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Facile control of RGD-alginate/hyaluronate hydrogel formation for cartilage regeneration

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ABSTRACT

Hydrogels are attractive carriers for delivery of cells in tissue engineering as cell/polymer constructs can be injected into the body in a minimally invasive manner. In this study, we prepared hydrogels using only RGD-alginate and hyaluronate in the presence of primary chondrocytes without additional chemical cross-linking reagents. RGD peptides were introduced to alginate chains to increase the specific interactions with cells, and hyaluronate was used to enhance the cellular interactions and resultant gel formation. The gelation of RGD-alginate/hyaluronate hydrogels was confirmed by rheological measurements. These gels were useful to regenerate cartilage tissues *in vivo*, which was confirmed by analysis of glycosaminoglycan secretion and chondrogenic gene expression in both qualitative and quantitative manner. This approach to controlling the gelation behavior using only cells and cell-interactive polymers could be useful for delivery of cells and therapeutics in biomedical applications.

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1. Introduction

Cartilage regeneration is essential for patients suffering from damaged or degraded cartilage, and native cartilage has limited capability for self-repair (Buckwallter & Mankin, 1997; Hunziker, 2002). Autologous cell transplantation is a currently accepted treatment method for these patients. However, this method still has limitations, including the small number of available cells for isolation, morbidity of the isolated site, limited proliferation of isolated cells, and mutation of the cells during the procedure (Risbud & Sittinger, 2002). Recently, tissue engineering approaches have shown potential in cartilage regeneration by delivery of cartilage-forming cells using polymer scaffolds.

Tissue engineering provides artificial tissues or organs to patients who suffer from tissue or organ defects (Hoffman, 2002; Langer & Vacanti, 1993; Lee & Mooney, 2001), and this approach typically requires a three dimensionally-structured scaffold that can support the adhesion, proliferation, and differentiation of cells to be engineered. For cartilage regeneration, various types of scaffolds such as sponges (Fujisato, Sajiki, Liu, & Ikada, 1996; Quintavalla et al., 2002), microspheres (Jaklenec, Wan, Murray, & Mathiowitz, 2008; Mercier, Costantino, Tracy, & Bonassar, 2005), and hydrogels (Bryant & Anseth, 2001; Temenoff, Athanasiou, Lebaron, & Mikos, 2002) have been exploited. Cartilage tissues have a high water content between 75% and 80% by wet weight (Darling & Athanasiou, 2003), and a hydrogel is an appropriate scaffold which has viscoelastic properties and water content similar to those of native cartilage tissues. Additionally, a hydrogel is a potential candidate for injectable delivery of therapeutic drugs including cells because it can be injected into the body in a minimally invasive manner.

Alginate is a natural polymer that is broadly used in biomedicine, as it has good biocompatibility and low toxicity, and requires mild gelation conditions to form a cross-linked structure (Prang et al., 2006; Smidsrod & Skjakbraek, 1990; Thornton, Alsberg, Albertelli, & Mooney, 2004). Alginate is a linear copolymer composed of homopolymeric blocks of β-D-mannuronate (M) and α-L-guluronate (G) residues (Frei & Preston, 1962; Haug, Larsen, & Smidsrod, 1966). Alginate has been extensively exploited to fabricate hydrogels and their needs for many biomedical applications, including tissue engineering applications are growing. Alginate typically forms gels by either chemical (e.g., covalent cross-linking) or physical cross-linking (e.g., ionic cross-linking). However, the ability of cells to contribute to gel formation has been largely ignored. Interestingly, it was reported that cross-linked network structures can be prepared using only cells and cell-interactive alginate. Hydrogels were formed by simply mixing either MC3T3-E1 mouse pre-osteoblasts (Lee, Kong, Larson, & Mooney, 2003) or NIH3T3 mouse fibroblasts (Park, Kang, Kim, Mooney, & Lee, 2009) with an RGD-alginate solution, due to specific interactions between integrin receptors of the cells and adhesion ligands coupled to an alginate backbone. However, primary chondrocytes, freshly

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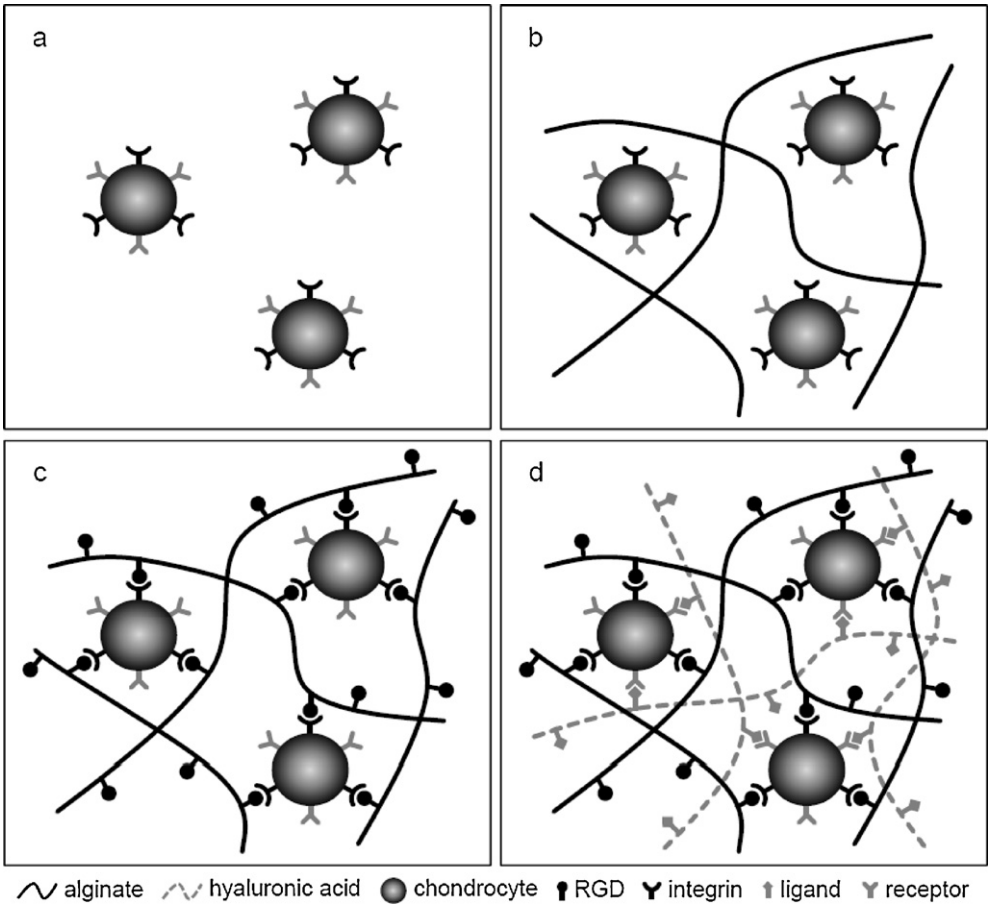

Fig. 1. Schematic description of cellular cross-linked hydrogels. Illustration of cells mixed with (a) media only, (b) non-modified alginate, (c) RGD-modified alginate, and (d) RGD-modified alginate/hyaluronate solution.

isolated from animals, do not effectively form a cellular cross-linked structure with the RGD-alginate because these cells do not express a sufficient amount of integrin receptors on their surface membranes. Thus, the mixture of RGD-alginate solution and primary chondrocytes lacks sufficient viscoelastic properties to remain at the *in vivo* injection site.

For this context, we hypothesized that combination of RGD-alginate and hyaluronate could be useful to form hydrogels in the presence of primary chondrocytes (Fig. 1d), because an addition of hyaluronate could improve the viscoelastic properties of the system and provide additional specific interactions with CD44 on the cell surface (Isacke & Yarwood, 2002; Knudson & Knudson, 2004; Kurtis et al., 2001). Hyaluronate is an important component of the extracellular matrices (ECMs) of native cartilage tissues, and is a non-sulfated member of glycosaminoglycans composed of the repeating disaccharides unit, β-1,4-D-glucuronic acid-β-1,3-*N*-acetyl-D-glucosamine (Fraser, Laurent, & Laurent, 1997). In this study, we prepared hydrogels using a RGD-alginate/hyaluronate solution cross-linked with primary chondrocytes, and investigated the viscoelastic properties of the gels *in vitro*. We also tested the efficacy of RGD-alginate/hyaluronate gels in cartilage regeneration in mice.

2. Materials and methods

2.1. Preparation of peptide-modified alginate

Sodium alginate (FMC Biopolymer) was dissolved in a 2-(*N*-morpholino) ethanesulfonic acid (Sigma–Aldrich) solution at room temperature (pH 6.5, 0.3 M NaCl). A peptide with the sequence of $(glycine)_4$-arginine-glycine-aspartic acid-serine-proline (G_4RGDSP) was purchased from Anygen and added to an alginate solution in the presence of 1-ethyl-3-(dimethylaminopropyl)carbodiimide (Sigma–Aldrich) and *N*-hydroxysulfosuccinimide (Thermo). The peptide-modified alginate was purified by dialysis against deionized water for 4 days (molecular weight cut-off = 3500), activated charcoal treatment, and sterilization with a 0.22-μm filter. Non-modified alginate was also prepared using the same purification procedure without peptide coupling as a control.

2.2. Cell isolation and culture

Primary chondrocytes were isolated from articular knee cartilage of New Zealand white rabbits (4-week-old, Samtako). After washing the fragments with PBS, they were digested with 0.05 wt% collagenase type II in DMEM/F-12 cell culture media containing 10% FBS and 1% penicillin–streptomycin for 10 h. Isolated cells were washed with PBS and cultured in DMEM/F-12 medium containing 10% FBS and 1% penicillin–streptomycin at 37 °C under a 5% CO_2 atmosphere. NIH3T3 mouse fibroblasts were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin at 37 °C under a 5% CO_2 atmosphere.

2.3. Preparation of cell cross-linked hydrogels

Alginate and hyaluronate were dissolved in DMEM/F-12 and transferred to a syringe. Either primary chondrocytes or NIH3T3

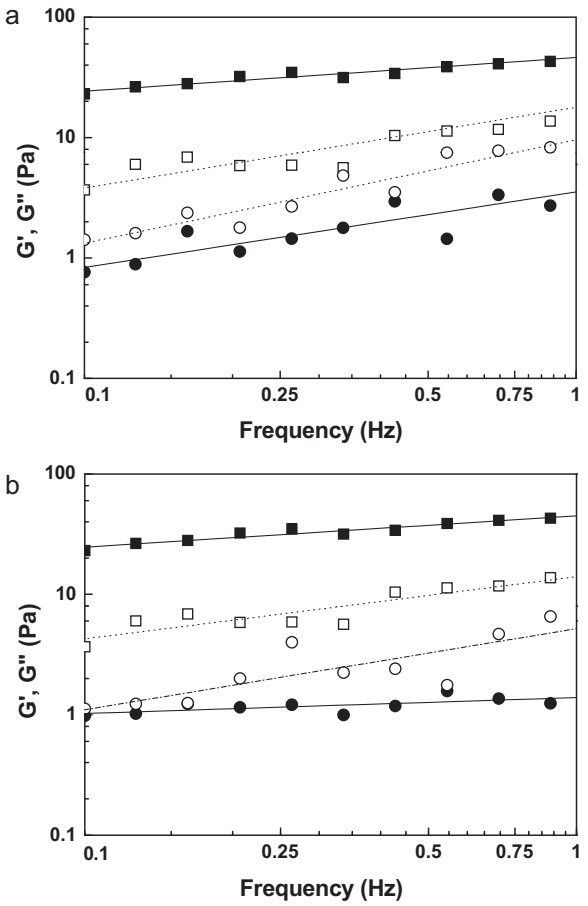

Fig. 2. Changes in the storage modulus (G', filled symbols) and loss modulus (G'', open symbols) of polymer/cell mixtures. (a) Viscoelastic properties of primary chondrocytes mixed with a solution of either RGD-alginate/hyaluronate (squares; [polymer] = 2.5 wt%; alginate/hyaluronate = 4/1, w/w) or RGD-alginate (circles; [polymer] = 3 wt%). (b) Viscoelastic properties of primary chondrocytes (squares) and NIH3T3 cells (circles) mixed with a solution of RGD-alginate/hyaluronate (squares; [polymer] = 2.5 wt%; alginate/hyaluronate = 4/1, w/w). The cell density was kept constant at 1.25×10^7 cells/ml.

cells were transferred to another syringe containing 7×10^{-3} M $CaSO_4$ solution. This concentration of $CaSO_4$ was not sufficient to form an ionically cross-linked alginate gel and was used to increase the viscosity of alginate solution. The two syringes were combined with a female connector and mixed rapidly.

2.4. Rheological measurements

The storage modulus (G') and loss modulus (G'') of the cell-polymer mixtures were measured using a rotational rheometer (Gemini 150; Malvern) equipped with a cone and plate fixture (4° cone angle, 20 mm diameter). The viscoelastic properties were measured using the frequency sweep mode in the range of 0.1–1 Hz. The operating temperature was maintained at 37 °C.

2.5. In vivo tissue regeneration

All of the procedures were in compliance with Hanyang University Guidelines for the care and use of laboratory animals. BALB/c nude mice (6-week-old, Central Lab Animal) were anesthetized with an intramuscular injection of ketamine hydrochloride (8 mg/kg body weight). Primary chondrocytes (5.0×10^7 cells/ml) were mixed with media only, non-modified alginate ([polymer] = 3 wt%), RGD-alginate ([polymer] = 3 wt%),

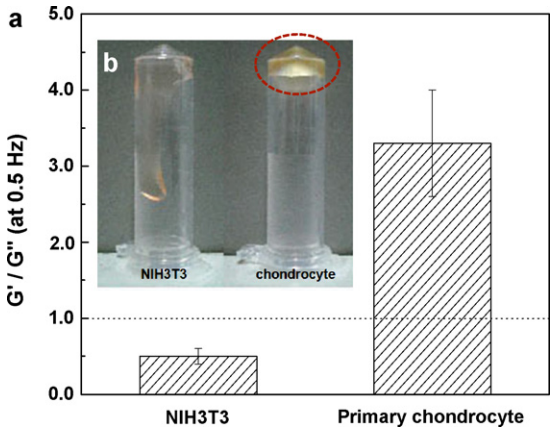

Fig. 3. (a) Viscoelastic properties of cell/polymer mixtures. The dotted line indicates the cross-over point where $G' = G''$ and provides a measure of the gelation threshold. (b) Images of cell/polymer mixtures taken after 10 min of mixing ([cell] = 5.0×10^7 cells/ml; [polymer] = 2.5 wt%; alginate/hyaluronate = 4/1, w/w). Gels were indicated by the dotted line.

and RGD-alginate/hyaluronate solution ([polymer] = 2.5 wt%; alginate/hyaluronate = 4/1, w/w). A subcutaneous injection of each mixture (injection volume, 150 μl) into the dorsum of a mouse was then performed ($n = 4$ per treatment group). Six weeks after injection, all the mice were sacrificed and the regenerated tissues were retrieved.

2.6. Histological analysis

Retrieved tissues were washed with cold PBS (4 °C) and were embedded in Tissue-Tek® O.C.T. compound (Sakura Finetechnical) for histological analysis. Tissue sections with 10 μm thick were obtained using a cryotome (Leica) and were stained with Safranin-O.

2.7. Quantification of glycosaminoglycan content

Tissue sections were lyophilized, weighed, and added to deionized water (100 μl). A dissolving buffer (55 mM sodium citrate, 30 mM EDTA, and 0.15 M sodium chloride) was then added to the solution to remove any remaining alginate gel. The supernatant was removed after centrifugation, followed by incubation with 500 μl of papain solution (300 μl/ml) dissolved in 20 mM PBS containing 5 mM EDTA and 2 mM dithiothreitol at 60 °C for 24 h. For quantification of sulfated glycosaminoglycan (sGAG), the Blyscan™ sGAG assay kit (Biocolor) was used. Briefly, 1,9-dimethylmethylene blue reagent (1 ml) was added to the extracted solution (100 μl) and allowed to react for 30 min with shaking. After elimination of the supernatant by centrifugation at $10{,}000 \times g$ for 10 min, the sGAG–dye complexes were dissolved with dissociation reagent (1 ml). Absorbance of the dye in each sample and the chondroitin-4-sulfate standards along with a blank were measured at 645 nm using a spectrophotometer (Molecular Devices).

2.8. mRNA isolation and PCR

For PCR analyses, RNA was isolated from retrieved tissues using the RNAiso plus kit (Takara). The amount of RNA was determined by an UV-VIS spectrometer at 260 nm, and the concentration and quality were checked on an agarose gel. Isolated RNA samples were reverse transcribed to cDNA using a Maxime RT PreMix kit (iNtRON Biotechnology). Expression of chondrogenic marker genes (collagen type II, aggrecan, and SOX-9), hypertropic marker gene (collagen type X), and housekeeping gene (GAPDH) were

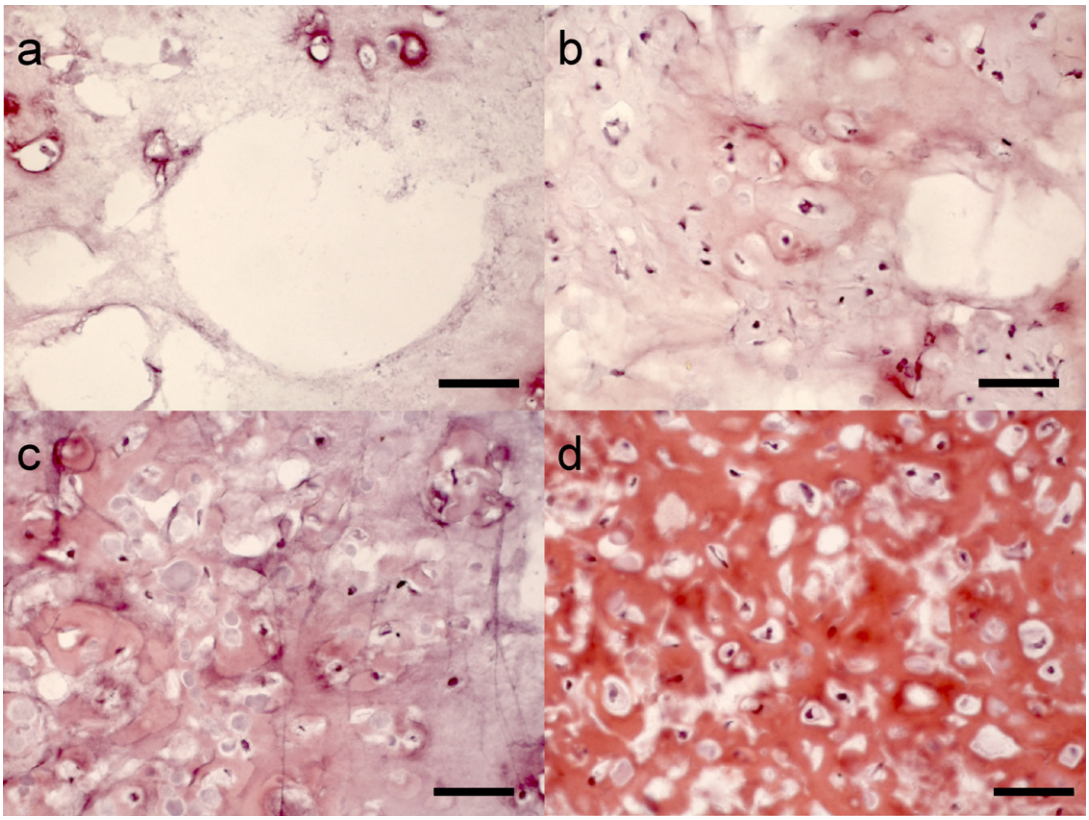

Fig. 4. Images of Safranin-O-stained tissue sections retrieved from mice (scale bar, 50 μm). Cells were transplanted with (a) media only, (b) non-modified alginate, (c) RGD-modified alginate, and (d) RGD-modified alginate/hyaluronate.

investigated using RT-PCR (Takara). PCR products were analyzed by conventional agarose gel electrophoresis. The sequences of primers used are as follows (Cosmogenetech): collagen type II, 5′-AACACTGCCAACGTCCAGAT-3′, 5′-CTGCAGCACGGTATAGGTGA-3′; collagen type X, 5′-CCCTATGCCATAAAGAGTAAAGG-3′, 5′-TCCCTGTTGTCCAGGTTTTC-3′; aggrecan, 5′-GAGGTCGTGGTGAAAGGTGT-3′, 5′-GTGTGGATGGGGTACCTGAC-3′; SOX-9, 5′-ACCTCAAGAAGGAGAGCGAAGA-3′, 5′-CGGGTGGTCTTTCTTGTGCT-3′; GAPDH, 5′-TCACCATCTTCCAGGAGCGA-3′, 5′-CACAATGCCGAAGTGGTCGT-3′. PCR amplification and real-time fluorescence detection of collagen type II (5′-GTCCAGGCAGAGGCAGGAA-3′, 5′-GACACGGAGTAGCACCATCG-3′) was also performed by ABI Prism 7500 real-time PCR system (Applied Biosystems) using SYBR® Premix Ex Taq™.

2.9. Statistical analysis

All data are presented as mean ± standard deviation ($n = 4$). Statistical analyses were performed using Student's t-test. *P-values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Preparation and characterization of RGD-alginate/hyaluronate hydrogels

We first tested whether the RGD-alginate solution could form a cross-linked structure with primary chondrocytes. A measure of viscoelastic properties of cell/polymer mixtures provides a useful means to investigate the cross-linked structure formation (Lee et al., 2003; Park et al., 2009). When a RGD-alginate solution ([polymer] = 3 wt%) was mixed with chondrocytes ([cell] = 1.25×10^7/ml), the loss modulus of the mixture (G'') was higher than the storage modulus (G') (circles in Fig. 2a), indicating that the mixture does not produce a gel structure due to a lack of interactions between the polymers and the cells. The number of integrins on the surface of primary chondrocytes was less than those on immortalized cells (Loeser, Sadiev, Tan, & Goldring, 2000). This may explain why primary chondrocytes do not form a cross-linked structure with the RGD-alginate, even though primary chondrocytes are known to interact with RGD peptides (Tan, Huang, Lao, & Gao, 2009).

Surprisingly, G' was higher than G'' when the mixture of RGD-alginate/hyaluronate solution ([polymer] = 2.5 wt%; alginate/hyaluronate = 4/1, w/w) and chondrocytes ([cell] = 1.25×10^7 cells/ml) were measured (squares in Fig. 2a). This finding clearly supports that the RGD-modified alginate/hyaluronate solution could form a cross-linked structure with primary chondrocytes (Fig. 1d). Hyaluronate interacts with chondrocytes through the CD44 expressed on the cell surface (Aruffo, Stamenkovic, Melnick, Underhill, & Seed, 1990; Isacke & Yarwood, 2002; Knudson, 2003). Adding hyaluronate not only increased the interactions with chondrocytes, but increased viscosity as well. The complex viscosity (η^*) of RGD-alginate and RGD-alginate/hyaluronate (alginate/hyaluronate = 4/1, w/w) solutions at 37 °C were 8.0 ± 0.2 and 32.9 ± 1.1 Pa s, respectively ([polymer] = 2.0 wt%). However, when the concentration of alginate/hyaluronate mixtures was increased over 2.5 wt% and mixed with the cells, the system did not form a cellular cross-linked structure, likely due to the high viscosity of the alginate/hyaluronate solution and thus inhomogeneous mixture formation (data not shown).

To further investigate the specific interactions between hyaluronate and the primary chondrocytes, a RGD-alginate/hyaluronate solution was mixed with NIH3T3 cells. Interestingly, the mixture did not produce gel structures (circles in Fig. 2b), irrespective of the same polymer concentration and same

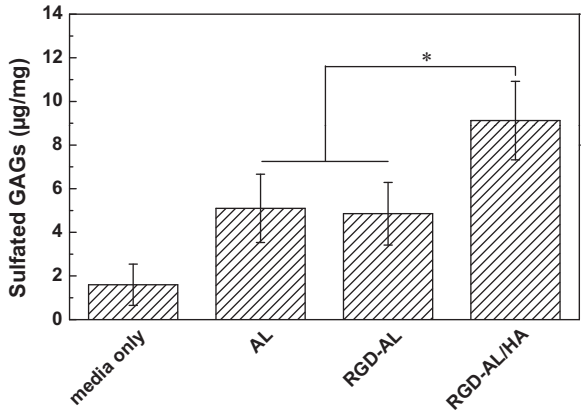

Fig. 5. Quantitative analysis of sulfated GAGs from regenerated cartilage tissues (*$P < 0.05$). Cells were transplanted with media only, non-modified alginate (AL), RGD-alginate (RGD-AL), and RGD-alginate/hyaluronate (RGD-AL/HA).

number of cells in the system. As such, we found that hyaluronate did not have a direct effect on NIH3T3 binding as it did with chondrocytes. The viscoelastic properties of the cell/polymer mixtures were remarkably different between the mixture with chondrocytes and that with NIH3T3 cells (Fig. 3a). The cross-over point where $G' = G''$ provides a measure of the gelation threshold, and the G'/G'' value of a polymer/chondrocyte mixture was greater (3.3 ± 0.7) than that of a polymer/NIH3T3 cells (0.5 ± 0.1). Primary chondrocytes mixed with the RGD-alginate/hyaluronate solution formed the gel structure, as clearly shown in Fig. 3b. A mixture of NIH3T3/polymer flowed, indicating that it still had liquid properties due to its failure to form a cross-linked structure.

3.2. In vivo cartilage regeneration

We next tested the efficacy of RGD-alginate/hyaluronate hydrogels in cartilage regeneration in mice. Primary chondrocytes ([cell] = 5.0×10^7 cells/ml) were mixed with either non-modified alginate ([polymer] = 3 wt%), RGD-modified alginate ([polymer] = 3 wt%), or RGD-alginate/hyaluronate ([polymer] = 2.5 wt%; alginate/hyaluronate = 4/1, w/w) and subcutaneously injected into the dorsum of mice. Cells suspended in media only were also injected into mice as a control. The total volume of retrieved cell/polymer constructs did not change from the initial injected volume. However, the volume for cells delivered with media only was drastically reduced. This demonstrates that a polymer scaffold is essential for the desired cartilage regeneration. Tissue sections stained with Safranin-O clearly show that the RGD-alginate/hyaluronate gels were effective to form extracellular matrices, as indicated by the large amount of sulfated glycosaminoglycans (GAGs) in newly formed tissues (Fig. 4d). Chondrocytes in lacunae were clearly observed when cartilage tissues were formed using RGD-alginate/hyaluronate hydrogels.

The content of sulfated GAGs in the tissues was next quantified and normalized by the lyophilized tissue weight (Fig. 5). The group treated with media only had the least amount of extracted sulfated GAGs (1.5 ± 1.0 μg/ml), as the cartilage tissues regenerated only minimally. There was no difference between non-modified alginate group (5.1 ± 1.6 μg/mg) and RGD-alginate group (4.8 ± 1.4 μg/mg). Interestingly, the sulfated GAGs of the RGD-alginate/hyaluronate group were most often detected (9.1 ± 1.8 μg/mg), compared to the other groups. GAGs are essential components of the ECM of cartilage tissues; the more GAGs were produced, the more active were the injected chondrocytes. Moreover, a higher concentration of GAGs created a more favorable environment for injected chondrocytes. From quantification of sulfated GAGs, we also confirmed

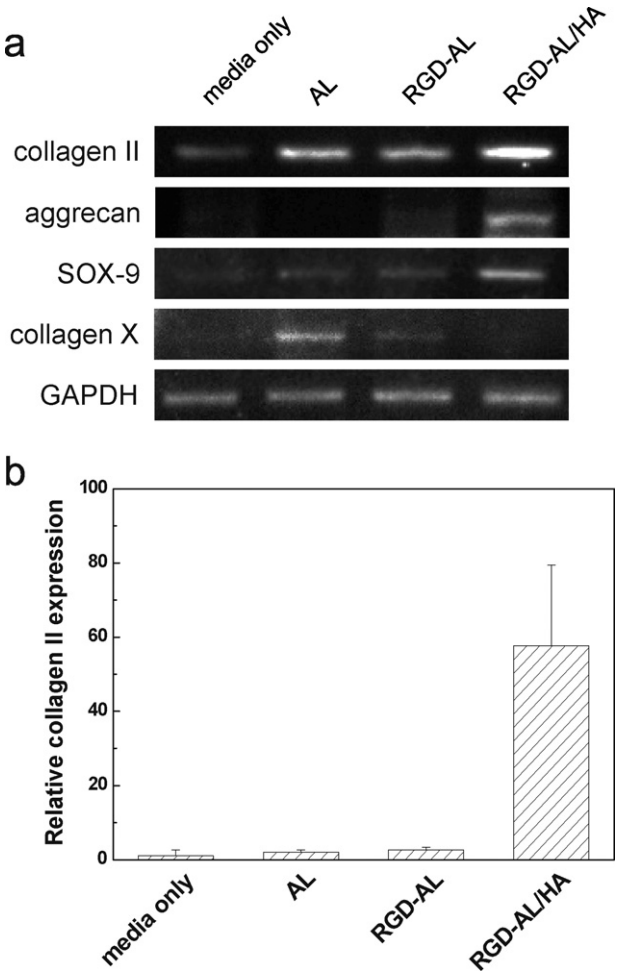

Fig. 6. (a) Expression of chondrogenic marker genes (aggrecan, collagen type II and SOX-9) and hypertrophic marker gene (collagen type X) in regenerated cartilage tissues. (b) Quantitative analysis of collagen type II expression in the tissues. Cells were transplanted either with media only, non-modified alginate (AL), RGD-alginate (RGD-AL), and RGD-alginate/hyaluronate (RGD-AL/HA).

that the RGD-alginate/hyaluronate gel was adequate for the regeneration of cartilage tissues.

3.3. Chondrogenic gene expression

We further investigated gene expression of collagen type II, aggrecan, and SOX-9, and collagen type X (Fig. 6a). Collagen type II (COL2A1) and aggrecan are well-known major structural components of cartilage, especially articular cartilage; and SOX-9 is expressed in all cartilage tissues and binds to directly influence the collagen type II gene (Bell et al., 1997; Lefebvre, Huang, Harley, Goodfellow, & de Crombrugghe, 1997; Ng et al., 1997). Collagen type X (COL10A1) is expressed by hypertrophic chondrocytes during endochondral ossification (Kielty, Kwan, Holmes, Schor, & Grant, 1985; Von Der Mark et al., 1992). The RGD-alginate/hyaluronate group has the highest levels of aggrecan, collagen type II, and SOX-9 gene expression. There was no difference in chondrogenic gene expression between the non-modified alginate group and the RGD-alginate group. These genes were rarely expressed in the group treated with cell culture media only. This finding also demonstrates that the RGD-alginate/hyaluronate group provides an appropriate environment to chondrocytes. As such, they effectively form the ECMs and regenerate cartilage tissues. On the other hand, collagen type X was most highly expressed in the group treated with non-modified alginate. This implies that

injected chondrocytes in a mixture of non-modified alginate lost their inherent chondrogenic characteristics and underwent maturation (Kameda, Watanabe, & Iba, 1997). The RGD peptide greatly influences the activities of chondrocytes (e.g., adhesion, growth) (Chen, Curran, Curran, & Hunt, 2006; Jeschke et al., 2002; Tan et al., 2009), and the ossification of the injected chondrocytes in a non-modified alginate mixture could be accelerated, likely due to the lack of adhesion peptides in the system. The quantitative analysis of collagen type II gene expression also supports that the RGD-alginate/hyaluronate hydrogel is most efficient for the regeneration of cartilage tissues (Fig. 6b).

4. Conclusions

We demonstrated that gelation behavior of RGD-alginate/hyaluronate hydrogels cross-linked with primary chondrocytes can be controlled by regulating the specific interactions between the polymer and the cells. Not only did the addition of hyaluronate increase the viscosity of the polymer solution, it also improved the interactions with primary chondrocytes. RGD-alginate/hyaluronate hydrogels provided an appropriate environment for cartilage regeneration *in vivo*. These hydrogels may be widely used in biomedical applications through a minimally invasive procedure as an injectable system for delivery of cells and drugs without the need of chemical gelation reagents.

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