Effects of Growth Factors on Heparin-Carrying Polystyrene-Coated Atelocollagen Scaffold for Articular Cartilage Tissue Engineering

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Abstract: The specific aim of our investigation is to study the potential use of a collagen/heparin-carrying polystyrene (HCPS) composite extracellular matrix for articular cartilage tissue engineering. Here, we created a high-performance extracellular matrix (HpECM) scaffold to build an optimal extracellular environment using an HCPS we originally developed, and an atelocollagen honeycomb-shaped-scaffold (ACHMS-scaffold) with a membrane seal. This scaffold was coated with HCPS to enable aggregation of heparin-binding growth factors such as FGF-2 and TGF-β1 within the scaffold. Three-dimensional culture of rabbit articular chondrocytes within the HpECM-scaffold and subsequent preparation of a tissue-engineered cartilage were investigated. The results showed remarkably higher cell proliferative activity within the HpECM-pretreated-FGF-2 scaffold and the sustenance of phenotype within the HpECM-pretreated-TGF-β1 scaffold. It was thought that both FGF-2 and TGF-β1 were stably immobilized in the HpEMC-scaffold since HCPS generated an extracellular environment similar to that of heparan sulfate proteoglycan within the scaffold. These results suggest that an ACHMS-scaffold immobilized with HCPS can be a HpECM for cartilage regeneration to retain the heparin-binding growth factors within the scaffolds. © 2007 Wiley Periodicals, Inc.
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INTRODUCTION

The three key ingredients for tissue engineering are morphogens, extracellular matrix scaffolding, and responding cells.[1] Morphogens induce tissue differentiation and cell proliferation. The scaffold provides a substratum for cell adhesion, proliferation, and differentiation. Chondrocytes can survive and grow within the extracellular matrix whose main components are various kinds of collagens and proteoglycans in articular cartilage. Proteoglycans in both the extracellular matrix and the cell surface consist of a core protein to which multiple glycosaminoglycan chains are attached at specific sites. It is important for articular cartilage tissue engineering to select the proper scaffold. The atelocollagen honeycomb-shaped scaffold (ACHMS-scaffold) with a membrane seal (Table I) was developed for interver-

TABLE I. List of Scaffolds and Materials in this Study

ACHMS	An atelocollagen honeycomb-shaped-scaffold with a membrane seal
HCPS	Heparin-carrying polystyrene
HpECM	A high-performance extracellular matrix, ACHMS-scaffold coated with HCPS
HpECM+pre-FGF-2 scaffold	HpECM-scaffold pretreated with FGF-2
HpECM+pre-TGF-β1 scaffold	HpECM-scaffold pretreated with TGF-β1

tebral disc tissue engineering,[2–4] and this same scaffold was also found suitable for maintaining articular chondrocyte phenotype[5,6] and for chondrogenic[7] and osteogenic potential in adipose tissue-derived stromal cells.[8,9]

Heparin/heparan sulfate are members of the glycosaminoglycans and normally are present as proteoglycans, in which a number of heparin/heparan sulfate-chains are covalently attached to a core protein. Although heparin/heparan sulfate is widely distributed on cell surfaces and in extracellular matrices in most animal tissues, heparin is synthesized by mast cells in connective tissue and stored in cytoplasmic granules.[10,11] Heparin is isolated on a commercial basis from animal tissue (pig or bovine intestinal mucosa, or bovine lung, etc.) and has been extensively used as an antithrombotic drug for a long time.[12] The biological role of heparin/heparan sulfate is highly diverse. Aside from its well-known anticoagulant action, as shown in Table II, the molecules are found to be associated with growth factors and cytokines in various biological processes, as well as involved in cell adhesion, recognition, migration, and regulation of various enzymatic activities.[10,11,13] In addition, recent progress in growth factor research suggests that many growth factors are able to function in a nondiffusible way when immobilized on the extracellular matrix or the cell surfaces through binding with heparan sulfate proteoglycan.[14] The ability of the matrix or cell surface heparan sulfate proteoglycan to immobilize heparin-binding growth factors provides an additional tool for controlling the cellular effects of growth factors.[10,12,13,15,16]

Periodate-treated, nonanticoagulant heparin-carrying polystyrene (HCPS, Table I) has been described previously as a synthetic water-soluble glycoconjugate with an amphiphilic structural unit consisting of hydrophilic polysaccharides and hydrophobic polystyrene moieties and as a heparan sulfate proteoglycan mimic.[12] It has been estimated that the molecular size of HCPS is $\sim$80–120 kDa, and that it consists of more than 10 periodate-oxidized, alkaline-degraded, low-molecular-weight (IO$_4$-LMW-) heparin chains enriched with trisulfated disaccharide structures linked to its polystyrene core.[12,16] In this work, the potential use of a collagen/HCPS composite extracellular matrix with FGF-2 and TGF-β1 for articular cartilage tissue engineering has

been evaluated as a high-performance extracellular matrix (HpECM, Table I) scaffold. The HCPS has the binding sites of heparin-binding molecules such as FGF-2 and TGF-β1 with the lowest anticoagulant activity.[12,15,16] The specific aim of this investigation is to assess the potential use of HpECM with the heparin-binding growth factors for articular cartilage tissue engineering.

MATERIALS AND METHODS

All procedures using animals in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) published by the National Institutes of Health, USA, and Guidelines of Tokai University on Animal Use.

Preparation of HCPS

Heparin-carrying polystyrene (HCPS) was prepared as reported previously.[12,16] Briefly, heparin from porcine intestine (25 g) dissolved in 400 mL of 0.1M NaIO$_4$ in 0.05M sodium acetate buffer (pH 5) was stirred at 4°C for 3 days. The unreacted NaIO$_4$ was then neutralized by the addition of glycerol (25 mL), and the reaction mixture was subsequently dialyzed and lyophilized. The product (periodate-oxidized heparin) was then degraded in an alkaline solution (pH 12) at room temperature for 30 min, and the degraded product was recovered after dialysis and lyophilization as periodate-oxidized, alkaline-degraded (IO$_4$-LMW) heparin.[12]

IO$_4$-LMW heparin (500 mg) and N-p-vinylbenzylamine (250 mg) were dissolved in 20 mL of 50 mM N,N,N',N'-tetramethyl-ethylenediamine (pH 4.75), after which 1 mL of 0.8 mM NaCNBH$_3$ was added. The reaction mixture was stirred for 24 h at room temperature, dialyzed, and lyophilized to yield a white powder (heparin-carrying monostyrene). This powder (100 mg) and 2 mg of potassium

TABLE II. Heparin-Binding Proteins

Growth factors	FGF-1(aFGF), FGF-2(bFGF), FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, HGF (hepatocyte growth factor), HBEGF (heparin binding epidermal growth factor), VEGF (vascular endothelial growth factor), TGFβ, and so forth
Adhesive matrix proteins	Fibronectin, vitronectin, laminin, collagens, thrombospondin, and so forth
Enzymes involved in lipid metabolism	Lipoprotein lipase, hepatic lipase, phospholipase, apolipoprotein B, apolipoprotein E, and so forth
Serine protease inhibitors	Antithrombin III, heparin cofactor II, protease nexins, and so forth
Other proteins	Superoxide dimustase, elastase, platelet factor 4, N-CAM, transcription factors, DNATopoisomerase, RNA polymerase, tumor necrosis factor, and so forth

peroxodisulfate were dissolved in 1 mL of distilled water and the polymerization was performed at 60°C for 24 h under dried N_2 gas. The reaction solution was then poured into an excess amount of ethanol to obtain polymers as a precipitate using ultrafiltration, and the HCPS was obtained as white powder after lyophilization. The weight fraction of heparin component in HCPS was estimated to be 92% using carbazole assay.[12,16]

HCPS solution was sterilized by a syringe-driven 0.22 μm filter unit using MILLEX[TM] (MILLIPORE, MA).

Binding of HCPS to ACHMS Scaffold and Preparation of High-Performance Extracellular Matrix

Atelocollagen honeycomb-shaped-scaffolds (ACHMS-scaffolds) were treated at 130°C for 20 min in vacuum for sterilization. Round ACHMS (diameter: 11 mm, thickness: 2 mm, average pore size: 200 μm)-scaffolds in 48-well tissue culture plates (Koken) were coated overnight at 4°C with 100 μL of the 0.1 wt/wt % HCPS aqueous solution.[3,4] The HCPS solution was subsequently removed from the wells by sucking, and the scaffolds were washed three times with 0.5M NaCl in PBS containing 1 wt/wt % bovine serum albumin (BSA-PBS) and three times with BSA-PBS. The ACHMS-scaffolds coated with HCPS (high-performance extracellular matrix, HpECM) were then desiccated for 48 h. We previously reported that about 15 μg of HCPS was immobilized on 1 cm^2 of a surface of type I-collagen membrane in 24-well tissue culture plates (area, 64 mm^2; Koken, Tokyo, Japan) using carbazole assay.[16] In this work, the HCPS attached to a round ACHMS-scaffold (diameter: 11 mm; thickness: 2 mm) was estimated to be 40–50 μg/scaffold.

The HCPS attached to the scaffold was not degraded in the absence of cells for at least 7 days. In addition, the biological activity of the immobilized growth factors did not reduce for at least 7 days (data not shown).

Immobilization of FGF-2 and TGF-β1 to ACHMS or HpECM Scaffold

Various concentrations (5, 10, 20, 40, 80, and 160 ng/mL) of FGF-2 (human recombinant FGF-2; R&D Systems, Minneapolis, MN) and TGF-β1 (human recombinant TGF-β1; R&D Systems) in 0.5 mL of DMEM/F12 (Iwaki, Tokyo, Japan), supplemented with 10% fetal bovine serum, were added to the ACHMS- or HpECM-scaffold and immobilized overnight at 4°C. The immobilized growth factors were estimated by ELISA for FGF-2 and TGF-β1 by measuring FGF-2 and TGF-β1 remaining in the culture medium[16] (Table III).

Isolation and Culturing of Chondrocytes Within ACHMS- or HpECM-Scaffold in Sterile Condition

Twelve knee joints were obtained from 4-week-old female Japanese white rabbits, each weighing about 900 g. Articular cartilage was separated from the joint with a scalpel

TABLE III. Immobilization of FGF-2 and TGFβ to ACHMS- or HpECM-Scaffold

Conc. (ng/mL)	FGF-2 (ng/scaffold)		TGFβ (ng/scaffold)	
	HpECM	ACHMS	HpECM	ACHMS
5.0	0.9 ± 0.3	0.1 ± 0.0	0.7 ± 0.2	0.2 ± 0.1
10.0	2.0 ± 0.5	0.2 ± 0.1	1.4 ± 0.4	0.3 ± 0.1
20.0	3.6 ± 1.1	0.3 ± 0.2	3.0 ± 0.8	0.4 ± 0.2
40.0	7.0 ± 2.0	0.5 ± 0.2	5.5 ± 1.4	0.5 ± 0.2
80.0	12.4 ± 3.8	0.7 ± 0.3	10.2 ± 3.1	0.7 ± 0.3
160.0	18.8 ± 5.2	0.9 ± 0.4	15.6 ± 5.0	0.8 ± 0.3

Data represent the means ± SD of quadruplicate determinations.

and digested for 4 h in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 0.0125% (w/v) bacterial collagenase P (Boehringer Mannheim GmbH, Mannheim, Germany) and 0.05% Actinase E (Kaken Pharmaceutical, Tokyo, Japan).[19] The digested tissue was passed through a cell strainer (Becton Dickinson Labware, NJ) with a pore size of 40 μm. The filtrate was centrifuged at 1500 rpm for 10 min to separate the cells. Next, cells were seeded at high density (2 × 10^6 cells/scaffold) into ACHMS- and HpECM-scaffolds in 48-well plates (Sumitomo Bakelite, Tokyo, Japan) by centrifugation at 500 rpm for 5 min. The cells were then cultured in DMEM/F12 (Iwaki, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2 in air and 100% relative humidity.[2–4]

RNA Isolation and cDNA Synthesis

Cultured chondrocytes using ACHMS- or HpECM-scaffold were recovered, and the total mRNA was extracted with a RNAqueous kit (Ambion) according to the manufacturer's instructions. RNA quality from each sample was assured by the A260/280 absorbance ratio and by electrophoresis of 1.2% agarose formaldehyde gel. About 1.0–2.0 μg of total RNA was reverse transcribed into single strand cDNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). RT reaction was carried out for 60 min at 42°C and 5 min at 95°C in a thermocycler.

Primer Design and Real-Time PCR

All oligonucleotide primer sets of type I collagen, type II collagen, Aggrecan, SRY-box containing gene 9 (SOX9), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed based upon published mRNA sequences. Expected amplicon lengths were from 180–490 bp. Oligonucleotide primers used in this study are listed in Table IV. The real-time PCR was performed in a SmartCycler II (Cepheid, Sunnyvale, CA) using SYBR$^®$ Green PCR Master Mix (Applied Biosystems, Foster City, CA). About 2–2.5 μL of cDNA template was used for real-time PCR in a final volume of 25 μL. cDNA was amplified according to the following condition: 95°C for 15 s and 60°C for 60 s from 35 to 45 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. Melt-

TABLE IV. List of Rabbit-Specific Primers Used in Real-Time PCR

Primer ID	Sequence	Expect Size (bp)	References
Collagen type I A2-F	TTC TTG GTG CTC CTG GCA TTC	489	Guehring et al.[17]
Collagen type I A2-R	GCA ATC CGT TGT GTC CCT TTA TG		
Collagen type II A1-F	GTC TCC ATA GCT GAA GTG G	386	Guehring et al.[17]
Collagen type II A1-R	CCA TGC AGT ACA TGC GGG		
Aggrecan-F	ACC CCTGAG GAA CAG GAG TT	189	Guehring et al.[17]
Aggrecan-R	GTG CCA GAT CAT CAC CAC AC		
SOX9-F	AAC GCA CAT CAA GAC GGA GC	208	Qiao et al.[18]
SOX9-R	ATG TAG GTG AAG GTG GAG TAG AG		
GAPDH-F	GGT GAA GGT CGG AGT GAA	399	Guehring et al.[17]
GAPDH-R	TTC ACG CCC ATC ACA AAC A		

ing curve analysis was performed (0.5°C/s increase from 55–95°C with continuous fluorescence readings) at the end of each cycle to ensure that single PCR products were obtained. Amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. All reactions were repeated in six separate PCR runs using RNA isolated from six sets of samples. Results were evaluated with the SmartCycler II software. GAPDH primers were used to normalize samples. To monitor crossover contaminations of PCR, RNase-free water (Qiagen, Valencia, CA) was included in the RNA extraction and used as a negative control. To ensure quality of data, a negative control was always applied in each run.

Western-Blotting Analysis of Type II Collagen Accumulation

Type II collagen was extracted from three different samples of cultured chondrocytes using ACHMS- and HpECM-scaffold on day 14. SDS polyacrylamide gel electrophoresis of the collagen preparations was carried out, and the gel was stained with Coomassie blue, as previously described.[20] Western-blotting analysis for type II collagen was performed according to the method of Kumagai et al.[21] Briefly, type II collagen was separated on a SDS polyacrylamide gel (35 min, 200 V), the protein bands were electroblotted on nitrocellulose membrane, and then reacted with an anticollagen type II monoclonal antibody (II-4CII; Fuji Chem. Ind., Tokyo, Japan). Immunoreaction was visualized according to the avidin-biotinylated horseradish peroxidase (HRP) complex method.

Fluorometric Assay of DNA Content for Cell Proliferation

Cell proliferation activities of six samples from each group were evaluated by DNA contents, as determined from papain digests of the cell and matrix components by a fluorometric assay.[3] Briefly, 15 μL of the papain digest was mixed with 300 μL of Hoechst 33258 (Polyscience, Warrington, PA) solution in a 96-well fluoroplate. Emission and excitation spectra were measured in a Titertek multi-

scan spectrofluorometer (Labsystem) at 456 and 365 nm, respectively. DNA concentrations were determined against a standard curve of calf thymus DNA (Sigma) ranging from 0.625 to 40 μg/mL.[22]

Histological Study

The four respective specimens were fixed for 24 h in 20% formaldehyde and embedded in paraffin, and 7 μm sections were cut through the entire specimen. Alternate sections were stained with safranin O (Merck), and hematoxylin and eosin, and then examined histologically. Microscopic examination evaluated the matrix staining characteristics and intensity, cellular morphology, and cell density.

Statistical Analysis

The statistical analysis was performed concerning both the results of real-time PCR (Figure 1) and cell proliferation (Figure 3). Data are expressed as means with S.D. for six samples. Differences in the change of values over time for each group were tested using a one-way analysis of variance (ANOVA), followed by individual *post hoc* comparisons (Bonferroni/Dunn). A difference was accepted as significant if the probability was less than 5% ($p < 0.05$).

RESULTS

Immobilization of FGF-2 and TGF-β1 to ACHMS or HpECM Scaffold

FGF-2 and TGF-β1, which specifically interact with heparin, were examined with respect to their immobilizations to ACHMS- and HpECM- (HCPS-coated ACHMS) scaffold. Both FGF-2 and TGF-β1 were found to be immobilized in the ACHMS-scaffold in a concentration-dependent manner (Table III). The amounts of immobilized FGF-2 and TGF-β1 did not change after a 3-day incubation period at 37°C (data not shown). However, significantly smaller amounts of these two growth factors were bound to the ACHMS-scaf-
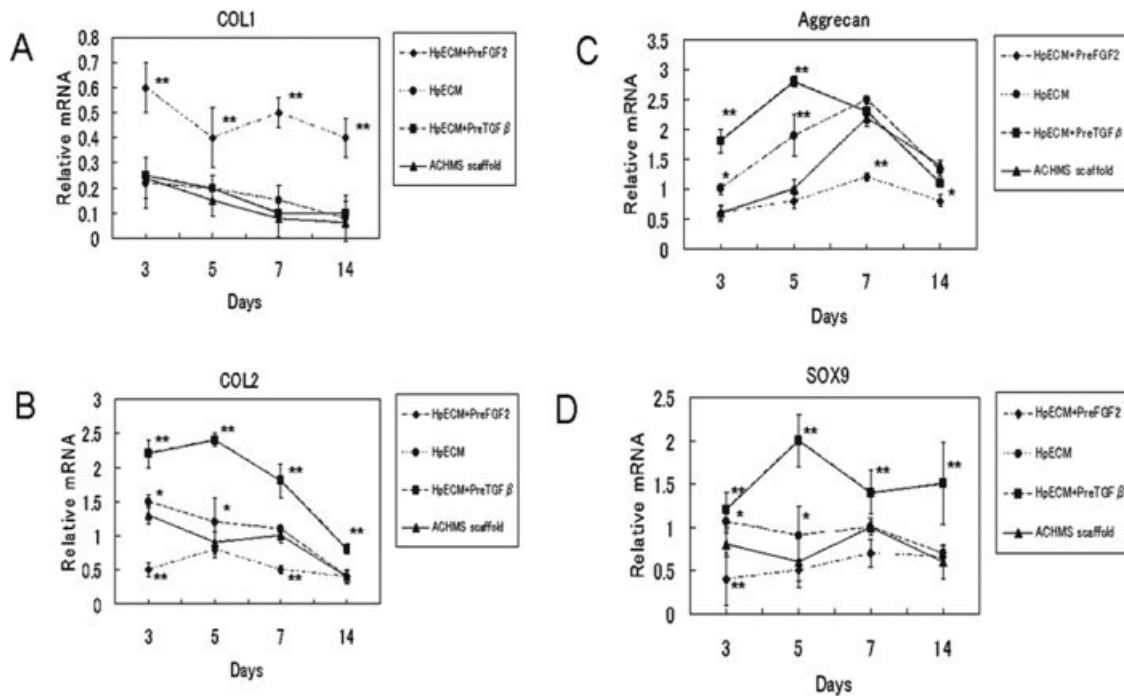
Figure 1. Gene expression levels measured by real-time PCR. Expression levels were normalized by GAPDH housekeeping gene at each point during the culture period ($n = 6$). (A) The relative expression of type I collagen. (B) The relative expression of type II collagen. (C) The relative expression of *aggrecan*. (D) The relative expression of SOX9. Results were expressed as means with S.D. for six samples. $*p < 0.05$ and $**p < 0.01$ when compared with ACHMS group.

fold without treatment with HCPS. Thus, HpECM-scaffold is an excellent biomaterial to enable heparin-binding growth factors to be adsorbed and retained within the scaffold. On the basis of these results, we pretreated HpECM-scaffold using 30.0 ng/mL FGF-2 or TGF-β1 in DMEM/F12 (Iwaki, Tokyo, Japan) supplemented with 10% fetal bovine serum to prepare HpECM-scaffold pretreated with FGF-2 (HpECM+pre-FGF-2 scaffold, Table I) and one pretreated with TGF-β1 (HpECM+pre-TGF-β1 scaffold, Table I).

Cultured Chondrocyte Phenotype

Figure 1 shows mRNA expression of each scaffold during the cultured period. The expression of collagen type I in the HpECM+pre-FGF-2-scaffold was at a high level during the culture period. Concerning the collagen type II expression, that of the HpECM+pre-TGF-β1 scaffold has been higher than that of the other scaffold. HpECM- and ACHMS-scaffolds exhibited a moderately high level of expression at the beginning of culture. The aggrecan expression in the HpECM+pre-TGF-β1 and HpECM-scaffold was higher than that of the other up to 7 days of culture. The SOX9 expression in HpECM+pre-TGF-β1 scaffold reached a high level during the culture period, and at the beginning of culture, the expression was also high in the HpECM-scaffold.

Figure 2 shows Western blotting of type II collagen accumulation in each scaffold. A clear difference was observed by the expression of type II collagen by Western

blotting. Thus, the levels of the expression were in the order of HpECM+pre-TGF-β1, HpECM, ACHMS, and HpECM+Pre-FGF-2 scaffolds.

Cell Proliferations

The chondrocyte proliferation in HpECM-scaffold was significantly increased as of 3 days after cultivation. Cell proliferation increased in the HpECM-scaffold alone, but the increase was more evident in the HpECM+pre-FGF-2-scaffold. Cell proliferation, however, was suppressed in the HpECM+pre-TGF-β1 scaffold (Figure 3).
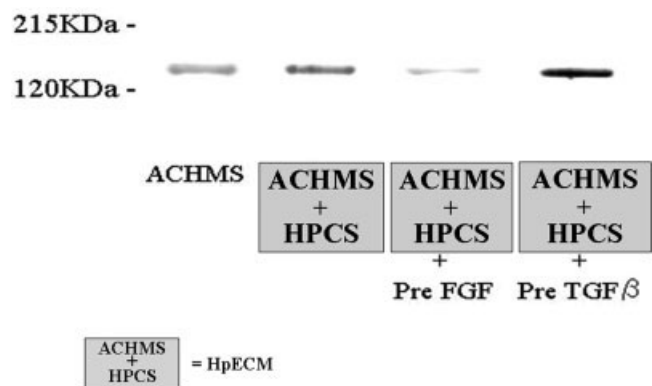

Figure 2. Western blotting of type II collagen. Type II collagen was extracted from the tissue-engineered cartilage on day 14. Western-blotting analysis of type II collagen was carried out as described in the Methods section. Amounts of type II collagen expression varied with the scaffold and culture conditions ($n = 3$).
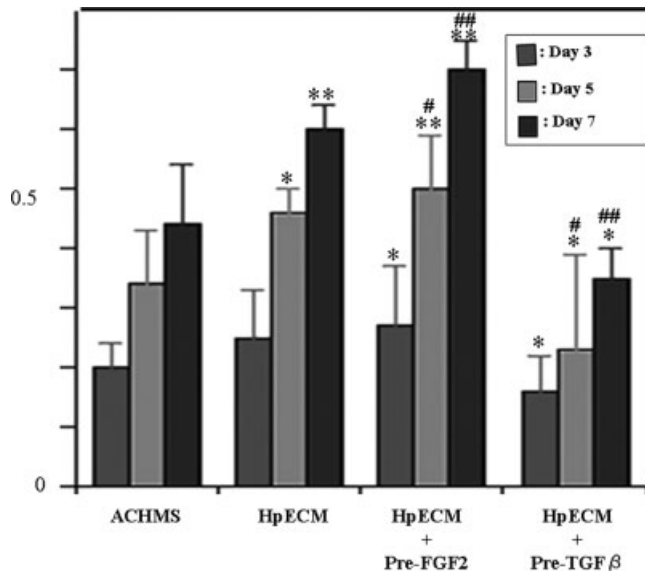
Figure 3. Cell proliferation activities. Cell proliferation activities increased only in HpECM-scaffold. The increase was more evident in the HpECM precoated with FGF-2 scaffold. When precoated with TGF-β1, cell proliferation activities were slightly inhibited. Results were expressed as means with S.D. for six samples. *$p < 0.05$ and **$p < 0.01$ when compared with ACHMS group. #$p < 0.05$ and ##$p < 0.01$ when compared with HpECM group.

Histological Study

Properties of tissue-engineered cartilage *in vitro* cultured for 14 days were histologically different from each other. All cartilages in HpECM, ACHEMS, HpECM+pre-TGF-β1, and HpECM+pre-FGF-2 scaffolds were chondrogenetic in terms of safranin-O staining, but stronger stainings were shown in the HpECM-scaffold and HpECM+pre-TGF-β1 scaffold groups. Abundant accumulation of matrix was noted in these groups. In HpECM+pre-FGF-2 scaffold group, the cell shape became like fibroblastic morphology, and the staining of safranin-O was weak (Figure 4).

DISCUSSION

We previously reported the ACHMS-scaffold applicability in terms of stability, cytocompatibility, uniform, and high-density seeding of chondrocytes, and accumulation of matrix components.[6] The culture of chondrocytes in the ACHMS-scaffold resulted in a high-density, three-dimensional culture, like a pellet or micromass culture. Chondrocytes at a density of 2×10^6 cells could be seeded into an ACHMS-scaffold by gentle centrifugation without spillage of cells due to the scaffold membrane seal.[2–7] In this study, cell proliferation increased only in the HCPS-treated ACHMS (HpECM)-scaffold, and the increase was even more evident in the HpECM pretreated with FGF-2. On the contrary, the cell proliferation was suppressed in the HpECM pretreated with TGF-β1. Those results showed HpECM can adsorb and retain the heparin-binding growth factors such as FGF-2 and TGF-β1 in the pericellular microenvironment (Figure 5).

Collagen, either pure or in combination with other biomolecules, is an important natural biomaterial now used in
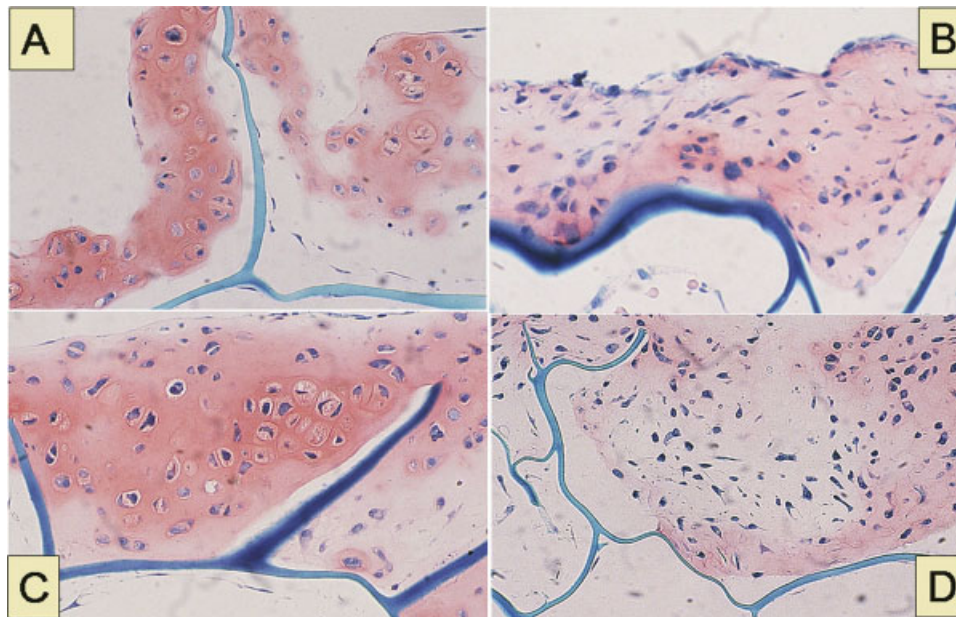
Figure 4. Tissue-engineered cartilage. Properties of tissue-engineered cartilage *in vitro* cultured for 14 days. (A) HpECM-scaffold, (B) ACHMS-scaffold, (C) HpECM + pre-TGF-β1-scaffold, and (D) HpECM + pre-FGF-2-scaffold. Chondrocytes within each HpECM- and ACHMS-scaffold retain their chondrocyte phenotype, yet each tissue-engineered cartilage is different. Therefore, ECM plays an important role in expressing the phenotype (safranin-O staining, ×100). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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          │   ACHMS-scaffold        │
          │   Atelocollagen         │
          │   chondrogenesis        │
          └─────────────────────────┘
                     │ HCPS immobilization
                     ▼
          ┌─────────────────────────────────┐
          │   HpECM                         │
          │  heparin-binding protein        │
          │  absorption ↑                   │
          │  signal transduction like       │
          │  a HSPG                         │
          │  chondrogenesis                 │
          └─────────────────────────────────┘
      Pre-coating FGF-2  ╱          ╲  Pre-coating TGF-β1
                        ╱            ╲
  ┌──────────────────────┐    ┌──────────────────────────┐
  │ HpECM+pre-FGF-2      │    │ HpECM+pre-TGF-β1 scaffold │
  │ scaffold            │    │ proliferation activity ↓  │
  │ proliferation       │    │ chondrogenesis ↑          │
  │ activity ↑          │    │                           │
  │ chondrogenesis      │    │                           │
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          │ Efficient and effective utilization  │
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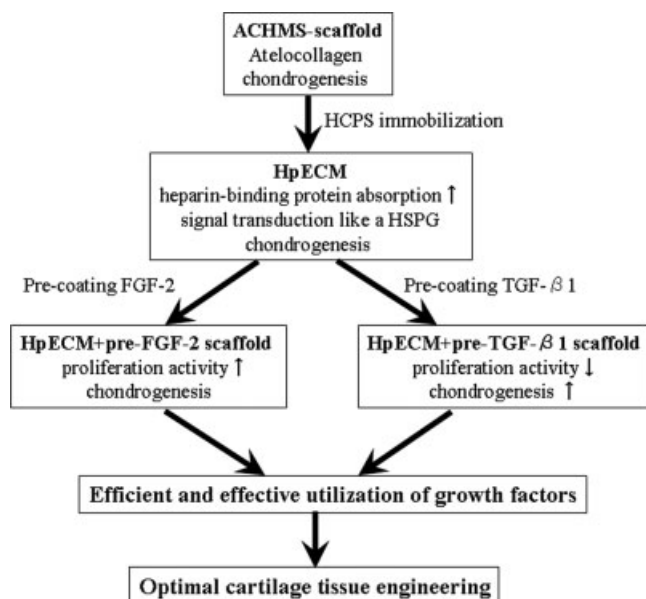
Figure 5. The logic thinking flow. We made an HpECM, which has much heparin-binding protein absorption, by immobilization of HCPS to the ACHMS-scaffold made from atelocollagen with chondrogenesis. A HpECM works like heparin-sulfate proteoglycan. We think precoating FGF-2 or TGF-β1 of HpECM is useful for the cartilage tissue engineering because of the efficient and effective utilization of growth factors.

a variety of tissue engineering applications. Previous studies have shown that HCPS is efficiently adsorbed on polymetric surfaces immobilizing various heparin-binding growth factors, and that several types of cells such as fibroblast and endothelial cells are able to grow by immobilized HCPS and preservation of heparin-binding growth factors on the HCPS-immobilized polymeric surface.[12,17] We have also found that the HCPS efficiently and stably attaches to collagen surfaces, such as collagen membrane and collagen sponges, probably through a hydrophobic interaction between the HCPS and collagen molecules.[10,11] HCPS-coating of the collagen membrane did not affect adhesion of chondrocytes and stimulated the cell proliferation. Thus, HCPS-bound collagen materials such as HpECM scaffold, in combination with various heparin-binding growth factors, will provide novel biomaterials in cell and tissue engineering fields, controlling cellular activities such as growth and differentiation (Figure 5).

Some studies have investigated heparin-binding or the heparin-immobilized collagen scaffold as carriers of exogenous growth factors.[23–27] Kanematsu et al.[25] demonstrated that collagenous matrices function as release carriers of growth factors using bladder cells, and served to actively control the tissue regeneration. This is the first study using HCPS and collagen complex to create tissue-engineered cartilage.

In this study, we created a HpECM to build an optimal extracellular environment using our originally-developed HCPS and ACHMS-scaffolds. Both scaffolds allow three-dimensional culturing of articular chondrocytes and subse-

quent preparation of tissue-engineered cartilage. We investigated the effect of FGF-2 on cell proliferation and the effect of TGF-β1 in inducing differentiation. These effects were because of the fact that both have heparin-binding sites and are stably fixed in the HpEMC since the HCPS built an extracellular environment similar to that of heparan sulfate proteoglycan within the collagen. As a result, the matricrine and juxtacrine effects were displayed along with their respective activities. At the same time, endogenous growth factors, cytokines, and other substances were also maintained around this artificial extracellular matrix. Histological findings seemed to correspond to the results of Western blotting.

Ability of growth factors to be immobilized by heparan sulfate proteoglycan on either the cell surface or within the extracellular matrix accentuates their actions by (1) promoting the accumulation of growth factors at high concentrations in the appropriate location to encounter their target cells; (2) activating growth factors by inducing conformational changes in the bound growth factors; (3) promoting conformation-dependent association or polymerization of growth factors and their cell-surface receptors and facilitating the assembly of the appropriate molecular complex to initiate signal transduction; and (4) protecting growth factors from both chemical and physiological degradation. Furthermore, many interactions between heparan sulfate proteoglycan and growth factors are highly specific. Thus, involvement of heparan sulfate proteoglycan in promoting paracrine, autocrine, juxtacrine, and matricrine functions provides a powerful and sophisticated model for regulating growth factor-mediated cellular activities.[14] In the present study, the regulation of chondrocyte proliferation and differentiation by immobilized heparin-binding growth factors was demonstrated. In other words, this type of stimulation by nondiffusional growth factors makes it possible to regulate tissue formation with artificial biomaterials.

The expression of collagen type II, aggrecan, SOX9 in HpECM- and HpECM-pre-TGF-β1 scaffold, maintained a high level. These preferable conditions for chondrocytes effect the accumulation of cartilageous extracellular matrix. On the other hand, the low expression of these in HpECM+preFGF2 scaffold means that immobilized FGF2 effected the proliferation and differentiation of chondrocytes during the culture period. We think that there are two reasons why the effect of both FGF-2 and TGF-β1 lasted for more than 7 days. One is that a powerful and sophisticated model[14] function like an HSPG has occurred. And the other is that the initial inductions to cultured chondrocyte have an impact on subsequent differentiation. The results suggested that HpECM-scaffold could adsorb and retain the heparin-binding growth factors in the pericellular microenvironment and modify the chondrocyte via signal transduction. The histological findings corresponded to the result of Western blotting of collagen type II. In this study, we were unable to directly measure the total amount of heparin-binding protein existing around each scaffold.

Although we should further study the mechanism of signal transduction of HpECM, HpECM-scaffolds still have a beneficial effect on maintaining chondrocyte phenotype. We observed the optimal condition of HpECM *in vitro*, but we should determine whether the same phenomena would occur *in vivo*.

In future, tissue engineering approaches, increasing importance will likely be placed on building an optimal extracellular environment.

The main conclusion of this study is that an ACHMS-scaffold immobilized with HCPS can be a HpECM scaffold for cartilage regeneration to retain the heparin-binding growth factors within the scaffolds.

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