

## **An Effectiveness Study of Hyaluronic acid [Hyabest® (J)] in the Treatment of Osteoarthritis of the Knee on the Patients in the United States**

Toshihide Sato\*

Hiroshi Iwaso\*\*

\*Fine Chemical Division, Q.P. Corporation, Tokyo, Japan 150-0002

\*\*Department of Orthopaedic Sports Surgery, Kantoh Ropsai Hospital,  
Kanagawa, Japan 211-8510

### **Abstract :**

Patients of knee osteoarthritis are also increasing in other area than Japan. In the United States, for instance, number of the patients are estimated to reach 21 million, however so far there are no study reports available on the effectiveness of oral intake of hyaluronic acid for the American patients. We have conducted a placebo controlled double blind test on 37 patients, who were age 40 or above and lived in the United States, by orally feeding them with 200mg/day of high purity hyaluronic acid (Hyabest® (J)) for 8-weeks. Effectiveness in the reduction of pain in the knee joints was assessed by the changes in WOMAC (Western Ontario McMaster universities Osteoarthritis Index) scores. At the same time safety of the oral intake of Hyabest (J) was examined by blood tests conducted at pre-and-aft administration.

Significant improvement in WOMAC scores was observed after 4-weeks of oral intake in the group of Hyabest (J). As the group of placebo also showed decline in WOMAC scores there was no statistically significant differences observed between the two groups. However, in the analysis of the scores of patients only suffering from severe pain the Hyabest (J) group has shown significant improvement over the placebo group. No adverse changes for safety were detected from the blood test readings. The above study result suggests that oral intake of high purity hyaluronic acid (Hyabest (J)) is effective in the treatment of American patients of knee osteoarthritis.

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Key words : Hyaluronic acid, American patients, knee osteoarthritis, double blind test, test by oral intake

## I. Preface

As the population of senior citizens has been increasing, number of knee osteoarthritis patients who are suffering from degeneration in the joint cartilage or in the subcartilaginous bone tissue with pain, tenderness or inflammation has been sharply rising. It is reported that in the United States currently about 40% of the population of age 60 or elder have some symptoms and about 10% are complaining of difficulties in their daily life <sup>1)</sup>.

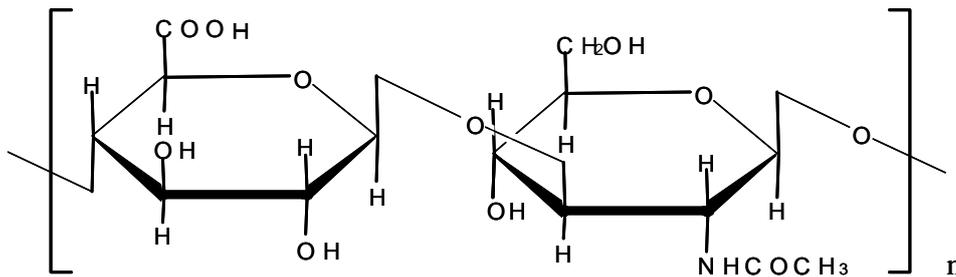


Fig. 1. Chemical structure of Hyaluronic acid

Joint cartilages are composed of type II collagen and proteoglycan containing hyaluronic acid, and synovial fluid filling joint cavity contains hyaluronic acid secreted from synovial membrane <sup>2)</sup>. It has been confirmed that hyaluronic acid level particularly in synovial fluid decreased by aging <sup>3)</sup>. Hyaluronic acid is a kind of high molecular poly-saccharides with tens of thousands to millions of molecular weight, having linear structure of N-acetyl-glucosamine and D-glucuronic acid alternate chains (Fig. 1. ). It is known that direct injection of hyaluronic acid to joint cavity improves symptom of osteoarthritis and it is now commonly used for injection to the patient's joints.

The writers have already published a report concerning a human test conducted in Japan, and confirmed the effectiveness of oral intake of 240 mg/day high purity hyaluronic acid (Hyabest (J)) in the treatment of knee osteoarthritis <sup>4)</sup>.

While number of patients of knee osteoarthritis has also been increasing in the western society and it is estimated to have reached 21 mil. in the United States only <sup>5)</sup>, so far no reports on the test result of oral intake of hyaluronic acid to American patients of knee osteoarthritis are available. Now we have conducted placebo controlled test of 8-weeks oral intake of Hyabest(J) on American patients with knee osteoarthritis for

the assessment of its effectiveness. Since our previous test conducted in Japan with 240mg/day dose has clearly demonstrated effectiveness of oral intake of Hyabest(J) in the treatment of knee osteoarthritis, we have carried out the test with reduced dose of 200mg/day of Hyabest(J) this time.

## II. Target subjects

Test subjects are male and female patients with knee osteoarthritis at their age 40 or elder and are living in the States, who are at the same time classified into the grade II or III of Kellgren-Lawrence classification by the radio-graph. In the meantime informed consent by the subjects were obtained in writing at the time of screening and only those who confirmed their consent to participate in the test were enrolled in the program.

The test was conducted after its protocol and relevant documents were evaluated and approved by the institutional review board in San Diego, and after written consent of each subject was obtained after providing them individually with full explanation of the test program prior to the test, in compliance with the Principle of Helsinki Declaration.

Table 1. Analysis of Hyabest(J)  
(%)

Ingredient	Analytical value
Protein	0.01
Fat	0.01
Hyaluronic acid	98.49
Moisture	1.49

## III. Test methods

### 1. Test sample (food)

Analysis result of high purity Hyaluronic acid (Q.P.Corp. product name: Hyabest(J)) made by microbial fermentation method is exhibited in Table 1.

3-hard capsules of test sample which in total contains 200mg of Hyabest(J) and cornstarch were fed after breakfast to each subject of Hyabest(J) Group, while apparently identical 3-capsules but in which Hyabest(J) was substituted with

cornstarch were given to each subject of Placebo Group after breakfast. In view of religious reasons hard capsules were all made of vegetable materials.

## 2. Test design and test period

The test was programmed as placebo controlled double blind test and conducted for the period of 8-weeks. 3-observation time points were programmed; pre-administration, 4-weeks of administration and 8-weeks of administration.

**Table 2. List of questionnaires by WOMAC**

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### **Section A Pain**

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Based on your experience in the last 48 hours, state the degree of pain which you felt in each action shown below.

1. Walking on level place
  2. Ascending or descending stair way
  3. While sleeping (pain interrupting your sleep)
  4. Sitting down or laying down
  5. Standing straight
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### **Section B Stiffness**

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Based on your experience in the last 48 hours, state the degree of stiffness (not pain but stiff feeling in the knee which makes movement of the joint uneasy) which you felt in each action shown below.

6. Stiffness you feel when you wake up in the morning.
  7. Stiffness you feel in the daytime when you sit down, laying down or after resting a while.
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### **Section C Difficulties in the Daily Living**

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State the degree of difficulties which you felt in each of the following action.

8. Descending stairway
9. Ascending stairway
10. Standing up from sitting position on a chair
11. Standing still
12. Crouching down to pick something on a floor up
13. Walking on level place
14. Stepping in or out from a car or a bus
15. Going out for shopping
16. Putting socks or stockings on by yourself
17. Sitting up from a bed
18. Taking socks or stockings off by yourself
19. Stay laying down on a bed
20. Going in a bathtub or coming out of bathtub
21. Sitting down on a chair
22. Using toilet (a stool)
23. Doing heavy housework (like cleaning the floor, carrying heavy stuff or wiping the floor)
24. Doing light housework (like tidying up own belongings or cleaning up a dining table)

### 3. Method of assessment

Assessment at each time point was made according to the scoring of WOMAC (Western Ontario McMaster universities Osteoarthritis Index) <sup>6)</sup>. Questionnaires in the WOMAC scoring are listed in Table 2. There are 5 questionnaires relevant to “Pain”, 2 to “Stiffness” and 17 to “Difficulties in the activities of Daily Living (hereafter stated as “ADL”)” and point 0 represents lightest symptom and point 4 the severest symptom for each questionnaire. That means total score of the severest symptom for “Pain” will be 20, for “Stiffness” will be 8 and for “ADL” will be 68 and consequently “Total” will be 96. Score for each category and for “Total” will decline when the symptom is improved. Differences in the score between pre-administration and two other time points, also between Hyabest Group and Placebo Group were analyzed for assessment.

### 4. Blood test

Safety of the oral intake of Hyabest (J) was assessed by blood tests conducted twice, at pre-administration and on completion of the test (8-weeks). Test items are; blood cell's components (white cell count, red cell count, hemoglobin and hematocrit) , urea nitrogen, creatinine, AST(GOT), ALT(GPT), alkaline phosphatase, blood sugar level, sodium and potassium.

### 5. Methods of statistical processing

Within-group comparison of WOMAC scores for each category and the total score was tested by Wilcoxon's signed rank test (with multiple comparison by Bonferroni's inequality) and between-groups comparison of the same was tested by Mann-Whitney's U-test. Within-group comparison of blood test results was tested by paired t-test and between-groups comparison by unpaired t-test. Dr.SPSS II for Windows by SPSS Co., Ltd. was used as statistical software and significant level for each test was set at risk less than 5%.

IV. Test result

1. Test subjects

The test was commenced with 40 potential subjects, but 3 were failed due to personal reasons and finally total 37 subjects were enrolled into the test, of which 20 were assigned to Hyabest Group and 17 to Placebo Group. Demographic characteristic of 37 subjects ( 8-males and 29-females) are exhibited in Table 3

Table 4. Changes in WOMAC scores

		(Points)		
	Group	Pre-administ.	4-weeks	8-weeks
Pain	Hyabest (J ) Group	10.7 ± 1.1	8.3 ± 1.0*	6.2 ± 1.0*
	Placebo Group	10.6 ± 0.8	7.1 ± 0.9*	6.5 ± 1.0*
Stiffness	Hyabest (J ) Group	4.8 ± 0.4	3.3 ± 0.4*	2.8 ± 0.5*
	Placebo Group	4.7 ± 0.4	3.2 ± 0.4*	3.2 ± 0.5*
ADL	Hyabest (J ) Group	40.3 ± 3.5	28.8 ± 3.1*	22.4 ± 3.6*
	Placebo Group	38.5 ± 2.2	26.3 ± 3.1*	24.6 ± 3.1*
Total	Hyabest (J ) Group	55.7 ± 4.9	40.4 ± 4.4*	31.3 ± 5.1*
	Placebo Group	53.9 ± 3.1	36.5 ± 4.3*	34.4 ± 4.5*

Mean +/- Standard error

\*: P<0.05 vs Pre-administration

2. Assessment by WOMAC scoring

Changes in the WOMAC score for each assessment category are shown in Table 4. Significant decrease of the scores were observed at 4-weeks and afterwards in both Hyabest(J) Group and Placebo Group. Although no statistically significant difference was observed between Hyabest (J) Group and Placebo Group, mean score of each assessment category at 8-weeks was smaller in Hyabest(J) Group.

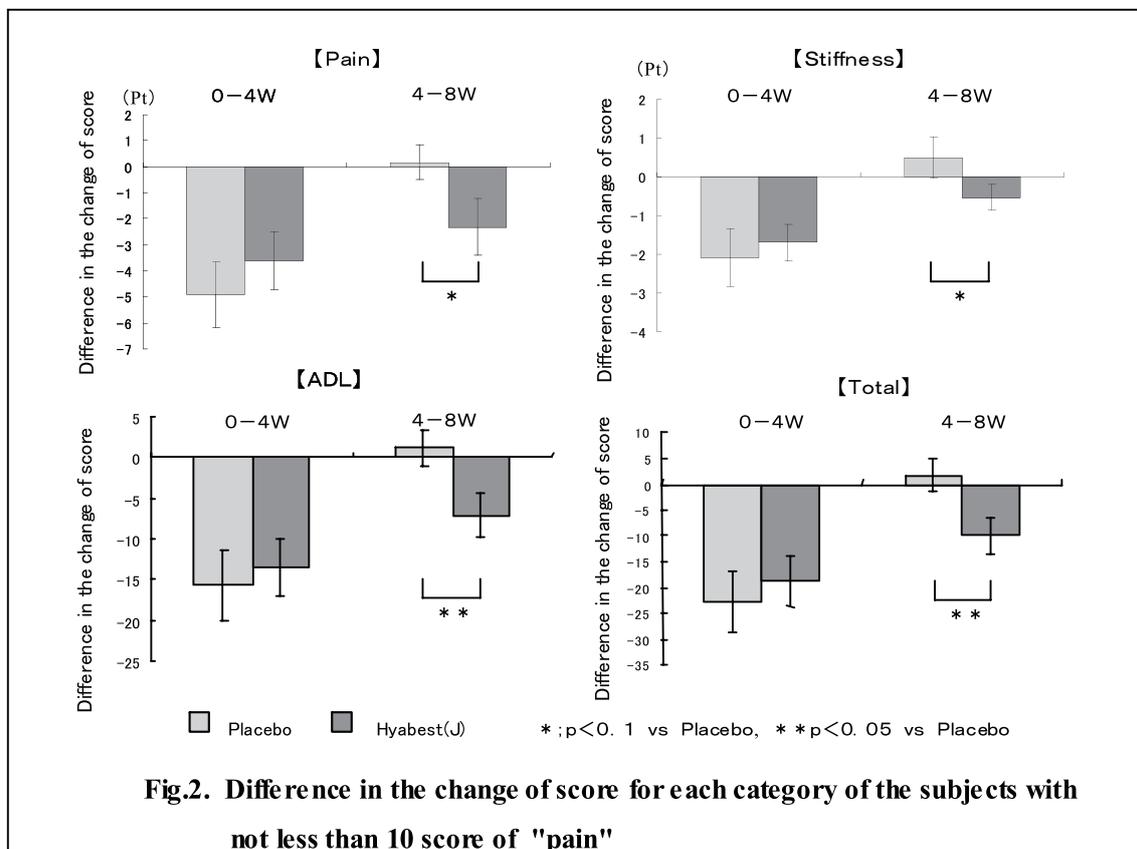
3. Blood test

Compared with the pre-administration readings, statistically significant increase of alkaline phosphatase and decrease of ALT (GPT) both in Placebo Group and significant decrease of white blood cell count in both Groups were observed at 8-weeks time point, however these were not such as clinically important. Thorough-out the whole test period there were no significant changes between Hyabest(J) Group and Placebo Group nor any adverse event attributable to the test sample.

V. Consideration

Effectiveness of oral intake of Hyabest(J) in the treatment of knee osteoarthritis of American patients was assessed by WOMAC scoring. Significant improvement of the scores was observed in Hyabest(J) Group at 4-weeks time point and afterwards compared with pre-administration. On the other hand ,significant improvement in the scores was also observed in Placebo Group at 4-weeks and afterwards ,which indicates no significant differences existed between Placebo Group and Hyabest Group. However, mean of the scores at 8-weeks of Hyabest Group was smaller than that of Placebo Group.

NIH (National Institutes of Health) has conducted a study on efficacy of oral intake of Glucosamine and Chondroitin sulfate together on knee osteoarthritis assessed by WOMAC index. In this study there reported no significant differences between the test group and the placebo group , however in the strata analysis of two groups, patients with low scores in “Pain” and those with high scores it was reported that intake of glucosamine and chondroitin sulfate was effective to alleviation of adverse symptom of osteoarthritis<sup>7)</sup> . In view of the outcome of this study we have also made stratum analysis targeting at the test subjects with not less than 10 score of “Pain”.



The target stratum consisted of 13 subjects of Hyabest Group and 12 of Placebo Group. In consideration of the influence of placebo effects which is deemed to be evident for the initial period up to 4-weekw, we have assessed “the scores between pre-administration and 4-weeks” and “4-weeks and 8-weeks”. Minus score means improvement in symptoms. The changes are exhibited in Fig. 2. Comparison of the scores of two groups tested by Mann-Whitney’s U-test showed significant improvement in Hyabest(J) Group against Placebo Group for “ADL” and “Total” scores in “4-weeks and 8-weeks” though no significant differences were seen in “the score between pre-administration and 4-weeks”. Also the analysis showed improving tendency for “Pain” and “Stiffness” in Hyabest(J) Group against Placebo Group.

The above strata analysis suggests that at 4-weeks and afterwards oral intake of Hyabest(J) is effective in the treatment of knee osteoarthritis of American patients, though in the initial 4-weeks period no significant differences were observed between Hyabest(J) Group and Placebo Group possibly due to the influence of placebo effects.

The writers of this report have already made a human trial of oral intake of Hyabest (J) 240mg/day on Japanese patients of osteoarthritis and reported its effectiveness in the improvement of the symptom<sup>4)</sup>. From the test result on American patients this time which also suggested improvement of symptom of knee osteoarthritis, it is deemed that Hyabest(J) is a food material with which improvement of the symptom is expected at 200mg/day dose level.

Direct injection of hyaluronic acid into joint cavity is commonly used for the treatment of knee osteoarthritis and inhibition of cartilaginous degeneration<sup>8)</sup>, protection of cartilaginous surface<sup>9)</sup>, normalization of synovial fluid level<sup>10)</sup> and alleviation of pain by various anti-inflammatory action<sup>11)</sup> are known. Although action mechanism in the improvement of knee osteoarthritis by oral intake of Hyabest (J) has not been clarified yet, the test result confirming effectiveness in the improvement of the symptom in rather short period of 4-weeks of the intake suggests that its anti-inflammatory multi-function contributes to the effectiveness.

Oral intake of Hyabest (J) is expected to contribute to reducing burden of “regular visit to specialized institute” or of “side-effects of medicine” on the patients which are the important issues of currently prevailing major treatments of knee osteoarthritis; physiotherapy, pharmacotherapy and orthotherapy.

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# Oral Administration of High Molecular Weight Hyaluronan (900 kDa) Controls Immune System via Toll-like Receptor 4 in the Intestinal Epithelium

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Akira Asari<sup>‡</sup>, Tomoyuki Kanemitsu<sup>¶</sup> and Hitoshi Kurihara<sup>¶</sup>

*From the ‡Hyaluronan Research Institute Inc., 4-5-9 Kichijojiminami-cho, Musashino-shi, Tokyo 180-0003, the §Division of Biochemistry, Department of Hard Tissue Engineering, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8459, and the ¶Q.P. Corporation, 5-13-1 Sumiyoshi-cho, Fuchu-shi, Tokyo 183-0034, Japan*

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Low molecular weight hyaluronan enhances or induces inflammation through toll-like receptor 4 (TLR-4). However, the effects of high molecular weight hyaluronan (HA900) on TLR-4 are unknown. In this study, HA900 (900 kDa) was administered orally to MRL-lpr/lpr mice, a Th-1-type autoimmune disease model. Lymphoaccumulation of double-negative T cells, which is enhanced by proinflammatory cytokines, was suppressed by HA900 treatment. Cytokine array analysis showed that HA900 treatment enhanced production of interleukin-10, an anti-inflammatory cytokine, and down-regulated chemokine production. HA900 colocalized with TLR-4 on the luminal surface of epithelial cells in the large intestine. These cells are parts of the immune system and express cytokines. DNA array analysis of the tissue from the large intestine showed that HA900 treatment up-regulated suppressor of cytokine signaling 3 (SOCS3) expression and down-regulated pleiotrophin expression. Treatment of cultured double-negative T cells from MRL-lpr/lpr mice with pleiotrophin rescued these cells. SOCS3, which is known to suppress inflammation, was enhanced by HA900 treatment. In TLR-4 knockdown HT29 cells (a cell line derived from large intestinal cells), HA900 did not bind to HT29 cells and did not up-regulate SOCS3 expression. Our results suggest that oral administration of HA900 modulates Th-1-type autoimmune disease and inflammation by up-regulating SOCS3 expression and down-regulating pleiotrophin expression via TLR-4 in intestinal epithelial cells.

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Many reports have shown that low molecular weight hyaluronan (HA)<sub>2</sub> enhances or induces inflammation via toll-like receptor 4 (TLR-4) (1, 2). It is well known that the functions of HA are molecular weight-dependent (3, 4). Therefore, we hypothesized that the actions of HA via TLR-4 depend on its molecular weight. MRL-lpr/lpr mice (3) are characterized by lymphoaccumulation of double-negative T cells (DNT cells), i.e. lymph node enlargement in their lymph nodes and spleens,

and proliferation of these cells is enhanced by proinflammatory cytokines (5, 6).

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<sup>1</sup> To whom correspondence should be addressed: E-mail: [a.asari@hyaluronan.co.jp](mailto:a.asari@hyaluronan.co.jp).

<sup>2</sup> The abbreviations used are: HA, hyaluronan; PB5, phosphate-buffered saline; HRP, horseradish peroxidase; TLR-4, toll-like receptor 4; RT, reverse transcription; oligo, oligonucleotide; DNT, double-negative T cell; PMA, phorbol 12-myristate 13-acetate; IL, interleukin; LPS, lipopolysaccharide; SCF, stem cell factor; VEGF, vascular endothelial growth factor, DW, distilled water.

TLR-4 is a type of HA receptor found on the surface of large intestinal epithelial cells, which form part of the immune system and express cytokines. SOCS3 expression suppresses inflammation and arthritis (7,-,9).

In this study, we showed that oral administration of high molecular weight HA (HA900) up-regulated suppressor of cytokine signaling 3 (SOCS3). In large intestinal epithelial cells, HA900 bound to TLR-4 on the cell surface and was not systemically absorbed.

Furthermore, HA900 suppressed pleiotrophin expression, which is implicated in inflammation, in intestinal tissue and in arthritis. Pleiotrophin mRNA in the spinal cord is significantly up-regulated after induction of experimental autoimmune encephalomyelitis (10, 11), and pleiotrophin is expressed in adults with inflammatory diseases, particularly in rheumatoid arthritis (12). These findings suggest that HA900 controls the immune system through up-regulation of SOCS3 and down-regulation of pleiotrophin. This is the first study to show a relationship between TLR-4 and high molecular weight HA.

## **EXPERIMENTAL PROCEDURES**

*Materials*—HA900 and HA oligosaccharides (HA oligos prepared by HCl degradation; Mr 3200–15,000) were obtained from Q.P. Corp. (Tokyo, Japan).

*Mice*—Four-week-old female MRL-lpr/lpr mice were purchased from Charles River Japan (Yokohama, Japan) and cared for at our animal facility. All animal protocols were approved by the Animal Experiment Committee at Hyaluronan Research Institute, Inc.

*Animal Experiments*—Female MRL-lpr/lpr mice were randomly placed into three groups. One group received water (control group); one received HA900 (200 mg/kg/day), and one received HA oligos (200

mg/kg/day). The substances were administered orally in water bottles to mice between 14 and 18 weeks of age. The volume of water ingested by each mouse (about 7 ml/day) was used to calculate the dose; each mouse received 29 mg/ml HA900 or HA oligos. One day after the final administration, all animals were sacrificed by exsanguination under general anesthesia. Their lymph nodes were weighed to assess the effects of treatment on DNT cells.

*Quantification of Serum HA Concentration*— HA polymer (average Mr of 757,000; Calbiochem) was dissolved in 50 mm phosphate/citrate buffer (pH 5.0) at 0.01 mg/ml as a stock coating solution, and 50  $\mu$ l of this solution was poured into each well of a 96-well amine-conjugated microtiter plate (Sumitomo Bakelite, Japan). A volume of 10  $\mu$ l of sodium cyanoborohydrate solution (10 mg/ml) was added to each well and incubated for 1 h at 25 °C. After washing three times with 10 mm phosphate-buffered saline (pH 6.8) containing 0.05% Tween 20 (PBS/Tween), 100  $\mu$ l of diluted Block Ace® (DS Pharma Biomedical, Japan) solution (diluted 1:4 with pure water) was poured into each well and incubated for 1 h at 37 °C. After washing with PBS/Tween, 50  $\mu$ l of different dilutions of standard HA solutions as well as diluted serum samples were poured into the wells followed by 50  $\mu$ l of biotinylated HA-binding protein (Hokudo, Japan) solution (0.1 mg/ml) in diluted Block Ace® solution (diluted 1:10 with pure water). After incubation for 1 h at 37 °C followed by washing with PBS/Tween, 100  $\mu$ l of horseradish peroxidase (HRP)-conjugated streptavidin (Avidin-PO, Calbiochem) solution (0.05 mg/ml) in diluted Block Ace® solution (1:10 dilution) was poured into each well, and the plates were incubated for 1 h at 37 °C. Finally, after washing with PBS/Tween, 100  $\mu$ l of TMB peroxidase substrate was poured into each well and incubated for 20 min at room temperature before 100  $\mu$ l of 0.1 n HCl was added. Absorbance at 450:630 nm was measured using an MTP-300 Microplate Reader (Corona Electric, Japan).

*Estimation of Molecular Weight of Serum HA*—The molecular weight of serum HA was estimated by agarose gel electrophoresis. In brief, samples were diluted to give HA concentrations of 30–35  $\mu$ g/ml and mixed with an equal volume of 40% glycerol. A 0.75% agarose gel (10  $\times$  10  $\times$  0.2 cm) was prepared in 40 mm Tris-HCl buffer (pH 8.0) containing 50 mm sodium acetate and 9 mm EDTA (TAE buffer). Samples (10  $\mu$ l) were loaded on the agarose gel, and electrophoresis was performed at a constant voltage of 25 V for 5 h at room temperature. After electrophoresis, the gel was soaked in TAE buffer containing 2 mm l-ascorbic acid and 0.1 mm iron sulfate for 30 min before being washed twice with TAE buffer for 15 min. Hyaluronan in the gel was transferred to a positively charged nylon membrane (10  $\times$  10 cm) (Hybond™-N+, GE Healthcare) at a constant electric current of 100 mA for 2 h in a semi-dry transfer unit (Hoefler® SemiPhor™, GE Healthcare). After the transfer, the membrane was soaked in 10% skimmed milk in 50 mm Tris-HCl (pH 7.5) containing 0.1 m sodium chloride (TBS) overnight and washed twice with TBS. The membrane was then soaked in Block Ace® solution (diluted

1:10) containing 0.1 mg/ml biotinylated HA-binding protein and incubated for 1 h at room temperature. After washing three times with PBS/Tween, the membrane was soaked in Block Ace® solution (diluted 1:10) containing 0.05 mg/ml Avidin-PO. After washing three times with PBS/Tween, the membrane was rinsed with PBS and pure water to remove detergent and reacted with a chemical luminescent reagent mixture (SuperSignal, Pierce) for 5 min. The results were recorded on x-ray film (RX-U; Fuji Photo Film, Japan).

*DNA Array Analysis*—Large intestinal tissues from control and HA900 groups were used in DNA array analyses (Takara Bio, Japan). To confirm the results, mRNA transcripts for SOCS3 were detected by RT-PCR, and quantification was performed using three samples. The intensities observed were typical, and clear statistical differences were found.

*RT-PCR*—Total RNA was isolated from large intestinal tissues of mice and HT29 cells using TRIzol reagents (Invitrogen); 1 µg of total RNA was used for the reverse transcript reactions. PCR amplification was performed for 35 cycles.

For RT-PCR of the intestinal tissues, the SOCS3 primer sequences used in the study were as follows: forward, 5'-GCGAGAAGATTCCGCGGTA-3'; reverse, 5'-CCGTTGACAGTCTTCCGACAA-3'. For RT-PCR of the HT29 cells, the SOCS3 primer sequences used in the study were as follows: forward, 5'-CTTCAGCTCCAAGAGCGAGTA-3'; reverse, 5'-GAGCTGTCGCGGATCAGAAAG-3'. Glyceraldehyde-3-phosphate dehydrogenase primer sequences used were as follows: forward, 5'-ACCACAGTCCATCAC-3'; reverse, 5'-TCCACCACCCTGTTGCTGTA-3'.

*Histopathology*—Frozen sections of lymph nodes taken from mice were treated with water or HA900 and immunostained with an antibody to pleiotrophin (Acris) followed by HRP-labeled rabbit IgG (Lab Vision Corp.). Frozen sections of large intestinal tissues and submandibular lymph nodes were stained with biotinylated HA-binding protein (Hokudo, Japan) followed by streptavidin-HRP (Lab Vision Corp.) and diaminobenzidine. Streptomyces hyaluronidase digestion of tissue sections was used as a control. The frozen sections of large intestinal tissues were also used for double staining with biotinylated HA-binding protein and anti-TLR-4 antibody (Abnova Corp.). Frozen sections of submandibular lymph nodes were analyzed for apoptosis using the terminal dUTP nick end-labeling method.

*Western Blotting for SOCS3 in HT29 Cells*—Confluent HT29 cells were left untreated or were treated with HA oligos or with HA900 for 24 h. The cell lysates were obtained using a lysate kit (Funakoshi, Japan). The lysates were electrophoresed using Tris acetate gel. The samples were then transferred to

a polyvinylidene difluoride membrane. The membrane was immunostained for SOCS3 (first antibody, anti-human SOCS3 (Cell Signaling Technology, Japan); second antibody, HRP-anti-mouse IgG (Abnova)). Signals were detected using FCL solution (GE Healthcare) and recorded on x-ray film.

*In Vitro Study of DNT Cells*—DNT cells were obtained from lymph nodes of MRL-*lpr/lpr* mice and treated ( $4 \times 10^5$  cells/well) with pleiotrophin (0, 1, 10, and 100 ng/ml) or phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) for 6 h. A methanethiosulfonate assay was then performed using incubation with Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan) for 1 h. Data are shown as means  $\pm$  S.E. of triplicate values. Each experiment was repeated at least three times.

*Binding of HA900 or HA Oligos to Large Intestinal Cells or HT29 Cells*—Confluent HT29 cells were incubated with 1  $\mu$ g/ml HA900 or HA oligos for 1 h at room temperature. Large intestinal tissues from MRL-*lpr/lpr* mice treated orally with HA900 or HA oligos were frozen and sectioned. HT29 and large intestinal cells were then treated with biotinylated HA-binding protein. HT29 cells were then treated with anti-TLR-4 antibody followed by Texas Red anti-mouse IgG (GenWay Biotech Inc.). The same experiments were performed using TLR-4 knockdown HT29 cells.

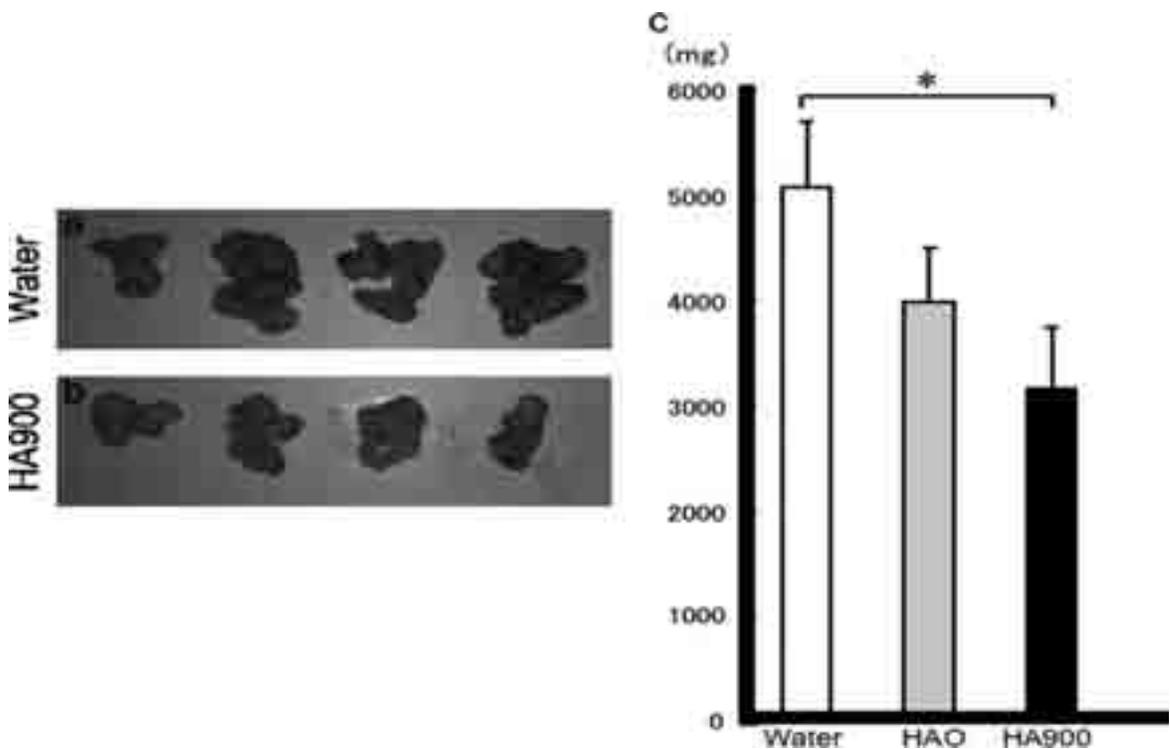
*SOCS3 Expression in HT29 Cells*—HT29 cells were left untreated or were treated with 1  $\mu$ g/ml HA900 or HA oligos for 24 h at 37 °C. The samples were then used in Western blotting for SOCS3 antibody. Immunostaining for SOCS3 was performed in HT29 cells left untreated or treated with 1  $\mu$ g of HA oligos or 0.01, 0.1, and 1  $\mu$ g of HA900. The cells were then treated with a secondary antibody labeled with HRP (Abnova) followed by diaminobenzidine. The intensity of staining was analyzed by NIH Image.

*TLR-4 Knockdown*—Lipofectamine (Invitrogen) was mixed with three types of TLR-4 RNA (CAGACTTGCGGGTTCTACATCAAAT, CGGATGGCAACATTTAGAATTAGTT, and GATCTCAGTAGAAATGGCTTGAGTT). The solution was added to HT29 cells in 24 wells of a 96-well plate. The plate was then incubated for 48 h at 37 °C. Then HA900 or HA oligos were added, and cells were incubated for 24 h. The knockdown and wild-type HT29 cells were immunostained for TLR-4, HA, and SOCS3.

*Array Analysis of Cytokines and Chemokines*—Serum (10  $\mu$ l) was taken from each mouse, and sera from each group were pooled. Sera taken from mice who received water as well as those that received HA900 were used for array analyses of cytokines and chemokines (enzyme-linked immunosorbent assay, Raybio; n = 2). This experiment was repeated twice (n = 4).

## RESULTS

Treatment with HA900 (200 mg/kg/day) suppressed weight increases in submandibular (Fig. 1, a and b), mesenteric, inguinal, and axillary lymph nodes compared with controls, whereas treatment with HA oligos showed a trend toward lower weights but did not reach statistical significance (Fig. 1c). Cytokine and chemokine arrays showed up-regulation of IL-10 and down-regulation of MCP-5, MIP-2, regulated on activation normal T cell expressed and secreted, P-selectin, stem cell factor (SCF), and VEGF (Fig. 2). In the serum of C57 BL/6 mice (C57), the levels of MCP-5, SCF, and VEGF were not detected, because the C57 BL/6 mice were normal.



**FIGURE 1.** a and b, HA900 suppressed submandibular lymph node enlargement. c, total weight of lymph nodes in the HA900 group was less than that in the control group.  $n = 6$ ; \*,  $p < 0.05$ ; Tukey's multiple comparison test. HAO, hyaluronan oligosaccharides.

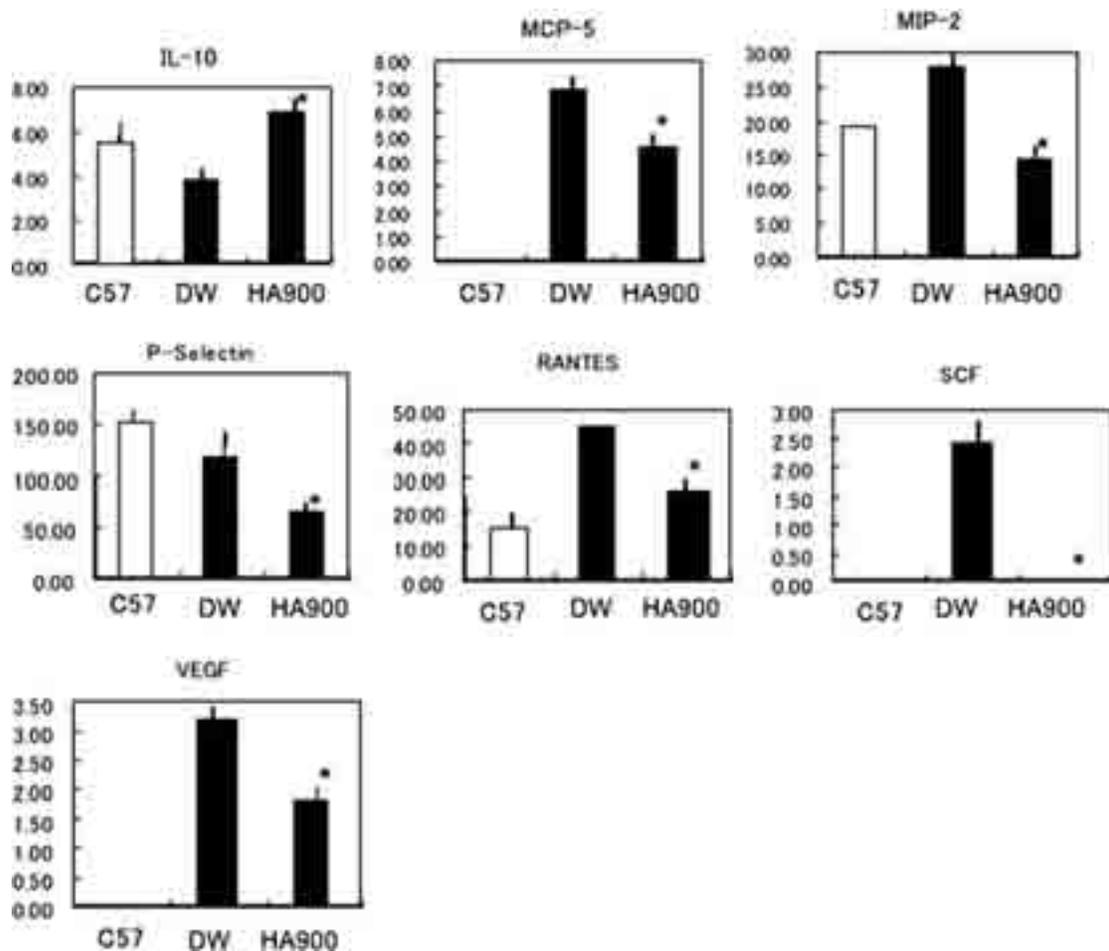
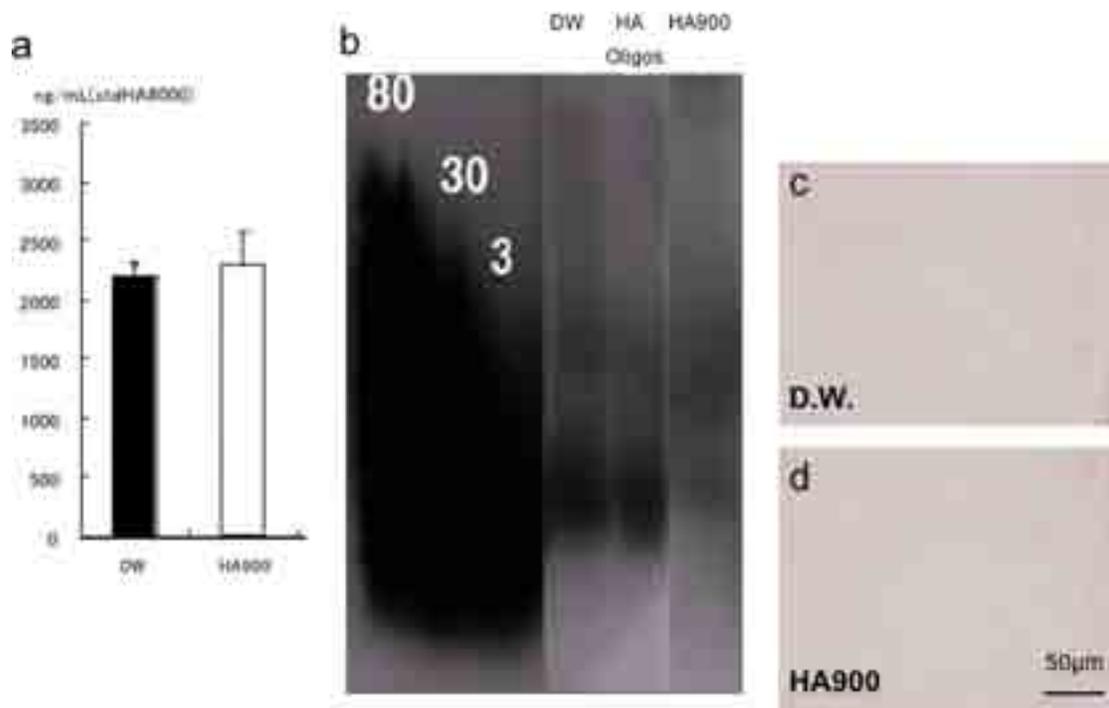


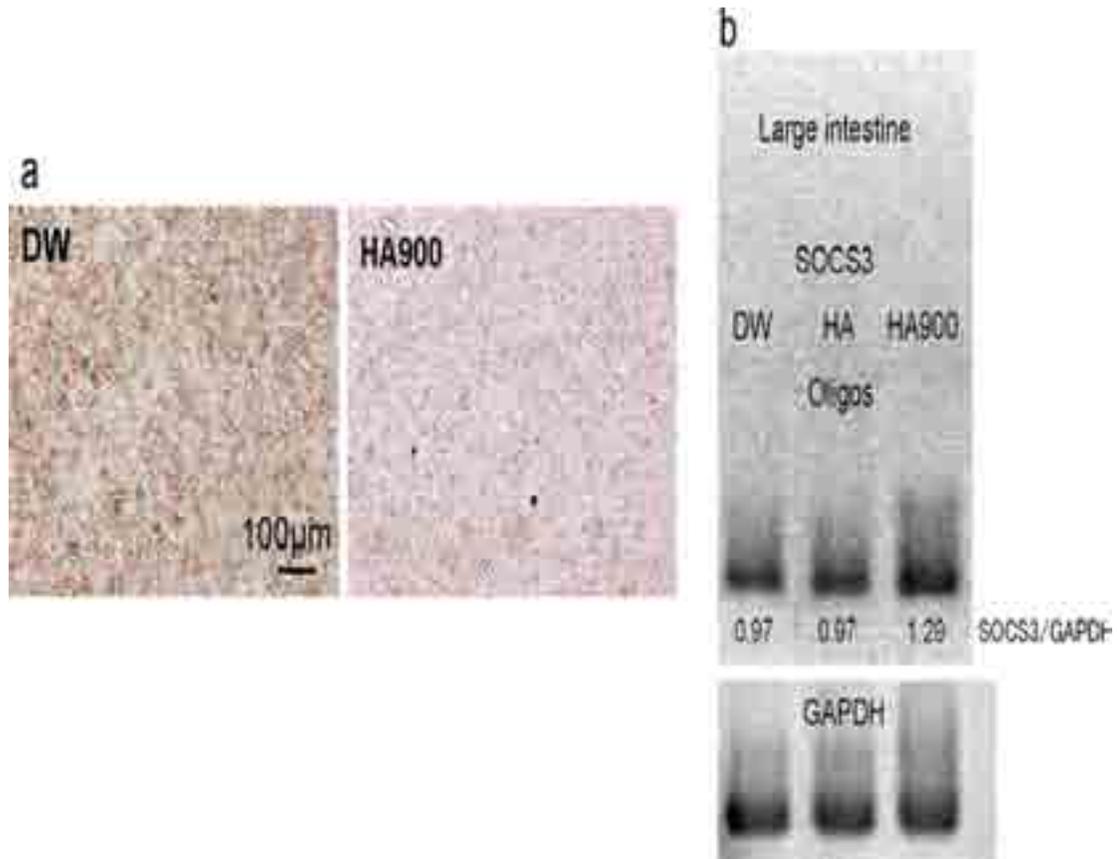
FIGURE 2. Results of cytokine and chemokine array analysis of serum. Most of the humoral factors in serum or MRL-*lpr/lpr* mice, except for IL-10, were decreased by the treatment with HA900. The y axis indicates relative intensity of the expression. C57, serum from normal C57 BL/6 mice.  $n = 6$ ; \*,  $p < 0.05$ ; Tukey's multiple comparison test. RANTES, regulated on activation normal T cell expressed and secreted

Treatment with HA900 did not elicit any changes in the concentration or molecular weight of HA in plasma (Fig. 3, *a* and *b*). Furthermore, HA was not detected in lymph nodes of control or HA900 groups (Fig. 3, *c* and *d*).



**FIGURE 3.** *a*, HA concentration in serum. HA900 treatment did not change HA concentration. Standard HA, 8 kDa. *b*, molecular weight of HA in serum. HA900 treatment did not change the molecular weight of HA. Standard HA, 80, 800 kDa; 30, 300 kDa; 3, 30 kDa. *c* and *d*, HA staining in submandibular lymph nodes was not detected.

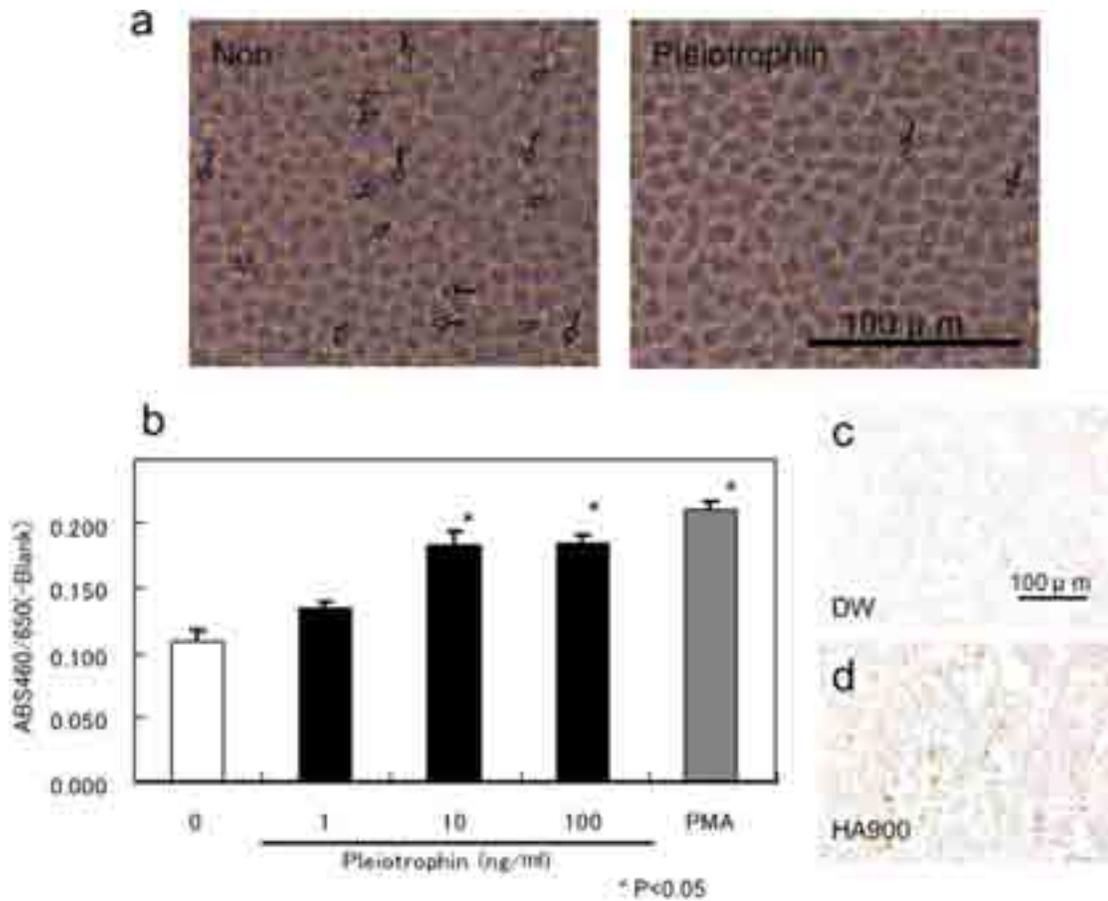
DNA array analysis of large intestinal tissues showed that HA900 treatment up-regulated SOCS3 (ratio of HA900/DW, 2.0) and down-regulated pleiotrophin (ratio of HA900/DW, 0.5). Immunostaining for pleiotrophin in lymph nodes was stronger in the control group than in the HA900 group (Fig. 4*a*). RT-PCR analysis showed that SOCS3 mRNA expression in large intestines was stronger in the HA900 group than in the control group (Fig. 4*b*).



**FIGURE 4. Immunostaining for pleiotrophin in lymph nodes (a) and RT-PCR of SOCS3 in large intestinal tissues (b).** Pleiotrophin expression was diminished by the treatment with HA900 (a). Ratio of the intensity (SOCS3/glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)) showed that HA900 treatment up-regulated mRNA expression of SOCS3 (b).

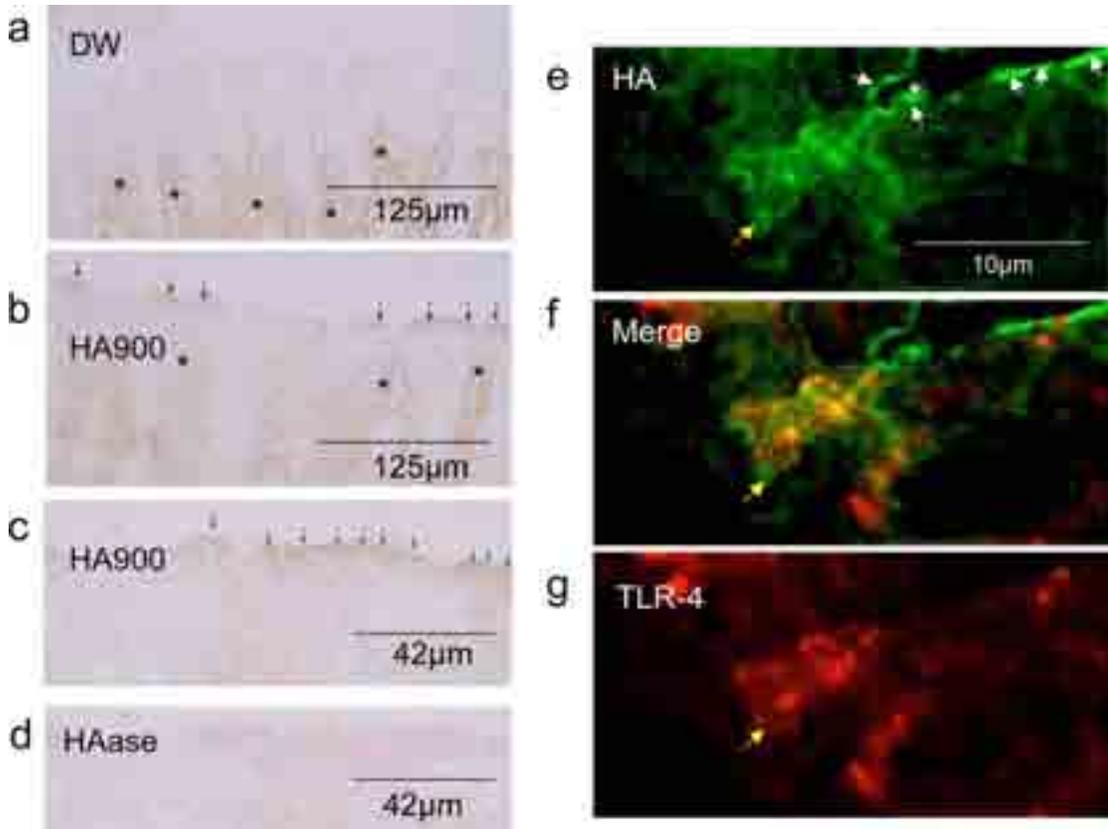
DNT cells were obtained from lymph nodes of MRL-*lpr/lpr* mice by mincing the lymph nodes. It is well known that more than 90% of the lymphocytes of lymph nodes are DNT cells (5, 13).

A large amount of cell debris (indicative of cell death of DNT cells) was observed among untreated controls (Fig. 5a). In contrast, only a small amount of debris was observed in control lymphocytes after treatment with pleiotrophin (Fig. 5a) or PMA (data not shown). PMA was used because PMA generally activates lymphocytes. Furthermore, a methanethiosulfonate assay also showed that treatment of DNT cells from MRL-*lpr/lpr* mice with pleiotrophin suppressed cell death as did treatment with PMA (Fig. 5b). Apoptosis in lymph node tissues from MRL-*lpr/lpr* mice increased after treatment with HA900 as indicated by the brown-stained cells (Fig. 5d), but this did not occur in the control group treated with distilled water (Fig. 5c).



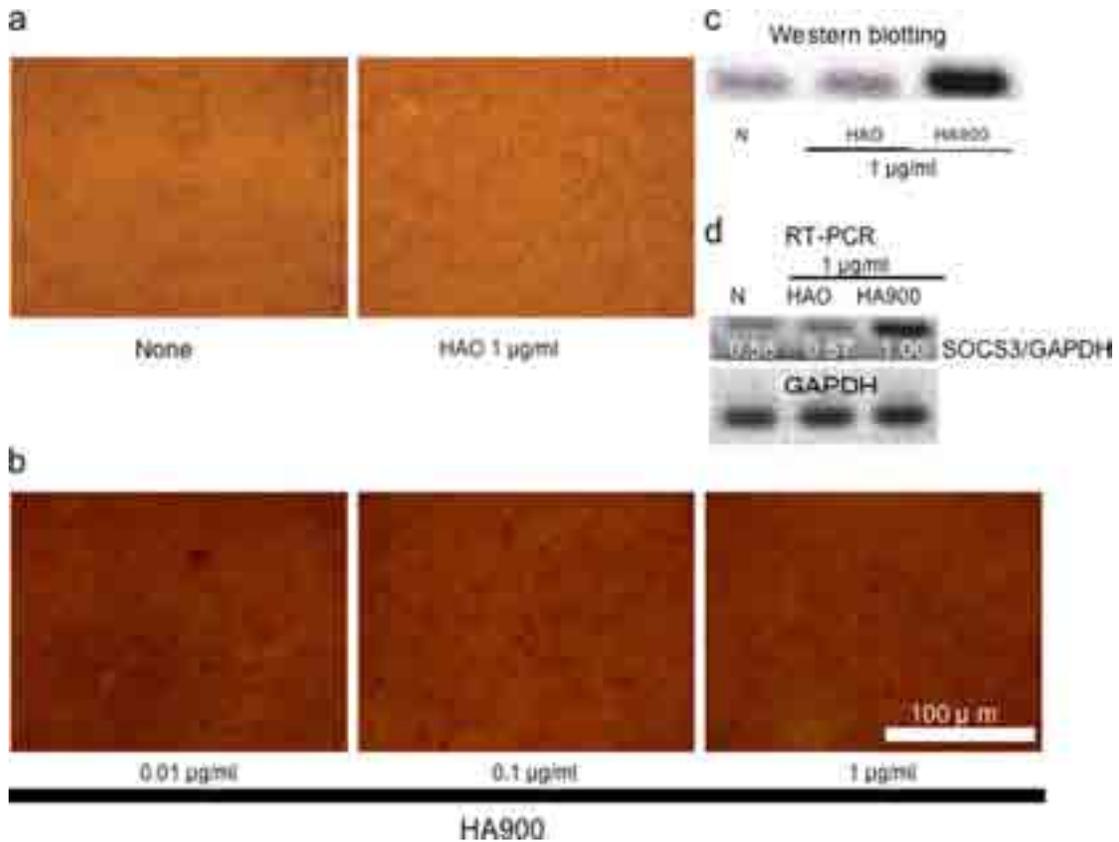
**FIGURE 5.** *a*, morphology of cell death in submandibular lymph node tissues. Fragmented cell debris (*arrows*) was seen in untreated cultures, and a small amount of debris was observed in the presence of pleiotrophin. *b*, appearance of fragmented cell debris and methanethiosulfonate assay show that treatment with pleiotrophin suppressed cell death in DNT cells. On the other hand, terminal dUTP nick end-labeling method shows that HA900 treatment induced apoptosis in lymph nodes (*c* and *d*). Data are shown as mean  $\pm$  S.E. \*,  $p < 0.05$ , Tukey's multiple comparison test.

HA was detected in the lamina propria in intestinal tissues of control and HA900 groups (Fig. 6, *a–c*) but was also selectively detected on the luminal surface and intracellular space of intestinal epithelial cells in the HA900 group (Fig. 6, *b* and *c*) where it was often colocalized with TLR-4 (Fig. 6, *f* and *g*). The colocalization was restricted to this area. Most of the endogenous subepithelial HA was not colocalized with TLR-4 (Fig. 6, *e–g*). Treatment with *S. hyaluronidase*, specific for HA, abolished HA (Fig. 6*d*).



**FIGURE 6.** HA staining in tissues from the large intestine (*a–d*). HA was detected on the luminal surface of intestinal epithelial cells (*arrows*) in the HA900 group but not in the control group. *d*, pretreatment with *Streptomyces hyaluronidase* abolished HA. Vertical section of large intestinal tissue was stained with biotinylated HA-binding protein (*green*, *e*) and anti-TLR-4 (*red*, *g*). *f*, in the merge, *yellow* and *orange* indicate the colocalization of HA900 and TLR-4. *Yellow arrows* indicate the apical surface of the intestine. *White arrows* indicate endogenous HA in the villi connective tissue. \* indicates lamina propria.

Immunostaining for SOCS3 showed that HA900 treatment up-regulated SOCS3 expression (Fig. 7*b*) compared with control and HA oligo groups (Fig. 7*a*). Western blotting and RT-PCR showed increased SOCS3 expression in HT29 cells after treatment with HA900 but not when treated with HA oligos (Fig. 7, *c* and *d*).



**FIGURE 7.** SOCS3 immunostaining (*a* and *b*), Western blotting for SOCS3 (*c*), and RT-PCR for SOCS3 (*d*) in HT29 cells. In any case, HA900 up-regulated the SOCS3 expression. *N*, nontreatment control; *HAO*, hyaluronan oligosaccharide; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Wild-type HT29 cells expressed TLR-4 that was colocalized with HA900 (Fig. 8) or HA oligos (data not shown). On the other hand, neither HA900 (Fig. 9) nor HA oligos (data not shown) bound TLR-4 knockdown HT29 cells. Furthermore, HA900 did not change SOCS3 expression in the knockdown HT29 cells (Fig. 9).

*High Molecular Weight HA Controls Immune System via TLR-4*

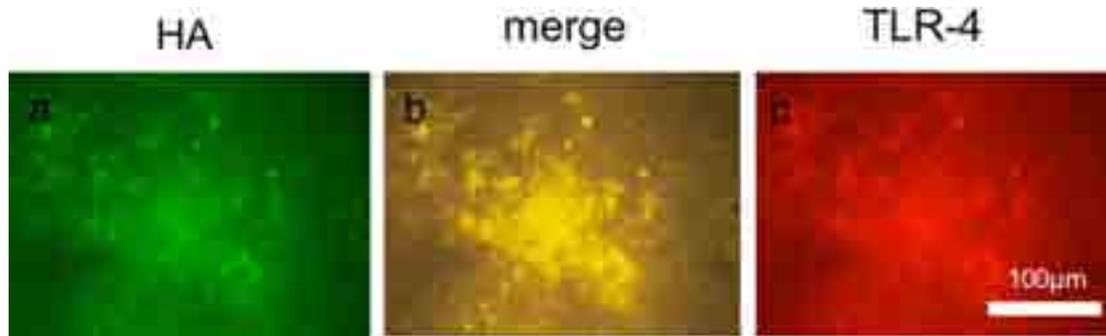


FIGURE 8. In wild-type HT29 cells treated with HA900, HA (*green*) was colocalized with TLR-4 (*red*). In the merge, *yellow* also indicated the colocalization.

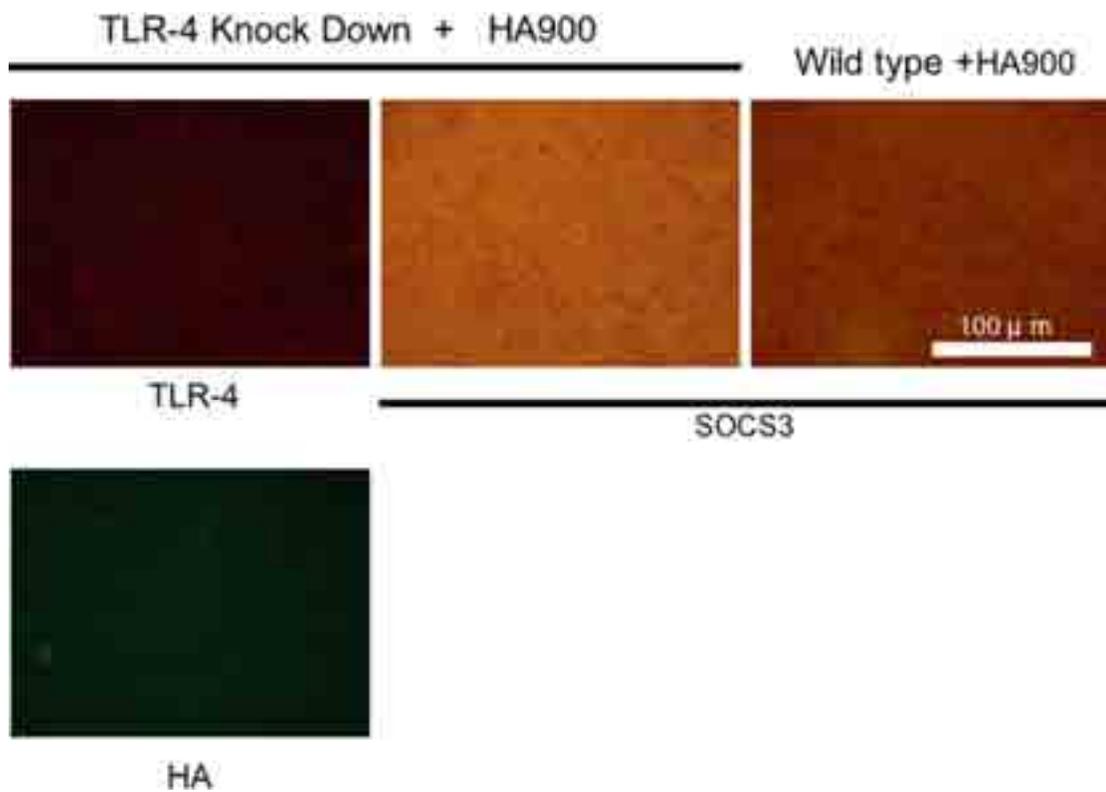
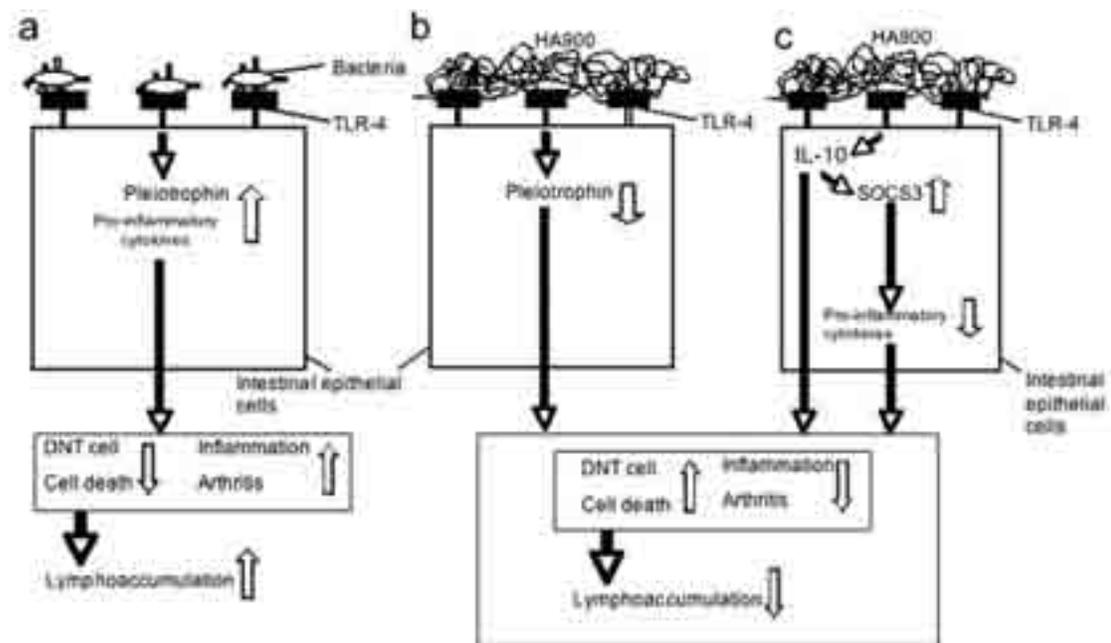


FIGURE 9. Effects of TLR-4 knockdown in HT29 cells. Neither immunostaining of TLR-4 (*red*) nor HA staining (*green*) was detected. SOCS3 expression was not changed by the treatment with HA900.

## DISCUSSION

Fig. 10 shows our model based on the studies in this report. TLR-4 receptors respond to bacterial endotoxins by up-regulation of pleiotrophin and pro-inflammatory cytokines. This increases inflammatory responses, including DNT lymphoaccumulation. Our studies support the model in which HA900 interacts with TLR-4 by a mechanism that blocks expression of pleiotrophin and up-regulates SOCS3. This would be consistent with the study showing that HA injections in LPS-treated mice promoted survival (14). The mechanism may be related to HA interaction with CD44 capturing the kinase required for LPS-TLR-4 receptor activation. Otherwise, our model would have to find a way to distinguish between TLR-4 interaction with bacterial endotoxin and HA. Thus, cooperation from another receptor (CD44) may be crucial



**FIGURE 10.** *a*, intestinal bacteria stimulate TLR-4 to induce inflammation and lymphoaccumulation via pleiotrophin. *b*, HA900 down-regulates pleiotrophin expression in intestinal epithelial cells, leading to suppression of inflammation, arthritis, and lymphoaccumulation. *c*, serum IL-10 concentration was reduced by HA900 treatment. It is speculated that HA900 up-regulates IL-10 expression, which induces SOCS3 expression in intestinal epithelial cells, leading to suppression of inflammation and arthritis. *White arrows*, up-regulation or down-regulation.

Lymphoaccumulation reflects inflammation by Th-1-type cytokines in MRL-*lpr/lpr* mice (5, 6). Therefore, it is adequate and convenient to observe the lymphoaccumulation to examine the effect of substances on the immune system.

Treatment with HA900 did not elicit any changes in the molecular weight or concentration of HA in serum. Furthermore, staining for HA in lymph nodes was not changed by treatment with HA900, which suggests that HA900 can modulate the function of the intestinal epithelium as a part of the immune system. Cultured DNT cells from MRL-*lpr/lpr* mice exhibited apoptotic programmed cell death without stimulation (3, 15); whereas treatment of DNT cells with pleiotrophin suppressed cell death. Furthermore, HA900 treatment induced apoptosis in the lymph node tissues. These results suggest that HA900 induces cell death of DNT cells by down-regulating pleiotrophin expression. Many reports have shown that SOCS3 suppresses inflammation, that IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of SOCS3 (16), and that induction of the SOCS3-CIS3 complex is a strategy for treating inflammatory arthritis (17). Our studies show that HA900 treatment of MRL-*lpr/lpr* mice increased SOCS3 mRNA and IL-10 consistent with its ability to suppress inflammatory responses. Intra-articular injection of HA900 suppresses inflammation in osteoarthritis (18).

In this study, oral administration of HA900 reduced lymphoaccumulation in MRL-*lpr/lpr* mice, which are a well established model for autoimmune diseases. These mice develop lymphoaccumulation (lymphadenopathy), hypergammaglobulinemia, serum autoantibodies, and generalized autoimmune disease, including glomerulonephritis and arthritis, and have also been used as a model for systemic lupus erythematosus (3). This lymphoaccumulation disorder involves a marked increase in DNT cells (CD4<sup>+</sup>CD8<sup>-</sup>) in *lpr* mice and is explained by defects in Fas, which mediates apoptosis (19). Lymphoaccumulation also occurs due to hyperproduction of proinflammatory cytokines (5, 6).

Most endogenous HA does not bind to TLR-4. Exogenous HA was found in apical epithelial cells of the upper villi of the large intestine, and colocalization of HA900 and TLR-4 was observed in these cells. HA900 may be taken up by intestinal epithelial cells via TLR-4. These results suggest that exogenous HA affects intestinal epithelial cells.

We had speculated that oral administration of HA900 would stimulate intestinal epithelial cells, which produce cytokines as protection against infection following exposure to bacteria. When administered, HA900 colocalized with TLR-4 on intestinal epithelial cells, but HA900 did not bind TLR-4 knockdown HT29 cells, suggesting that HA900 is bound to TLR-4. TLR-4 is known to be expressed on the surface of intestinal epithelial cells (20, 21) and is an HA receptor (1). Coexpression of TLR4 and MD-2 is necessary and sufficient for LPS responsiveness in intestinal epithelial cells (20), and expression of MD-2 is blocked by the STAT inhibitor SOCS3 (20). HA900 may prevent MD-2

binding to TLR-4 in commensal pathogen-associated molecular patterns such as LPS, or HA900-induced SOCS3 may reduce lymphoaccumulation because SOCS3 is known to suppress production of proinflammatory cytokines and improve arthritis (10).

The biological activity of HA is dependent on its molecular weight (3). Low molecular weight HA enhances inflammation via TLR-4 (3), whereas the present results suggest that HA900 may suppress inflammation involving chemokines, leukocyte and lymphocyte adhesion, P-selectin, and SCF expression via TLR-4. Overexpression of proinflammatory cytokines causes autoimmune disease and inflammation. MRL-*lpr/lpr* mice do not exhibit these conditions in a clean environment because of diminished levels of intestinal bacterial flora (22). Array analysis of cytokines (a total of 37) and chemokines showed that HA900 treatment up-regulated IL-10, and suppressed MCP-5, MIP-2, regulated on activation normal T cell expressed and secreted, P-selectin, SCF, and VEGF. IL-10 suppresses chemokine production (23, 24) and enhances SOCS3 expression (25). Furthermore, down-regulation of P-selectin suppresses the infiltration of lymphocytes and leukocytes (26, 27). The absolute suppression of SCF indicates that arthritis may be suppressed because SCF is implicated in processes in the arthritic synovium (28). The suppression of VEGF also suggests potential improvement of arthritis (29). Low molecular weight HA activates the innate immune response via TLR-2 in an MyD88-, IL-1R-associated kinase-, tumor necrosis factor receptor-associated factor-6-, protein kinase C $\zeta$ -, and NF- $\kappa$ B-dependent pathway. Furthermore, intact high molecular weight HA can inhibit TLR-2 signaling (30).

Arthritis did not occur in MRL-*lpr/lpr* mice in this study because these mice were too young. However, up-regulation of SOCS3 and suppression of pleiotrophin by HA900 treatment should suppress arthritis. Immunostaining, Western blotting, and RT-PCR for SOCS3 in HT29 cells showed that SOCS3 expression was enhanced by treatment with HA900 but not with HA oligos.

HA900, which was added to HT29 cells, showed colocalization with TLR-4 in these cells. During treatment with TLR-4 small interfering RNA, TLR-4 protein expression was diminished. HA900 neither bound to TLR-4 knockdown HT29 cells nor up-regulated SOCS3 in these cells compared with wild-type HT29 cells treated with HA900. We conclude that oral administration of HA900 suppresses inflammation and Th-1-type autoimmune disease by up-regulating SOCS3 and down-regulating pleiotrophin via TLR-4 in intestinal epithelial cells.

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