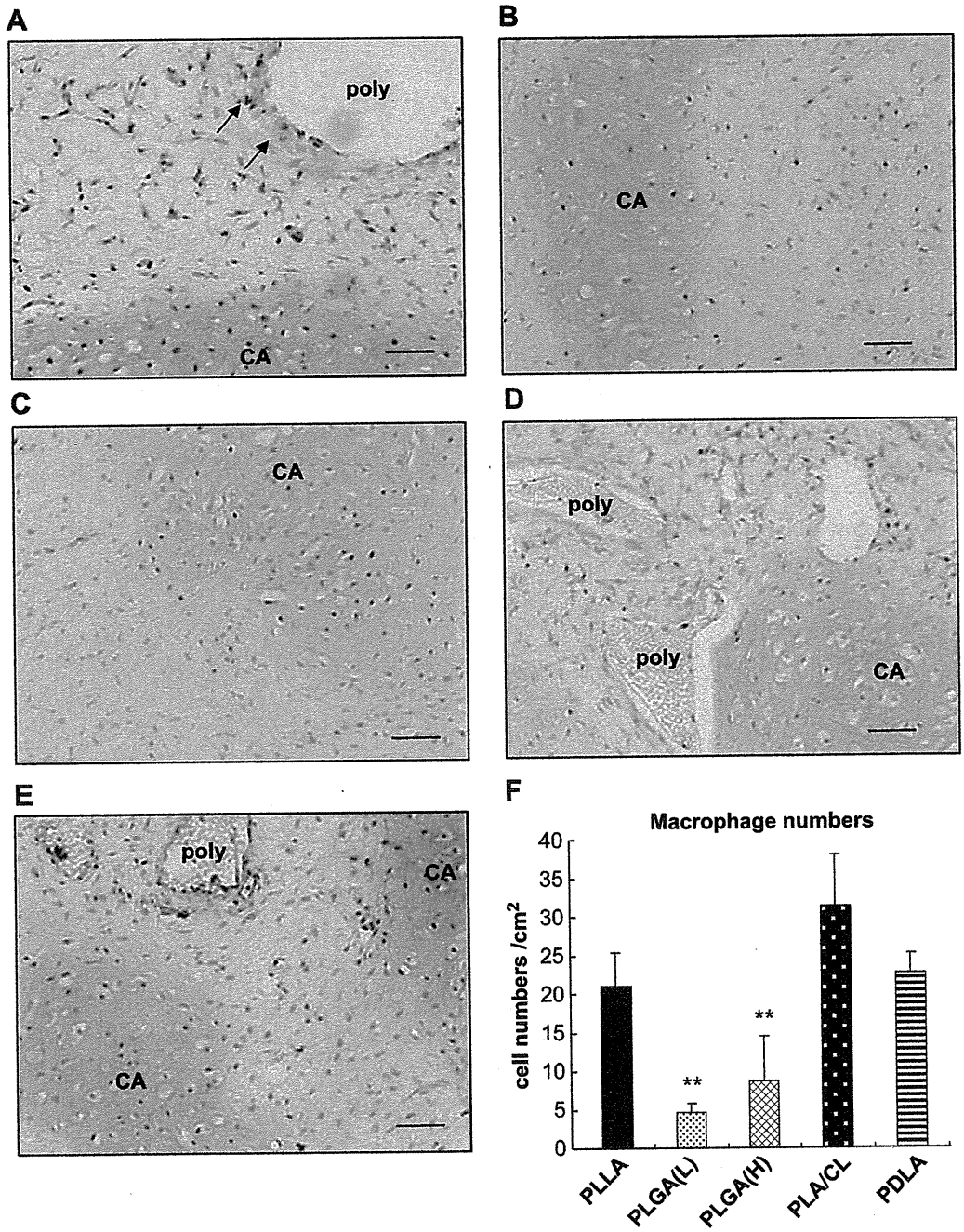

Fig. 9. The immunohistochemical localization of the macrophages in the constructs using the scaffold consisting of various polymers. A–E: The immunohistochemical localization of the macrophages by F4/80. There were polymer remnants (poly) in PLLA (A), PLA/CL (D) and PDLA (E), in which the localization of the macrophages accumulated around the remnants, but not in the regenerative cartilage (CA). Especially, multinucleated cells (arrows) were noted around the remnants. B: PLGA (L), C: PLGA (H). Bar = 100 μm. F: The number of macrophages in all constructs of each scaffold. All values are presented as mean plus standard deviation of 3 samples per group. Statistics were assessed using the Dunnett significance test (**$P < 0.01$ vs PLLA).

5. Conclusions

The structure optimal for the porous scaffold in combination with the atelocollagen was regarded to be that with the porosity of 95% and pore size of 0.3 mm made by the sugar leaching method, which effectively kept the chondrocytes/atelocollagen mixture in the scaffolds and indicated a fair cartilage regeneration. Regarding the comparison among PLLA, PDLA, PLA/CL and PLGA, PLGA and PLLA were superior to the others, when the tissue-engineered cartilage using each polymer was transplanted in nude mice. Although either of polymers has been currently recommended for the scaffold of cartilage, the polymer for which biodegradation is exactly synchronized to the cartilage regeneration would improve the quality of the tissue-engineered cartilage.

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References

[1] Marlovits S, Zeller P, Singer P, Resinger C, Vecsei V. Cartilage repair: generations of autologous chondrocyte transplantation. Eur J Radiol 2006;57:24–31.
[2] Caldamone AA, Diamond DA. Long-term results of the endoscopic correction of vesicoureteral reflux in children using autologous chondrocytes. J Urol 2001;165:2224–7.
[3] Uchio Y, Ochi M, Matsusaki M, Kurioka H, Katsube K. Human chondrocyte proliferation and matrix synthesis cultured in atelocollagen gel. J Biomed Mater Res 2000;50:138–43.
[4] Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–24.
[5] Malemud CJ, Stevenson S, Mehraban F, Papay RS, Purchio AF, Goldberg VM. The proteoglycan synthesis repertoire of rabbit chondrocytes maintained in type II collagen gels. Osteoarthritis Cartilage 1994;2:29–41.
[6] Gerard C, Catuogno C, Amargier-Huin C, Grossin L, Hubert P, Gillet P, et al. The effect of alginate, hyaluronate and hyaluronate derivatives biomaterials on synthesis of non-articular chondrocyte extracellular matrix. J Mater Sci Mater Med 2005;16:541–51.
[7] Ushida T, Furukawa K, Toita K, Tateishi T. Three-dimensional seeding of chondrocytes encapsulated in collagen gel into PLLA scaffolds. Cell Transplant 2002;11:489–94.
[8] Hutmacher DW, Ng KW, Kaps C, Sittinger M, Klaring S. Elastic cartilage engineering using novel scaffold architectures in combination with a biomimetic cell carrier. Biomaterials 2003;24:4445–58.
[9] Hannouche D, Terai H, Fuchs JR, Terada S, Zand S, Nasseri BA, et al. Engineering of implantable cartilaginous structures from bone marrow-derived mesenchymal stem cells. Tissue Eng 2007;13:87–99.
[10] Yamaoka H, Tanaka Y, Nishizawa S, Asawa Y, Takato T, Hoshi K. The application of atelocollagen gel in combination with porous scaffolds for cartilage tissue engineering and its suitable conditions. J Biomed Mater Res A 2009;93:123–32.
[11] Sakai D, Mochida J, Yamamoto Y, Nomura T, Okuma M, Nishimura K, et al. Transplantation of mesenchymal stem cells embedded in atelocollagen gel to the intervertebral disc: a potential therapeutic model for disc degeneration. Biomaterials 2003;24:3531–41.
[12] Yamaoka H, Asato H, Ogasawara T, Nishizawa S, Takahashi T, Nakatsuka T, et al. Cartilage tissue engineering using human auricular chondrocytes embedded in different hydrogel materials. J Biomed Mater Res A 2006;78:1–11.
[13] Chou CH, Cheng WT, Kuo TF, Sun JS, Lin FH, Tsai JC. 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. J Biomed Mater Res A 2007;82:757–67.
[14] Shangkai C, Naohide T, Koji Y, Yasuji H, Masaaki N, Tomohiro T, et al. Transplantation of allogeneic chondrocytes cultured in fibroin sponge and stirring chamber to promote cartilage regeneration. Tissue Eng 2007;13:483–92.
[15] Girotto D, Urbani S, Brun P, Renier D, Barbucci R, Abatangelo G. Tissue-specific gene expression in chondrocytes grown on three-dimensional hyaluronic acid scaffolds. Biomaterials 2003;24:3265–75.
[16] Kang JY, Chung CW, Sung JH, Park BS, Choi JY, Lee SJ, et al. Novel porous matrix of hyaluronic acid for the three-dimensional culture of chondrocytes. Int J Pharm 2009;369:114–20.
[17] Yamane S, Iwasaki N, Kasahara Y, Harada K, Majima T, Monde K, et al. Effect of pore size on in vitro cartilage formation using chitosan-based hyaluronic acid hybrid polymer fibers. J Biomed Mater Res A 2007;81:586–93.
[18] Komura M, Komura H, Kanamori Y, Tanaka Y, Suzuki K, Sugiyama M, et al. An animal model study for tissue-engineered trachea fabricated from a biodegradable scaffold using chondrocytes to augment repair of tracheal stenosis. J Pediatr Surg 2008;43:2141–6.
[19] Masuoka K, Asazuma T, Ishihara M, Sato M, Hattori H, Yoshihara Y, et al. Tissue engineering of articular cartilage using an allograft of cultured chondrocytes in a membrane-sealed atelocollagen honeycomb-shaped scaffold (ACHMS scaffold). J Biomed Mater Res B Appl Biomater 2005;75:177–84.
[20] Woodfield TB, Malda J, de Wijn J, Peters F, Riesle J, van Blitterswijk CA. Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique. Biomaterials 2004;25:4149–61.
[21] Kang Y, Yang J, Khan S, Anissian L, Ameer GA. A new biodegradable polyester elastomer for cartilage tissue engineering. J Biomed Mater Res A 2006;77:331–9.
[22] Aoyagi R, Yoshida T. Freuqency equations of an ultrasonic vibrator for the elastic sensor using a contact impedance method. Jpn J Appl Phys 2004;5B: 3204–9.
[23] Miller RA, Brady JM, Cutright DE. Degradation rates of oral resorbable implants (polylactates and polyglycolates): rate modification with changes in PLA/PGA copolymer ratios. J Biomed Mater Res 1977;11:711–9.
[24] Matsusue Y, Yamamuro T, Oka M, Shikinami Y, Hyon SH, Ikada Y. In vitro and in vivo studies on bioabsorbable ultra-high-strength poly(L-lactide) rods. J Biomed Mater Res 1992;26:1553–67.
[25] Laitinen O, Tormala P, Taurio R, Skutnabb K, Saarelainen K, Iivonen T, et al. Mechanical properties of biodegradable ligament augmentation device of poly (L-lactide) in vitro and in vivo. Biomaterials 1992;13:1012–6.
[26] Wang Z, Wang S, Marois Y, Guidoin R, Zhang Z. Evaluation of biodegradable synthetic scaffold coated on arterial prostheses implanted in rat subcutaneous tissue. Biomaterials 2005;26:7387–401.
[27] Rich J, Kortesuo P, Ahola M, Yli-Urpo A, Kiesvaara J, Seppälä J. Effect of the molecular weight of poly(epsilon-caprolactone-co-DL-lactide) on toremifene citrate release from copolymer/silica xerogel composites. Int J Pharm 2001; 212:121–30.
[28] Takahashi T, Ogasawara T, Kishimoto J, Liu G, Asato H, Nakatsuka T, et al. Cell Transplant 2005;14:683–93.
[29] Tanaka Y, Ogasawara T, Asawa Y, Yamaoka H, Nishizawa S, Mori Y, et al. Growth factor contents of autologous human sera prepared by different production methods and their biological effects on chondrocytes. Cell Biol Int 2008;32: 505–14.
[30] Furukawa KS, Ushida T, Toita K, Sakai Y, Tateishi T. Hybrid of gel-cultured smooth muscle cells with PLLA sponge as a scaffold towards blood vessel regeneration. Cell Transplant 2002;11:475–80.
[31] Fujihara Y, Asawa Y, Takato T, Hoshi K. Tissue reactions to engineered cartilage based on poly-L-lactic acid scaffolds. Tissue Eng Part A 2009;15:1565–77.
[32] Klocke NW, Snyder PW, Widmer WR, Zhong W, McCabe GP, Breur GJ. Detection of synovial macrophages in the joint capsule of dogs with naturally occurring rupture of the cranial cruciate ligament. Am J Vet Res 2005;66:493–9.
[33] Smith MD, Triantafillou S, Parker A, Youssef PP, Coleman M. Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. J Rheumatol 1997;24:365–71.
[34] Goldring MB. The role of the chondrocyte in osteoarthritis. Arthritis Rheum 2000;43:1916–26.
[35] Au RY, Al-Talib TK, Au AY, Phan PV, Frondoza CG. Avocado soybean unsaponifiables (ASU) suppress TNF-alpha, IL-1beta, COX-2, iNOS gene expression, and prostaglandin E(2) and nitric oxide production in articular chondrocytes and monocyte/macrophages. Osteoarthritis Cartilage 2007;15: 1249–55.
[36] Makino K, Ohshima H, Kondo T. Mechanism of hydrolytic degradation of poly (L-lactide) microcapsules: effects of pH, ionic strength and buffer concentration. J Microencapsul 1986;3:203–12.

The application of atelocollagen gel in combination with porous scaffolds for cartilage tissue engineering and its suitable conditions

H. Yamaoka,[1,2] Y. Tanaka,[1] S. Nishizawa,[1] Y. Asawa,[1] T. Takato,[2] K. Hoshi[1,3]
[1]*Department of Cartilage & Bone Regeneration (Fujisoft), Graduate School of Medicine, The University of Tokyo Hospital, The University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo, Japan*
[2]*Division of Tissue Engineering, The University of Tokyo Hospital, The University of Tokyo, Hongo 7-3-1, Bunkyo-Ku, Tokyo, Japan*
[3]*Department of Plastic, Oral and Maxillofacial Surgery, Teikyo University school of Medicine, Kaga 2-11-1, Itabashi-ku, Tokyo, Japan*

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Abstract: For improving the quality of tissue-engineered cartilage, we examined the *in vivo* usefulness of porous bodies as scaffolds combined with an atelocollagen hydrogel, and investigated the suitable conditions for atelocollagen and seeding cells within the engineered tissues. We made tissue-engineered constructs using a collagen sponge (CS) or porous poly(L-lactide) (PLLA) with human chondrocytes and 1% hydrogel, the concentration of which maximized the accumulation of cartilage matrices. The CS was soft with a Young's modulus of less than 1 MPa, whereas the porous PLLA was very rigid with a Young's modulus of 10 MPa. Although the constructs with the CS shrank to 50% in size after a 2-month subcutaneous transplantation in nude mice, the PLLA constructs maintained their original sizes. Both of the porous scaffolds contained some cartilage regeneration in the presence of the chondrocytes and hydrogel, but the PLLA counterpart significantly accumulated abundant matrices *in vivo*. Regarding the conditions of the chondrocytes, the cartilage regeneration was improved in inverse proportion to the passage numbers among passages 3–8, and was linear with the cell densities (10^6 to 10^8 cells/mL). Thus, the rigid porous scaffold can maintain the size of the tissue-engineered cartilage and realize fair cartilage regeneration *in vivo* when combined with 1% atelocollagen and some conditioned chondrocytes.
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Key words: chondrocyte; scaffold; atelocollagen; poly(L-lactide); tissue engineering

INTRODUCTION

Cartilage possesses abundant extracellular matrices specifically consisting of type II collagen (COL2) and progeoglycan, and functions to accomplish smooth joint motion or to support body shape. Once the cartilage is injured by trauma, age-related diseases, inflammatory diseases, congenital anomalies, or other disease, people become severely limited due to pain, reduction of joint motion, and deterioration of morphological integrity. Because cartilage has poor regenerative capacity by itself, its regeneration using a tissue engineering technique significantly benefits the treatment of these various cartilage disorders. As a clinical protocol of cartilage tissue engineering, autologous chondrocyte implantation (ACI) has been practically applied for the purpose of treating focal joint defects[1] or the augmentation of nasal cartilage.[2] This cell-based therapy involves several procedures, that is, the harvesting of a small cartilage biopsy, the isolation and proliferation of chondrocytes *in vitro*, and the subsequent transplantation of proliferated autologous chondrocytes into the lesions. The ACI material principally belongs to a cell suspension or occasionally a cell mixture with hydrogels which are produced by the cells during the culture. Since 1987, the number of patients that have been treated by the ACI method has reached more than 12,000 internationally.[3] However, the

Correspondence to: K. Hoshi; e-mail: pochi-tky@umin.net
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clinical indication of the present ACI is confined to the focal cartilage defects in joints or noses mainly due to athletic trauma or a cosmetic complaint. To broaden the indication range of cartilage tissue engineering to larger or more complicated cartilage defects, for example, the end stage of osteoarthritis or a congenital anomaly of the craniofacial region, we should make an implant-type tissue-engineered cartilage with greater firmness and a 3D structure that corresponds to the properties of the defective cartilage.

For the purpose of increasing the mechanical properties of tissue-engineered cartilage, we should improve the scaffold adequately for the regenerative cartilage constructs. In our basic concept, we think that the chondrocytes should be sufficiently proliferated in the monolayer culture before the *in vivo* transplantation, after which those cells are harvested from the monolayer culture plates, administered into the scaffold at the high cell density, and are transplanted into the body within the scaffold. We chose the 2D system in cell proliferation, because the cell number can be precisely counted in the monolayer culture and the cell density in the tissue-engineered cartilage constructs will be well controlled. Thereafter, the sufficient numbers of cells contained within the scaffold under the 3D condition would begin to make abundant cartilage matrices in the body, finally resulting in the regenerative cartilage constructs. Therefore, the material design of the scaffold should effectively retain the chondrocytes without cell outflow, favorably provide the biological 3D environment for the chondrocytes, and maintain the gross 3D shape and mechanical strength of the whole construct. To date, two types of scaffolds have been used for cartilage tissue engineering. The first is a hydrogel, whereas the second is a porous body. Although the latter can provide load-bearing capacity and mechanical strength, such scaffolds are often associated with a relatively low cell-seeding efficiency, inadequate cell distribution, and an increase in chondrocyte dedifferentiation. In contrast, it is well known that encapsulation within hydrogel materials, such as agarose,[4] atelocollagen,[5] and type II collagen,[6] improves the biological environment for cultured chondrocytes, and stimulates their matrix synthesis. We previously examined the matrix synthesis of human chondrocytes which were 3D-cultured within various hydrogel scaffolds.[7] We focused on the atelopeptides of collagen (atelocollagen), alginate, and synthetic peptides PuraMatrix™, as typical hydrogels that are or will soon be clinically available. The accumulation of cartilaginous matrices was remarkable in the chondrocytes within the atelocollagen hydrogel. The chondrocytes embedded in the atelocollagen showed a high expression of the β1 integrin, seemingly promoting cell-matrix signaling, while the N-cadherin expression was inhibited, implying a decrease in cell-to-cell contacts and the maintenance of chondrocyte solitariness in the atelocollagen. Considering the biological effects and clinical availability, atelocollagen may be accessible for clinical use. Actually, as a modified protocol of ACI, autologous chondrocytes were cultured within atelocollagen, while a gel material consisting of a mixture of atelocollagen and chondrocytes was placed into a focal joint defect.[8] It could reduce the possible risk of chondrocytes leaking from the site of the graft after load-bearing resumption, which was occasionally observed when chondrocytes were transplanted in a cell suspension.[8]

However, the mechanical properties of the atelocollagen hydrogel, even if the matrix production of chondrocytes is biologically enhanced within the hydrogel, are not sufficiently strong *in vitro*. Based on our previous data, even when the accumulation of cartilaginous matrices in tissue-engineered cartilage pellets generated by human chondrocytes and atelocollagen was strongly enhanced by stimulation from bone morphogenetic protein (BMP)-2, insulin, and the thyroid hormone, the mechanical properties of the cartilage pellets were much weaker than those of native human cartilage.[9]

In previous studies, the development of composite scaffolds have been attempted to improve the poor mechanical properties of typical biological scaffolds, collagen. The collagen scaffolds had been reinforced by the incorporation of poly(glycolide) (PGA) fiber, which was used by itself or in hybridization with other biomaterials for the culture of MSCs.[10–12] Some other researchers have overcome the mechanical weakness of the hydrogel by combining it with a biodegradable porous scaffold. It was also reported that a collagen gel was used in combination with a nonwoven mesh made of poly(L-lactide) (PLLA),[13] a mesh of PGA,[14] and a nonwoven fabric of PGA coated with PLLA.[15] In these studies, cell retainment within the constructs using the collagen gel with these porous bodies and their fair cartilage regeneration under *in vitro* conditions were confirmed, but their *in vivo* effects have not yet been reported.

With a focus on clinical applications, we examined the *in vivo* cartilage regeneration of the constructs using some porous bodies in combination with human chondrocytes and atelocollagen. In this study, we selected the collagen sponge and porous PLLA for the porous bodies, both of which are components or materials of clinically available medical products. The collagen sponge is included in the artificial dermis Pelnac® (Gunze, Kyoto, Japan) used to maintain wound moisture, retain growth factors, or enhance cell attachment. PLLA is widely used as raw material for absorbable bone screws and plates. We also investigated the suitable conditions for ate-

locollagen and the seeding cells, such as their densities or doubling populations for making higher-quality tissue-engineered cartilage, when they are used in combination with the porous scaffolds. The objectives of this study are to elucidate the usefulness of porous bodies as the scaffolds for the cartilage tissue engineering when they are used *in vivo* in combination with atelocollagen, and to determine the specificities of atelocollagen and human chondrocytes for improving the quality of tissue engineered cartilage.

MATERIALS AND METHODS

Chondrocyte preparation

All procedures for the present experiments were approved by the ethics committee of the University of Tokyo Hospital (#622). Auricular cartilage remnants were obtained from five microtia patients (age range of 10–15 years) who underwent operations at the University of Tokyo Hospital, after receiving permission from the institutional ethics committee and the informed consent of the patients. The chondrocytes were isolated by digestion of the cartilage remnants with 0.15% collagenase (Wako Pure Chemical Industries, Osaka, Japan). The isolated chondrocytes were cultivated as previously reported.[16] Briefly, the isolated chondrocytes were seeded in a 100-mm plastic tissue culture dish at a density of 2500 cells/cm^2 and cultured in a medium containing Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (Sigma Chemical, St. Louis, MO), 5% human serum (Sigma Chemical), 100 ng/mL of FGF-2 (kindly provided by Kaken Pharmaceutical, Tokyo, Japan), and 5 μg/mL of insulin (Roche Diagnostics GmbH, Mannheim, Germany), then incubated at 37°C/5% CO_2. The medium was changed three times per week. Passages were performed by treatment with a trypsin-EDTA solution (Sigma Chemical) when the cells were approaching confluence on day 7 of the culture.

Preparation of porous scaffolds

The collagen sponge was purchased from Gunze as Pelnac®, a part of which was used for the experiment (Fig. 1). The porous PLLA was prepared from raw material (MW: 300,000, Polysciences, Warrington, PA) by the sugar-leaching method described in a previous paper (Fig. 1).[17] The mechanical properties of the scaffolds were measured using a Venustron tactile sensor (Axiom, Fukushima, Japan). With computer control, the motor-driven sensor unit automatically presses the objects down up to 0.5 mm in depth from the surface, and provides compression strength and a decrease in the resonant frequency. Young's modulus was calculated by the compression strength and the frequency decrease using Venus 42 (Axiom) software, based on a previous report.[18] The retainment of the DMEM/F-12 or atelocollagen solution was measured as the (wet weight − dry weight)/volume of the porous body.

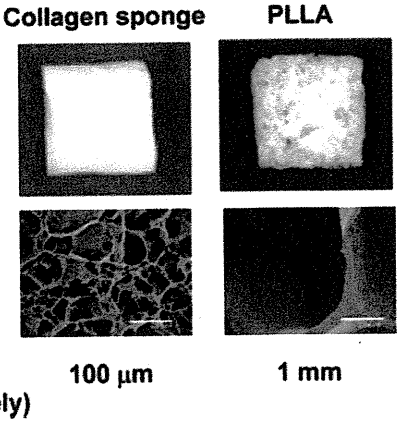

Figure 1. The morphology and the specificities of the porous scaffolds used in this study. Top, gross appearance (1 cm × 1 cm); bottom, scanning microscopic images. Bar = 200 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Tissue-engineered cartilage

For tissue-engineered cartilage pellets, cutlured chondrocytes (passage 4) were suspended in an atelocollagen solution (Koken, Tokyo, Japan) at a density of 10^7 cells/mL. Twenty microliters of the cell/atelocollagen suspension (total 2 × 10^5 cells) were placed in the bottom of a 15-mL conical tube to form a gel after a 1-h incubation at 37°C. The atelocollagen pellets were cultured in a DMEM/F-12 medium with or without recombinant human BMP-2 (200 ng/mL, kindly provided by Astellas Pharma, Tokyo), insulin (5 μg/mL, MP Biomedicals, Irvine, CA), and triiodothyronine (10^{-7}M, T3, EMD Bioscience, San Diego, CA) for 1–3 weeks.[9] Throughout the experiment, the medium was changed three times/week.

To make the tissue-engineered cartilage construct, a 100-μL atelocollagen aliquot containing the chondrocytes was added to the collagen sponge or PLLA (φ5 × 3 mm) to form a gel after a 1-h incubation at 37°C. The constructs using the porous bodies with or without the human chondrocytes or atelocollagen were then subcutaneously transplanted into nude mice (6-week-old male) for 2 months. The animal experiments were performed according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

Total RNA extraction and real time RT-PCR

Total RNA was isolated from the chondrocytes using the chaotropic Trizol method (Nippon-gene, Tokyo). Total mRNA (1 μg) was reverse transcribed using the Super Script reverse transcriptase with a random hexamer (Takara Shuzo, Otsu, Japan). Full-length or partial-length cDNA of the target genes, including the PCR amplicon sequences, was amplified by PCR, cloned into pCR-TOPO

Zero II or pCR-TOPO II vectors (Invitrogen, Carlsbad, CA), and used as standard templates after linearization. The QuantiTect SYBR Green PCR Master Mix (Invitrogen) was used, and SYBR Green PCR amplification and real-time fluorescence detection were performed using an ABI 7700 Sequence Detection system. We measured three aliquots of the reverse transcription products for each sample by real-time RT-PCR. All experiments were run in every sample from four patients and the results that could be tetraplicated are shown in this study. The sequence of the primers we used in the real-time PCR to detect COL1A1, COL2A1, and GAPDH were as follows: COL1A1 F:5′-CTCCTCGCTTTCCTTCCTCT-3′, R:5′-GTGCTAAAGGTGC CAATGG T-3′; COL2A1 F:5′-GAGTCAAGGGTGATCGT GGT-3′, R: 5′-CACCTTGGTCTCCA GAAGGA-3′; GAPDH F: 5′-GAAGGT GAAGGTCGGAGTCA-3′, R: 5′-GAAGAT GGTGATGGGATTTC-3′.[7] GAPDH was used as the housekeeping gene.

Histology

For the histology analysis, the tissue-engineered cartilage was fixed with 4% paraformaldehyde in 0.1*M* phosphate buffer (pH 7.4) for 2 h at 4°C and successively immersed in 10% sucrose in phosphate-buffered saline (PBS), 20% sucrose in PBS, and a 2:1 mixture of 20% sucrose in PBS and the OCT compound in succession. The samples were flash-frozen in liquid nitrogen, cryosectioned to a thickness of 10 μm, and stained with toluidine blue-O.

Biochemical measurement of type I and II collagens (COL1 and COL2) and proteoglycan

The cell/gel construct was dissolved in 10 mg/mL of pepsin/0.05*M* acetic acid at 4°C for 48 h and then in 1 mg/mL of pancreatic elastase/0.1*M* Tris-0.2*M* NaCl-5 m*M* $CaCl_2$ at 4°C overnight. In the case of the monolayer culture, the cells attached to the culture plates were harvested by a cell scraper and were treated as above. The cell debris and insoluble material were removed by centrifugation at 6000*g* for 30 min. The collagen proteins were solubilized and quantified by ELISA according to the protocol of the human type I and II Collagen (COL1 and COL2) Detection Kit (Chondrex, Redmound, WA). The collagen proteins were captured by polyclonal anti-human type I or type II collagen antibodies and detected by the biotinylated counterparts and streptavidin peroxidase. OPD and H_2O_2 were added to the mixture and the spectrophotometric absorbance of the mixture was measured at a wavelength of 490 nm. Proteoglycan was measured using the Alcian blue-binding assay for the sulfated glycosaminoglycan (GAG) content (Wieslab AB, Lund, Sweden). GAG in the supernatant was precipitated with an Alcian blue solution, and the sediments, after centrifugation at 6000*g* for 15 min, were redissolved in a 4*M* GuHCl-33% propanol solution. The spectrophotometric absorbance of the mixture was measured at a wavelength of 600 nm. The collagen or GAG content in the tissue-engineered cartilage was adjusted by the total protein content of the pellets. The total protein content of the samples was measured using the Bio-Rad Dc Protein

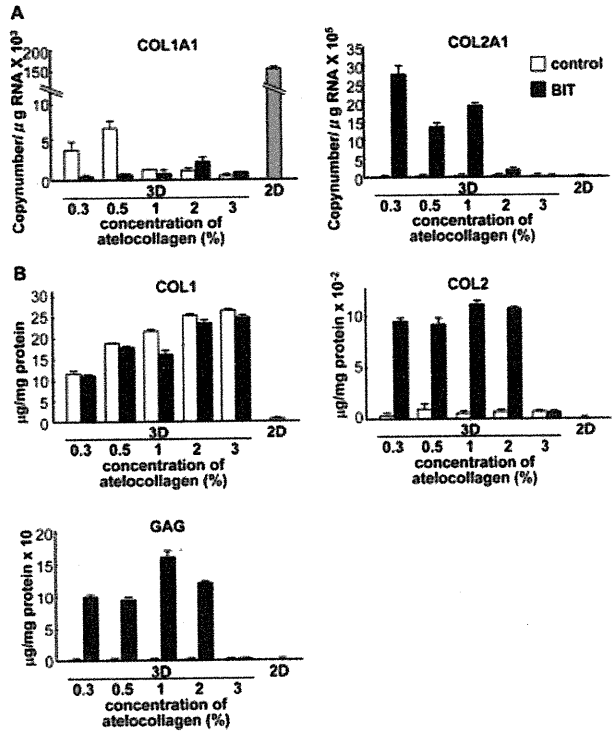

Figure 2. Concentration of atelocollagen suitable for cartilage matrix accumulation. A: Gene expressions evaluated by real time RT-PCR. B: Biochemical measurements of COL1, COL2, and GAG. Human auricular chondrocytes (200,000 cells) at passage 4 were embedded within different concentrations of atelocollagen (20 μL, 3D) and were cultured *in vitro* for one (A) or 3 (B) weeks with BMP-2, insulin, and T3 (BIT) or without BIT (control). For comparison, the gene expressions and matrix production were examined in the chondrocytes cultured in the monolayer culture (2D) at passage 4. All values are presented as mean plus standard deviation.

Assay (Bio-Rad Laboratories, Hercules, CA). We measured three aliquots of the enzyme-digested solution for each sample. All experiments were run in every sample from four patients and the results that could be tetraplicated are shown in this study.

RESULTS

Concentration of atelocollagen

We first examined the concentration of atelocollagen suitable for the matrix accumulation in the tissue-engineered cartilage. The human auricular chondrocytes cultured in the monolayer at the passage of four remarkably decreased the gene expression or matrix production of cartilage matrices, including type II collagen or proteoglycan [Fig. 2(2D)]. In contrast, when they were embedded within atelocollagen and were incubated *in vitro*, the production of

the cartilage matrices was stimulated, especially with BMP-2, insulin, and T3 (BIT)[9] [Fig. 2(3D)]. Although the gene expression of *COL1A1* was up-regulated in 0.3–0.5% atelocollagen gel without BIT (control), it was suppressed according to the increase in the gel concentration [Fig. 2(A)]. The *COL1A1* expression was also inhibited by the stimulation of BIT [Fig. 2(A)]. The *COL2A1* expression was significantly up-regulated between the 0.3–1% gel concentrations by the stimulation of BIT, although it was suppressed in a more than 2% gel concentration with BIT, or in all concentrations without BIT [Fig. 2(A)]. On the other hand, the accumulation of the COL1 protein in the tissue-engineered cartilage was increased when the gel concentration became higher. In spite of the high *COL1A1* gene expression in the control groups of the 0.3–0.5% gel, the COL1 accumulation of the control group was almost similar to that of the BIT groups and rather lower than that of the 1–3% gel, suggesting that the construct with the low gel concentration could not sufficiently retain abundant matrix production. The accumulation of COL2, like proteoglycan, was abundantly observed in the 1–2% gel with BIT, although neither of the cartilage matrices were up-regulated in the chondrocytes embedded within the 3% gel at the gene or protein level, possibly due to deterioration in the diffusion of nutrients or growth factors necessary for the cartilaginous matrix production [Fig. 2(B)]. Therefore, we chose the 1% gel concentration for chondrocyte encapsulation in later experiments.

Usefulness of porous bodies

To support the mechanical strength of the tissue-engineered cartilage using 1% atelocollagen and provide it with a 3D shape, we attempted to use porous bodies in combination with this gel material. We chose the collagen sponge and porous PLLA for typical biodegradable porous scaffolds (Fig. 1), and compared their usefulness. We examined the mechanical properties of the porous scaffolds using a tactile sensor. Both the compression strength and Young's modulus of the porous PLLA were significantly greater than those of the collagen sponge (Fig. 3). The retainment of the culture basal medium (DMEM/F-12) or the 1% atelocollagen solution within a unit volume of the porous scaffolds was measured for the purpose of evaluating the affinity of DMEM/F-12 and atelocollagen, either of which is a material composed of a chondrocyte suspension or chondrocytes/atelocollagen mixture, respectively. As a result, PLLA retained less DMEM/F-12 than the collagen sponge. However, the atelocollagen retainment in PLLA was almost similar to that in the collagen sponge; possibly because atelocollagen

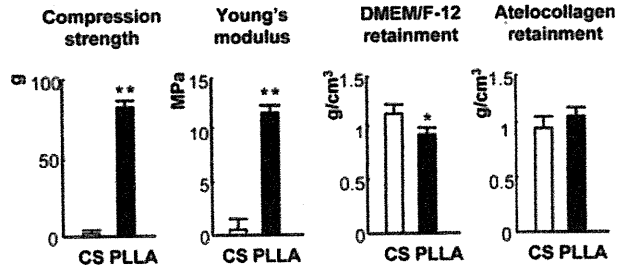

Figure 3. Biophysical properties of the porous scaffolds. Mechanical properties and the retainment of DMEM/F-12 or 1% atelocollagen were compared between collagen sponge (CS) and porous PLLA. All values are presented as mean plus standard deviation. Statistics were assessed using Student's *t*-test (*$p < 0.05$, **$p < 0.01$ vs. CS).

possesses a high viscosity that prevents cells from flowing out of the porous scaffolds (Fig. 3).

We then made the tissue-engineered cartilage constructs having the porous bodies administered with or without the mixture of human chondrocytes (10^7 cells/mL) or 1% atelocollagen gel, and subcutaneously transplanted them into the backs of nude mice. Regardless of the use of the atelocollagen gel or administration of the chondrocytes, all constructs of the collagen sponges could not maintain their initial size and shape [Fig. 4(A)]. Quantitative measurements of the construct sizes showed that downsizing of the collagen sponge constructs was suppressed by 50% after transplantation when containing both the chondrocytes and atelocollagen [cells (+) gel (+)], while the constructs without cells or gel shrank by more than 60% [Fig. 4(B)]. In contrast, the PLLA, with or without the atelocollagen or chondrocytes, almost maintained their original sizes (Fig. 4).

We also evaluated the histological and biochemical properties of the tissue-engineered cartilage constructs after a 2-month transplantation. Both of the porous scaffolds showed dense metachromasia in the presence of the chondrocytes and atelocollagen [cells (+) gel (+)] [Fig. 5(A)]. Although the collagen sponge constructs did not maintain their sizes, the metachromasia was partially observed in the constructs with the chondrocytes [cells (+) gel (+) and cells (+) gel (−)]. The PLLA constructs of cells (+) gel (+) showed round chondrocytes with abundant metachromatic matrices, and exhibited the characteristic for cartilage tissues [Fig. 5(A)]. Biochemical measurements of the collagen sponge constructs showed protein accumulation of COL2 and proteoglycan in the presence of cells [cells (+) gel (+) and cells (+) gel (−)], although COL1 was also abundant in the cells (+) gel (+) [Fig. 5(B)]. With the PLLA, the constructs with the chondrocyte/atelocollagen mixture [cells (+) gel (+)] contained abundant cartilage matrices, especially proteoglycan, when com-

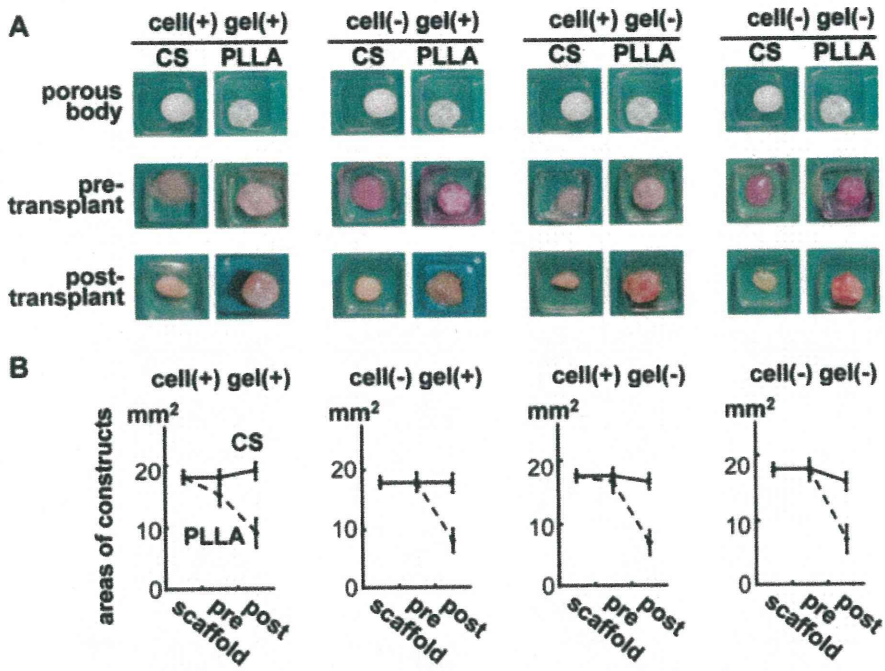

Figure 4. *In vivo* changes in the sizes of tissue-engineered constructs using atelocollagen and porous bodies. A: Macroscopic observation of tissue-engineered constructs. Constructs with (+) or without (−) chondrocytes (cell) or atelocollagen (gel) were observed before (pre-transplant) and after (post-transplant) the subcutaneous transplantation into nude mice. Mold size, 10 × 10 mm. CS, collagen sponge; PLLA, porous PLLA. B: Morphometrical changes in the sizes of the constructs. Solid line, construct with porous PLLA (PLLA); dashed line, construct with collagen sponge (CS). pre and post, before and after subcutaneous transplantation into nude mice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

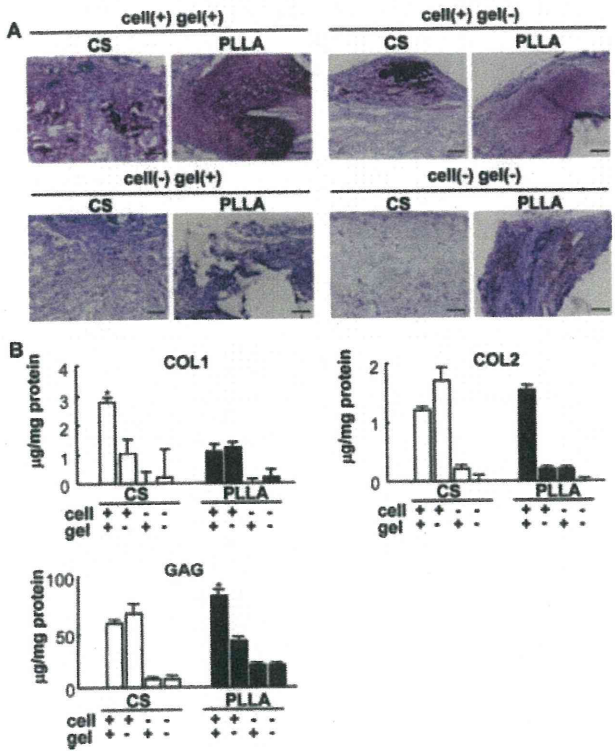

Figure 5. Histological and biochemical changes of tissue-engineered constructs using atelocollagen and porous bodies. A: Histological observation. Toluidine blue staining. Metachromasia (purple color) was prominently observed in the construct of porous PLLA (PLLA) with chondrocytes and atelocollagen [cell (+) gel (+)]. Bar = 100 μm. CS, collagen sponge. B: Biochemical measurements of COL1, COL2, and GAG. All values are presented as mean plus standard deviation. Statistics were assessed using Student's *t*-test [$*p < 0.01$ vs. CS cell (+) gel (−)]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pared with the collagen sponge constructs. Based on these results, porous PLLA would be favorable for maintaining the sizes and shapes of the tissue engineered construct and accumulate the cartilage matrices.

Cell density and passage number of chondrocytes

Finally, we examined the chondrocyte conditions suitable for the cartilage matrix accumulation in the PLLA construct with human chondrocytes and 1% atelocollagen. When we compared the quality of cartilage regeneration among the tissue-engineered

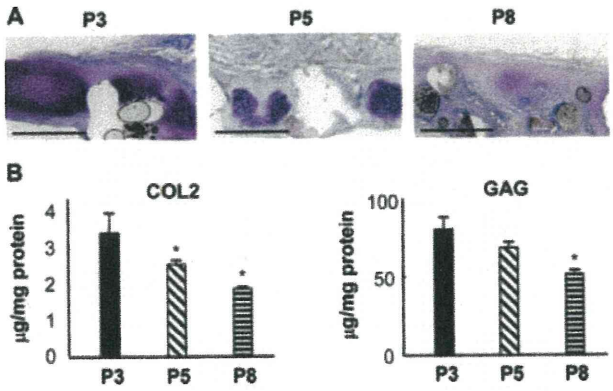

Figure 6. Tissue-engineered cartilage generated by human auricular chondrocytes with various passage numbers (passages 3–8) and subcutaneously transplanted into nude mice for 2 months. A: Histological images. The areas of dense metachromasia decreased according to the increase in the passage number. Bar = 1 mm. B: Biological measurement. The amount of COL2 and proteoglycan decreased when the passage number increased. All values are presented as mean plus standard deviation. Statistics were assessed using Student's *t*-test (*$p < 0.01$ vs. P3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

constructs using human chondrocytes of passage 3 (corresponding to a 10^3-fold increase in cell number), passage 5 (10^5-fold increase), and passage 8 (10^8), the areas of cartilage regeneration showing dense metachromasia decreased in accordance with the increase in the passage number [Fig. 6(A)]. The biological measurements of COL2 and proteoglycan also decreased in inverse proportion to the passage numbers, whereas both COL2 and proteoglycan significantly decreased in passage 8 when compared with passage 3 [Fig. 6(B)].

As for the cell density in the constructs, the areas of cartilage regeneration gradually improved in accordance with the increase in cell density [Fig. 7(A)], corresponding to the results of the biological measurements of COL2 and proteoglycan [Fig. 7(B)]. Constructs using more than 5×10^8 cells/mL in cell density were hardly realized due to the limitation of the sizes in the chondrocytes. Fragments of PLLA were observed in the constructs of all cell densities examined [Fig. 7(A, arrows)], implying that polymer remnants were still present even at 2 months after transplantation.

DISCUSSION

The atelocollagen gel is a promising material for cartilage tissue engineering because it possesses adequate visco-elasiticity and exhibits good chondrocyte compatibility.[7] Atelocollagen is prepared by treatment with protease, showing low immunogenicity, and is usually used as a medical device for the treatment of tissue defects.[19] The atelocollagen solution forms a firm gel at 37°C with neutral pH, the conditions of which correspond to those suitable for maintaining live mammalian cells. When chondrocytes were embedded within this kind of hydrogel in a 3D environment, the gene expressions of cartilage matrices and their production were significantly up-regulated, when compared with those of a 2D culture (Fig. 2). The promotive effects of the 3D culture on cell differentiation have been reported in various kinds of cells,[10–12] and such effects were prominently noted in the chondrocytes. The chondrocytes in native cartilage are surrounded by abundant extracellular matrices in all directions and are isolated in their own lacunae. It implies that chondrocytes are constantly exposed to cell-matrix interactions and, in contrast, that they maintain cell solitariness under physiological conditions. The 3D environment of the atelocollagen gel possibly represents these situations to the cultured chondrocytes through the enhancement of cell-matrix interactions

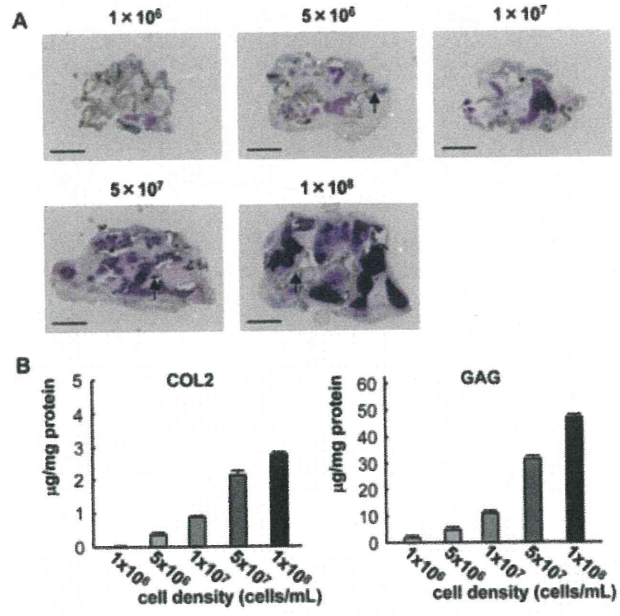

Figure 7. Tissue-engineered cartilage generated by human auricular chondrocytes with various cell densities (1×10^6 to 1×10^8 cells/mL) and subcutaneously transplanted into nude mice for 2 months. A: Histological images. Wider areas of dense metachromasia were observed in the higher cell density groups. Note that remnants of PLLA were observed as indicated by arrows. Bar = 1 mm. B: Biological measurement. Accumulation of COL2 and proteoglycans increased according to the increase in cell density. All values are presented as mean plus standard deviation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

or even 3D distribution of the cells within the hydrogel.

Although the biological hydrogel functions provide suitable 3D conditions for the cells, it may interfere with substance transfers necessary for cell metabolism and survival. However, the atelocollagen gel shows a good diffusion of nutrients or growth factors, providing a suitable 3D environment for the maintenance of chondrocyte activity. This gel is known to consist of an interconnected network, which possesses mesh estimated to be several tens of nanometers in size.[20] The gel of 0.35% bovine collagen showed a matrix mesh size of 57.7 nm.[21] Such a mesh appears to permit the free diffusion of nutrients. The glucose diffusion coefficient within the collagen gel of ~0.2% was measured to be 1.3×10^{-6} cm^2 s^{-1}, a value between an aqueous solution (9.2×10^{-6} cm^2 s^{-1}) and a biological matrix (islet of Langerhans, 3.8×10^{-7} cm^2 s^{-1}).[22] Practically, the three-dimensional (3D) culture of a 0.3% of collagen gel maintained human chondrocyte proliferation under repeated passage to realize a 1000-fold increase in the cell number at passage 3.[23] However, a lower expression and accumulation of cartilage matrices were observed in the tissue-engineered cartilage pellets made of 3% atelocollagen gel [Fig. 2(B)]. This suggested that such a high concentration of the atelocollagen gel may interfere with the diffusion of nutrients or growth factors for the chondrocytes. Too low a concentration gel could hardly retain either the chondrocytes or accumulation of matrices within the construct. Therefore, we recommended a concentration of around 1% for tissue-engineered cartilage.

Regarding the specificities of the porous scaffolds, a sufficient compression strength and Young's modulus are prerequisites for mechanical support of the atelocollagen gel when it is transplanted *in vivo*. The collagen sponge is a very soft material allowing the chondrocyte/atelocollagen mixture to gradually permeate into the collagen sponge by repeated manual compression and recovery. However, the stiffness of such an animal-derived material seemed too low to maintain size and 3D shape during *in vivo* transplantation. However, the porous body made of a biodegradable polymer, PLLA, possessed sufficient mechanical strength. The present data suggested that porous scaffolds with ~80 g of compression strength and 10 MPa of Young's modulus appeared to sufficiently maintain size and 3D shape for 2 months in the subcutaneous areas of the nude mouse back. When the viscous material of the chondrocyte/atelocollagen mixture is administered into inelastic PLLA, a sufficient pore size in the porous scaffold is needed. The pore sizes of the present PLLA scaffolds were ~1 mm (Fig. 1), which allowed viscous chondrocyte/atelocollagen mixture to infiltrate into the center of the scaffold and retain the mixture as much as the elastic collagen sponge material (Fig. 3).

The histological findings of the constructs using chondrocytes, atelocollagen, and porous PLLA after a 2-month implantation exhibited maturation of the tissue-engineered cartilage, while the porous scaffold of PLLA still remained without any significant degradation (Fig. 7). Maturation of the tissue-engineered cartilage increases its mechanical strength, as the tissue is sufficiently firm due to the abundant extracellular matrices, in which the chondrocytes in the engineered tissues produce. Therefore, it is ideal that the porous scaffold should be degraded during maturation of the tissue-engineered cartilage. Biodegradation of the scaffold would be synchronized to the speed of cartilage regeneration. However, the half-life of PLLA is as long as 1 year.[24] The long-term life of the porous bodies may be rather harmful because it may impair the regenerated cartilage or the adjacent host tissues. To obtain the tissue-engineered cartilage of higher quality and safety, faster biodegradability of the polymers used for the porous scaffolds should be considered.

Moreover, we examined the chondrocyte conditions, including the cell densities and passage numbers for *in vivo* cartilage regeneration using porous PLLA and atelocollagen. Various cell densities (2×10^6 to 1.25×10^8) were evaluated for cartilage tissue engineering using the scaffold system in combination with hydrogels and porous bodies.[13–15,25–33] However, the optimal cell density for human chondrocytes has not been sufficiently examined. We examined the relation between the density of human chondrocytes and *in vivo* cartilage regeneration. As a result, the highest cell density of 10^8 cells/mL was found to be the most effective for increasing the quality of the tissue-engineered cartilage. As the concentration of the atelocollagen solution could not be more than 3% due to its high viscosity, and as the average size of human auricular chondrocytes in the cell suspension was $\sim 8.5 \times 8.5 \times 8.5$ μm^3 (nearly equal to 0.6×10^{-8} mL) according to the data of this study, we could not prepare a chondrocyte/atelocollagen mixture with a cell density higher than the final density of 10^8 cells/mL in the 1% atelocollagen gel.

Thus, with a combination of the atelocollagen gel and the PLLA porous scaffold, we could prepare a hybrid scaffold with effective retainment of administered cells, good biocompatibility for the chondrocytes, and sufficient mechanical strength, which correspond to the material design of the scaffold for tissue-engineered cartilage. If an implant-type tissue-engineered cartilage with greater firmness and a 3D structure is made with this hybrid scaffold under the conditions determined in this study, it can be used for large cartilage defects that are not sur-

rounded by intact cartilage or that are located in sites suffering from severe mechanical loading, which are often observed in the final stage of osteoarthritis or various craniofacial anomalies. More detailed studies of the structure and composition of biodegradable polymers for porous scaffolds would improve the quality and safety of tissue-engineered cartilage.

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References

1. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–895.
2. Yanaga H, Yanaga K, Imai K, Koga M, Soejima C, Ohmori K. Clinical application of cultured autologous human auricular chondrocytes with autologous serum for craniofacial or nasal augmentation and repair. Plast Reconstr Surg 2006;117: 2019–2030; discussion 2031–2032.
3. Marlovits S, Zeller P, Singer P, Resinger C, Vecsei V. Cartilage repair: Generations of autologous chondrocyte transplantation. Eur J Radiol 2006;57:24–31.
4. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–224.
5. Uchio Y, Ochi M, Matsusaki M, Kurioka H, Katsube K. Human chondrocyte proliferation and matrix synthesis cultured in Atelocollagen gel. J Biomed Mater Res 2000;50: 138–143.
6. Malemud CJ, Stevenson S, Mehraban F, Papay RS, Purchio AF, Goldberg VM. The proteoglycan synthesis repertoire of rabbit chondrocytes maintained in type II collagen gels. Osteoarthritis Cartilage 1994;2:29–41.
7. Yamaoka H, Asato H, Ogasawara T, Nishizawa S, Takahashi T, Nakatsuka T, Koshima I, Nakamura K, Kawaguchi H, Chung UI, Takato T, Hoshi K. Cartilage tissue engineering using human auricular chondrocytes embedded in different hydrogel materials. J Biomed Mater Res A 2006;78:1–11.
8. Ochi M, Uchio Y, Kawasaki K, Wakitani S, Iwasa J. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. J Bone Joint Surg Br 2002;84:571–578.
9. Liu G, Kawaguchi H, Ogasawara T, Asawa Y, Kishimoto J, Takahashi T, Chung UI, Yamaoka H, Asato H, Nakamura K, Takato T, Hoshi K. Optimal combination of soluble factors for tissue engineering of permanent cartilage from cultured human chondrocytes. J Biol Chem 2007;282:20407–20415.
10. Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Tabata Y. Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly(glycolic Acid) fiber. Tissue Eng 2005;11:1476–1488.
11. Hosseinkhani H, Hosseinkhani M, Tian F, Kobayashi H, Tabata Y. Ectopic bone formation in collagen sponge self-assembled peptide-amphiphile nanofibers hybrid scaffold in a perfusion culture bioreactor. Biomaterials 2006;27:5089–5098.
12. Hosseinkhani H, Hosseinkhani M, Gabrielson NP, Pack DW, Khademhosseini A, Kobayashi H. DNA nanoparticles encapsulated in 3D tissue-engineered scaffolds enhance osteogenic differentiation of mesenchymal stem cells. J Biomed Mater Res A 2008;85:47–60.
13. Ushida T, Furukawa K, Toita K, Tateishi T. Three-dimensional seeding of chondrocytes encapsulated in collagen gel into PLLA scaffolds. Cell Transplant 2002;11:489–494.
14. Hannouche D, Terai H, Fuchs JR, Terada S, Zand S, Nasseri BA, Petite H, Sedel L, Vacanti JP. Engineering of implantable cartilaginous structures from bone marrow-derived mesenchymal stem cells. Tissue Eng 2007;13:87–99.
15. Terada S, Yoshimoto H, Fuchs JR, Sato M, Pomerantseva I, Selig MK, Hannouche D, Vacanti JP. Hydrogel optimization for cultured elastic chondrocytes seeded onto a polyglycolic acid scaffold. J Biomed Mater Res A 2005;75:907–916.
16. Takahashi T, Ogasawara T, Kishimoto J, Liu G, Asato H, Nakatsuka T, Uchinuma E, Nakamura K, Kawaguchi H, Chung UI, Takato T, Hoshi K. Synergistic effects of FGF-2 with insulin or IGF-I on the proliferation of human auricular chondrocytes. Cell Transplant 2005;14:683–693.
17. Furukawa KS, Ushida T, Toita K, Sakai Y, Tateishi T. Hybrid of gel-cultured smooth muscle cells with PLLA sponge as a scaffold towards blood vessel regeneration. Cell Transplant 2002;11:475–480.
18. Aoyagi R, Yoshida T. Freuqency equations of an ultrasonic vibrator for the elastic sensor using a contact impedance method. Jpn J Appl Phys 2004;43:3204–3209.
19. Sakai D, Mochida J, Yamamoto Y, Nomura T, Okuma M, Nishimura K, Nakai T, Ando K, Hotta T. Transplantation of mesenchymal stem cells embedded in Atelocollagen gel to the intervertebral disc: A potential therapeutic model for disc degeneration. Biomaterials 2003;24:3531–3541.
20. Wallace DG, Rosenblatt J. Collagen gel systems for sustained delivery and tissue engineering. Adv Drug Deliv Rev 2003; 55:1631–1649.
21. Rosenblatt J, Rhee W, Wallace D. The effect of collagen fiber size distribution on the release rate of proteins from collagen matrices by diffusion. J Control Release 1989;9:195–203.
22. Rong Z, Cheema U, Vadgama P. Needle enzyme electrode based glucose diffusive transport measurement in a collagen gel and validation of a simulation model. Analyst 2006;131: 816–821.
23. Takahashi T, Ogasawara T, Asawa Y, Mori Y, Uchinuma E, Takato T, Hoshi K. Three-dimensional microenvironments retain chondrocyte phenotypes during proliferation culture. Tissue Eng 2007;13:1583–1592.
24. Suzuki M, Ikada Y. Biodegradable polymers in medicine. In: Reis R, Roman JS, editors. Biodegradable Systems in Tissue Engineering and Regenerative Medicine. New York: CRC Press; 2005. p 3–12.
25. Rotter N, Aigner J, Naumann A, Planck H, Hammer C, Burmester G, Sittinger M. Cartilage reconstruction in head and neck surgery: Comparison of resorbable polymer scaffolds for tissue engineering of human septal cartilage. J Biomed Mater Res 1998;42:347–356.
26. Marijnissen WJ, van Osch GJ, Aigner J, Verwoerd-Verhoef HL, Verhaar JA. Tissue-engineered cartilage using serially passaged articular chondrocytes. Chondrocytes in alginate, combined in vivo with a synthetic (E210) or biologic biodegradable carrier (DBM). Biomaterials 2000;21:571–580.
27. Ameer GA, Mahmood TA, Langer R. A biodegradable composite scaffold for cell transplantation. J Orthop Res 2002;20: 16–19.
28. Haisch A, Klaring S, Groger A, Gebert C, Sittinger M. A tissue-engineering model for the manufacture of auricular-shaped cartilage implants. Eur Arch Otorhinolaryngol 2002; 259:316–321.
29. Hutmacher DW, Ng KW, Kaps C, Sittinger M, Klaring S. Elastic cartilage engineering using novel scaffold architec-

tures in combination with a biomimetic cell carrier. Biomaterials 2003;24:4445–4458.
30. Ruszymah BH, Chua K, Latif MA, Hussein FN, Saim AB. Formation of in vivo tissue engineered human hyaline cartilage in the shape of a trachea with internal support. Int J Pediatr Otorhinolaryngol 2005;69:1489–1495.
31. Lee CR, Grad S, Gorna K, Gogolewski S, Goessl A, Alini M. Fibrin-polyurethane composites for articular cartilage tissue engineering: A preliminary analysis. Tissue Eng 2005;11: 1562–1573.
32. Eyrich D, Wiese H, Maier G, Skodacek D, Appel B, Sarhan H, Tessmar J, Staudenmaier R, Wenzel MM, Goepferich A, Blunk T. In vitro and in vivo cartilage engineering using a combination of chondrocyte-seeded long-term stable fibrin gels and polycaprolactone-based polyurethane scaffolds. Tissue Eng 2007;13:2207–2218.
33. Liao E, Yaszemski M, Krebsbach P, Hollister S. Tissue-engineered cartilage constructs using composite hyaluronic acid/ collagen I hydrogels and designed poly(propylene fumarate) scaffolds. Tissue Eng 2007;13:537–550.

短期集中連載　欧州臨床試験の最前線〈1〉

～UHCTアライアンス-EFPIA Japan共同欧州施設訪問から学ぶ～

はじめに
～ハイレベルの臨床研究基盤構築を目指して～

荒川　義弘*

昨秋，大学病院臨床試験アライアンス（UHCTアライアンス）と欧州製薬団体連合会(EFPIA)との共同で，臨床試験をリードする欧州各国の大学病院，臨床研究支援センター，規制当局，製薬会社等を訪問し，臨床試験の基本的枠組や多施設・多国間共同臨床試験への取り組み等について聴取する機会を得た。

欧州では医薬品に係わるすべての介入試験に対しGCP（Good Clinical Practice）が適用されており，すべての臨床試験は規制当局と倫理委員会の承認がなければ開始することはできない。一方，欧州では日常診療の中に臨床研究が組み込まれており，それを支える幅広いスタッフの活躍があった。また，臨床研究の支援体制の整備と国内および国際間のネットワーク化も進められている。日本も国際標準を取り入れ，連携可能な体制の整備が望まれる。

大学病院臨床試験アライアンス（**UHCTアライアンス**）[1]は2006年2月に設立され，治験の実績が高く治験環境の改善に意欲の高い関東地区の7つの国立大学（東京大学，信州大学，千葉大学，筑波大学，東京医科歯科大学，群馬大学，新潟大学）の臨床試験事務局の代表で構成されている。アライアンス内で手順の標準化・簡便化を図る一方，申請段階からスタッフの研修まで幅広く連携を行い，その活動を環境改善のパイオニアとして積極的に公表している。

アライアンスのミッションの1つは国際化，国際連携であり，今回の欧州訪問の目的は，日本での試験環境の改善を積極的にアピールするとともに，欧州での実際を直接見聞きし，日本での臨床試験の環境改善に資することである。

欧州製薬団体連合会 日本技術委員会（**EFPIA Japan**）の方でも，治験の実施体制の現状と調和・連携の動きについて，日本との違いを，その根底となる考え方も含めて聴取したいとの動きがあり，今回の共同訪問が実現した。

訪問した国，施設は**表1**の通りである。

欧州では，**EU臨床試験指令**（EU Clinical Trials Directive 2001/20/EC）[2]の2004年導入により域内の**GCP**（Good Clinical Practice）の調和が進められる一方，治験以外の臨床試験にもGCPが適用されるようになり，臨床試験の数は激減したと言われる[3]。しかし，一方で最近は支援体制の整備やネットワーク化も急速に進められている。

今回の訪問では，こういった現状も含めて，あらかじめ7つの参加大学とEFPIAからの参加者にそれぞれの課題を持って調査していただくことになった。本連載は今回より3カ月にわたり掲載していくが，執筆者が異なるため，各人の調査内

*東京大学医学部附属病院臨床試験部・准教授/副部長（あらかわ・よしひろ）

表1 大学病院臨床試験アライアンス（UHCTアライアンス）-欧州製薬団体連合会 日本技術委員会（EFPIA Japan）共同欧州施設訪問での訪問施設
（期間：2009年10月28日～11月6日）

欧州ではすべての臨床試験にGCP（Good Clinical Practice）が適用され，各国の規制当局と地域ごとにおかれた倫理委員会の承認を得て試験の実施が可能となる．そのため，欧州域内の規制要件の調和や多施設共同研究支援センターの整備が強力に進められている．オックスフォード大学CTSUはグローバルな1万例規模のイベントスタディを自ら多数実施する施設である．また，訪問した病院はそれぞれの国で臨床試験の実績の高い病院ばかりであり，地域のネットワークを形成し，多施設共同研究支援センターの機能も備えていることが多い．訪問した企業も欧州に本拠を置くビッグファーマであり，グローバルな視点で臨床試験を展開している．

＜規制当局＞
・フランス医薬品庁（Agence française de sécurité sanitaire des Produits de Santé；AFSSAPS）
＜多施設共同試験支援センター＞
・（イギリス）オックスフォード大学臨床試験支援ユニット（Clinical Trial Service Unit & Epidemiological Studies Unit；CTSU）
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・（ドイツ）シャリテーベルリン大学病院（Charité - Universitätsmedizin Berlin）臨床研究コーディネーティングセンター（Koordinierungszentrum für Klinische Studien；KKS）
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容をもとにそれぞれ報告する。そのため報告者間で多少重複する部分もあると思われるが，意見聴取する人により感じ方も異なることもあり，あえて調整は行っていない。

文　献

1) 大学病院臨床試験アライアンス http://plaza.umin.ac.jp/~UHCTA/index.html
2) EU臨床試験指令DIRECTIVE 2001/20/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 4 April 2001 - on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use http://www.wctn.org.uk/downloads/EU_Directive/Directive.pdf
3) Hemminki A and Kellokumpu-Lehtine P-L. Harmful impact of EU clinical trials directive. BMJ **332** (7540)：501-502, 2006.

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The Optimal Conditions of Chondrocyte Isolation and Its Seeding in the Preparation for Cartilage Tissue Engineering

Kazumichi Yonenaga, D.D.S.,[1,2] Satoru Nishizawa, B.S.,[1] Yuko Fujihara, D.D.S., Ph.D.,[1] Yukiyo Asawa, D.V.M., Ph.D.,[1] Kanazawa Sanshiro, D.D.S.,[1] Satoru Nagata, M.D., Ph.D.,[3] Tsuyoshi Takato, M.D., Ph.D.,[2] and Kazuto Hoshi, M.D., Ph.D.[1]

To optimize the chondrocyte numbers obtained after collagenase digestion for cartilage tissue engineering, we examined the enzyme concentration and incubation time for collagenase digestion. The appropriate cell density in the chondrocyte primary culture was also verified. The collagenase digestion conditions that maximized the viable cell numbers were 24 h in 0.15% and 0.3% collagenase, 6 h in 0.6%, and 4 h in 1.2%, leading to $\sim 5\times10^5$ cells from 0.05 g. When seeded at 10,000 cells/cm^2, all of these cells became almost confluent within 1 week. Cells digested in 0.3% for 24 h or 0.6% for 6 h and seeded at 3000 cells/cm^2 may also be acceptable, and similarly reached confluence within 1 week. Results regarding expression of the p53, tumor necrosis factor-α, and interleukin-1β genes, as well as apoptosis enzyme-linked immunosorbent assay results, show that excessive collagenase exposure may decrease chondrocyte viability or activity. We recommend a 24-h incubation in 0.3% collagenase or 6 h in 0.6% collagenase, and a cell-seeding density of 3000–10,000 cells/cm^2. These conditions maximize the harvest of isolated chondrocytes from a small amount of biopsied tissue and significantly aid in obtaining a large quantity of cultured cells in a short period.

Introduction

In tissue engineering, cells are isolated from tissues/organs and cultured under biochemical and biophysical stimulation, eventually producing functionally equivalent tissues/organs. This procedure requires both a sufficient number of cells isolated from the tissues/organs and a high success rate of the primary culture. Although chondrocytes, which have been successful in various fields of regenerative medicine, are the major cell source for cartilage tissue engineering, they are difficult to isolate from cartilage because they contain abundant collagen-based matrices that require thorough digestion by collagenase.[1] Collagenase may decrease chondrocyte activity, not only because it damages the cell structure but also because chondrocytes separated from their native matrices show a decrease in proliferation, survival, and differentiation.[2] Because collagenase digestion might be a necessary evil during chondrocyte isolation, the concentration of collagenase and its incubation time should be restricted to a minimum. In previous reports, collagenase concentrations and incubation times during chondrocyte isolation varied widely (Table 1). The collagenase concentrations ranged from 0.03% to 0.6% (<1150 U/mL), and the incubation time ranged from 2 to 20 h.[3–30] Under these conditions, $\sim 1\times10^6$ cells/g of viable chondrocytes were harvested.[26] However, human cells are known to contain 1×10^8 cells/g,[31] which is 100 times more than the number of chondrocytes actually isolated. To increase the number of cells obtained after collagenase digestion, we attempted to determine the collagenase concentration and incubation time at which human chondrocytes are optimally digested.

The seeding density has a significant influence on cell viability and proliferation efficacy in the primary culture. If the seeding density is too low, the autocrine/paracrine system may barely work, which leads to slow proliferation. On the other hand, an overdose of chondrocytes may interfere with sufficient cell attachment to the substrate in the culture dish or evoke early contact inhibition, both of which lead to a less effective cell culture. However, the optimum seeding density in chondrocyte cultures for tissue engineering has not yet been investigated in detail. In this study, the appropriate cell density for the chondrocyte primary culture was also verified.

Materials and Methods

Chondrocyte isolation

All procedures were approved by the Ethics Committee of the University of Tokyo Hospital (ethics permission number

Departments of [1]Cartilage and Bone Regeneration (Fujisoft) and [2]Sensory and Motor System, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.
[3]Nagata Microtia and Reconstructive Plastic Surgery Clinic, Saitama, Japan.

TABLE 1. COLLAGENASE CONCENTRATIONS FOR CHONDROCYTE ISOLATION IN PREVIOUS REPORTS

Collagenase concentration	*Unit (U/mL)*	*Time (h)*	*Collagenase subtype*	*Origin of cartilage*	*Digested cartilage*	*Reference*
0.6%	—	12	Type 2	Human	Nasal	3, 4
0.5%	1150	6–8	Normal	Human	Auricular	5
0.3%	690	4	Normal	Human	Auricular	6
0.3%	690	8–12	Normal	Human	Auricular	7
0.3%	690	8–12	Type 2	Human	Auricular	8–11
0.3%	690	12–18	Normal	Cow	Articular	12
0.25%	575	4–6	Normal	Human	Articular	13
0.2%	460	8	Type 2	Human	Auricular	14
0.2%	460	10–14	Type 2	Human	Nasal	15
0.2%	—	4	Type 2	Human	Articular	16
0.15%	291	Overnight	Normal	Human	Auricular	17–19
0.15%	—	Overnight	Type B	Rabbit	Auricular	20
0.1%	—	2	Type 1	Rabbit	Auricular	21
0.1%	—	3	Type A	Rabbit	Articular	22
0.1%	230	12	Type 2	Human	Nasal auricular	23
0.1%	230	14–16	Type 2	Pig	Nasal auricular Articular	24
0.1%	—	16	—	Human	Articular	25
0.08%	960	16–20	Normal	Human	Articular	26
0.07%	100	3	Type 2	Goat	Articular	27
0.04%	—	Overnight	—	Human	Articular	28
0.03%	—	12–18	Type P	Human	Nasal	29
0.03%	—	16	Type P	Human	Nasal	30

Dashes indicate lack of data.

622). Remnant auricular cartilage from three microtia patients was obtained during surgery in accordance with the Helsinki Principles. The cartilage tissue was thoroughly minced with scissors and tweezers into fragments of 250–1000 μm (Fig. 1A). Approximately 3 mL of collagenase solution (Wako Pure Chemical Industries) at concentrations of 0.15%, 0.3%, 0.6%, or 1.2% was poured into a 5 mL tube (BD Falcon). Four tubes were prepared for each concentration, for a total of 16 tubes. Approximately 0.05 g of cartilage fragments was put into each tube, which was then placed in a 37°C water bath and shaken at 150 cycles/min. For each concentration, the number of total cells and viable cells as well as the cell viability were measured with a Nucleo-Counter (ChemoMetec) after 2, 4, 6, and 24 h.

Chondrocyte culture

The viable cells were seeded in 6.4-mm plastic culture dishes coated with collagen type 1. Cells were seeded at densities of 30,000; 10,000; 3000; 1000; 300; and 100 cells/cm^2 for the evaluation of the optimal cell-seeding density for the primary culture. For the analysis of gene expression, 35-mm plastic culture dishes coated with collagen type 1 were used for the culturing of cells digested under certain conditions. The culture medium was Dulbecco's modified Eagle's medium Nutrient Mixture F-12 HAM (Sigma Chemical Co.) containing 5% human serum (Sigma Chemical Co.), 100 ng/mL fibroblast growth factor-2 (Kaken Pharmaceutical Co., Ltd.), and 5 μg/mL insulin (MP Biomedicals).[32]

Real-time reverse transcription (RT)-polymerase chain reaction analysis

Total RNA was isolated from the chondrocytes with ISOGEN (Wako Pure Chemical Industries) following the supplier's protocol. cDNA was synthesized from 1 μg of the total RNA with the PrimeScript® RT-PCR Kit Perfect Real Time (Takara Shuzo). The full-length or partial-length cDNA of the target genes, including the polymerase chain reaction (PCR) amplicon sequences, was amplified by PCR, cloned into pCR-TOPO Zero II or pCR-TOPO II vectors (Invitrogen), and used as standard templates after linearization. Using the QuantiTect SYBR Green PCR Master Mix (Invitrogen) and the ABI 7700 Sequence Detection System, we performed real-time fluorescence detection with the following protocol: initial denaturation for 10 min at 94°C followed by 40 cycles of 15 s at 94°C and 1 min at 60°C. All reactions were run in quadruplicate. The sequences of the primers were 5′-CCA GCCAAAGAAGAAACCAC-3′ and 5′-CTCATTCAGCTCTC GGAAC-3′ for p53, 5′-CCCCAGGGACCTCTCTCTAATC-3′ and 5′-GGTTTGCTACAACATGGGCTACA-3′ for tumor necrosis factor alpha (TNF-α), 5′-CCTGTCCTGCGTGTT GAAAGA-3′ and 5′-GGGAACTGGGCAGACTCAAA-3′ for interleukin 1beta (IL-1β), 5′-AGAACCTTGTGTGACAAAT GAGAAC-3′ and 5′-TACCCATTAGACATATCCAGCTT GA-3′ for bcl-2, and 5′-GAAGGTGAAGGTCGGAGTCA-3′ and 5′-GAAGATGGTGATGGGATTTC-3′ for glyceraldehyde-3-phosphate dehydrogenase.[33–36]

Photometry analysis of ssDNA apoptosis enzyme-linked immunosorbent assay

Five thousand cells were transferred into each well of a 96-well microplate, and the microplate was centrifuged at 200 *g* for 5 min. The medium was removed, and 200 μL of fixative (80% methanol in phosphate-buffered saline) was added to each microwell. The microplate was incubated at room temperature for 30 min, after which the fixative was removed, and the microplate was kept at room temperature for 1–2 h to

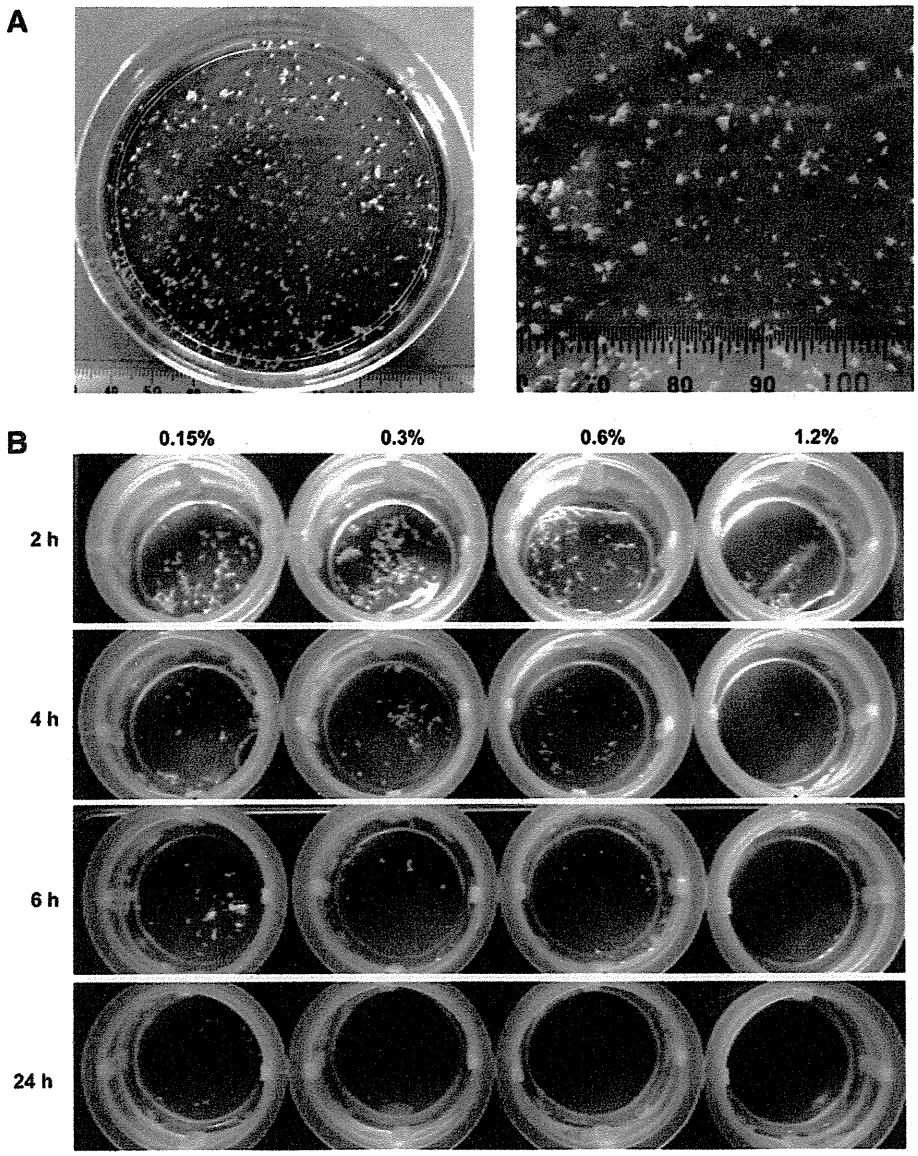


FIG. 1. **(A)** Cartilage fragment after manual mincing. We measured the sizes of the fragments ($n = 100$) and determined them to be 643 ± 381 μm (mean ± standard deviation [SD]). This size and variation seemed almost average because the previous studies also reported fragment sizes of 250–1000[14] or 250–500 μm.[29,30] Left, higher magnification; right, lower magnification. **(B)** Collagenase digestion of the cartilage fragments. (Upper) At all concentrations, the sizes of the collagenase digests decreased as the incubation periods increased (2–24 h). (Lower) The chart shows the degree of cartilage digestion. Many fragments clearly remained (+); some fragments visible (±); all were digested (–).

	0.15%	0.3%	0.6%	1.2%
2 h	+	+	+	±
4 h	+	+	±	-
6 h	+	±	-	-
24 h	±	-	-	-

allow attachment of the cells to the plate. Cell apoptosis was evaluated according to the manufacturer's protocol of the ssDNA Apoptosis ELISA Kit (Chemicon® International Inc.). The enzyme-linked immunosorbent assay absorbance was measured using a standard microplate reader at 405 nm.

Results

We examined the effects of collagenase concentration and incubation time on cartilage digestion. For all concentrations, the volume of the collagenase digests decreased as the incubation period increased (Fig. 1B). Although the cartilage pieces were somewhat visible after 24 h in the 0.15% collagenase, they had disappeared after the same amount of time in collagenase concentrations >0.3% (Fig. 1B). The amount of time for the cartilage remnants to disappear was shortened to 6 and 4 h in collagenase concentrations of 0.6% and 1.2%, respectively (Fig. 1B).

In all concentrations, both the total number of cells and the number of viable cells increased in parallel to the

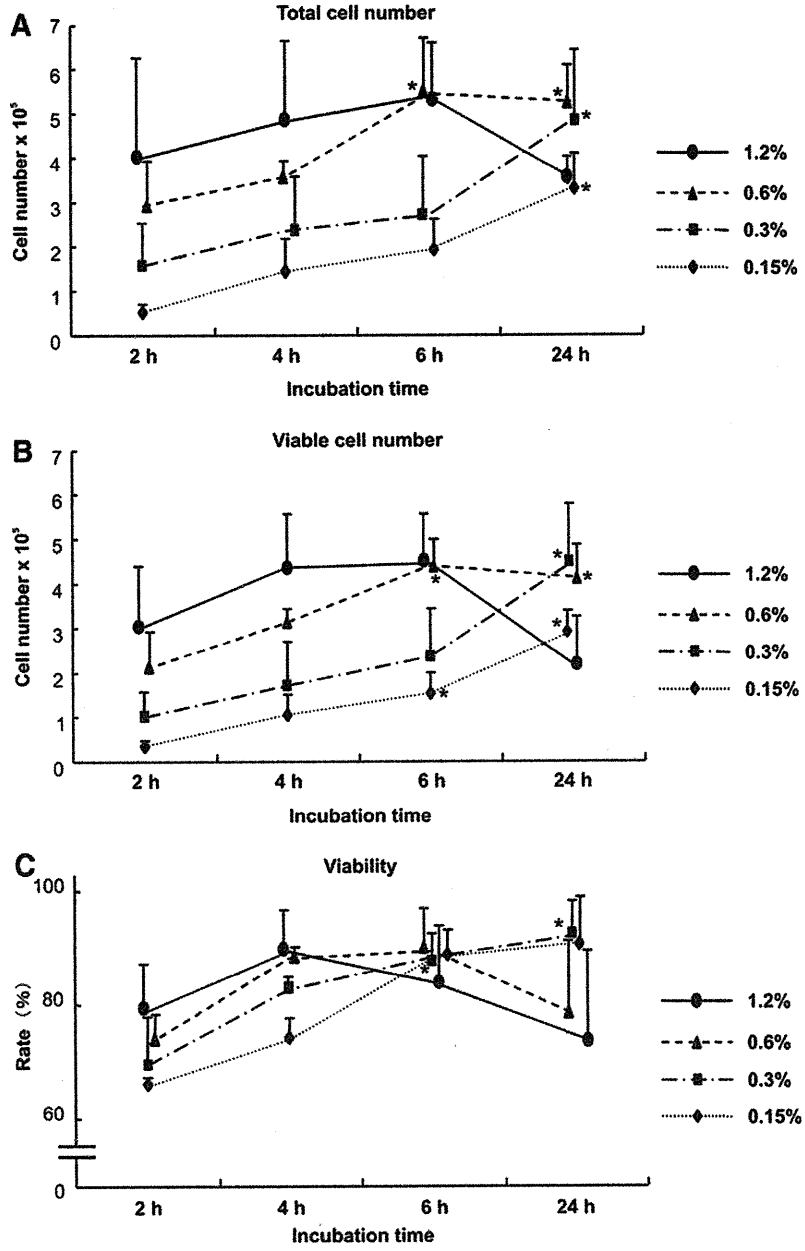


FIG. 2. Cell numbers and viability after collagenase digestion. Both the total **(A)** and viable **(B)** cell numbers increased with the increase in incubation time, except for the 1.2% collagenase. **(C)** Cell viability in 0.15% and 0.3% collagenase became ~100% at 24 h, whereas that in 0.6% collagenase reached maximum at 6 h. All values are presented as mean ± SD of three samples per group. Statistics were assessed using the Dunnett's test (*$p < 0.05$, vs. 2 h in each concentration of collagenase).

increase in the incubation time, with the exception of the sample with 1.2% collagenase for 24 h (Fig. 2A and B). The maximum number of viable cells, 4–5×10^5 cells obtained from ~0.05 g tissue, occurred at 4 h in 1.2% collagenase, 6 h in 0.6% collagenase, and 24 h in 0.3% collagenase. However, the sample with 0.15% collagenase provided <3×10^5 cells even after 24 h, possibly because of the abundant remnants of the cartilage digests (Fig. 2A and B). The total cell numbers and viable cell numbers under conditions of 24 h and 0.15% collagenase, 24 h and 0.3% collagenase, and 6 h and 0.6% collagenase were significantly higher than those at 2 h for each dose of collagenase (Fig. 2A and B). The cell numbers had decreased after 24 h in 1.2% collagenase, as the viability deteriorated (Fig. 2C). Therefore, we determined which conditions of collagenase digestion maximized the viable cell numbers and found that the incubation time can be reduced to 24 h in 0.15% collagenase and 0.3% collagenase, 6 h in 0.6% collagenase, and 4 h in 1.2% collagenase.

Next, we investigated the effect of cell density during seeding on cell growth (Fig. 3). Chondrocytes that had been digested by collagenase under suitable conditions as determined above were seeded at several cell densities (100–30,000 cells/cm^2). All of those seeded at 10,000 cells/cm^2 became almost confluent in 1 week. Seeding at 3000 cells/cm^2 may be acceptable for the chondrocytes digested with 0.3% collagen for 24 h and 0.6% for 6 h, but the cells digested under other conditions could not sufficiently proliferate at 3000 cells/cm^2 (Fig. 3B). The cells were aggregated with each other and formed cell clusters at 30,000 cells/cm^2 in the samples digested with 0.6% collagenase and 1.2% collagenase (Fig. 3A and B). When we counted the cell numbers, the cells seeded at a density of 3000–10,000 cells/cm^2 after di-

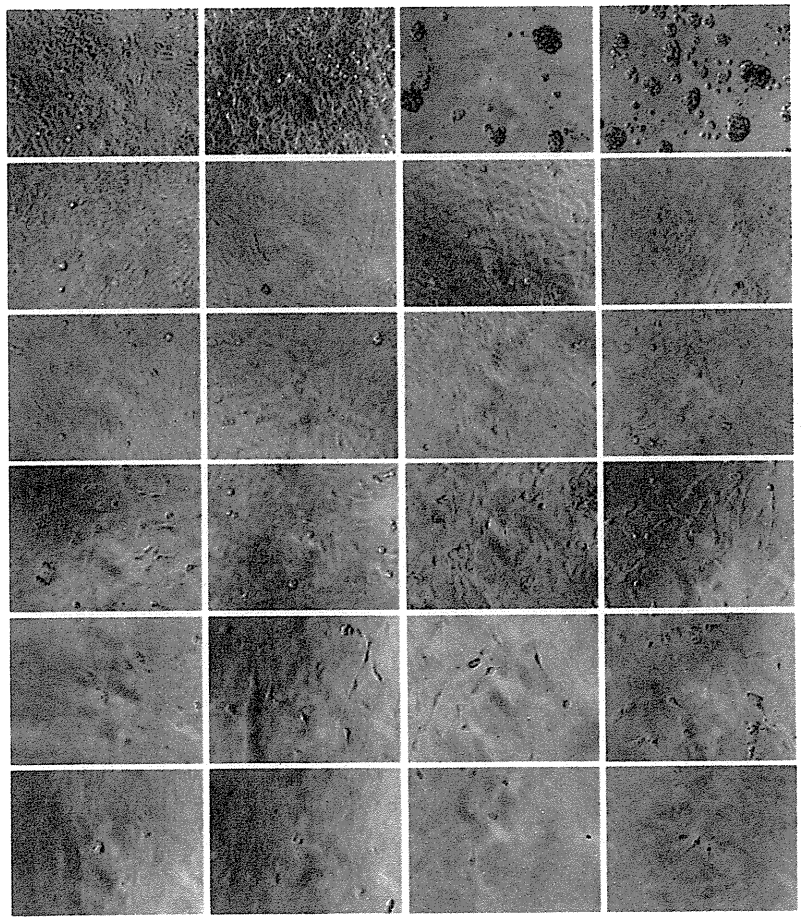
gestion in 0.3% collagenase for 24 h and 0.6% for 6 h reached the maximum cell numbers at 1 week (Fig. 4A), which supported the results shown in Figure 3. However, the cells seeded at <1000 cells/cm^2 became confluent as well after >2 weeks (Fig. 4B)

The possibility that the chondrocytes may fall into apoptosis or catabolic situations after the destruction of the native matrices by collagenase digestion could be not ignored. Thus, we examined gene expression of the apoptosis-related molecules and inflammatory cytokines in the chondrocytes for each set of collagenase digestion conditions. As shown in Figure 5, the proapoptotic factor p53 was likely upregulated as collagenase concentration increased, although we could not detect Bcl-2. The inflammatory cytokines TNF-α and IL-1β were scarcely detected in the chondrocytes digested in 0.15% collagenase for 24 h and in 0.3% collagenase for 24 h. Expression of these genes tended to increase in the chondrocytes with the higher collagenase concentrations (Fig. 5), suggesting that a collagenase concentration higher than 0.6% may somewhat affect chondrocyte viability or metabolism. Actually, the results of apoptosis assays also showed that apoptosis tended to be upregulated when the exposure time became longer in the higher concentrations of collagenase (0.6% or 1.2%). In contrast, the chondrocytes in the lower concentrations (0.15% and 0.3%) exhibited minimum apoptosis at 24 h (Fig. 6), which was consistent with the viability of chondrocytes (Fig. 2C) and the results of p53 expression (Fig. 5).

Discussion

Each laboratory engaged in the research and development of cartilage tissue engineering prepares various unique protocols according to their previous findings and experiences. Although they may independently work well, some kinds of standards are needed when we attempt to obtain stable results. However, thus far, no systematic analyses on collagenase

A



B

	0.15%/24h	0.3%/24h	0.6%/6h	1.2%/4h
30,000	○	○	×	×
10,000	○	○	○	○
3,000	○	○	○	○
1,000	△	△	△	△
300	△	△	△	△
100	△	△	△	△

FIG. 3. Optimal cell-seeding density to provide favorable cell growth. **(A)** The cells seeded at a density of 3000–10,000 cells/cm^2 became almost confluent in all the collagenase concentrations within 1 week. The cells at the density of <1000 cells/cm^2 could not sufficiently proliferate, whereas the cells tended to be aggregated at 30,000 cells/cm^2. **(B)** The chart shows the degree of cell growth. ○, confluent; Δ, did not reach confluence; ×, became aggregated.

doses or incubation times have been found. Although the cartilage includes different tissues and donor ages or diseases affect the cellularity or protein compositions of the tissues, the amount of collagen, which is the major inhibitory factor for chondrocyte isolation, seems almost constant.[37] This implies the possibility of a standard protocol. Thus, we regard the investigation of the optimal conditions for collagenase digestion to be a pivotal task for increasing the steadiness and safety of regenerative medicine.

On the basis of the results of the present study, by the time-course counting of cell numbers in various concentrations of collagenase, the maximum number of viable cells—5×10^5—were harvested from 0.05 g of original tissue at 24 h in 0.15% collagenase, 6 h in 0.6%, and 4 h in 1.2% (Fig. 2B). The incubation time at which the viable cell numbers reached a maximum corresponded to the point at which the collagenase digests completely disappeared (Fig. 1B). The reason why the harvested cell numbers in the 0.15% collagenase did not reach the maximum even at 24 h may be because the remaining cartilage digests in the solution at that time. The viability of the cells harvested at 2 h was lower than that of the later harvests. The cells harvested in this short incubation period had been located at the surface of the cartilage tissues that had been minced to $<1\,mm^3$ before the collagenase di-

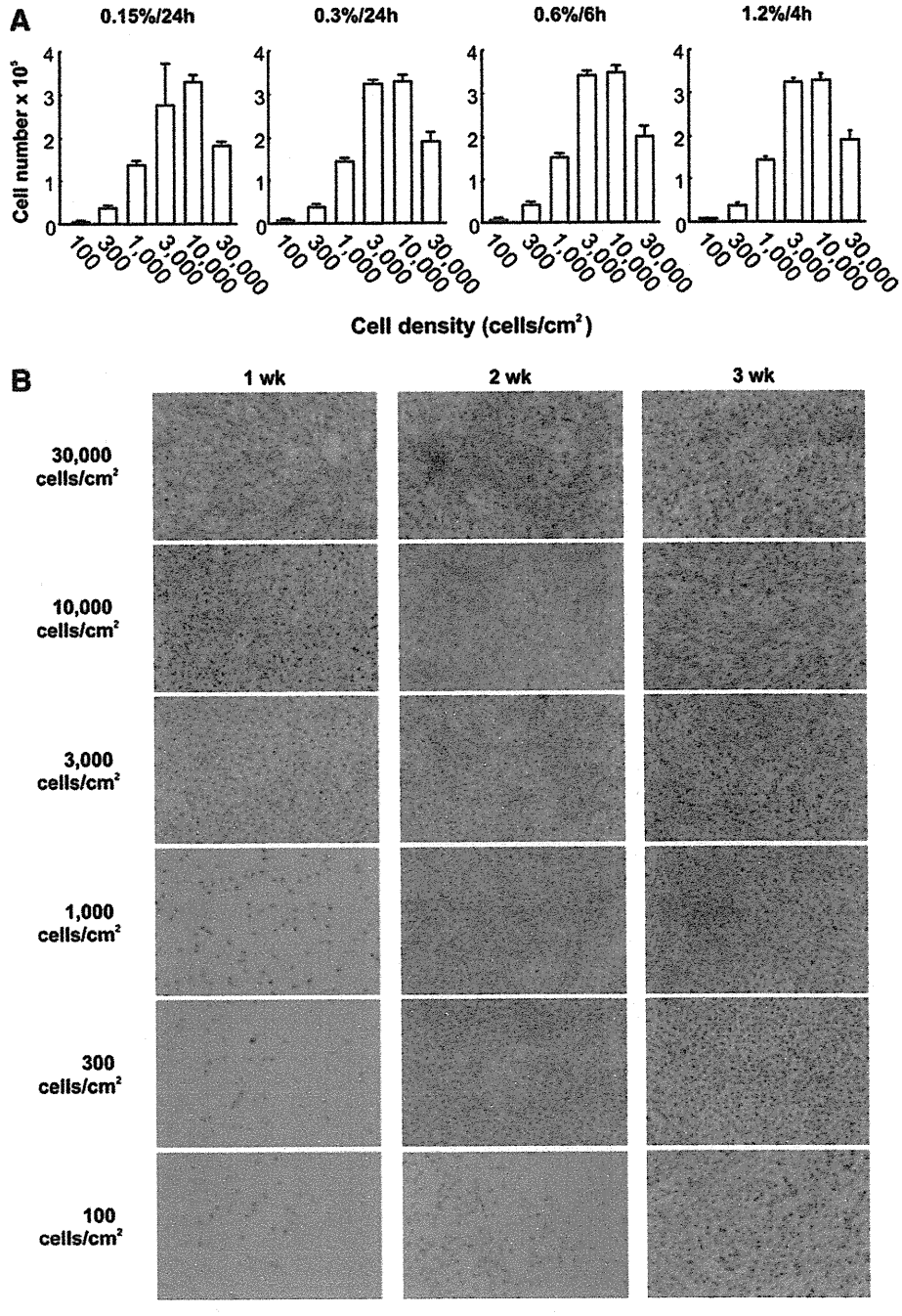


FIG. 4. Optimal cell-seeding density for obtaining sufficient cell numbers. **(A)** The cells seeded at a density of 3000–10,000 cells/cm^2 reached the maximum cell numbers at 1 week. All values are presented as mean ± SD of three samples per group. **(B)** The cells after digestion in 0.3% collagenase for 24 h were cultured for 3 weeks. They became confluent at >2 weeks when seeded at <1000 cells/cm^2. Color images available online at www.liebertonline.com/ten.