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ARTICLE

Effect of High Molecular Weight Hyaluronic Acid on Chondrocytes Cultured in Collagen/Hyaluronic Acid Porous Scaffolds

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Shangwu Chen,[a,b] Qin Zhang, PhD,[a,b] Naoki Kawazoe, PhD,[b] and Guoping Chen, PhD[a,b*]

Engineering cartilage tissue by culturing chondrocytes in porous scaffolds is one promising method to repair or restore the functions of diseased cartilage. Hyaluronic aid (HA) was used in porous scaffolds or hydrogels to promote the proliferation of chondrocytes and synthesis of cartilage extracellular matrix (ECM). However, whether HA in scaffolds has a beneficial effect on chondrocytes remains uncertain, possibly due to the uncontrolled pore structure and inhomogeneous HA in scaffolds. In this study, homogeneous collagen/HA scaffolds with well-controlled and interconnected pore structure were prepared by suppression of polyion complex formation between collagen and HA and using ice particulates as porogen. The pore structure and mechanical property of collagen/HA scaffolds and collagen scaffolds could be well controlled. High molecular weight HA in collagen/HA scaffolds inhibited the cellular proliferation, synthesis of sulfated glycosaminoglycan and cartilage ECM, compared with the results of collagen scaffolds. This study should provide additional information on the effects of HA in porous scaffolds on the chondrocyte behaviour in 3D culture.

1. Introduction

Human articular cartilage has limited self-repair because of the avascular nature, low rate of chondrocyte proliferation and matrix turnover [1-3]. Tissue engineering has been developed to engineer cartilage tissue to repair and restore the function of diseased cartilage. In cartilage tissue engineering, chondrocytes from the non-load bearing parts of cartilage are isolated, expanded *in vitro*, seeded in a three-dimensional (3D) scaffolds and cultured to engineer cartilage tissue. 3D porous scaffolds can provide the space for the adhesion and proliferation of chondrocytes and formation of extracellular matrix of cartilage [4-6].

Porous collagen scaffolds have been widely used to engineer various types of tissues such as skin, muscle and cartilage [7-12]. Collagen scaffolds with a high porosity and good interconnectivity are required to enable the homogeneous seeding of chondrocytes in the whole scaffolds and nutrient/oxygen transfer during cell culture. Porous collagen scaffolds can be prepared by using freeze-drying a collagen aqueous solution and cross-linking treatment. To enable good interconnectivity of collagen scaffolds, ice particulates have been used as porogen which can also initiate ice crystallization during freezing of collagen solution for the formation of interconnected pore structure after freeze-drying [13, 14].

Hyaluronic acid (HA) is abundant in the synovial fluid which functions to lubricate the surface of articular cartilage of joints. HA is also contained in cartilage and low concentrations of HA has important biological effects [15, 16]. HA has been used to maintain the phenotype, promote the proliferation of chondrocytes and synthesis of cartilage ECM. For example, chondrocytes have been cultured in hydrogels that contained HA of different concentrations and the concentration of HA affected the cell proliferation and synthesis of aggrecan or chondroitin sulfate[17, 18]. HA has also been supplemented in culture medium to culture chondrocytes in hydrogels or porous scaffolds, and HA in the medium affected cell proliferation and ECM synthesis in a dose-dependent manner [19, 20]. However, the effects of HA in collagen scaffolds or hydrogels remains controversial. While some studies have shown high molecular weight (MW) HA can promote the proliferation of chondrocytes and the synthesis of sulfated glycosaminoglycan (sGAG), some other studies have shown that high MW HA suppresses the expression of chondrogenesis-related genes such as the genes for collagen type II and aggrecan [21-23]. The inhomogeneous HA in scaffolds due to formation of polyion complex (PIC) between collagen and HA and uncontrolled pore structure from normal freeze-drying might confound the effect of HA on chondrocytes.

In this study, homogeneous collagen/HA scaffolds were prepared to study the effect of high MW HA on the proliferation and ECM synthesis of chondrocytes in 3D culture. Firstly, a homogeneous suspension of collagen/HA was prepared by suppression of PIC formation between collagen and HA. Secondly, the homogeneous collagen/HA suspension was used in combination with ice particulates to prepare collagen/HA scaffolds with well-controlled and interconnected pore structure. Finally, the porous scaffolds were used to culture bovine articular chondrocytes and the cell proliferation, ECM synthesis and tissue formation were examined to study the effect of high MW HA in collagen scaffolds for cartilage tissue engineering.

[a] *Tissue Regeneration Materials Unit, International Center for Materials Nanoarchitectonics, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan*
[b] *Department of Materials Science and Engineering, Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8571, Japan*

2. Materials and methods

2.1. Preparation of porous collagen/HA scaffolds

An aqueous solution (pH = 3.0) of type I collagen (2 wt.%) from porcine skin (Nippon Meat Packers, Inc.) and an aqueous solution of high MW hyaluronic acid (HA) (2 wt.%) from rooster comb (MW, ~ 1 × 10^6 Da; Wako Pure Industries, Ltd.) were used to prepare aqueous suspensions of collagen/HA mixture. 10 v.% of ethanol was used to dissolve collagen and HA. Before the two solutions were mixed, sodium chloride (NaCl) granules were added separately to the collagen and HA aqueous solutions, and the solutions were gently stirred at 4 °C for 12 h. The collagen and HA aqueous solutions were then mixed at a ratio of 9 : 1 (collagen : HA, v/v) and gently stirred at 4 °C for 24 h. Type I collagen (2 wt.%) solution without NaCl addition was used to prepare collagen scaffolds. The transmittance of the collagen/HA suspensions at 500 nm with different concentrations of NaCl was measured using an UV-Vis spectrophotometer (V-660, JASCO, Inc.).

Collagen/HA scaffolds and collagen scaffolds were prepared by using ice particulates and freeze-drying [11]. Briefly, ice particulates were prepared by spraying pure water into liquid nitrogen and sieving to control their diameter to 150 - 250 μm. Images of the ice particulates were imported into ImageJ software to measure the diameter of ice particulates. Collagen/HA suspension (NaCl concentration: 0.2 M) and collagen solution were cooled and mixed with pre-prepared ice particulates at the ratio of 1:1 (volume of liquid (ml): weight of ice particulates (g)) at -5 °C. The mixtures were transferred into a silicone mold (60 mm × 40 mm × 5 mm) on a copper plate wrapped with perfluoroalkoxy (PFA) film (Universal Co., Ltd). The mixture in the mold was flattened by covering the silicon frame with a glass plate wrapped with PFA film. The entire construct was frozen at -80 °C for 9 h. After glass plate and copper plate were removed from the construct, the frozen constructs were freeze-dried under a vacuum of less than 5 Pa for 24 h by using a freeze dryer (FDU-2200, Tokyo Rikakikai Co., Ltd.) to obtain porous scaffolds. The porous scaffolds were crosslinked using a solution of 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Peptide Institute, Inc.) and 20 mM N-hydroxysuccinimide (NHS, Wako Pure Chemical Industries, Ltd.) in an ethanol/water mixture (80/20, v/v) for 8 h as reported previously [24]. The crosslinked scaffolds were washed with MilliQ water for six times, soaked in MilliQ water, frozen and freeze-dried again under the same conditions.

2.2. Scanning electron microscopy and compression test

Crosslinked scaffolds were cut with a blade and their cross sections were sputter-coated with platinum. The cross sections were observed at 10 kV with a scanning electron microscope (SEM) (JSM-5610, JEOL, Ltd.). SEM images were imported in ImageJ software to measure the pore size of scaffolds. Three images of each type of scaffolds were imported and at least 60 pores per image were measured. For unconfined compression test, crosslinked scaffolds were cut into disks with a diameter of 6 mm and a height of 3 mm using a biopsy punch. Mechanical property of scaffolds in dry state and hydrated state were tested. The dry scaffolds were immersed in phosphate buffered saline (PBS) for 2 hours at room temperature to get hydrated scaffolds. Each sample was placed on the testing platform (Heavy Duty Platform, Stable Micro System) of a texture analyzer (TA.XTPlus, Texture Technologies Corp.) and compressed with a 20-mm diameter cylinder probe (P/20, Stable Micro System). The compression test started with a trigger force of 0.1 g. Samples were compressed at the rate of 0.1 mm/s until reaching 80% strain. Stress and strain values were acquired at the sampling frequency of 50 Hz by using a TA.XTPlus software to generate stress-strain curves. Young's modulus was calculated from the initial linear region of the stress-strain curve and sample dimension. A minimum of four samples were tested for each type of scaffold.

2.3. Culture of cells in scaffolds

Bovine articular chondrocytes (BACs) were isolated from the articular cartilage of the knees of 9-week old female calves from a local slaughterhouse as reported previously [11]. Cell culture medium was prepared from high-glucose Dulbecco's Modified Eagle's Medium (D6546, Sigma-Aldrich Co.) supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 μg/ml ascorbic acid. The primary BACs were subcultured in tissue culture flasks with the culture medium. P2 chondrocytes were harvested and suspended in the culture medium to get the suspension of BACs (3.33 × 10^7 cells/ml) for cell seeding.

Porous scaffolds were punched into disks (6 mm in diameter and 3 mm in thickness), sterilized with 70(v/v)% ethanol aqueous solution, washed with PBS for 6 times and conditioned with culture medium at 37 °C for 2 hours. After the culture medium in the scaffold disks was absorbed away using sterilized Kimwipe paper, 60 μl of the chondrocyte suspension was pipetted onto each scaffold disk and incubated at 37 °C. After 3 hours of incubation, the scaffolds were turned upside down and the medium inside was absorbed away. Another 60 μl chondrocyte suspension was pipetted onto each scaffold disk and incubated at 37 °C for 3 hours and culture medium was added. After 1 day culture, all the cell/scaffold constructs were transferred to 25-cm^2 tissue culture flasks and cultured in the medium under shaking (60 rpm) for 4 weeks.

2.4. Live/dead staining

Live/dead staining was performed to evaluate cell viability using calcein-AM and propidium iodide staining reagents (Cellstain Double Staining Kit, Dojindo Laboratories). After culture for 1 day, 1 week, 2 weeks or 4 weeks, the cell/scaffold constructs were washed with PBS and incubated in PBS-diluted calcein-AM (2 μM) and propidium iodide (4 μM) for 30 minutes. After being rinsed with PBS, the constructs were observed with a fluorescence microscope.

2.5. Quantification of DNA and sGAG and compression test of cultured constructs

Proliferation of the chondrocytes in scaffolds was evaluated by quantifying the DNA amount as described previously [24]. Briefly, cell/scaffold constructs cultured for 1 week, 2 weeks, 3 weeks or 4 weeks were harvested, freeze-dried and digested with papain solution. Aliquots of the papain digests were used to measure the DNA and sGAG amount of each sample. The DNA amount was quantified by using Hoechst 33258 dye (Sigma-Aldrich) and an FP-6500 spectrofluorometer (JASCO, Tokyo, Japan). The sGAG content was quantified by using Blyscan™ Glycosaminoglycan Assay (Biocolor Ltd., UK) according to the manufacturer's instructions.

Cell/scaffold constructs cultured for 1 week, 2 weeks or 4 weeks were harvested and kept in culture medium before compression test. The compressive modulus of cell/scaffold constructs was determined by using the compressive test described above. Four samples were used to calculate the average and standard deviation.

2.6. Histological staining

Cell/scaffold constructs cultured for 4 weeks were washed in PBS and fixed in 10% neutral buffered formalin for 2 days at room temperature. The constructs were dehydrated in ethanol series, embedded in paraffin and sectioned to get the vertical cross sections of constructs (8-µm thick). The cross sections were deparaffinized and stained with hematoxylin/eosin staining or safranin O/light green staining as described in previous work [11].

2.7. Statistical analysis

Student-t test was used to compare two groups of data. Significant differences were considered when $p < 0.05$.

3. Results and discussion

Collagen/HA scaffolds were prepared by suppressing formation of polyion complex (PIC) between collagen and HA and using ice particulates as porogen. Before collagen and HA solutions were mixed, sodium choloride (NaCl) was added into each solution to increase their ionic strength so that the formation of PIC in collagen/HA suspension could be suppressed. Formation of PIC was dependent on the ionic strength of collagen/HA suspensions (Fig. 1). Collagen/HA suspension without NaCl treatment was opaque due to the formation of PIC and the inhomogeneous suspension made it difficult to prepare a homogeneous scaffold. Collagen/HA suspensions with a high NaCl concentration (0.20 - 0.30 M) were transparent due to the suppression of PIC formation. Sodium and chloride ions in these suspensions shielded the electronic interaction of oppositely charged collagen and HA molecules and

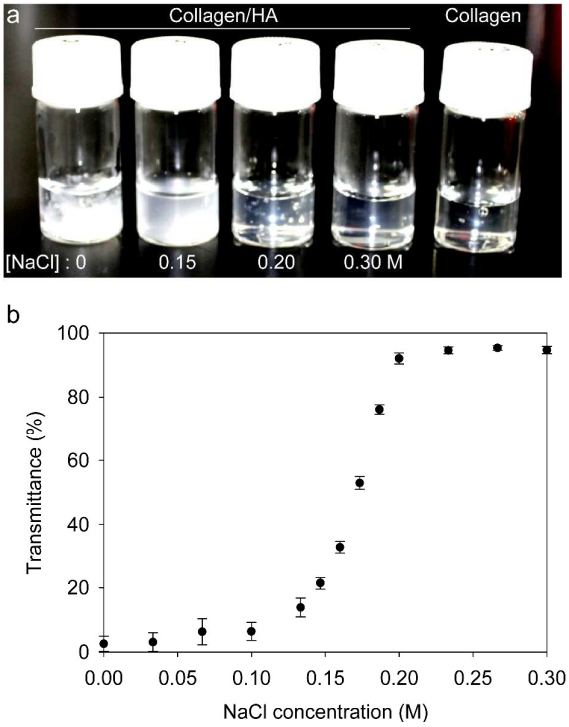

Fig. 1. Suppression of polyion complex formation in collagen/HA suspension by adding NaCl. (a) Collagen solution and collagen/HA suspensions with different concentration of NaCl ([NaCl] = 0 - 0.3 M). (b) Transmittance at 500 nm of collagen/HA suspensions with different concentration of NaCl. Means ± SD, N = 3.

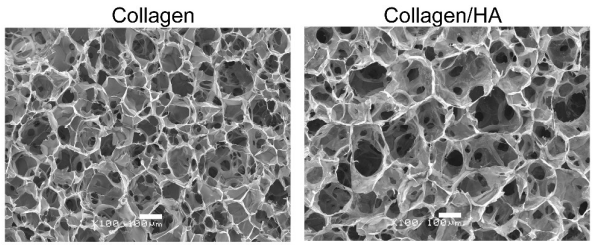

Fig. 2. SEM images of cross sections of collagen and collagen/HA scaffolds after crosslinking. Scale bar = 100 µm.

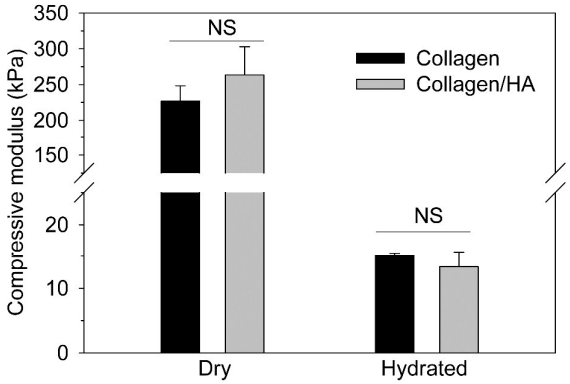

Fig. 3. Compressive modulus of crosslinked collagen and collagen/HA scaffolds in dry state or hydrated state. Means ± SD, N = 4. NS, no significant difference.

suppressed the formation of PIC. A homogeneous collagen/HA suspension (NaCl concentration = 0.2 M) was chosen to prepare collagen/HA scaffolds. Collagen solution without HA was used to prepare collagen scaffolds as the control group.

Collagen scaffolds and collagen/HA scaffolds had homogeneous, interconnected pore structure which were well controlled by using ice particulates and freeze-drying (Fig. 2). The ice particulates used to prepare scaffolds had the diameter of 179 ± 33 µm. The macropores of collagen scaffolds and collagen/HA had the diameter of 172 ± 38 µm and 187 ± 45 µm, respectively, which were controlled by the size of ice particulates (Fig. S1 and Fig. S2). The macropores were interconnected by the micropores due to the ice crystallization between neighbouring ice particulates [13]. The mechanical property of these scaffolds in dry state or hydrated state was quantified using a compression test. The two types of scaffolds in dry state or hydrated state had similar compressive moduli (Fig. 3). The dry scaffolds had higher compressive moduli than hydrated scaffolds. The two types of scaffolds had the same scaffold density (both prepared from 2 wt.% polymer solution). Collagen scaffolds were prepared from 2 wt.% collagen solution and collagen/HA scaffolds were prepared from the solution with 1.8 wt.% collagen and 0.2 wt.% HA. The polymer mass of the solutions used to prepare two types of scaffolds was the same. In addition, the two types of scaffolds had similar pore structure which was controlled by using ice particulates as porogen. The similarity in scaffold density and pore structure might explain the similar compressive modulus of two types of scaffolds. These results showed that the pore structure and mechanical property of two types of scaffolds were controlled by using ice particulates as porogen.

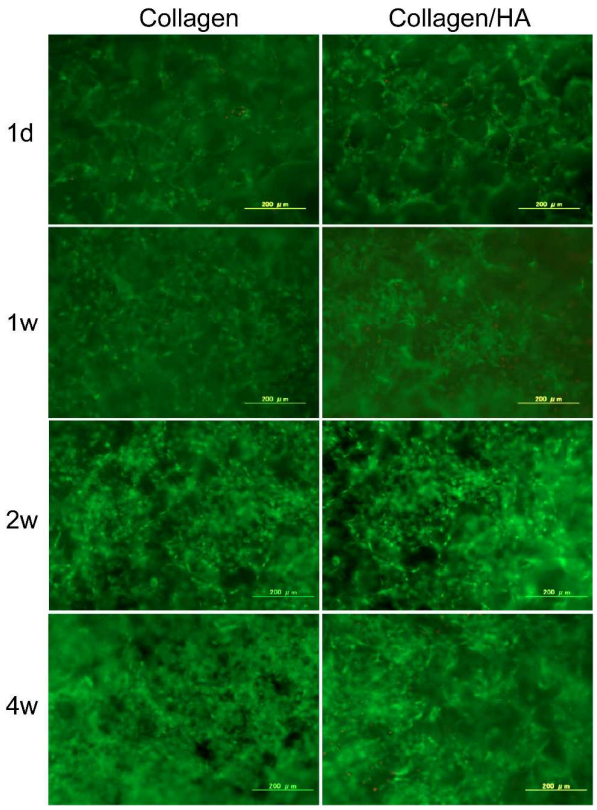

Fig. 4. Live/dead staining of chondrocytes cultured in collagen and collagen/HA scaffolds for 1 day (1d), 1 week (1w), 2 weeks (2w) and 4 weeks (4w). Green: live cells; red: dead cells. Scale bar = 200 µm.

Bovine articular chondrocytes were seeded in the porous scaffolds to study the effects of high MW HA in porous collagen scaffolds on the functions of chondrocytes. Live/dead staining of the cells cultured in the collagen and collagen/HA porous scaffolds showed that cell proliferated and maintained a high viability during the 4-week culture (Fig. 4). The proliferation of chondrocytes in the porous scaffolds was measured using DNA quantification (Fig. 5a). The cells proliferated in the two types of porous scaffolds during the 4-week culture, with a greater proliferation rate in collagen scaffolds than that in collagen/HA scaffolds. The DNA amount in collagen scaffolds was significantly higher than that in collagen/HA scaffolds after 2-week culture. Synthesis of sGAG by chondrocytes in the porous scaffolds were also measured (Fig. 5b). The chondrocytes cultured in collagen scaffolds synthesized significantly higher amount of sGAG than those in collagen/HA scaffolds. The compressive modulus of cell/scaffold constructs was measured after cell culture for different time (Fig. 5c). The compressive modulus of cell/scaffold constructs increased with culture time. Cell/scaffold constructs from collagen scaffolds had significantly higher compressive modulus than the constructs from collagen/HA scaffolds.

The formation of cartilage tissue in cell/scaffold constructs were examined using HE staining and safranin O staining. HE staining of the cell/scaffold constructs cultured for 4 weeks showed that the chondrocytes cultured in collagen scaffolds synthesized ECM that filled the pores of collagen scaffolds (Fig. 6). In contrast, the chondrocytes cultured in collagen/HA scaffolds synthesized less ECM, most of which were synthesized in the peripheral region of porous scaffolds. Similarly, the safranin O staining of cell/scaffold constructs from collagen scaffolds showed more intense staining of sGAG compared with those from collagen/HA scaffolds (Fig. 7). These results showed that the chondrocytes cultured in collagen scaffolds synthesized more cartilage tissue than those cultured in collagen/HA scaffolds.

Whether addition of high MW HA in hydrogel or porous scaffolds has a beneficial effect on chondrocytes is controversial. For example, when high MW HA was added in the culture medium to culture chondrocytes-seeded gelatin sponge, the DNA amount and sGAG synthesis increased with the concentration of HA [22].

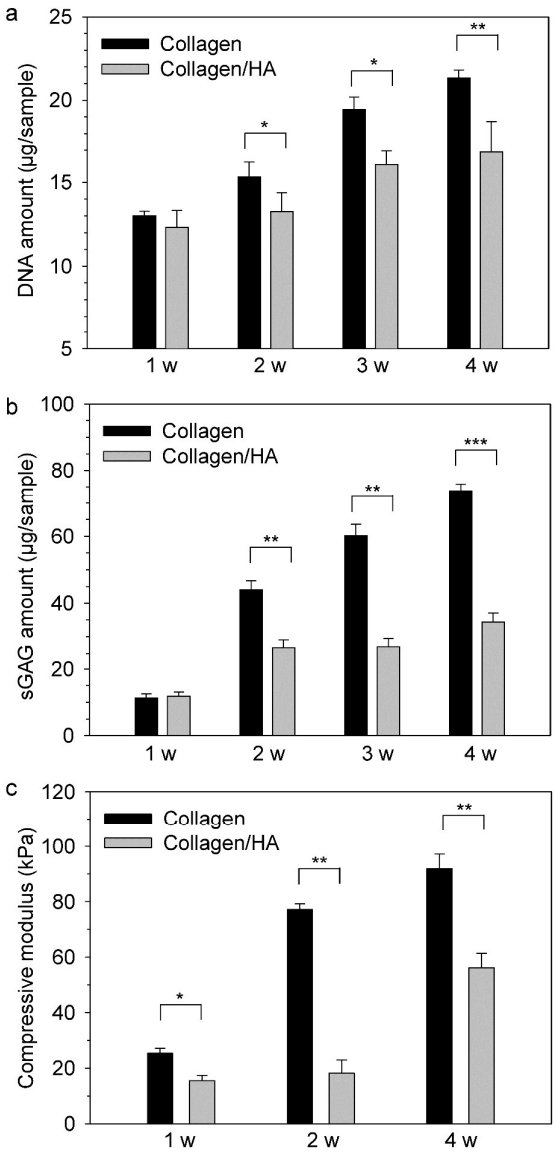

Fig. 5. The proliferation, synthesis of sGAG by chondrocytes and the mechanical property of cultured constructs. (a, b) The amount of DNA and sGAG in cell/scaffold constructs cultured for 1 week (1w), 2 weeks (2w), 3 weeks or 4 weeks (4w). (c) The compressive modulus of cell/scaffolds constructs cultured for 1 week, 2 weeks or 4 weeks. Means ± SD, N = 4. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

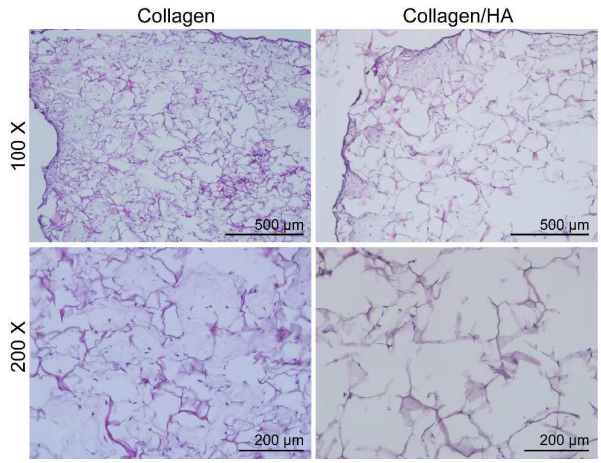

Fig. 6. HE staining of cell/scaffolds constructs cultured for 4 weeks *in vitro*. Images were taken at low and high magnifications.

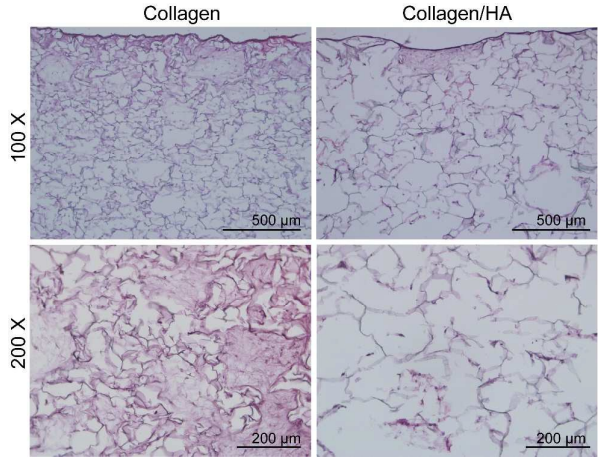

Fig. 7. Safranin O staining of cell/scaffolds constructs cultured for 4 weeks *in vitro*. Images were taken at low and high magnifications.

When high MW HA was used to prepare collagen/HA scaffolds or hydrogel for chondrocyte culture, lower amount of HA (2%, 5%) promoted the synthesis of sGAG or mRNA of type II collagen and aggrecan but a greater amount of HA (10%, 14%) inhibited the synthesis of sGAG and cartilage ECM [18, 21]. It was found that increasing amount of HA in collagen/HA scaffolds led to reduction of pore size of porousscaffolds which could impede the migration, proliferation and function of chondrocytes [21]. For high concentrations of HA in hydrogels, HA molecules would overlap with each other forming entanglements and hydrophobic patches. These physical properties of HA were suggested to inhibit the synthesis of cartilage ECM [18]. However, other studies pointed out that HA affected chondrocyte behaviour through its chemical properties rather than its physical properties. For example, Toole et al. found that very low concentrations of HA in culture medium (as low as 1 ng/ml) inhibited chondrogenesis on chondrocytes [16]. Solursh et al. found that not only HA in culture medium inhibited chondrogenesis, the oligosaccharides derived from HA by using hyaluronidase caused more potent inhibition effect [25]. It is noteworthy that the uncontrolled pore structure of porous scaffolds and inhomogeneous distribution of HA in porous scaffolds or hydrogel could confound the effects of HA on the proliferation and ECM synthesis of chondrocytes.

In the present study, homogeneous collagen/HA mixture suspension were prepared by suppression of PIC formation between collagen and HA. In addition, ice particulates were used as porogen to control the pore structure of collagen/HA scaffolds. The effects of high MW HA on chondrocytes were examined using the 3D collagen/HA scaffolds with well-controlled pore structure and homogeneous HA. We hypothesized that these collagen/HA scaffolds wound favour the chondrogenic behaviour of chondrocytes. However, the results showed that high MW HA in the porous scaffolds inhibited the cellular proliferation, synthesis of sGAG and formation of cartilage ECM from chondrocytes. Due to the inhibition of ECM formation within collagen/HA scaffolds, the mechanical property of cultured tissue from collagen/HA scaffolds were significantly lower than those from collagen scaffolds. It was also found that a lower amount of high MW HA (2%, 5%) in collagen/HA scaffolds prepared using ice particulates and suppression of PIC formation also caused inhibition effect on chondrocytes, with a trend of higher amount of HA causing greater inhibition (Fig. S3). Our results were in agreement with previous studies that used high MW HA in the medium for two-dimensional culture of chondrocytes [25-28]. Different concentrations of High MW HA (from 1×10^{-4} μg/ml to 100 μg/ml) was applied homogenously into culture medium, which inhibited the sGAG synthesis and accumulation of synthesized proteoglycan in the chondrocyte layer, with higher concentration of HA caused greater inhibition effect. By excluding the interfering factors such as uncontrolled pore structure and PIC formation in porous scaffolds, we also found that the homogenous HA in collagen/HA scaffolds inhibited the chondrogenesis in a similar dose-dependent manner. This inhibition effect from HA were found to be specific to chondrocytes but not to skin fibroblasts [26], which is in agreement of our previous study that showed collagen/HA scaffolds promoted the proliferation of skin fibroblasts [24].

4. Conclusions

Collagen/HA scaffolds with well-controlled pore structure and homogenous HA were prepared by using ice particulates as porogen and suppression of polyion complex formation. The homogeneous mixture suspension of collagen and HA were achieved by using NaCl to suppress the formation of polyion complex. The pore structure and mechanical property of collagen and collagen/HA scaffolds could be controlled by using ice particulates. Compared with collagen scaffolds, high MW HA in the porous collagen/HA scaffolds inhibited the proliferation, synthesis of sGAG and formation of cartilage ECM from chondrocytes. This study should provide additional information on the effects of high MW HA in porous scaffolds on the behaviour of chondrocytes in 3D culture.

Acknowledgements

This work was supported by the World Premier International Research Center Initiative on Materials Nanoarchitectonics and a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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