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FULL PAPER

Effects of ascorbic acid on proliferation and biological properties of bovine chondrocytes in alginate beads

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Abstract

Bovine chondrocytes were cultured in monolayers and alginate beads with or without ascorbic acid (Asc) for 16 days. Cell proliferation was examined every 4 days by staining with Hoechst 33258 dye. The gene expression of aggrecan, and collagen type I and II was analyzed at 16 days by reverse transcription and polymerase chain reaction. Cell morphology and the production of extracellular matrix (ECM) were evaluated by cytochemical, immunocytochemical and electron microscopical methods. Cells were continuously cultured in alginate beads with Asc for 2 months, and the cell morphology and ECM were examined. The proliferation of chondrocytes was significantly stimulated with Asc in both monolayers and alginate beads at 16 days. Expression of the collagen type I gene in both cultures was increased, and that of the collagen type II gene in alginate beads was decreased, by Asc. There were no significant cytochemical and immunocytochemical differences between the cultures in alginate beads with or without Asc at 16 days. In alginate beads cultured with Asc for 2 months, proliferating cells were observed mainly at the periphery of the beads, and glycosaminoglycan and collagen type II were found around the cells. These results suggest that Asc stimulated the proliferation of chondrocytes and maintained the chondrogenic properties of the cells in an alginate beads culture.

Keywords : Alginate, Bovine, Cartilage tissue engineering, Chondrocytes

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With 3 tables and 4 figures
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Introduction

Damaged articular cartilage which is an avascular, aneural and alymphatic tissue has usually very limited potential for repair[33,42]. Autologous chondrocyte transplantation has been reported to be one of the most effective methods of accelerating the regeneration of damaged cartilage and to reestablish the articular surface with hyaline cartilage[6,7,16]. To apply this method, chondrocytes that maintain their properties need to proliferate in *vitro*.

The proliferation and properties of chondrocytes have been investigated in a variety of culture systems. Monolayer cultured chondrocytes attach to the culture plate and dedifferentiate rapidly into spindle-shaped cells and lose the ability to produce cartilage-specific collagen type II[2,28,44]. Three-dimensional (3-D) culture of chondrocytes in agarose or alginate has become popular for the maintenance of chondrogenic properties[3,4,17,18] and for the redifferentiation of dedifferentiated chondrocytes[24,28]. Chondrocytes can be cultured in ionically gelled alginate beads with calcium ions or other multivalent ions, and alginate beads can be rapidly solubilized by the addition of a calcium chelator. Alginate has been also used as a 3-D scaffold for the transplantation of chondrocytes in plastic and reconstructive surgery[14,34].

Ascorbic acid (Asc), an antioxidant nutrient, was reported to have an inhibitory effect on the progression of osteoarthritis in guinea pigs and human[29,31,39]. The growth of chondrocytes and the production of extracellular matrix (ECM) were influenced by Asc under different culture conditions[1,9,37]. Ascorbic acid was reported to stimulate the growth of chondrocytes in many studies[19,37,46], though an inhibitory effect was also reported[10]. Moreover, there are many controversial studies on ECM components such as aggrecan[5,30], and collagen type I and II[9,13,37]. Effects of Asc on cell proliferation and ECM production have not yet been elucidated, and no reports have analyzed the effects of Asc on chondrocytes cultured with alginate beads.

The aim of the present study was to analyze the effects of Asc on the proliferation and biological properties of bovine chondrocytes in 3-D cultures using alginate beads. In addition, the data were compared with those obtained from a monolayer culture with Asc.

Materials and methods

Chondrocyte isolation : Articular cartilage was harvested from 4 clinically healthy adult Holstein-Friesian cows within 24 hr after death at a local slaughterhouse. Bovine chondrocytes were isolated individually as described previously with a slight modification[22,27]. Briefly, cartilage pieces were harvested from the carpal and tarsal joints and were digested with 1 mg/ml of collagenase (Wako Pure Chemical, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand island, New York, USA) for 18hr at 37℃ with gentle stirring. Cells were filtered through a 100 μm nylon mesh and centrifuged at 200×*g* for 5 min. The medium used in this experiment contained 63.5 μg/ml of penicillin G, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B.

Monolayer culture : Cells were resuspended in DMEM containing 10% fetal bovine serum (FBS) with or without 50 μg/ml of Asc (L(+)-Ascorbic Acid, Kanto Chemical, Tokyo, Japan) and were cultured at a density of 2×10^5cells/well in four 6-well culture plates for each cow. Cells were cultured at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed daily for 16 days. At intervals of 0, 4, 8, 12 and 16 days,

cells in a well were harvested with 0.25% trypsin (Nacalai Tesque Inc., Kyoto, Japan) and were placed in a 1.5 ml centrifuge tube for the cell proliferation assay. Cells in two wells were used for ribonucleic acid (RNA) extraction at 16 days.

Alginate culture : The alginate culture was prepared as described by Guo et al.[17] with slight modifications. A solution of low-viscosity sodium alginate (2.4%, Wako Pure Chemical) in 0.15M sodium chloride was sterilized by filtering through a 0.45-μm pore size filter (Millipore, Bedford, Massachusetts, USA). Cells were resuspended individually in DMEM at a density of $4 \sim 8 \times 10^6$ cells/ml and mixed (1 : 1) with a 2.4% solution of sodium alginate. The cell suspension in alginate solution (1.2%) at a density of $2 \sim 4 \times 10^6$ cells/ml was dropped slowly using a yellow pipette (1-200μl, Corning, Corning, New York, USA) into 30 ml of a 102mM calcium chloride solution in a 90-mm dish (Clinical Test Ware™, Iwaki Glass, Tokyo, Japan) agitated gently. After instantaneous gelation, approximately 90 beads from each cow were allowed to polymerize for 10 min at room temperature. Beads were washed three times with 0.15 M sodium chloride and then cultured in a 100-mm plastic tissue culture dish (Corning) in DMEM containing 10% FBS with or without 50 μg/ml of Asc. The diameter of the beads was measured using a vernier caliper and was approximately 2.5mm. The culture medium was changed daily for 16 days. Every 4 days, 3 beads for each cow were resolved in a 1.5ml centrifuge tube with 200 μl of a solution containing 50mM ethylenediaminetetraacetic acid and 10mM 2 [4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, and were used for the cell proliferation assay. Cells from 6 beads for each cow were used for RNA extraction and those from 6 beads for the evaluation of cell morphology and ECM development at 16 days.

Beads for 2 cows were cultured with Asc continuously in a new culture plate for 2 months and were used for the examination of cell morphology and ECM. The culture medium was changed every 2 days and the plate was changed every 6 or 8 days.

Cell proliferation assay : The proliferation of cells was assayed using the Hoechst 33258 fluorescent dye technique[23]. Cells in a 1.5 ml centrifuge tube were collected by centrifugation (600×*g* for 10 min). After removal of the supernatant solution in the centrifuge tubes, cells were digested over night at 60℃ with 1 ml of papain (125 μg/ml, Wako Pure Chemical) in 0.01M Dulbecco's phosphate-buffered saline (pH7.4) containing 5mM ethylenediaminetetraacetic acid disodium salt and 5mM L-cysteine hydrochloride. The amount of deoxyribonucleic acid (DNA) was measured in duplicate using Hoechst 33258 dye (Wako Pure Chemical). Calf thymus DNA (Sigma Chemicals, Saint Louis, Missouri, USA) was used as the standard. Excitation (365nm) and emission (440nm) were measured with a spectrofluorometer (FP 777, Jasco, Tokyo, Japan).

Reverse transcription and polymerase chain reaction (RT-PCR) : Total RNA was extracted from cells in a well of monolayer culture and 3 alginate beads respectively using TriZol reagent (Gibco BRL) at 16 days. Reverse transcription of 0.5μg of total RNA and PCR amplification of DNA was performed in duplicate using a commercially available kit (RNA PCR Kit (AMV) Ver. 2.1; Takara Shuzo, Otsu, Shiga, Japan). PCR primers for bovine aggrecan, and collagen type I and II were prepared as described in a previous report[38] with slight modifications. PCR primer pairs for β-actin were designed from a reference as

Table 1. Primer sequences used in reverse transcription and polymerase chain reaction.

Gene	Primer sequence	Product size	Primer source*
Collagen type I	F, 5'-TGCTGGCCAACCATGCCTCT	491bp	AB008683
	R, 5'-ATTGCACAATGCTCTGATCA		
Collagen type II	F, 5'-CCACTGCAAGAACAGCATTG	463bp	X02420
	R, 5'-CCAGTTCAGGTCTCTTAGAG		
Aggrecan	F, 5'-CACTGTTACCGCCACTTCCC	303bp	U76615
	R, 5'-GACATCGTTCCACTCGCCCT		
β-actin	F, 5'-CGCACCACTGGCATTGTCAT	227bp	K00622
	R, 5'-TCCAAGGCGACGTAGCAGAG		

* Gene Bank accession number

Table 2. Effect of ascorbic acid (Asc) on cell proliferation in monolayer and alginate beads cultures (%of Day 0)

		Day 0	Day 4	Day 8	Day12	Day16
Monolayer	without Asc	100	135±14	321±22	414±15	464±26
	with Asc	100	159±25	422±23*	694±56*	816±59*
Alginate beads	without Asc	100	107± 4	102± 9	119±11	132±16
	with Asc	100	109± 5	124±18	145±15**	163±32**

Chondrocytes were cultured with or without 50 μg/ml Asc. Data represent the mean (standard deviation of 4 independent experiments. * $P<0.05$ when compared with monolayer culture without Asc on the same day. ** $P<0.05$ when compared with alginate beads culture without Asc on the same day.

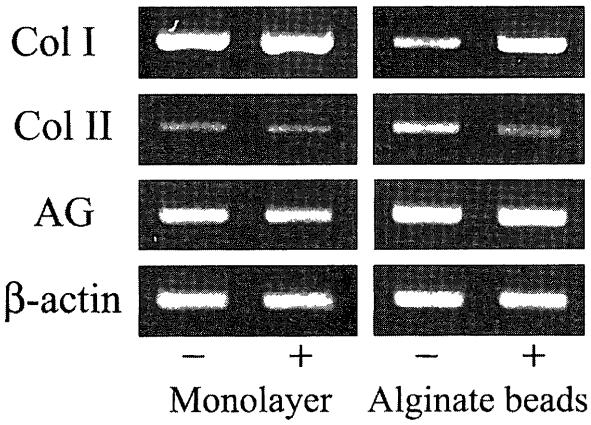

Fig. 1. Reverse transcription and polymerase chain reaction analysis of specific genes in bovine chondrocytes cultured in monolayers and alginate beads. Cells were cultured with Asc (+) and without Asc (−) for 16 days. The gene of collagen type I; Col I, collagen type II; Col II, aggrecan; AG, bovine β-actin; β-actin.

shown in Table 1 [11]. After checking the linear formation of PCR production using 20, 25 and 30 cycles, 25 cycles was chosen. The thermocycle program included one cycle of 94℃ for 2 min of initial heating, followed by repeated cycles of 94℃ for 45 sec, 60℃ for 45 sec and 72℃ for 90 sec. Final extension was carried out at 72℃ for 7 min. PCR products were separated in 2% agarose gel and visualized with ethidium bromide. PCR bands were analyzed using Scion Image software (Beta 4.02 for Windows).

Cytochemical and immunocytochemical studies : Beads were fixed in 10% formalin for 2 days, then washed, dehydrated and embedded in paraffin at 60℃. Sections were cut to a thickness of 5 μm and were stained with hematoxylin-eosin for morphological examination of the cells and with alcian blue (pH 1.0) for the detection of sulfated glycosaminoglycan. For the detection of collagen type I and II, dehydrated sections were treated with 2.5% hyaluronidase (Type I-S, Sigma Chemicals) for 1 hr at 37℃ and a 3% hydrogen peroxide methanol solution for 30 min. Sections

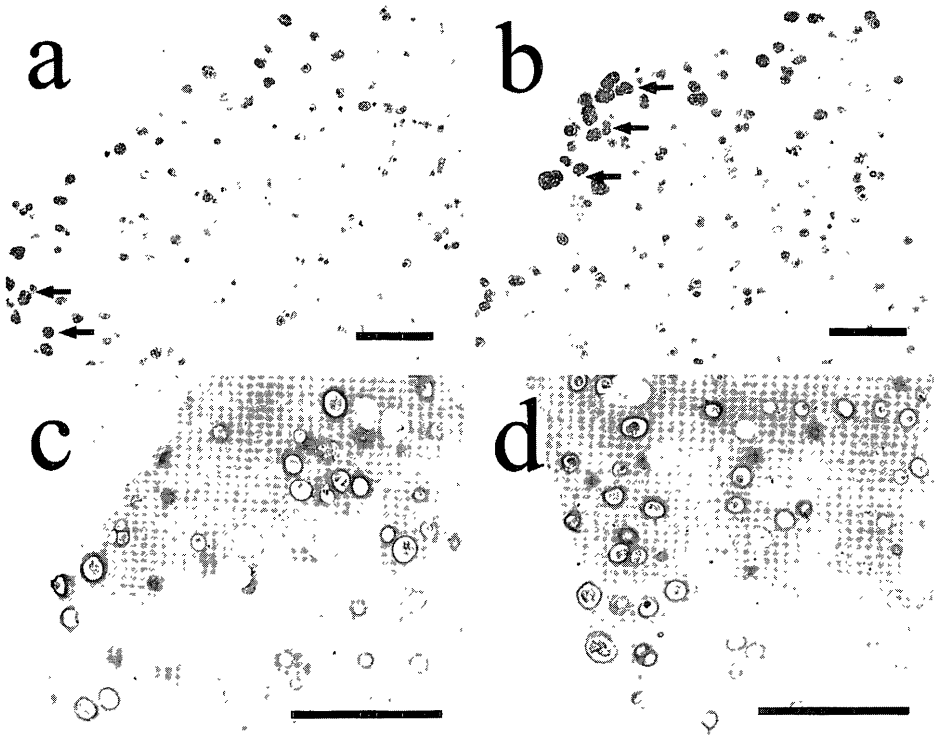

Fig. 2. Photomicrograph of a section of alginate beads at 16 days. Ascorbic acid stimulates the proliferation of chondrocytes (a, b). Cell proliferation (arrows) is marked at the periphery of alginate beads. Glycosaminoglycan was detected in pericellular matrix (c, d). a, c: Alginate beads cultured without Asc. b, d: Alginate beads cultured with Asc. a, b: Hematoxylin-eosin stain. c, d: Alcian blue stain. Scale bars = 100 μm.

were incubated with 10% normal goat serum for 1 hr at 37℃. Anti-bovine collagen type I and II (LSL, Tokyo, Japan) diluted 1 : 500 and 1 : 1,000 respectively in 0.01 M phosphate-buffered saline (pH7.4) were applied as a primary antibody at 4 ℃ overnight, and sections were then treated with anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark) as a secondary antibody, and visualized using a rabbit PAP kit (DAKO). Collagen type I and II were finally detected with 0.05% diaminobenzidine containing 0.01% hydrogen peroxide. The negative control was treated without primary antibody. Thirty cells per gel were randomly selected and cells immunoreactive for collagen type I or II were counted.

Transmission electron microscopy : The fixation of beads was performed as described in the literature[44]. Beads were prefixed for 24 hr with 2% glutaraldehyde and 2% tannic acid in 0.1M sodium-cacodylate to which 10 mM calcium chloride was added. After a wash with 0.1M sodium-cacodylate buffer, beads were postfixed with 1% osmium tetroxide in 0.1M sodium-cacodylate for 1 hr. They were then embedded in epon and processed for electron microscopy, and sections were stained with ethanolic uranyl acetate and aqueous lead citrate. All electron micrographs were obtained using a JEOL-1210 (JEOL, Tokyo, Japan).

Data analysis : The data obtained from the

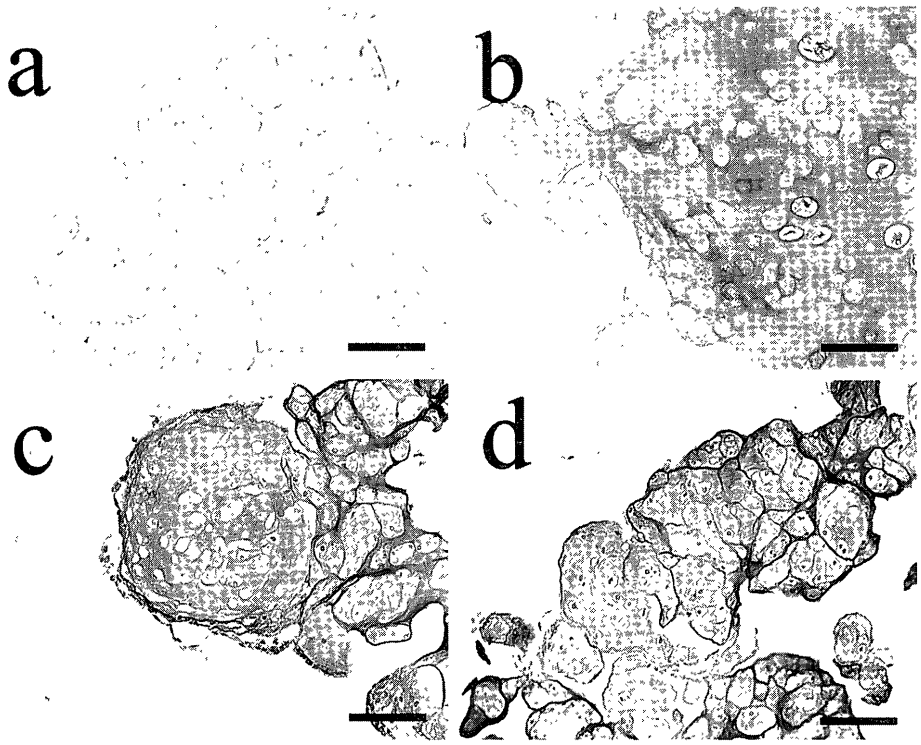

Fig. 3. Photomicrograph of a section of alginate beads after 2 months. The negative control of immunohistochemical stain was treated without primary antibody (a). Pericellular matrix stained with alcian blue is clearly visible at the periphery of alginate beads (b). Collagen type I (c) and II (d) are detected in the pericellular matrix of a majority of the cells. a, c, d : immunohistochemical stain. b : Alcian blue stain. Scale bars = 100 μm.

Table 3. Percentage of cells immunoreactive for collagen type I or II at 16 days in alginate beads.

	Collagen type I	Collagen type II
without Asc	68%	80%
with Asc	71%	79%

Thirty cells per gel were counted randomly. Data represent percentages of 4 independent experiments. There was no significant difference on culturing with or without Asc.

cell proliferation assay were compared by the paired t-test at a level of significance of $P < 0.05$.

Results

Chondrocyte proliferation : Asc stimulated the proliferation of chondrocytes both in the monolayer and the alginate beads (Table 2). After 16 days, DNA concentration of cells had increased approximately 8.2 fold in the monolayer culture and 1.6 fold in the alginate beads compared with the concentration on day 0.

Analysis of chondrocyte gene expression : Expression of the aggrecan, collagen type I and II genes was analyzed at 16 days. As shown in Fig. 1, a marked band of the collagen type I gene was observed in chondrocytes of a monolayer culture compared with that in cells of the alginate beads culture. In alginate beads, the aggrecan and collagen type II genes of cells were expressed at higher levels than in the monolayer culture. The ra-

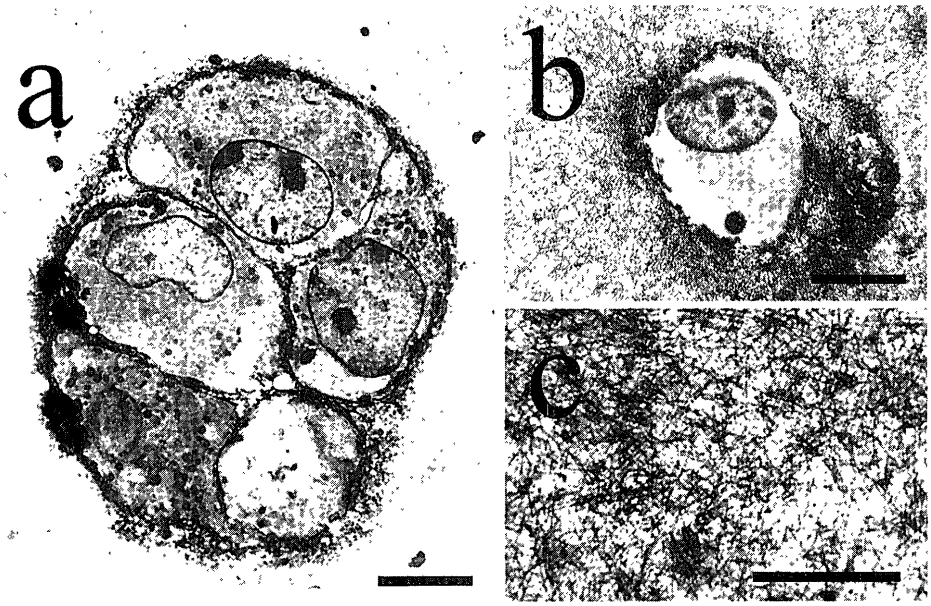

Fig. 4. Electron micrographs of bovine chondrocytes cultured in alginate beads. Chondrocytes as clusters of 4 - 7 cells were observed at the periphery of the beads at 16 days (a). More extracellular matrix and collagen fibers are observed in alginate beads cultured for 2 months (b). Higher magnification image of the dense collagen fibers (c). Scale bars = 5 μm.

tio (R) was calculated as the density of specific messengerRNA/density of β-actin messenger-RNA, and the mean R and standard deviation of 4 independent experiments were obtained. Expression of the collagen type I gene in each of the 4 cows in monolayer ($R=1.29\pm0.11$) and alginate beads ($R=1.01\pm0.09$) cultures seemed to be higher with as compared ($R=1.22\pm0.13$ and $R=0.90\pm0.14$, respectively) to without Asc. Expression of the collagen type II gene ($R=0.88\pm0.17$) in alginate beads culture seemed to be lower with as compared ($R=0.98\pm0.12$) to without Asc. However, ratios were not significantly different with or without Asc.

Cytochemical and immunocytochemical evaluation : Chondrocytes cultured in alginate beads were observed as round to oval cells at 16 days. Cell proliferation was marked at the periphery of the alginate beads, while cells in the central area were seen as individuals or small clusters (Figs. 2a and 2b). Glycosaminoglycan (Figs. 2c and 2d), and collagen type I and II were detected in the pericellular matrix with or without Asc in culture at 16 days. The percentage of cells immunoreactive for collagen type I was approximately 70% and for collagen type II, 80% (Table 3). No clear difference in the synthesis of the ECM was observed between alginate cultures with or without Asc at 16 days.

In alginate beads cultured for 2 months, an intensive proliferation of cells was also observed at the periphery of the alginate beads, and several prominent cell clusters were formed on the surface of the beads (Fig. 3). Cells were round and often observed at primitive lacunae. Pericellular matrix stained with alcian blue was clearly visible at the periphery of alginate beads (Fig. 3b). Collagen type I and II were also detected in the pericellular

matrix of the majority of the cells (Figs. 3c and 3d).

Transmission electron microscopy: Chondrocytes were observed as clusters of 4 - 7 cells at the periphery of alginate beads at 16 days (Fig. 4a). Extracellular matrix was formed around clusters and individual cells, while collagen fibers were not observed at 16 days. No clear difference in the synthesis of the ECM was observed between alginate cultures with or without Asc at 16 days. More ECM and collagen fibers were observed in alginate beads cultured for 2 months (Fig. 4b).

Discussion

A lot of chondrocytes that maintain the chondrogenic phenotype are necessary for transplantation to damaged cartilage[10]. In this study, the effects of Asc on the proliferation and biological properties of chondrocytes were analyzed in alginate beads cultured up to 2 months.

The proliferation of primary bovine chondrocytes was stimulated with Asc in monolayer cultures and alginate beads. This result was the same as the reports that Asc stimulates the growth of chondrocytes cultured in monolayer and collagen cultures[19,37,46]. The hypertrophy of chondrocytes and incorporation of thymidine required for cell proliferation were stimulated with Asc[46]. The inhibitory effect on the proliferation of primary bovine chondrocytes in the other study[10] was however not observed in our study.

Cell proliferation was marked at the periphery of alginate beads compared to the central area. It is conceivable that the cells at the periphery were more exposed to the culture medium. This result has an important implications for better outcomes with alginate beads culture. Beads are usually made by the gelation of alginate using a needle (19 gauge to 25) or yellow pipette of 200 μl. The beads used in the present study were almost the same diameter (2.5mm) as in previous reports[17,26]. Therefore, more small beads or a flat alginate bed may help in exposing a larger number of cells to the medium. Recently, modifications to alginate cultures such as a layer culture have been reported[21,36]. However, the effect on cell proliferation in this type of alginate culture has not been analyzed yet.

The gene expression of chondrocytes in the alginate beads culture was examined by RT-PCR and compared with the result in the monolayer culture at 16 days. Expression of the collagen type I gene in the monolayer and alginate beads increased, and that of the collagen type II gene in alginate beads decreased, in the presence of Asc. Similar data on the expression of the collagen type I and II genes were obtained in monolayer culture of bovine chondrocytes[15,19]. By contrast, the expression of collagen type II of bovine fatal epiphyseal chondrocytes was increased after 1 month of 3 -D culturing in collagen sponges with Asc[37]. It is thought that this is due to the change of metabolic activities with age and/or the form of collagen sponges. It was also reported that the attachment, proliferation and metabolism of chondrocytes are affected by ECM components such as collagen type I and II , fibronectin and chondronectin[8,25,40].

Glycosaminoglycan, and collagen type I and II were detected in the pericellular matrix at 16 days, but a clear difference in the synthesis of the ECM was not detected between alginate cultures with or without Asc. It is thought that for chondrocytes, a long period is needed to evaluate ECM production by histological methods. The percentage of cells immunoreactive for collagen type I was 70% in alginate beads at 16 days. This result was different from previous reports[12,35,44]. In our

study, chondrocytes isolated from the cartilage of adult cattle were used, and the articular cartilage layer was very thin. It was reported that collagen type I was detected by an immunohistochemical examination in the cartilage of aged animals with osteoarthritis, while normal articular hyaline cartilage with a relatively thick layer contains only collagen type II in the ECM[20,32]. However, the reason for the high percentage of cells immunoreactive for collagen type I in our culture cannot be explained from the present data and information from previous reports.

In alginate beads cultured with Asc for 2 months, glycosaminoglycan and collagen type II, markers of the chondrogenic phenotype, were detected in the pericellular matrix. Collagen type I, a marker of the dedifferentiation of chondrocytes, was also detected. Chondrocytes dedifferentiated by *in vitro* culturing can produce hyaline cartilage after *in vivo* implantation[28,41,43]. It is conceivable that chondrocytes need to maintain a more chondrogenic phenotype for fast redifferentiation after implantation.

It was thought that the proliferation of cells in alginate beads was too slow to be applied in transplantation. In the primary culture of chondrocytes, monolayer culturing is therefore an effective method for the proliferation of cells, which were tolerably able to maintain a chondrogenic phenotype.

In conclusion, the present study suggests that Asc stimulates the proliferation of chondrocytes, and the cells retain chondrogenic properties until 2 months in alginate beads with Asc.

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