

Potential of exogenous cartilage proteoglycan as a new material for cartilage regeneration

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Abstract

Background

Although proteoglycan (PG) is one of the major components of cartilage matrices, its biological function is not fully elucidated.

Methods

The objectives of this study were to investigate the proliferation and differentiation of chondrocytes embedded in atelocollagen gel with exogenous cartilage PG (PG-atelocollagen gel) in vitro, and also to evaluate the repair of cartilage defects by PG-atelocollagen gel in vivo. In the in vitro study, rabbit chondrocytes were cultured in the PG-atelocollagen gel. Cell proliferation and mRNA expression levels were measured, and gels were histologically evaluated. In the in vivo study, cultured PG-atelocollagen gel containing chondrocytes were transplanted into full-thickness articular cartilage defects in rabbit knees, and evaluated macroscopically and histologically.

Results

For the in vitro study, chondrocyte proliferation in 5.0 mg/ml PG-atelocollagen gel was enhanced, and the gene expression of *Col2a1* and *Aggrecan* were decreased. In contrast, chondrocyte proliferation in 0.1 and 1.0 mg/ml PG-atelocollagen gel was not enhanced. The gene expression of *Aggrecan* in 0.1 and 1.0 mg/ml PG-atelocollagen gel was increased. For the in vivo study, the histological average total score of the 0.1 mg/ml PG-atelocollagen gel was significantly better than that of the group without PG.

Conclusions

Although the appropriate concentration of PG has not been defined, this study suggests the efficacy of PG for cartilage repair.

Introduction

Articular cartilage damage or defects are unable to regenerate because of a limited capacity for spontaneous healing. Numerous techniques and basic research have been performed to promote the repair of articular cartilage defects [1–4]. Autologous chondrocyte transplantation (ACT) reported by Brittberg et al. is a technique to use a patient's own chondrocytes for regeneration of the defect and has been clinically applied as an effective treatment [5–7]. Although long-term clinical observation of patients who have received ACT has confirmed improvement in their quality of life [7], histological results have not been satisfactory and cartilage-specific gene expressions such as type II collagen and aggrecan were reduced [8]. The development of more effective culture methods to maintain chondrocyte differentiation is required for the purpose of successful repair of wide cartilage damage/defects.

Various extracellular matrix components such as collagen, proteoglycan (PG) and hyaluronan (HA), maintain homeostasis of cartilage tissue through not only protecting the chondrocytes from mechanical damage from joint loading, but also by regulating modulators such as cytokine and growth factor to maintain cell functions [9]. PG is a family of glycoconjugates of a core protein covalently linked to one or more glycosaminoglycan (GAG) sugar chains, and it is an important macromolecule that affects physiological functions such as cell attachment [10], migration [11], differentiation [12, 13], and morphogenesis [14, 15].

Various kinds of synthetic/natural high polymers have been developed as scaffolds to maintain the differentiation of chondrocytes transplanted to articular cartilage defects. Several studies have shown that type II collagen and GAG such as HA and chondroitin sulphate, which are major extracellular matrix components in articular cartilage, are useful as a cell scaffold material for three-dimensional (3D) culture using chondrocytes [16–18]. In addition, several studies have reported that PG potentiated chondrogenic differentiation of mesenchymal stem cells or transdifferentiation of adult dermal fibroblasts [19, 20]. However, there have been few studies that investigated the potential for exogenous cartilage PG as a candidate material for 3D culture using chondrocytes.

The 3D culture method using atelocollagen gel reported by Ochi et al. has been clinically applied to the treatment of full-thickness defects of cartilage in knees based on the result of in vitro and in vivo studies [21–23]. In our study, chondrocytes were cultured in atelocollagen gel which contained exogenous cartilage PG (PG-atelocollagen gel). The objectives of this study were to investigate the proliferation and differentiation of chondrocytes embedded in PG-atelocollagen gel in vitro, and also to evaluate the repair of a full-thickness articular cartilage defect in a rabbit model by cultured PG-atelocollagen gel containing chondrocytes in vivo. The hypotheses under study were that PG-atelocollagen gel enhances chondrocyte differentiation, and promotes the repair of articular cartilage defects.

Materials and methods

The study protocol was accepted by the Institutional Ethics Committee on Animal Experiments.

Preparation of PG-atelocollagen gel in the in vitro and in vivo studies

Atelocollagen gel (3% atelocollagen implant, Koken Co., Tokyo, Japan) was mixed with cartilage PG [24] dissolved in RPMI1640 culture medium (Gibco, NY, USA) containing 10% foetal bovine serum, 100 µM ascorbic acid, 100 units/ml of penicillin, and 100 µg/ml streptomycin. Then, passage one chondrocytes isolated from articular cartilages of ten-week-old Japanese white rabbits (Hokudo Co., Sapporo, Japan) were embedded in the PG-atelocollagen gel (final concentration of 1.5% collagen). PG dissolved in RPMI1640 culture medium was sterilised by a 0.20-µm disposable syringe filter (Iwaki Glass Co., Tokyo, Japan) for the experiments.

Cell culture within PG-atelocollagen gel in the in vitro and in vivo studies

A quantity of 50 µl of the PG-atelocollagen gel containing chondrocytes was placed on 35-mm diameter culture dishes (Iwaki Glass Co., Tokyo, Japan) and then gelled by incubation at 37°C for 30 min. The gel thus formed was layered with 2 ml of RPMI1640 culture medium containing 10% foetal

bovine serum, 100 μ M ascorbic acid, 100 units/ml of penicillin, and 100 μ g/ml streptomycin. Culture medium was exchanged every three days.

In the in vitro study, first, a 50- μ l mixture of 6.0×10^4 cells (1.2×10^6 cells/cm³; final concentrations of PG: 0.1, 1.0, and 5.0 mg/ml) was cultured for 21 days. Next, a 50- μ l mixture of 1.0×10^5 cells (2.0×10^6 cells/cm³; final concentrations of PG: 0.1 and 1.0 mg/ml) was cultured for 35 days to examine the effect of PG at a longer culture period. In the in vivo study, a 50- μ l mixture of 1.0×10^5 cells (2.0×10^6 cells/cm³; final concentrations of PG: 0.1 mg/ml) was cultured. After 21 days of culture, PG-atelocollagen gels containing chondrocytes were carefully detached from the culture dish and prepared for transplantation. In the control group PG was not mixed in the atelocollagen gel.

Cell proliferation in the in vitro study

Chondrocytes cultured in the PG-atelocollagen gel were released by incubation with collagenase (Collagenase S-1; Nitta Gelachin, Tokyo, Japan). Then the cell numbers were counted in a haemocytometer and their viability was estimated with the trypan blue exclusion test [5].

Gene expression of *Col2a1* and *Aggrecan* in the in vitro study

Real-time quantitative reverse transcriptase-PCR (qRT-PCR) amplification was performed to quantify the gene expression levels of *Col2a1* and *Aggrecan*. Chondrocytes cultured in the PG-atelocollagen gel were released by incubation with collagenase, and then total RNAs from chondrocytes were extracted using an RNeasy Kit (Qiagen, Victoria, Australia). Each 1 μ g of total RNA was reverse-transcribed using a SuperScriptTM First-Strand Synthesis System with oligo (dT) priming methods (Invitrogen, Carlsbad, CA, USA). All primers and probes (TaqMan MGB probes) were designed from the GenBank sequences using the Primer Express Software (Applied Biosystems, Foster City, CA, USA). The sequences of primers and probes are listed in Table 1. The reactions were heated at 50°C for two minutes and 95°C for ten minutes followed by 40 cycles of 95°C for 15 sec and 60°C for one min using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). For each primer/probe set, a Ct value was determined by the cycle number at which the fluorescence intensity reached a specific value in the middle of the exponential region of amplification. Relative gene expression levels were determined by employing the comparative C_t method (ABI User Bulletin 2) and standardising levels to that of *Gapdh*.

Table 1

Sequences used for real-time quantitative reverse transcriptase-PCR

Gene		Sequences	Amplicon size	Sequence accession number
<i>Col2a1</i>	Sense	5'-CCCCACGCCCACTCG-3'	67	S83370
	Antisense	5'-CCCAGCTTTTGTTCAGTCT-3'		
	Probe	5'-CCCAGTTCAGGTCTCTTG-3'		
<i>Aggrecan</i>	Sense	5'-CTGCAGGCTGCCTACGA-3'	64	L38480
	Antisense	5'-CAGTCTGATCAGCCAGCCA-3'		
	Probe	5'-CCACCAGTGCACGCC-3'		
<i>Gapdh</i>	Sense	5'-CGACCACTTCGGCATTGTG-3'	76	L23961
	Antisense	5'-CCCGTCCACGGTCTTCTG-3'		
	Probe	5'-CCACGGTGACGCCAT-3'		

Gapdh Glyceraldehyde-3-phosphate dehydrogenase

Histological evaluation in the in vitro study

PG-atelocollagen gels were stained with alcian blue and also stained immunohistochemically. Then, the expression of GAG and type II collagen in gels were microscopically evaluated. The cultured PG-atelocollagen gel containing chondrocytes was rinsed twice with PBS, fixed in 10% formalin, dehydrated through a graded series of ethanol, infiltrated with isoamyl alcohol, and embedded in paraffin. Sections of 4- μ m thickness through the centre of the gel were cut and stained. The expression of type II collagen in PG-atelocollagen gel containing chondrocytes was immunohistochemically stained using mouse anti-human type II collagen monoclonal antibody (Daiichi Fine Chemical Co., Toyama, Japan) and Vectastain Elite ABC Mouse IgG kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturers' instructions.

Transplantation of PG-atelocollagen gel in the in vivo study

The cultured PG-atelocollagen gel containing chondrocytes was transplanted in a full-thickness articular cartilage defect (5 mm in diameter and 3 mm in depth) created in the patellar groove of the femur of a ten-week-old Japanese white rabbit. Six male rabbits weighing between 2.7 kg and 3.4 kg were used in this experiment. The surgery on the rabbits was performed under general anaesthesia by intravenous injection of 50 mg/kg of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, Illinois). After a medial parapatellar incision, each patella was dislocated laterally and a full-thickness articular cartilage defect was created on the patellar groove of the femur. The defect was filled with a cultured PG-atelocollagen gel containing chondrocytes and covered with a periosteal flap (6 $\times$ 6 mm). One knee of each rabbit was assigned randomly to be a control while the other received PG-atelocollagen gel. The periosteal flap which had already been harvested from the ipsilateral medial proximal tibia was sutured to the peripheral cartilage rim of defects by six interrupted 6–0 nonresorbable nylon sutures with the cambium layer facing the patella [22]. After the operation, all animals were allowed to walk freely in their cages without splints.

Macroscopic and histological evaluation of reparative tissue in the in vivo study

Grafted areas were macroscopically and histologically evaluated to assess the quality of reparative tissue. When the animals were killed at 12 weeks after operation, the surface of the grafted areas was graded on a scale of 0–8 as follows: 0 = normal, 1 = focal surface roughness, 2 = widespread surface irregularity, 3 = beginning surface fibrillation, 4 = severe surface fibrillation, 5 = beginning erosion, 6 = severe erosion, 7 = slight ulceration, and 8 = severe ulceration (proposed by Wachsmuth et al. [25]). After macroscopic observation, the distal part of the femur was excised and fixed with 10% buffered formalin for seven days. Each specimen was decalcified in 10% ethylenediaminetetraacetic acid (EDTA) in PBS for four weeks and embedded in paraffin. Sections (5 μ m thick) were cut through the grafted area sagittally and stained with safranin O and fast green. Immunohistochemical staining of type II collagen was also performed in a method similar to the in vitro study. The specimens containing the grafted area were evaluated according to the histological grading scale described by Wakitani et al. [26]. Toluidine blue staining, which was originally used for assessment of PG content of the repair matrix, was replaced with safranin O staining in this study.

Statistical analysis

All statistical evaluations were performed using SPSS software (version 12.0J, SPSS, Chicago, USA). Values are presented as mean $\pm$ standard deviation (SD). Statistical analysis of the data was performed by *t*-test and one-way analysis of variance followed by Tukey HSD test. A *P*-value less than 0.05 was considered to be significant.

Results

Cell proliferation in the in vitro study

The number of viable chondrocytes cultured in the 5.0 mg/ml PG-atelocollagen gel was significantly higher than that of the other three groups for each day (Fig. 1). The number of viable chondrocytes in the 5.0 mg/ml PG-atelocollagen gel was increased by 2.95 fold compared to control group at 21 days. There were no significant stimulatory effects of chondrocyte proliferation in 0.1 and 1.0 mg/ml PG-atelocollagen gel at seven and 21 days.

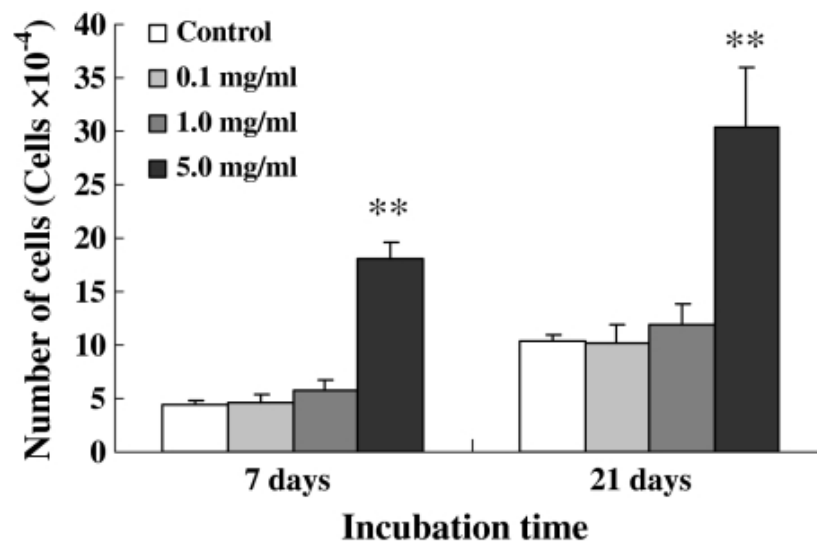


Fig. 1

Effect of exogenous cartilage PG on proliferation of chondrocytes in the in vitro study. Data represent averages of four independent experiments $\pm$ SD (***P* < 0.01 compared to control group)

Gene expression of *Col2a1* and *Aggrecan* in the in vitro study

Gene expression of *Col2a1* in PG-atelocollagen gel was not changed compared to that of the control group except for 5.0 mg/ml PG-atelocollagen gel (Fig. 2a, c). Gene expression of *Col2a1* in 5.0 mg/ml PG-atelocollagen gel was significantly decreased by 0.43 fold compared to that of the control group. Gene expressions of *Aggrecan* in 0.1 and 1.0 mg/ml PG-atelocollagen gel were increased by 2.34 and 2.05 fold compared to that of the control group, respectively, at 35 days of culturing (Fig. 2d). Although there was no statistical significance, gene expressions of *Aggrecan* in 0.1 and 1.0 mg/ml PG-atelocollagen gel were slightly decreased by 0.8 and 0.73 fold compared to that of the control group, respectively, at 21 days of culturing (Fig. 2b). Gene expression of *Aggrecan* in 5.0 mg/ml PG-atelocollagen gel was significantly decreased by 0.55 fold compared to that of the control group.

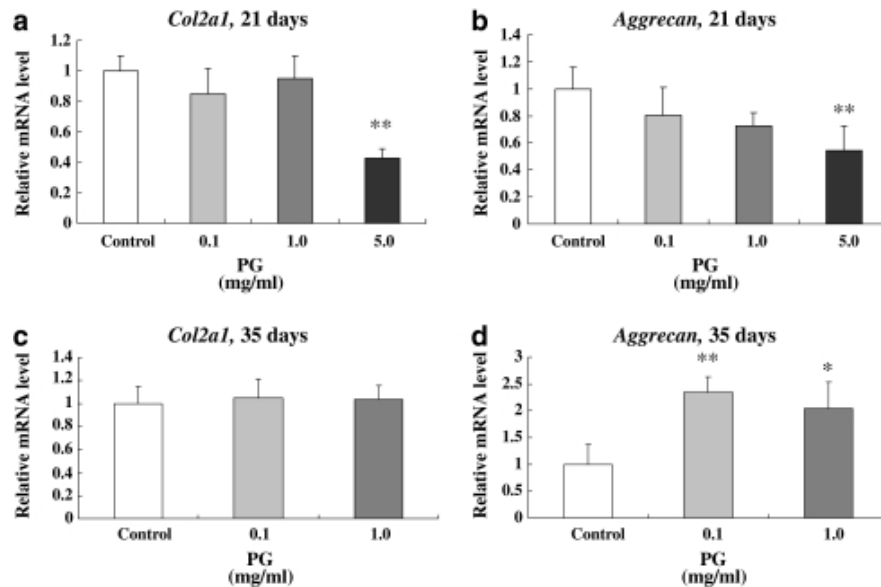


Fig. 2

Effect of exogenous cartilage PG on chondrocyte-specific gene expression in the in vitro study. Quantification of gene expression of *Col2a1* (a, c) and *Aggrecan* (b, d) was estimated by real-time PCR analysis at 21 (a, b) and 35 (c, d) days of 3D culture. The results are expressed as the fold increase of expression in relation to the control group. Data represent an average of four independent experiments $\pm$ SD (* $P < 0.05$, ** $P < 0.01$ compared to control group)

Histological evaluation in the in vitro study

Histological findings of 0.1 and 1.0 mg/ml PG-atelocollagen gels were similar to that of the control group at 21 days of culturing. Cells in the 5.0 mg/ml PG-atelocollagen gel were more densely formed than in other groups (Fig. 3a, b). Although GAG chains and type II collagen were clearly stained around cells in each gel, there were many cells in which pericellular staining was weak in the 5.0 mg/ml PG-atelocollagen gels (Fig. 3a, b). Pericellular staining with alcian blue in the 0.1 and 1.0 mg/ml PG-atelocollagen gel tended to be strong compared to the control group at 35 days of culturing although staining of areas without cells was similar between groups (Fig. 3c). Immunohistochemical staining of type II collagen at 35 days of culturing was similar between control and 0.1, 1.0 mg/ml PG-atelocollagen gel.

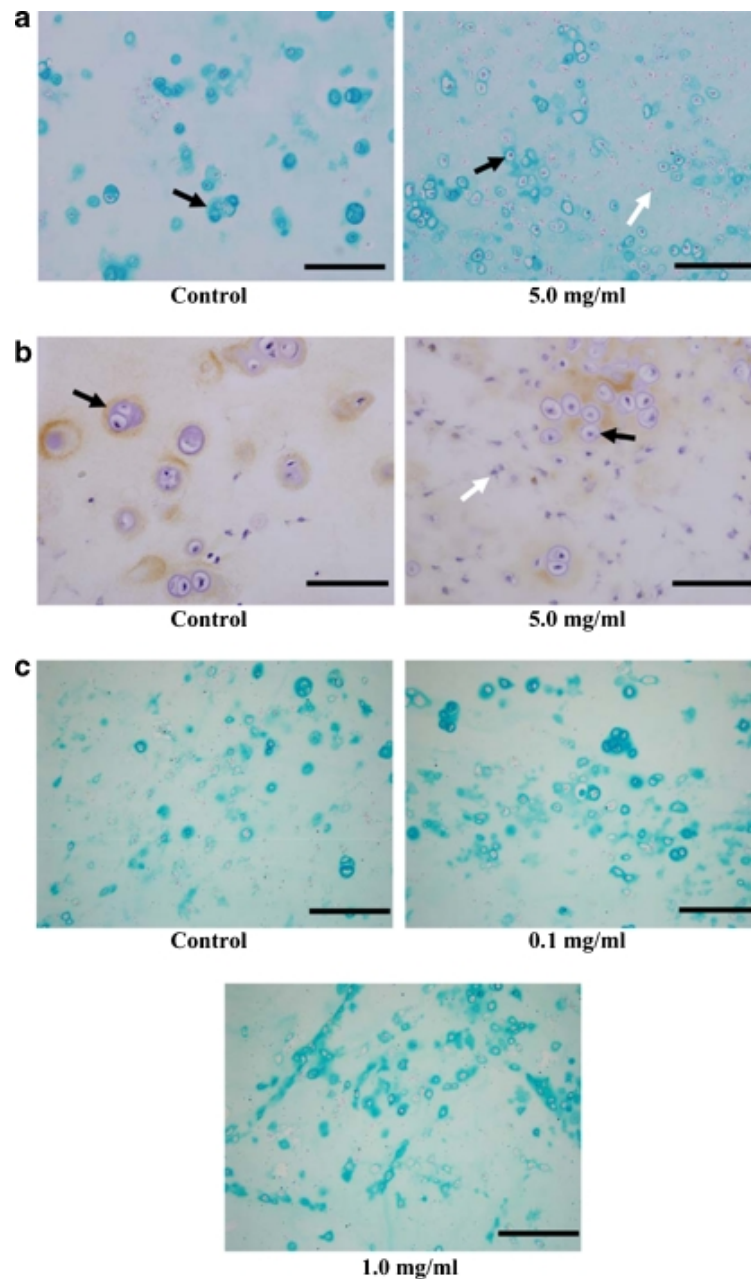


Fig. 3

Effect of exogenous cartilage PG on cartilage matrix formation in the in vitro study. At 21 (**a, b**) and 35 (**c**) days of culturing, 4- μ m sections of gels were stained with alcian blue for detecting GAG chains (**a, c**), and immunohistochemically stained for detecting type II collagen (**b**). *Black arrows* indicate cells in which pericellular staining is strong. *White arrows* indicate cells in which pericellular staining is weak. Scale bar: 200 μ m (**a, c**), 100 μ m (**b**)

Macroscopic and histological evaluation in the in vivo study

The defect was filled with reparative tissue which had a white, smooth and glossy surface at 12 weeks after surgery in the control and 0.1 mg/ml PG-atelocollagen gel groups (Fig. 4). Macroscopically, the surface of reparative tissue seemed to be connected to the adjacent normal cartilage, but most of the reparative tissues had a margin distinguishable from surrounding normal cartilage. In the control group, one knee was classified as grade 1 and five as grade 2. In the 0.1 mg/ml PG-atelocollagen gel group, three knees were classified as grade 1 and three as grade 2. There were no significant differences between groups. In both groups, no abnormal reactions occurred on the opposite patellar cartilage, and no sign of synovitis, adhesions, or adverse reactions were evident.

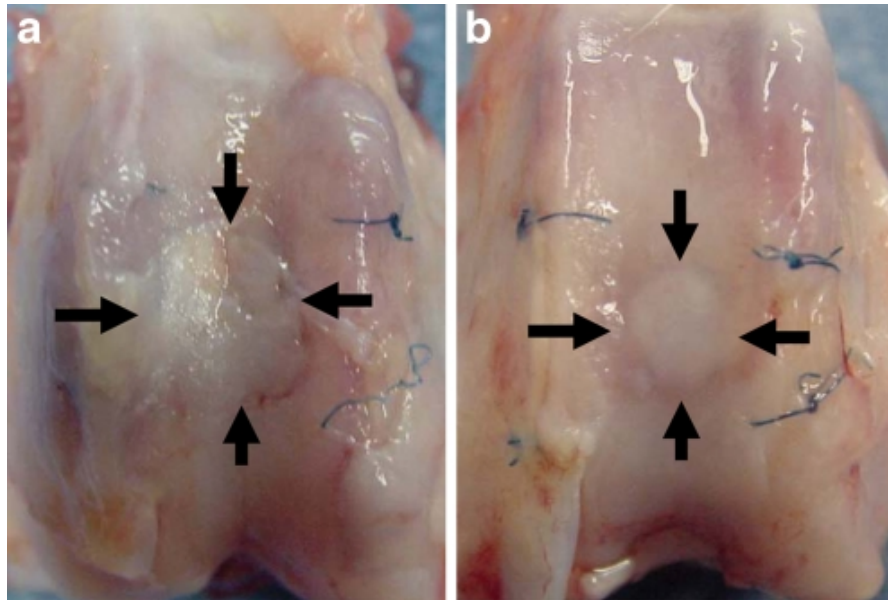


Fig. 4

Macroscopic appearance of the reparative tissues with transplantation of cultured PG-atelocollagen gel containing chondrocytes and periosteal flap at 12 weeks after surgery. **a** Control group. **b** 0.1 mg/ml PG group. The four *arrows* show the transition between repair and normal cartilage

The defect was filled with hyaline cartilage-like tissue which had small round cells, rich extracellular matrices which stained with safranin O, and stained immunohistochemically for type II collagen in the control and 0.1 mg/ml PG-atelocollagen gel groups at 12 weeks after surgery (Fig. 5). The reparative tissues were faintly stained in the superficial layer, and were well stained in the middle and deep layers. Many of them were thicker than adjacent normal cartilage. The average total score of the 0.1 mg/ml PG-atelocollagen gel group at 12 weeks after surgery was significantly better than that of the control group ($p = 0.028$; Table 2). Although there were no significant differences of each evaluated item, the score of surface regularity of the 0.1 mg/ml PG-atelocollagen gel group tended to show lower value than that of the control group ($p = 0.092$). The reparative tissue of the negative control group without gel implantation was fibrous at 12 weeks after operation (data was not shown) as Katsube et al. [22] reported similar results.

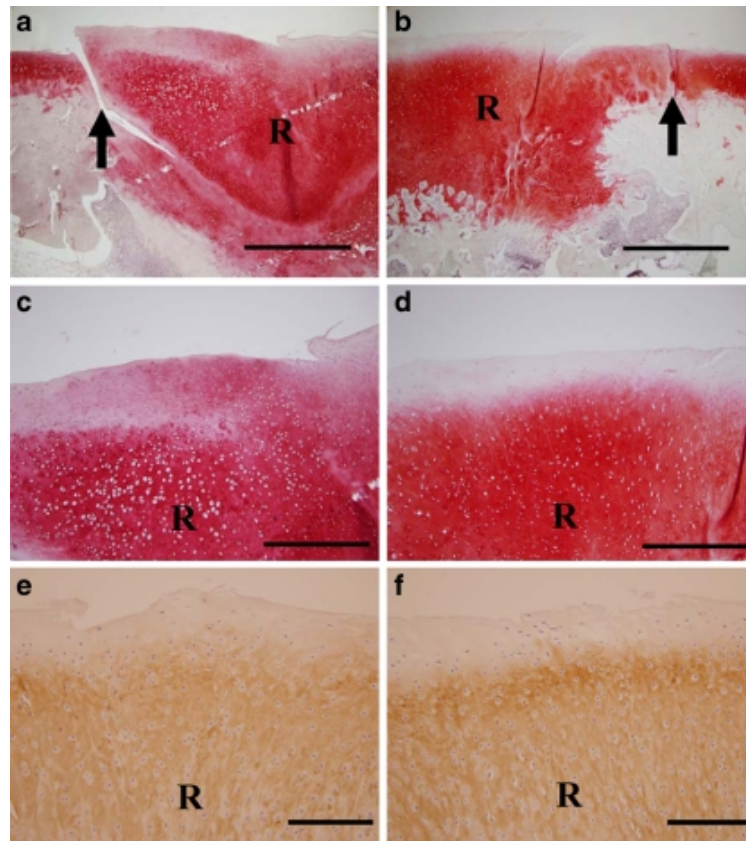


Fig.5

Histological sections of the reparative tissue with transplantation of cultured PG-atelocollagen gel containing chondrocytes and periosteal flap at 12 weeks after surgery in the control (a,c,e) and 0.1 mg/ml PG (b,d,f) groups. (a–d) Safranin O staining. (e,f) Immunohistochemical staining of type II collagen. *R* reparative tissue. Each arrow shows the transition between repair and normal cartilage. Scale bar: 1.0 mm (a,b), 500 μ m (c,d), 200 μ m (e,f)

Table 2

Histological scale scores for each group studied

Evaluation	Control (<i>n</i> = 6)	0.1 mg/ml PG (<i>n</i> = 6)
Cell morphology	0.67 $\pm$ 0.52	0.33 $\pm$ 0.52
Matrix staining	0.67 $\pm$ 0.52	0.67 $\pm$ 0.52
Surface regularity	0.83 $\pm$ 0.41	0.33 $\pm$ 0.52
Thickness of cartilage	0.50 $\pm$ 0.55	0.17 $\pm$ 0.41
Integration of donor with host adjacent cartilage	1.00 $\pm$ 0.63	0.67 $\pm$ 0.52
Average total score	3.67 $\pm$ 0.82	2.17 $\pm$ 1.17

Discussion

This study showed that PG-atelocollagen gel increased the gene expression of *Aggrecan* in cultured chondrocytes in vitro and resulted in an improvement in the average total score of reparative tissues in vivo. Previously, there have been few studies that investigated the potential for exogenous cartilage PG as a candidate material for 3D culture using chondrocytes. Currently, the atelocollagen gel culture method is clinically applied for the repair of chondral defects of the knees in Japan [23]. Recently, Tohyama et al. reported satisfactory clinical and arthroscopic outcomes of a prospective multicentre clinical trial in patients undergoing atelocollagen-associated autologous chondrocyte implantation [27]. Our results suggest that a new culture method, which contained exogenous cartilage PG in atelocollagen gel, improves clinical outcomes of cartilage repair.

In the in vitro study, high concentration of PG-atelocollagen gels (5.0 mg/ml) enhanced chondrocyte proliferation, but decreased the gene expression of *Col2a1* and *Aggrecan*. These results suggest that a high concentration of PG is unfavourable for maintaining quality of chondrocytes in 3D culture, and may even induce chondrocyte dedifferentiation. Previously, Zhang et al. reported that exogenous PG enhanced chondrocyte proliferation in monolayer culture [28]. They demonstrated that the G1 domain of recombinant versican protein, a member of the chondroitin sulfate proteoglycan family, stimulated chondrocyte proliferation by destabilising chondrocyte adhesion. Although our culturing method was the 3D culture, there is a possibility that the same mechanism had an effect on our results.

On the other hand, low concentration PG-atelocollagen gels (0.1 and 1.0 mg/ml) did not enhance chondrocyte proliferation. Gene expressions of *Aggrecan* in low concentration PG-atelocollagen gels were slightly decreased regardless of maintenance of gene expression of *Col2a1* compared to the control group at 21 days of culturing. There is a possibility that the synthesis of endogenous PG was inhibited because exogenous cartilage PG contained in atelocollagen gel formed aggregates with HA and link proteins which were produced around cultured chondrocytes in the early culture period. Subsequently, the gene expressions of *Aggrecan* were significantly increased in low concentration PG-atelocollagen gels in longer culture periods. Several studies have reported the effect of GAG or monosaccharides composing GAG on gene expression and accumulation of type II collagen and aggrecan in 3D culture of chondrocytes [16–18]. In addition, French et al. reported that the core protein of heparin sulphate PG had an important role in enhancing a chondrogenic differentiation pathway of C3H10T1/2 cells [19]. Although the action mechanisms of GAG and core protein were still unclear, degradation products of exogenous cartilage PG such as GAG, monosaccharides, and fragments of core protein, might affect the differentiation of chondrocytes cultured for longer culture periods.

In the in vivo study, 0.1 mg/ml PG-atelocollagen gel, which mostly increased the gene expression of *Aggrecan* in vitro, showed an improvement in the histological average total score of reparative tissues compared to the control group. Pericellular matrix formation caused by enhancement of gene expression of *Aggrecan* might induce the early maturation of repair tissues. Our results indicate that 0.1 mg/ml PG-atelocollagen gel is more favourable for maintaining quality of chondrocytes in vitro and in vivo than the atelocollagen gel without PG.

There were several limitations to this study. First, the sequence of core protein of the cartilage PG used in this study has not been defined. Previous analysis of the composition of GAG chains in that cartilage PG showed they are composed of 58% 6-sulphated unsaturated disaccharide units, 26% 4-sulphated unsaturated disaccharide units, 8.6% nonsulphated unsaturated disaccharide units and 7% disulphated unsaturated disaccharide units [24]. It will be necessary to investigate the sequence of core protein of cartilage PG, and to examine what components of core protein as well as GAG chains works for proliferation and differentiation of chondrocytes. Second, allogeneic chondrocytes were used in the in vivo study. Although allogeneic chondrocytes as well as core protein of exogenous cartilage PG have the possibility of immunogenic rejection, there were no signs of synovitis, adhesions, or adverse reactions. Katsube et al. reported that cultured allogeneic chondrocyte transplantation embedded in atelocollagen gel was effective in repairing an articular cartilage defect in a rabbit model [22]. They stated that allogeneic chondrocytes embedded in atelocollagen gel is protected from immunogenic rejection by the produced matrix and atelocollagen. The antigenicity of core protein may be masked in the same way. Third, the degree to which exogenous cartilage PG should be contained in atelocollagen

gel was not examined. Our results indicated a high concentration of PG is unfavourable for maintaining the quality of chondrocytes. It will be necessary to examine the effect of low concentration PG in detail on differentiation and matrix production of chondrocytes.

Despite these limitations, this study showed the potential effect of exogenous cartilage PG contained in atelocollagen gel with chondrocytes on repair of a full-thickness articular cartilage defect. Future study is needed to clarify the sequence of core protein and the optimal concentration of cartilage PG contained in atelocollagen gel. Elucidation of the biological function of PG may aid the development of newer approaches to promote the healing of cartilage defects.

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