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Novel porous matrix of hyaluronic acid for the three-dimensional culture of chondrocytes

Jeong Yeon Kang[a], Chung Wook Chung[a], Jong-Hyuk Sung[b], Byung-Soon Park[b], Je-Yong Choi[c], Seung Jin Lee[d], Byung-Chul Choi[e], Chang-Koo Shim[a], Suk-Jae Chung[a], Dae-Duk Kim[a,*]

[a] College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, South Korea
[b] Prostemics Research Institute, Division of Stem Cell Research, Seoul 135-010, South Korea
[c] Department of Biochemistry and Cell Biology, Cell & Matrix Research Institute, School of Medicine, Kyungpook National University, Daegu 700-422, South Korea
[d] College of Pharmacy, Ewha Womans University, Seoul 120-750, South Korea
[e] College of Pharmacy, Chung-Ang University, Seoul 156-756, South Korea

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ABSTRACT

A novel three-dimensional (3D) scaffold of chemically unmodified hyaluronic acid (HA) with minimum cross-linkage was developed for the culture of chondrocytes, thereby to promote cartilage repair. The porous structure of the scaffold was observed by scanning electron microscopy (SEM), and the pore size was controlled by fabrication conditions including swelling time and composition of the HA matrix. Rabbit primary chondrocytes and human chondrocytic cell lines (C-20/A4) were cultured in the HA matrix to investigate whether they can be applied to construct the cartilage tissue *in vitro*. The chondrocytes retained chondrocytic spherical morphology in this HA matrix. Moreover, results from the MTT assay showed good cellular viability within the HA matrix; optical density increased for up to 28 days, demonstrating that the cells continued to proliferate inside the HA matrix. Phenotypic analysis (RT-PCR, Alcian blue staining and quantification of s-GAG) showed that chondrocytes, when three-dimensionally cultured within the HA matrix, expressed transcripts encoding collagen type II and aggrecan, and produced sulfated glycosaminoglycans (s-GAG), indicating chondrogenic differentiation. The new HA matrix therefore appears as a potentially promising scaffold for the three-dimensional culture of chondrocytes for cartilage tissue engineering.

1. Introduction

Osteoarthritis (OA) is a disease that causes articular cartilage to wear off and shows local degenerative change (Adams, 1988). When damaged, the articular cartilage neither has an ability to restore its tissue back to the original condition nor differentiate inside the joint area. This limited ability for self-repair is mainly due to the absence of nerves and blood vessels in the articular cartilage (Kuettner, 1992; Messner and Gillquist, 1996; Poole et al., 2001).

Autologous chondrocyte implantation (ACI) is one of the promising approaches for OA treatment. The autologous chondrocytes implanted over the lesion eventually produce a form of cartilage that is very much like the original one (Grande et al., 1989). However, despite numerous research trials since 1968, the success rate of ACI is still less than 40% due to the difficulty in retaining the cells in the defect area for a long enough time to allow initiation of extracellular matrix production (Aston and Bentley, 1986; Wakitani et al., 1998). Furthermore, prolonging the cell culture before injection tended to cause the formed cartilage to be increasingly fibrous in nature (Rodriguez and Vacanti, 1998).

It is well known that chondrocytes grown in monolayer culture (*i.e.*, two dimensions) undergo characteristic processes of dedifferentiation, marked by loss of collagen type II and aggrecan core protein as well as induction of collagen type I expression (Takigawa et al., 1987; Hering et al., 1994; Lefebvre et al., 1994). However, when grown in a porous three-dimensional (3D) scaffold, chondrocytes are known to maintain their differentiated phenotype and function. It is also known that the pore size in the scaffold is crucial for retaining the cells in the scaffold (Sibylle et al., 2003). Since scaffolds can encourage the proliferation of chondrocytes without losing important functions of differentiation, three-dimensional scaffold and chondrocyte-based strategies in cartilage tissue engineering are currently regarded as one of the most promising approaches for the treatment of OA.

An attractive building block with applications in drug delivery, tissue engineering, and viscosupplementation (Balazs and Denlinger, 1989) for arthritis and wound healing is hyaluronic acid (HA) (Miyauchi et al., 1996). HA, a major component in synovial

* Corresponding author. Tel.: +82 2 880 7870; fax: +82 2 873 9177.
E-mail address: ddkim@snu.ac.kr (D.-D. Kim).

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fluid (Laurent, 1987) and cartilage surface, inhibits chondrocytic chondrolysis (Homandberg et al., 1997). It is biocompatible, biodegradable and non-immunological (Freed et al., 1994; Peppas and Langer, 1994), which makes it beneficial for transplantation and injection. Moreover, HA itself is used as therapeutic aids in the treatment of OA to improve the lubrication of articulating surfaces and to reduce joint pain (Balazs and Denlinger, 1989; Hunt et al., 1989; Balazs et al., 1991). However, direct injection of uncross-linked soluble HA is not effective enough because of its poor mechanical properties, rapid degradation and clearance *in vivo* (Barbucci et al., 2002). Thus, when the cell-carrier HA is used as a scaffold for the chondrocyte culture, one can expect better effectiveness in the chondrocyte implantation resulting in better cartilage repair. Although there have been many studies in the past decades that used HA matrix and chondrocyte culture for cartilage repair, all of them utilized the HA matrix with chemical modification and/or cross-linkage using glutaraldehyde (Tomihata and Ikada, 1997a) or carbodiimide (Tomihata and Ikada, 1997b), which may face toxicity problems.

The goal of this study was to examine the feasibility of a novel porous 3D scaffold for chondrocyte culture using chemically unmodified HA which was minimally cross-linked using polyethyleneglycol diglycidylether (PEGDG). The pore size of the matrix was controlled by fabrication conditions, including swelling time and composition of the scaffold. The viability, proliferation and differentiation of rabbit primary articular chondrocytes and human chondrocytic cell lines (C-20/A4) in porous HA scaffold have been systematically investigated with the hope of obtaining deeper insight into the porous 3D matrix of chemically unmodified HA for articular cartilage regeneration and eventually for the treatment of OA.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA) was purchased from Shandong Freda Biochem Co., LTD. (1040 kDa, Jinan, China). The cross-linker, poly (ethylene glycol) diglycidyl ether [PEGDG, CH_2OCH-$(CH_2CH_2O)_n$–$CHOCH_2$, $n = 200$, MW = 8886 Da], was purchased from Polysciences (Warrington, PA). Trypsin/EDTA, type II collagenase (290 units/mg) and isopropanol were purchased from Sigma–Aldrich (St-Louis, USA). Dulbecco's modified Earle's medium (DMEM, 4.5 g/L glucose & L-glutamine) and Ham's F-12 (L-glutamine) were obtained from BioWhittaker (Wakersville, MD). All other chemicals were from standard laboratory suppliers and were of the highest purity available.

2.2. Synthesis of hyaluronic acid matrix

HA (0.5, 1, and 2 g) was first dissolved in 5.6 mL of NaOH of varying concentrations from 0.1 to 0.5 N. After adding excess amount of PEGDG (2.0 mL) as a cross-linker, the solution was thoroughly mixed using a mechanical stirrer (Overhead Stirrer, HT 120AX, DAIHAN scientific, Korea). The gel-like mixture was then put into 24-well tissue culture plates (Nunc, Roskilde, Denmark) and was placed at 60 °C for 3 h to form a hydrogel by gelation. After thoroughly rinsing out the excess PEDGD with distilled water, the resulting hydrogels were transferred to 6-well plates and swelled in distilled water at room temperature. After swelling for 1, 4 or 7 days, the swelled hydrogels were frozen in a deep freezer (−80 °C) for 24 h, and then freeze-dried under vacuum for 48 h. Final HA matrices were sealed and kept at room temperature until used for further experiments.

2.3. Cell culture in hyaluronic acid matrix

2.3.1. Chondrocyte culture

2.3.1.1. Isolation of rabbit articular chondrocytes. Five week old New Zealand white rabbits (Samtako, Osan, Korea) were humanely sacrificed under general ether anesthesia, and articular cartilage slices were gathered from the knee joints of each rabbit under sterile condition. Cartilage specimens were cut into small slices and minced like paste and then washed three times in sterile PBS. Then, the chondrocytes were isolated with 0.25% trypsin (Difco Lab., Detroit, MI) in sterile PBS for 30 min followed by 0.2% collagenase (Sigma Chemical Co.) in DMEM supplemented with 10% fetal calf serum (FCS) (Invitrogen Corp., Carlsbad, CA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 mg/mL fungizone (BioWhittaker, Walkersville, MD) for 18 h at 37 °C in a culture plate for digestion. Finally, the isolated cells were collected by centrifugation (1200 rpm, 5 min) and washed three times with the culture medium (Shintaro et al., 2005). The suspended cells were cultured in DMEM supplemented with 10% FCS, and 1% penicillin/streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and the culture medium was changed every 2–3 days.

2.3.1.2. Human chondrocytic cells lines. Human chondrocytic cell lines (C-20/A4) were cultured in DMEM/Ham's F-12 medium that is supplemented with 10% FCS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Culture medium was changed every 2–3 days.

2.3.2. Cells seeding into hyaluronic acid matrix

The HA matrices were cut into small cubes (8 mm × 8 mm × 3 mm) and were transferred to a 24-well tissue culture plate and then pre-sterilized with 0.2-μm-filtered 70% (v/v) ethanol solution at 4 °C for 3 h. Residual ethanol was removed by N_2 flow and washed several times using the culture media. Sterilized HA was pre-wetted with fresh culture media by incubating for 2 h at 37 °C in a humidified atmosphere of 5% CO_2. After removing this media, 20 μL of cell suspension containing 1×10^6 cells was loaded onto each matrix and allowed to penetrate into the matrix. The matrix was left undisturbed in an incubator at 37 °C under 5% CO2 condition for 4 h to allow cell attachment to the matrix, after which 1 ml of culture media was added to each well and kept in the incubator. Culture medium was changed every 2–3 days.

2.4. Cell morphology

For morphological observation, primary chondrocytes and C-20/A4 were seeded on HA matrix at a density of 1×10^6 cells/matrix in 24-well plates. After 14 and 21 days of incubation, the morphology of cells cultured on the HA matrix was examined by SEM (JSM-5310LV, JEOL, Tokyo, Japan). The cells adhering to the matrix were washed once with PBS, and then fixed with Karnovsky's fixative and osmium tetraoxide at 4 °C for 2 h each. After dehydration with a series of graded alcohols, the cells were critical point dried using CO_2. The matrix was then gold sputtered in vacuum and examined by SEM.

2.5. Cell viability and proliferation

Cell viability and proliferation during the culture in the HA matrix for up to 28 days were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] mitochondrial reduction. The HA matrix seeded with cells was carefully transferred into new 24-well culture plate and 1 ml of fresh culture media was added. After adding 100 μL of MTT solution (5 mg/ml in Dulbecco's PBS) to each well, the HA matrices were incubated for 4 h at 37 °C in a 5% CO_2 incubator. The unreduced MTT and medium were then discarded. Each well was washed twice with PBS and the staining of the viable cells was observed under light microscopy.

In order to quantitatively determine the cell proliferation, the MTT formazan crystals were dissolved with 1.0 mL of DMSO. Plates were shaken for 20 min, and then 100 μL of each solution was transferred to a 96-well plate. Absorbance was read at 560 nm using the microplate reader (Emax, Molecular Devices Corporation, Sunnyvale, CA). Control wells were HA matrices without chondrocytes seeding. At least three matrices were analyzed for each condition for 7, 14, 21 and 28 days.

2.6. Cell differentiation

2.6.1. RT-PCR

After culturing primary chondrocytes and C-20/A4 in HA matrix for 7, 14, 21 and 28 days, total RNA of each HA matrix was isolated using Qiagen RNeasy Plus Kit (Chatsworth, USA) and stored at −80 °C. Reverse transcriptase reactions were performed with 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (PerkinElmer, Norwalk, CT, USA) and oligo-(dT) priming. Polymerase chain reactions were performed under the following conditions: denaturation for 30 s at 94 °C, annealing for 30 s at 62 °C, elongation for 1 min at 72 °C. The specific primer pairs and PCR conditions of each gene are summarized in Table 1. The parallel amplification of cDNA for the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

2.6.2. GAG production

2.6.2.1. Alcian blue staining. The production of sulfate-glycosaminoglycan (s-GAG) was observed from primary chondrocytes and C-20/A4 cultured either in HA matrix or in culture plate for 7 days. s-GAG production was confirmed colorimetrically using an Alcian blue staining (Vinatier et al., 2005). Briefly, samples were fixed in 4% paraformaldehyde after washing with PBS. Then, they were stained with 0.1% Alcian blue/0.1 M HCl solution at room temperature. After overnight incubation, cells were thoroughly rinsed with distilled water. The deep blue staining of the s-GAG was observed under light microscopy.

2.6.2.2. Dimethylmethylene blue method. After the rabbit primary chondrocytes and C-20/A4 were cultured in HA matrix placed in 24-well tissue culture plates for 7, 14, 21 and 28 days, the total amount of s-GAG was quantitatively determined by the 1,9-dimethylmethylene blue (DMMB) dye method using shark chondroitin sulfate C as a

Table 1
Sequences of primer pairs, annealing temperatures, size of PCR products, and cycle numbers used for RT-PCR analyses of various gene expression in chondrocytes.

Gene	Primer sequences (5′→3′)	Annealing temperature	Product length	Cycle number
Aggrecan	TGAGGAGGGCTGGAACAAGTACC GGAGGTGGTAATTGCAGGGAACA	62 °C	350 bp	38
Collagen II	CCGAGGCAACGATGGTCAGC TGGGGCCTTGTTCACCTTTGA	62 °C	359 bp	38
Collagen I	GGTGGTTATGACTTTGGTTAC CAGGCGTGATGGCTTATTTGT	62 °C	702 bp	30
GAPDH	TGGTATCGTGGAAGGACTCATGAC ATGCCAGTGAGCTTCCCGTTCAGC	60 °C	190 bp	30

standard (Farndale et al., 1986). The samples were first digested with papain (Sigma Co., USA) for 48 h at 60 °C in buffer solution (pH 6.8) composed of 0.1 M NaH_2PO_4, 5 mM Na_2EDTA, and 5 mM cysteine·HCl. The dye solution was prepared by dissolving 16 mg of DMMB in 1.0 L of distilled water containing glycine (3.04 g), NaCl (2.37 g) and 0.1 M HCl (95 mL). An aliquot (100 μL) of each sample digested with papain was placed in a polystyrene tube and 2.5 mL DMMB reagent was added on the sample. Mixing was completed by pouring the solution into a disposable cuvette, and then the absorbance was measured at 560 nm on a microplate reader.

3. Results

3.1. Synthesis of the HA matrix

Fig. 1 shows the SEM images of the cross-section of the freeze-dried HA scaffolds. The left column (a, b, c) shows the effect of NaOH concentration while the swelling period (1 day) and HA amount (1 g) are fixed. The middle column (d, e, f) shows the effect of swelling period (1, 4, 7 days) with fixed NaOH concentration (0.3 N) and HA amounts (1 g). The right column (g, h, i) shows the effect of the amount of HA (0.5, 1, and 2 g) with fixed swelling period (1 day) and NaOH concentration (0.3 N). As shown in the figure, the HA matrix exhibited macroporous structure with interconnected open pores, and pore size varied from 30 to 500 μm. The size of the pores in the HA matrix was proportional to the swelling time, but reversely proportional to the NaOH concentration and the amount of the HA. Pore size in the scaffold is known to be critical for the growth of cells (Allemann et al., 2001; Sibylle et al., 2003). HA scaffold prepared with 1 g HA, 0.3 N NaOH, and 1 day of swelling period showed the optimal pore size (between 50 and 300 μm), and thus was selected for further evaluation.

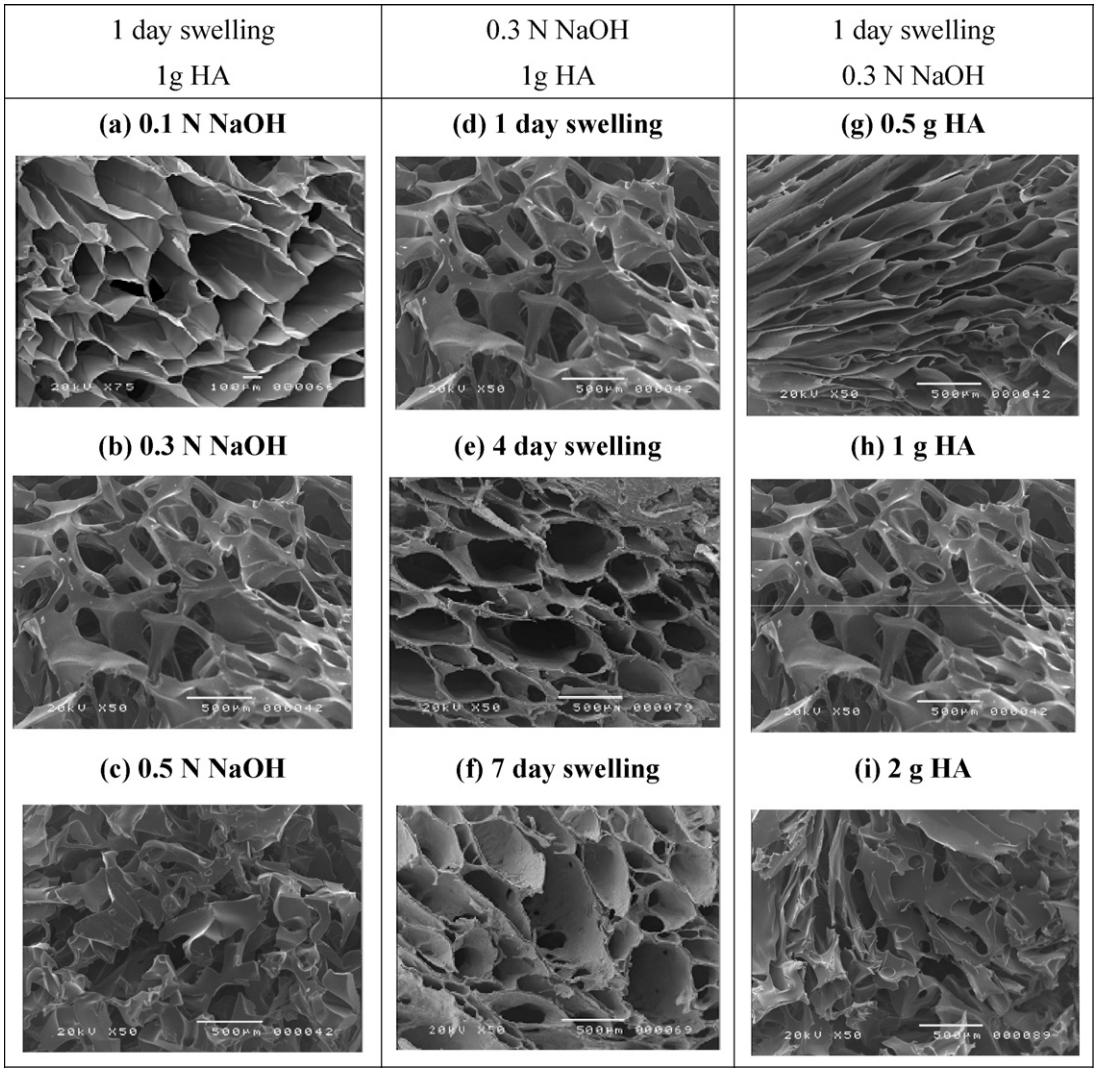

Fig. 1. Scanning electron microscopic images of the cross-section of the HA matrix with variation of pore size at different NaOH concentration level, different swelling time and different amounts of hyaluronic acid.

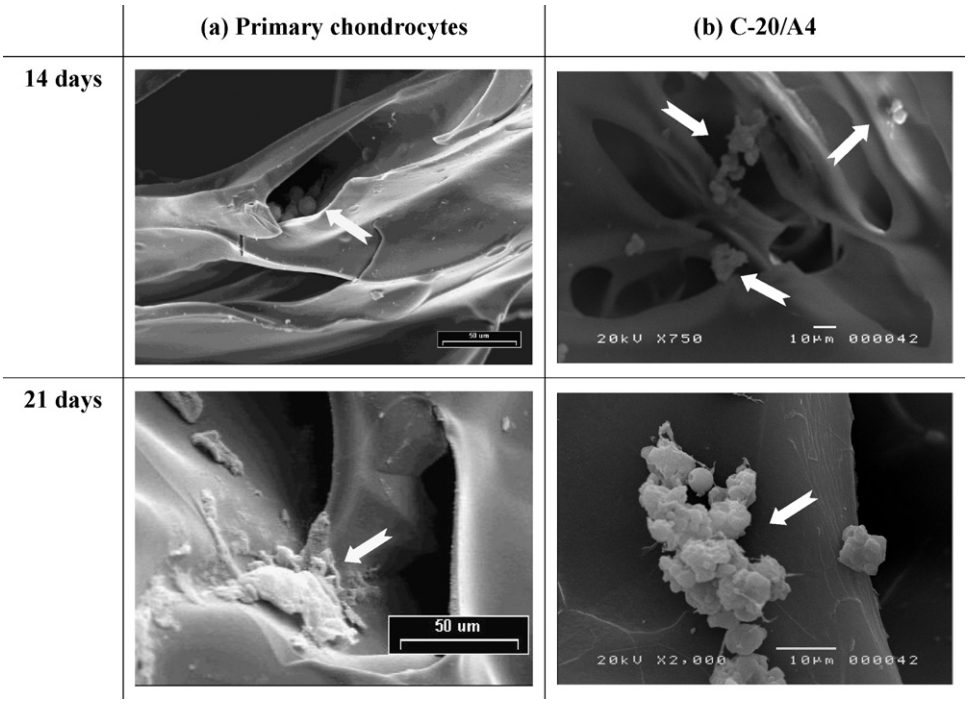

Fig. 2. Scanning electron micrographs of the (a) primary chondrocytes and (b) C-20/A4 cultured in the HA matrix for 14 and 21 days. The HA matrices were fabricated with 0.3 N NaOH, 1 day swelling, and 1 g HA. The characteristic round morphology of the chondrocytes (white arrows) and the dense fibers of the extracellular matrix can be found around the chondrocytes.

3.2. Cell morphology

The morphology of primary chondrocytes and C-20/A4 of the HA matrix after incubation for 14 and 21 days are shown in the cross-sectional view in Fig. 2. In the left column of the figure, it is shown that most of the cells were attached on the HA matrix structure and maintained the roundness characteristic after incubation for 14 days. After incubation for 21 days, cells grown on the HA matrix acquired predominantly spherical or some fusiform shape. Some cells were attached on the polymer by filapodia, but most of the cells were connected to each other by forming cell aggregations. The right column of the figure shows the cell morphology of the C-20/A4, which was used for direct comparison with the primary chondrocytes. There was no significant morphological difference between primary chondrocytes and C-20/A4. As shown in this figure, spherical shapes were apparent with 14 day incubation, and obvious cell aggregation was also observed with 21 day incubation.

3.3. Cell viability and proliferation

The viability of primary chondrocytes and C-20/A4 cultured in the HA matrix for 7 and 28 days were observed under the microscope after MTT treatment. After 4 h of incubation, viable cells in the HA matrix were stained with the MTT reagent. Fig. 3 shows the viability of the primary chondrocytes and C-20/A4 cells. As shown in the images, the two cases did not indicate much difference. Chondrocytes seeded in the HA matrix were dispersed individually and retained the spherical shapes, which was consistent with the SEM observation (Fig. 2).

It is apparent that the primary chondrocytes and C-20/A4 were stained by MTT treatment, indicating that cells were alive during the whole culture period in the HA matrix and formed nodular structure as cell culture period increased from 7 to 28 days. Moreover, Fig. 4 shows the proliferation of primary chondrocytes and C-20/A4 in the HA matrix, which was quantitatively determined by optical density (OD) at 560 nm after MTT treatment. The OD value increased over incubation time, demonstrating the increase of cellular proliferation inside the HA matrix.

3.4. Cell differentiation

RT-PCR, Alcian blue staining and dimethylmethylene blue (DMMB) methods were used for the phenotypic analysis. The result showed that the mRNA expression of collagen type II and aggrecan increased with the culture period, and s-GAG was produced in the 3D HA matrix. These results indicate the chondrogenic differentiation of both the primary chondrocytes and C-20/A4 cells cultured in the HA matrix.

3.4.1. RT-PCR

In order to investigate temporal changes in collagen type I, II and aggrecan mRNA expression, chondrocyte cells cultured in the HA matrix were analyzed by semiquantitative RT-PCR at different time points. Fig. 5 shows the results of (a) rabbit primary cell and (b) C-20/A4. The 1st column is the ladder marker. The 2nd column is the RT-PCR result of the 2D culture of 7 days. From the 3rd column to 7th column, each column shows the result of 3D cultures after 7, 14, 21, and 28 days, respectively. The collagen type I expression of 3D culture illustrated a marked down-regulation compared to the 2D culture in both primary chondrocytes and C-20/A4 cases, suggesting a loss of chondrocyte phenotype. On the contrary, collagen type II and aggrecan mRNA expression increased from day 7 to 28 for both primary chondrocytes and C-20/A4 cases compared to the 2D culture. Moreover, type II collagen expression, the major differentiation marker of chondrocytes, significantly increased in primary chondrocytes compared to C-20/A4. These results indicate that the HA matrix maintains the phenotype in chondrocytes and promotes cell differentiation.

3.4.2. GAG production

3.4.2.1. Alcian blue staining. Glycosaminoglycan (s-GAG) is a differentiation marker of chondrocytes and the major components of

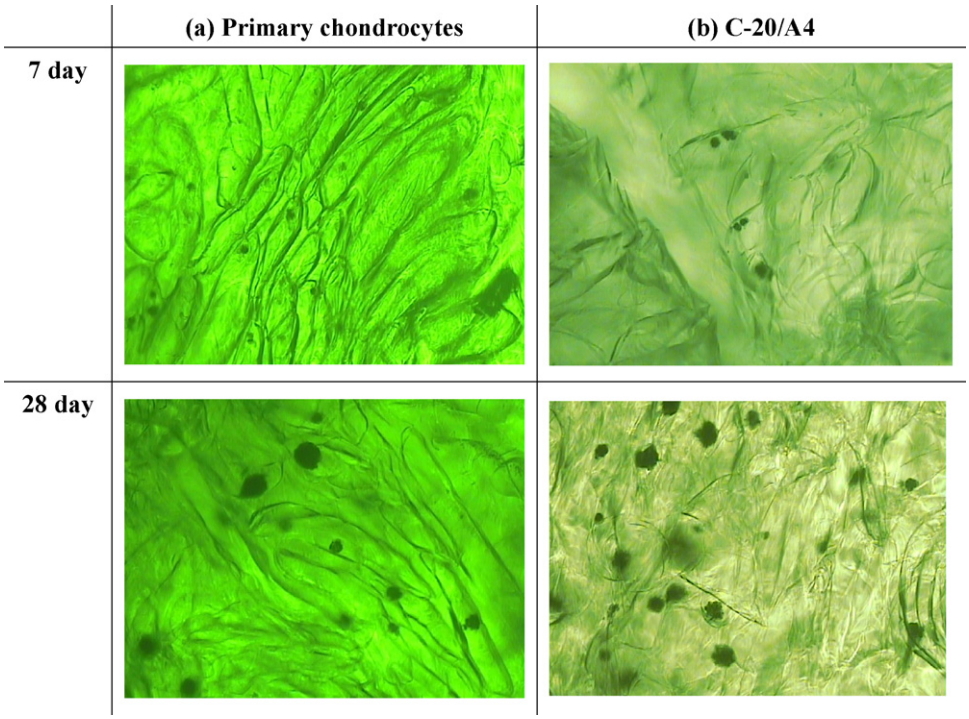

Fig. 3. MTT staining of HA matrix 7 and 28 days after seeding of (a) primary chondrocytes and (b) C-20/A4. The size of cell clusters is bigger after 28 days cultivation than that after 7 days.

cartilage tissue. The effect of 3D culture of primary chondrocytes and C-20/A4 on s-GAG production was determined by the Alcian blue staining after 7 days of culture in the 2D tissue culture plates and in the 3D HA matrix. As shown in Fig. 6, cells cultured in 2D showed blurred appearance, whereas those cultured in 3D showed strong positive blue staining. This implies the accumulation of s-GAG in the extracellular matrix of chondrocytes cultured in 3D HA matrix. Thus, the 3D HA matrix seems to provide better conditions and environments than the 2D culture for the production of s-GAG.

3.4.2.2. Dimethylmethylene blue method. The production of s-GAG during the culture of chondrocytes in the HA matrix was quantitatively determined by DMMB methods. As shown in Fig. 7, the amount of secreted s-GAG increased as the incubation time increased from 7 to 28 days, indicating that the HA matrix enhanced the ability of primary chondrocytes and C-20/A4 to synthesize s-GAG with increasing cultivation time. However, the amount of s-GAG between the primary chondrocytes and C-20/A4 was not significantly different.

4. Discussion

Three-dimensional scaffolds and chondrocyte-based strategies in cartilage tissue engineering are currently regarded as promising approaches for restoring hyaline cartilage for the treatment of OA. Chondrocytes are known to undergo a phenotypic dedifferentiation when cultured in a 2D system, and therefore new approaches focus on the use of natural or synthetic 3D scaffolds. In this study, a new and improved method of preparing the 3D scaffold has been attempted by using chemically unmodified HA with minimum cross-linking to support the regeneration of functional cartilage.

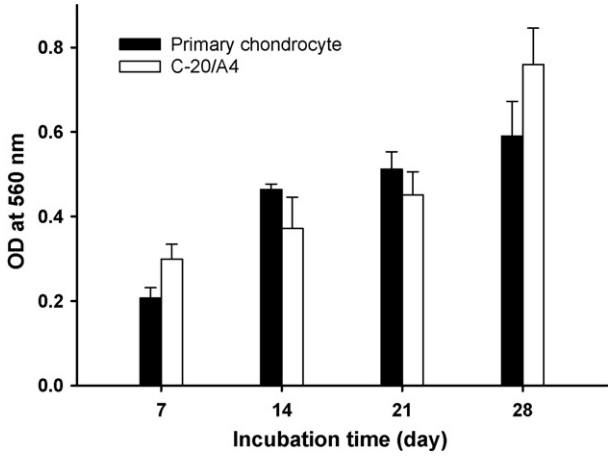

Fig. 4. Cell proliferation of primary chondrocytes and C-20/A4 cultured in HA matrix for 7, 14, 21 and 28 days, analyzed by the MTT assay. Data are presented as mean ± standard error ($n = 3$).

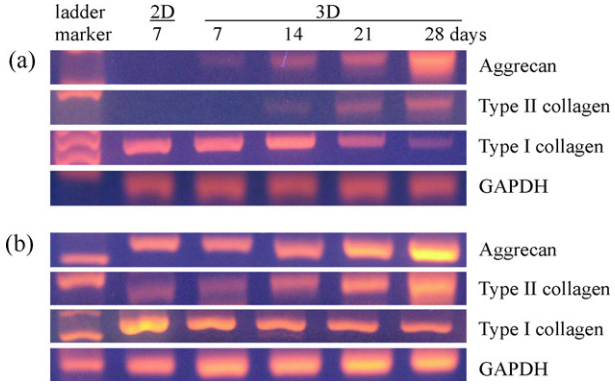

Fig. 5. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the (a) primary chondrocytes and (b) C-20/A4. Lane 1, ladder marker; Lane 2, 2D culture for 7 days; Lane 3, 3D culture in the HA matrix for 7 days; Lane 4, 14 days; Lane 5, 21 days; Lane 6, 28 days.

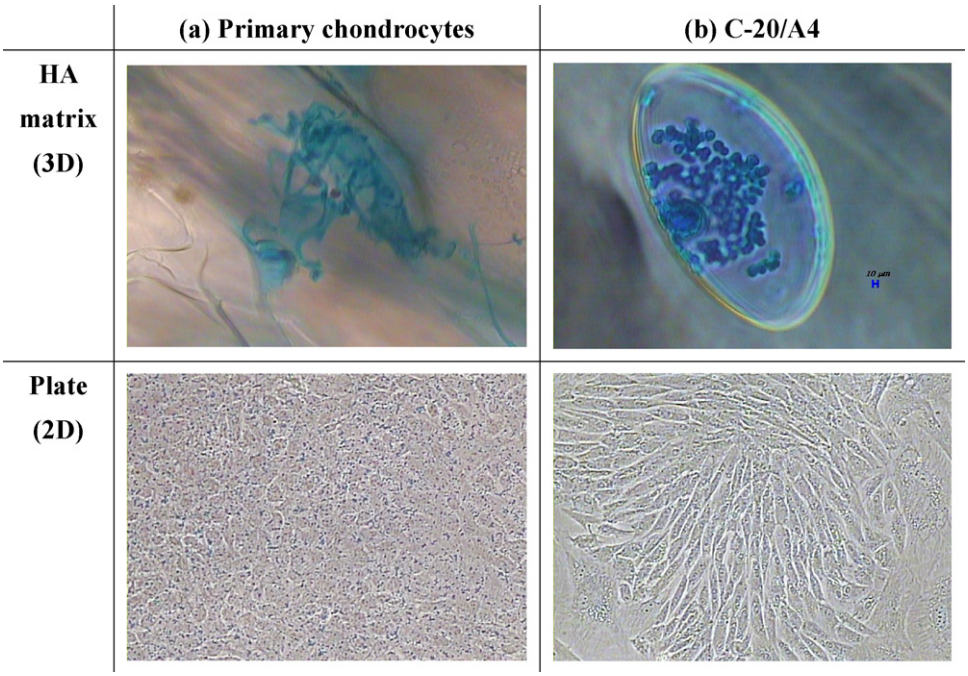

Fig. 6. s-GAG production by Alcian blue staining. Primary chondrocytes (a) and C-20/A4 (b) were cultured for 7 days in 3D HA matrix and 2D tissue culture plate. The stain of the extracellular matrix of the cartilage-like tissue was stronger in the 3D HA matrix than in the 2D plate culture. Samples were observed by a light microscope. Original magnification of HA matrix: x 200; of tissue culture plate × 100.

Retaining macromolecules of the cell in the scaffold is critical in cell-based strategies and this can be controlled by the size of pores in the scaffold. Too large a pore size could cause insufficient initial attachment of cells; enlargement of the pore size might decrease the proportion of cells that adhere to the scaffold. Even though chondrocytes are metabolically active, they seem to lack the appropriate environment or proper stimuli to produce extracellular matrix in large pore size scaffolds (Sibylle et al., 2003). In contrast, when the pore size in the scaffold is too small, it may potentially limit the access of nutrients (Allemann et al., 2001) and impede the permeation of cell waste that needs to be secreted and eliminated. In addition, pores too small may interfere with a homogeneous cell distribution within the scaffold and increase the edge effect (Sibylle et al., 2003).

The pore size of the HA matrix used in this study was managed by adjusting the NaOH concentration, the amounts of HA contents, and swelling period. As shown in Fig 1, it was possible to control and regulate the pore size of HA matrix from 30 to 500 μm. Based on the SEM study, the combination of 0.3 N NaOH and 1 g HA, with 1 day of swelling period (b, d, h) turned out to be the condition for the most appropriate pore size for the chondrocytes culture in the HA matrix; the actual pore sizes were between 50 and 300 μm.

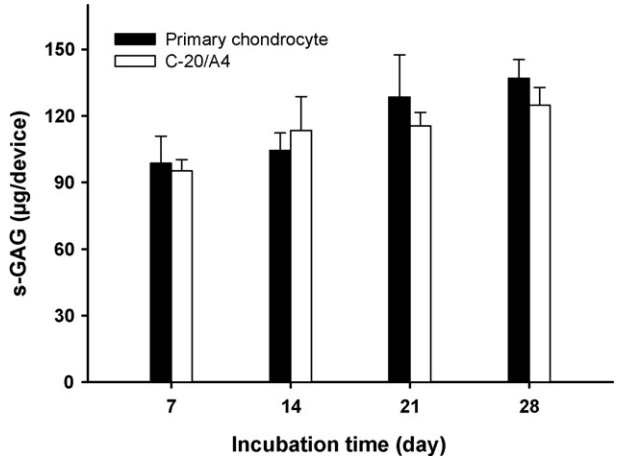

Fig. 7. Quantitative determination of s-GAG synthesis from (a) primary chondrocytes and (b) C-20/A4 cultivated within the HA matrix analyzed by the dimethylmethylene blue dye method. Error bars mean standard deviations ($n = 4$).

Another important aspect in the development of a novel scaffold is the evaluation of cytotoxicity. To improve mechanical properties, degradation rate, and clearance, HA is usually chemically modified or cross-linked to form hydrogel materials. The conventional cross-linkers such as glutaraldehyde and carbodiimde that have been used in other studies have demonstrated high toxicity that could cause several side effects (Gough et al., 2002). In this study, PEGDG was used in order to minimize possible risk of toxicity, based on a previous report which reported that diepoxy cross-linkers exhibited lower cytotoxicity than formaldehyde, glutaraldehyde, and a water-soluble carbodiimide (Nishi et al., 1995). As shown in Figs. 2, 3 and 4, the results not only showed that chondrocytes attached well and retained their good viability in the 3D HA matrix, but also suggested that they proliferated well and aggregated into distinct nodules containing large clusters of spherical cells. These results clearly proved that the 3D HA matrix prepared in this study was safe and toxic free with good cellular viability and proliferation.

A major pitfall in cartilage cell-based therapy is the dedifferentiation of chondrocytes, which occurs during *in vitro* culture. Chondrocytes lose their round morphology and obtain a more fibroblast-like shape during monolayer culture (Glowacki et al., 1983). This process is accompanied by an increase of collagen type I expression, but at the same time by the decrease of collagen type II and aggrecan expression which are the distinctive markers of chondrocytic phenotype. When the chondrocytes were cultured in the 3D scaffold, chondrocytes retained their spherical morphology and showed an up-regulation of collagen type II and aggrecan expression as well as a down-regulation of collagen type I expression (Fig. 5). Chondrocytes expanded in the HA matrix produced more collagen type II and aggrecan which were well-documented differentiation markers of chondrocytes as the culture period increased.

On the other hand, collagen type I, which is mainly expressed by fibroblastic cells and usually found in monolayer cell cultures, was gradually reduced in chondrocytes grown in the HA matrix. These results, therefore, confirmed that the 3D HA matrix was capable of allowing a differentiation process.

Synthesis of s-GAG is one of the important functions of chondrocytes and plays a significant role in regulating the chondrocyte phenotype. In the present study, culturing chondrocytes in the 3D HA matrix led to a marked production of s-GAG, as shown in Figs 6 and 7. These results obviously demonstrate that HA matrix provides an environment that allows chondrocytes to be cultured into a 3D HA matrix enabling synthesis of s-GAG.

Overall, the results demonstrated that the 3D matrix of the chemically unmodified HA was successfully prepared with minimum cross-linking, and seemed to be a suitable scaffold for the 3D culture of chondrocytes. This scaffold permitted the growth of phenotypically stable chondrocytes, and enabled the synthesis of cartilage-like ECM. Whether this HA matrix could support cartilage repair *in vivo* remains to be further investigated in adapted animal models.

5. Conclusions

A simple and novel porous matrix for the three-dimensional culture of chondrocytes was successfully prepared by using an HA matrix using PEGDG for minimal cross-linking. Pore size of the matrix could be optimized by changing the NaOH concentration, swelling period, and amount of HA contents. Compared to that prepared by the 2D plate culture, this scaffold permitted the growth of phenotypically stable chondrocytes and synthesis of cartilage-like extracellular matrix. These results suggest that the new porous HA matrix could be an effective scaffold for chondrocytes delivery for the treatment of articular cartilage defects.

Acknowledgements

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