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関節軟骨の再生医療

ティッシュエンジニアリング　2006

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Regeneration of articular cartilage defect

関節軟骨欠損の治療法として，約1世紀前に同種骨軟骨移植，数十年前に骨髄刺激法，1990年代になりモザイクプラスティーおよび自己軟骨細胞移植が開発された．しかしながら，いまだに確立された軟骨修復法は存在せず，より有効な方法の開発が望まれている．

近年，前駆細胞，特に骨髄間葉系細胞が注目されている．筆者らが骨髄間葉系細胞移植でヒト関節軟骨欠損修復を行ったところ，修復は促進されたが完全な硝子軟骨による修復は得られなかった．骨髄間葉系細胞に成長因子投与，あるいは遺伝子を導入する研究が行われている．ほかにも，羊膜細胞，筋肉サテライト細胞，脂肪細胞，滑膜細胞，胚性幹細胞などからの軟骨再生研究が行われているが，臨床に応用できるものはいまだにごく少数である．

軟骨欠損修復の臨床的問題点としては，自然経過が明らかでないこと，あるいは評価方法が確立されていないことであり，これらを明らかにすることが重要である．

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Key words：軟骨欠損自然経過，軟骨細胞移植，前駆細胞移植，成長因子，遺伝子導入

関節軟骨は可動関節の相対する骨表面を覆い，軟骨下骨にかかる外圧を分散・吸収するショックアブソーバー，および関節表面の摩擦係数を低下させ滑動性をよくする役割を持っている．関節軟骨は疎な軟骨細胞と豊富な軟骨基質からなり，軟骨基質が無構造で硝子のようであることから組織学的に硝子軟骨とよばれる．関節軟骨は，血管，神経，リンパを欠く．軟骨基質は含水性に富み約70%が水分である．血管を持たない関節軟骨は関節液からの拡散により栄養されており，荷重による関節軟骨圧縮時の関節液の移動により，関節軟骨細胞の栄養が促進される．

関節軟骨の修復能力は非常に弱い．その原因として血流が乏しいこと，細胞周囲に密度の高い基質が存在していること，あるいは軟骨細胞自体が高度に分化しておりほとんど分裂増殖しないこと，などが考えられている．

関節軟骨欠損の治療法として，約1世紀前に同種骨軟骨移植，数十年前に骨髄刺激法，自己骨軟骨移植が開発された．しかしながら，骨髄刺激法(marrow stimulation technique)以外はさまざま

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な問題があり広く行われてはいなかった．1990年代になり，自己骨軟骨移植の改良方法であるモザイクプラスティーが開発され，いくつかの問題はあるが，これまでの方法にくらべはるかに有効であることが明らかになった．また，同じころ，組織再生工学の進歩により，自己軟骨細胞移植が開発され，これも世界中でこれまでに1万例以上に行われ，これで関節軟骨欠損修復の問題が解決されたかと思われた．しかしながら，ドナーサイド，あるいは有効性の評価が一定しないなどの問題があり，さらに良好な方法の開発が望まれている．これらの問題を解決するために，骨軟骨の前駆細胞の移植が注目されている．

本稿では関節軟骨の再生医療を紹介し，これらのさまざまな問題点，およびこれから広く行われるために必要なことなどを解説する．

これまでの関節軟骨修復法

1．骨髄刺激法

関節軟骨欠損を修復する方法として，約半世紀前から骨髄刺激法が行われてきた．この方法は，軟骨損傷部の軟骨下骨の連続性を断ち，骨髄からの出血を生じさせ，軟骨前駆細胞および成長因子を損傷部に供給し，軟骨修復を促進させる方法である．簡便であり侵襲も少ないことから現在も広く行われている方法であるが，再生されるのは本来の硝子軟骨ではなく主として線維軟骨であると考えられている．

線維軟骨による修復の是非については論争中である．動物実験モデルにて，線維軟骨による修復は長期経過において関節症性変化を引き起こすとの報告[1]もみられるが，ヒトでは，少なくとも短期成績には問題がないとの報告が多く，その簡便性から組織再生工学などの手法を行う前にまず行ってみる価値のある方法である．

2．モザイクプラスティー

欧米では同種骨軟骨移植が行われているが，免疫反応，感染症，あるいは保存による軟骨細胞の死滅が問題である．自己骨軟骨移植はかつては一部の施設で行われていたが，自己骨軟骨片の採取による欠損部ができるという問題があった．

約10年前から，自己骨軟骨移植の改良法であるモザイクプラスティーが行われるようになり，良好な成績が報告されている．この方法では，小さな自己骨軟骨組織を関節の荷重にあまり関与しない周辺部から複数個採取し，それを骨軟骨欠損部にモザイク状に移植する．関節軟骨採取部を分散させるため大きな骨軟骨欠損をつくらないこと，荷重しない部位からの骨軟骨片の採取が可能であることなどの利点があるが，本来の曲率の関節軟骨表面形状を再現するのが技術的に困難であること，採取できる骨軟骨片には限りがあること，周辺部とはいえ正常軟骨部に欠損が生じること，移植軟骨の層状剥離などが問題として指摘されている[2]．

軟骨細胞移植による関節軟骨再生

1．同種軟骨細胞移植

1968年，家兎の実験系で同種軟骨細胞移植が報告された[3]．これは，同種の関節軟骨細胞あるいは成長軟骨細胞を血清に浮遊させて骨軟骨欠損部に移植する方法であるが，細胞の欠損部への固定が不十分であり良好な修復が得られなかった．その後，筆者らは細胞の固定性をよくするためにコラーゲンゲルを利用すると成績が向上することを報告した[4,5]．この方法は動物実験では，現在でも最も良好な修復が得られる方法の一つであるが，臨床応用に当たっては組織採取，感染症，あるいは拒絶反応の問題があり実用化が困難であった．

アメリカではtissue banking systemが確立されており，ヒトの関節軟骨の採取が可能である．Advance Tissue Science社がヒトでの同種軟骨細胞移植商品の開発を行っていたが，2002年の同社の倒産により，その開発は遅れ，現在でもいまだ実用化されていない．

2．自己軟骨細胞移植

自己軟骨細胞移植は1989年にGrandeらが家兎の実験系で報告し[6]，1994年にBrittbergらがヒト

に臨床応用した[7]．自家軟骨片を非荷重部から採取し，軟骨細胞を単離後，単層培養した軟骨細胞を，骨膜で縫着した軟骨欠損部に浮遊液の状態で注入，移植する方法である．1997年，米国食品医薬品局の認可を受け，Genzyme Biosurgery社により商業ベースで提供され，欧米ではすでに１万例以上に行われた．

この方法の利点は，自己細胞移植であるため免疫反応あるいは感染症が問題とならず，臨床応用が容易なことである．欠点としては，軟骨採取のために正常組織に欠損をつくること，自己組織採取のために別に１回手術が必要であること，細胞を液体に浮遊させ，骨膜で覆った欠損部に移植するため，細胞の固定性が不十分であること，培養で細胞を増殖させる過程で脱分化を生じることなどがあげられる．

その有効性については，当初からさまざまな報告がなされ，いまだに論争中である．2004年，自己軟骨細胞移植と骨髄刺激法の一つであるmicrofracture法の比較研究が行われ，２年の経過で有意差がないとの報告がなされた[8]．今後，自己軟骨細胞移植の有効性の再評価が必要であると考えられる．

前駆細胞

すでに分化した細胞を採取すると組織欠損が生じる．また，分化した細胞を増殖させると脱分化して，本来の機能を失う．そこで，小さな組織欠損で採取可能であり，増殖させたあとでも分化を誘導することのできる前駆細胞が注目されている．

整形外科分野で最も早くから注目されていた骨軟骨前駆細胞として，骨髄間葉系細胞がある．

１．骨髄間葉系細胞

骨髄を採取し，そのなかの接着細胞をdiffusion chamberに入れて動物の皮下に移植すると骨軟骨が出来ることは，すでに20年以上前から報告されていた[9]．骨髄血中の有核細胞を培養するとごく一部の細胞が接着・増殖する．この細胞を継代培養すると紡錘型の細胞がほとんどを占めるようになり，この細胞から骨，軟骨，筋肉，脂肪などの間葉系細胞が分化誘導されるため，間葉系幹細胞とよばれる[10]．また，1999年，骨髄間葉系細胞から内胚葉由来である肝細胞[11]，外胚葉由来である神経細胞[12]が分化誘導されることが報告され，間葉系のみならずあらゆる組織再生の細胞源として注目されている．筆者らは，これらの細胞が均一な細胞ではなく，さまざまな細胞の集まりであること，間葉系の細胞に分化するのみならず他の胚葉の組織にも分化すること，および生体から採取した骨髄血中の接着細胞は株化した細胞と異なり分裂能力に限界があることなどから間葉系幹細胞の定義に当てはまらないと考え，骨髄間葉系細胞とよぶ．骨髄血の採取は局所麻酔で可能であり，さらに培養で増殖させることが可能であるために，臨床応用に適した細胞である．骨再生，末梢循環障害改善をはじめさまざまな分野での再生医療に応用が試みられている．

筆者らは，この自己骨髄間葉系細胞を骨軟骨欠損部に移植すると修復が促進されることを家兎の実験系で報告した[13]．この結果を踏まえ，筆者らは２例のヒト膝蓋骨軟骨欠損症例に骨髄間葉系細胞移植を施行した[14]．２例とも７～８週後に施行した関節鏡で軟骨組織の修復を認め，術後臨床症状の改善は顕著であった．しかし，臨床では細胞を移植しないコントロール群の設定が困難であるため，本当に細胞移植が有効であったかを判断できない場合が多い．臨床症状の改善が組織の再生のためか，あるいは手術につづく安静，リハビリテーションのために改善されたのかはわからない．そこで筆者らは，内側型変形性膝関節症に対し高位脛骨骨切り術(HTO)を受ける患者24症例を対象に，細胞移植群と非移植群の２群を作製し比較することにより，骨髄間葉系細胞移植の有効性を検討した[15]．手術時平均年齢は64歳(49～70歳)であった．

HTO時に膝関節を展開し，12例に対して大腿骨内顆荷重部の関節軟骨欠損部軟骨下骨をabrasionし，コラーゲンゲルに包埋した骨髄間葉系細胞を充填し，脛骨内側より採取した骨膜にて

被覆した(細胞移植群)．他の12例に対しては同様に，HTO施行時に細胞の入っていないコラーゲンゲルを充填して骨膜で被覆した(コントロール群)．

臨床成績は細胞移植群，コントロール群ともに術前にくらべて有意に改善したが，両群間で改善度に有意差はなかった．抜釘時，同意が得られた症例で関節鏡を施行し，鏡視下および組織学的に修復組織を点数化し評価した．移植後7週，42週，いずれにおいても細胞移植群が有意に良好な修復であった．この方法は変形性関節症のような比較的広範囲の欠損に対しても応用が可能な方法であるが，今後も長期にわたり良好な機能を維持できるかは不明であり，注意深い経過観察が必要である．

2．骨髄間葉系細胞移植の改良点

骨髄間葉系細胞移植で欠損修復は促進されたが，完全な硝子軟骨による修復は得られなかった．筆者らの症例では，培養骨髄間葉系細胞にはなにも処置を加えずそのまま移植し，移植部位での自然な分化にゆだねたため，分化が不充分であったと考えられる．骨髄間葉系細胞を軟骨細胞に積極的に分化誘導する方法が成績改善に有効である可能性がある．現在，*in vitro*で小さな細胞塊しか軟骨に分化させることはできず，また，その場合でも中心部が石灰化するという問題が残る[10]．骨髄間葉系細胞移植後に骨髄からの間葉系細胞を動員する効果のあるfibroblast growth factor-2(FGF-2)を持続投与することによる欠損修復の試み[16]，骨髄間葉系細胞の培養中に軟骨形成促進作用のある成長因子(bone morphogenetic protein：BMP，insulinlike growth factor：IGF，transforming growth factor-β：TGFβ，cartilage derived matrix protein-1：CDMP-1など)を投与[17]，あるいはそれらの遺伝子導入により骨髄間葉系細胞の分化・増殖をコントロールする研究も行われており，これにより軟骨修復が促進されることが報告された[18]．また，軟骨の初期分化に関与する因子(Sry-related HMG box (SOX)-5, 6, 9, parathyroid hormone(PTH)/PTH related protein, hedgehog family)[19]や，成長因子の構成的活性型受容体，細胞内情報伝達物質(BMPにおける*Smads*など)，などのさまざまな遺伝子導入が試みられている．また，細胞の分裂能力を維持する目的でテロメラーゼ遺伝子を導入する報告もある．

さまざまな細胞を体外に取り出し，遺伝子を導入して関節内に戻すという，いわゆる*ex vivo*の遺伝子導入においては，移植する細胞自体が組織欠損を修復することも期待できるが，移植細胞が導入された遺伝子の産物を関節内に供給することも考えられる．この方法を使って軟骨修復を促進する成長因子を関節内に供給し，軟骨再生を促進するのみならず，変性を促進する因子を阻害する物質を供給し，変性の進行を防ぐことも可能であるため，最も注目されている分野の一つである．

3．その他の細胞による関節軟骨修復

骨髄間葉系細胞のほかにもさまざまな自己細胞使用による関節軟骨修復法が研究されている．生体組織内には種々の前駆細胞が存在することが明らかになり，羊膜細胞，筋肉サテライト細胞[17]，脂肪細胞，滑膜細胞などからの軟骨再生研究が行われており，*in vitro*の軟骨形成能の確認，動物実験での軟骨再生の有効性が示され，臨床応用が近いものもある．そのなかで滑膜細胞は軟骨形成能が最も高いことが示されたが[20]，滑膜採取のために関節鏡手術が必要であることなどの問題があり，現段階では骨髄間葉系細胞に大きくまさるものはない．

胚性幹細胞(ES細胞)，2003年に報告されたmultipotent adult progenitor cell(MAPC)[21]は他の組織再生と同様に，軟骨再生においても期待されている．これらの細胞を軟骨細胞にのみに分化させる方法の開発が重要である．筆者らは，ES細胞を関節腔内に注入すると奇形腫をつくり関節を破壊するが[22]，骨軟骨欠損部に移植すると奇形腫をつくらず骨軟骨に分化することを明らかにしたが[23]，そのメカニズムは不明であり，今後の解明が待たれる．

分化度の低い細胞を移植に使った場合，軟骨修

復にかぎらずすべての細胞移植共通の問題であるが，がん化の問題[24]，あるいは異種蛋白(ウシ胎児血清，担体に使うコラーゲン)などの使用による免疫原性の問題[25]がある．

関節軟骨欠損の臨床的問題点

関節軟骨修復のための細胞移植法は，皮膚とともに最も早くから開発された組織工学医療技術の一つである．しかしながら，その後の進歩が少ない．その原因としては自然経過が明らかでないこと，および治療効果の評価方法が確立されていないことによる．

1．関節軟骨欠損の自然修復能

一般的には，関節軟骨層にとどまる浅い損傷の場合は修復されず，関節軟骨下骨まで及ぶ深い損傷の場合は本来の関節軟骨組織である硝子軟骨ではなく線維軟骨で修復されると古くから考えられている．実際には関節軟骨の自然修復は動物種，年齢，損傷の性状，その部分にかかる荷重の大きさなどにより異なることもあり詳細は不明である．

ヒトにおける関節軟骨修復能力は一般的に動物より乏しいと考えられていたが，近年，関節鏡，MRIなどの診断技術の進歩により関節軟骨欠損の自然経過を追うことが可能になり，弱いながらも自己修復能力があることが明らかになってきた．今後，さらに軟骨欠損の自然経過が明らかになり，どのような年齢の，どの部位の欠損は放置しても治り，どのような欠損は変形性関節症へ進行するため積極的に修復すべきか，という手術適応が明らかにされることが望まれる．

2．関節軟骨欠損に対する手術の適応

外傷，関節リウマチなどの関節炎，変形性関節症などの退行性変性により関節軟骨が欠損すると関節機能の障害(疼痛，関節水腫，関節可動域制限など)が生じると考えられている．しかし，関節軟骨は神経を欠くために，関節軟骨自体が疼痛を感じることはない．外傷性軟骨欠損，関節リウマチ，あるいは変形性関節症により関節軟骨の消失した人で痛みを感じずに歩いている人は大勢いる．関節軟骨欠損の疼痛発生のメカニズムは解明されておらず，痛みのみを手術適応とするには問題がある．逆に，痛みがなくても，力学的破綻が存在することによる将来の関節の破壊が予想される場合には，予防のために手術する可能性もある．

筆者らは関節軟骨欠損修復のための移植手術はなるべく適応を絞り，あらゆる保存療法に抵抗した症例，あるいは将来関節機能が破綻する可能性が高いと考えられるような軟骨欠損を対象にすべきであると考えている．

3．関節軟骨修復の評価方法

前述の骨髄刺激法では，軟骨欠損部は本来の関節軟骨である硝子軟骨では修復されず，線維軟骨であると考えられている．修復組織が硝子軟骨であっても線維軟骨であっても短期臨床症状には関係しないとの報告もあるが，10～20年の長期の経過を追うと，差が出るようである．

ヒトの場合，修復組織の生検が困難であり，組織学的修復を評価しにくいという問題がある．そのために臨床症状の改善が主な評価とならざるをえない．しかし臨床症状だけであると，症状の改善が組織の再生のためか，あるいは手術およびそれにつづく安静(一般的に関節症状は関節を使わなければ軽快することが多い)のために改善されたのかわからない．すなわち，sham手術のコントロール群をつくって比較できないという問題があり，臨床症状で治療成績を正確に判断できないことも大きな問題である．

現在，筆者が考える評価方法としては，痛みなどの臨床症状の軽減，MRIなどによる欠損部の物理的充填の確認(硝子軟骨であることが望ましい)，および将来の進行するであろう変形性関節症の予防(現在，確立された評価法はない)，の三つである．これを確実に示すことにより，関節軟骨再生組織工学医療技術として認められると考える．

おわりに

近代細胞工学の進歩により軟骨再生分野は目覚ましい発展を遂げている．しかしながら，多くは実験における進歩であり，臨床に応用できるものはいまだにごく少数である．臨床に使える方法の開発が望まれる．

今後の課題としては，関節軟骨損傷に対し特殊な技術を必要とせず，患者に対する侵襲が少なく，確実に硝子軟骨で修復できる方法の開発であろう．

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

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Abstract: A source of morphologically and functionally available human cartilagenous tissue for implantation is required in the field of tissue engineering. To achieve this goal, we evaluated the effects of hyaluronic acid (HA-810 and 1680 kDa), and chondroitin sulfate (CS-A 16 and C-34 kDa) on human articular chondrocytes (HC) in micromass and rotation culture conditions. Cell proliferation was increased by CS-A 16 kDa under micromass and rotation cultures, while cell differentiation was increased under rotation but not micromass conditions. Proliferation and differentiation due to CS-C 34 kDa were very similar to the control under both culture conditions. With HA, cell proliferation was increased depending on the molecular weight under micromass and rotation conditions. In contrast, chondrocyte differentiation was enhanced under rotation conditions, but decreased under micromass conditions depending on the molecular weight of HA. In both culture conditions, aggrecan gene was continuously expressed. However, the collagen type II gene was more weakly expressed in rotation than the micromass culture conditions. Thus, the chemical structures of polysaccharides, and the culture condition, rotation or micromass, caused differences in chondrogenesis. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 80A: 257–267, 2007

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

INTRODUCTION

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

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

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tion[32] and it enhanced proliferation, as well as chondroitin sulfate (CS) synthesis, of rabbit articular cartilage.[33]

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

MATERIALS AND METHODS

Chondrocytes and medium

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

Preparation of materials

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

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

Preparation of siliconized vessel

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

Cell culture

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

Cell morphology assay

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

Measurement of wet weight

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

Proliferation assay

Alamar blue method

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

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

Crystal violet staining

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

Differentiation assay

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

Reverse transcription and polymerase chain reaction

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

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

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

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

Statistical analysis

Student's *t* tests were used to assess whether differences observed between the polymers treated and the control

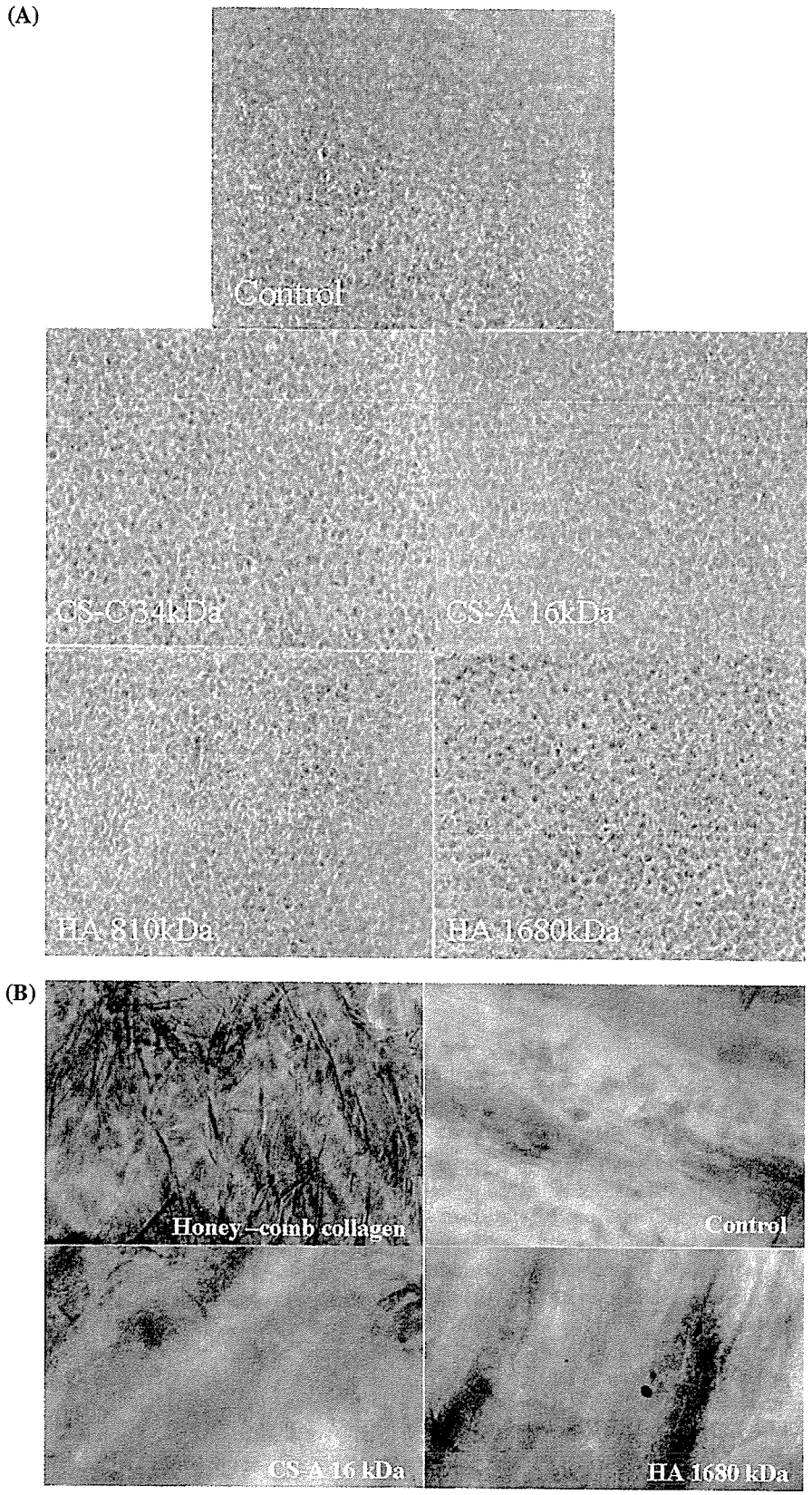

Figure 1. Light microscopic appearance of cultured HC in (A) micromass and (B) 3-D honeycomb rotation conditions spotted as high density cultures treated with different molecular weights and molecular structures of HA and CS for 4 weeks (after alcian blue staining, original magnification ×200).

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

RESULTS

Cell morphology

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

Wet weight

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

Cell proliferation assay

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

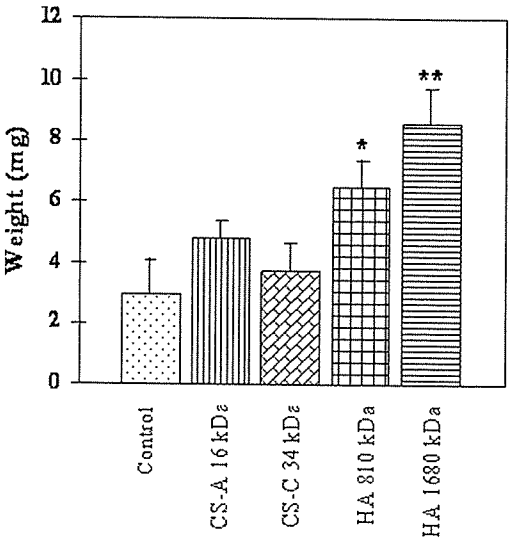

Figure 2. Wet weight of tissue constructs obtained by culturing HC on collagen honeycomb in rotation condition and treated with different molecular weights and structures of HA and CS for a period of 4 weeks. HA 810 kDa (*$p < 0.05$), and HA 1680 kDa (**$p < 0.01$) are significantly different compared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

Cell differentiation

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

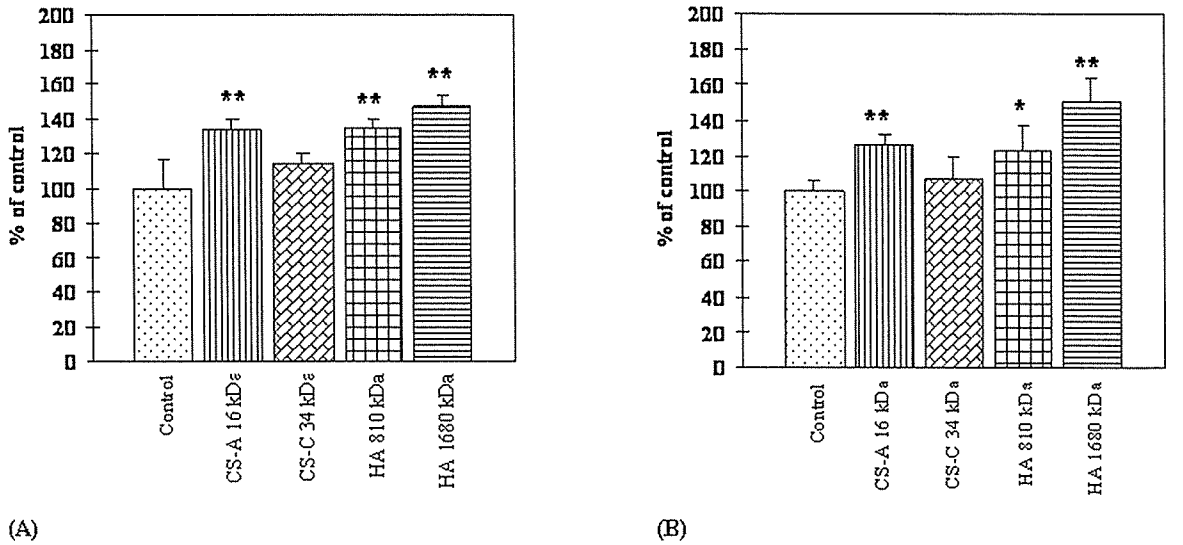

Figure 3. Cell proliferation of HC estimated by alamar blue method. (A) Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. CS-A 16 kDa, HA 810 kDa, and HA 1680 kDa are significantly different compared with control ($^{**}p < 0.01$). (B) In rotation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

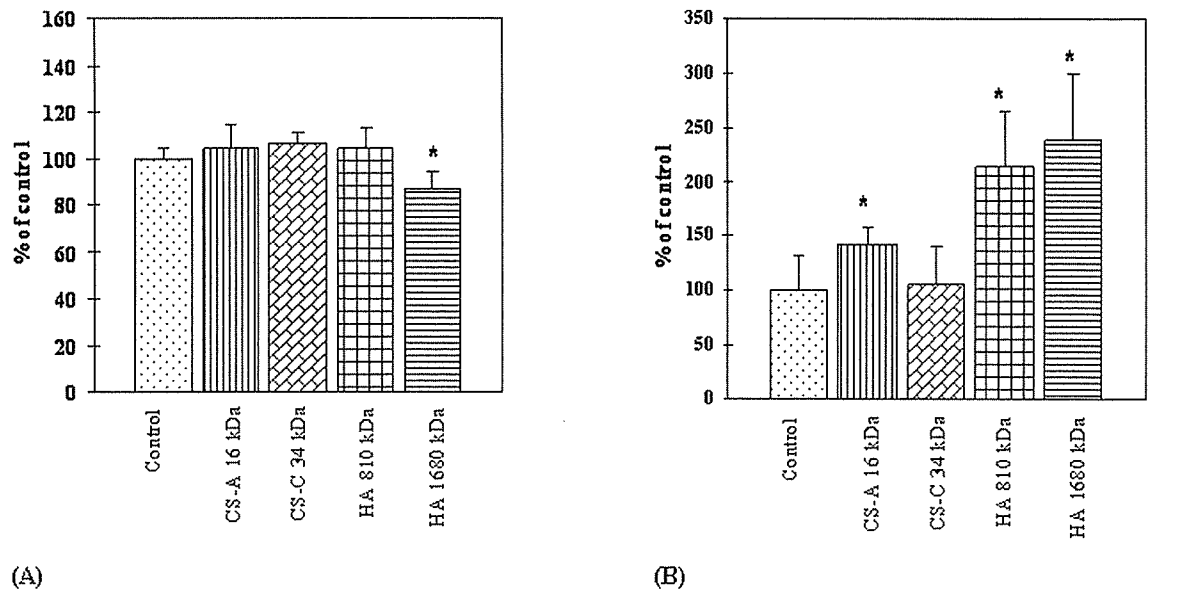

Figure 4. Cell differentiation of HC estimated by alcian blue method. (A) Under micromass conditions in control, CS-A 16 kDa, CS-C 34 kDa, HA 810 kDa, and HA 1680 kDa for 4 weeks. HA 1680 kDa is significantly different compared with control ($^{*}p < 0.05$). (B) In rotation conditions, cultures were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

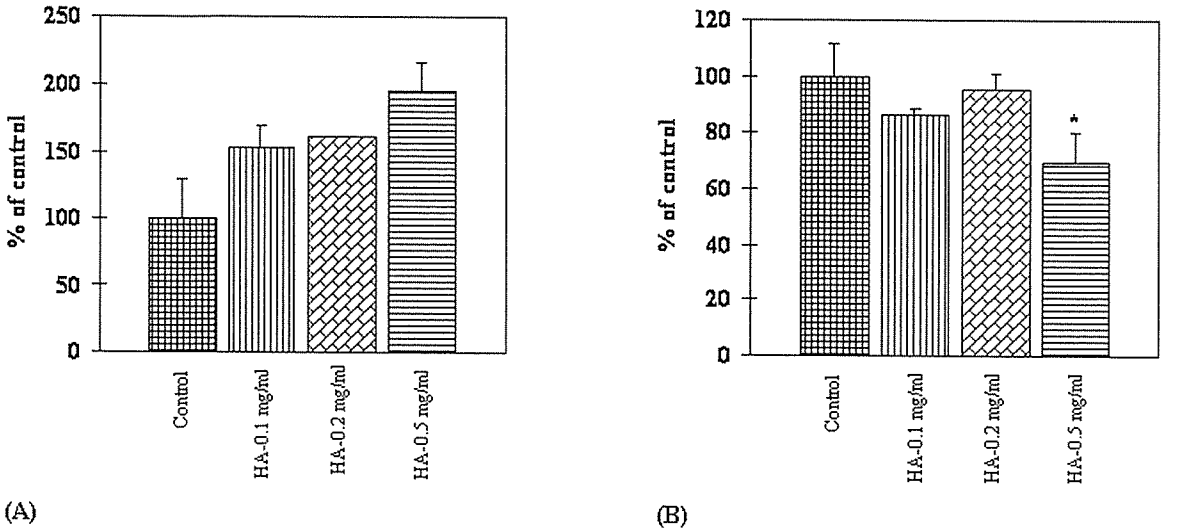

Figure 5. Chondrogenic effect of HA (1680 kDa) with different concentrations under micromass conditions for 4 weeks. (A) As demonstrated by crystal violet staining, cell proliferation was increased with 0.1, 0.2, and 0.5 mg/mL of HA 1680 kDa respectively, compared with the control culture. (B) Cell differentiation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

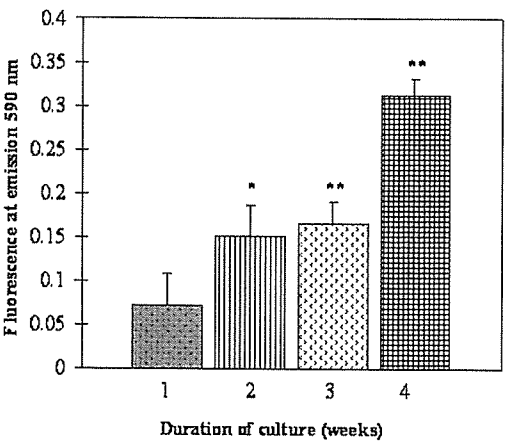

Figure 6. Time course of cell proliferation under micromass conditions for 4 weeks, estimated by crystal violet staining. Cell proliferation was significantly increased after 2 weeks ($*p < 0.05$), 3 weeks ($**p < 0.01$), and 4 weeks ($**p < 0.01$) of culture, compared with the chondrocytes cultured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

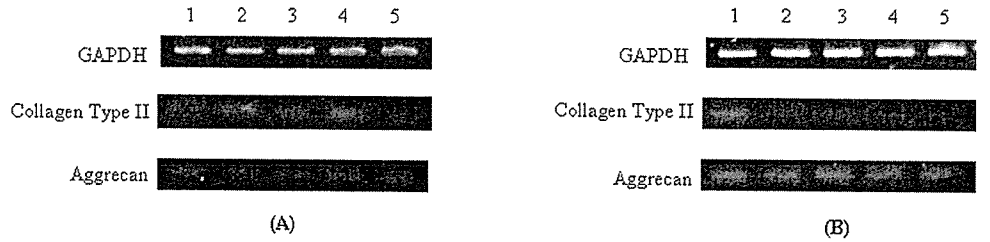

Figure 7. (A) Reverse transcription-polymerase chain reaction amplification of GAPDH, collagen type II, and aggrecan in cultures of HC with four different types of CS and HA for 4 weeks in micromass conditions. Lane 1, control; lane 2, CS-A 16 kDa; lane 3, CS-C 34 kDa; lane 4, HA 810 kDa; and lane 5,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

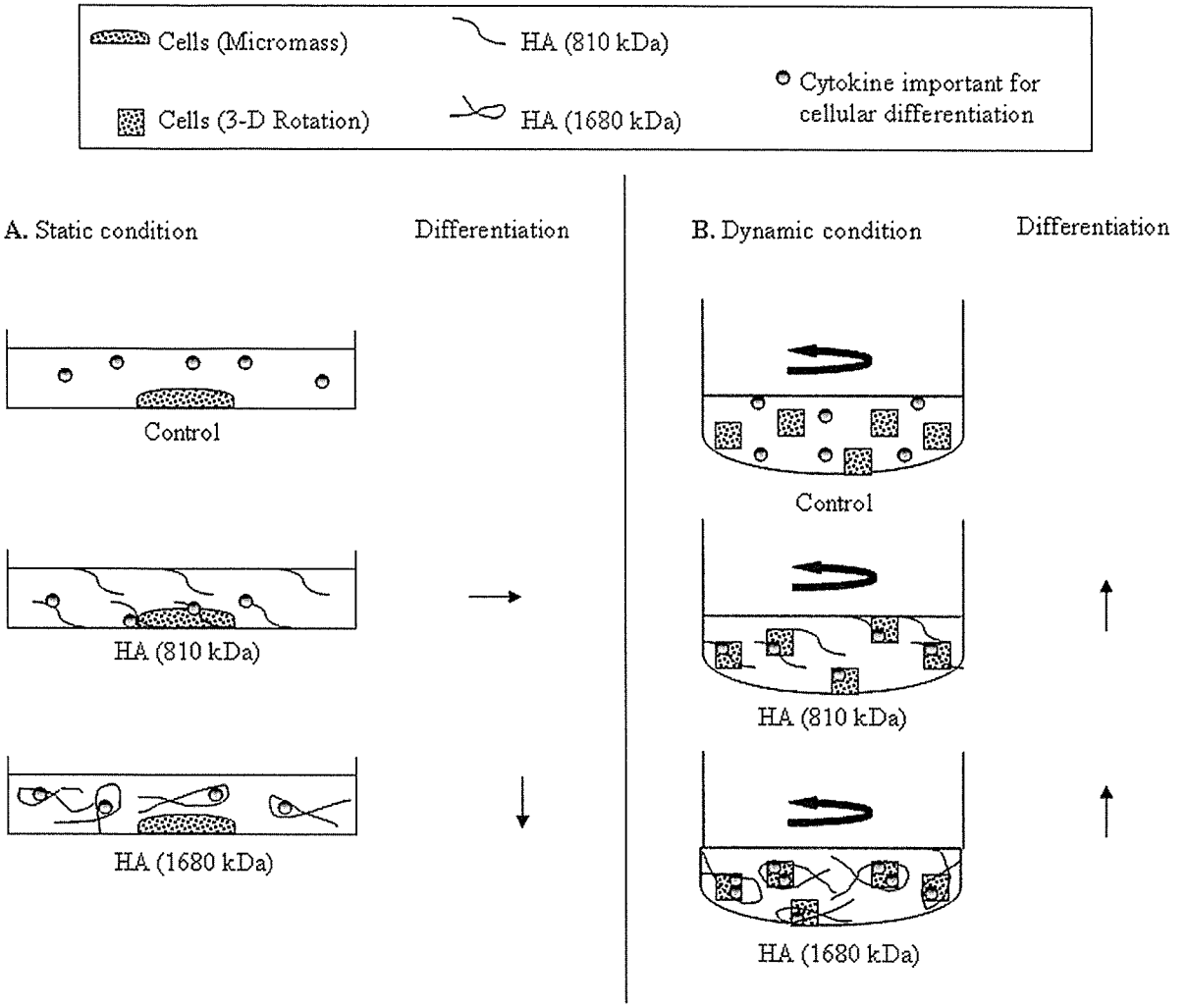

Figure 8. Schematic representation of the effects of different molecular weights of HA on the differentiation of HC. (A) Static condition (micromass). (B) Dynamic condition (rotation culture).

(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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日本の医療機器の研究開発と制度の動向

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日本の技術力を礎に，新たな医療機器の研究開発が進められているが，そのシステムには様々な課題も指摘されている．開発の推進と審査の迅速化を目指し，平成17年に厚生労働省と経済産業省の共同で「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」が設置された．評価指標の策定によって，医療機器の開発は今後どのように進むのか？　再生医療をはじめとする新たな技術はどこまで実用化しているのか？　そこで本対談では，次世代医療機器検討会の審査ワーキンググループ事務局長を務める土屋利江氏と，厚生労働省で医療機器の承認申請の制度改革に従事される俵木登美子氏をお迎えし，日本の医療機器の動向をうかがった．（編集部　一戸敦子）

医療機器とは？

―医療機器の定義をお教えください．

俵木　医療機器は安全性，有効性と品質を確保して提供されるよう，薬事法によって規制を受けています．基本的には医薬品と同じように目的で定義されており，疾病の診断・治療・予防のために使われるもの，または身体の構造，機能に影響を与えるものが該当します．また，具体的に政令で1つ1つ種類が規定されていて，そこに該当するものが医療機器になるのです．それにはメスや救急絆創膏のようなものから，ペースメーカや陽子線治療器のような大型の治療装置までが含まれることになります．

―かなり幅広い範囲に渡っていますが，すべて同じような承認申請が行われているのですか？

俵木　医療機器はリスクに応じて国際的にクラス分類されています．世界的に医療機器の規制を整合しようとする会合（GHTF：Global Harmonization Task Forth）が日米欧豪加の5極で行われ，その中でリスクに応じた医療機器の4分類が整合されました．我が国でも平成14年の薬事法改正で規制の中に取り込んでいます（図1）．

クラスⅠは非常にリスクの低いもので，企業自らが安全と品質を確認して市場に出すことができる自己認証となります．クラスⅡはⅠよりややリスクがありますが，国の定めた基準をもと