Tissue Engineering of Articular Cartilage Using an Allograft of Cultured Chondrocytes in a Membrane-Sealed Atelocollagen Honeycomb-Shaped Scaffold (ACHMS Scaffold)

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Abstract: The aim of this study was to investigate with tissue engineering procedures the possibility of using atelocollagen honeycomb-shaped scaffolds sealed with a membrane (ACHMS scaffold) for the culturing of chondrocytes to repair articular cartilage defects. Chondrocytes from the articular cartilage of Japanese white rabbits were cultured in ACHMS scaffolds to allow a high-density, three-dimensional culturing for up to 21 days. Although the DNA content in the scaffold increased at a lower rate than monolayer culturing, scanning electron microscopy data showed that the scaffold was filled with grown chondrocytes and their produced extracellular matrix after 21 days. In addition, glycosaminoglycan (GAG) accumulation in the scaffold culture was at a higher level than the monolayer culture. Cultured cartilage *in vitro* for 14 days showed enough elasticity and stiffness to be handled *in vivo*. An articular cartilage defect was initiated in the patellar groove of the femur of rabbits and was subsequently filled with the chondrocyte-cultured ACHMS scaffold, ACHMS scaffold alone, or nonfilled (control). Three months after the operations, histological analysis showed that only defects inserted with chondrocytes being cultured in ACHMS scaffolds were filled with reparative hyaline cartilage, and thereby highly expressing type II collagen. These results indicate that implantation of allogenic chondrocytes cultured in ACHMS scaffolds may be effective in repairing articular cartilage defects. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 75B: 177–184, 2005

Keywords: regeneration; articular cartilage; chondrocytes; scaffold; tissue engineering

INTRODUCTION

Articular cartilages show a limited reparative capacity after injury. Full-thickness cartilage injuries typically heal using fibrocartilage. This tissue, however, lacks the biochemical and mechanical properties necessary to yield a complete and durable repair.[1] Initially, the repair tissue may appear and function like hyaline cartilage; it deteriorates with time, provoking a degenerative process that may eventually involve the entire joint.[2,3] This inability to reconstitute the cartilage tissue by an intrinsic healing process has promoted studies of various composite materials in chondrocyte-seeded implants in the treatment of cartilage lesions.

The implantation of chondrocyte–collagen composites into cartilage defects has proved a promising method of cartilage repair.[4,5] The high-density, three-dimensional culture system using the scaffold is able to maintain the phenotype of chondrocytes and their ability to produce extracellular molecules required for tissue reconstruction.[6,7] In fact, chondrocytes cultured in three-dimensional collagen gels produce an extracellular matrix consisting of proteoglycans and type II collagen having an architecture similar to hyaline cartilage.[4,5] However, the collagen gel is mechanically too weak to maintain a desired shape and structure during culturing, and requires some complicated procedures to retain the chondrocyte–collagen gel *in vivo* in the

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cartilage lesions. Although numerous tissue engineering approaches have been studied to repair damaged cartilage using such scaffolds as agarose,[8] alginate,[9] chitosan,[10] hyaluronic acid,[11] fibrin,[12] and synthetic polymers,[13–15] an improved and more reliable strategy is desired for cartilage repair.

A new atelocollagen honeycomb-shaped scaffold containing a membrane sealing (ACHMS scaffold) has been reported to allow a high-density, three-dimensional culturing of various cells as tissue engineering scaffold.[16,17] Furthermore, previous reports indicated the useful application of this scaffold as a biomaterial for tissue engineering of intervertebral discs using annulus fibrosus (AF) cells.[18,19] The AF cells were able to grow *in vitro* and maintain its phenotype in the ACHMS scaffold. In addition, the AF cells cultured in an ACHMS scaffold maintained the viability and ability to produce type II collagen and proteoglycan.[17] The main purpose of this work is to evaluate the ACHMS scaffold in a three-dimensional culturing of chondrocyte cells for tissue engineering of articular cartilage.

MATERIALS AND METHODS

Preparation of ACHMS Scaffold

Preparation of the atelocollagen honeycomb sponge has been described previously[16] and has been obtained from KOKEN Inc. Tokyo, Japan. Briefly, type I atelocollagen from bovine dermis in solution (1%, pH 3) was poured into a shallow flask and exposed to ammonia gas in order to neutralize. The resulting white gel was then rinsed with distilled water to wash out the excessive ammonia and salt produced during the neutralization reaction. The gel freeze-dried resulted in honeycomb-shaped sponge. The pore diameter of the sponge was controlled by changing the concentration of collagen solution and ammonia gas. When a high concentration of ammonia gas was used, small pores were produced, and vice versa. In this study, we used the scaffold with average 350 μm diameter pores. The slices were irradiated with ultraviolet (UV) light to yield an insoluble honeycomb-shaped sponge material.[16] To prepare the collagen–membrane sealing, type I atelocollagen solution (1%, pH 3) was poured into the flask and exposed to ammonia gas to neutralize. The honeycomb-shaped sponges were placed on the produced thin gel and air-dried in a clean bench. After making the membrane insoluble by UV irradiation, the honeycomb-shaped sponges with membrane sealing at the bottom side were cut into round shapes having a diameter of 6 mm using a punch. Finally, the round atelocollagen honeycombs with a membrane sealing were treated at 130°C for 20 min in vacuum for sterilization.[17]

Culturing of Chondrocytes from Joints of Rabbits

Articular cartilage slices from joints (hip, knee, and shoulder joints) were obtained from 4-week-old male Japanese white rabbits weighing about 1 kg. These cartilage slices were minced and then digested in F12/DMEM (Iwaki Inc., Tokyo, Japan) containing 0.4% (w/v) pronase E (Kaken Pharmaceutical Inc., Tokyo, Japan) for 1 h and F12/DMEM containing 0.025% (w/v) bacterial Collagenase P (Boehringer Mannheim Co. Ltd., Mannheim, Germany) for 4 h. The digested tissue was passed through a cell strainer (Becton Dickinson Labware Co. Ltd.) with a pore size of 40 μm. The filtrate was centrifuged at 1500 rpm for 10 min to separate the cells. The cells (0.2 mL) were then seeded at a high density (2×10^6 cells/scaffold) on the top (open side) of the round ACHMS scaffold (diameter, 6 mm; thickness, 2 mm) in 96-well plates (Sumitomo Bakelite Corp., Tokyo, Japan) by centrifugation at 500 rpm (45 g) for 5 min and cultured in F12/DMEM supplemented with 10% fetal bovine serum (Iwaki) and 50 μg/mL ascorbic acid (Sigma Corp., St, Louis, MO) at 37°C in an atmosphere of 5% CO_2 in air and 100% relative humidity. After the indicated period of incubation, the cultures were studied by (i) a scanning electron microscope, (ii) determination of the DNA content as indicator of cell proliferation, as well as (iii) determination of glycosaminoglycan (GAG) accumulation as marker of matrix formation.[17] Prior to transplantation of the chondrocyte-containing ACHMS scaffold into an articular cartilage defect *in vivo*, the chondrocytes were cultured for 14 days in the scaffold. The cells also have been plated at a density of 2×10^6 cells/dish (Falcon culture dish; diameter, 10 cm; Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) and monolayer cultured under the same conditions as described above.

Scanning Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde (Wako Pure Chemical Corp., Osaka, Japan) and 1% osmium acid (Wako

TABLE I. Histological Grading Scale for Defect Cartilage

Category	Points
A. Cell Morphology	
Hyaline cartilage	0
Mostly hyaline cartilage	1
Mostly fibrocartilage	2
Mostly noncartilage	3
Noncartilage only	4
B. Matrix Staining (Metachromasia)	
Normal (compared with host adjacent cartilage)	0
Slightly reduced	1
Markedly reduced	2
No metachromatic stain	3
C. Surface Regularity	
Smooth (>3/4)	0
Moderate (>1/2–3/4)	1
Irregular (1/4–1/2)	2
Severely irregular (<1/4)	3
D. Thickness of Cartilage	
>2/3	0
1/3–2/3	1
<1/3	2
E. Integration of Donor with Host Adjacent Cartilage	
Both edges integrated	0
One edge integrated	1
Neither edge integrated	2
Maximum Total	14

This table was extracted from Wakitani et al.'s histological grading scale.[22]

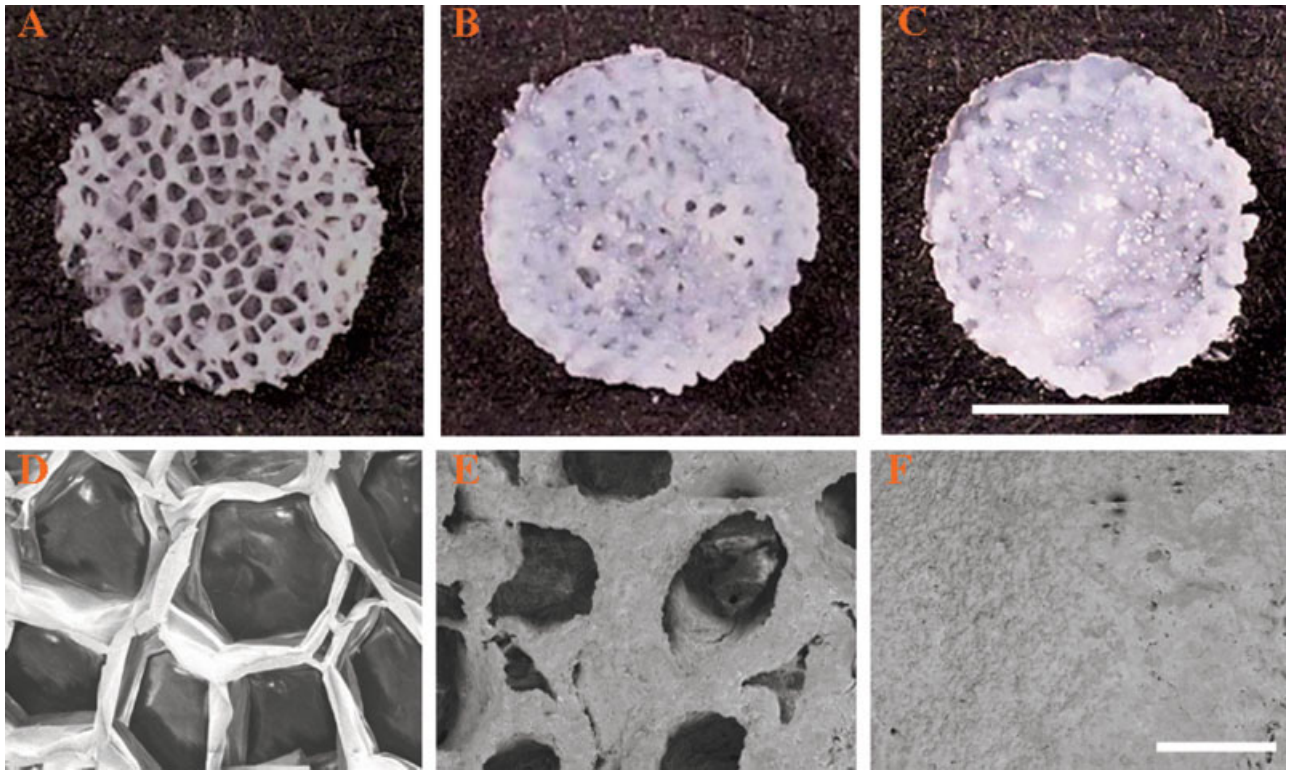

Figure 1. Appearance of the chondrocyte-seeded honeycomb-shaped atelocollagen scaffold with membrane sealing (ACHMS scaffold). Upper panel: Microscopic appearance of the chondrocyte-seeded ACHMS scaffold (original magnification, 4×). Bar represents 5 mm. Lower panel: Scanning electron microphotographs of the chondrocyte-seeded ACHMS scaffold (original magnification, 100×). Bar represents 300 μm. (A, D) Before seeding the cells, (B, E) cultured for 14 days, (C, F) cultured for 21 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Pure Chemical). After dehydration through a graded series of water–ethanol and ethanol–isoamyl acetate solutions, the samples were critical point-dried (HCP-2, Hitachi Corp., Tokyo, Japan). Samples were subsequently shadowed with gold using an ion sputter (JFC1100, Nihon Denshi Corp., Tokyo, Japan) and observed with a scanning electron microscope (JSM-840, Nihon Denshi Corp.).[17,19]

Fluorimetric Assay of DNA

The DNA content in papain digests of the cells and matrix components were determined using a fluorimetric assay.[20] Briefly, 15 μL of the papain digest was mixed with a 300 μL solution of Hoechst 33258 (Polyscience Corp., Warrington, PA) in a 96-well fluoroplate (Sumiron R; Sumitomo Bakelite Corp.). Emission and excitation spectra were obtained with a Titertek Multiscan Spectrofluorometer (Lab Systems Corp., Helsinki, Finland) at 456 nm 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.

Assay to Determine the GAG Content

To examine the GAG content in cultured chondrocytes in the ACHMS scaffold, the cell–matrix component was assayed using the 1,9-dimethylmethylene blue (DMB) method, as described by Farndale and colleagues.[21] Briefly, the cell cultures within the honeycomb-shaped scaffold on day 2, 7, 14, and 21 were digested with 300 μg/mL papain in 50 m*M* phosphate buffer, pH 6.5, containing 2 m*M* *N*-acetyl cysteine and 2 m*M* EDTA at 60°C, overnight. Also the cells cultured in a monolayer were harvested using a scraper and digested with papain as described above. The sample solutions (140 μL) were gently mixed with an equal volume of DMB solution in a 96-well microtiter plate and the absorbance at 530 nm was measured immediately using a Titertec Multiscan Spectrophotometer (Lab Systems). Absorbance values were quantified against a standard curve of shark chondroitin sulfate C (Seikagaku Kogyo Corp., Tokyo, Japan), ranging from 0.625 to 20 μg/mL. The absorbance value of the digestion of the scaffold without cells with papain was defined as 0 point.

Transplantation of Chondrocytes in the ACHMS Scaffold

Twelve Japanese white rabbits (male, 16–18 weeks old, average weight 3 kg) were used in this study. The rabbits were anesthetized with intramuscular injections of 120 mg ketamine and 9 mg xylazine. After a medial parapatellar incision of both legs, each patella was dislocated laterally and

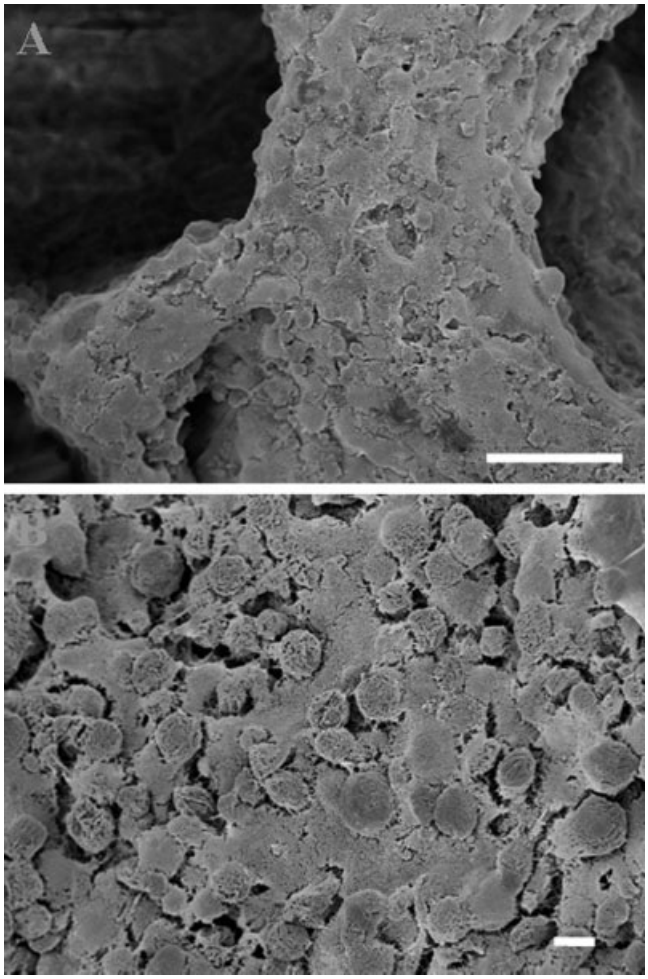

Figure 2. Scanning electron microphotograph of the chondrocyte-seeded ACHMS scaffolds. (A) SEM of the surface of the scaffold cultured chondrocytes for 14 days (original magnification, 250× bar length, 100 μm). (B) SEM of the surface of the scaffold cultured chondrocytes for 21 days (original magnification, 700× bar length, 10 μm). Both samples were observed at the center part of the chondrocyte-seeded scaffold.

one full thickness cylindrical defect (4 mm in diameter and 3 mm deep) was created on the patellar groove of the femur in both legs using a low-speed drill (Takagi Corp., Niigata, Japan). The rabbits were classified into the following three recipient groups: (i) the cells-containing scaffold insertion group, in which chondrocytes cultured in an ACHMS scaffold were allografted into the created full-thickness articular cartilage defect, (ii) the scaffold insertion group, in which only the ACHMS scaffold was inserted, and (iii) the noninsertion group. Before allograft transplantation, chondrocytes cultured in the scaffold and the scaffold without cells were cut to adjust to the size of defects. The implanted scaffolds (with and without chondrocytes) were inserted into the defects and fixed without any additional fixation such as fibrin glue. After the operation, all animals were allowed to walk freely without any splints in the cages. These animal experiments were approved and carried out following the guidelines for animal experimentation of the National Defense Medical College, Tokorozawa, Saitama, Japan.

Histological Evaluations *In Vitro* and *In Vivo* and Wakitani's Scale

The rabbits were sacrificed 3 months after the operation by an overdose intravenous anesthesia. The distal part of the femur was excised and fixed with 10% buffered formalin for a period of 7 days. The chondrocytes cultured in an ACHMS scaffold *in vitro* were also fixed with 10% buffered formalin for 7 days. Each specimen was decalcified with 10% DETA in distilled water (pH 7.4) for 3 weeks and embedded in paraffin. Sections about 6 μm thick were cut through the grafted area, stripped from paraffin, and stained with toluidine blue.

Sections were, after stripping from paraffin, also pretreated with 0.1 mg/mL actinase E (Kaken Pharmaceutical Inc.) in PBS at 37°C for 30 min. And after incubating the section with 10% pig serum at room temperature for 30 min, thus reducing the degree of nonspecific background staining, the sections were incubated overnight with 50 μg/mL mouse antihuman type II collagen monoclonal antibody (Daiichi Fine Chemical Corp., Toyama, Japan) in PBS containing 0.1% bovine serum albumin at 4°C. The sections were then incubated with biotinylated rabbit antimouse immunoglobins (Dako Co.) at dilution of 1:500 for 30 min and followed with peroxidase-conjugated streptavidin (Dako) at dilution of 1:500 for 30 min at room temperature. Finally, the sections were immunostained by incubating with 20 mg DAB (diaminobenzidine) and 5 μL hydrogen peroxide (30%) in 100 mL PBS for 5 min at room temperature. Sections were incubated with PBS instead of specific primary antibodies and stained as a negative control.

The sections were evaluated microscopically and scored according to a histological grading scale, consisting of 5 categories with a total score ranging from 0 to 14 points as described by Wakitani and colleagues (Table I).[22]

Statistical Analysis

To analyze quantitative DNA and GAG data, the Mann–Whitney *U* test was carried out. Histological results were analyzed using ANOVA, followed by the Dunn test as *post hoc* test. *p* values less than 0.05 were considered statistically significant.

RESULTS

Structure of ACHMS Scaffold

The upper surface of the ACHMS scaffold was found to be honeycomb shaped, as shown in Figures 1(A,D). The diameter of the pores could be controlled from about 100 to 1,000 μm upon varying the concentration of the collagen solution and ammonia gas. The lower surface was sealed with the same atelocollagen membrane. Scaffolds having a diameter of about 6 mm were used in the present study. The honeycomb-shaped parts consisted of thin multiple collagen membranes of less than 1 μm in thickness. It was found to be elastic and during the entire cell culture procedure did not deform or collapse upon forceps handling. Scaffolds could be

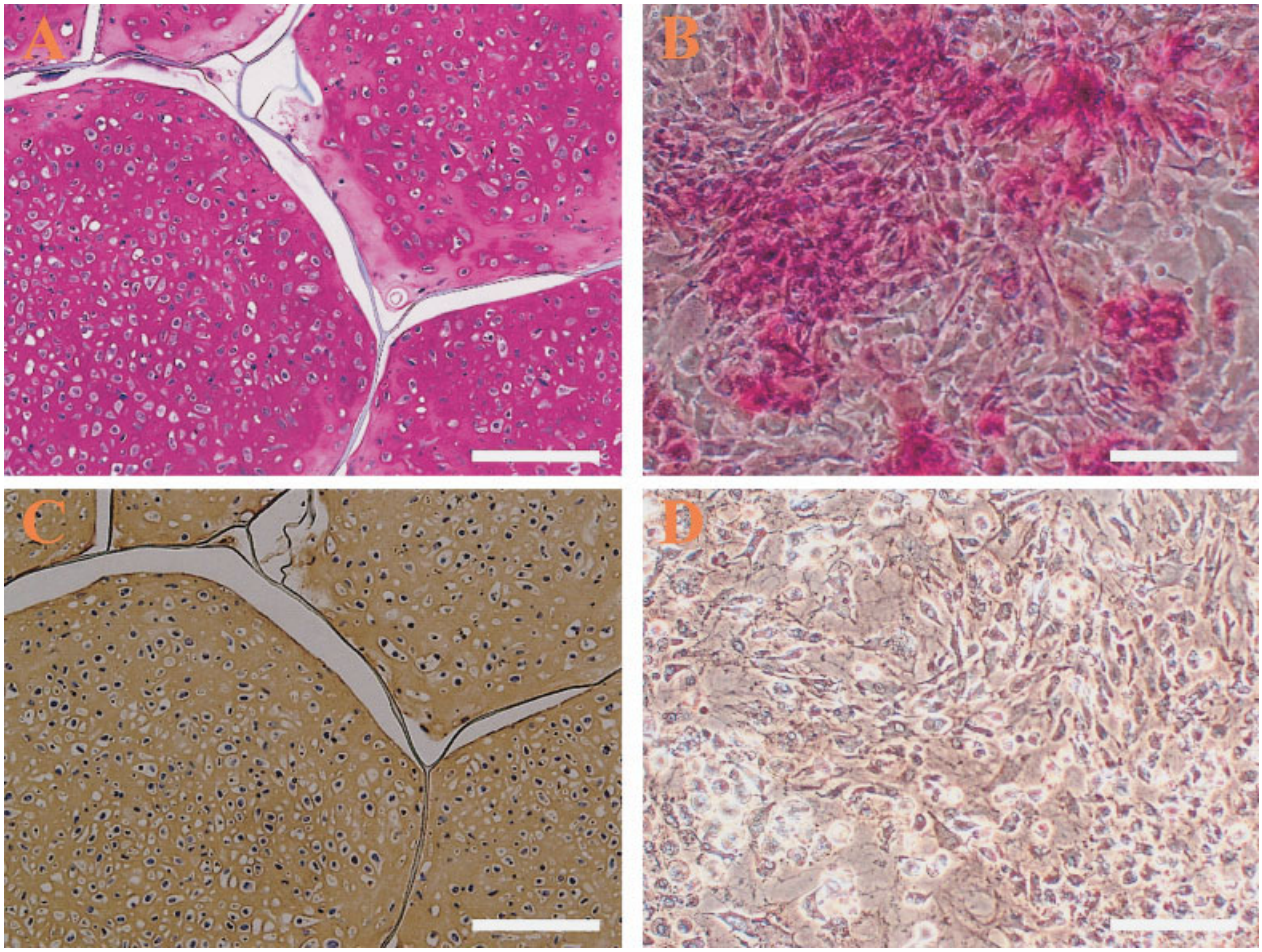

Figure 3. Histological observations of the chondrocyte-seeded ACHMS scaffolds *in vitro* (light micrograph) and the chondrocytes in monolayer culture (phase-contrast light micrograph). (A, B) Toluidine blue staining of the scaffold (A) and monolayer (B) cultured chondrocytes for 21 days (bar length, 100 μm). (C, D) Type II collagen immunostaining of the scaffold (C) and monolayer (D) cultured chondrocytes for 21 days (bar length, 100 μm). Each microphotograph is a representative of six samples stained with toluidine blue and anti-type II collagen in each group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

easily cut with scissors or sharp knives into a desirable shape being maintained during the cell culturing.

Culture of Chondrocytes in ACHMS Scaffolds *In Vitro*

High density of chondrocytes (2×10^6 cells/scaffold) could be seeded into the ACHMS scaffold by gentle centrifugation without spilling cells due to the scaffold membrane sealing. Scanning electron microscopy after day 14 and 21 of culturing (Figures 1 and 2) revealed the scaffold was filled with grown chondrocytes with their produced and accumulated matrix molecules. It was also found that chondrocytes in the scaffold retained their spherical shape, that is, a typical appearance of the chondrocyte cells, during the culturing period (Figure 2). Also Figure 3 shows microphotographs of the chondrocyte cultures inside the scaffold after 21 days. Chondrocytes were able to proliferate in the scaffold, resulting in a stronger toluidine blue staining [Figure 3(A)] and type II collagen immunostaining [Figure 3(C)] than monolayer culture [Figures 3(B,D)]. Figure 3 demonstrated that chondrocytes in the scaffold maintained their small and round figures. On the other hand, chondrocytes in the monolayer became large fibroblastlike cells. The tissue engineered cartilages *in vitro* were found elastic and stiff enough to be handled in *in vivo* experiments.

The DNA content of chondrocytes in the scaffold culturing increased at a lower rate when compared to the monolayer culturing during the initial 14 days [Figure 4(A)]. The increase of total GAG content from the cell–matrix components of the scaffold culture occurred at a higher rate than in the monolayer culture, and this difference was observed throughout the entire culture period for up to 21 days [Figure 4(B)]. Thus, chondrocytes are able to gradually grow for a longer period of time (at least 21 days), and although having a lower growth rate than in a monolayer culture, formation of matrix molecules in the scaffold culture is higher.

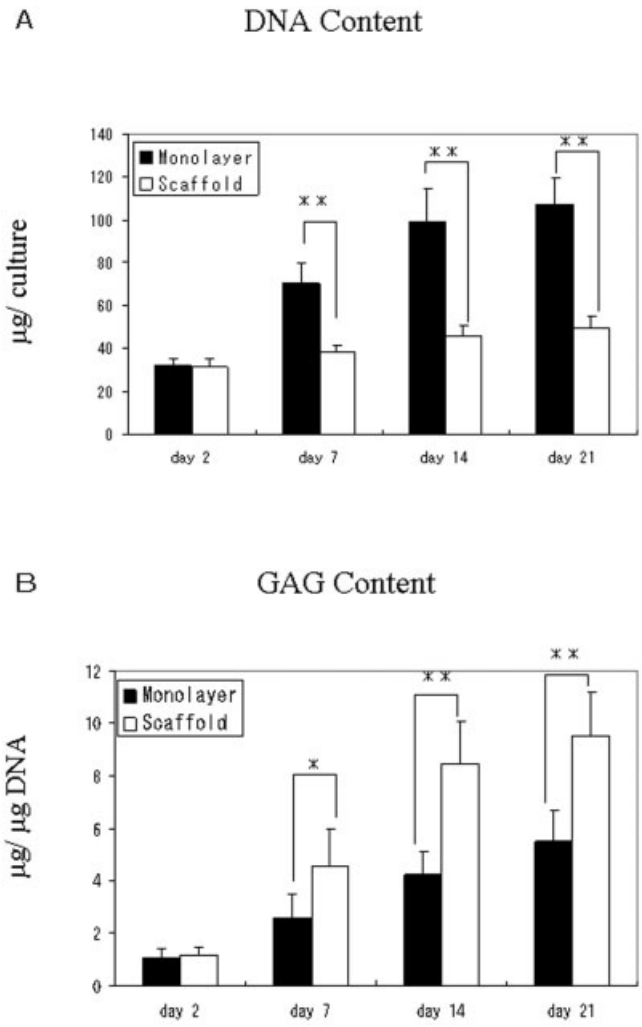

Figure 4. DNA and GAG content in the chondrocyte cultures using ACHMS scaffolds (white bars) and monolayer cultures (black bars). Measurements of DNA (A) and GAG (B) contents have been carried out on the indicated days, as described in the Materials and Methods section. Data represent the mean ± SD of six determinations. $*p < 0.05$, $**p < 0.01$.

Histological Evaluation and Wakitani's Scale

No sign of arthritis, for example, cartilage erosion or severe synovial proliferation, was observed in any operated knee. In the cells-containing scaffold insertion group, the defects were completely filled with reparative tissue resembling hyaline cartilage at 3 months after operation. The chondrocytes in the scaffold maintained their small round morphology, and the extracellular matrices, especially proteoglycans and type II collagen, were intensely stained by toluidine blue and anti-type II collagen, respectively. The reparative tissue showed a smooth surface being connected with adjacent normal cartilage at 3 months after operation [Figure 5(A)]. In contrast, the surface of the reparative tissue in the scaffold insertion group and noninsertion group was slightly irregular and the thickness of the tissue was smaller than the cell-containing scaffold insertion group [Figures 5(B,C)]. The intensity of toluidine blue and anti-type II collagen declined and the areas of staining were found lower in the scaffold insertion group and noninsertion group. Furthermore, the mean in Wakitani's score (Table I)[22] of the cell-containing scaffold insertion group at 3 month was found significantly more profound than the other two groups (Table II).

DISCUSSION

Collagen is the most abundant protein in mammals and the most frequently used material as scaffold in various mammalian cell cultures. In particular, three-dimensional cell cultures are commonly carried out using collagen in gels, sponges, films, and sandwich structures of these materials.[23,24] In general, a scaffold for cartilage tissue engineering is expected to fulfill the prerequisites concerning structure and biocompatibility and should enable a preformed shape prior to transplantation.[25] Furthermore, the scaffold should be noncytotoxic and nonantigenic, guarantee a uniform cell distribution, maintain cell viability and phenotype, and allow the synthesis of extracellular matrix components as is required for the development of solid connective tissue.[25,26] The present study demonstrates the applicability of the ACHMS scaffold in terms of stability, cytocompatibility, uniform and high-density seeding of chondrocytes, and accumulation of matrix components.

Although the proliferation of chondrocytes in the scaffold appeared to be lower than the monolayer culture during 21 days (Figure 4), the scanning electron microscopic data showed grown chondrocytes retaining their spherical shape and typical cell appearance with an abundant deposition of matrix products after 21 days (Figure 2).

Another essential aspect for a scaffold of chondrocytes concerns the influence of the scaffold material on the phenotype of cultured chondrocytes. In case of monolayer culturing of chondrocytes, the cultured cells were expanded showing fibroblastic morphologies [Figure 3(B,D)], resulting in dedifferentiation of the cells. The synthesis of type II collagen and proteoglycan has been considered to be sensitive metabolic markers to investigate the phenotype level, as has been described for chondrocytes isolated from hyaline cartilage tissue.[27–29] Therefore in this study, the expression of type II collagen and proteoglycans (glycosaminoglycans) by chondrocytes was evaluated as a metabolic marker of the phenotype of chondrocytes. Immunostaining (for type II collagen) demonstrates that chondrocytes cultured in the scaffold retain a higher ability to deposit type II collagen [Figure 3(C)], as well as glycosaminoglycans [Figure 4(B)], when compared to monolayer culturing [Figure 3(D)]. Thus, a high-density, three-dimensional culturing system using a scaffold is able to maintain the phenotype of chondrocytes and their ability to produce extracellular molecules required for tissue regeneration.

The tissue-engineered cartilages *in vitro* were found to be elastic and stiff enough to be handled in subsequent *in vivo* experiments and implanted without any additional fixation. In the present study, articular cartilage defects implanted with chondrocytes embedded in ACHMS scaffolds *in vivo* showed

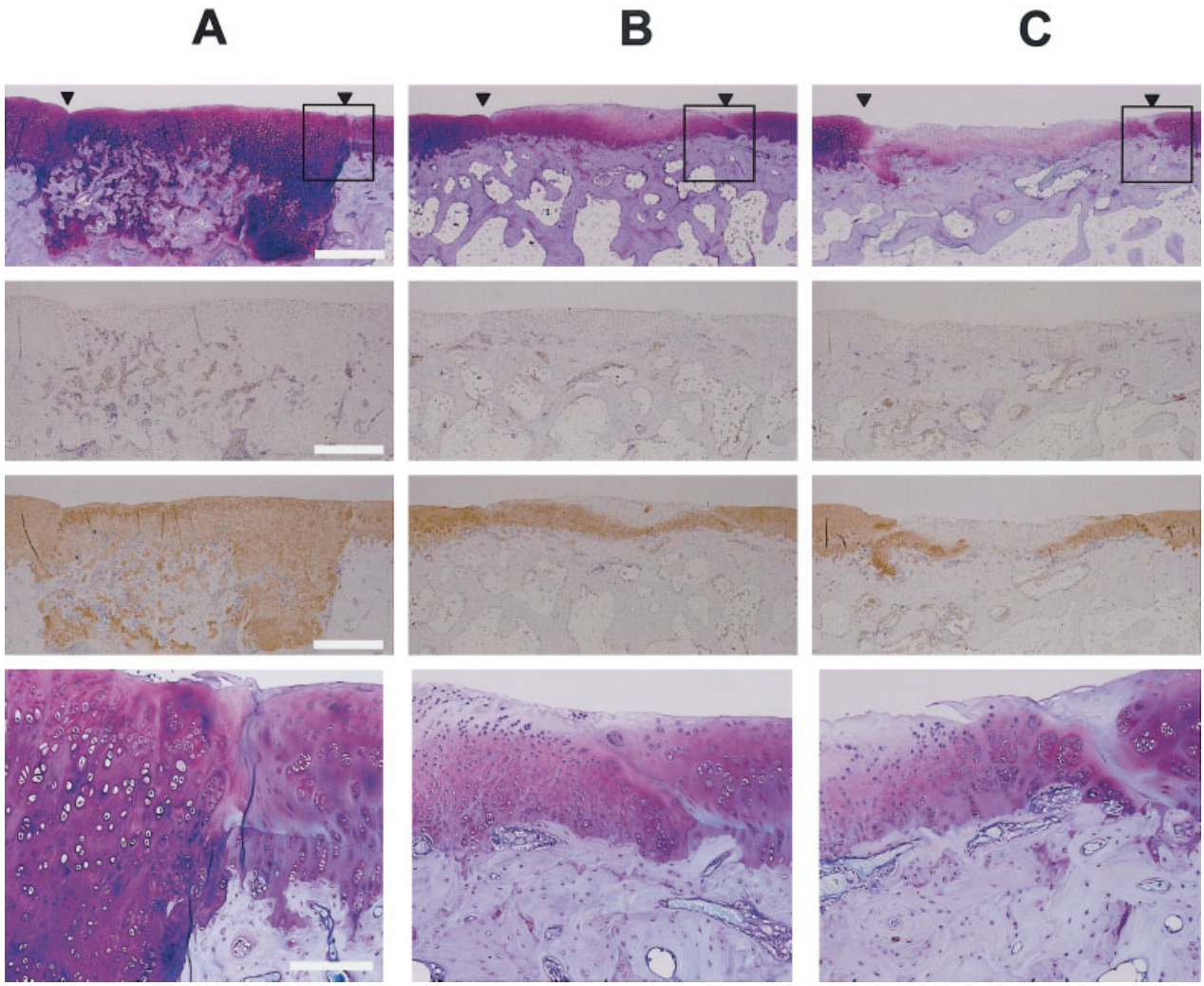

Figure 5. Histological observations of the chondrocyte-seeded ACHMS scaffolds 3 months after allografting. (A) Chondrocyte-containing ACHMS scaffold insertion group. (B) ACHMS scaffold insertion group. (C) Noninsertion group. Upper (first line) panels: Toluidine blue staining of the implanted scaffold (bar length, 1 mm). Upper middle (second line) panels: Negative control immunostaining (PBS instead of the type II collagen antibody) of the implanted scaffold (bar length, 1 mm). Lower middle (third line) panels: Type II collagen immunostaining of the implanted scaffold (bar length, 1 mm). Lower (fourth line) panels: Toluidine blue staining of the implanted scaffold (bar length, 200 μm). Each microphotograph is a representative of eight tissue samples stained with toluidine blue and anti-type II collagen in each group, and the squares (upper panel) show the sites of the microphotographs in the lower panel. The triangles show defected area of the cartilage. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. Results of Histological Grading Scale

		Scores on Histologic Grading Scale					
	n	A	B	C	D	E	Total
The Cell containing Scaffold	8	0.8 (0–2)	0.6 (0–1)	1.0 (0–2)	0.4 (0–1)	1.1 (0–2)	3.8 (2–6)*
The Scaffold	8	1.9 (1–3)	1.6 (1–2)	1.4 (0–2)	1.0 (0–2)	1.3 (0–2)	7.1 (4–11)
The Noninsertion (control)	8	1.9 (1–3)	1.8 (1–3)	1.9 (1–3)	1.1 (0–2)	1.4 (1–2)	8.0 (5–12)

Each data represents mean and range (parentheses) of the scales.
* $p < 0.01$, versus the scaffold and the noninsertion (control).

improved histological findings [Figure 5(A) and Table II]. With grafted chondrocytes proliferating slowly and forming an extracellular matrix similar to hyaline cartilage, thereby maintaining the chondrocyte phenotype. Furthermore, in the implantation sites filled with the chondrocyte-cultured ACHMS scaffolds, no signs were observed of immunologic rejection and degeneration of the reparative tissues up to 6 month (data not shown). The goal of regenerating articular cartilage is not only to achieve anatomic morphology, but also to restore its function. The allografted chondrocytes exhibited a proliferated activity resulting in the formation of hyaline cartilage. However, the mechanical strength properties of regenerated articular cartilage remains to be investigated.

In summary, we used tissue engineering methods to carry out allograft implantations in order to regenerate articular cartilage. The allografted chondrocytes exhibited a proliferated activity showing both *in vitro* and *in vivo* extracellular matrix production, thereby maintaining the phenotype of chondrocytes. These results suggest the possibility to achieve regeneration of articular cartilage through allografting of chondrocytes in an ACHMS scaffold.

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