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Biocompatibility of cross-linked hyaluronate (Gel-200) for the treatment of knee osteoarthritis

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SUMMARY

Objective: To compare the biocompatibility and immunogenicity of two intra-articular hyaluronan formulations, Gel-200 (Gel-One®) and hylan G-F 20 (Synvisc® series).
Experimental design: A comparison of the biocompatibility of Gel-200 and hylan G-F 20 was made using a rat subcutaneous air pouch model and the knee joint of normal rabbits. Immunogenicity was evaluated using a homologous passive cutaneous anaphylaxis (PCA) assay in guinea pigs.
Results: In the air pouch model in rats, characteristic fibrous belts formed in the subcutaneous tissue. Injection of hylan G-F 20 into the air pouch induced granulomatous nodules primarily composed of macrophages, multinucleated giant cells, and eosinophils accompanied with the test material in the center of the nodules in the fibrous belt. Furthermore, the thickness of the fibrous belt in the hylan G-F 20 group increased significantly compared to the saline group. Injection of Gel-200 into the air pouch induced neither granulomatous inflammation nor significant thickening of fibrous belt, while foamy macrophages containing the test material were observed. Intra-articular injection of hylan G-F 20 into the rabbit knee joints induced granulomatous inflammation, eosinophil infiltration, and significant increase in the number of cells in the synovial fluid, while these findings were absent in the Gel-200 group. In the immunogenicity assay, hylan G-F 20 induced a positive PCA reaction, but the Gel-200 did not.
Conclusion: Gel-200 showed more favorable biocompatibility and less immunogenicity compared to hylan G-F 20. Gel-200 is expected to be a single injection hyaluronan product with less safety concerns for the treatment of knee osteoarthritis (OA) pain.

Introduction

Osteoarthritis (OA) of the knee is a common joint disorder in the aging population. Intra-articular hyaluronic acid (HA) products have been widely accepted as a viscosupplements for the treatment of knee OA pain[1,2]. HA products are divided into two major types, native HA products and cross-linked HA products. Native HA products are injected 3 to 5 times per treatment course and their safety has been established based on the results of long-term clinical use[3]. In the cross-linked HA category, there are three products on the US market, Gel-One®, Synvisc® and Synvisc-One®. Synvisc and Synvisc-One are composed of hylan G-F 20, which is a mixture of two cross-linked HA derivatives. Synvisc-One was the first single injection product approved in the US and contains three times the volume of hylan G-F 20 as Synvisc, which requires three injections. However, there is growing clinical evidence to suggest that hylan G-F 20 may be associated with an increased incidence of pseudosepsis or granulomatous synovitis[4–13]. Furthermore, in non-clinical studies, evidence of an immune response to hylan G-F 20 has been demonstrated. In particular, it has been reported that hylan G-F 20 exhibited immunogenicity in guinea pigs and certain strains of mice[14], and induced an inflammatory tissue response in the air pouch in mice[15]. The air pouch model is a well-established model to test local inflammatory and immunological effects[16]. Furthermore, it has been reported that normal rabbit knee joints treated with hylan G-F 20 were slightly inflamed[17]. It appears likely that the adverse responses in clinical practice may be attributable to a component of hylan G-F 20. Therefore, safer products requiring fewer injections would be expected to improve the quality of life for OA patients.

Gel-One® is a recently approved single injection product for the treatment of knee OA pain on the US market and is composed of Gel-200, a novel cross-linked HA hydrogel manufactured by photo-gelation technology. The strands of HA in Gel-200 are

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bound to each other via dimers of cinnamic acid with ultraviolet irradiation resulting in a clean cross-linked highly viscoelastic hydrogel. The highly purified photocurable HA derivative chemically linked with cinnamic acid, an intermediate of Gel-200, can be cross-linked by using only ultraviolet irradiation without any additional condensation reagents. The photo-gelation technology achieves high purification without any elimination process for residual reagents and/or by-products after cross-linking. 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 associated pain for over 13 weeks[18]. There have been no reported incidents of pseudosepsis from Gel-200 in clinical use. The absence of allergic reactions or pseudosepsis and the low incidence of adverse events associated with this treatment support the favorable safety profile of Gel-200 for treatment of symptomatic OA of the knee[18].

The objective of this study was to compare the biocompatibility and immunogenicity of Gel-200 and hylan G-F 20 in non-clinical models.

Materials and methods

Animals

Female Sprague–Dawley rats (8 weeks old; Charles River Laboratories Japan, Inc., Kanagawa, Japan), male New Zealand White rabbits (11 weeks old; Oriental Yeast Co., Ltd, Tokyo, Japan), and male Hartley guinea pigs (6 weeks old; Japan SLC, Inc., Shizuoka, Japan) were utilized in this study. The animals were quarantined and acclimatized to the environmental conditions for 1 week. The study protocols were reviewed by the In-house Animal Experiment Ethics Committee, and after approval, performed under the animal husbandry/management system in an appropriate environment with animal protection/welfare in mind.

Air pouch model in rats

Seventy-two rats were anesthetized with isoflurane and 20 mL of air sterilized with a 0.22 μm filter was injected subcutaneously into the back. Three days later, an additional 10 mL of air was injected into the air pouch to maintain the cavity. Six days after the first injection, the rats were allocated to four groups ($n = 18$) based on the body weights. Saline, carrageenan (1 w/v%; λ-Carrageenan, Sigma Aldrich, MO, USA), hylan G-F 20 (8 mg/mL; Synvisc®, Genzyme Corporation, NJ, USA), or Gel-200 (10 mg/mL; Gel-One®, Seikagaku Corporation, Tokyo, Japan) was administered into the air pouch of animals at 2 mL/body. Fourteen, 28 and 56 days after the administration, six rats from each group were euthanized. The air pouch was washed with 10 mL of phosphate buffered saline (PBS) with 1 mM ethylenediaminetetraacetic acid (EDTA), the pouch fluid was collected, and the total number of cells in the pouch fluid was counted with a light microscope. For characterization of the cell types, the pouch fluid was smeared on a slide, stained with Diff-Quick (Sysmex Corporation, Hyogo, Japan), and observed under a light microscope. The proportion of the cell profiles corresponding to neutrophils, eosinophils, monocytes or lymphocytes was recorded ($n = 6$).

For histological examination, pouch tissue was harvested immediately after the pouch fluid collection, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (HE) or Movat's pentachrome (MP). Measurements of the thickness of fibrous belt and histological scoring were performed independently by two evaluators under blinded conditions. Intra-observer and inter-observer reproducibility of the histological scoring were confirmed. Fibrous belts are observed characteristically in air pouch models in rats and mice[15,16,19] and their thickness is measured to evaluate the inflammatory reaction of biomaterials. Fibrous belt is defined as a belt-like structure composed of fibroblasts and collagenous matrices and formed between the subcutaneous connective tissue and pouch cavity; the thickness in representative area of each slide was measured by cellSens Standard software (Olympus Corporation, Tokyo, Japan). Furthermore, as granulomatous inflammation with multinucleated giant cells, foamy macrophage infiltration, and eosinophil infiltration were characteristic reactions in the fibrous belt, these findings were graded 0–3, according to the scores shown in Table I. In each group, some slides had to be excluded from histological examination due to absence of a fibrous belt, which had possibly sloughed off during lavage fluid collection or the sectioning procedures. The numbers of evaluated samples were $n = 3$–6 per each group.

Biocompatibility study in normal rabbit knee joints

Thirty-six rabbits were allocated to six groups ($n = 6$) based on the body weights and anesthetized by an intravenous injection of mixed anesthesia using midazolam (Dormicum, Astellas Pharma Inc, Tokyo, Japan), xylazine (Selactar 2%, Bayer Yakuhin Ltd., Osaka, Japan), butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and saline (1:2:1:2) at a volume of 2 mL/body. Three groups of animals received a single intra-articular injection of 0.25 mL of either saline, Gel-200, or hylan G-F 20 into both knee joints and the other three groups of animals received three consecutive weekly intra-articular injections of 0.25 mL of either saline, Gel-200, or hylan G-F 20 into both knee joints. Fifteen days after the first injection, all the rabbits were euthanized and both knee joints were collected. From the right knee joints, synovial fluid was collected by washing the joint cavity three times with 0.5 mL lavage solution (PBS with 1 mM EDTA). For each sample the number of total cells, monocytes, lymphocytes, and heterophils in the synovial fluid were determined by the same method as in the air pouch model in rats ($n = 6$).

From the left knee joints, the synovium was collected and fixed in 10% neutral buffered formalin. Fixed samples were processed, embedded in paraffin, sectioned, and stained with HE or MP for histological examination. Histological scoring was performed

Table I
Histological scoring

	Histological finding	Score
Granulomatous inflammation with multinucleated giant cells*	None	0
	Slight	1
	Moderate	2
	Severe	3
Foamy macrophage infiltration*	None	0
	Slight	1
	Moderate	2
	Severe	3
Eosinophil infiltration*	None	0
	Slight	1
	Moderate	2
	Severe	3
Synovial thickening†	Lining cell layer 1–2 cells thick	0
	Lining cell layer 3–5 cells thick	1
	Lining cell layer 6–8 cells thick and/or mild increase in cellularity	2
	Lining cell layer >9 cells thick and/or severe increase in cellularity	3

* Score used for rat air poch model and rabbit knee joint model.
† Score described previously[20] and used for rabbit knee joint model.

independently by two evaluators under blinded conditions. Intra-observer and inter-observer reproducibility of the histological scoring were confirmed. Granulomatous inflammation with multinucleated giant cells, foamy macrophage infiltration, and eosinophil infiltration was scored on a 4-point scale of Grades 0 (No change) to 3 (Severe change). Synovial thickening was scored using criteria given in a previous report[20] (Table I). The numbers of evaluated samples were $n = 6$ per each group.

Immunogenicity study in guinea pigs

Immunization and serum collection

An immunogenicity study in guinea pigs was performed as described by Nagami *et al.*[21] with some modifications. Thirty-four guinea pigs were allocated to six groups ($n = 6$ except for the positive control group, $n = 4$) and immunized by subcutaneous injection of the test material once a week for 3 weeks as follows: (1) Gel-200 at 2.5 mg/kg, (2) Gel-200 at 2.5 mg/kg + an equal volume of Freund's complete adjuvant (FCA, Difco Laboratories Inc, MI, USA), (3) hylan G-F 20 at 2.5 mg/kg, (4) hylan G-F 20 at 2.5 mg/kg + FCA, (5) ovalbumin (OVA, Sigma Aldrich) at 1.5 mg/animal + FCA as a positive control, and (6) non-immunized as a negative control. FCA was used only for the first immunization, and incomplete adjuvant was used for the second and third immunizations. Sixteen days after the final immunization, blood was drawn from each animal and separated sera were stored at −80°C until analyses described below.

Homologous passive cutaneous anaphylaxis (PCA)

Twelve naive guinea pigs each received six intradermal injections into the dorsal skin of 0.1 mL sera from immunized animals. Twenty-four hours after injection, each animal was administered intravenously a corresponding antigen (Gel-200 5 mg/kg, hylan G-F 20 5 mg/kg, or OVA at 1 mg/animal) and Evans blue dye. Thirty minutes after the elicitation, the animals were euthanized and the diameters of blue spots that had developed at the serum injection sites were measured. When a blue spot was >5 mm in diameter, the PCA reaction was considered positive and further diluted serum was tested in other naive animals to determine the maximal PCA titer of the serum. Each serum was tested in two naive recipients to confirm the reproducibility of the reactions.

Statistical analyses

Statistical analyses were performed using SAS (SAS Institute Inc., NC, USA). The effects from treatment with saline, Gel-200, hylan G-F 20, or carrageenan were analyzed by the following multiple comparison tests. One-way analysis of variance (ANOVA) with Dunnett's test was used for the thickness of the fibrous belt and cell numbers in the lavage fluid. Kruskal–Wallis with Steel's test was used for histological scores. Results were represented as mean and 95% confidence intervals (CI). Any P-values of <0.05 were considered statistically significant.

Results

Histological analysis of the rat air pouch model

In the saline group at day 14, there were distinctive fibrous belts composed of fibroblasts and collagenous matrices, which might be the wall of the air pouch. Average thickness of the fibrous belt at day 14 was 205 μm ($n = 5$; 95% CI, 78–333), which decreased at subsequent time points [Fig. 1(A) and (E)].

In the Gel-200 group, the average thickness of the fibrous belt at day 14 (343 μm ($n = 4$; 95% CI, 101–585)) was not significantly different from that in the saline group. Granulomatous inflammation and eosinophil infiltration were not observed. Significant infiltrations of foamy macrophages containing test material were observed in and on the surfaces of the fibrous belts at days 14 and 28 [Fig. 1(C, G, and H)].

In the hylan G-F 20 group, the average thickness of the fibrous belt at day 14 (789 μm ($n = 3$; 95% CI, 289–1289)) was significantly higher than that in the saline group ($P = 0.002$). Thickening of the fibrous belt tended to resolve at subsequent time points. Furthermore, significant granulomatous inflammation with multinucleated giant cells (days 14, 28, and 56) and infiltration of eosinophils (days 28 and 56) were observed [Fig. 1(B, D, I and J)].

In the carrageenan group, the average thickness of the fibrous belt at day 14 (738 μm ($n = 5$; 95% CI, 426–1050)) was significantly higher than that in the saline group ($P = 0.002$). Thickening of the fibrous belt did not resolve at subsequent time points. Foamy macrophages (days 14, 28, and 56) and eosinophil infiltration (days 14 and 28) were also significant [Fig. 1(C) and (D)]. Granulomatous inflammation could not be scored in the same grading system as in the other groups because large-sized and eosinophilic macrophages without prominent multinucleated giant cell accumulation were prevalent in the carrageenan group and, though the degree of inflammation was severe [Fig. 1(F)], these findings were different from the typical granulomatous inflammation seen in the hylan G-F 20 group.

Pouch fluid cell count

In the Gel-200 group, the average number of total cells, lymphocytes, and monocytes increased at day 14, and decreased after day 28, though these changes were not significant compared to the saline group (Table II).

In the hylan G-F 20 group, the average number of total cells, lymphocytes, and monocytes increased at day 14, and decreased after day 28, though these changes were not significant compared to the saline group. Eosinophil infiltration was observed in only the hylan G-F 20 group at days 14 and 28.

In the carrageenan group, the average number of total cells, lymphocytes, monocytes, and neutrophils were significantly increased compared to the saline group at multiple time points.

Histological analysis of normal rabbit knee joints

In the saline group, no abnormalities were detected in the synovium in both the single- and three-injection regimens [Fig. 2(A–E)].

In the Gel-200 group, slight proliferation of the synovial cells and fragments of the test material in the adipose tissue with infiltration of macrophages were seen [Fig. 2(A) and (F)]. Foamy macrophage infiltration score was increased significantly compared to the saline group in the three-injection regimen [$P = 0.01$; Fig. 2(C)]. Granulomatous inflammation and eosinophil infiltration were not observed.

In the hylan G-F 20 group, the synovial cells showed similar changes to the Gel-200 group. Granulomatous inflammation with multinucleated giant cells emerged in both single- [Fig. 2(G)] and three-injection (photomicrographs not shown) regimens, though changes in the scores were not significantly different from the saline group. Eosinophil infiltration score was significantly higher than the saline group in the three-injection regimen [$P = 0.004$; Fig. 2(D)], while foamy macrophage infiltration was not detected.

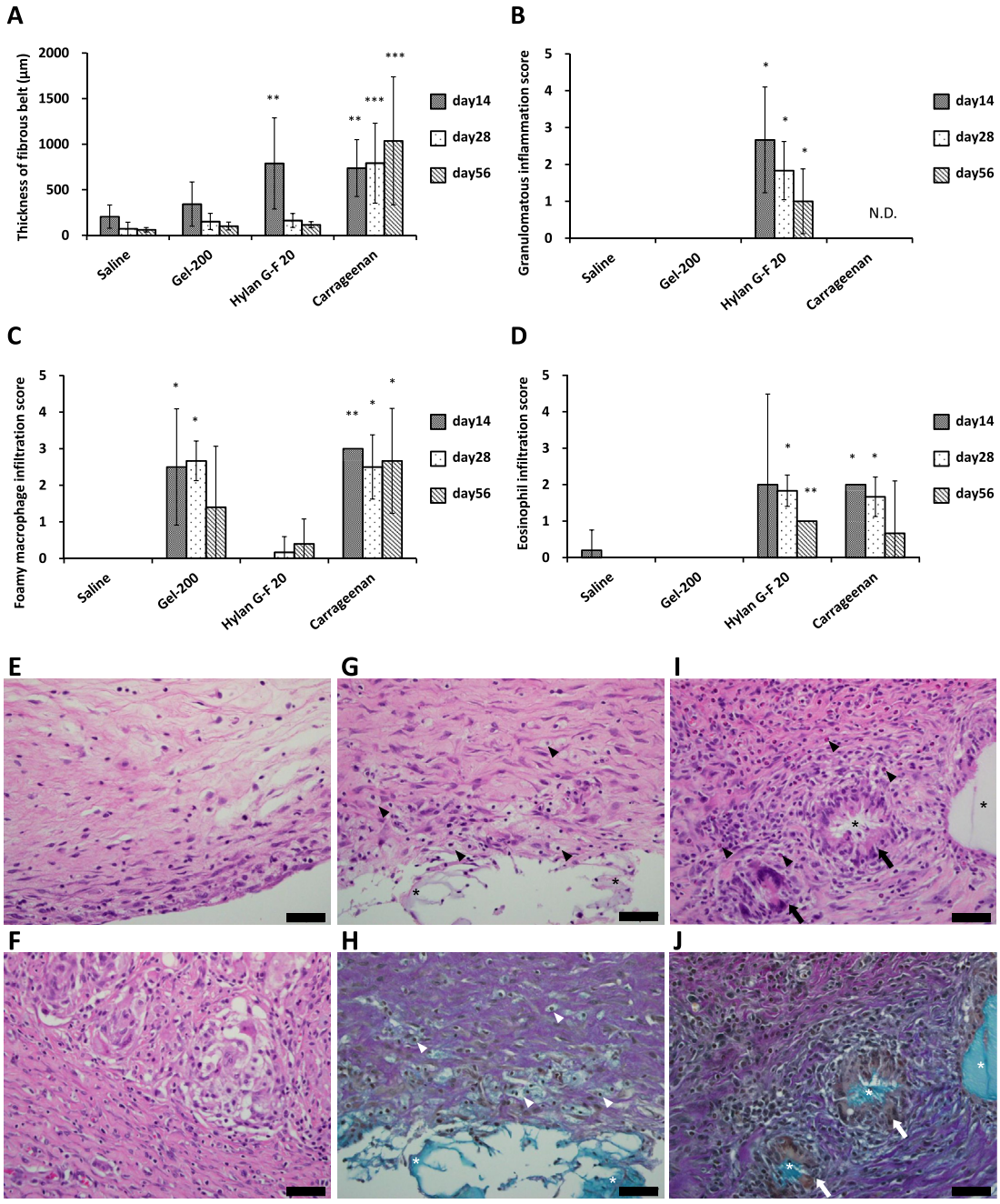

Fig. 1. Histological evaluation of the air pouch in rats after injection of four different samples. Values represent the means ± 95% CI ($n = 3$–6). (A) Thickness of fibrous belt was increased in the hylan G-F 20 (**$P = 0.002$) and carrageenan group (**$P = 0.002$) compared to the saline group at day 14. On days 28 and 56, only the carrageenan group showed significant increase (***$P < 0.001$ for both). (B) The score for granulomatous inflammation with multinucleated giant cells was higher in all three time points in the hylan G-F 20 (*$P = 0.02$, 0.01, and 0.02 at days 14, 28, and 56, respectively) compared to the saline group. N.D.: not determined. (C) The foamy macrophage infiltration score was increased in the Gel-200 group at days 14 (*$P = 0.02$) and 28 (*$P = 0.02$) and in the carrageenan group at days 14 (**$P = 0.008$), 28 (*$P = 0.02$), and 56 (*$P = 0.02$) compared to the saline group. (D) The eosinophil infiltration score was increased in the hylan G-F 20 group at days 28 (*$P = 0.01$) and 56 (**$P = 0.005$) and in the carrageenan group at days 14 (*$P = 0.01$) and 28 (*$P = 0.02$) compared to the saline group. (E–J) Histological appearance of the rat air pouches at day 14. (E) In the saline group, fibrous belt composed of fibroblasts and collagenous matrices is seen (HE × 40). (F) In the carrageenan group, accumulation of large-sized macrophages and granulomatous inflammation are seen in the upper-half of the figure. A mature fibrous belt with inflammatory cells is seen in the lower-half of the figure (HE × 40). (G and H) In the Gel-200 group, foamy macrophages (arrowheads) containing the test article (asterisks, stained blue in (H)) are seen in and on the surface of the fibrous belt (G; HE stain × 40, H; MP stain × 40). (I and J) In the hylan G-F 20 group, granulomatous nodules (arrows) primarily composed of macrophages, multinucleated giant cells, and eosinophils (arrowheads) accompanied with the test article (asterisks, stained blue in (J)) in the center of the nodules are seen in the fibrous belt (I; HE stain × 40, J; MP stain × 40). Scale bars: 50 μm (E–J).

Synovial fluid cell count

In the Gel-200 group, neither single- nor three-injection induced significant increase of any types of cells in the synovial fluid (Fig. 3).

In the hylan G-F 20 group, single-injection tended to increase the average number of cells in the synovial fluid but not significantly (total cells; $P = 0.06$) compared to those of the saline [Fig. 3(A)]. Three consecutive weekly injections of hylan G-F 20 significantly increased the average number of total cells (3.8×10^6

Table II
Cell recruitment in the air pouch after injection of four different samples

	The number of cells/days after administration		
	14 days	28 days	56 days
Total cell			
Saline	$7.0 \pm 5.0 \times 10^4$	$7.8 \pm 9.5 \times 10^3$	$4.6 \pm 1.9 \times 10^3$
Gel-200	$4.4 \pm 2.4 \times 10^6$	$4.3 \pm 6.1 \times 10^4$	$9.0 \pm 18.0 \times 10^4$
Hylan G-F 20	$5.3 \pm 2.9 \times 10^6$	$5.7 \pm 7.0 \times 10^4$	$6.9 \pm 10.4 \times 10^4$
Carrageenan	$5.3 \pm 2.7 \times 10^{7***}$	$3.8 \pm 4.4 \times 10^{7*}$	$2.1 \pm 3.1 \times 10^7$
Lymphocyte			
Saline	$2.7 \pm 3.2 \times 10^4$	$3.1 \pm 3.1 \times 10^3$	$1.4 \pm 0.6 \times 10^3$
Gel-200	$3.5 \pm 1.9 \times 10^5$	$3.7 \pm 2.1 \times 10^3$	$2.5 \pm 5.1 \times 10^4$
Hylan G-F 20	$6.4 \pm 5.9 \times 10^5$	$9.9 \pm 6.9 \times 10^3$	$2.1 \pm 3.2 \times 10^4$
Carrageenan	$9.5 \pm 11.9 \times 10^{6*}$	$5.0 \pm 3.4 \times 10^{6***}$	$6.5 \pm 10.6 \times 10^6$
Monocyte			
Saline	$4.3 \pm 2.5 \times 10^4$	$4.8 \pm 6.5 \times 10^3$	$3.2 \pm 1.4 \times 10^3$
Gel-200	$4.0 \pm 2.3 \times 10^6$	$4.0 \pm 5.9 \times 10^4$	$6.4 \pm 13.0 \times 10^4$
Hylan G-F 20	$3.9 \pm 1.8 \times 10^6$	$4.4 \pm 6.8 \times 10^4$	$4.7 \pm 7.2 \times 10^4$
Carrageenan	$3.8 \pm 1.6 \times 10^{7***}$	$2.9 \pm 3.5 \times 10^{7*}$	$1.2 \pm 1.7 \times 10^7$
Neutrophil			
Saline	0	0	0
Gel-200	0	0	0
Hylan G-F 20	0	0	0
Carrageenan	$5.5 \pm 5.5 \times 10^{6**}$	$4.2 \pm 7.2 \times 10^6$	$2.4 \pm 3.4 \times 10^6$
Eosinophil			
Saline	0	0	0
Gel-200	0	0	0
Hylan G-F 20	$7.9 \pm 13.3 \times 10^5$	$2.9 \pm 5.7 \times 10^3$	0
Carrageenan	0	0	0

Values represent the means ± 95% CI ($n = 6$). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs saline for each time point.

($n = 6$; 95% CI, 0.8–6.8 $\times 10^6$); $P = 0.01$), monocytes (2.2×10^6 ($n = 6$; 95% CI, 0.7–3.7 $\times 10^6$); $P = 0.008$), and heterophils (1.0×10^6 ($n = 6$; 95% CI, −0.2–2.3 $\times 10^6$); $P = 0.03$) compared to the saline group [Fig. 3(B)].

Homologous PCA

All animals in the positive control group challenged with OVA showed positive PCA reactions, which confirmed adequate immunization (Table III). In both the Gel-200 and Gel-200 + FCA groups, sera from the immunized animals induced no PCA reaction. On the other hand, sera from animals immunized with hylan G-F 20 (1/6) or hylan G-F 20 + FCA (5/6) induced positive PCA reactions in the recipient guinea pigs. Maximal PCA titers of the sera were 10 and 1 in the hylan G-F 20 and hylan G-F 20 + FCA groups, respectively.

Discussion

Non-cross-linked HA has long been used to reduce pain in OA patients with a favorable safety profile[22,23]. Cross-linked HA is a newer class of intra-articular injection for the treatment of knee OA. Cross-linking is intended to render longer-lasting efficacy to HA by increasing the viscoelasticity and resistance to degradation in the knee joint tissues. The first cross-linked HA product, Synvisc® composed of hylan G-F 20, has been approved and used in the US since 1997[24]. However, there is growing evidence to suggest that hylan G-F 20 may be associated with an increased incidence of severe acute inflammatory reaction that is clinically distinct from the local inflammatory reactions seen with non-cross-linked HA[4–10]. Recently, another type of cross-linked HA product, Gel-200, has been approved. Therefore, it was decided to assess the biocompatibility and immunogenicity of Gel-200 and hylan G-F 20 in non-clinical studies.

In both the rat subcutaneous air pouch and rabbit knee joint models, Gel-200 showed higher biocompatibility compared to hylan G-F 20. Histologically, there was no evidence of apparent toxicity due to Gel-200. Tissue reactions such as phagocytosing foamy macrophages in the rat air pouch model and slight proliferation of synovial cells in the rabbit knee joint seen in the Gel-200 groups were within the limits of what should be expected with a normal biological response against biodegradable and semi-solid materials[25,26]. As for the cell counts in synovial fluid in rabbits, the pattern of cell recruitment in Gel-200-treated animals was almost the same as in the saline group, not only after single injection but also after repeated injections. Schiavinato *et al.* evaluated the biocompatibility of non-cross-linked HA products, Hyalgan and Artz, in the same rabbit model as used in this study and revealed that both materials did not increase the number of inflammatory cells in the synovial fluid[17]. These results suggest that biocompatibility of Gel-200 may be comparable to non-cross-linked HA products, which requires further study for direct comparison of Gel-200 with non-cross-linked HA products.

Compared to Gel-200, hylan G-F 20 showed several different findings in both the rat and rabbit models. First, significant increase in fibrous belt thickness was seen after the injection of hylan G-F 20 into the rat air pouch, which may be attributed to formation of granulomatous nodules in the fibrous belt. In the Gel-200 group, thickening of the fibrous belt was not statistically significant. Similar results were reported in the mouse air pouch model[15,19], where marked fibroblastic responses together with air pouch membrane thickening were detected in hylan G-F 20-injected animals. Second, granulomatous inflammation with multinucleated giant cells appeared in the rat air pouch and in the rabbit knee joint. Hylan G-F 20-related granulomatous reactions were reported in guinea pigs after intradermal administration[27] and in patients treated with repeated intra-articular administrations[11–13]. However, the association between hylan G-F 20 and granulomatous reaction in the clinical setting remains controversial since a recent report argues that granulomas can be expected to occur in the progression of OA without hylan G-F 20 injection[28]. Given the limited opportunities for histological examination in patients, the non-clinical models used in this study will help in the prediction of clinical safety profiling of intra-articular HA products. Third, as for the recruitment of cells in pouch lavage fluid, eosinophils were observed only in the hylan G-F 20 group. Furthermore, hylan G-F 20 caused significant cell increase after repeated injection into the rabbit knee joints, which was not observed in the Gel-200 group. Similar observations were reported by Schiavinato *et al.*[17], where three weekly injections of hylan G-F 20 into rabbit knee joints increased total cell numbers in the synovial fluid at 15 days post-injection whereas those of native HA products did not.

In the immunogenicity assay in guinea pigs, serum from Gel-200-sensitized animals did not induce a PCA reaction even when combined with adjuvant. Guinea pigs are one of the most effective species for detecting the immunogenicity of non-human macromolecules with a molecular weight greater than 5000[29]. PCA has the advantage of measuring not only the biologically active antibodies but also the consequences of allergen/antibody interactions leading to inflammatory mediator release from mast cells and the expression of cutaneous anaphylaxis. Therefore, the lack of immunogenicity of Gel-200 in guinea pigs indicated that the risk of allergic reaction caused by Gel-200 is expected to be very low.

Serum from hylan G-F 20-sensitized guinea pigs induced positive PCA reactions in recipient animals, which was comparable to results of previous studies showing positive PCA reaction by hylan G-F 20 in mice and guinea pigs[14,27]. Guinea pig homologous PCA is used as a biological assay of sera to detect antigen-specific IgG1 and IgE[30]. For IgE production, Th-2 cytokines such as IL-4, and 13 are essential[31]. In addition, another Th-2 cytokine, IL-5, has been recognized as the major maturation and differentiation factor for

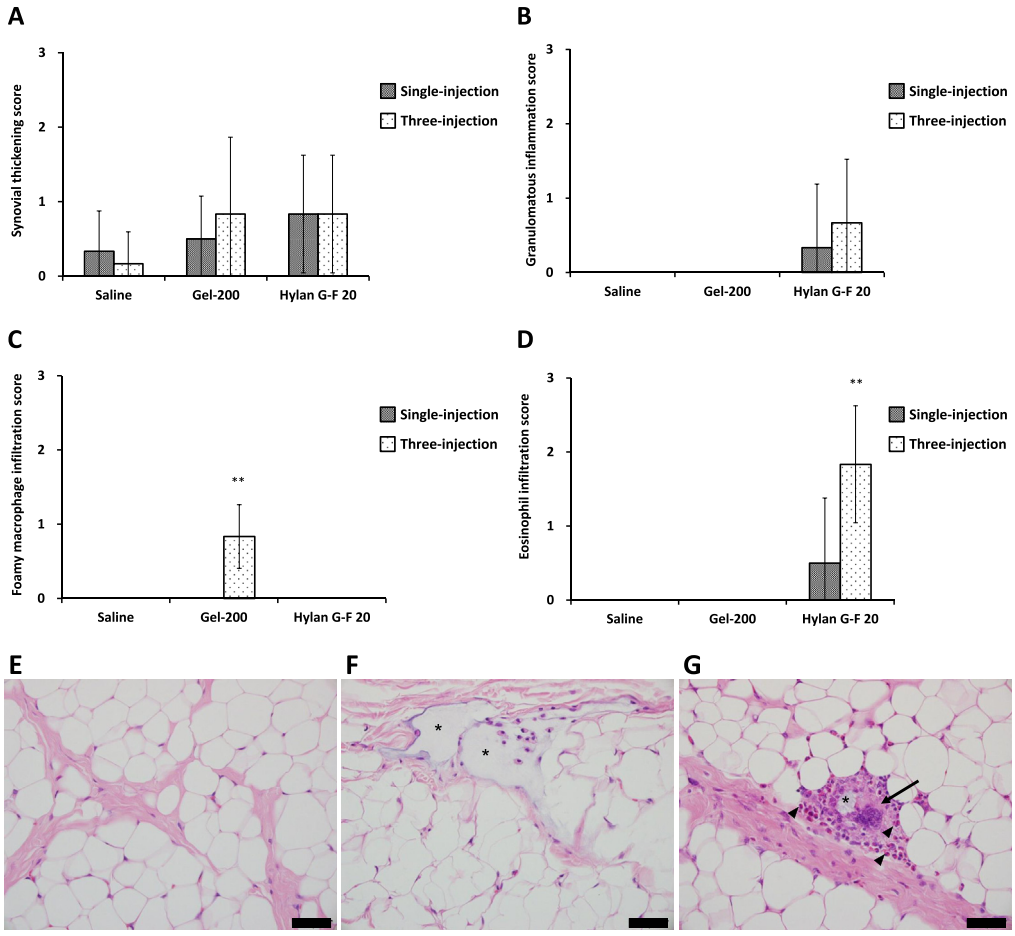

Fig. 2. Histological evaluation of rabbit knee joints after injection of three different samples. Values represent the means ± 95% CI ($n = 6$). (A) The average synovial thickening score was increased in the Gel-200 and hylan G-F 20 group, but not significantly so compared to the saline group. (B) Granulomatous inflammation with multinucleated giant cells was observed only in the hylan G-F 20 group but change in the score was not significant. (C) Foamy macrophage infiltration score was increased significantly in the Gel-200 group compared to the saline group in three-injection regimen (**$P = 0.0098$). (D) Eosinophil infiltration was observed in the hylan G-F 20 group and significantly higher than the saline group in three-injection regimen (**$P = 0.004$). (E–G) Histological appearance of characteristic tissue reaction around test samples 15 days after single administration (HE × 40). (E) In the saline group, no abnormalities were observed. (F) In the Gel-200 group, fragments of the test article (asterisks) with infiltration with macrophages in the adipose tissue are seen. (G) In the hylan G-F 20 group, granulomatous nodules with multinucleated giant cells (arrow) and eosinophils (arrowheads, distinguished from neutrophils histologically), which were accompanied by the test article (asterisk) in the center of the nodules, were seen in the adipose tissue. Scale bars: 50 μm (E–G).

eosinophils[32]. Given that both positive PCA reaction and eosinophil infiltrations were observed in the present study, it is suggested that hylan G-F 20 may induce Th-2 type response.

The differences in biocompatibility and immunogenicity between Gel-200 and hylan G-F 20 shown in this study may be due to a difference in the cross-linking technology between the two products. Gel-200 is composed of cross-linked HA, a derivative of a highly purified sodium HA product extracted from chicken combs and strands of HA are bound to each other via dimers of cinnamic acid resulting in increased viscoelasticity[25]. On the other hand, hylan G-F 20 is composed of 80% hylan A and 20% hylan B. Hylan A is prepared by bridging HA molecules using formaldehyde and proteins derived from chicken combs, which results in viscous fluid fraction[33]. Hylan B is prepared by forming cross-links between hylan A or HA using vinylsulfone, which results in globule gel-like fraction[33–35]. The different link structures bridging HA, namely, cinnamic acid derivative in Gel-200 and protein and vinylsulfone derivatives in hylan G-F 20, may have resulted in the biocompatibility differences seen in rats and rabbits.

A possible explanation for the difference in macrophage response between Gel-200 and hylan G-F 20 is the difference in physicochemical character such as particle size. In fact, biomaterial with small particle size (<10 μm) is considered to be degraded within phagosomes after phagocytosis, while biomaterial with large particle size (between 10 μm and several hundred micrometers) could not be phagocytosed by single-nucleated macrophages, leading to fusion of macrophages into multinucleated foreign body giant cells to engulf the particle[26]. Particle-free property of Gel-200 might result in the infiltration of foamy phagocytosing macrophages whereas relatively large particle size (≈500 μm) of hylan B gel in hylan G-F 20[33,36] might result in the formation of multinucleated giant cells.

The differences in immunogenicity between Gel-200 and hylan G-F 20 may be attributed to residual protein amount, which is supported by results of previous studies[4,23,35,37]. Puttick *et al.* were the first to document severe acute inflammatory reactions in patients treated with hylan G-F 20 and to detect significant antibody titers to chicken serum proteins in the serum of one of his patients[4]. Bucher *et al.* described the presence of anti-chicken protein antibody in the serum of hylan G-F 20 immunized rabbits[37]. Furthermore, Ohshima *et al.* observed that residual protein amounts were larger with hylan G-F 20 than with other HA products[35]. From these points of view, it was assumed that the relatively higher levels of chicken protein used for cross-linking may have caused the

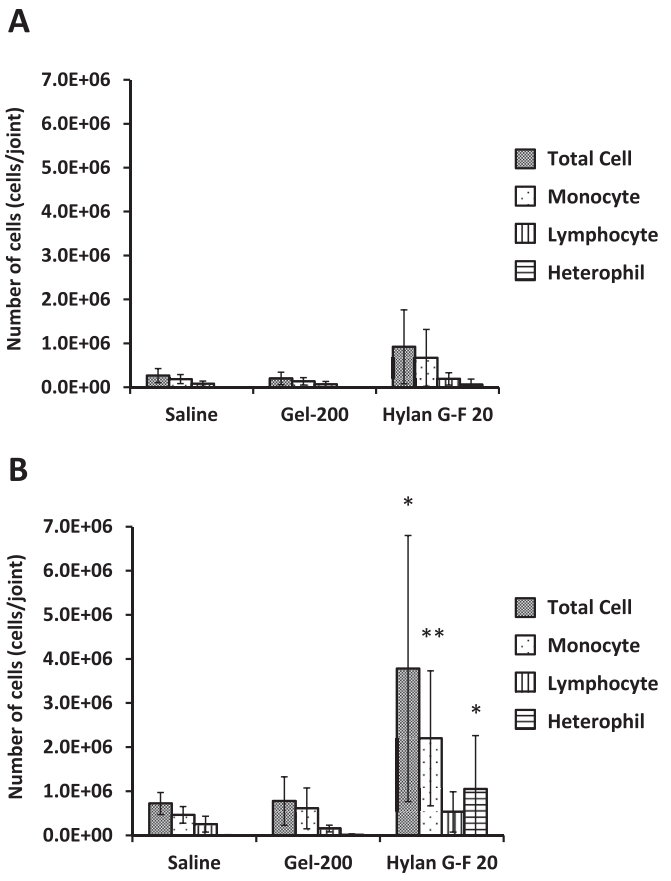

Fig. 3. Cell recruitment in the synovial fluid in rabbits after single (A) or three weekly (B) injections of three different samples. Values represent the means ± 95% CI ($n = 6$). (A) Marginal increases compared to the saline group were observed for total cells ($P = 0.06$), monocytes ($P = 0.06$), and lymphocytes ($P = 0.09$) in the hylan G-F 20 group. (B) Total cells ($^*P = 0.01$), monocytes ($^{**}P = 0.008$), and heterophils ($^*P = 0.03$) were significantly increased in the hylan G-F 20 group compared to the saline group. Gel-200 did not showed significant increases in cell numbers in either treatment regimen.

increased immunogenicity to hylan G-F 20. In Gel-200, chicken proteins are not used as cross-linkers, which may explain the absence of immunogenicity in the present study.

One of the limitations of the current study is the relatively small sample size (six animals per group). Especially in the rabbit knee joint study, occurrence of granulomatous reaction was limited to 1–3 per six animals in the hylan G-F 20 group. Thus, the change was not statistically significant. Low incidence of the granulomatous reaction in the rabbit knee joint may reflect the clinical results of hylan G-F 20 with generally well-tolerated profile with incidental reports of severe acute inflammatory reactions[8]. Another limitation is that, in the rat air pouch model, the lavage fluid collection and histological sectioning were performed on the same sample, which may be the reason for the loss of several samples for histological evaluation, leading to sample size of 3–6 per group. Nevertheless, clear differences of histological scores between groups in air pouch model suggest that sample size difference would not change the conclusion of the present study.

In conclusion, Gel-200 showed a well-tolerated profile in animal models where hylan G-F 20 induced inflammation and showed immunogenicity to a similar extent as previously reported. Coupled with a recent report from a randomized controlled trial of Gel-200, where pseudosepsis and allergic reactions were not reported for any of the 249 analyzed patients[18], the biocompatible and non-immunogenic findings of Gel-200 in this study support a favorable safety profile for this cross-linked HA product for the treatment of knee OA.

Table III
Homologous PCA responses in naïve guinea pigs

Immunization, mg/kg BW	Challenge, mg/kg BW	No. of positive sera	Maximal PCA titers
Gel-200, 2.5	Gel-200, 5	0/6	–
Gel-200, 2.5 + FCA	Gel-200, 5	0/6	–
Hylan G-F 20, 2.5	Hylan G-F 20, 5	01/6	10
Hylan G-F 20, 2.5 + FCA	Hylan G-F 20, 5	5/6	1
OVA* + FCA	OVA†	4/4	1000–10,000
Non-immunized	Gel-200, 5	0/2	–
	Hylan G-F 20, 5	0/2	–
	OVA†	0/2	–

* 1.5 mg/animal.
† 1 mg/animal. BW: body weight.

Contributions

M. Ishikawa: study conception and design, acquisition of data, interpretation of data, drafting the article, final approval of the article to be published.
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.
K. Urano: drafting and revising the article, final approval of the article to be published.
Y. Tanaka: study conception and design, interpretation of data, drafting and revising the article, final approval of the article to be published.
T. Hatanaka: drafting and revising the article, final approval of the article to be published.
A. Nii: acquisition of data, interpretation of data, drafting and revising the article, final approval of the article to be published.

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This study was conducted by Seikagaku Corporation.

Conflict of interest
M. Ishikawa, K. Yoshioka, K. Urano, Y. Tanaka, T. Hatanaka and A. Nii are employees of Seikagaku Corporation working in the Research & Development Division.

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