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Pharmacological effects of novel cross-linked hyaluronate, Gel-200, in experimental animal models of osteoarthritis and human cell lines

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SUMMARY

Objective: To study the pharmacological effects of Gel-200, cross-linked hyaluronate.
Experimental design: We examined the chondroprotective, anti-inflammatory and analgesic effects of Gel-200 in experimental animal models for osteoarthritis (OA) and in a human synovial sarcoma cell line and normal human articular chondrocytes.
Results: In the OA model, a single-dose intra-articular (IA) injection of Gel-200 significantly suppressed cartilage degeneration and reduced synovitis of the knee joint. In the joint pain model, Gel-200 significantly suppressed pain responses for 4 weeks after injection. The residual property of Gel-200 in the knee joint tissue was investigated in rabbits. The mean residual ratio of injected Gel-200 in the synovium was 3.3% (95% confidence interval [CI], 2.4–4.2) at 28 days after the injection. The long-lasting analgesic effect of Gel-200 might be explained by its high residual ratio in the joint. In addition, we investigated the mechanism of action of Gel-200 in a human synovial sarcoma cell line and normal human articular chondrocytes. Gel-200 inhibited IL-1β-induced production of MMP-1, 3 and 13 in human chondrocytes and production of prostaglandin E_2 in human synoviocytes in a concentration-dependent manner, respectively.
Conclusion: A single-dose IA injection of Gel-200 exerts chondroprotective and anti-inflammatory effects in the experimental OA model, and long-lasting analgesia in the joint pain model, suggesting the beneficial multimodal function of Gel-200 against symptomatic OA patients.

Introduction

Osteoarthritis (OA) of the knee is the most common joint disorder throughout the world and is one of the leading causes of disability in the elderly. Intra-articular (IA) injection of hyaluronic acid (HA) is a recognized treatment for pain associated with symptomatic knee OA[1–3]. HA products are divided into two major types, native HA products and cross-linked HA products. Native HA products are injected three to five times per one treatment course and its safety has been established based on long time clinical experiences[4]. In the cross-linked HA category of the US market, there are three products, Gel-One, Synvisc and Synvisc-One.

Gel-One is the most recently approved single injection product in the US market and is composed of Gel-200 which is a novel cross-linked hyaluronate hydrogel manufactured by photo-gelation technology. The polysaccharide chains of hyaluronan in Gel-200 are bound to each other via dimers of cinnamic acid with ultraviolet irradiation resulting in a clean cross-linked highly viscoelastic hydrogel. The photocurable hyaluronan derivative chemically linked with cinnamic acid, intermediate of Gel-200, is purified before the cross-linking reaction. Gel-200 is cross-linked by only ultraviolet irradiation without any additional condensation reagents. This photo-gelation technology need not required elimination of residual reagents and/or by-products after cross-linking to achieve high purification. In a multi-center randomized controlled trial in patients with symptomatic OA of the knee, a single injection of Gel-200 was well tolerated and relieved pain associated with symptomatic OA of the knee over 13 weeks[5]. In this study, we investigated the pharmacological effects of Gel-200 in animal models (rabbits and rats), the human synovial sarcoma cell line, and normal human articular chondrocytes.

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Materials and method

Animals

Animals were quarantined and acclimatized to the environmental conditions for 1 week. The animal studies were reviewed and approved by the In-house Animal Experiment Ethics Committee, performed under the animal husbandry/management system in an appropriate environment with animal protection/welfare in mind.

OA model in rabbits

Twenty-four male Japanese White rabbits (16 weeks-old) were obtained from Oriental Yeast Co., Ltd. The rabbit anterior cruciate ligament (ACL) transection model was used in this study. The rabbit ACL transection model is accepted as an arthritis model which produces cartilage degeneration similar to OA in humans[6]. Following induction of anesthesia by a solution containing equal proportions of ketamine (Veterinary Ketalar50, 50 mg/mL, Sankyo Yell Yakuhin Co., Ltd., Tokyo, Japan) and xylazine (Selactal 2% injection solution, Bayer Co., Osaka, Japan), rabbits were anesthetized by the inhalation of isoflurane (Forane, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The incision area was sterilized with 70% ethanol and Isodin solution (Meiji Seika Kaisha Ltd., Tokyo Japan). An arthrotomy was performed in only the left knee using a lateral parapatellar approach with 2 cm skin incision. The ACL was visually transected with a # 15 blade. After the knee was repositioned, the synovial membrane and skin were sutured with 4-0 nylon. The animals were allocated to two groups at 4 weeks after ACL transection surgery. Animals without abnormalities were allocated to two groups ($n = 12$) based on the body weights and the width of the operated knee joints, using stratified continuous randomization. Gel-200 (manufactured by Seikagaku Corporation) or phosphate buffered saline (PBS) was administered in a single dose into the joint cavities of the left hindlimbs at a volume of 50 μL/kg/joint. Necropsy was performed at 9 weeks after surgery. Blood was drawn from the heart using a heparinized 21G needle under ketamine general anesthesia and animals were euthanized by exsanguination. Plasma was obtained by centrifugation at 3,000 rpm for 15 min, and was stored below −20°C.

The left knee was dissected and 2 mL physiological saline was injected into the joint cavity. The lavage fluid was aspirated and collected. These procedures were repeated twice on each joint. The synovium and femur were removed for histopathological assessment and fixed in 10% neutral buffered formalin solution.

Gross morphological assessment of cartilage degeneration was performed according to the reported procedure[7]. The severity of the degeneration was scored based on the criteria shown in Table I (a total of 12 joints, 24 scores, in each group). The damaged length and width were measured using a digital caliper.

Table I
Cartilage degeneration scoring criteria

Findings	Grade
Intact surface (No staining by Indian ink)	1
Minimal fibrillation (Surface retains the ink as elongated specks)	2
Overt fibrillation	3
Erosion (Loss of cartilage exposing the sub-cartilaginous bone)	4
0 mm < Erosion ≤ 2 mm in length	4a
2 mm < Erosion ≤ 5 mm in length	4b
5 mm < Erosion	4c
5 mm × 2 mm < Erosion (mm in length × width)	4d

The volume of the synovial fluid (SFV)[8] was determined from the volume of the recovered SF and the calcium concentrations in the recovered SF and plasma. The calcium concentration was measured using a commercial assay kit (Calcium-C test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan): SFV (mL/joint) = calcium content in SF (μg/joint)/calcium concentration in plasma (μg/mL).

The total protein content in the SF was measured by a commercial assay kit (BCA Protein Assay Kit, PIERCE, USA). The prostaglandin E_2 (PGE_2) content in the SF was measured using a commercial assay kit (PGE_2 EIA kit [Enzo Life Sciences, Inc., PA, USA]). Paraffin sections were prepared from formalin-fixed synovium and stained with hematoxylin-eosin (HE). Using the synovium preparations, the severity of the inflammation was evaluated histopathologically from: (1) the cuboidal/stratified synovial lining cells, (2) cellular infiltration, (3) fibrosis/edema, and (4) hemorrhage in the synovial tissue. Following EDTA (Life Technologies Corporation, CA, USA) decalcification and safranine O staining, changes in the femur were observed in: (1) areas of the cartilage matrix with unstained/decreased staining for safranine O, (2) fissure formation in the cartilage matrix, (3) fibrillation of the cartilage matrix, (4) cartilage defect, (5) increase in the number of chondrocytes, (6) decrease in the number of chondrocytes, (7) remodeling of the sub-cartilaginous bone, and (8) blood vessel invasion in the cartilage matrix. These severities were scored on a 4-point scale of Grade 0 (No change) to 3 (Severe change).

Bradykinin induced-arthritic pain model in rats

Male Sprague–Dawley rats were obtained from Charles River Laboratories Japan Inc. (Tokyo, Japan). We used the rat joint pain model, induced by injecting bradykinin (an endogenous hyperalgesic substance) with PGE_2 (a pain enhancer), which has been used to assess the analgesic effects of hyaluronan preparations based on the behavioral manifestations of joint pain in gait such as "lifting the foot", "claudication" and "walking on three legs"[9,10] or pain-related behaviour using a dynamometer and weight bearing[11]. Rats were given general anesthesia by an isoflurane small animal anesthetizer. Gel-200 or PBS was administered in a single dose with a 29G needle-tipped syringe into the joint cavity at a volume of 50 μL/joint ($n = 12$, each evaluation point), which dose is generally used for injection into the articular cavity of rats[9,10]. Bradykinin (Peptide Institute, Inc., Osaka, Japan) was dissolved and diluted with PBS at a concentration of 16 μg/mL. PGE_2 (Cayman Chemical Company, MI, USA) for enhancing bradykinin-induced pain was dissolved in ethanol and the solution was diluted with PBS at a concentration of 4 μg/mL. The bradykinin solutions were prepared from equal volumes of bradykinin and PGE_2 solutions. A Fast Green FCF was added to bradykinin solutions to detect leakage from the joint cavity. The colored solutions were sterilized by filtration using a 0.22-μm filter and used for injection.

The bradykinin solutions were injected at 1, 2 and 4 weeks after Gel-200 or PBS administration to induce pain. To assess the pain response accurately, no anesthesia was used. The bradykinin solution was injected into the rats that received the test materials. Under blinded conditions, each animal was observed walking for 2 min after the bradykinin solution injection. The severity of pain was scored using criteria by Gotoh *et al.*[9] (Table II). After the analgesic assessments, all the rats were sacrificed by exsanguination under general anesthesia without pain. The knee joint was exposed through an incision and the injection site was confirmed by the green-colored bradykinin solution in the joint cavity.

Table II
Criteria for assigning pain scores

Behavioral manifestation	Score
Normal or claudication for ≤ 5 s	0
Claudication for 6–30 s	1
Showing one of the following two manifestations: Claudication for ≥ 31 s Lifting the foot for ≤ 5 s	2
Showing one of the following two manifestations: Lifting foot followed by claudication Walking on three legs for ≤ 5 s	3
Walking on three legs for ≥ 6 s	4

Residual ratio of Gel-200 in the joint cavity and synovium of the knee in rabbits

Thirty female Japanese White rabbits (15–18 weeks-old) were obtained from Oriental Yeast Co., Ltd. Anesthesia was induced by a solution containing equal proportions of ketamine, xylazine, and physiological saline. Gel-200 was injected in a single dose into the joint cavities of both hindlimbs under anesthesia ($n = 5$, 10 knee joints per group) at a volume of 50 μL/kg/joint. Necropsy was performed at 1, 3, 5, 7, 14 and 28 days after the injection of Gel-200. Animals were sacrificed by exsanguination under ketamine general anesthesia. After the knee was dissected, the synovial fluid (SF) was collected by washing the joint cavity 2 times with 2 mL physiological saline. The synovium was removed and digested at 55°C for 40 h with 5 mL of 2 mg/mL proteinase K (Sigma–Aldrich Co., MO, USA) solution (43 units/mg protein). The residual ratio of Gel-200 in the SF and the synovium was calculated using the amount of injected Gel-200 and the quantified trans-cinnamic acid contents, a component of Gel-200, in preparations recovered from the test animals. Preparation of the samples of the SF and the synovium digest, as well as the experimental conditions for analysis were performed in a preliminary study; the trans-cinnamic acid was extracted under alkaline conditions from the SF and the synovium digest, and then quantified by high-performance liquid chromatography (HPLC) with an octadecylsilyl (ODS) column.

Measurement of matrix metalloproteinase-1, -3, and -13 production by human chondrocytes

Normal human articular chondrocytes (NHAC-Kn, Lonza Walkersville, Inc., MD, USA), were seeded in 24-well plates at a density of 2×10^5 cells/well in DMEM/F-12 (Life Technologies Corporation, CA, USA) medium containing 10% heat-inactivated FBS (Sigma–Aldrich Co., MO, USA) and incubated at 37°C under 5% CO_2. After 24 h, the cells were preincubated with or without Gel-200 in the concentration range of 0.1–3.0 mg/mL for 30 min. The cells were stimulated with 10 μL of rhIL-1β (final concentration, 10 ng/mL) to induce MMP-1, -3 and -13 production in the presence or absence of Gel-200. After 16 h, the MMPs contents in the conditioned media were determined with Quantiline enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, MN, USA).

Measurement of prostaglandin E_2 production by human synoviocytes

Human synovial sarcoma cells (SW982, American Type Culture Collection, VA, USA), were seeded in 48-well plates at a density of 3×10^4 cells/0.5 mL/well in RPMI 1640 medium containing 10% heat-inactivated FBS and 1% penicillin/streptomycin, incubated at 37°C under 5% CO_2. After 48 h, the culture supernatants were removed and the solution of the test materials with IL-1β was then added to each well. The concentration of Gel-200 was 0.003–3.0 mg/mL, and it was incubated for 42 h. PGE_2 levels were measured after stimulation of the cells with rhIL-1β (final concentration, 10 ng/mL) at 37°C for 42 h. After incubation, samples of the medium were collected for determination of PGE_2 with PGE_2 ELISA kits (Assay Designs Inc., MI, USA). Three independent experiments were performed. For another set, SW982 was placed in a 48-well plate and incubated for 44 h. The cells were incubated with or without anti-CD44 antibody IM7 (BD Biosciences, CA, USA) at 50 μg/mL for 4 h. After 4 h, the supernatants were removed and Gel-200 (final concentration, 3 mg/mL) and IL-1β (final concentration, 10 ng/mL) were added to each well. They were incubated for 42 h as previously described.

Statistical analyses

Statistical analyses were performed using SAS (SAS Institute Inc., NC, USA). Direct effects from treatment with Gel-200 were assessed using a Wilcoxon rank sum test for gross morphological assessment of cartilage degeneration and analgesic assessment of the joint pain model in rats. Student's *t*-test was performed for assessment of the volume of SF, protein content and PGE_2 content in the SF. Williams' test was performed for assessment of PGE_2, MMP-1, -3, and -13 production *in vitro*. Results of the measurements in each group were represented as the mean and 95% confidence intervals (CI). Any *P*-values of <0.05 were considered statistically significant.

Results

We investigated the chondroprotective and anti-inflammatory effects of Gel-200 in the rabbit OA model, the analgesic effects in a rat joint pain model, the inhibitory effects of prostaglandin E_2 and MMPs production in a human synovial sarcoma cell line, and normal human articular chondrocytes to reveal the mechanism of action of Gel-200.

Gross morphological assessment of cartilage degeneration in the rabbit OA model

In the macroscopic picture, the damaged areas of the femoral condyle, which were stained by India ink were more severe in the control group [Fig. 1(A)]. The percentage of animals with mild degeneration (Grade 1–3) was higher in the Gel-200 group [Fig. 1(B)]. The percentage of animals with ≥Grade 4 (exposure of sub-cartilaginous bone) was 67% (16/24 of total points) in the control group vs 16% (4/24 of total points) in the Gel-200 group. Progression of the cartilage degeneration was significantly suppressed in the Gel-200 group compared to the control group ($P < 0.0001$, Wilcoxon rank sum test).

Histopathological examination of the articular cartilage in the rabbit OA model

Histopathological changes were observed in: the areas of the cartilage matrix unstained/decreased staining for safranin O, fissure formation in the cartilage matrix, fibrillation of the cartilage matrix, the cartilage defect, increase in the number of chondrocytes, decrease in the number of chondrocytes, remodeling of sub-cartilaginous bone and blood vessel invasion in the cartilage matrix. A lesion with fibrillation exhibited decreased staining for safranin O and a decrease in chondrocytes. Decrease in the number of chondrocytes was observed as cluster formations and found around degenerative lesions. Bone remodeling in the sub-cartilaginous bone and blood vessel invasion in the cartilage matrix were observed beneath a severe degenerative lesion in the cartilage. These changes were less severe in the Gel-200 group

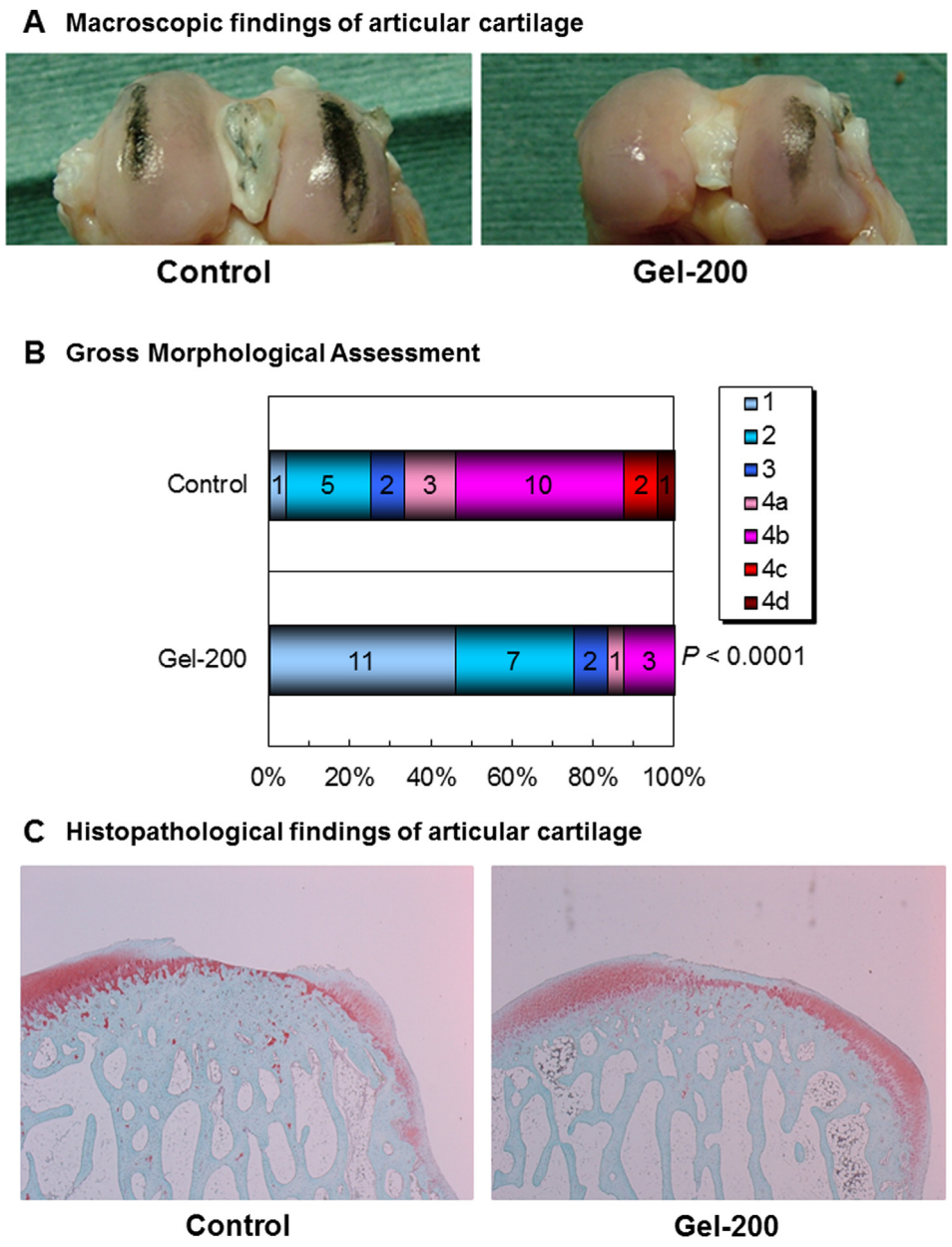

Fig. 1. (A) These representative macroscopic pictures of articular cartilage of the femoral condyles were taken at 9 weeks after ACL transection surgery. In the control group, the surface of the femoral condyle had severe cartilage erosion which stained with India ink. However, in the Gel-200 group, the erosion area was seen to be less than in the control group. (B) Morphological assessment of cartilage damage. As the severity of cartilage lesions increases, so does the score obtained using the previous report. The control group was administered PBS after surgery. Gel-200 groups were administered Gel-200 once. *P*-values were determined by the Wilcoxon rank sum test ($n = 12$ [24 points] per group). (C) These representative photomicrographs were taken at 9 weeks after ACL transection, the stain used is Safranin O and Fast green, and the magnification is ×40. The Safranin O stain displays GAGs content within the cartilage (GAGs: red, Bone and collagen fibers: green). In the Gel-200 group, the severity of the cartilage degeneration was less severe compared with that of the control group.

compared to the control group [Fig. 1(C)]. Mean total histological scores for the articular cartilage were 13.0 ($n = 12$; 95% CI, 11.7–14.3) in the control group and 9.8 ($n = 12$; 95% CI, 7.9–11.7) in the Gel-200 group, respectively. The mean histological score of the Gel-200 group was statistically significantly lower than the control group (Student *t*-test, $P = 0.01$).

Volume of the synovial fluid (SFV), total protein and PGE_2 content in the SF

The mean SFV was 0.901 mL/joint ($n = 12$; 95% CI, 0.779–1.023) in the control group, and 0.558 mL ($n = 12$; 95% CI, 0.442–0.674) in the Gel-200 group, which showed significant suppression of the SFV compared to that in the control group [Fig. 2(A), Student *t*-test, $P < 0.001$]. The mean protein content in the SF was 15.4 mg/joint (95% CI, 12.3–18.5) in the control group, whereas it was 9.3 mg/joint (95% CI, 6.7–11.9) in the Gel-200 group [Fig. 2(B)]. The decreases in the protein contents in the Gel-200 group were statistically significant compared to that in the control group (Student *t*-test, $P = 0.007$). The mean PGE_2 content in SF was 519 pg/joint (95% CI, 381–657) in the control group [Fig. 2(C)], whereas that in Gel-200 group was 278 pg/joint (95% CI, 148–408). Gel-200 significantly decreased PGE_2 content in SF (Student *t*-test, $P = 0.021$).

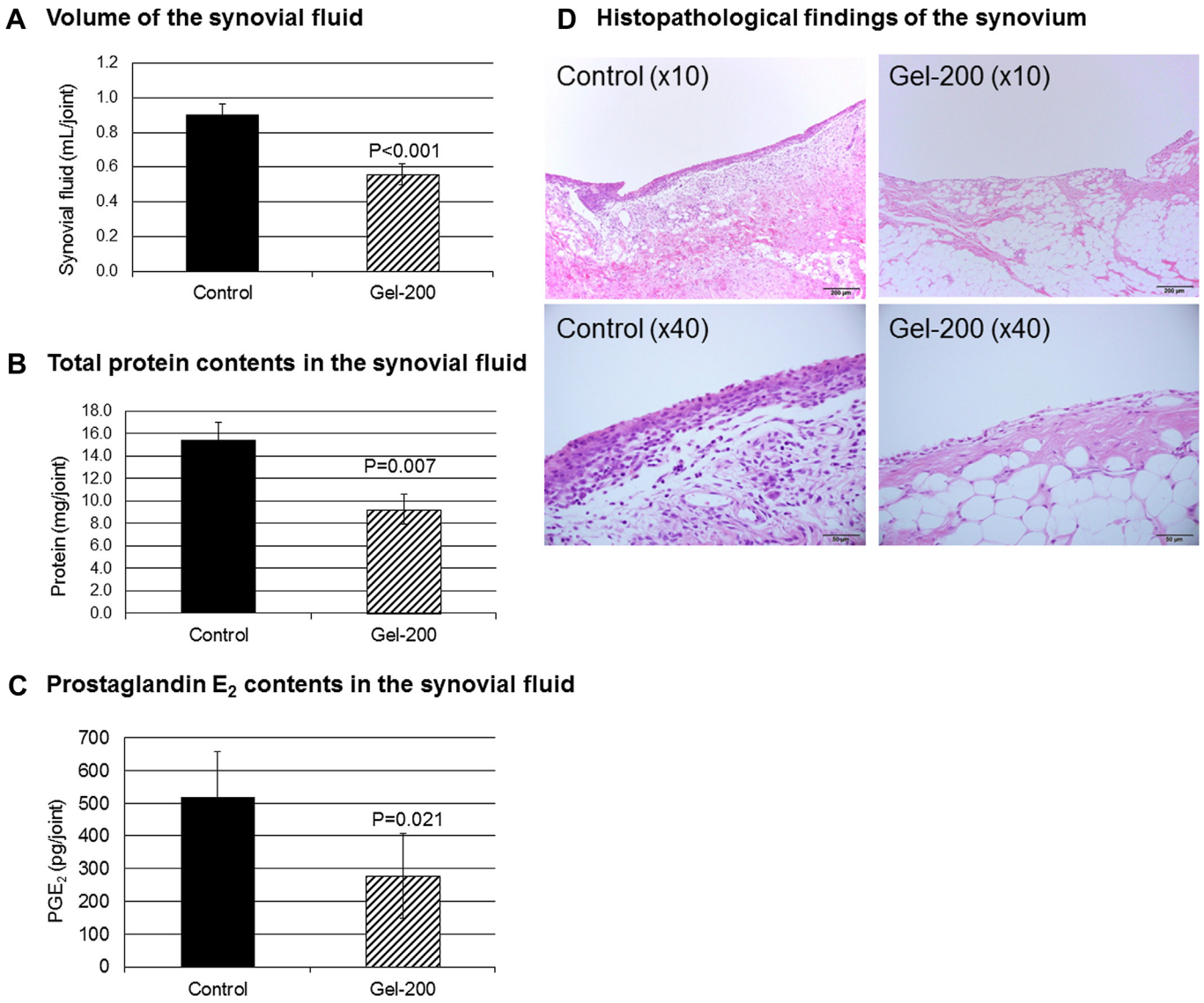

Fig. 2. (A) The volume of SF in the joint cavities at 9 weeks after ACL transection. Four weeks after ACL transection, the test materials were administered into the joint cavities at a volume of 50 μL/kg/joint. Nine weeks after ACL transection, synovial fluid (SF) was collected by washing the joint cavity 2 times with 2 mL physiological saline. Gel-200 significantly suppressed the increase in volume of the SF. (B) The total protein content in synovial fluid. The total protein content in synovial fluid was measured using a commercial assay kit (BCA Protein Assay Kit, PIERCE, USA). Values represent the means ± 95% CI. *P*-values were determined by the Student's *t*-test ($n = 12$ per group). (C) The PGE_2 content in synovial fluid. The PGE_2 content in synovial fluid was measured using a commercial assay kit (PGE_2 EIA Kit, Enzo Life Sciences, Inc., USA). Values represent the means ± 95% CI. *P*-values were determined by the Student's *t*-test ($n = 12$ per group). (D) These representative photomicrographs were taken at 9 weeks after ACL transection. Paraffin sections were made from formalin-fixed synovium and stained with HE. The magnification is ×10 or ×40. The following histopathological observations were carried out and scored for synovium: cuboidal/stratified synovial lining cells, cellular infiltration, fibrosis/edema, hemorrhage and calcium deposition in synovial tissue. The severity of the changes was slighter in the Gel-200 group.

Histopathological examination of the synovium

The histopathological changes were observed and scored based on a 4-point scale of Grade 0 (No change) to 3 (Severe change) in the synovium of: cuboidal synovial lining cells, stratified synovial lining cells, cellular infiltration, fibrosis/edema, and hemorrhage in the synovial tissue. The severity of the changes was slightly less in the Gel-200 group [Fig. 2(D)]. The mean total histological score for the synovium in the control group was 8.8 ($n = 12$; 95% CI, 8.0–9.6), whereas it was 7.4 ($n = 12$; 95% CI, 5.9–8.9) in the Gel-200 group. A trend of lower scores was seen in the Gel-200 treated group, though this difference was not statistically significant.

Bradykinin induced-arthritic pain model in rats

The pain scores using the 5-point scale are shown in Fig. 3. Gel-200 reduced bradykinin-induced arthritic pain with statistically significant differences as compared to the control group at 1, 2, and 4 weeks after the injection ($n = 12$ each evaluation point, Wilcoxon rank sum test, $P < 0.001$, $P = 0.003$, and $P = 0.016$, respectively).

Residual ratio of Gel-200 in the joint cavity (SF) and synovium of the knee in rabbits

The residual property of Gel-200 in the SF and the synovium was investigated ($n = 5$, 10 knee joints each evaluation point).

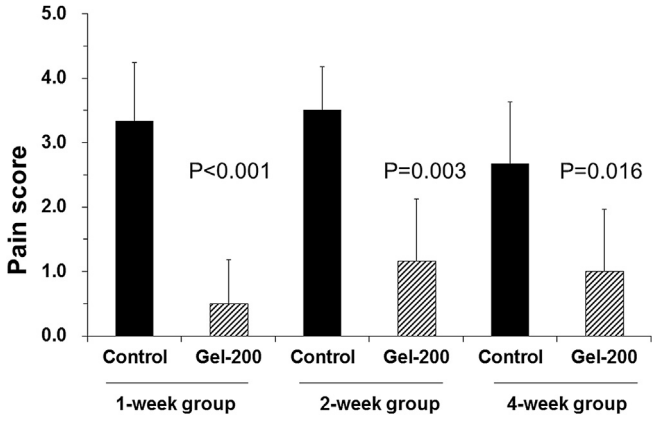

Fig. 3. Gel-200 or PBS was injected in a single dose into the joint cavities of the left hindlimb at a volume of 50 μL/joint. One, 2, or 4 weeks after the injection, walking of each animal was observed for 2 min after injection of the bradykinin solutions under blinded conditions. The severity of pain was scored according to the 4-point scale. Values represent the means ± 95% CI. *P*-values of 1-, 2- and 4-week group were determined by the Wilcoxon's *t*-test ($n = 12$ per group).

Most of the administered Gel-200 disappeared from the SF immediately between 1 and 28 days, as shown in Fig. 4. The residual ratio decreased up to 7 days after Gel-200 injection and subsequently Gel-200 was not detected in the SF. In the synovium, the administered Gel-200 was detected between 1 and 28 days after the injection. The mean residual ratio of injected Gel-200 in the synovium was 3.3% (95% CI, 2.4–4.2) at 28 days after the injection.

Measurement of matrix metalloproteinase-1, -3, and -13 production by human chondrocytes

The production of MMP-1, MMP-3 and MMP-13 is stimulated by IL-1β in human chondrocytes. When human chondrocytes were incubated with IL-1β in the presence of Gel-200, Gel-200 significantly inhibited the production of MMP-13 and MMP-3 in human chondrocytes at concentrations from 0.1 to 3 mg/mL in a concentration-dependent manner [Fig. 5(A) and (B)]. Gel-200 showed a trend toward inhibition in MMP-1 production. Only at the maximum concentration (3 mg/mL), its inhibitory effect was significant [Fig. 5(C)].

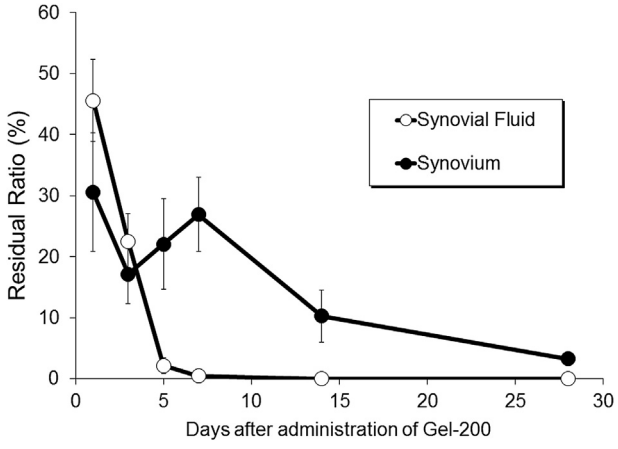

Fig. 4. Gel-200 was administered into the joint cavities of both hindlimbs at a volume of 50 μL/kg/joint in rabbit. Necropsy was performed on days 1, 3, 5, 7, 14 and 28 after injection of the test material. The residual ratio of Gel-200 in the synovial fluid and the synovium was calculated by quantifying trans-cinnamic acid, a component of Gel-200, in preparations recovered from the test rabbits. Values represent the means ± 95% CI ($n = 5$, 10 knee joints per group) (○) synovial fluid; (●) synovium.

Measurement of PGE_2 production by human synoviocytes

The production of PGE_2 is stimulated by IL-1β in human synoviocytes. When human synoviocytes were incubated with IL-1β in the presence of Gel-200, the levels of secreted PGE_2 were decreased in a concentration-dependent manner [Fig. 6(A)]. Gel-200 suppressed PGE_2 production in the range of 0.03–3 mg/mL in a concentration-dependent manner. The percent inhibition of 0.03, 0.3 and 3 mg/mL Gel-200 was 18.4, 44.9% and 49.2%, respectively. When human synoviocytes were incubated for 44 h after 4 h of preincubation with 50 μg/mL of anti-CD44 antibody IM7, the inhibitory effect of 3 mg/mL of Gel-200 was partially reversed [Fig. 6(B)].

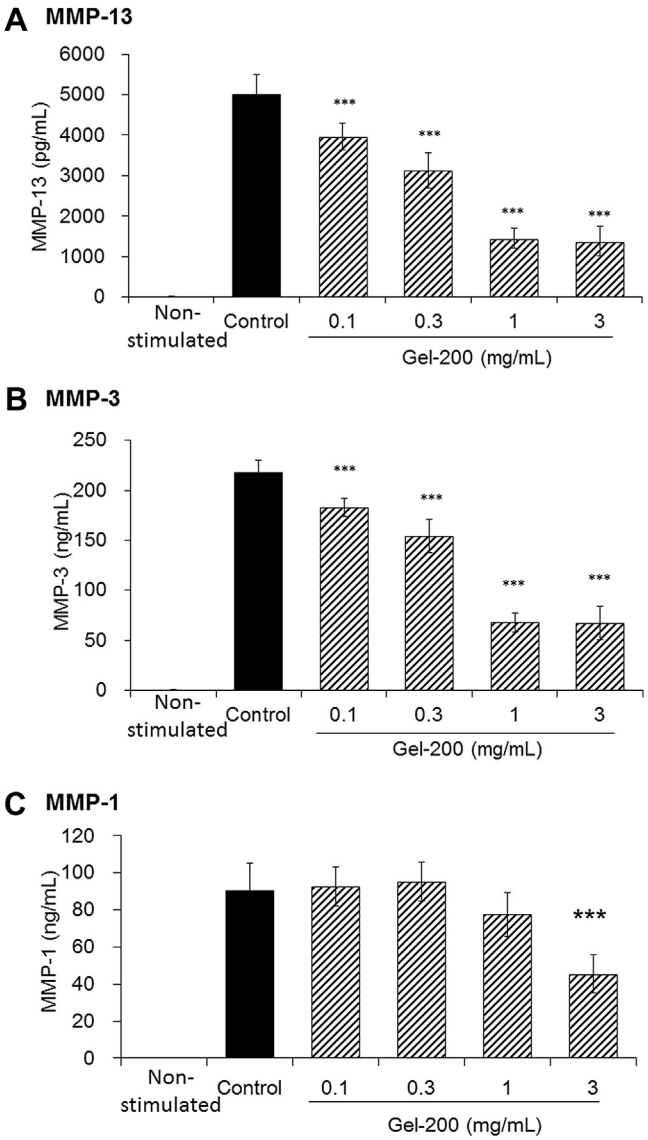

Fig. 5. Human chondrocytes were incubated with 10 ng/mL IL-1β in the absence or presence of Gel-200 at various concentrations (0.1, 0.3, 1, 3 mg/mL) for 16 h. Secreted MMP-1, MMP-3 and MMP-13 were measured by ELISA. Values represent means ± 95% CI in six wells. Data shown are representative of three independent experiments. ***$P < 0.0001$, significant difference from the control group by the Williams test.

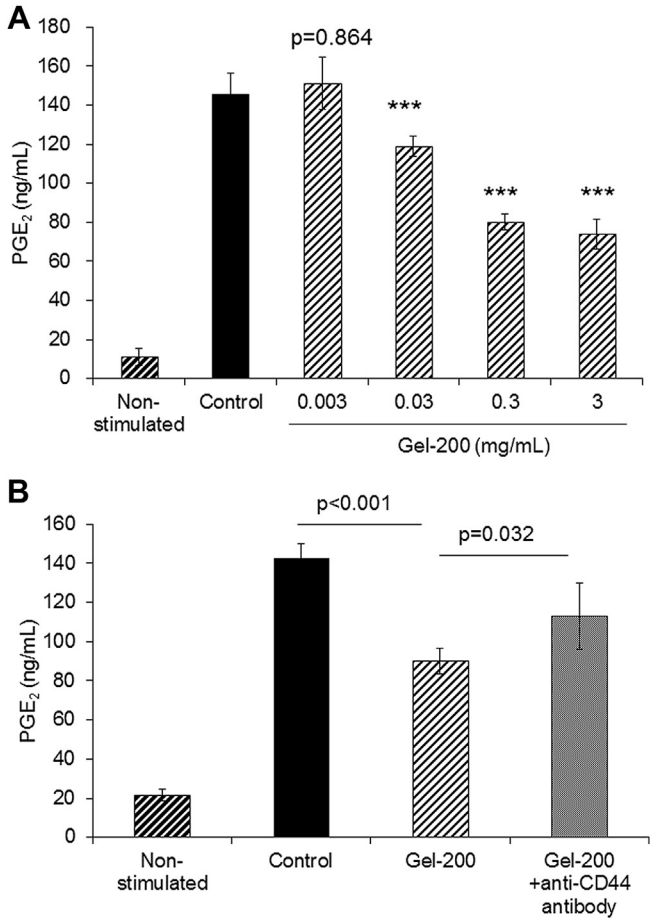

Fig. 6. Human synoviocytes were incubated with 10 ng/mL IL-1β in the absence or presence of Gel-200 at various concentration (0.003, 0.03, 0.3, 3 mg/mL) for 42 h. Secreted PGE_2 in the supernatants was measured by ELISA. Values represent means ± 95% CI in six wells. Data shown are representative of three independent experiments. ***$P < 0.0001$, significant difference from the control group by the Williams test.

Discussion

The effect of Gel-200 was comprehensively evaluated by comparing the morphological assessment of the parameters of cartilage degeneration, indicators of hydrarthrosis including the SFV, total protein content, PGE_2 content and histopathological examination using a rabbit OA model. These results demonstrate that single-dose IA injection of Gel-200 prevented cartilage degeneration more effectively than PBS. The OA model used in the present study is produced by transecting the ACL, resulting in irreversible degeneration of the cartilage over an extended period of time. The cartilage changes are similar to those observed in human OA[12]. Therefore, the ACL transection model has been frequently used as an arthritis model for the evaluation of hyaluronan preparations[13–15] or glucosamine[16]. In the previous studies, it was reported that three IA injections of hylan G-F 20[15] and HYADD 4-G[17] or five IA-HA injections[13,14] had chondroprotective effects in this model. It is considered that HA acts as a shock absorber or mechanical stabilizer for the nociceptors in the joints[18]. Belmonte C *et al.* report that IA-HA reduces pain through its effect on peripheral pain receptors, increasing the viscoelastic properties of the SF[19]. Furthermore, as the possible mechanism, HA itself may trap NO molecules, and protect against chondrocyte apoptosis during the development of OA[20]. Since NO production in the meniscus and synovium of the HA treated group were significantly lower than that in the control, the inhibition of NO production might be part of the mechanism of the therapeutic effect of HA on OA[21]. Hashimoto *et al.* report that there are significant correlations between NO release and chondrocyte apoptosis and between OA severity and chondrocyte apoptosis[22]. Additionally, since HA inhibits MMP-3 and IL-1β production in the synovium in the ACL transection model, one mechanism of HA might be down-regulation of MMP-3 and IL-1β production in the synovium during the early development of OA[23].

This study is the first report of the chondroprotective effects of HA with a single-dose IA injection. This effect appeared to result from the high viscosity and elasticity of Gel-200, which produces sustained lubrication and protective action against abrasion and physical stress from its sustained presence in the joint cavity for a longer period of time than unmodified hyaluronan. In addition, Gel-200 inhibited IL-1β-induced MMP production in human chondrocytes in a concentration-dependent manner. MMP is expressed by chondrocytes and synovial cells in human OA and is thought to play a critical role in cartilage destruction[24–27]. Mehraban F *et al.* demonstrated that MMP-3 is initially upregulated in the synovium which may play a pivotal role in the pathogenesis of cartilage, however, chondrocyte-derived MMP-3 is upregulated in the later phase, contributing further to progression of the cartilage lesions[28]. We speculate that the chondroprotective effect of Gel-200 is due to the inhibition of MMP production. The minimal effective concentration of Gel-200 for MMP-1 (3 mg/mL) was higher than for MMP-3, 13 (100 μg/mL). It has been reported that HA inhibits phosphorylation by p38 mitogen-activated protein kinase (MAPK) via its principal receptor, CD44, and exerts an anti-inflammatory effect[29,30]. Gel-200 weakly inhibited MMP-1 compared with the other MMPs, because p38 MAPK contributed slightly to the production of MMP-1. Okada A *et al.* describes that MMPs are involved in cartilage degeneration via the fragmentation of aggrecan and collagen, and are increased in the SF of OA patients[25]. In the present study, the inhibitory effects of MMP production by Gel-200 were considered to contribute to the articular cartilage protection in knee OA.

Gel-200 appeared to improve synovitis, as judged from the reduction in the increase in SFV, total protein and PGE_2 content in SF. Since cartilage degeneration is milder when synovitis is not severe, these changes induced by Gel-200 may interact beneficially to reduce the progression of the pathological changes. The histopathological observations also support this conclusion. In fact, glycosaminoglycan (GAG) in the cartilage matrix and surviving chondrocytes was maintained to a greater degree in the Gel-200 group and the cartilage degeneration was mild. On the other hand, Gel-200 improved the cuboidal/stratified synovial lining cells, cellular infiltration, fibrosis/edema and hemorrhage in the synovial tissue and suppressed the inflow of blood components into the joint cavity by alleviating the synovitis. These results suggest that Gel-200 may suppress the synovitis associated with knee OA.

In the second study, Gel-200 exerted a long-lasting analgesic effect for up to 4 weeks in rats. The mechanism by which Gel-200 acts in the pain model is unclear. However, the analgesic effect assessed by this pain model was reported to be associated with the concentration of hyaluronan in synovial tissue[9,10]. We examined the local retention of Gel-200 in the synovium and SF after a single-dose IA injection in rabbits. Gel-200 was retained in the synovium at least at 28 days [Fig. 4]. We speculate that Gel-200 administered into the joint cavity penetrated to the synovium, and was retained there for a long time. Although the remaining amount of Gel-200 may be small, it was thought that sufficient Gel-200 for the pharmacological effect to be shown remained in the synovium at 28 days after injection. Furthermore, Gel-200 decreased PGE_2

production in a concentration-dependent manner in human synoviocytes. The inhibitory effect of Gel-200 was partially reversed by pretreatment with anti-CD44 antibody IM7. We considered the mechanism of Gel-200 action was biologically mediated by CD44, but not the rheological properties. It has been reported that HA inhibits PGE_2 production, and that pretreatment with OS/37, a monoclonal antibody specific for the hyaluronate-binding epitope on CD44, reverses the inhibitory effects of HA[31]. The inhibition of PGE_2 production by HA was also confirmed in a clinical study[32]. These findings suggest that Gel-200 exerts an analgesic effect via multimodal functions, and the effects of Gel-200 are similar to those observed with other HA preparations. We consider that the long-lasting pharmacological effect is associated with the sustained local retention in the synovium and SF. Moreover, in order to calculate the half-life of Gel-200, additional examination with a radioisotope is required.

In conclusion, results in our non-clinical studies showed that the single-dose IA injection of Gel-200 exerted chondroprotective, anti-inflammatory effects, and long-lasting analgesia, suggesting the beneficial multimodal function of Gel-One® in patients with symptomatic OA.

Contributions

K. Yoshioka: study conception and design, acquisition of data, interpretation of data, drafting and revising the article, final approval of the article to be published.

Y. Yasuda: acquisition of data, revising the article, final approval of the article to be published.

T. Kisukeda: acquisition of data, revising the article, final approval of the article to be published.

R. Nodera: acquisition of data, revising the article, final approval of the article to be published.

Y. Tanaka: study conception and design, drafting and revising the article, final approval of the article to be published.

K. Miyamoto: study conception and design, acquisition of data, drafting and revising the article, final approval of the article to be published.

Conflict of interest

K. Yoshioka, Y. Yasuda, T. Kisukeda, R. Nodera, Y. Tanaka and K. Miyamoto are employees of Seikagaku Corporation working in the Research & Development Division.

Role of the funding source

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