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**INTERACTIONS BETWEEN JELLYFISH MUCIN AND
HYALURONAN IN HUMAN CHONDROCYTES**

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ABSTRACT

We previously investigated the effects of jellyfish mucin and hyaluronan on degeneration in the knee joints of white rabbits. Here, the effects of jellyfish mucin and hyaluronan were examined in cultured human chondrocytes. We used a freeze-dried solid extract of jellyfish mucin from jellyfish mucin (from Nomura's jellyfish, *Nemopilema nomurai*) that had been purified using ion exchange chromatography. Knee articular chondrocytes obtained from operations were isolated enzymatically and cultured with interleukin-1-alpha added when the cells were subconfluent. The cells were transferred to culture medium with or without hyaluronan or jellyfish mucin. The cells were collected at various times, and the gene expression levels of collagen types I and II, SOX9, matrix metalloproteinase-3, and aggrecan were evaluated using real-time polymerase chain reaction (PCR). The concentrations of collagen type II, MMP-3, and transforming growth factor- β (TGF β) β secreted into the medium were measured using enzyme-linked immunosorbent assays (ELISAs). Real-time PCR showed that the gene expression levels of collagen type II, SOX9, and aggrecan increased in cells exposed to both jellyfish mucin and hyaluronan. Collagen type I gene expression was reduced by hyaluronan but was not affected by jellyfish mucin. MMP-3 gene expression in the absence of hyaluronan was reduced by jellyfish mucin in a dose-dependent manner. ELISAs showed that the concentration of transforming growth factor β with or without hyaluronan treatment was increased by jellyfish mucin in a dose-dependent manner, but there was no effect of mucin or hyaluronan on the concentrations of collagen type II or MMP-3. These results suggest that jellyfish mucin and hyaluronan may have beneficial effects on the physiology of cultured human chondrocytes and might contribute to cartilage repair and regeneration.

Key Words: jellyfish mucin, hyaluronan, chondrocyte, cultured cells.

INTRODUCTION

Osteoarthritis (OA) is one of the most common of the arthropathies and is characterized by cartilage degeneration that progresses slowly over a long time. OA appears most commonly in the load-bearing joints of the lower limbs, mainly the knees. There is a strong correlation between the onset of OA and patient age, and it is a main

cause of pain and joint function disorder in the elderly. (Felson, 1988) Moreover, young people often develop OA after an injury such as a joint fracture or an injury to the knee cartilage, anterior cruciate ligament, or meniscus. (Allen *et al.*, 1984; Jacobsen, 1977)

Current conservative treatment of OA focuses primarily on pain relief with systemic painkiller treatment and local intra-articular treatments. Nonsteroidal anti-inflammatory drugs (NSAIDs) are used widely as systemic painkillers. (Hochberg *et al.*, 2012) However, some studies have reported that the use of NSAIDs might promote

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further pathological conditions. (Huskisson *et al.*, 1995; Rashad *et al.*, 1989; Dougados *et al.*, 1996; Reijman *et al.*, 2005) A potential positive effect of the intra-articular injection of hyaluronan—a widely used local treatment—is that it increases the viscosity of the synovial fluid and inhibits destruction of the articular cartilage. (Petrella *et al.*, 2002; Dougados *et al.*, 1993; Salk *et al.*, 2006; Kobayashi *et al.*, 1994)

A thin membrane on the surface of articular cartilage is involved in protecting and reducing friction on the surface. The membrane comprises glycoproteins with a mucin-type structure similar to tribonectin and lubricin. (Rhee *et al.*, 2005; Jay *et al.*, 2007) Human glycoproteins in joint have tandem repeat regions of 7–8 amino acids in the mucin domain, and more than 90% of the mucin-type sugar chain with binding affinity to amino acids is subject to glycosylation. The sugar chain is short (glycoform) with as few as 2–3 glucose molecules and includes sialic acid residues. The characteristics are very similar to those of jellyfish mucin, which was used in this study. (Masuda *et al.*, 2007)

Figure 1 shows a swarm of Nomura's jellyfish (*Nemopilema nomurai*). Takayuki Baba, a collaborator in this study, discovered jellyfish mucin in many jellyfish, including Nomura's jellyfish, and purified it successfully. (Masuda *et al.*, 2007) Its purity is so high that homogeneous products can be prepared on an industrial scale. In our previous studies, we reported that jellyfish mucin and hyaluronan administration reduced articular cartilage degeneration in the knee joints of Japanese white rabbits. (Ohta *et al.*, 2009) Here, the interaction between jellyfish mucin and hyaluronan was examined in cultured human chondrocytes.

METHODS

Human articular chondrocytes

The Institutional Review Board of Tokai University School of Medicine approved this study. After obtaining informed consent. Human articular chondrocytes were obtained during total knee replacement in the affiliated hospital of Tokai University School of Medicine and were isolated by enzymatic treatment. The patients were one man and two women with an average age of 70.3 (range 68–73) years.

Reagents

Artz® (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) was used as a high-polymer hyaluronan. The mean molecular weight of this hyaluronan is approximately 800,000 Da. It was used at a concentration of 25 mg/2.5 mL. Jellyfish mucin was prepared by dissolving the extract derived from Nomura's jellyfish in saline at 1 mg/mL.

Extraction and purification of jellyfish mucin

The mesoglea, the major part of the umbrella in Nomura's jellyfish, was cut into small pieces and

suspended in water. After removing insoluble material by centrifugation at $10,000 \times g$, ethanol was added to the supernatant at one-third of the volume of the pellet. The resulting precipitate was harvested by centrifugation at $10,000 \times g$ and dissolved in water. The supernatant was collected by centrifugation at $10,000 \times g$, dialyzed against water, and lyophilized. The lyophilized material was then dissolved in phosphate-buffered saline (PBS) and incubated with anion-exchange gel beads derivatized with diethylaminoethyl (DEAE) resin (Toyoparl DEAE-650M; Tosoh, Tokyo, Japan) for 1 h. The beads were washed well with phosphate buffer, and the bound proteins were eluted with elution buffer (phosphate buffer plus 0.5 M NaCl). The eluent was collected by filtration, dialyzed against water, and lyophilized.

Cell culture

Cartilage samples from patients were cut into slices about 1 mm thick and were treated enzymatically in 0.4% (w/v) of Actinase E (Kaken Pharmaceutical Co., Ltd.) in Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12; Gibco, Carlsbad, CA, USA) for 1 h. Additional enzymatic treatment continued for 6 h in 0.016% (w/v) bacterial collagenase P (Roche Diagnostics GmbH, Mannheim, Germany) in DMEM/F12 medium. After enzymatic treatment, the cartilage tissues were filtered through a 100- μ m cell strainer (Becton Dickinson Labware Co. Ltd, Franklin Lakes, NJ, USA), seeded at 1×10^4 cells per 100-mm dish, and cultured. Monolayer primary cultured cells (P0) were treated with 0.05% trypsin/EDTA (Gibco) at 37°C for about 10 min. The obtained cells were washed in PBS, suspended in culture medium, and subcultured at 1×10^4 cells per dish. Passage 1 cells were seeded at 1.0×10^4 cells per 100-mm dish and cultured in the AA+AB+/DMEM F12 culture medium with or without hyaluronan or jellyfish mucin at the concentrations shown in Table I (n = 6).

For real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) procedures, passage 1 cells were seeded at 1×10^4 cells per dish and cultured. When the cells became subconfluent, they were transferred into culture medium containing interleukin 1 α (IL-1 α) (Perotech Inc., Rocky Hill, NJ, USA) with 20% fetal bovine serum/AA+/AB+/DMEM/F12. After 12 h, the culture medium was removed, and the cells were washed twice in PBS. The cells were assigned to six testing groups: a control group(A), 100 μ g/mL of jellyfish mucin only group (B), 300 μ g/mL of jellyfish mucin only group(C), hyaluronan only group(D), hyaluronan + 100 μ g/mL of jellyfish mucin group(E) and hyaluronan + 300 μ g/mL of jellyfish mucin group(F) (Table I). For real-time PCR (n = 6), the cells in each group were collected at 0, 6, 12, 24, and 48 h. The cells were washed twice in PBS and then dissolved in 1 ml of TRIzol reagent (Life Technologies Gaithersburg, MD, USA). For ELISAs (n = 4), the culture media for each

group were obtained at 0, 12, 24, 48, and 72 h, and stored in sample tubes at -20°C .

RNA isolation and cDNA synthesis

Total RNA was extracted using RNeasy Mini kits (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA quality from each sample was determined using the A260/280 absorbance ratio and by electrophoresis on 1.2% agarose formaldehyde gels. Aliquots of 1.0–2.0 μg of total RNA were reverse transcribed into single-strand cDNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The reverse transcription reaction was performed in a thermocycler at 42°C for 60 min and then at 95°C for 5 min.

Primer design and real-time PCR

In this study, the cells in the six groups shown in Table I were collected at 0, 6, 12, 24, and 48 h, washed twice in PBS, and then dissolved in 1 ml of TRIzol. All oligonucleotide primer sets were designed based upon the published mRNA sequences. The expected amplicon lengths ranged from 70 to 200 bp. The oligonucleotide primers used in this study are listed in Table II. Real-time PCR was performed in a SmartCycler II (Cepheid, Sunnyvale, CA, USA) with SYBR® Green PCR Master Mix (Applied Biosystems). Aliquots of 2–2.5 μl of cDNA template were used for real-time PCR in a final volume of 25 μl . cDNA was amplified according to the following condition: 95°C for 15 s and 60°C for 60 s for 35–45 amplification cycles.

Fluorescence changes of SYBR Green were monitored after every cycle. A melting curve analysis was performed (0.5 $^{\circ}\text{C}/\text{s}$ increase from 55°C to 95°C with continuous fluorescence monitoring) at the end of cycling to ensure that single PCR products were obtained. The amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. All reactions were repeated in six separate PCR runs using RNA isolated from four sets of human samples. The results were evaluated using SmartCycler II software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize samples. To monitor any crossover contaminations of PCR, RNase-free water (Qiagen Inc.) was included in the RNA extraction as a negative control. To ensure the quality of data, a negative control was always applied in each run.

Evaluations of humoral factors

The culture media from the six groups shown in Table I were obtained at 0, 12, 24, 48, and 72 h. For collagen type II, matrix metalloproteinase-3 (MMP-3), and transforming growth factor- β 1 (TGF- β 1), the supernatants from each group were assayed by ELISA according to the protocol in each kit (Collagen Type II ELISA Kit, Catalogue No. M036000, Whittaker-MA Bioproducts,

Walkersville, MD, USA; Human Total MMP-3 Kit, Catalogue No. DMP300, R&D Systems. Minneapolis, MN, USA; Human TGF- β 1 Kit, Catalogue No. DB100B, R&D Systems). The absorbance values of the ELISA samples were measured at 450 nm with a spectrophotometer and were compared with the standard curves provided in the collagen type II, MMP-3, and TGF- β 1 kits.

Cell proliferation activity

Proliferation was measured using a 3-(4,5-dimethyl-2-thiazolyl) -2,5- diphenyl-2 H - tetrazolium bromide (MTT; Dojindo, Kumamoto, Japan) assay on culture days 3, 5, 7, 9, 11, and 14. There were six replicates per experimental condition. The cells were collected on days 3, 5, 7, 9, 11, and 14, and washed twice in PBS; 100 μl of MTT solution was added, and the cells were incubated in air with 5% CO_2 at 37°C for 2 h. The supernatant was absorbed, suspended in 100 μl of dimethyl sulfoxide, and moved to a round plate to measure light absorbance (optical density at 590 nm wavelength) using a plate reader (SoftMax Pro; Molecular Devices Corp., Sunnyvale, CA, USA). The culture medium was changed every 2–3 days.

Statistical analysis

All data are expressed as the mean \pm standard error. Factorial analysis of variance (ANOVA) was applied for comparisons. When an ANOVA was significant, individual differences between treatments were tested by the multiple-comparison method of Scheffe. $P < 0.05$ was considered significant.

RESULTS

Real-time PCR results

Genes important for maintaining characteristics of articular cartilage such as COL2, SOX9, AGC were expressed in a different manner. COL1 and MMP-3 were also expressed uniquely.

Collagen type I (COL1)

In the presence of hyaluronan (Groups D, E, and F), the overall expression of COL1 was reduced. There was no effect of jellyfish mucin concentration (Figure 2A).

Collagen type II (COL2)

In the absence of hyaluronan (Groups A, B, and C), the expression of COL2 was unchanged, and there was no effect of jellyfish mucin concentration. However, the expression of COL2 increased significantly in the presence of hyaluronan (Groups E and F). A marked increase was seen in Group E (100 $\mu\text{g}/\text{mL}$ of jellyfish mucin; Figure 2B).

SRY-related HMG Box 9 (SOX9)

In the absence of hyaluronan (Groups A, B, and C), the expression of SOX9 increased significantly only in

Group B, in which 100 µg/mL of jellyfish mucin was added. However, in the presence of hyaluronan (Groups D, E, and F), the expression of SOX9 increased with added mucin in a dose-dependent manner (Figure 2C).

Matrix metalloproteinase-3 (MMP-3)

In the absence of hyaluronan (Groups A, B, and C), the expression of MMP-3 was reduced by the added jellyfish mucin in a dose-dependent manner. However, in the presence of hyaluronan (Groups D, E, and F), the expression of MMP-3 decreased both with and without added mucin (Figure 2D).

Aggrecan (AGC)

In the absence of hyaluronan (Groups A, B, and C), the expression of AGC was reduced by the addition of jellyfish mucin. However, in the presence of hyaluronan (Groups D, E, and F), the expression of AGC increased significantly following the addition of 300 µg/mL mucin (Figure 2E).

Analysis of humoral factors by ELISA

Collagen type II

There were no appreciable changes in the quantity of collagen type II secreted from the cells over time, with or without hyaluronan or jellyfish mucin (Figure 3A).

MMP-3

MMP-3 secretion was not influenced by treatment with hyaluronan or jellyfish mucin, and the amount of MMP-3 secreted reached a steady state at 24 h (From 22.16±1.42 ng/ml (group F of 24hours) to 26.73±0.50 ng/ml (group D of 72hours) (Figure 3B).

TGF-β

The amount of secreted TGF-β was not influenced by the presence or absence of hyaluronan, but it increased

over time with added jellyfish mucin in a dose-dependent manner (Figure 3C).

Cell proliferation rates

Cell proliferation increased following the introduction of hyaluronan and mucin. However, treatment with jellyfish mucin increased the cell proliferation activity more than did hyaluronan. Cell proliferation increased further when both agents were added (Figure 4).

DISCUSSION

Hyaluronan in the synovial fluid contributes to the consistency and elasticity of synovial fluid, which serves as a lubricant and shock absorber. Increasing content of low-molecular-weight hyaluronan in the joints decreases the consistency and elasticity of the synovial fluid in inflammatory states such as OA. (Pritzker *et al.*, 2006) Hyaluronan is involved in anti-inflammatory actions, analgesia, reduction in cartilage degeneration, and healing. (Kontinen *et al.*, 1991; Schiavinato *et al.*, 1989)

Lubricin, which is included in the mucin-containing glycoprotein/tribonectin group of molecules (superficial zone protein), has been recently identified as a component of synovial fluid and the joint surface. (Chang *et al.*, 2008) Lubricin controls lubrication in the articular cartilage. Its coefficient of friction is very low at 0.005–0.03. It has been hypothesized that lubricin contributes to reducing joint friction. (Chang *et al.*, 2008) The effects of mucins in the human body go beyond surface protection and reducing friction over the articular cartilage. These molecules provide various functions, such as protection of the ocular surface by a mucin film, (Davidson *et al.*, 2004) protection against infection by *Helicobacter pylori* in the gastric mucosa, (Kawakubo *et al.*, 2004) and protection of the early embryo in the Fallopian tube. (Lapensee *et al.*, 1997).

Figure 1. Nomura's jellyfish. The world's largest jellyfish grows to over 1 m in diameter and weighs more than 200 kg (photo by Mr. Ryoichi Kanzaki; SEA EGG DIVERS).



Figure 2. Real-time PCR. The overall gene expression of COL1 was reduced in the presence of hyaluronan (A). In the absence of hyaluronan, COL2 gene expression was almost constant and was unrelated to the concentration of added jellyfish mucin. However, in the presence of hyaluronan, COL2 gene expression increased significantly with the addition of mucin; the percentage increase was greatest at 100 $\mu\text{g}/\text{mL}$ mucin (B). In the presence of hyaluronan, SOX9 gene expression increased in a dose-dependent manner with added mucin and increased significantly with 100 $\mu\text{g}/\text{mL}$ of mucin. (C). In the absence of hyaluronan, MMP-3 gene expression was reduced by mucin in a dose-dependent manner. However, in the presence of hyaluronan, MMP-3 gene expression was not influenced by the presence or absence of mucin (D). In the absence of hyaluronan, adding jellyfish mucin reduced AGC gene expression. However, AGC gene expression was significantly increased by the addition of 300 $\mu\text{g}/\text{mL}$ of mucin in the presence of hyaluronan (E).

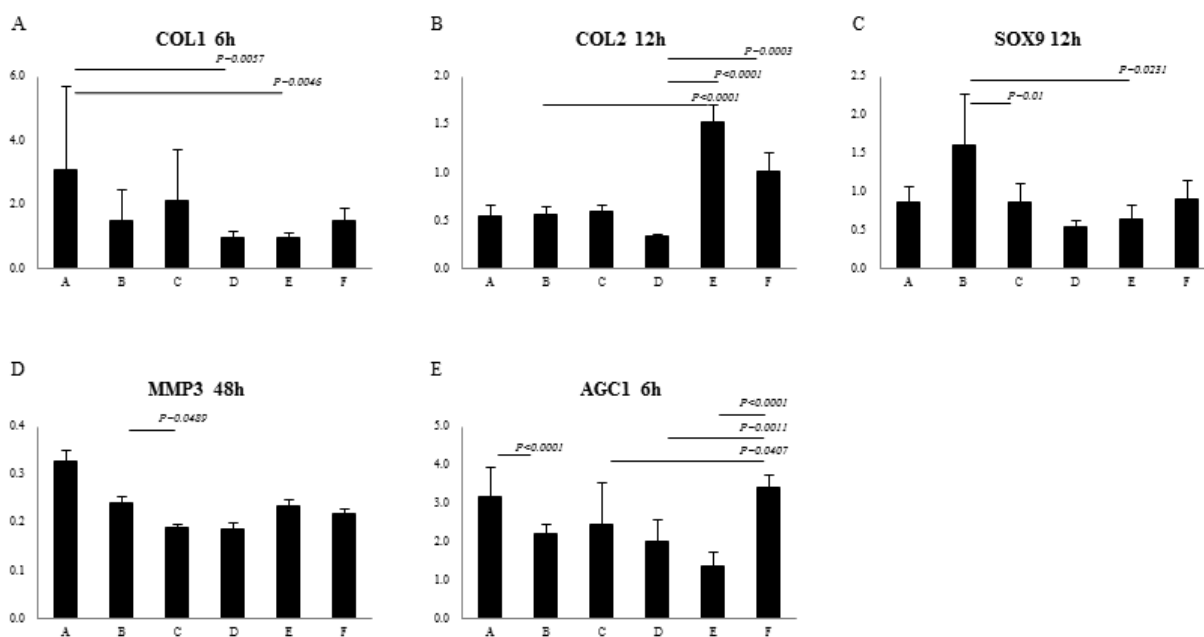


Figure 3. ELISA. The quantity of collagen II secreted by the cells did not change over time with or without hyaluronan or mucin treatment (A). MMP-3 secretion was not influenced by hyaluronan or mucin, and its secretion level reached a steady state at 24 h. At 24 h, significant difference were found between group C and F (*) ($P < 0.05$)(B).

TGF- β secretion was not influenced by the presence or absence of hyaluronan. The level of TGF- β secretion increased with added mucin in a dose-dependent manner and over time. At 24, 48, and 72 h, significant differences were found between groups A, B, C, E, and F (*), between groups B, C, and F (\dagger), between groups D, E, and F (\S), and between groups E and F (\ddagger) (all $P < 0.0001$)(C).

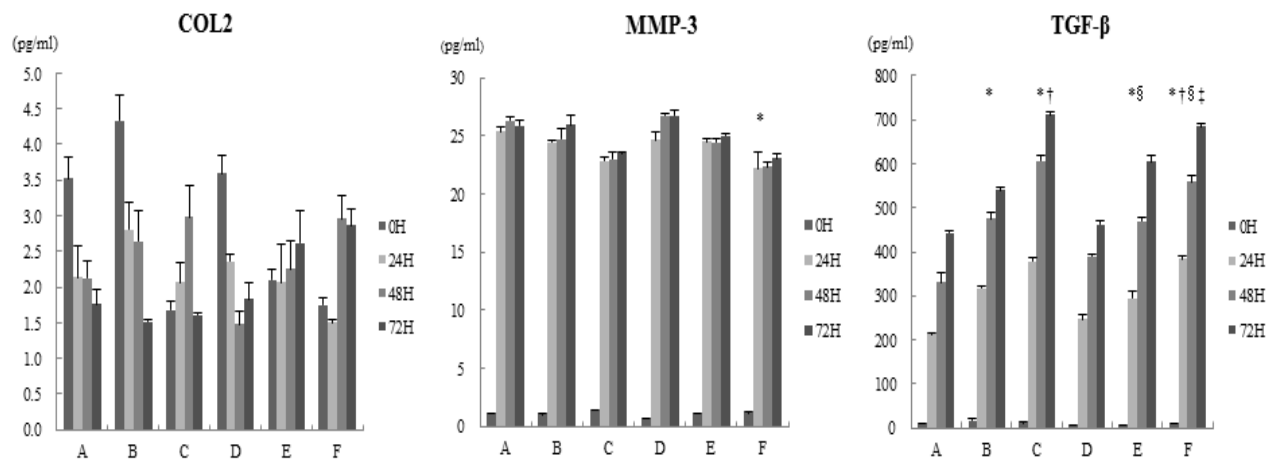


Figure 4. MTT assay. Adding hyaluronan and mucin increased chondrocyte proliferation, although mucin treatment increased cell proliferation more than did hyaluronan. Cell proliferation was increased further by adding both hyaluronan and mucin. Significant differences were found between groups A, B, C, E, and F (* $P < 0.01$) on day 3. On day 5, significant differences were found between groups A and E ($\dagger P < 0.01$), between groups B and E ($\ddagger P < 0.01$), and between groups D, E and F ($\S P < 0.001$).

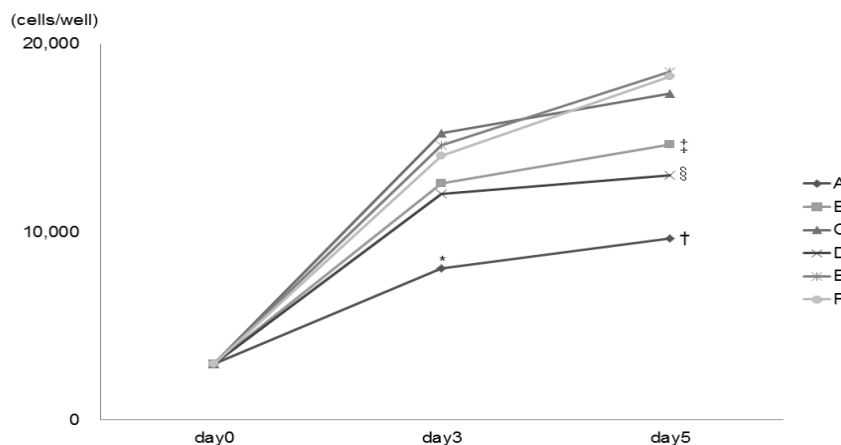


Table 1. Experimental Groups

	A	B	C	D	E	F
Hyaluronan (500 $\mu\text{g/mL}$)	-	-	-	+	+	+
Mucin ($\mu\text{g/mL}$)	-	100	300	-	100	300

Table 2. List of Primers Used in Real-time PCR

Primer ID	Accession No.	Sequence	Expected Size(bp)
COL 1-F	NM_000088	AAG GGT GAG ACA GGC GAA CAA	170
COL 1-R		TTG CCA GGA GAA CCA GCA AGA	
COL 2-F	NM_033150	GGA CTT TTC TTC CCT CTC T	113
COL 2-R		GAC CCG AAG GGT CTT ACA GGA	
SOX 9-F	NM_000346	AAC GCC GAG CTC AGC AAG A	138
SOX 9-R		CCG CGG CTG GTA CTT GTA ATC	
MMP-3-F	NM_002422	ATT CCA TGG AGC CAG GCT TTC	138
MMP-3-R		CAT TTG GGT CAA ACT CCA ACT GTG	
Aggrecan-F	NM_001135	TCG AGG ACA GCG AGG CC	94
Aggrecan-R		TCG AGG GTG TAG GCG TGT AGAGA	
GAPDH-F	NM_002046	GCA CCG TCA AGG CTG AGA AC	142
GAPGH-R		ATG GTG GTG AAG ACG CCA GT	

Many types of glycoprotein mucins appear as ingredients of mucus and are widely distributed in animals and plants. Although various forms of mucins are found in plants such as lotus, okra, and yam, many such molecules have a short peptide chain or a long sugar chain, which places them in a group of materials very different from animal-derived mucins. By contrast, all animals maintain mucins as ingredients of mucus and digestive fluids. The sequence of the core peptide and the structure of sugar chains and non-mucin-type domains vary between different animals. Mucins are commonly derived from domestic animals and can be mass-produced but, currently, there are few examples of animal-derived mucins that are mass-produced as materials. However, mass production of mucins tends to be avoided because it is impossible to

prevent completely contamination by prions, which cause bovine spongiform encephalopathy. Mucins derived from snails, starfish, sponges, squids, and other species are distributed commercially (Grundy *et al.*, 2000; Kimura *et al.*, 2003; Adikwn *et al.*, 2007) but in limited amounts. Organic chemical construction methods to obtain mucin-type high polymers with a simple structure require substantial costs and are not practical.

Nomura's jellyfish can interfere with fishing in the Japanese Sea by swamping fishing nets. These jellyfish also accumulate in the waterways from which seawater is piped for cooling power stations located on the coast, thereby causing water flow reductions that can cause plant shutdowns. Removal of these jellyfish is routine work at power stations, industrial facilities, and ports located in

coastal areas. It is difficult to deal with the large numbers of jellyfish that are collected and then need to be discarded.

Jellyfish mucin has a simple structure comprising almost solely mucin sequences and is economically viable for mass production. (Matsuda *et al.*, 2007) It has been confirmed that the protein structure of jellyfish mucin is similar to that of typical human mucin (MUC5AC). Consequently, it has potential to be used widely in materials for therapeutic treatments. The tandem repeat structure comprises only eight amino acids (sequence VVETTAAP or VIETTAAP). Moreover, the sugar chains are short, there are few kinds of constituent sugars and sialic acids, and there are no peptide sequences present except for that of the mucin. Therefore, it is expected that jellyfish mucin's antigenicity and allergenicity will be mild when used therapeutically. The effects of jellyfish mucin on friction reduction were as expected, and we have reported that administration of jellyfish mucin along with hyaluronan reduces articular cartilage degeneration in white rabbit knee joints. (Ohta *et al.*, 2009)

In the present study, the effects were examined *in vitro* in human cultured chondrocytes. Real-time PCR showed that the gene expression of COL2, SOX9, and AGC was increased in the presence of both mucin and hyaluronan. Hyaluronan reduced COL1 gene expression, but jellyfish mucin had no effect on this gene. In the absence of hyaluronan, MMP-3 gene expression was reduced by mucin in a dose-dependent manner. The ELISA results showed that, although hyaluronan and mucin did not affect COL2 and MMP-3 concentrations, the concentration of TGF- β was increased by jellyfish mucin in a dose-dependent manner with or without hyaluronan. In addition, chondrocyte proliferation increased significantly in the presence of both hyaluronan and jellyfish mucin. Therefore, we hypothesize that the ability of jellyfish mucin to reduce cartilage degeneration might reflect the promotion of the effects of an anabolic factor. The effects of mucin were increased further when administered in combination with hyaluronan. As the effect of catabolic factors, the expression of MMP-3 was reduced, but there was no effect on COL1 expression, although an effect of hyaluronan was found. Hyaluronan had no effect on TGF- β , but its expression was increased by jellyfish mucin in a dose-dependent manner. This suggests that the growth-

stimulating effects of TGF- β on chondrocytes might have been a side effect of the jellyfish mucin used in this study. TGF- β is a multifunctional cytokine (Sporn *et al.*, 1988) that controls the growth and differentiation of cells, and protein synthesis in the extracellular matrix. (Madri *et al.*, 1988) TGF- β inhibits the growth of osteoclast-like cells, stimulates endochondral ossification, and promotes osteogenesis. (Chenu *et al.*, 1988; Critchlow *et al.*, 1995) TGF- β -induced osteogenesis is mediated by stimulation of subperiosteal pluripotent cells, which can differentiate into chondrogenic cells. (Joyce *et al.*, 1990; Miettinen *et al.*, 1994) Recent reports on the *in vivo* effects of TGF- β found that its activity is closely associated with mesenchymal tissues such as connective tissue, cartilage, and bone. (Andrew *et al.*, 1993; Bourque *et al.*, 1993) There is one report of the potential of TGF- β to promote fracture healing, (Critchlow *et al.*, 1995) but this effect was short term, and its high cost limits its clinical application. Therefore, a method to prolong the effect of endogenous TGF- β for a longer time is required for articular cartilage repair. Jellyfish mucin, which was used in this study, might become one of the solutions to this problem.

CONCLUSIONS

Jellyfish mucin and hyaluronan appear to influence the physiology of cultured human chondrocytes and may contribute to cartilage repair and regeneration.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

TT and TK performed most of the experiments. TT, TK, and TB performed the PCR analyses. KK and YK performed the statistical analyses. MS and JM designed and coordinated the study, and helped draft the manuscript. All authors approved the final manuscript.

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