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Effect of Chondroitin Sulfate and Hyaluronic Acid on Gene Expression in a Three-Dimensional Culture of Chondrocytes

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NOTES

The effect of glycosaminoglycan addition on a three-dimensional (3D) culture of porcine chondrocyte cells was investigated with a view to use in cartilage regenerative medicine. Chondroitin sulfate C increased the mRNA expression of type 2 collagen, while chondroitin sulfate A did not. Hyaluronic acid of high molecular weight markedly decreased the mRNA expression of both aggrecan and type 2 collagen, although hyaluronic acid of low molecular weight showed no apparent effect.

[**Key words:** chondrocyte, chondroitin sulfate, hyaluronic acid, RT-PCR]

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Although several three-dimensional (3D) cultivation methods of chondrocyte cells were developed with a view to their use in regenerative medicine, sufficient accumulation of extracellular matrices of aggrecan and type 2 collagen has not yet been achieved. While the addition of several cytokines such as transforming growth factor (TGF)- β and bone morphogenetic protein (BMP) was shown to increase the production of these extracellular matrices, they are too expensive. On the other hand, the addition of glycosaminoglycans, which are components of aggrecan and not expensive, was investigated (1-3). Several oral supplements of glycosaminoglycans containing hyaluronic acids of high and low molecular weight, which are claimed to effect cartilage repair, are also commercially available. However, there is no report on the influence of glycosaminoglycans such as hyaluronic acids of high and low molecular weight on the gene expression of aggrecan and type 2 collagen.

Recently, a 3D culture method combining a collagen gel and a copolymer mesh of polylactate and polyglucuronic acid (PLGA), which realizes both uniform cell distribution and mechanical strength without shrinkage during culture, was developed (4).

In the present study, the effect of the addition of several glycosaminoglycans on the gene expression of aggrecan and type 2 collagen in chondrocyte cells during 3D culture combining a collagen gel and a PLGA mesh was studied.

Articular cartilage was harvested aseptically from the femoropatellar

grooves of the knee joints of pigs (4). Chondrocyte cells were cultivated three-dimensionally combining a collagen gel (0.5%, pH 3, Kokencellgen I-PC™; Koken, Tokyo) and PLGA mesh (6 mm ϕ , 0.25 mm thickness, interval between bundles of fibers; approximately 400 μ m, Vicryl Mesh 910™; Johnson & Johnson, Tokyo) (4). The culture medium consisted of MEM (Gibco, NY, USA), 10% FCS, 2500 U/l penicillin, 2.5 mg/l streptomycin, and 50 μ g/ml L-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka). Several glycosaminoglycans, namely, chondroitin sulfate C sodium salt from shark cartilage (Sigma, St. Louis, MO, USA), chondroitin sulfate A sodium salt from bovine trachea (Sigma), high-molecular-weight hyaluronic acid sodium salt from rooster comb (Mw; 1,300,000 – 2,000,000; Sigma), low-molecular-weight hyaluronic acid sodium salt from pig skin (Mw; 100,000 - 150,000; Seikagaku, Tokyo), heparin sodium salt from pig small intestine (Wako Pure Chemicals), and dermatan sulfate (Sigma), were added to the culture medium. The gelling medium contained 30.5 g/l MEM, 35.7% FCS, 8930 U/l penicillin, 8.93 mg/l streptomycin, and 179 μ g/ml L-ascorbic acid 2-phosphate.

The 3D gel culture was hydrolyzed at 37°C for 3 h using 2.5 g/l collagenase. Cell concentration was determined by the Trypan Blue method after hydrolysis. Total RNA was extracted from cells after the gel cultures (n=3) using a RNeasy Mini kit (Qiagen, Victoria, Australia). DNase-treated RNA was used to produce cDNA using Omniscript and

Sensiscript RT kits (Qiagen) and the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR was performed with the cDNA using a HotStar Taq Master Mix kit (Qiagen) and ABI PRISM 7700 (Applied Biosystems) using actin as the standard. The sequences of primers and probes are listed in Table 1. The ratios of the mRNA expression of aggrecan and type 2 collagen to that of actin were calculated.

The collagen-gel culture was rinsed twice with PBS, fixed in 20% formalin, dehydrated through a graded series of ethanol, infiltrated with isoamyl alcohol, and embedded in paraffin. Sections of 3 μ m thickness were cut through the center of the gel, rinsed with xylene and ethanol, immersed in hydrogen peroxide (0.3%) and methanol, and treated with hyaluronidase (1 mg/ml, Sigma). Each section was stained with a mouse anti-type 2 collagen antibody (RDI, Flanders, NJ, USA) employing an ImmunoPure Ultra-sensitive ABC mouse IgG staining kit (Pierce, Rockford, IL, USA) and DAB/Metal Concentrate (Pierce).

Three-dimensional cultures were performed for 14 d employing the culture medium supplemented with several glycosaminoglycans (chondroitin sulfate C, chondroitin sulfate A, hyaluronic acid of high molecular weight, hyaluronic acid of low molecular weight, heparin, and dermatan sulfate), and the degree of expression of aggrecan and type 2 collagen mRNA was determined (Fig. 1). There was almost no change following the addition of heparin and dermatan sulfate (data not shown). The addition of glycosaminoglycans did not influence cell number in the gel

(Figs. 1 and 2). Hyaluronic acid of high molecular weight markedly suppressed the expressions of aggrecan and type 2 collagen mRNA, while there was no apparent change in the expression with the addition of hyaluronic acid of low molecular weight (Fig. 1).

Chondroitin sulfate A did not affect the expression of type 2 collagen mRNA, while there was a slight increase in aggrecan mRNA expression with the addition of chondroitin sulfate A (10 mg/l) (Fig. 2). On the other hand, chondroitin sulfate C (100 mg/l) markedly increased the mRNA expression of type 2 collagen and slightly increased the mRNA expression of aggrecan (Fig. 2). Lower (10 mg/l) and higher (1000 mg/l) concentrations of chondroitin sulfate C had no apparent effect (data not shown).

Sections of cultures with or without the addition of chondroitin sulfate C were stained at 2 weeks with the anti-type 2 collagen antibody (Fig. 3). The three-dimensional culture with the addition of chondroitin sulfate C gave better staining compared with that without the addition.

The increase in total protein during 4 weeks of 3D culture was approximately one fifth of the total protein in primary cartilage tissue (data not shown). Thus, improvement of the cultivation conditions may be necessary to obtain appropriate cartilage tissue *in vitro* for regenerative medicine, while the addition of chondroitin sulfate C was effective to enhance the gene expression and accumulation of type 2 collagen.

A receptor (annexin 6) for chondroitin sulfate was found on the surface of dermal cancer cells (5), although there is little research on receptors for

glycosaminoglycans. The results obtained here suggested that chondrocyte cells also have a cell surface receptor for chondroitin sulfate C and that the binding of chondroitin sulfate C to this receptor resulted in the increase of the expression of type 2 collagen mRNA. This receptor may not bind chondroitin sulfate A, because there was almost no effect following the addition of chondroitin sulfate A (Fig. 2). The reason for the difference between the two types of hyaluronic acids was not clear.

Consequently, among several glycosaminoglycans; namely, chondroitin sulfates C and A, hyaluronic acids of high and low molecular weights, heparin, and dermatan sulfate; chondroitin sulfate C markedly increased the expression level of type 2 collagen mRNA in a 3D culture of chondrocytes, while hyaluronic acid of high molecular weight suppressed both the expression of aggrecan and type 2 collagen mRNAs. Addition of chondroitin sulfate C might contribute to the accumulation of type 2 collagen during 3D culture of chondrocyte cells with a view to their use in cartilage regenerative medicine, while the influence of the addition of chondroitin sulfate C on the accumulation of type 2 collagen should be determined.

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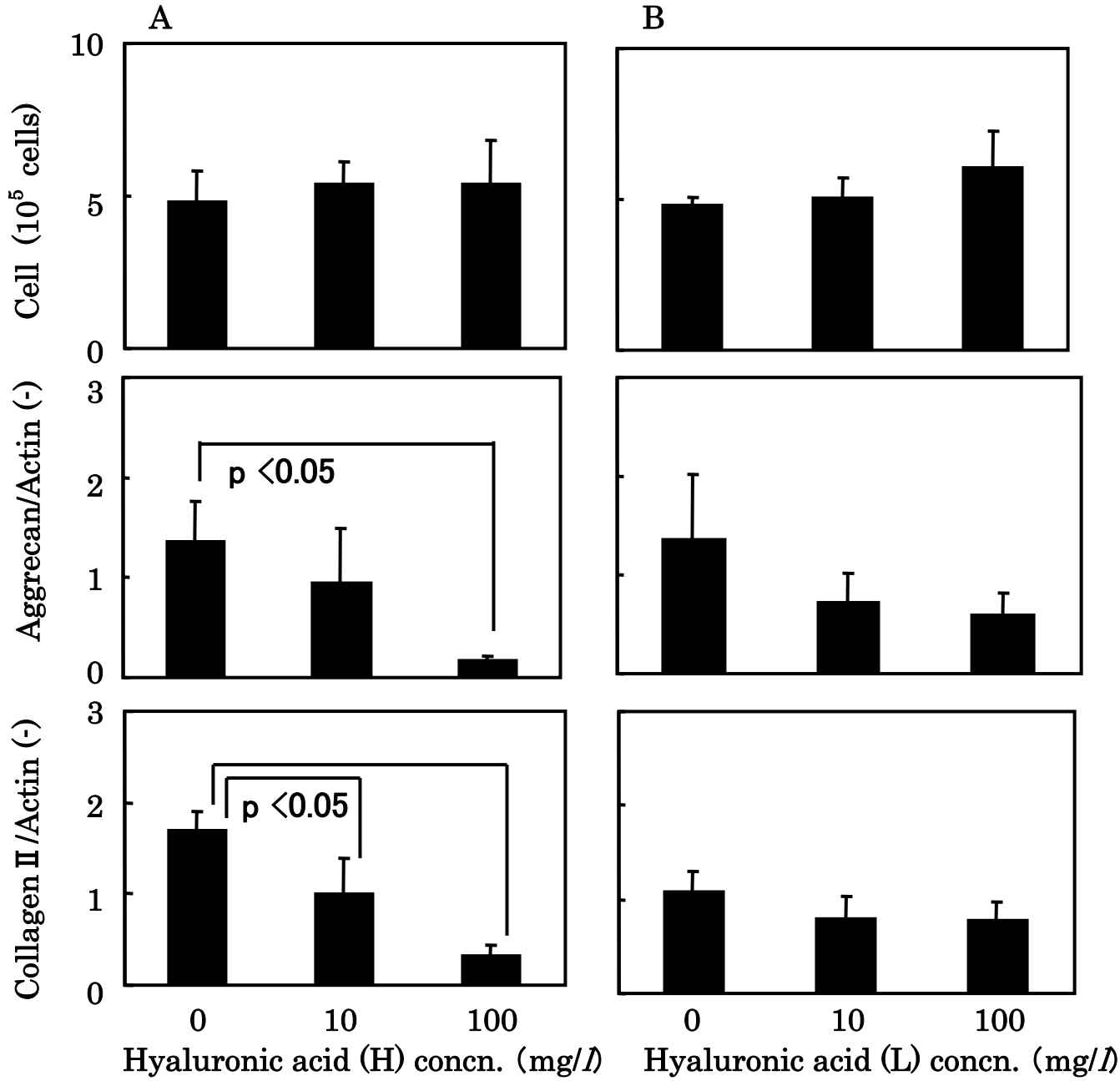
TABLE 1. Sequences used in PCR

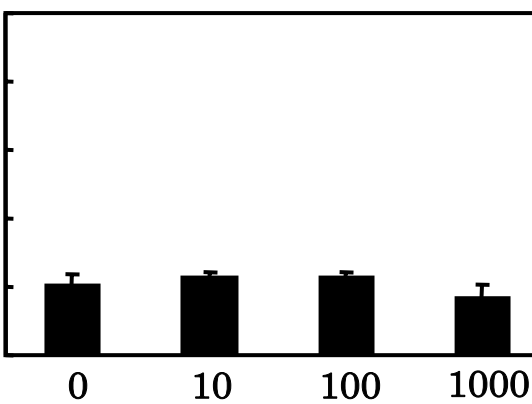
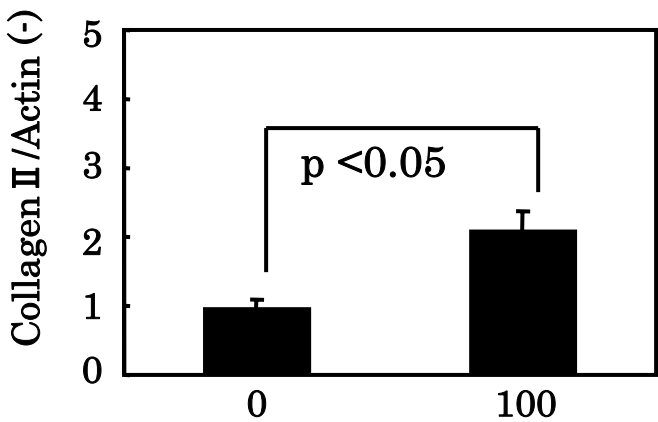
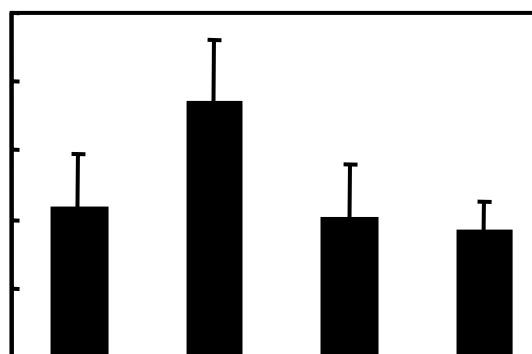
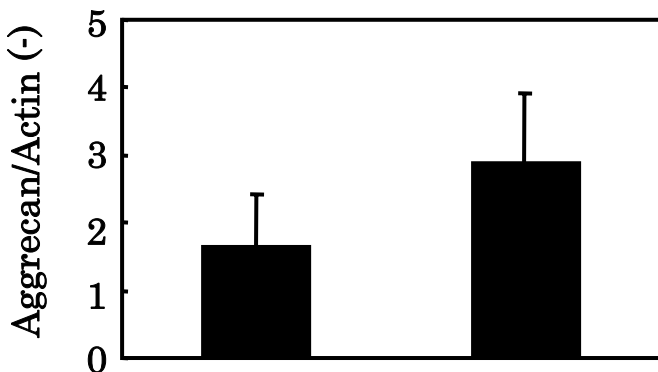
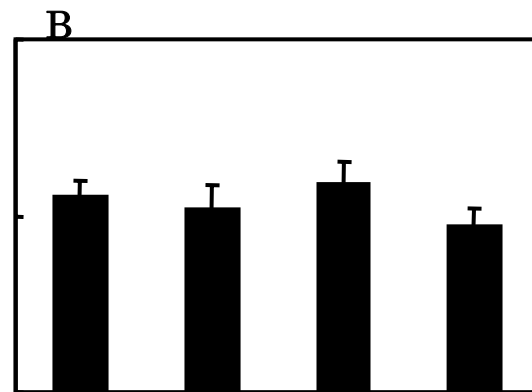
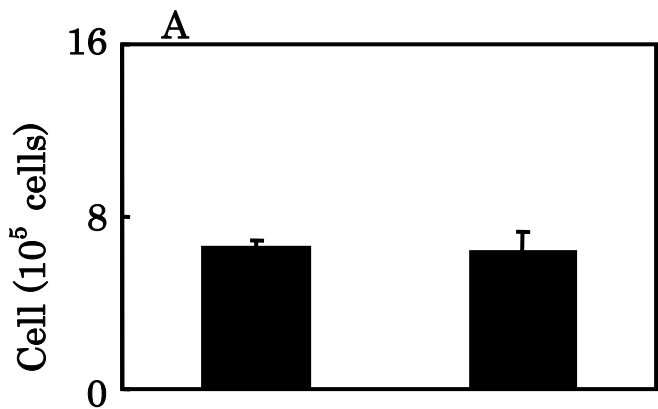
Gene	Sequences
Porcine Aggrecan (AF201722)	Sense: 5'-TGCAGGTGACCATGGCC-3' Antisense: 5'-CGGTAATGGAACACAACCCCT-3' Probe: 5'-CCCTGGGCAGCCACGACTTCC-3'
Porcine Type 2 collagen α 1 (AF201724)	Sense: 5'-CCATCTGGCTTCCAGGGAC-3' Antisense: 5'-CCACGAGCCAGGAGCT-3' Probe: 5'-ACCAGGAACGCCCTGATCACCTGG-3'
Porcine Actin (SSU07786)	Sense: 5'-TGCAGGTGACCATGGCC-3' Antisense: 5'-CGGTAATGGAACACAACCCCT-3' Probe: 5'-CCCTGGGCAGCCACGACTTCC-3'

FIG. 1. Effect of hyaluronic acids on the expression of aggrecan and type 2 collagen mRNA in a three-dimensional culture of porcine chondrocyte cells. (A) Hyaluronic acid (H), hyaluronic acid of high molecular weight; (B) hyaluronic acid (L), hyaluronic acid of low molecular weight.

FIG. 2. Effect of chondroitin sulfates on the expression of aggrecan and type 2 collagen mRNA in a three-dimensional culture of porcine chondrocyte cells. (A) Chondroitin sulfate C; (B) chondroitin sulfate A.

FIG. 3. Effect of chondroitin sulfate C on type 2 collagen accumulation in 3D culture. The sections of 3D cultures with (A) or without (B) the addition of chondroitin sulfate C were stained with the anti-type 2 collagen antibody.

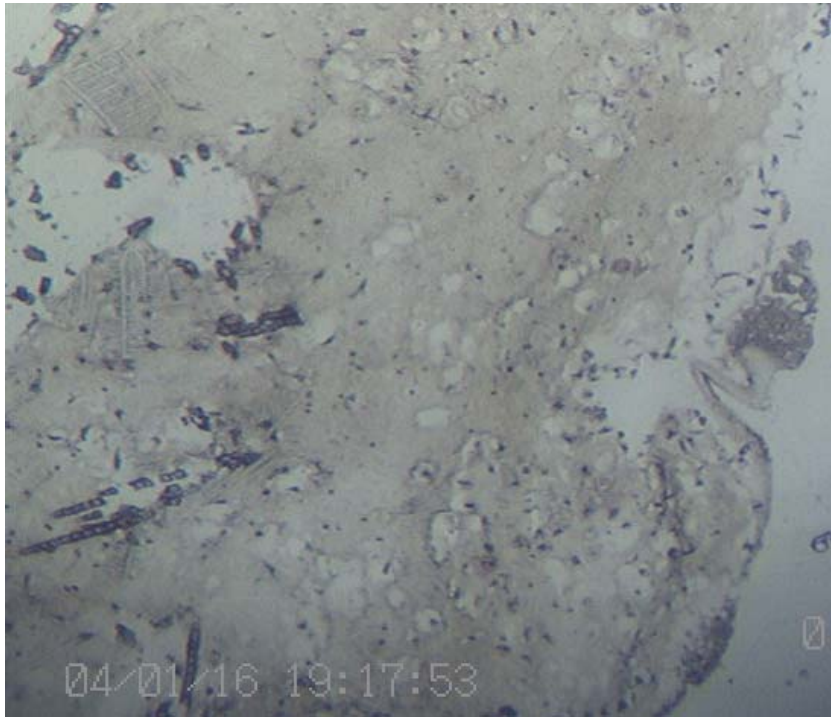




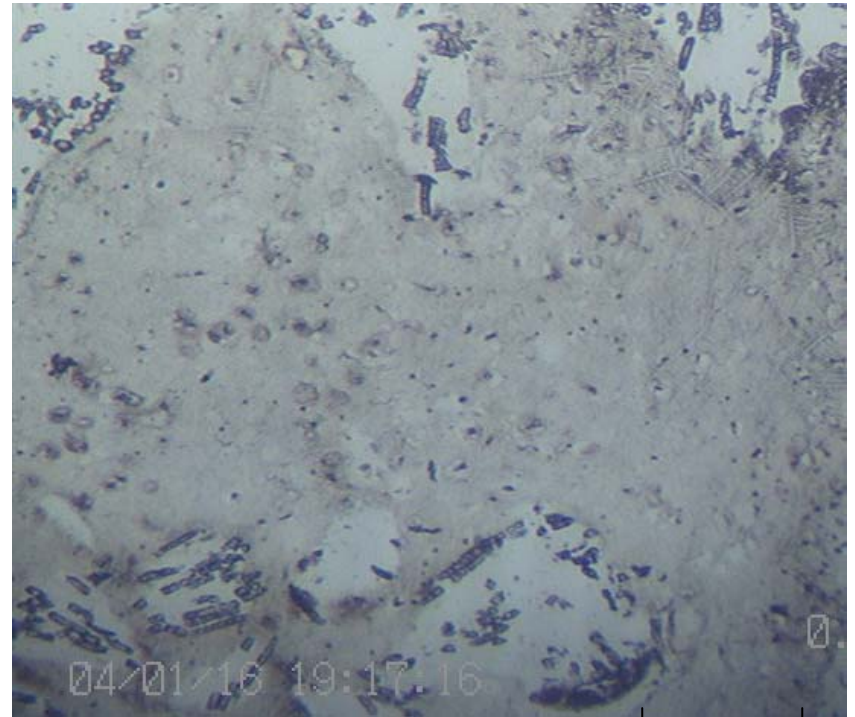
Chondroitin sulfate C concn. (mg/l)

Chondroitin sulfate A concn. (mg/l)

A



B



200 μ m