An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc

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Abstract: The aim of this study was to investigate the possibility of using the atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus (AF) cells in tissue engineering procedures of intervertebral disc repair. AF cells from the intervertebral discs of Japanese white rabbits were cultured for up to 3 weeks in the ACHMS-scaffold to allow a high density, three-dimensional culture. Although the DNA content in the scaffold increased at a lower rate than in the monolayer culture, scanning electron microscopy data showed that the scaffold was filled with the grown AF cells and produced extracellular matrix on day 21. The amount of type II collagen and its mRNA expression by the scaffold cultured cells were determined using Western blotting and Northern blotting analyses, respectively, and remained at a higher level than in the monolayer cultured cells. Furthermore, glycosaminoglycan (GAG) accumulation in the scaffold culture was at a higher level than in the monolayer culture. Western blot analysis for extracted proteoglycans from the scaffold culture also exhibited a much higher proteoglycan accumulation than the monolayer culture. These results indicate that the AF cells are able to grow and remain phenotypically stable in the scaffold. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 64A: 249–256, 2003

Key words: tissue engineering; cultured cell; scaffold; intervertebral disc; annulus fibrosus; phenotype

INTRODUCTION

Intradisc treatments, such as posterior hernitomy, percutaneous nucleotomy, laser disc decompression, and chemonucleolysis, are all intended to accelerate disc degeneration. Although treated patients usually obtain relief from sciatica after the procedures, postoperative acceleration of disc degeneration is often observed and ultimately may lead to a loss of motion, as well as persistent low back pain.[1–3] Although spinal column allografts have been studied for the treatment of segmental defects in a canine model, preservation of the transplanted allograft discs was variable and the discs were not morphologically or metabolically normal.[4–6] On the other hand, tissue engineering concepts have been applied to biomaterials, yielding chondrocyte-seeded implants for cartilage repair.

The intervertebral disc, like the articular cartilage, is avascular and has no direct contact with other body fluids.[7] The disc has two distinct anatomic regions: the annulus fibrosus (AF) is the fibrous ring forming the circumference of the intervertebral disc; and the nucleus pulposus (NP) is the remnant of the notochord central portion of the intervertebral disc. Each region contains an abundant extracellular matrix surrounding cells with different morphologies.[1,7] Besides the large amount of type I collagen in the layers of parallel-oriented bundles of dense connective tissue, most of the intervertebral matrix molecules are cartilage type (large aggregates of proteoglycans and type II collagen).[7,8] In fact, intervertebral disc cells, that is, AF cells, express mainly the cartilage-type matrix molecules, having quantitative differences as a function of cell morphology. The AF cells remain phenotypically stable in three-dimensional culture in alginate beads

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or agarose gels,[7,8] and AF cells produce abundant cartilage-type matrix molecules, such as proteoglycan[9] and type II collagen.[10,11] It has been suggested that the phenotype of AF cells is chondrocytic cells at a slightly different stage of differentiation than articular chondrocytes.[7]

A new atelocollagen honeycomb has been reported allowing a high density, three-dimensional culturing of various cells as a tissue engineering scaffold.[12] A modified scaffold covered with a collagen membrane (ACHMS-scaffold) has been prepared in the present study. This ACHMS-scaffold is able to effectively retain a large number of cells, allowing a three-dimensional cell culture. The main purpose of this work was to evaluate the *in vitro* use of the ACHMS-scaffold for a three-dimensional culturing of AF cells.

MATERIALS AND METHODS

Preparation of ACHMS-scaffold

Preparation of the atelocollagen honeycombs has been described previously,[12] and they were obtained from KOKEN, Inc. (Tokyo, Japan). Briefly, type I atelocollagen from bovine dermis in solution (1%, pH 3) was poured into a shallow tray and exposed to ammonia gas in order to neutralize it. The resulting white gel was then rinsed with distilled water in order to wash out excessive ammonia and salt that was produced during the neutralization reaction. The white gel was then freeze dried and the resulting honeycomb-shaped sponge was sliced into pieces having a thickness of 2 mm. The slices were irradiated by ultraviolet (UV) light to yield an insoluble honeycomb-shaped sponge material.[12]

To prepare the collagen-membrane seal, type I atelocollagen solution (1%, pH 3) was poured into the tray and exposed to ammonia gas to neutralize it. 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 a membrane seal were cut, using a punch, into round shapes having a diameter of 11 mm. Finally, the round atelocollagen honeycombs with a membrane seal were treated at 130°C for 20 min in vacuum for sterilization.

Culturing of AF cells from intervertebral discs

Intervertebral discs from lumbar spines were obtained from 4-week-old female Japanese white rabbits weighing about 1 kg. After NP was separated from the disc with a spatula, the remaining AF was shredded with scissors and digested for 4 h in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 0.4% (w/v) bacterial Collagenase P (Boehringer Mannheim, Mannheim, Germany). 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. The cells (0.5 mL) were then seeded at a high density (2×10^6 cells/scaffold) into the round-shaped atelocollagen honeycomb sponge (diameter: 11 mm, thickness: 2 mm) having a membrane seal as a scaffold in 48-well plates (Sumitomo Bakelite, Tokyo, Japan) by centrifugation at 500 rpm (45 g) for 5 min and then cultured in F12 (Iwaki, Tokyo, Japan)/DMEM supplemented with 10% fetal bovine serum (Iwaki) 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 using scanning electron microscope, Northern and Western blotting for type II collagen expression, DNA content as an indicator of cell proliferation, as well as glycosaminoglycan (GAG) and proteoglycan accumulation as markers of matrix accumulation. The cells also were plated at a density of 2×10^6 cells/dish into culture dishes (diameter: 10 cm; Falcon; Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) and cultured as the monolayer culture under the same conditions described above.

Scanning electron microscopy

Samples were fixed in 2.5% glutaraldehyde (Wako Pure Chemical, Osaka, Japan) and 1% osmium acid (Wako 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, Tokyo, Japan). Samples were cross-sectionally cut with a sharp knife to observe the intrascaffold and subsequently shadowed with gold using an ionspatter (JFC1100; Nihon Denshi Corp., Tokyo, Japan) and observed with a scanning electron microscope (JSM-840; Nihon Denshi Corp.).

Fluorimetric assay of DNA

The DNA contents in the papain digests of the cells and matrix components were determined by a fluorimetric assay.[13] Briefly, 15 μL of the papain digest was mixed with 300 μL of Hoechst 33258 (Polyscience, Warrington, PA) solution in a 96-well fluoroplate (Sumiron®; Sumitomo Bakelite). Emission and excitation spectra were obtained with a Titertek multiscan spectrofluorometer (Lab Systems, Helsinki, Finland) at 456 nm and 365 nm, respectively. DNA concentrations were determined against a standard curve of calf thymus DNA (Sigma) ranging from 0.625–40 μg/mL.

Northern blotting analysis for type II collagen mRNA

Nonradioactive Northern blotting analysis for type II collagen mRNA was performed as described previously[14,15] and according to the protocol of the manufacturer using a NorthernMax kit (Ambion Inc., Austin, TX). The cDNA probe for the Northern blotting analysis also was prepared according to Takaishi et al.[14] using the polymerase chain reaction. Briefly, scaffold- and monolayer-cultured AF cells were recovered and the total mRNA was extracted with a RNAqueous kit (Ambion). The extracted mRNA was suspended in the RNAsecure reagent (Ambion) and quantified by measuring the absorbance at 260 nm. After heat denaturation, samples of mRNA (6 μg) were electrophoresed in de-

naturing formaldehyde and 1% agarose gel at 105 V until bromophenol blue dye migrated to the edge of the gel, and then they were transferred to a BrightStar-Plus Membrane (Ambion). The mRNA was fixed to the membrane by heating at 80°C for 15 min. Hybridization was performed with heat-denatured biotinylated probes at 42°C overnight under gentle rotation according to the procedure given by the manufacturer (NorthernMax kit; Ambion). The membrane was sequentially washed with low stringency wash solution #1 and high stringency wash solution #2. Detection of the probe was performed using the BrightStar BioDetect Kit (Ambion), and the chemiluminescent signals were measured during 34 min of exposure. The chemiluminescent signals were quantitatively analyzed using the Chemi Genius Plus System (Syngene Ltd., Cambridge, UK) and compared to the signal at day 0.

Assay to determine the GAG content

To examine the GAG content in AF cells, the cell-matrix component was assayed using the 1,9-dimethylmethylene blue (DMB) method described by Farndale et al.[16] Briefly, the cell cultures within the honeycomb-shaped scaffold on days 2, 5, 10, and 14 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. The cells cultured in a monolayer were also 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 Co., Tokyo, Japan), ranging from 0.625 to 20 μg/mL.

Western blotting analysis of type II collagen and proteoglycan accumulation

Type II collagen was extracted from both monolayer-cultured AF cells and AF cells within the scaffold on day 21. SDS polyacrylamide gel electrophoresis of the collagen preparations was carried out and the gel was stained with Coomassie blue, as previously described.[17] Western blotting analysis for type II collagen was performed according to the method of Kumagai et al.[18] Briefly, type II collagen was separated on a SDS polyacrylamide gel (35 min, 200 V), and the protein bands were electroblotted on nitrocellulose membrane and reacted with an anticollagen type II monoclonal antibody (II-4CII; Fuji Chem. Ind., Ltd., Tokyo, Japan). Immunoreaction was visualized according to the avidin-biotinylated horseradish peroxidase (HRP) complex method.

Noncollagenous proteins containing proteoglycans were extracted from both monolayer-cultured AF cells and AF cells within the scaffold after 21 days with 4 *M* guanidine HCl (Wako Pure Chemical) containing protease inhibitors (0.1 *M* 6-aminohexanoic acid, 5 m*M* benzamidine hydrochloride, 1 m*M* phenylmethylsulphonylfluoride) in 50 m*M* Tris HCl (pH 7.4) at 4°C, overnight, according to the method of Takagi et al.[19] The extract was centrifuged at 5,000*g* for 10 min and supernatants were pooled. The guanidine extractions were repeated four times, and all supernatants were combined and termed *G1 extract*. The remaining cell pellets were then extracted four times with 0.2 *M* EDTA containing the protease inhibitors as described above, and all these supernatants were combined and termed *E extract*. The pellets were again extracted with 4 *M* guanidine HCl containing protease inhibitors in 50 m*M* Tris HCl to prepare the G2 extract, as described above. G1, E, and G2 extracts were dialyzed against 0.1 *M* ammonium bicarbonate containing 0.005% Brij using Spectra/Por MWCO 50,000 (Spectrum Medical Industries, Inc., CA) and concentrated using a ultra filtrating device (Ultrafree-4; Millipore Japan, Tokyo, Japan). SDS polyacrylamide gel electrophoresis of the extracts (10 μL of sample, which was identical to 1.6 μg of cultured cell DNA) was carried out (35 min, 200 V) in a 5–20% gradient cross-linked polyacrylamide gel according to the method of Takagi et al.,[19] and the gel was stained with Gelcode Blue Reagent (Pierce, Rockford, IL). A G1 extract was also digested with 0.1 U of protease-free chondroitinase ABC (Seikagaku Co.) at 37°C for 1 h and electrophoresed as described above. The Western blotting analysis was performed using a monoclonal antiproteoglycan △Di-6S (3-B-3; Seikagaku Co.) and biotinylated antimouse IgG (H+L) (Funakoshi Inc., Tokyo, Japan) as a second antibody. Immunoreactions were visualized according to the avidin-biotinylated HRP complex method.

RESULTS

Structure of the ACHMS-scaffold

The upper surface of the ACHMS-scaffold was found to be honeycomb shaped, as shown in Figure 1(A). The diameter of the pores could be controlled from about 100 to 1000 μm upon changing the concentration of collagen solution and ammonia gas. The lower surface was sealed with the same atelocollagen membrane [Fig. 1(B,C)]. The scaffold with a diameter of about 400~600 μm was used in the present study. The honeycomb-shaped partitions were composed of thin multiple collagen membranes of less than 1 μm thickness. They were found to be elastic and did not deform or collapse upon forceps handling during the entire cell culture procedure. The scaffold could be easily cut with scissors or a sharp knife. Therefore, the creation of a desirable shape was easy and the shapes could be maintained.

Cell proliferation in the ACHMS-scaffold

A high density of AF cells (2×10^6 cells/scaffold) could be seeded into the ACHMS-scaffold by gentle centrifugation without spilling cells due to the scaffold membrane seal [Fig. 2(A)]. The DNA content in the scaffold culture of AF cells increased at a slower rate when compared to the monolayer culture during 14 days (Fig. 3). However, scanning electron microscopy revealed the scaffold was filled with grown AF cells with their produced and accumulated matrix mol-

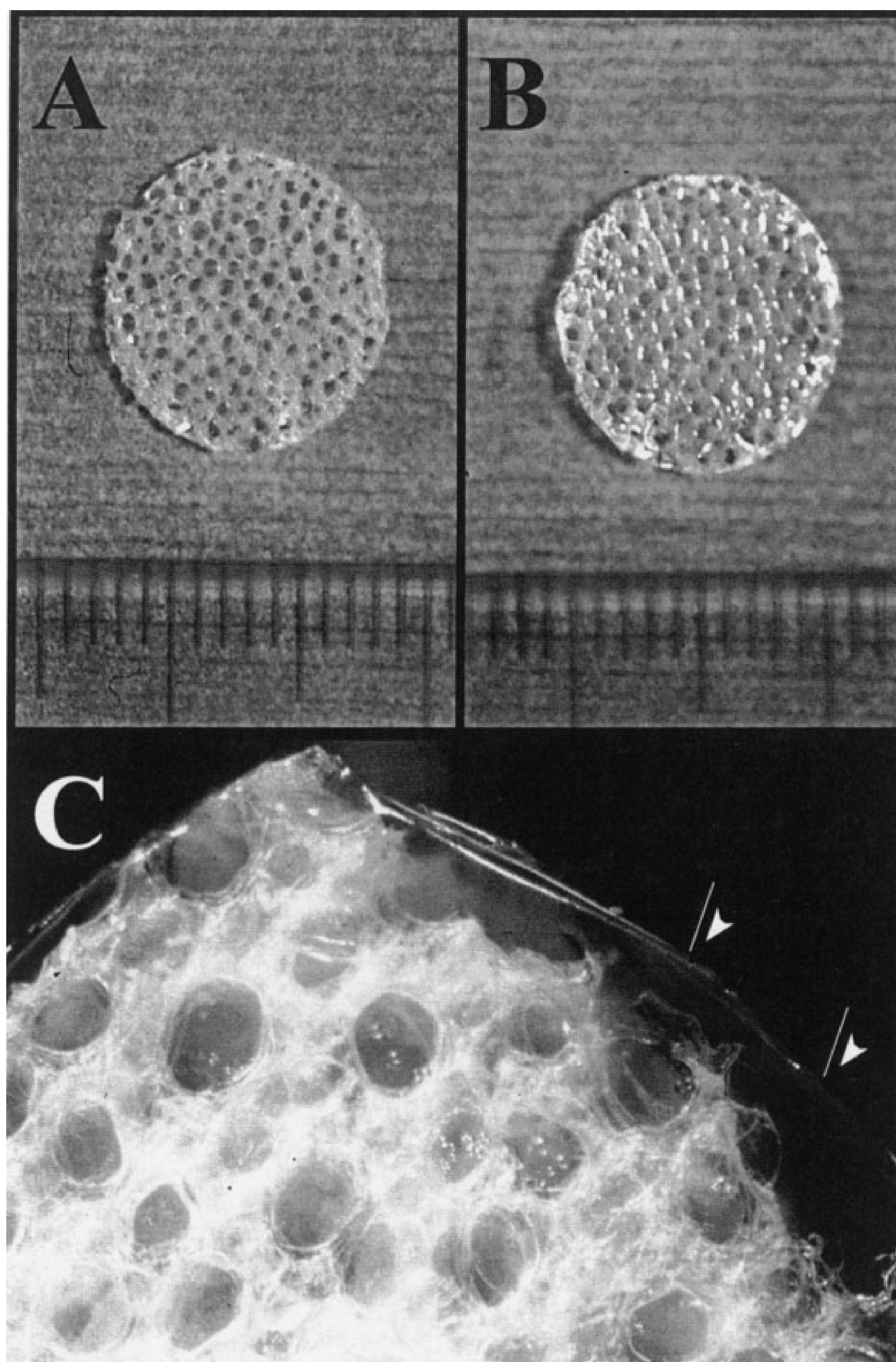

Figure 1. Appearance of the honeycomb-shaped atelocollagen scaffold with a membrane seal (ACHMS-scaffold). (A) Top of the scaffold, (B) bottom of the scaffold with the membrane seal, (C) microscopic appearance of the bottom of the scaffold showing the membrane seal (original magnification ×8). Arrowheads represent 1 μm membrane seal.

ecules on day 21 [Fig. 2(B,C)]. It was also found that AF cells in the scaffold retained their spherical shape and typical chondrocytic appearance during the culture period (data not shown). It was also observed that cartilage lacunae were formed around the cells [Fig. 2(C)]. Thus, AF cells are able to survive and gradually grow for a longer period of time (at least 3 weeks), having a lower growth rate than a monolayer culture and producing matrix molecules in the scaffold culture.

Northern blotting of type II collagen mRNA and Western blotting of type II collagen

The amount of type II collagen mRNA in the ACHMS-scaffold and monolayer cultures was mea-

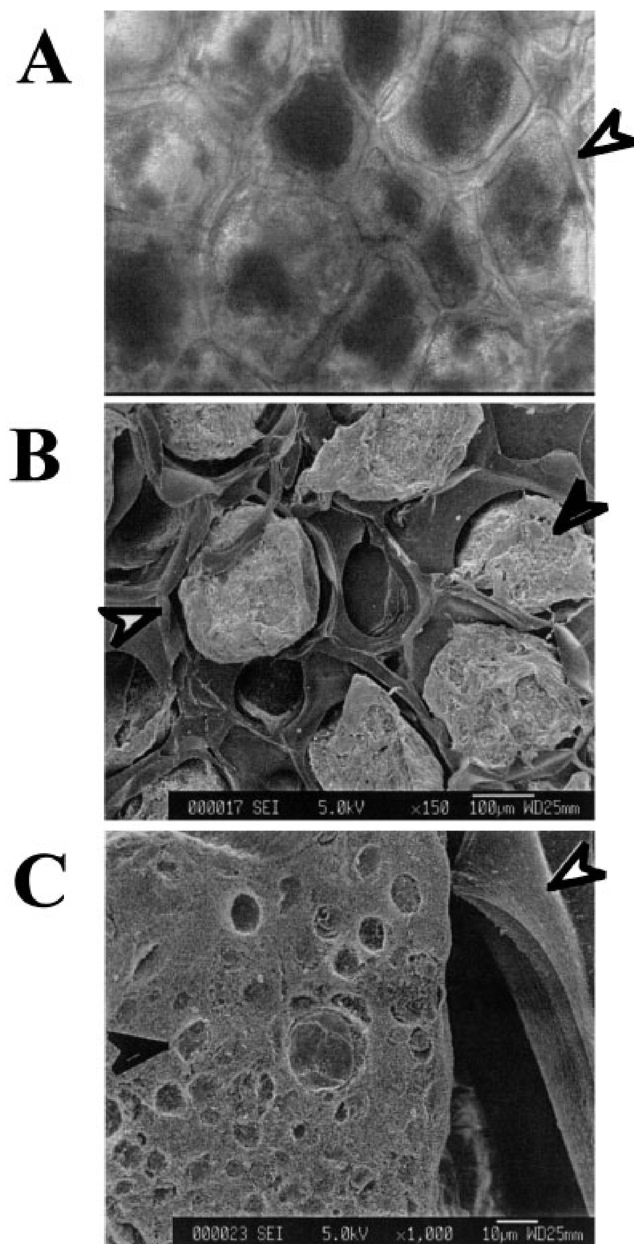

Figure 2. Microphotographs of the AF cell-seeded scaffolds. (A) A phase-contrast microphotograph of the scaffold after plating 2×10^6 AF cells/scaffold (original magnification ×100). (B) Scanning electron microphotograph of the scaffold cultured AF cells after 21 days (original magnification ×150). (C) Scanning electron microphotograph of the scaffold cultured AF cells after 21 days (original magnification ×1000). White arrowheads represent the atelocollagen honeycomb and black arrowheads represent a complex of cells and matrix.

sured on day 0, 3, 7, 10, and 14 using Northern blotting (Fig. 4). While the amount of type II collagen mRNA in the monolayer culture during the first 3 days significantly decreased to about 18% of the initial amount (day 0), the mRNA in the scaffold culture retained a high level (about 74%). Although the mRNA concentration increased in the monolayer culture with start-

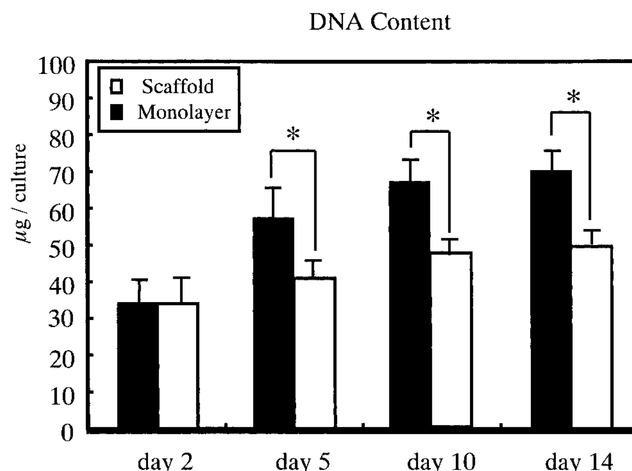

Figure 3. DNA content in the scaffold AF cell cultures determined using the scaffold (white bars) and monolayer cultures (black bars). Measurement of DNA content was carried out on the indicated days as described in the Materials and Methods section. Results are expressed as means with S.D. for 6 samples. $p < 0.05$, monolayer cultures compared with scaffold cultures.

ing multilayer overgrowth of the cells after postconfluence (from day 7), it was retained at a lower level than in the scaffold culture. Western blotting of type II collagen also showed that accumulation of type II collagen within the scaffold culture was much higher than within the monolayer culture (Fig. 5).

GAG accumulation and Western blotting of proteoglycans

The increase rate of the total GAG content from the cell-matrix component of the scaffold culture was higher than the monolayer culture from an early stage (day 2), and this difference was observed throughout the entire culture period for up to 14 days (Fig. 6).

Figure 7 shows SDS-PAGE [Fig. 7(A)] and Western blotting of proteoglycans accumulated in the scaffold and monolayer cultures [Fig. 7(B)]. Almost all noncollagenous molecules extracted with 4 *M* guanidine from the scaffold and monolayer cultures were found in the G1 pool. The bands for the extracted molecules in the G1 pool of the scaffold culture were much more intense than those in the monolayer culture, as shown in Figure 7(A), indicating an efficient accumulation of large amounts of matrix noncollagenous molecules in the scaffold culture. The monoclonal antibody (△Di-6S) recognizes stubs including the 6-O-sulfate group of the galactosamine residue at the nonreducing end of cleaved chondroitin sulfate chains of proteoglycan with chondroitinase ABC. Figure 7(B) shows a significantly higher accumulation of chondroitin sulfate proteoglycan in the scaffold culture compared to the monolayer culture.

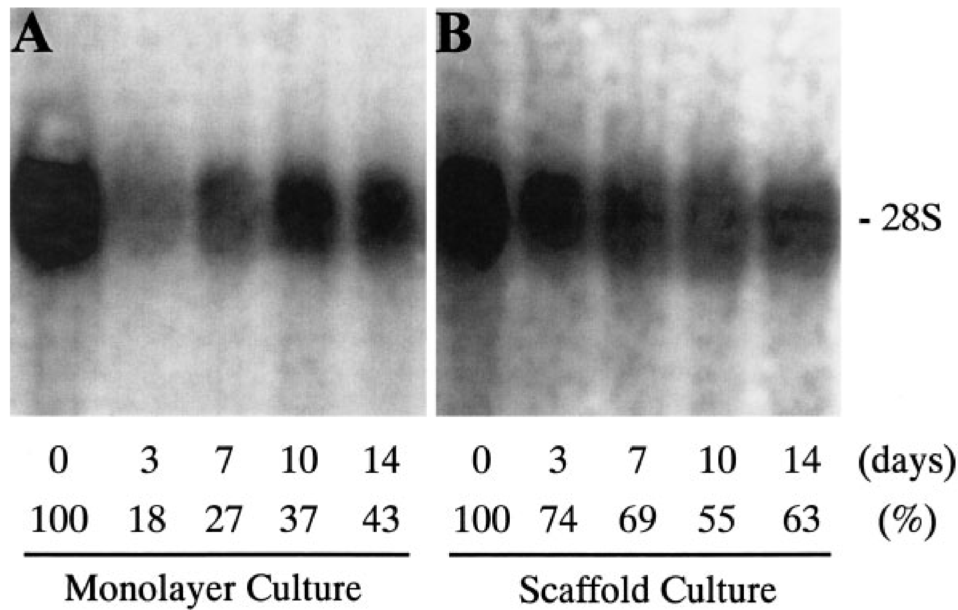

Figure 4. Northern blotting of type II collagen mRNA. Nonradioactive Northern blottings for type II collagen mRNA from scaffold AF cell cultures and monolayer cultures on day 0, 3, 7, 10, and 14 were carried out as described in Materials and Methods. The chemiluminescent signal strength (%) is represented as a percentage of the signal on day 0 (100%), as described in the Materials and Methods section.

DISCUSSION

Collagen is the most abundant protein in mammals and the most frequently used material as a scaffold for various mammal cell cultures. In particular, three-dimensional cell cultures are commonly performed using collagen gels, collagen sponges, collagen films, and sandwich structures of these materials.[20–23] Generally, a scaffold for cartilage tissue engineering is expected to fulfill the prerequisites concerning structure and biocompatibility. Such a scaffold should enable a preformed shape prior to transplantation.[24] Further-

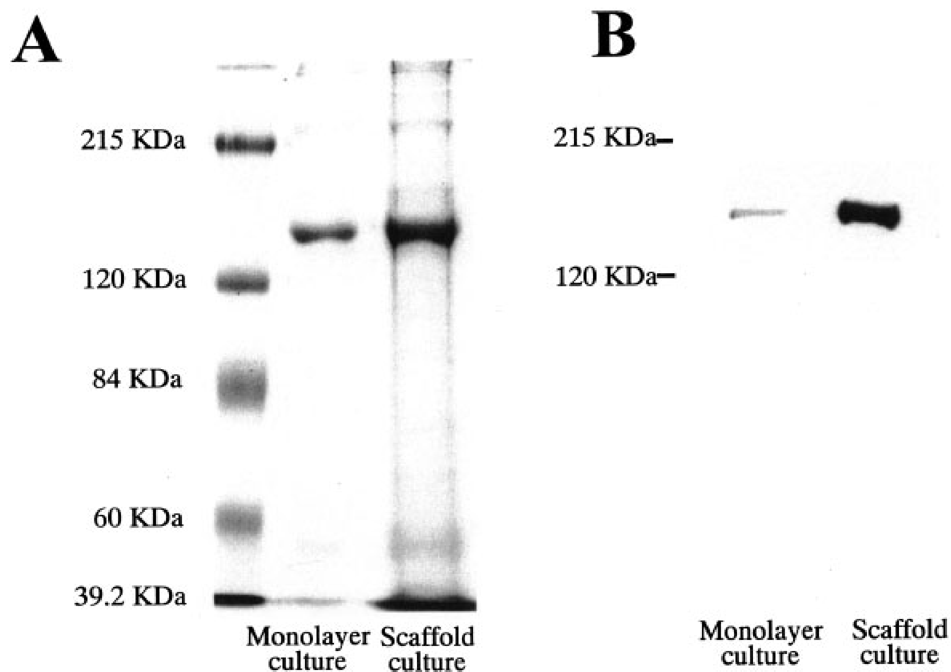

Figure 5. Western blotting of type II collagen. Type II collagen was extracted from the monolayer and scaffold AF cell cultures on day 21. SDS polyacrylamide gel electrophoresis for the collagen extractions (A) and Western blotting analysis of type II collagen (B) were carried out as described in the Materials and Methods section.

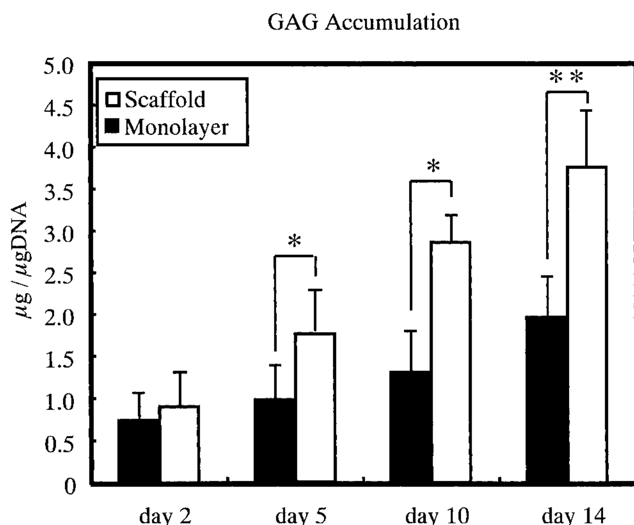

Figure 6. Glycosaminoglycan (GAG) accumulation in monolayer (black bars) and scaffold (white bars) AF cell cultures was measured on the indicated days. Results are expressed as means with S.D. for 6 samples. $^{*}p < 0.05$, $^{**}p < 0.01$, scaffold cultures compared with monolayer cultures.

more, a scaffold should be noncytotoxic and nonantigentic, 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.[24–26] The present study demonstrates a good performance of the ACHMS-scaffold in terms of stability, cytocompatibility, highly dense and uniform seeding of cells, and accumulation of produced matrix components.

Although the proliferation of AF cells in the scaffold appeared to be slower than in the monolayer culture during 14 days (Fig. 3), the scanning electron microscopy data showed the filled grown AF cells retained their spherical shape and typical chondrocytic appearance with an abundant deposition of matrix products after 21 days (Fig. 2).

Another essential aspect for a scaffold of AF cells concerns the influence of the material composing the scaffold on the phenotype of cultured AF cells. In the case of monolayer culturing of AF cells, the cultured AF cells were expanded and showing fibroblastic morphologies (data not shown), resulting in the dedifferentiation of the cells. The syntheses of type II collagen and proteoglycan have been considered to be sensitive metabolic markers to investigate the phenotype level in studies dealing with chondrocytes isolated from hyaline cartilage tissue.[27–29] It is known that the phenotype of AF cells is a chondrocytic cell, which mainly expresses type II collagen and proteoglycans.[1,7] Therefore, the expression of type II collagen and proteoglycans by AF cells was assayed as a metabolic marker of the phenotype of AF cells in this study. Also, the data presented demonstrate that when compared with the monolayer culture, AF cells cultured in the scaffold retain a stronger ability to express type II collagen mRNA (Fig. 4) and to deposit type II collagen (Fig. 5), as well as proteoglycans (Figs. 6 and 7). Thus, the high density, three-dimensional culture system using the scaffold is able to maintain the phenotype of AF cells and their ability to produce extracellular molecules required for the tissue reconstruction.

In summary, we have demonstrated the usefulness of the atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) to serve as an AF cell culture of the rabbit intravertebral disc. Although the growth of AF cells in the scaffold appeared to be slower than in a monolayer culture, the scaffold culture maintained the viability and capability of produc-

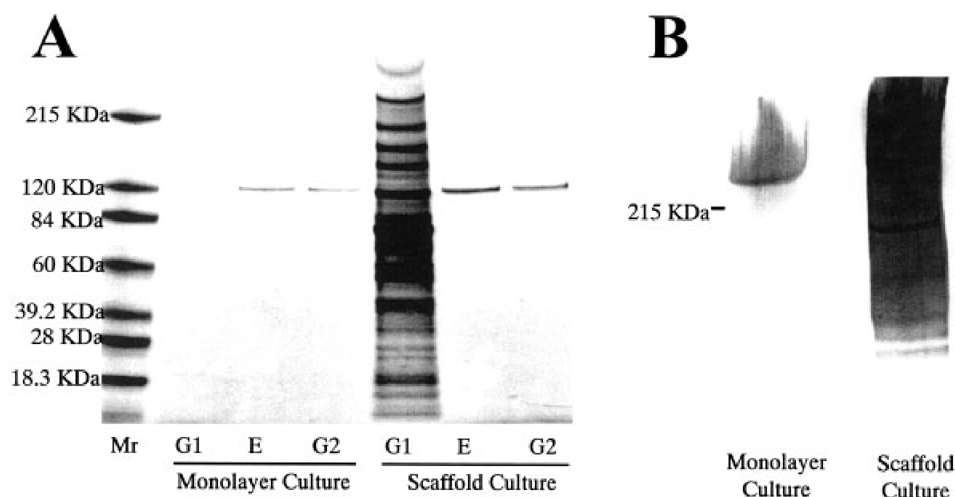

Figure 7. Western blotting of proteoglycans. Proteins in G1-, E-, and G2-extracts from monolayer and scaffold AF cell cultures were applied to gradient (5–20%) SDS polyacrylamide gel electrophoresis (A). The lanes in (A) are as follows: Mr, protein molecular weight standards; G1, G1 extract; E, E extract; G2, G2 extract. The proteins in the G1 extract were also analyzed with Western blotting of proteoglycans using the antiproteoglycan △Di-6S monoclonal (B).

ing type II collagen and proteoglycans. The particular nature of the extracellular environment of the scaffold in which the AF cells are cultured enhances the maintenance of phenotypically differentiated AF cells and entrapment of their extracellular matrix products.

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