Cartilage tissue engineering using human auricular chondrocytes embedded in different hydrogel materials

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Abstract: To seek a suitable scaffold for cartilage tissue engineering, we compared various hydrogel materials originating from animals, plants, or synthetic peptides. Human auricular chondrocytes were embedded in atelopeptide collagen, alginate, or PuraMatrix™, all of which are or will soon be clinically available. The chondrocytes in the atelopeptide collagen proliferated well, while the others showed no proliferation. A high-cell density culture within each hydrogel enhanced the expression of collagen type II mRNA, when compared with that without hydrogel. By stimulation with insulin and BMP-2, collagen type II and glycosaminoglycan were significantly accumulated within all hydrogels. Chondrocytes in the atelopeptide collagen showed high expression of β1 integrin, seemingly promoting cell–matrix signaling. The N-cadherin expression was inhibited in the alginate, implying that decrease in cell-to-cell contacts may maintain chondrocyte activity. The matrix synthesis in PuraMatrix™ was less than that in others, while its Young's modulus was the lowest, suggesting a weakness in gelling ability and storage of cells and matrices. Considering biological effects and clinical availability, atelopeptide collagen may be accessible for clinical use. However, because synthetic peptides can control the risk of disease transmission and immunoreactivities, some improvement in gelling ability would provide a more useful hydrogel for ideal cartilage regeneration. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 78A: 1–11, 2006

Key words: chondrocyte; scaffold; hydrogel; regenerative medicine; matrix

INTRODUCTION

Tissue engineering has been researched and developed to aid in the repair or reconstruction of defective or injured organs. In tissue engineering, the artificial tissues are composed of living cells, often with a suitable scaffold. The scaffold provides the seeded cells with the space for function and supports their activities. Because every scaffold possesses specific properties that fit some kinds of cells or mimic other tissues in mechanical strength, we should choose the optimal scaffold based on the characteristics of the target cells and tissues.

Cartilage is one of the expectative targets for tissue engineering, because cartilage differs from other tissues in its limited capacity for self-repair.[1] The difficulty in the self-repair of cartilage seems to be due to the lack of a sufficient supply of healthy chondrocytes to the defective sites or to the low productivity of matrices in regenerated chondrocytes. Cartilage tissue engineering could overcome such limitations by using

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ex vivo culture techniques and supportive artificial materials.

In principle, chondrocyte activities are maintained when they are placed in the proper 3D environment. During the development and growth of cartilage, the chondrocytes produce abundant matrices, encase themselves within cavities, and are eventually separated from each other.[2] In contrast, the chondrocytes deviated from the physiological 3D environment rapidly lose the typical phenotype and protein synthesis, which is termed dedifferentiation.[3] In cartilage tissue engineering, the chondrocytes isolated from their original tissues would be conditioned in a 3D environment, mimicking the physiological situation with favorable scaffolds so as to reproduce their functions and enhance protein synthesis.

To date, attempts have been made to use two types of scaffolds for cartilage tissue engineering. The first is a solid type of scaffold including a honeycomb, porous body, mesh, sponge, and unwoven fabric.[4] The solid-type scaffolds have some advantages in shaping the macroscopic structure of regenerated tissues or in supporting their mechanical strength. However, the use of solid-type scaffolds includes practical conflicts. The smaller the pore sizes are, the more difficult it will be for the cell suspension to infiltrate into the scaffold. In contrast, when the pore sizes of solid scaffolds are increased, the chondrocytes are attached to the walls of huge pores, but they are not placed in a 3D condition.

The second is a hydrogel. Various materials derived from animals or plants, for example, collagen type I gel,[5] atelopeptides of collagen,[6] fibrin glue,[7] gelatin,[8] agarose,[9] or alginate,[10,11] are classified as this type. Although the hydrogel lacks mechanical strength in itself, this type of material can be mixed with cells and can surround the seeded cells in all directions. The hydrogel type of scaffold could be used to reconstruct the 3D environment for the chondrocytes in cartilage tissue engineering. Many previous articles reported that those hydrogel materials were more effective in retaining chondrocyte functions or promoting matrix synthesis, when compared with monolayer culture.[6,9,12] However, the biological specificities of each hydrogel materials have seldom been compared with each other. Van Susante and coworkers compared the potentials of both collagen type I and alginate gels as carriers for bovine articular chondrocytes, and evaluated the proliferation and proteoglycan synthesis of chondrocytes when the cells were cultured in an encapsulation with each gel in media containing 10% fetus bovine serum. The collagen type I gel had promoted chondrocyte proliferation, but the alginate gel had an advantage in proteoglycan synthesis.[13] Because this collagen type I gel had been prepared in extraction with acetic acid,[5] it preserved some telopeptides of collagen and antigenicity. At present, atelopeptides of collagen that were treated with protease have been regarded to show even lower immunogenicity than does native collagen that has usually been used as a medical device for treatment of tissue defects or tissue engineering.[14]

In the present study, we selected some kinds of hydrogel scaffolds that are or will soon be clinically available, and examined proliferation and matrix synthesis of human chondrocytes in the encapsulation with them so as to provide information on the choice of a suitable scaffold for clinical application. We focused on atelopeptides of collagen and alginate, both of which have been used as materials for medical devices, and either of which is a typical hydrogel material derived from animals or plants. Because the atelopeptide collagen spontaneously polymerizes into a stable gel at neutral pH and physiological temperature, and because the alginate undergoes instant ionotrophic gelation in the presence of divalent cations such as Ca^{2+}, they are widely used for the 3D culture studies of chondrocytes.[6,10]

In addition to both materials derived from animals or plants, synthetic peptides were also evaluated as a potential candidate for a clinically-available hydrogel scaffold. The synthetic peptides, PuraMatrix™, have been recently designed to serve as substrates for cell growth, differentiation, and biological functions. PuraMatrix™ is composed of synthetic polypeptides (AcN-$(RADA)_4$-CNH_2).[15] The motif RAD was incorporated to mimic the known cell adhesion motif RGD that is found in many ECM proteins.[16] They assemble to form an *in vivo*-like 3D extracellular matrix hydrogel around 37°C at pH 7, suggesting characteristics similar to the atelopeptide collagen. This has been used for research on hepatic regeneration or nerve regeneration and has been reported to promote neurite outgrowth and synaptic formation in neural cells, as well as functional differentiation in hepatocyte-like cells.[15,17] At present, clinical trials of the PuraMatrix™ for use in the orthopedic field have been planned by the suppliers.

As the experimental design, human auricular chondrocytes were embedded within each hydrogel material at the identical concentration of 0.5% by weight. We examined the proliferation ability of the chondrocytes at Passage 2, which should be more expanded when large sizes of engineered tissues would be made. The cell/hydrogel constructs at low cell density (2×10^4 cells/mL) were incubated in a medium containing 10% fetal bovine serum (FBS). To evaluate matrix synthesis, we used the dedifferentiated chondrocytes of Passage 4, and made cell/hydrogel constructs at high cell density (10^7 cells/mL). The constructs were incubated with BMP-2 and insulin, either of which was reported to induce the redifferentiation of chondrocytes or effectively enhance matrix synthesis.[18,19] The properties of hydrogel or cell/hydrogel constructs

were examined cytologically, biochemically, histologically, and biomechanically. Moreover, to clarify the molecular effects of each hydrogel material, cell–matrix interactions or cell-to-cell contacts were examined through the gene expression pattern of β1 integrin or N-cadherin, both of which are major adhesion molecules in chondrocytes or their progenitors.[20,21]

MATERIALS AND METHODS

Chemicals and antibodies

Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM/F12), penicillin–streptomycin solution, and trypsin–EDTA solution were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase from *Clostridium histolyticum* and Isogen were from Wako Pure Chemical Industries (Osaka, Japan). Bullet kit chondrocyte growth medium (CGM) and sodium alginate were obtained from Cambrex Bioscience Walkersville (Walkersville, MD). Other reagents were atelopeptide collagen (Kawaken Fine Chemicals Co., Tokyo, Japan), fetal bovine serum (FBS, Thermo Electron, Melbourne, Australia), insulin (MP Biomedicals, Irvine, CA), OCT compound (Miles, Elkhart, IN), PuraMatrix™ (3DM, Cambridge, MA), and recombinant human bone morphogenetic protein-2 (BMP-2, kindly provided by Yamanouchi Pharmaceutical Co., Tokyo, Japan).

Chondrocyte isolation and preparation

All procedures for the present experiments were approved by the ethics committee of the University of Tokyo Hospital (ethics permission #622). With 0.15% collagenase digestion, human chondrocytes were isolated from remnant auricular cartilage of microtia patients who underwent the operation at the University of Tokyo Hospital, with informed consent. Isolated chondrocytes were seeded in a 100-mm plastic tissue culture dish at a density of 6400 cells/cm^2 and cultured in the CGM, in a 37°C/5% CO_2 incubator. The medium was changed three times per week. Passages were performed by treatment with trypsin-EDTA solution when the cells were approaching confluence.

Chondrocyte proliferation in each hydrogel

For the collagen-based 3D culture, chondrocytes (Passage 2) were suspended in 0.5% atelopeptide collagen solution (pH 7). The mixture was placed in each well of a six-well plate at 2 mL at a density of 2×10^4 cells/mL. The collagen formed a gel on 1 h incubation at 37°C, embedding the cells in a 3D condition (total 4×10^4 cells in 2 mL of gel). The medium containing DMEM/F-12 with or without 10% FBS was gently poured on the gel at a volume of 2 mL in a 37°C/5% CO_2 incubator. Throughout the experiment, the medium was changed three times per week. To release the cells, the gel was incubated in 0.3% collagenase at 37°C for 30 min.

In the case of alginate, chondrocytes (Passage 2) were resuspended in 0.5% sodium alginate. Two ml of the cell/alginate suspension at a density of 2×10^4 cells/mL was dropped into each well of a six-well plate filled with 102 m*M* $CaCl_2$. The beads formed within 10 min in the $CaCl_2$ solution (total 4×10^4 cells in 2 mL of gel). After removing the $CaCl_2$ solution from the beads and washing the beads with 155 m*M* NaCl, the medium containing DMEM/F-12 with or without 10% FBS was gently poured on the beads at a volume of 2 mL in a 37°C/5% CO_2 incubator. Throughout the experiment, the medium was changed three times per week. To release the cells, the gel was incubated in 55 m*M* Na citrate at 37°C for 30 min.

In the PuraMatrix™–based 3D culture, 1% of PuraMatrix™ (1 mL) was diluted with chondrocytes (Passage 2) resuspended in 20% sucrose (1 mL). Two milliliter of the mixture containing 4×10^4 cells (density: 2×10^4 cells/mL) was gently poured into the medium containing DMEM/F-12 with or without 10% FBS at a volume of 2 mL in a 37°C/5% CO_2 incubator. The PuraMatrix™ formed 2 mL of gel with a total of 4×10^4 cells within 1 h at 37°C. To release the cells, the gel and cell mixture were aspirated up and down. Throughout the experiment, the medium was changed three times per week. The resuspension was centrifuged at 1000*g* for 5 min to remove the media and the PuraMatrix™.

To evaluate the cell proliferation by cell count, the chondrocytes from three patients were individually incubated in three wells of a six-well plate within each hydrogel material. After a 2-week incubation, the cell numbers were counted by a hematocytometer, while the viability of cells was checked by trypan blue staining.

Chondrocyte culture at high cell density in each hydrogel

Dedifferentiated chondrocytes (Passage 4) were suspended in 0.5% of atelopeptide collagen solution, alginate solution, and PuraMatrix™ solution, at a density of 10^7 cells/mL. In the atelopeptide collagen, 20 μL of the cell/material suspension (total 2×10^5 cells) was placed into the bottom of a 15 mL conical tube to form a gel on 1 h incubation at 37°C. In the case of alginate, 20 μL of the cell/material suspension was dropped into a 15 mL tube filled with 102 m*M* $CaCl_2$. The gel formed within 10 min. The cell/material suspension of PuraMatrix™ (20 μL) was gently poured into the bottom of a 15 mL tube filled with the DMEM/F-12 to form a gel within 1 h at 37°C. The DMEM/F-12 medium was used at a volume of 2 mL for each gel and cultured in a 37°C/5% CO_2 incubator. To induce redifferentiation of the chondrocytes, 5 μg/mL insulin and 200 ng/mL BMP-2 were added to the medium, according to the previous report or its modification.[18,19] As a control without any hydrogel, a cell/medium suspension containing 2×10^5 chondrocytes was centrifuged at 500*g* in a 15 mL conical tube. The supernatant was removed and the resultant pellet was cultured in the DMEM/F-12 without any factors. Throughout the experiment, the medium was changed three times per week.

Total RNA extraction and real-time PCR

Total RNA was isolated from the cell/gel mixture with Isogen following the supplier's protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). For real-time PCR, the ABI Prism Sequence Detection System 7000 was used. Aliquots of first-strand cDNA (1 μg) were amplified with the QuantiTect SYBER Green PCR Kit (Qiagen, Osaka, Japan) under the following conditions: initial denaturation for 10 min at 94°C followed by 40 cycles consisting of 15 s at 94°C and 1 min at 60°C. Data analysis consisted of fold induction; the expression ratio was calculated from the differences in threshold cycles at which an increase in reporter fluorescence above a baseline signal could first be detected among three samples and was averaged for duplicate experiments. The sequence of primers we used in real-time PCR to detect Col1A1, Col2A1, β1 integrin, N-cadherin, and GAPDH were as follows:

Col1A1 F:5′-CTCCTCGCTTTCCTTCCTCT-3′, R:5′-GTGCTAAAGGTGCCAATGG T-3′; Col2A1 F:5′-GAGTCAAGGGTGATCGTGGT-3′, R: 5′-CACCTTGGTCTCCA GAAGGA-3′[22]; β1integrin F: 5′-AGTTGCAGTTTGTGGATCACTGAT-3′, R: 5′-AAA GTGAAACCCGGCATCTG-3′[23]; N-cadherin; F: 5′-GTGCCATTAGCCAAG GGATTCAGC-3′, R: 5′–GCGTTCCTGTTCCACTCATAGGAGG-3′[24]; GAPDH F: 5′-GAAGGT GAAGGTCGGAGTCA-3′, R: 5′-GAAGATGGTGATGGGATTTC-3′. GAPDH was used as the housekeeping gene.

Biochemical measurement of glycosaminoglycan and collagen types I and II

We evaluated the glycosaminoglycan (GAG) content using the Alcian blue-binding assay (Wieslab AB, Lund, Sweden), according to the supplier's protocol. After digestion in 0.3% collagenase for 1 h at 37°C, cell debris and insoluble material were removed by centrifugation at 6000*g* for 30 min. GAG in the supernatant was precipitated with Alcian blue solution, and the sediments after centrifugation at 6000*g* for 15 min were dissolved again in 4*M* GuHCl-33% propanol solution. The spectrophotometric absorbance of the mixture was measured at a wavelength of 600 nm.

The collagen proteins of the cell/gel construct were solubilized and quantified in ELISA according to the protocol of human Type I, II Collagen Detection Kit (Chondrex, Redmound, WA). The cell/gel construct was dissolved in 10 mg/mL pepsin/0.05*M* acetic acid at 4°C for 48 h and then in 1 mg/mL pancreatic elastase/1×TSB at 4°C overnight. In the mixture, the collagen proteins were captured by polyclonal anti-human type I or type II collagen antibodies and detected by 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.

Histology

The cell/gel constructs were fixed with 4% paraformaldehyde in 0.1*M* phosphate buffer (pH 7.4) for 2 h in 4°C and immersed in 10% sucrose in phosphate-buffered saline (PBS), 20% sucrose in PBS, and a 2:1 mixture of 20% sucrose in PBS and OCT compound in succession with rapid freezing with liquid nitrogen in preparation for cryosection. For the alginate, 0.1*M* cacodylate buffer containing 102 m*M* $CaCl_2$ was used instead of phosphate buffer or PBS. The specimens were cryosectioned at a thickness of 10 μm and were stained with toluidine blue-O.

Young's modulus of each hydrogel

Five hundred microliter of each hydrogel at 0.5% weight was prepared in three wells of a 24-well plate, and was incubated in 0.5 mL of DMEM/F-12 for 24 h in 37°C. After removal of medium, Young's modulus of each hydrogel was measured with a Venustron tactile sensor (Axiom, Fukushima, Japan). Under computer control, the motor-driven sensor unit automatically presses down on the surface of materials and provides an indentation force and a decrease in the resonant frequency. The resonant frequency of the sensor was set to 50 Hz, while the maximum depth of indentation was 1 mm. Young's modulus can be calculated by the indentation force and the decrease of the resonant frequency, based on the principles reported by Aoyagi and Yoshida.[25] The software Venus 42 provided by the manufacturer was used for calculation. Young's modulus was measured 9 times in three wells for each hydrogel.

RESULTS

Chondrocyte proliferation

At first, we examined the effects of atelopeptide collagen, alginate, and PuraMatrix™ on chondrocyte proliferation. Human auricular chondrocytes of Passage 2 were encapsulated in the three kinds of hydrogel materials and cultured for 2 weeks. The chondrocytes in atelopeptide collagen proliferated at ~10-fold with 10% FBS, although no proliferation was seen without FBS. The cells rather decreased in number with or without FBS, in alginate or PuraMatrix™ (Fig. 1).

Gene expression of chondrocytes in each hydrogel

Next, we observed the effects of those hydrogel materials on the gene expression of chondrocytes when the dedifferentiated chondrocytes (Passage 4) were embedded in the hydrogel at a high cell density of 10^7 cells/mL. At 1 week after incubation, the expression of Col1A1 in all of hydrogel materials ranged between half and 2-fold of that in high density culture without any hydrogel (gel(−)), when they were incu-

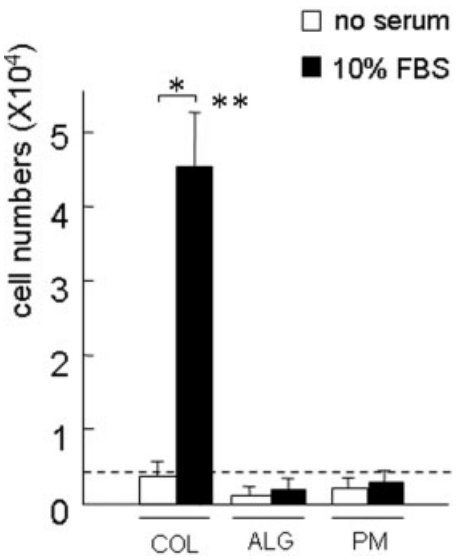

Figure 1. Effects of atelopeptide collagen, alginate, or PuraMatrix™ hydrogel on chondrocyte proliferation. In the atelopepide collagen (COL), chondrocytes of Passage 2 effectively increased in numbers not without (blank bar), but with (filled) FBS. The cell numbers in alginate (ALG) or PuraMatrix™ (PM) rather decreased either with or without FBS. The broken line indicates the number of chondrocytes at the start of incubation. All values are presented as mean plus standard deviation. Statistics were assessed using the Student *t* test (*: $p < 0.05$ with *vs.* without FBS; **: $p < 0.05$ when compared with alginate and PuraMatrix™).

bated in medium without any factors (control). Because of the induction of redifferentiation by a medium containing insulin and BMP-2, the Col1A1 expression in all hydrogels decreased, when compared with that in the control medium.

The expression of Col2A1 was more than 100 times larger than that of gel(−), when the cells were embedded in every kind of hydrogel material, even in the control medium. With the redifferentiation medium, those in all hydrogel exhibited over a 10^5 fold increase, when compared with that of gel(−). Among the three kinds of materials, the expression of both Col1A1 and Col2A1 in PuraMatrix™ tended to be high in the control medium, but response to the redifferentiation medium was rather low in Col2A1 expression (Fig. 2).

To examine the molecular effects of the hydrogel on chondrocytes, we measured the gene expression of β1 integrin and N-cadherin, which play major roles in cell–matrix interactions and cell-to-cell contacts, respectively, in chondrocytes.[20,21] At 1 week, β1 integrin was abundantly expressed in gel(−). Within all kinds of hydrogel materials, the β1 integrin expression was upregulated in the redifferentiation medium when compared with that in the control. β1 integrin of the atelopeptide collagen constructs showed the highest expression among three materials, not only in the redifferentiation media, but also in the control, suggesting abundant extracellular signaling through chondrocyte/collagen interaction. The enhancement of β1 integrin expression in both control and redifferentiation media seemed lower within PuraMatrix™ than within atelopeptide collagen, although PuraMatrix™ abundantly contains the RAD motif, which is regarded as the analog of RGD, the major integrin ligand in collagen.[16] Alginate did not enhance β1 in-

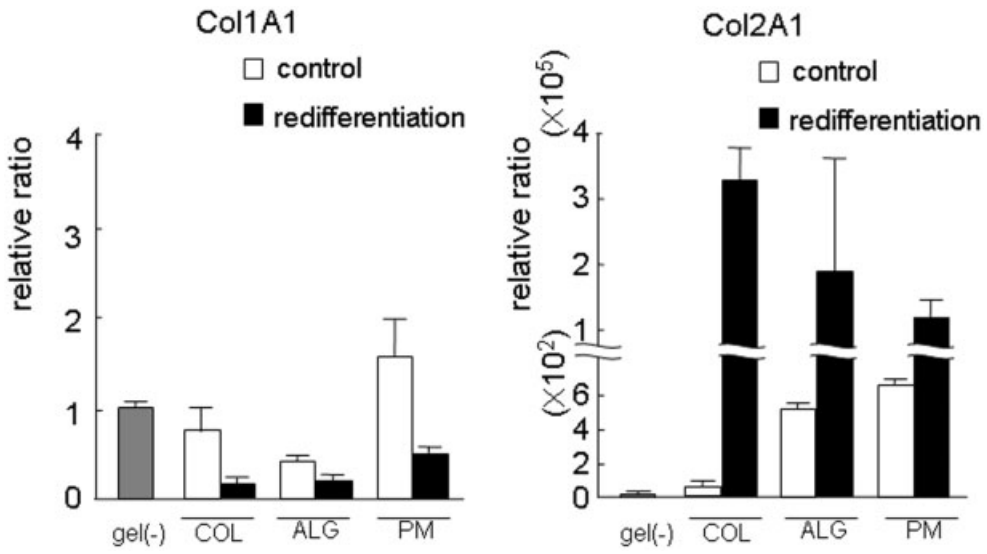

Figure 2. Gene expression of collagen types I and II in chondrocytes within each hydrogel. Within all hydrogel materials, the expression of Col2A1 was promoted in the redifferentiation medium (filled bar), when compared with the control (blank bar), although that of Col1A1 expression was decreased. The expression in the high-cell density culture without any hydrogel (gel(−)) was standardized to 1 (grey bar). All values are presented as mean plus standard deviation. COL, ateloeptide collagen; ALG, alginate; PM, PuraMatrix™.

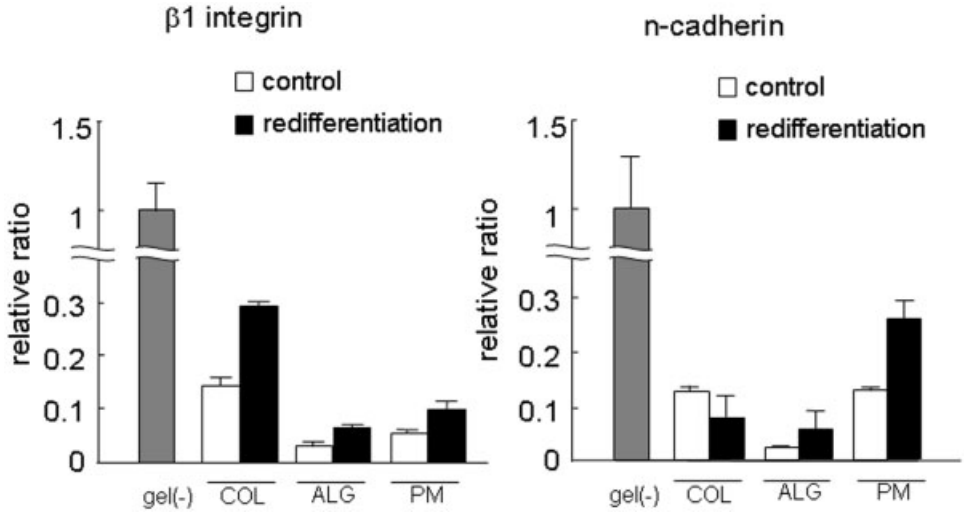

Figure 3. Gene expression of β1 integrin and N-cadherin in chondrocytes within each hydrogel. The atelopeptide collagen showed high expression of β1 integrin, not only in the redifferentiation media (filled bar), but also in the control (blank bar), although the alginate did not promote β1 integrin expression. The expression of N-cadherin was also inhibited within the alginate in both the control and redifferentiation media. The expression of high-cell density culture without any hydrogel (gel(−)) was standardized to 1 (grey bar). All values are presented as mean plus standard deviation. COL, ateloeptide collagen; ALG, alginate; PM, PuraMatrix™.

tegrin expression, perhaps due to the lack of cell–matrix interactions (Fig. 3, left).

Although the expression of N-cadherin was enhanced in the gel(−), its expression decreased in all hydrogel materials. Particularly, the upregulation of N-cadherin expression was inhibited within alginate in both control and redifferentiation media, suggesting that alginate can maintain isolation of each chondrocyte. In the atelopeptide collagen, although both control and redifferentiation media promoted N-cadherin expression when compared with that in alginate, it was downregulated according to the induction of redifferentiation. Contrarily, the chondrocytes in PuraMatrix™ rather increased the expression of N-cadherin, when the redifferentiation was induced (Fig. 3, right).

Matrix synthesis of chondrocytes in each hydrogel

At 3 weeks after the incubation of chondrocytes at a high density (10^7/mL), gel(−) accumulated collagen type I, although it was diminished in encapsulation with each hydrogel. Moreover, the protein content of collagen type I was decreased in the atelopeptide collagen and in PuraMatrix™ with the redifferentiation media, when compared with that in the control. The content of collagen type II or GAG in gel(−) and each hydrogel was hardly detectable in the control medium, but the redifferentiation medium significantly gained amounts of GAG and collagen type II, in all hydrogels. The accumulation of collagen type II was remarkable in the redifferentiation medium within the atelopeptide collagen hydrogel, while the GAG content was abundantly determined in alginate. Also in PuraMatrix™, the effects of the redifferentiation medium were noted on the synthesis of collagen type II and GAG, although the amount of both matrices was less than that in atelopeptide collagen or alginate (Fig. 4).

The constructs of chondrocytes with encapsulation in atelopeptide collagen, alginate, and PuraMatrix™ were examined histologically. All the constructs were metachromatically stained with toluidine blue, when they were cultured in the redifferentiation media. However, the findings for PuraMatrix™ showed a sparse and loose appearance in the middle area of the constructs, even in the redifferentiation medium, suggesting that the ability to support cells and newly-synthesized matrices in PuraMatrix™ was weak (Fig. 5). The aggregation of high-cell density culture without any hydrogel (gel(−)) was too small in size and too fragile to be prepared for histological examination.

Mechanical properties

While the hydrogel of atelopeptide collagen or alginate was firm, the PuraMatrix™ gel seemed rather soft when the hydrogel was probed by the tip of a microspatula. To examine mechanical properties of each hydrogel quantitatively, we measured Young's modulus by a Venustron tactile sensor. Each hydrogel reached maximal elasticity within 24 h of incubation in the culture media. Young's modulus of atelopeptide collagen was the highest (65.5 ± 4.1 kPa), following

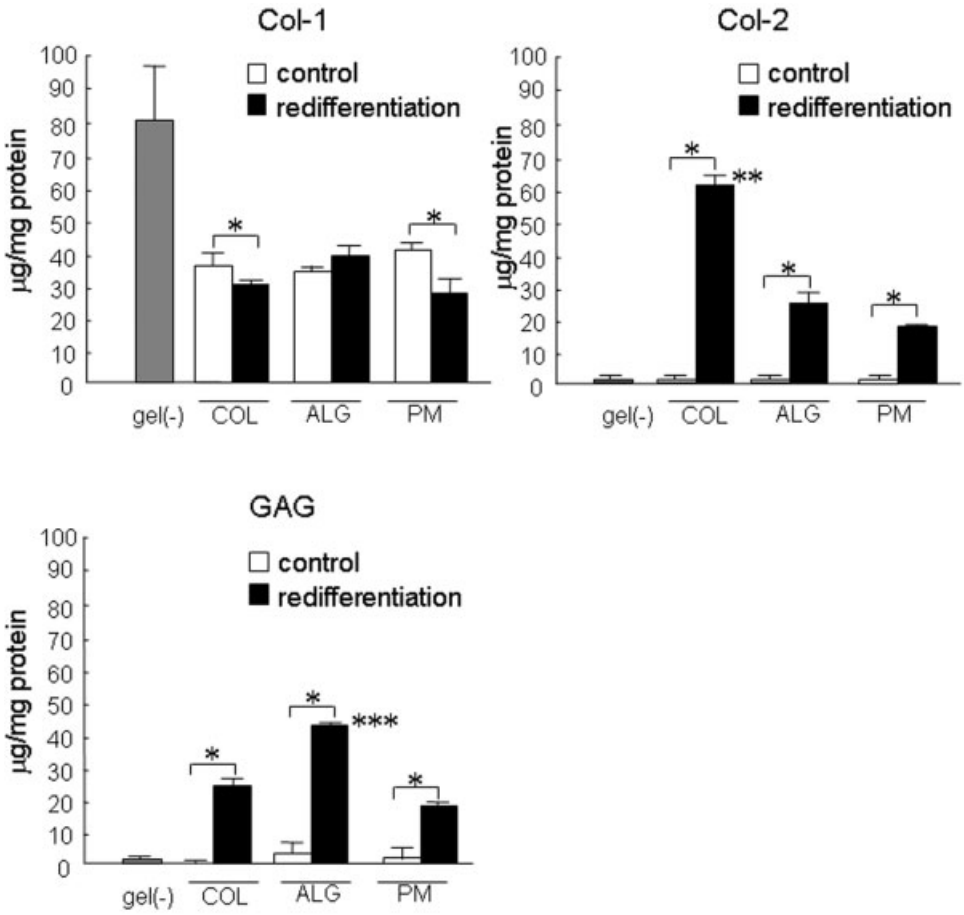

Figure 4. Matrix synthesis of chondrocytes in each hydrogel. Amounts of collagen type II and GAG were increased in the redifferentiation medium (filled bar), when compared with the control (blank bar), within all materials. The amount of collagen type I was decreased in the atelopeptide collagen (COL) and the alginate (ALG) hydrogel. All values are presented as mean plus standard deviation. Statistics were assessed using the Student *t* test (*: $p < 0.01$ control *vs.* redifferentiation; **: $p < 0.01$ when compared with alginate and PuraMatrix™ (PM); ***: $p < 0.01$ when compared with collagen and PuraMatrix™). Gray bar: high-cell density culture without any hydrogel (gel(−)).

those of alginate (36.3 ± 5.4 kPa) and PuraMatrix™ (16.7 ± 1.0 kPa) (Fig. 6).

DISCUSSION AND CONCLUSIONS

Chondrocytes in native cartilage are surrounded by an abundant extracellular matrix in all directions and are isolated into their own lacunae. This implies that chondrocytes are constantly exposed to cell–matrix interactions, and in contrast, that they are separated from each other to lose cell-to-cell contacts under physiological conditions. Cell–matrix interaction is essential for chondrocyte proliferation, differentiation, or survival.[26] Integrins are a major class of cell adhesion molecules and are expressed in chondrocytes. They consist of α/β heterodimers that associate with intracellular proteins on ligand binding.[27] Chondrocytes dominantly contain α1β1, α3β1, α5β1, α10β1, αvβ3, and αvβ5 integrins.[28] Integrins recognize the peptide motif "RGD" commonly contained in several extracellular matrix proteins such as fibronectin, collagen, and vitronectin. After engagement with the extracellular matrix components, integrin receives signal via multiple downstream effectors, including integrin-linked kinase (ILK), and exerts various functions. Mice with a chondrocyte-specific disruption of the gene encoding ILK by Cre-LoxP system developed chondrodysplasia, caused by impaired chondrocyte proliferation.[29,30]

The atelopeptides of type I collagen are a fiber protein made from a collagen, which is solubilized by protease.[31] The atelopeptide collagen seems to possess the ligands for integrins, RGD. In the present study, chondrocytes embedded in the atelopeptide collagen hydrogel proliferated rapidly with FBS and produced abundant collagen type II and GAG. This may suggest that the support or stimulation of cell growth and matrix synthesis is related to cell–matrix interaction through RGD-integrin signaling. On the other hand,

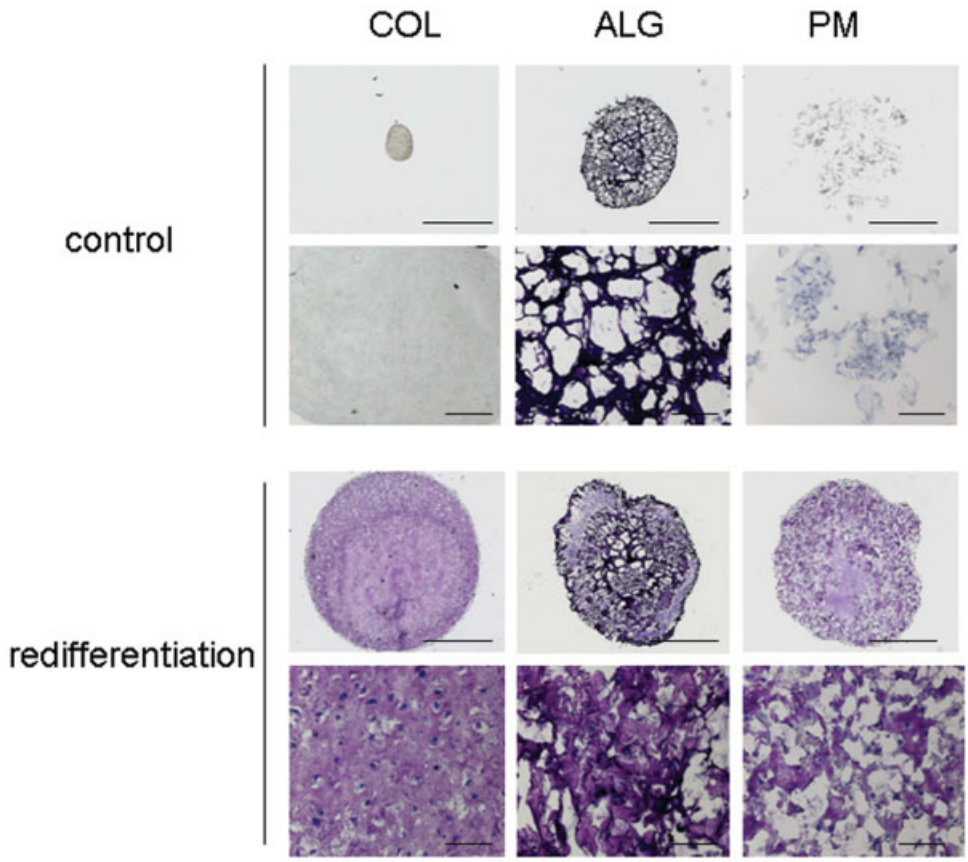

Figure 5. Histological findings for the chondrocyte/hydrogel constructs. The cell/gel constructs were metachromatically stained with toluidine blue, when they were cultured in the redifferentiation media. The internal areas of cell/PuraMatrix™ (PM) constructs showed a rather sparse and loose appearance, even in the redifferentiation medium. COL, atelopeptide collagen; ALG, alginate. Low magnification, bar = 1 mm; high = 100 μm.

the expression of β1-integrin was enhanced in high-cell density culture without any hydrogel, although it did not contain detectable amount of collagen type II and GAG. The reason why this method of culture did not accumulate cartilaginous matrices in spite of high β1-integrin expression may be that isolation of each chondrocytes was extremely diminished with cell-to-cell contacts increased, and that the 3D environment promoting chondrocyte activity could not be reproduced.

PuraMatrix™ consists of repeated sequences of RAD. The motif RAD mimics the known cell adhesion motif RGD. Actually, the RGD tripeptide of certain proteins in some species is replaced by RAD in counterparts of different species.[16] In the previous report using U251MG glioma cells, the recombinant fusion protein containing RGD promoted extensive cell attachment and spreading in cell adhesion assay, while mutation of RGD to RAD did not result in significant loss of either activity.[16] Those abundant reactive motives may also transduce the extracellular signaling into chondrocytes and induce cell growth or the gene expression of a cartilage matrix marker, such as collagen type II, in PuraMatrix™.

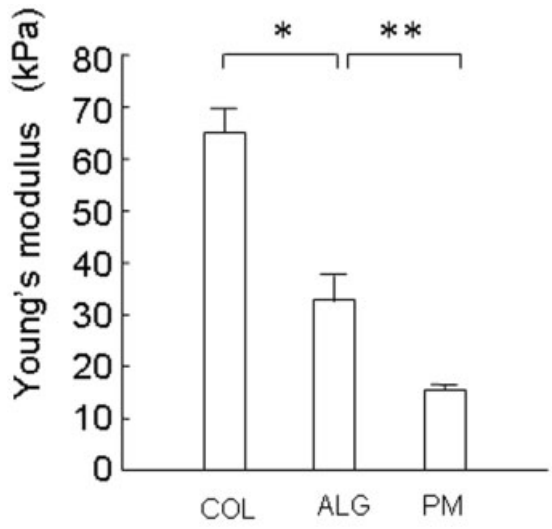

Figure 6. Young's modulus of each hydrogel. Young's modulus of each hydrogel was examined by a Venustron tactile sensor. The atelopeptide collagen showed the highest elasticity among all materials. All values are presented as mean plus standard deviation. Statistics were assessed using the Student *t* test (*: $p < 0.01$ atelopeptide collagen (COL) *vs.* alginate (ALG); **: $p < 0.01$ alginate *vs.* PuraMatrix™ (PM)).

However, the proliferation of chondrocytes or the accumulation of cartilaginous matrices, collagen type II and GAG, was rather smaller in PuraMatrix™ than in atelopeptide collagen or alginate. Histological findings also showed that the interior structure was sparse and loose in the cell/hydrogel construct using PuraMatrix™. One of the reasons was thought to be the weakness of the gelling ability and preservability for chondrocytes and matrices produced by chondrocytes within the cell/hydrogel constructs. Indeed, the gel of PuraMatrix™ showed the lowest Young's modulus among all hydrogel materials, and was soft enough to recover the cells by repeated pipetting of the cell/hydrogel construct.

On the other hand, alginate forms a firm gel. Alginate is a linear polysaccharide isolated from brown seaweed. Because it is composed of a linear co-polymer of two uronic acids, L-guluronic and D-mannuronic acid linked by β1, 4 and α1, 4 glucoside bonds,[10] it does not possess common adhesion molecules for mammalian cells. Because this kind of material hardly provides cell–matrix interactions for cells, extracellular signaling was reduced, resulting in the inhibition of chondrocyte proliferation. In contrast, a firm gel of alginate could preserve the isolation of each chondrocyte with cell-to-cell contacts decreased, as shown in Figure 3.

Cell-to-cell contacts are known to play some roles in the regulation of chondrocyte differentiation. The expression of N-cadherin in chondrocytes was changed according to the stages of differentiation. This was detectable in prechondrogenic cells, increased during cell aggregation, but became undetectable in hypertrophic chondrocytes that were embedded in abundant matrix and lost cell-to-cell contacts.[32] Also *in vitro*, N-cadherin is decreased during chondrocyte differentiation, in contrast to the upregulation of collagen type II.[33] Chondrocytes in conventional monolayer culture possess abundant cell-to-cell contacts and become dedifferentiated.[34] Also noted in the present data, the expression of N-cadherin was upregulated in the high-cell density culture without any hydrogel, suggesting an increase in cell-to-cell contacts, while the chondrocytes in this culture produced minimal amount of either collagen type II or GAG. In contrast, the 3D culture in hydrogel materials decreased the N-cadherin expression and enhanced the expression of Col2A1, even without BMP-2 and insulin. Particularly, alginate helps the chondrocytes reduce cell-to-cell contacts and maintain the cell shape and function. These properties may enhance the expression and accumulation of cartilaginous matrices such as collagen type II and GAG, in the alginate constructs.

Although alginate possesses the favorable property of inducing redifferentiation in chondrocytes and that it is widely used as a material for wound dressing on patients suffering from refractory ulcers, the application of alginate implants in the human body has not yet been clinically experienced. Because the immunoreactivity of this material during long-term intracorporeal usage has remained unknown, we should further evaluate its safety before it becomes available in clinics. On the other hand, the atelopeptide collagen has been confirmed to show even lower immunogenicity than native collagen, because telopeptides determining the antigenicity are removed from the collagen in protease digestion.[14] This kind of material has already been used to correct or repair depressed sites in soft tissue as an Atelocollagen™ implant or to compensate for a joint defect with autologous chondrocytes.[35] The atelopeptide collagen hydrogel showed some advantage for proliferation and matrix synthesis, especially collagen type II, when compared with other materials. Also in histology, the findings of cell/hydrogel constructs using the atelopeptide collagen showed abundant matrices metachromatically stained with toluidine blue, embedding round-shaped and isolated chondrocytes, which resemble physiological cartilage tissues. Therefore, the atelopeptide collagen may be accessible for clinical use in cartilage tissue engineering from the standpoints of biological properties and clinical availability.

However, the atelopeptide collagen has been prepared from animals. Although it is quality-controlled as a medical device to prevent disease transmission or to maintain a aseptic state, a discussion about the future risks for unknown disease transmission, as has been repeatedly considered for the use of FBS, may be inevitable. The use of FBS has been restricted for clinical application because it includes the risk for transmission of viral and other pathogens. Problems of a possible immune reaction against bovine protein in the serum have also been considered when the regenerated tissues cultured in the FBS-contained medium are transplanted into humans. The previous studies had shown immune response by antibody detection against bovine serum proteins in burn patients receiving keratinocyte grafts cultured from FBS.[36,37]

In Japan, the Ministry of Health, Labor and Welfare announced in 2000 that animal products from a country or zone with the occurrence of bovine spongiform encephalopathy (BSE) are prohibited for use as a medical device. According to the changes in occurrence of BSE or the policy of each nation, the supply for a medical device originating from cattle may expose the risk of arrest of supply. On this point, synthetic materials will have merits, because they may be able to control contamination or immunoreactivity. Therefore, the expectation of synthetic peptides would increase as substitutes for materials originating from organism products. Increase in gelling ability could improve supportability of cells or matrices. In the present study, we gently put the cell/PuraMatrix™ suspension into the medium adjusted to neutral pH to

form a gel. Usage of transwell dishes may induce immediate neutralization of the PuraMatrix™ and more rapid and firm gel formation, as the suppliers recommend, although we could not adopt it because we had to make uniform the experimental procedure to that of atelopeptide collagen or alginate in which the transwell dishes were not used. Some improvement of synthetic peptides would provide more useful hydrogel materials to create ideal regenerated cartilage.

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