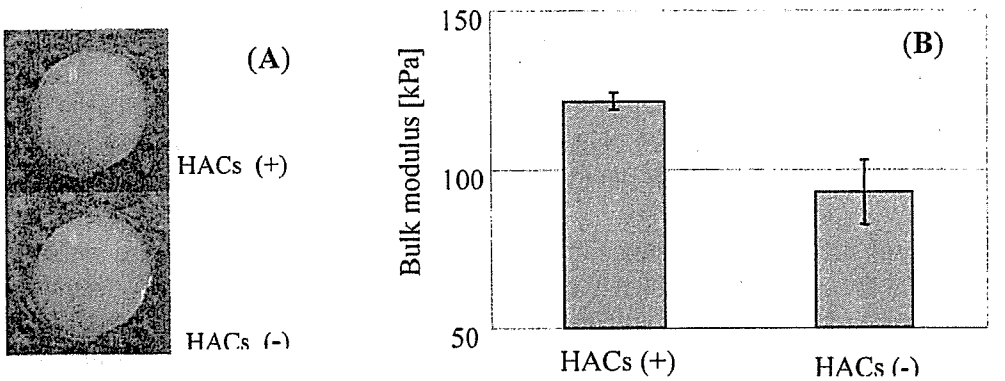


Fig. 3 Samples (A) and bulk moduli (B) of the cultivated cartilages grown with HACs and without HACs on collagen scaffolds for four weeks

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Biomaterials 28 (2007) 844–850
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ffects of sulfated hyaluronan on keratinocyte differentiation and Wnt and Notch gene expression

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Received 3 July 2006; accepted 24 September 2006

ıstract

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

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

1. Introduction

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

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

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

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

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doi:10.1016/j.biomaterials.2006.09.041

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

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

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

2. Materials and methods

2.1. Sulfated hyaluronan

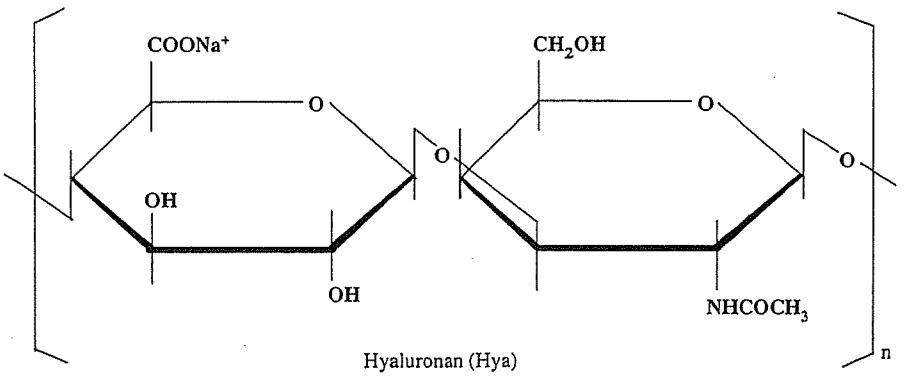
SHya was prepared by the method reported previously [4]. A solution of 2% Hya120 (molecular weight, 1.2×10^6) solution in *N*,*N*-dimethylformamide (DMF) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was mixed with trimethylamine (TMA)–SO_3 complex (Aldrich Chemical Co., Inc., Milwaukee, WI, USA)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

2.2. Cell culture

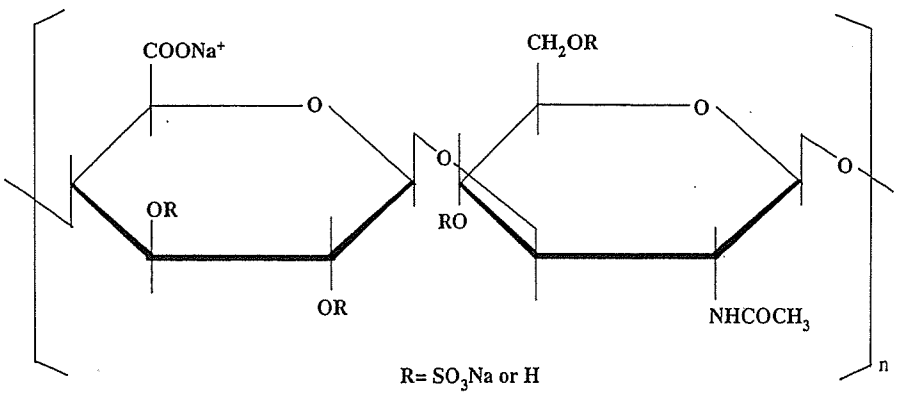
NHEKs isolated from neonatal human foreskins (Cambrex Bioscience, Walkersville, MD, USA) were cultured with K-110 Type II medium (Kyokuto, Tokyo, Japan) supplemented with 2% whole bovine pituitary extract, 50 IU penicillin G, 50 μg/ml streptomycin, and 0.03 mM $CaCl_2$ (low-calcium condition) at 37 °C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

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

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



Hyaluronan (Hya)



Sulphated hyaluronan (SHya)

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

4. Determination of cell numbers by crystal violet assay

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

5. Quantitative real time RT-PCR

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

2.6. Statistical analysis

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

3. Results

3.1. Adhesiveness of NHEKs to SHya

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

3.2. Effect of SHya coating on keratinocyte proliferation

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

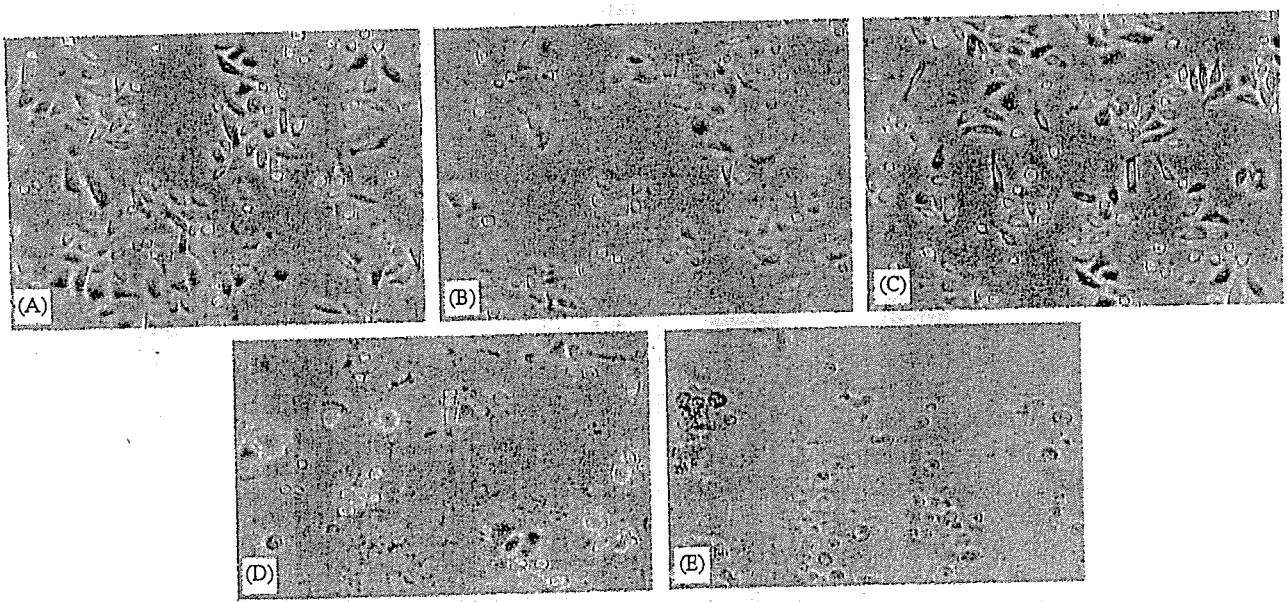


Fig. 2. The adhesion of NHEKs to SHya-coated surfaces. NHEKs were seeded at 1.0×10^4 cells/cm^2 onto SHya- and Hya-coated 24-well plates. Then NHEKs were incubated with non-coated (A), 0.4 mg/cm^2 SHya-coated (B), 0.8 mg/cm^2 SHya-coated (C), 0.4 mg/cm^2 Hya-coated (D), and 0.8 mg/cm^2 Hya-coated (E)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