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Fibrin glue mixed with gelatin/hyaluronic acid/chondroitin-6-sulfate tri-copolymer for articular cartilage tissue engineering: the results of real-time polymerase chain reaction

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Received 24 July 2006; revised 13 September 2006; accepted 3 November 2006
Published online 00 Month 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31186

Abstract: Autologous fibrin glue has been demonstrated as a potential scaffold with very good biocompatibility for neocartilage formation. However, fibrin glue has been reported not to provide enough mechanical strength, but with many growth factors to interfere the tissue growth. Gelatin/hyaluronic acid/chondroitin-6-sulfate (GHC6S) tri-copolymer sponge has been prepared as scaffold for cartilage tissue engineering and showed very good results, but problems of cell seeding and cell distribution troubled the researchers. In this study, GHC6S particles would be added into the fibrin glue to provide better mechanical strength, better cell distribution, and easier cell seeding, which would be expected to improve cartilage regeneration *in vitro*. Porcine cryo-precipitated fibrinogen and thrombin prepared from prothrombin activated by 10% $CaCl_2$ solution were used in two groups. One is the fibrin glue group in which porcine chondrocytes were mixed with thrombin–fibrinogen solution, which was then converted into fibrin glue. The other is GHC6S-fibrin glue in which GHC6S particles were added into the thrombin–fibrinogen solution with porcine chondrocytes. After culturing for 1–2 weeks, the chondrocytes cultured in GHC6S-fibrin glue showed a round shape with distinct lacuna structure and showed positive in S-100 protein immunohistochemical stain. The related gene expressions of tissue inhibitor of metalloproteinases-1, matrix metalloproteinase-2, MT1-MMP, aggrecan, decorin, type I, II, X collagen, interleukin-1 β, transforming growth factor-β 1 (TGF-β1), and Fas-associating death domain were checked by real-time PCR. The results indicated that the chondrocytes cultured in GHC6S-fibrin glue would effectively promote extracellular matrix (ECM) secretion and inhibit ECM degradation. The evidence could support that GHC6S-fibrin glue would be a promising scaffold for articular cartilage tissue engineering.
 J Biomed Mater Res 80A: 000–000, 2007

Key words: GHC6S; fibrin glue; articular cartilage; tissue engineering; real-time PCR

INTRODUCTION

It is a great challenge to repair a damaged articular cartilage due to poor blood circulation for nutrients support. Many methods have been developed clinically that tried to improve the regeneration ability of damaged articular cartilage, such as microfracture, abrassive, mosaicplasty, and so forth. They all showed a promising result at the initial stage of postoperation. However, none among those completely recovered had a normal cartilage texture when checked with histological examination. Recently, tissue-engineering method has been progressively developed to prepare a construct with functional cells for articular cartilage therapy.[1,2]

As known, the triads of tissue engineering are cells, scaffolds, and signaling systems. Many scaffolds have been prepared as the scaffold for the articular cartilage tissue engineering.[3,4] In the previous study, gelatin/hyaluronan/chondroitin-6-sulfate tri-copolymer (GHC6S) sponge has been developed and proven to be a suitable scaffold for cartilage tissue engineering because of the similar composition to the extracellular matrix of articular cartilage.[5–7] However, preformed GHC6S sponge is generally out of reach in homogenous cell distribution due to the problems of mass transportation and hypoxia

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in the deep area of the sponge. The cells inside the sponge would migrate to the sponge surface for better nutrients and oxygen supply, which causes inhomogeneous cell distribution in the whole sponge. Even cell seeded with a special method, such as spinner flask, cells still migrate out of the sponge after having cultured for a period of time.[8–10]

Gel form scaffold has been used for better cell distribution as a cell carrier for articular cartilage tissue engineering. There are many materials available to be prepared as gel form for cartilage regeneration, for example, collagen, alginate, agarose, and fibrin.[11,12] Fibrin is derived from fibrinogen that can be harvested and isolated from patient's peripheral blood. Fibrinogen can be cryo-precipitated and polymerized to form sticky glue by activated thrombin. Silverman et al. showed that fibrin glue might be used as an adequate scaffold for neocartilage formation.[13] However, they provided less mechanical strength for latter clinical applications.[14,15] Taking the mechanical strength into consideration, Peretti et al. added the devitalized cartilage chips to fibrin glue as scaffold for tissue engineering of articular cartilage to increase the mechanical strength and to prevent shrinkage after culturing for a period of time.[16–18]

In this study, GHC6S particles are going to be added into fibrin glue to evaluate the possibility as the scaffold for articular cartilage tissue engineering. Porcine chondrocytes will be seeded in the scaffold and then cultured for 1 and 2 weeks. The expression of aggrecan, decorin, type I, II, X collagens were used to evaluate the synthesis of extracellular matrix (ECM). Transforming growth factor-β 1 (TGF-β1) and Interleukin-1 β (IL-1β) would be used to elucidate possible pathways of the regulation on ECM secretion. Matrix metalloproteinase-2 (MMP-2), membrane type 1-metalloproteinase (MT1-MMP), and tissue inhibitor of metalloptoreinases-1 (TIMP-1) were used to check ECM degradation. Fas-associating death domain-containing protein (FADD) would be used to check the cell apoptosis/necrosis. These gene expressions were evaluated to prove whether chondrocytes still keep its phenotype and remain normal function in catabolism/anabolism. The histochemicomorphormetric examinations will be in conjunction with special stain, such as Alcian blue, Hematoxylin & Eosin, and S-100 protein, to further prove chondrocyte as a normal expression of ECM.

MATERIALS AND METHODS

Fibrinogen and thrombin preparation

Fresh whole blood was obtained from porcine in a single-used blood bag (CDPA-1 whole blood, Terumo Corporation, Japan). After centrifugation at 2200*g* for 10 min, plasma was separated as the supernatant from the whole blood. Then, plasma was frozen at the temperature of −20°C in a centrifuge tube for use. Ten milliliters of fibrinogen solution was obtained from 50 mL of plasma by cryo-precipitation. To activate the prothrombin, 0.6 mL of 10% $CaCl_2$ solution was added in to 10 mL of plasma. Subsequently, the solution was gently mixed by inversion and was kept at the room temperature for 15 min. Thrombin solution was obtained from the supernatant of the mixing solution after centrifugation at 2200*g* for 10 min. The concentrated fibrinogen solution and thrombin solution were ready for use.

Preparation of GHC6S particles

Gelatin (G-2500, Sigma Co., St. Louise, MO) was copolymerized with hyaluronic acid (HA) (Seikagaku Co., Tokyo, Japan) and chondroitin-6-sulfate (C6S) (C-4384, Sigma Co., St. Louise, MO)[5,6] by carbodiimide. Gelatin, HA, and C6S were sequentially dissolved and mixed well in 8 g of d.d.H_2O at a temperature of 40°C in 0.5 g, 0.5 mg, and 0.1 g of weight, respectively. Two milliliters of 1% cold 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (E1769, Sigma Co., St. Louise, MO) solution was added to the above well-mixed solution for cross-linking at a temperature of 25°C. The cross-linking reaction was performed with the presence of *N*-hydroxysuccinimideb (NHS) at the molar ratio of 0.2 of NHS to EDC. After gelation at the temperature of 4°C, the gel was frozen at a temperature of −20°C for 1 h followed by −80°C for 1 h, and then lyophilized for 72 h. After drying, the gel turned to a sponge structure, which was then soaked in 10 mL of 0.2% EDC solution at a temperature of 4°C for 48 h for further cross-linking. The sponge was dried as mentioned above to be frozen at a temperature of −20°C for 1 h followed by −80°C for 1 h, and then lyophilized for 72 h. The final sponge was quenched in liquid nitrogen and ground to small particles, called gelatin/hyaluronic acid/chondroitin-6-sulfate (GHC6S) particles. Finally, the particles were sterilized by EO gas.

Chondrocytes isolation

Hyaline cartilage was harvested from porcine knee joints and cut into small pieces about $5 \times 5 \times 0.5$ mm^3 in size. Immediately, the cartilage fragments were washed with PBS and sterilized by soaking in PBS containing antibiotics (250 μg/mL gentamycin, 1000 U/mL penicillin, and 2.5 μg/mL fungizon). The cartilage pieces were digested with 0.2% collagenase for 16 h at a temperature of 37°C in DMEM with 10% fetal calf serum addition. The isolated chondrocytes were resuspended and washed with PBS, then expanded by monolayer culture. Cell number was determined by hemocytometer with trypan blue dye.[5]

Cells seeded

After monolayer cultured for 1 week, the chondrocytes were trypsinized and resuspended in PBS, and mixed with fibrinogen solution to form the cell–fibrinogen solution. The activated thrombin solution was added into the cell–fibrinogen solution at a volume ratio of 1:1. The cell density of the cell–fibrinogen–thrombin solution was 2.86×105 cells/mL. Three hundred milligrams of GHC6S particles were immediately mixed with 10 mL cell–thrombin–fibrinogen solution as

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TABLE I
The Primers Designed for the Real-Time PCR[a]

Genes	Acc No.	Primer Sequence	Size
GAPDH	AF017079	Sense 5′-GTCATCCATGACAACTTCGG-3′; Antisense 5′-GCCACAGTTTCCCAGAGG-3′	103
Type I collagen	AF201723	Sense 5′-CAGAACGGCCTCAGGTACCA-3′; Antisense 5′-CAGATCACGTCATCGCACAAC-3′	101
Type II collagen	AF201724	Sense 5′-GAGAGGTCTTCCTGGCAAAG-3′; Antisense 5′-AAGTCCCTGGAAGCCAGAT-3′	118
Type X collagen	AF222861	Sense 5′-CAGGTACCAGAGGTCCCATC-3′; Antisense 5′-CATTGAGGCCCTTAGTTGCT-3′	117
Aggrecan	AF201722	Sense 5′-CGAAACATCACCGAGGGT-3′; Antisense 5′-GCAAATGTAAAGGGCTCCTC-3′	107
Decorin	AF140270	Sense 5′-GCATTTGCACCTTTGGTGAA-3′; Antisense 5′-GACACGCAGCTCCTGAAGAG-3′	102
MT1-MMP	NM_214239	Sense 5′-GCTGTGGTGTTCCAGACAAG-3′; Antisense 5′-GGATGCAGAAAGTGATCTCG-3′	111
MMP2	NM_214192	Sense 5′-GTTCTGGAGGTACAATGA-3′; Antisense 5′-ACCACGGCGTCCAGGTTA-3′	102
TIMP1	AF156029	Sense 5′-AACCAGACCGCCTCGTACA-3′; Antisense 5′-GGCGTAGATGAACCGGATG-3′	102
IL1-β	NM_001005149	Sense 5′-ACCTCAGCCCTCTGGGAGA-3′; Antisense 5′-GGGCTTTTGTTCTGCTTGAG-3′	102
TGF-β1	NM_214015	Sense 5′-GCACGTGGAGCTATACCAGA-3′; Antisense 5′-ACAACTCCGGTGACATCAAA-3′	114
FADD	NM_001031797	Sense 5′-CTCGGCAGCTTAAAGTGTCTGA-3′; Antisense 5′-GCCCTGAGCGCATCCA-3′	154

Acc No., gene bank accession number; size, size of PCR product in basepair.
[a]Mission Biotech, Taipei.

a batch. Then, 1 mL of cell–fibrinogen–thrombin solution with or without GHC6S particles was pipetted into each well of 24-well plates. The number of replicates is six. Cell-seeded GHC6S-fibrin glue was cultivated for 1 and 2 weeks. The cell-seeded fibrin glue (without GHC6S particle addition) would be the control group. The cultured constructs were harvested and evaluated by methods stated as follows.

Histological evaluation

The *in vitro* cultured specimens were fixed in 10% formalin and embedded in paraffin. Sections (6 μm in thickness) were stained with Hematoxylin and Eosin (H & E) and Alcian blue according to standard protocols.[5,19] Sections were incubated in 3% acetic acid for 10 min and washed by PBS. Then, sections were covered with Alcian blue (pH 1.0, MUTO PURE CHEMICALS Co., Japan) solution for 30 min to react with the sulfate groups of glycosaminoglycans as blue in color. AQ2

Immunohistochemical evaluation

After incubation in 3% H_2O_2, the sections were retrieved in citrate buffer solution (pH 7) at 95°C for 20 min. Goat serum was used to block nonspecific antigen on the sections, and they were then incubated for 1 h at room temperature with rabbit anticow S-100 protein antibodies (IgG, NCL-S100p from Novocastra Laboratories).[20] Subsequently, the sections were washed with PBS and incubated with the biotinylated goat antirabbit secondary antibody for 10 min. Enhanced horseradish peroxidase conjugated streptavidin was then bound to the biotinylated secondary antibody. Finally, the 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution was used to create an intense brown deposit around the antigen/antibody/enzyme complex in the section of the specimen.

Real-time PCR

First, total RNA was extracted for first strand synthesis after the constructs cultured for 1 and 2 weeks. Within the final volume of 13 μL, there were 11 μL of total RNA, 1 μL of 50 μ*M* oligo$(dT)_{20}$ (Y01212, Invitrogen Co. California), 1 μL of 10 m*M* dNTP (S1805,Clontech). After annealing at 65°C for 5 min, the mixture was chilled in ice for more than 1 min. Then, 4 μL First-Strand buffer, 1 μL of 0.1*M* DTT, 1 μL RNaseOUTTM, and 1 μL SuperScript III Ttase (Cat.No. 18064-014, Invitrogen Co. California) were added into the mixture. Subsequently, polymerization was allowed to occur at 50°C for 1 h followed by heating at 70°C for 15 min.[21] AQ3

The cDNA from the first strand reaction were used for real-time PCR. Real-time PCR was performed by ABI Prism 7000 (Applied Biosystems, Foster City, CA). The total volume of the universal reaction was 25 μL, which consisted of 12.5 μL of 2*X* SYBR green Master Mix (ABI no. 4309155), 5 μL of 1 μ*M* sense and antisense primer solution (Table I), and 7.5 μL of cDNA. The content of cDNA was 50 ng per reaction. To correct for pipetting errors, each cDNA sample was run in triplicate. After activation of SYBR green at a temperature of 50°C for 2 min, samples were then denatured at 95°C for 10 min. Subsequently, the samples were cycled 40 times in two stages, including a denaturation step at 95°C for 15 s and a following annealing/extension step at 60°C for 1 min. The gene expressions of GAPDH, aggrecan, decorin, MT1-MMP, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloptoreinases-1 (TIMP-1), type I, II, X collagen, interleukin-1 β (IL-1β), transforming growth factor-β 1 (TGF-β1), Fas-associating death domain (FADD) (Table I) were analyzed by number of threshold cycles (C_t). T1

Statistical analysis

All the samples in each group were analyzed by real-time PCR in three replicates to avoid pipetting error. The values of C_t of the genes are normalized by the value of C_t of GAPDH to shown as $-\Delta C_t$ shown as the means ± SD. Gene expression between two culture period was analyzed statistically by Student's *t* test with a level of significance of $\alpha = 0.01$.

RESULTS

Histological evaluation

Figure 1 showed the H&E stain pictures of chondrocyte cultured in fibrin glue or GHC6S-fibrin glue for 1 F1

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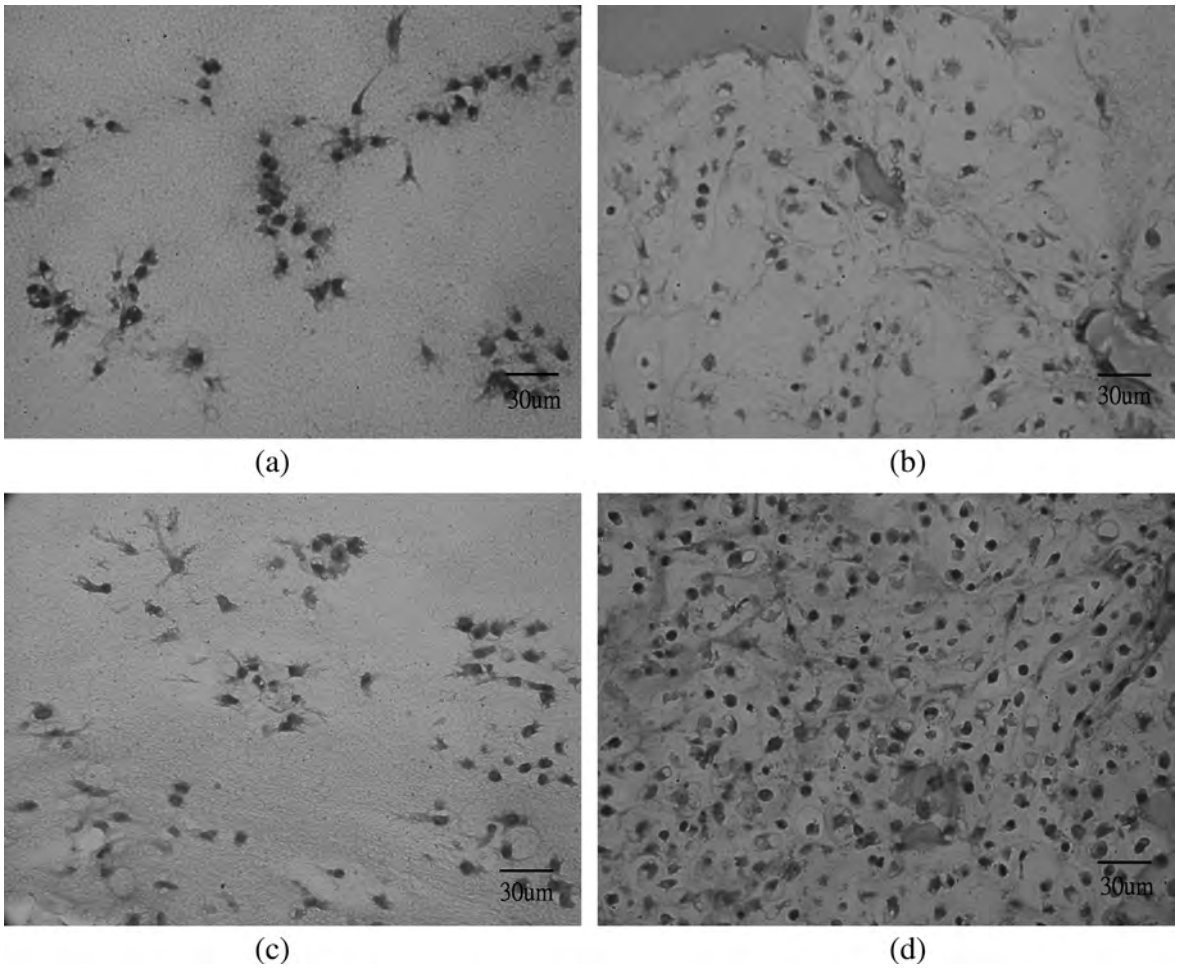

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Figure 1. After culturing for 1 week, constructs of chondrocytes cultured in fibrin glue (a) and in GHC6S-fibrin glue (b) were harvested. After culturing for 2 weeks, constructs in fibrin glue (c) and in GHC6S-fibrin glue (d) were prepared for parafilm sections. The prepared constructs were sectioned and stained with Hematoxyline and Eosin for histological examination. The cells cultured with only fibrin showed a fibrous-like shape. With apparent lacuna structure, the round shape chondrocytes were shown in GHC6S-fibrin glue after being cultured for 2 weeks. The scale bar shown in the figure is 30 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and 2 weeks. The cell distribution was not so homogeneous and more pseudopodia processes stretched out when cultured in fibrin glue for 1 week [Fig. 1(a)]. On the contrary, chondrocytes showed more homogeneous distribution and appeared in round shape when cultured in GHC6S-fibrin glue [Fig. 1(b)]. After culturing for 2 weeks, the chondrocytes cultured in fibrin glue showed more fibrous-like morphology [Fig. 1(c)]. If chondrocytes cultured in GHC6S-fibrin glue for 2 weeks, lacunas can be clearly examined in the picture [Fig. 1(d)].

F2 Figure 2 showed the Alcian blue stain of chondrocyte after culturing in GHC6S-fibrin glue or fibrin glue for 1 and 2 weeks. The results were quite similar to those of the H&E stain. When chondrocytes cultured in fibrin glue for 1 week, the sulfated glycosamninoglycans (GAGs) were secreted and surrounded by the cell clusters [Fig. 2(a)]. After culturing in GHC6S-firbin glue for 1 week, the GAGs distribution was more homogeneous than that of cultured in fibrin glue [Fig. 2(b)]. After culturing in fibrin glue for 2 weeks, chondrocytes secreted more sulfated GAGs and some of lacuna could be observed [Fig. 2(c)]. When chondrocytes were cultured in GHC6S-fibrin glue for 2 weeks, the lacuna was obviously observed with deep Alcin blue stain appeared in the extracellular matrix [Fig. 2(d)].

F3 Figure 3 showed the pictures of S-100 protein immunohischemical stain with hematoxylin counter stain. Most of the chondrocytes cultured in GHC6S-fibrin glue was observed in brown color, but not for those cultured in fibrin glue.

Gene expression of articular cartilage related collagens

In real-time PCR, the $-\Delta C_t$ values of genes were normalized by the house-keeping gene, GAPDH.

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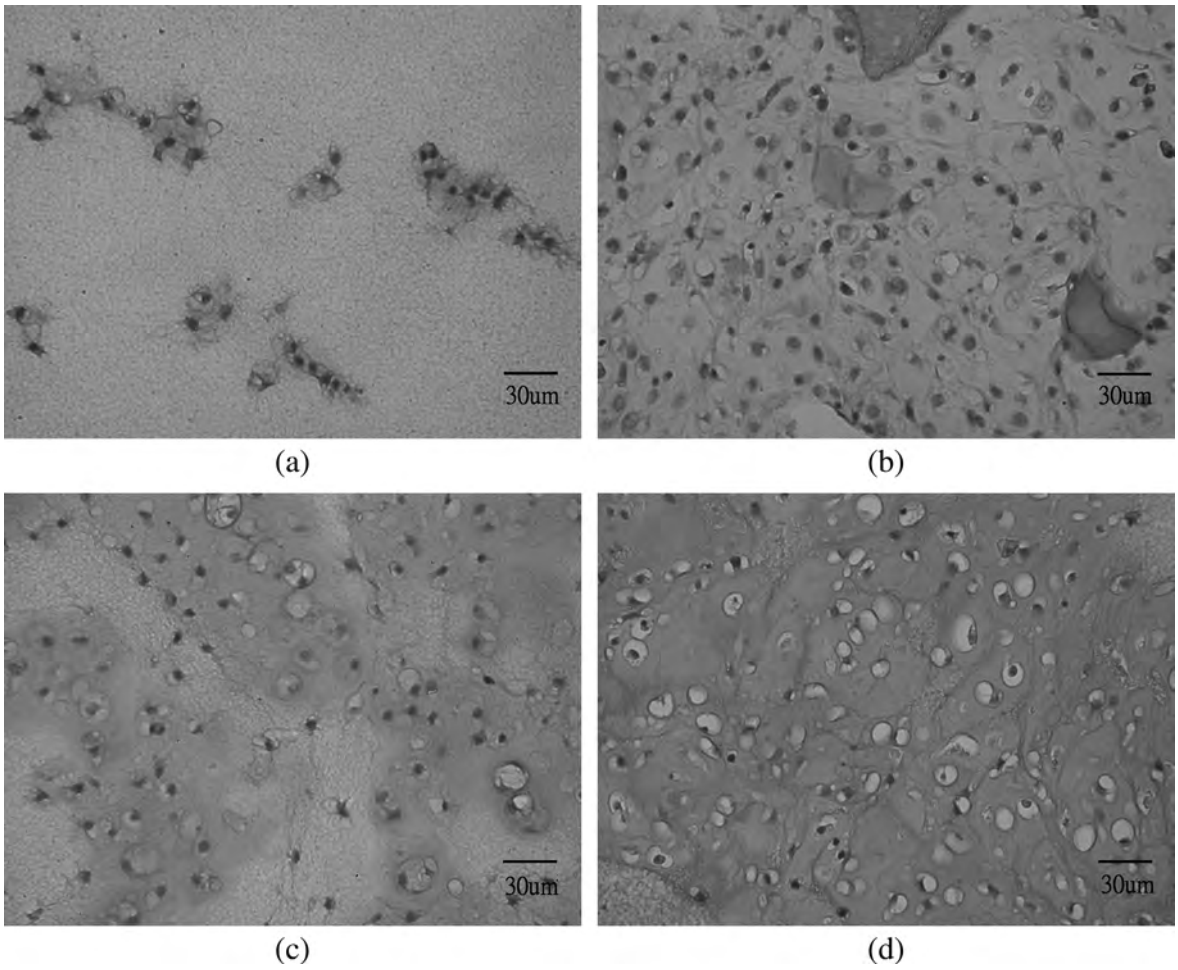

Figure 2. After culturing for 1 week, constructs of chondrocytes cultured in fibrin glue (a) and in GHC6S-fibrin glue (b) were harvested. After culturing for 2 weeks, constructs in fibrin glue (c) and in GHC6S-fibrin glue (d) were prepared for parafilm sections. The prepared constructs were sectioned and stained with Alcian blue to sulfated glycosaminoglycans. The cells were surrounded with secreted GAGs in (a) and (b). With the culture time, obviously GAGs dispersed to the adjacent area to form a homogenous distribution in both of (c) and (d). Especially, chondrocyte cultured in GHC6S-fibrin glue, the morphology is shown as hyaline cartilage with the structure of lacuna. All sections were counterstained with Hematoxylin. The scale bar shown in the figure is 30 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The expression of type II collagen, type I collagen, and type X collagen were shown in Figure 4(a–c). F4 Type II collagen, one of the major components in the extracellular matrix (ECM) of articular cartilage, increased with the culture time when chondrocytes was cultured in GHC6S-fibrin glue [Fig. 4(a)]. However, type II collagen expression decreased with culture time when cultured in fibrin glue [Fig. 4(a)].

No matter whether chondrocytes were cultured in fibrin glue or GHC6S-fibrin glue, type I collagen expression increased with the culture time [Fig. 4(b)].

When chondrocytes were cultured in fibrin glue, type X collagen expression was progressively increased with the culture time. If cultured in GHC6S-fibrin glue, chondrocytes kept very low level in type X collagen expression and there was no significant difference (p-value = $0.637 > 0.01$) in type X collagen expression in the two culture periods [Fig. 4(c)].

Proteoglycans expression

Figure 5 shows the results of aggrecan and decorin F5 expression after cultured in fibrin glue and GHC6S-fibrin glue for 1 and 2 weeks. Aggrecan expression was clearly increasing with the culture time when chondrocytes were cultured in fibrin glue, but no significant difference (p-value = $0.051 > 0.01$) for the chondrocytes cultured in GHC6S-fibrin glue [Fig. 5(a)]. In the decorin expression, the chondrocytes cultured in fibrin glue sharply increased with the culture time. The decorin expression was decreased with culture time, but only in small amount for the chondrocytes cultured in GHC6S-fibrin glue [Fig. 5(b)].

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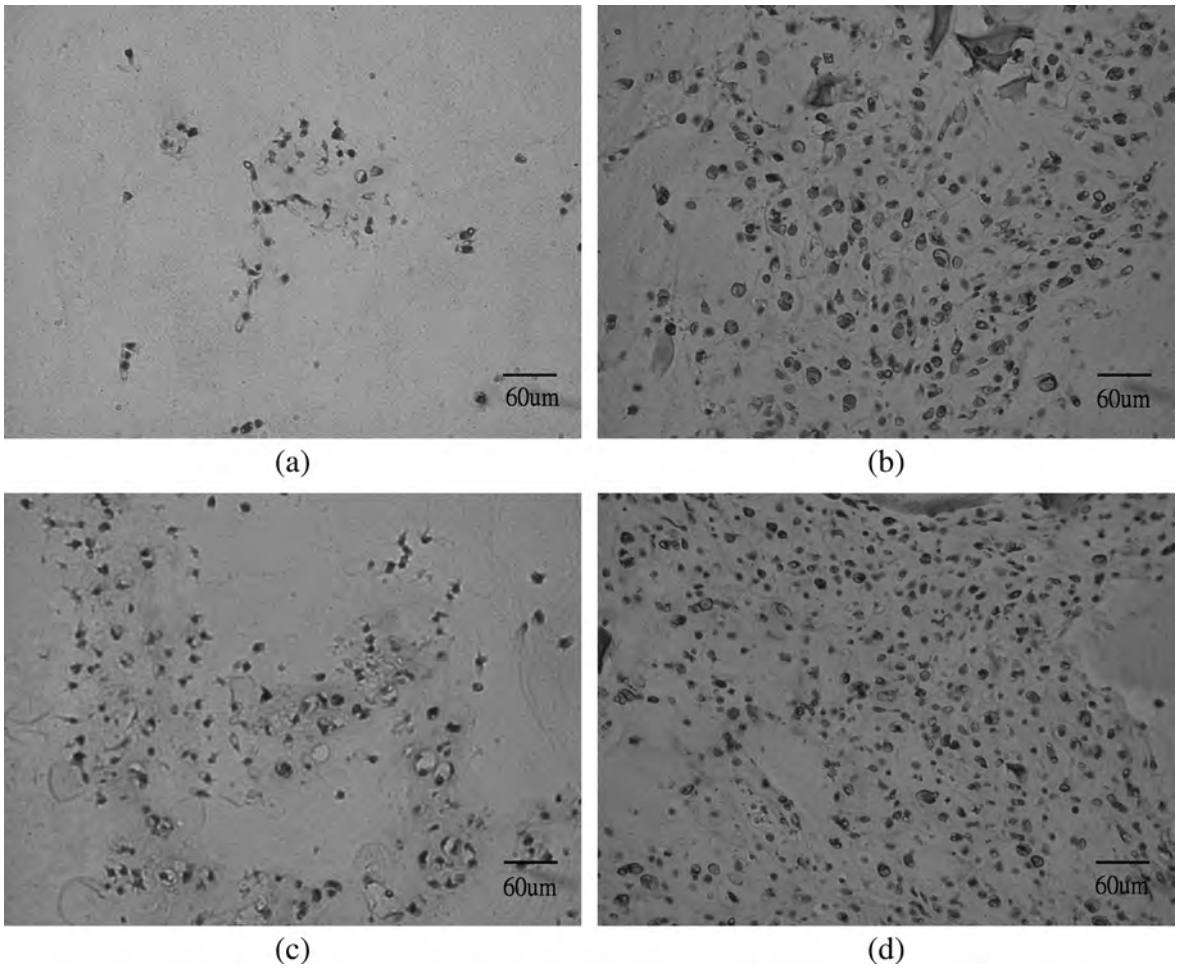

Figure 3. After culturing for 1 week, constructs of chondrocytes cultured in fibrin glue (a) and in GHC6S-fibrin glue (b) were harvested. After culturing for 2 weeks, constructs in fibrin glue (c) and in GHC6S-fibrin glue (d) were prepared for parafilm sections. The prepared constructs were sectioned and conjugated with rabbit monoclonal antibodies to S-100 protein. S-100 protein, the specific protein in chondrocytes, was stained as brown in cytoplasma. And all sections were counterstained with Hematoxylin. The scale bar shown in the figure is 60 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Gene expression related with extracellular matrix degradation

Three major factors related to extracellular matrix degradation were measured by RT-PCR with GAPDH normalization as shown in Figure 6. Chondrocytes cultured in fibrin glue for 2 weeks expressed more MMP-2 than those cultured for 1 week [Fig. 6(a)]. If cultured in GHC6S-fibrin glue, chondrocytes showed extremely low MMP-2 expression when cultured for 1 week and when checked no MMP-2 response even when cultured for 2 weeks Figure 6(a).

After culturing in fibrin glue, chondrocytes expressed much lower membrane-type 1 metalloproteinase (MT1-MMP) in the 1st week than that of in the 2nd week. As shown in Figure 6(b), MT1-MMP sharply increased in the 2nd week. There was no statistic difference (p-value = 0.346 > 0.01) in MT1-MMP expression in the two culture periods when the chondrocytes were cultured in GHC6S-fibrin glue. The chondrocytes cultured in GHC6S-fibrin glue kept very low expression in MT1-MMP [Fig. 6(b)].

The expression of inhibitors of metalloproteinase (TIMP-1) was increasing very much with the culture time when the chondrocytes cultured in fibrin glue. TIMP-1 expression showed no significant difference (p-value = 0.010 ≥ 0.01) in the two culture periods when chondrocytes cultured in GHC6S-fibrin glue [Fig. 6(c)].

Cytokines expression

In transforming growth factor-β1 (TGF-β1), the expression increased with the culture time when chondrocytes cultured in fibrin glue, but decreased when cultured in GHC6S-fibrin glue [Fig. 7(a)].

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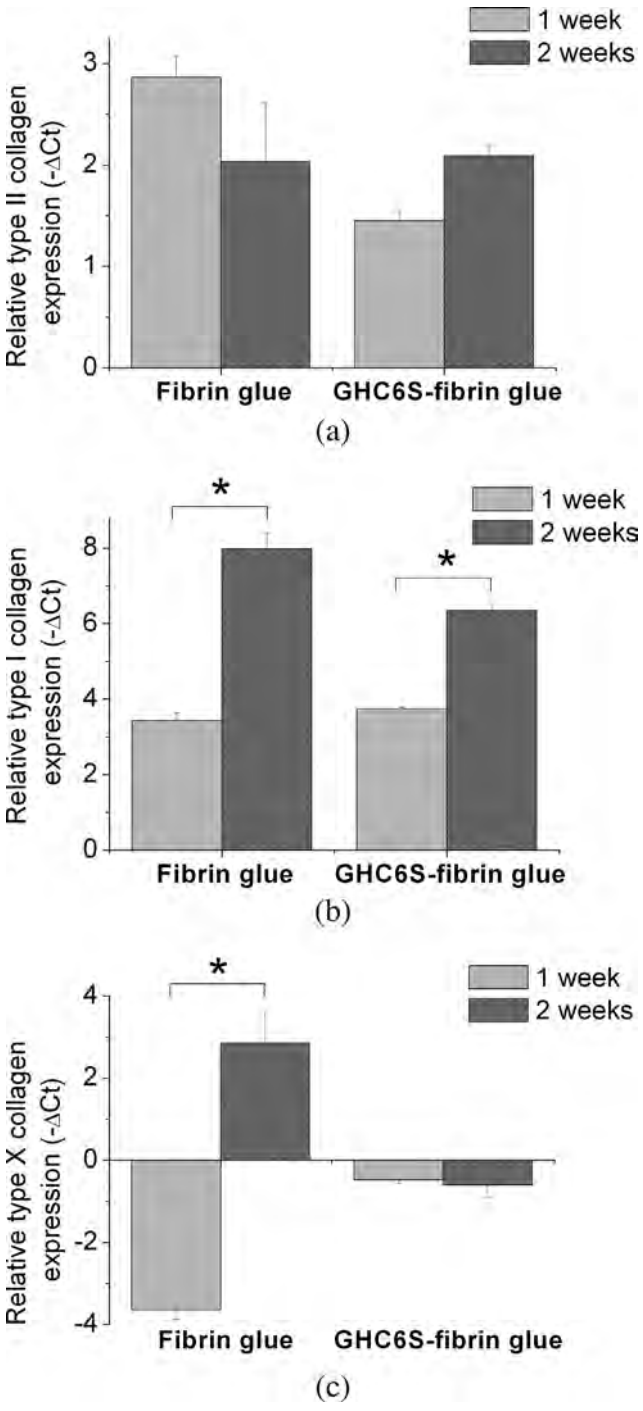

Figure 4. Constructs of chondrocytes cultured in fibrin glue or GHC6S-fibrin glue for 1 or 2 weeks were harvested and analyzed by real-time PCR. The values of $-\Delta C_t$ of relative gene expression in real-time PCR by (a) type II collagen, (b) type I collagen, and (c) type X collagen were normalized by GAPDH. In the bar charts, $-\Delta C_t$ was shown by mean with SD. (*means p-value < 0.01). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In the interleukin-1β (IL-1β), the expression sharply increased in 2nd week than that of in the 1st week when chondrocytes cultured in fibrin glue. When cultured in GHC6S-fibrin glue, chondrocytes showed only mild increase in IL-1β expression [Fig. 7(b)].

The expression of FADD was sharply increasing with the culture time when the chondrocytes cultured in fibrin glue [Fig. 7(c)]. The expression showed no statistical difference (p-value $= 0.331 > 0.01$) in the two culture periods even if chondrocytes were cultured in GHC6S-fibrin glue. The FADD expression of chondrocytes cultured in GHC6S-fibrin glue kept very low during the culture periods.

DISCUSSION

In normal hyaline cartilage, chondrocyte controls the homeostasis of ECM, where synthesis and degradation

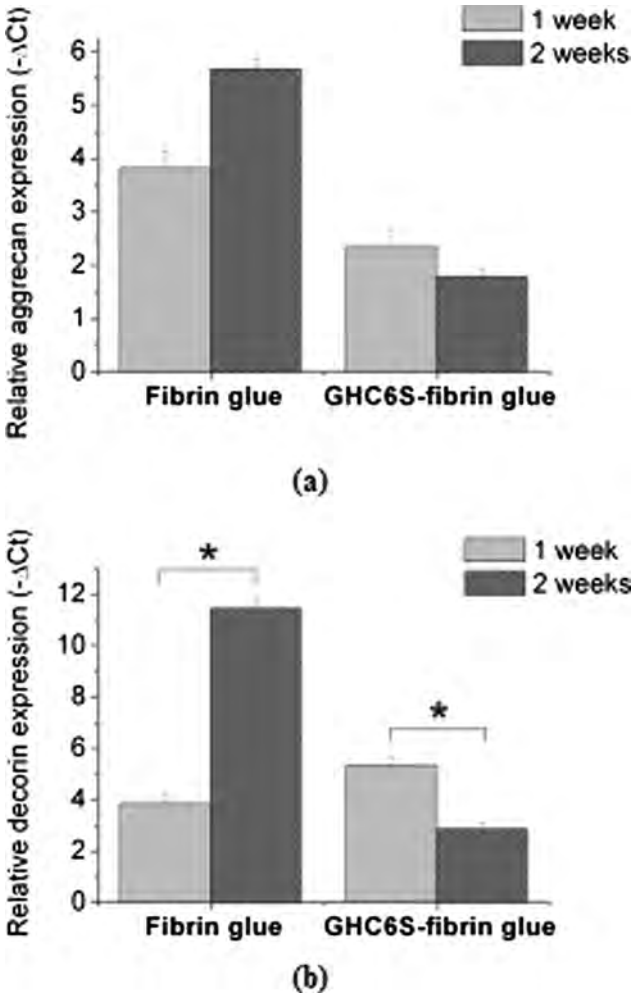

Figure 5. Constructs of chondrocytes cultured in fibrin glue or GHC6S-fibrin glue for 1 or 2 weeks were harvested and analyzed by real-time PCR. The values of $-\Delta C_t$ of relative gene expression in real-time PCR by (a) aggrecan and (b) decorin were normalized by GAPDH. In the bar charts, $-\Delta C_t$ was shown by mean with SD. (*means p-value < 0.01). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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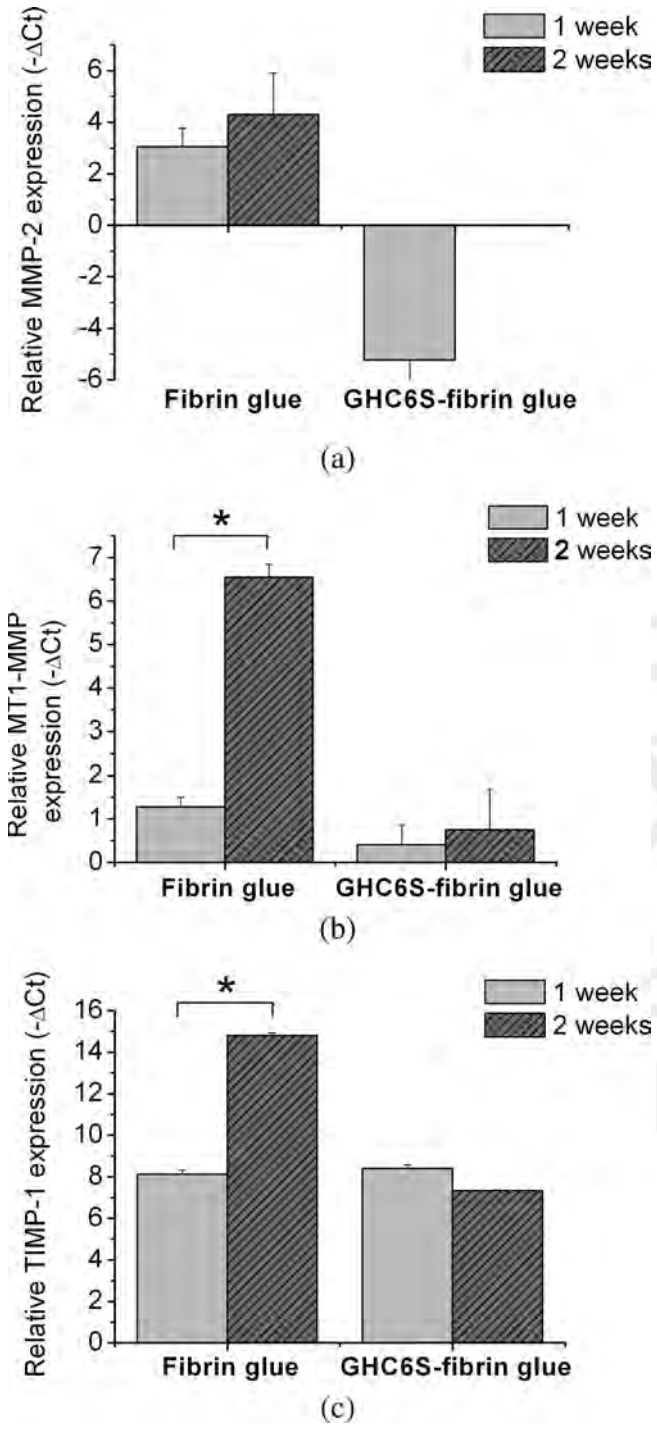

Figure 6. Constructs of chondrocytes cultured in fibrin glue or GHC6S-fibrin glue for 1 or 2 weeks were harvested and analyzed by real-time PCR. The values of $-\Delta C_t$ of relative gene expression in real-time PCR by (a) MMP-2, (b) MT1-MMP, and (c) TIMP-1 were normalized by GAPDH. In the bar charts, $-\Delta C_t$ was shown by mean with SD. (*means p-value < 0.01). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were kept in the same pace and in equilibrium condition. Once the equilibrium condition was damaged or when chondrocyte behavior somehow changed, the hyaline cartilage might not work in normal phase. For instance, IL-1β could not be detected in the plasma of osteoarthritis patients, but can be found with a prominent level in synovial fluid. It suggests that IL-1 most likely acts as local mediator to alter cartilage metabolism in synovial joint.[22]

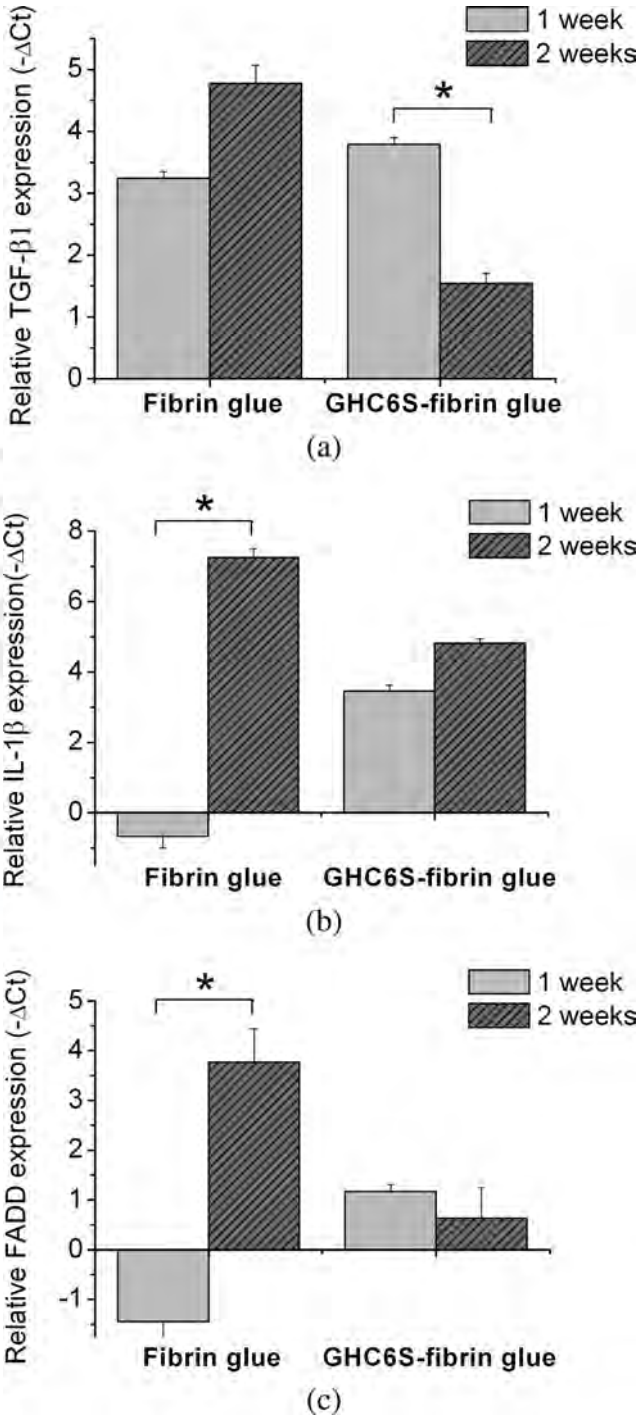

Figure 7. Constructs of chondrocytes cultured in fibrin glue or GHC6S-fibrin glue for 1 or 2 weeks were harvested and analyzed by real-time PCR. The values of $-\Delta C_t$ of relative gene expression in real-time PCR by (a) TGF-β1, (b) IL-1β, and (c) FADD were normalized by GAPDH. In the bar charts, $-\Delta C_t$ was shown by mean with SD. (*means p-value < 0.01). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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Chondrocyte may be stimulated by IL-1β in autocrine mechanism and expect to highly express metalloprotease to the matrix that is resulted in ECM breakdown and eventually causes defect on articular cartilage.[23]

In this study, IL-1β highly expressed in the chondrocytes cultured in fibrin glue for 2 weeks [Fig. 7(b)] that was in agreement with the results of MMP-2 and MT1-MMP expression [Fig. 6(a,b)]. MMP-2, one of protinases to degrade the elastin, aggrecan, and other matrix proteins is activated by MT1-MMP.[24–28] As shown in Figure 6(a,b), chondrocytes cultured in GHC6S-fibrin glue for 2 weeks showed a very low expression in MMP-2 and MT1-MMP. In the contrary, the chondrocytes cultured in fibrin glue for 2 weeks showed very high expression in the two protienases and sharply increased in expression when compared with those cultured in fibrin glue for 1 week. Furthermore, fibrin or fibrinogen has high-affinity to bind to IL-1-β and acts as a cofactor to enhance the cellular response, such as to activate endothelial cell nuclear factor κB (NF-κB), to stimulate monocyte chemoattractant protein-1 (MCP-1) secretion, and to promote nitric oxide (NO) synthesis.[29,30] If the chondrocytes were cultured in fibrin glue only, the activity of IL-1-β might be enhanced and high expression of MMP-2 and MT1-MMP induced. We think that the GHC6S particles might play part of the role to inhibit the two proteinases expression and might keep ECM from degradation.

TIMP-1, an inhibitor of MMPs, plays an important role to prevent the degradation of the ECM. It is secreted by many types of cells and can be upregulated by a variety of factors, such as serum, bFGF, EGF, TGF-β1, IL-6 family, IL-1, retinoids progesterone, and phobolester.[31] In this study, the expression of TIMP-1 sharply increased when chondrocytes cultured in fibrin glue for 2 weeks [Fig. 6(c)]. TIMP-1 expression showed no significant difference when chondrocytes cultured in GHC6S-fibrin glue for 1 and 2 weeks. The results might be in part due to negative feedback of expression high in MMP-2 and MT1-MMP.

In cartilage tissue engineering, it was important that the rate of ECM synthesis and degradation should be matched with each other or kept in equilibrium condition. For the ECM, the genes related to the fibrillar extracellular matrices, such as type I, type II, and type X collagens, are used to check the phenotype of the chondrocytes.[32–34] In normal cartilage, type II collagen is the major fibrillar ECM. If phenotype of chondrocyte is lost, the type I collagen will appear to replace type II collagen. The cells would differentiate toward hypertrophic chondrocytes or finally to fibrous-like cells. The type I collagen expression of chondrocytes cultured in GHC6S-fibrin glue for 2 weeks did not sharply increase as those cultured in fibrin glue [Fig. 4(b)].

Type X collagen is a key factor related to hypertrophilization of chondrocyte. In the study, the type X collagen expression of chondrocyte cultured in fibrin glue for 2 weeks increased very much, but the expression kept extremely low for the chondrocyte cultured in GHC6S for 2 weeks [Fig. 4(c)]. The results indicated that the fibrin glue added with GHC6S particles would inhibit the differentiation of chondrocyte towards undesired pathway.

In the histological examination, the cells cultured in GHC6S-fibrin glue showed a round shape with intact lacuna structure [Fig. 1(b,d)]. The distribution of cells cultured in GHC6S-fibrin glue was also homogenous, which was shown in the results of H&E staining in Figure 1(b,d). This indicated that hyaluronic acid within the particles helped the migration and proliferation of chondrocyes.[35,36] Results also showed positive stain in Alcian blue as well as in S-100 protein, which was observed in chondrocyte cytoplasma[37] (Figs. 2 and 3). Sulfated GAGs in the construct of cells cultured in fibrin were secreted and surrounded the cells. However, the distribution of sulfated GAGs in the construct of cells cultured in GHC6S-fibrin glue was obviously homogenous [Fig. 2(b,d)]. In mRNA level, the expression of aggrecan and decorin of the cells cultured in GHC6S-fibrin glue showed the ability of chondrocytes to reconstruct the ECM (Fig. 5). It is indicated that cells stayed in GHC6S-fibrin glue could prevent hypertrophilization and keep more chondrogenic phenotype.

In this study, the chondrocytes cultured in fibrin glue for 2 weeks showed much higher expression in TGF-β1 than those cultured GHC6S-fibrin glue [Fig. 7(a)]. The results were matched with the expression of type X collagen [Fig. 4(c)] and IL-1β [Fig. 7(b)] that indicate the chondrocytes toward hypertrophic differentiation. TGF-β1 could be expressed by chondrocytes to increase the expression of TIMP-1 and type II collagen.[38,39] The similar result was shown in the previous study with the immobilized TGF-β1 on the bioactive sponge made by GHC6S.[5] In the previous studies, TGF-β1 can down-regulate the expression of IL-1 receptors[40,41] and has the ability to inhibit the production of proteinases that finally suppress the cartilage degradation.

FADD is a protein containing a death domain homologous to the death domains of Fas and TNFR-1.[42] FADD binds protein caspase-8, which has a death domain as well as protease catalytic activity, and which may then trigger a common pathway.[43] Activation of caspase in cell death pathway would be induced by FADD, and then cause cell death. The expression of FADD played an important role to regulate the cell viability. Chondrocytes cultured in GHC6S-fibrin glue showed much lower expression in FADD than those of cultured in fibrin glue [Fig. 7(c)]. The GHC6S particles may inhibit the FADD expression.

CONCLUSION

The study showed that the addition of GHC6S particles to the fibrin glue could help chondrocyte to main-

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tain its phenotype. In the morphological examination, chondrocytes cultured in GHC6S-fibrin glue showed more distinct lacuna structure and round shape appearance than those of in fibrin glue. To check with mRNAs expression, type X collagen was suppressed due to GHC6S particles addition that prevented chondrocytes toward hypertrophic differentiation. The existence of the GHC6S particles in the fibrin glue could effectively mitigate the increasing of type I collagen. The expression of type I collagen and type X collagen significantly increased in chondrocytes cultured in fibrin glue with the cultured time. Type II collagen expression increased with cultured time when chondrocytes cultured in GHC6S-fibrin glue but decreased in fibrin glue. When cultured for 2 weeks, the chondrocytes in fibrin glue showed much higher expression in IL-1β, MMP-2, and MT1-MMP than those in GHC6S-fibrin glue. When chondrocytes cultured in GHC6S-fibrin glue for 2 weeks, the expression of MMPs was inhibited. The expression of FADD was sharply increasing with the culture time when the chondrocytes were cultured in fibrin glue. The expression showed no statistical difference in the two culture periods if chondrocytes cultured in GHC6S-fibrin glue. The FADD expression of chondrocytes cultured in GHC6S-fibrin glue was kept very low during the culture periods.

The results indicated that the chondrocytes cultured in GHC6S-fibrin glue would effectively promote ECM secretion and inhibit ECM degradation. The evidence could support that GHC6S-fibrin glue would be a promising scaffold for articular cartilage tissue engineering.

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