SHAP Potentiates the CD44-mediated Leukocyte Adhesion to the Hyaluronan Substratum

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CD44-hyaluronan (HA) interaction is involved in diverse physiological and pathological processes. Regulation of interacting avidity is well studied on CD44 but rarely on HA. We discovered a unique covalent modification of HA with a protein, SHAP, that corresponds to the heavy chains of inter-α-trypsin inhibitor family molecules circulating in blood. Formation of the SHAP-HA complex is often associated with inflammation, a well known process involving the CD44-HA interaction. We therefore examined the effect of SHAP on the CD44-HA interaction-mediated lymphocyte adhesion. Under both static and flowing conditions, Hut78 cells (CD44-positive) and CD44-transfected Jurkat cells (originally CD44-negative) adhered preferentially to the immobilized SHAP-HA complex than to HA. The enhanced adhesion is exclusively mediated by the CD44-HA interaction, because it was inhibited by HA, but not Iga, and was completely abolished by pretreating the cells with anti-CD44 antibodies. SHAP appears to potentiate the interaction by increasing the avidity of HA to CD44 and altering their distribution on cell surfaces. Large amounts of the SHAP-HA complex accumulate in the hyperplastic synovium of rheumatoid arthritis patients. Leukocytes infiltrated to the synovium were strongly positive for HA, SHAP, and CD44 on their surfaces, suggesting a role for the adhesion-enhancing effect of SHAP in pathogenesis.

Leukocyte cell-surface adhesion molecules play important roles in hematopoiesis, trafficking, recruitment, and the activation of leukocytes. Efforts toward identifying the adhesion molecules mediating the leukocyte-endothelial interaction have led to two families of adhesion molecules: the glycan-recognizing selectin family, which participates in the primary adhesion and rolling of leukocytes on the endothelium, and the integrin family members, which recognize the immunoglobulin superfamily molecules that are involved in the subsequent firm adhesion. The participation of these adhesion molecules has been well documented in leukocyte extravasation and function in diverse systems. Nevertheless, it is certain that there are more adhesion molecules involved in the regulation of these processes.

A promising candidate is the CD44-hyaluronan (HA)2 interaction that has attracted much attention since the late 1980s. CD44, first cloned as a lymphocyte surface antigen recognized by lymphocyte homing-interfering monoclonal antibodies (1), is a single polypeptide type I transmembrane protein encoded by a single gene. The standard form of CD44 consists of an N-terminal HA-binding domain, a stem region, a transmembrane domain, and a cytoplasmic domain. There are many CD44 variant forms with insertions at the stem region resulting from the alternative splicing of the exon 6–15 (or exon V1–V10) (2, 3). CD44 shares a highly conserved HA-binding domain, the link module, with HA-binding large proteoglycans (aggrecan, PG-M/versican, brevican, and neurocan), the link protein, and tumor necrosis factor-stimulated gene 6 product (TSG6) (1, 4). CD44 is also expressed in many kinds of cells other than leukocytes. Although CD44 interacts with a number of cell-surface or extracellular molecules, such as fibronectin, collagen, selectin, osteopontin, and chondroitin sulfate proteoglycans, its principal ligand is believed to be HA (3). The roles of CD44-HA interaction in lymphocyte extravasation (5, 6) and stromal interaction (7, 8) have become evident not only in cell adhesion systems in vitro with cell monolayer or tissue sections, but also in systems in vivo using specific antibodies or CD44-null mice. These studies implicated that the CD44-HA interaction may underlie the pathogenesis of a wide range of disorders such as the autoimmune diseases, including arthritis (9–11), encephalomyelitis (12), crescentic glomerulonephritis (13), and uveoretinitis (14); allergic diseases, including asthma (7) and contact dermatitis (15); inflammatory bowel disease (8, 16); graft rejection (17); atherosclerosis (18); hepatitis (19); and lung inflammation (20). Depending on the diseases, the consequences of the interaction may either be inflammatory, for example in the autoimmune diseases, where its role in the recruitment and activation of circulating leukocytes is predominant, or anti-inflammatory, e.g. in hepatitis and lung inflamma-

The abbreviations used are: HA, hyaluronan; HC, heavy chain; SHAP, serum-derived hyaluronan-associated protein; HABP, the aggrecan-link protein complex-derived HA-binding protein; HRP, horseradish peroxidase; TSG6, tumor necrosis factor-stimulated gene 6; RA, rheumatoid arthritis; Iga, inter-α-trypsin inhibitor; PBS, phosphate-buffered saline; CMV, cytomegalovirus.
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tion, where its principal role seems to be the induction of apo-
ptosis of leukocytes.

Given the diversity of the targeting tissue and effects of
CD44-HA interaction, it is not surprising that the interaction is
under the control of extremely complex mechanisms. It is well
known that most CD44-bearing leukocytes are actually not
adhesive to HA until they are activated. Extensive studies on the
activation-associated molecular modification of CD44 have
revealed that it is multifactorial, involving transcriptional up-
regulation (21), the expression of new variant forms (22), gly-
cosylation (N-glycan, O-glycan, and glycosaminoglycan) (23,
24), sialylation (25), sulfation (26), phosphorylation (27), palmi-
toylation (28), and dimerization/oligomerization (29, 30). The
regulation is strongly event- and cell type-dependent. Even
the same kind of modification may lead to opposite out-
comes under different circumstances; for example, the
blockage of N-glycosylation with tunicamycin or by amino
acid residue substitution enhances the CD44-HA interaction
in pre-B cells, fibroblasts, Chinese hamster ovary cells, and
melanoma cells (31–33) but reduces it in lymphoma cells,
ovarian tumor cells, and melanoma cells (34, 35).

In sharp contrast to CD44, little information is available
about the regulation of CD44-HA interaction on HA side. The
major reason is due to the structural simplicity of HA: the lin-
early repeating N-acetylgalactosamine-glucuronic acid disaccha-
dride units. In the absence of a core protein modification, sulfa-
tion, and epimerization, the chain length appears to be the sole
molecular variation of HA. In recent years, there are accumu-
lating data indicating that the degradation of HA macromole-
cules occurs during some inflammatory disorders and that the
resulting low molecular weight HA fragments exhibit different
biological activities from their precursors (36, 37). The size-de-
pendent avidity of HA to cell-surface CD44 has been examined
using HA oligomers. Monovalent binding was observed in 6- to
18-sugar-oligomers, and divalent binding with greatly and pro-
gressively increased avidity in 20- to 38-sugar-oligomers (38).
The relationship of such a mechanism to the pathological role
of naturally occurring HA fragments, which are usually longer
than the oligomers examined, remains to be further examined.
Additionally, TSG6 has been recently shown to modulate the
CD44-HA interaction, thus providing a new insight into the
functional regulation of CD44-HA interaction on HA side (39).

We have found that HA, ever thought to be a free molecule,
could also be covalently modified by a protein. The protein,
designated SHAP (serum-derived HA-associated protein), is
bound to HA via a unique ester bond between the carboxyl of its
C-terminal aspartate and the C-6 hydroxyl group of an internal
N-acetylgalactosamine of HA (40, 41). SHAP is identical to the
heavy chains of inter-α (trypsin) inhibitor (IαI) family mole-
cules (42), which link via the same type of ester bond to the
chondroitin sulfate chain of the IαI light chain, the bikunin
proteoglycan (43). The formation of the SHAP-HA complex is
believed to be a transesterification reaction, where HA substi-
tutes for chondroitin sulfate to form an ester bond with
HC/SHAP. IαI family molecules, principally synthesized and
secreted by the liver, occur constitutively at high concentra-
tions in the circulation. The SHAP-HA complex is most likely
formed between the extravasated IαI and the local HA, as best
exemplified by rheumatoid arthritis (RA) and ovulation process
(44, 45). The reaction is indissolable for the construction of
HA-rich extracellular matrix of the expanding cumulus oopho-
rus, oocyte transportation, and successful oocyte-sperm inter-
action (fertilization) (45). In addition, the complex is present in
large amounts in the synovial fluid of RA patients. Patients with
various inflammatory disorders often have an elevated plasma
level with the complex (44, 46). In this study, we examined the
effect of SHAP on CD44-HA interaction and found that SHAP
significantly increase the avidity of HA to the cell-surface
CD44.

MATERIALS AND METHODS

Cells, Antibodies, and Other Reagents—Human cutaneous T
lymphoma cell Hut78 and human acute T leukemia cell Jurkat
were purchased from ATCC. The human hepatoma cell line
HLF was from Health Science Research Resources Bank
(Osaka, Japan). Mouse IgG2a anti-human CD44 (Ab4, clone
156–3C11) was from NeoMarkers (Fremont, CA). Affinity-puri-
ified rat IgG2b anti-mouse CD44 (clone IM7) was from ebiosciences.
San Diego, CA). Biotinylated rat IgG2a anti-mouse CD44 (clone
KM81) was from Cedarlane Laboratories (Ontario, Canada). Goat
anti-human TSG6 polyclonal antibody was from R&D systems,
Inc. (Minneapolis, MN). Rabbit anti-human IαI immunoglobulin
and horseradish peroxidase (HRP)-conjugated rabbit anti-
goat immunoglobulin antibody were from Dako (Glostrup,
Denmark). Rabbit anti-human bikunin antiserum was pre-
pared in this laboratory (45). Alexa 594-conjugated-rabbit anti-
mouse IgG, Alexa 488-conjugated streptavidin, and R-phycoc-
yerythrin-conjugated streptavidin were from Molecular Probes
Inc. (Eugene, OR). HRP-conjugated goat anti-rabbit IgG anti-
body (affinity-purified IgG) and HRP-conjugated streptavidin
were from Jackson ImmunoResearch Laboratories (West
Grove, PA). RPMI 1640 cell culture medium, phorbol 12-my-
ristate 13-acetate, deferoxamine mesylate, protease inhibitor
cocktail, p3×FLAG-CMV-14 vector, and anti-FLAG M2 affin-
ity gel were from Sigma (St. Louis, MO). Cosmedium 001 was
from Cosmo Bio Co. Ltd. (Tokyo, Japan). G418 was from
Nacalai Tesque (Kyoto, Japan). HABP (the aggrecan-link pro-
ctein complex-derived HA-binding protein), sodium hyaluro-
nate (Alta®) and Streptomyces hyaluronidase were from Seika
gaku Corp. (Tokyo, Japan). Falcon tissue culture dishes were
from BD Bioscience. Superscript II RNase H reverse tran-
scriptase was from Invitrogen. Micro BCA Protein Assay Re-
agent kit was from Pierce. Silver stain II kit was from Wako Pure
Chemical Industries Ltd. (Osaka, Japan). Fugene 6 transfection
reagent was from Roche Applied Science (Indianapolis, IN).
The 3,3',5,5'-tetramethylbenzidine solution was from Kirkeg-
aard & Perry Laboratories (Gaithersburg, MD). Western blot
chemiluminescence reagent was from PerkinElmer Life Sci-
ences. Other reagents were from major suppliers.

Purification of the SHAP-HA Complex from RA Synovial
Fluid—The purification and characterization of the synovial
SHAP-HA complex has been described in detail elsewhere (47).
Briefly, the synovial fluid was subjected to sequential cesium
chloride isopycnic centrifugation in the presence of 4 m guani-
dine hydrochloride to separate hyaluronan from the non-co-
valently associated proteins. The final highly purified prepara-
tion contains HA molecules with an average molecular weight of 1.8 million, about half of which bears SHAP at an average ratio of three to five SHAPs per HA chain. To cleave the ester linkage between SHAP and HA, the SHAP-HA complex solution was supplemented with NaOH to reach a final concentration of 0.1 M and incubated at room temperature for 30 min, followed by neutralization with HCl solution. In control tubes, NaOH solution and HCl solution were mixed before adding to the SHAP-HA complex solution.

Purification of the SHAP-HA Complex from RA Hyperplastic Synovium—The hyperplastic synovium was obtained from RA patients receiving synovectomy. Synovium (6 g of wet weight) was homogenized with a glass grinder in 20 ml of phosphate-buffered saline (PBS) buffer containing 5 mM deferoxamine mesylate, 10 mM EDTA, and 6% protease inhibitor cocktail. The homogenates were mixed with an equal volume of 8 M guanidine hydrochloride and then stirred overnight at 4 °C. After removing the insoluble materials by centrifuging at 10,000 rpm for 30 min, the density of the solution was adjust to 1.38 g/ml with solid cesium chloride. The solution was then subjected to isopycnic centrifugation as described above. The resulting density gradient was partitioned into 20 fractions, which were then precipitated twice with 3 volume of 95% ethanol containing 1.3% potassium acetate and finally dissolved in 0.4 ml of MilliQ water. The protein and uronate contents in each fraction were measured by using MicroBCA kit and carbazole reaction, respectively.

Purification of IaI—Mouse IaI was purified from pooled serum from C57BL/6 mice as described before (45). The highly purified human IaI was a gift from the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan.

Enzyme-linked Immunosorbent Assay—The SHAP-HA complex and HA were quantified by enzyme-linked immunosorbent assay as previously described (44, 47). Briefly, the SHAP-HA complex or HA in the sample solutions were captured on HABP (2 μg/ml in 0.1 M NaHCO₃, pH ~9.5)-coated wells of MaxiSorp plates, and then detected with rabbit anti-IaI antibody and then HRP-conjugated goat anti-rabbit IgG antibody, or biotinylated HABP and then HRP-conjugated streptavidin, respectively. The color reaction was developed with 3,3′,5,5′-tetramethylbenzidine substrate and stopped with 1 N HCl. The absorbance value was read at 450 nm.

Cell Adhesion Assay—The cells were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 μg/ml streptomycin, and 6.25 μg/ml penicillin G. The floating cells were collected by centrifugation, washed with PBS for three times, counted, and then resuspended at proper densities. For the adhesion assay under static conditions, microplates were coated with HABP and then SHAP-HA complex or HA (250 or 1000 μg/ml) as mentioned above and assembled in the parallel flow chamber designed by Lawrence et al. (48). The SHAP-HA or HA-coated microplates were placed onto a transparent plastic plate with a silicon rubber gasket in parallel so that between the two surfaces a chamber with a thickness of 320 μm was formed, to which the inlet and outlet are connected. Rolling assays with the chamber were performed as described previously (49). Briefly the chamber was mounted on the stage of an inverted phase-contrast microscope (Model IX70, Olympus Products, Tokyo). Hut78 cells were introduced into the chamber at a concentration of 5 × 10⁵/ml at decreasing levels of fluid shear (1.13, 0.85, 0.57, 0.42, and 0.28 dyne/cm²). The shear stress in the chamber was controlled using a syringe pump (Model 944, Harvard Apparatus, South Natick, MA). The numbers of rolling or attached cells were determined from digital images recorded with a Model CS–220 video camera system (Olympus). The flow chambers were maintained at 37 °C with an air-curtain incubator (Olympus Products, Model IX-IBM) throughout the experiments. The wall shear stress (T) was calculated from the equation, $T = 3μQ/2a^2b$, where μ = coefficient of viscosity (1.0 centipoise), Q = volumetric flow rate (cm³/s), 2a = channel height (3.2 × 10⁻² cm), and b = channel width (1.3 cm).

Immunolabeling and Fluorescence Microscopy—Hut78 cell suspension was laid on the SHAP-HA or HA-coated cover glass (prepared as mentioned above), followed by incubating at room temperature for 20 min or 1 h. After washing, the adhered cells were fixed with 4% buffered neutral formaldehyde. The cover glass was blocked with 10% goat serum (Nichirei, Tokyo, Japan), incubated with PBS-T (0.1% Tween 20 in PBS) containing Ab4 antibody (1 μg/ml) and biotinylated HABP (0.5 μg/ml), and then with PBS-T containing Alexa 594-rabbit anti-mouse IgG (1:1,000 dilution) and Alexa 488-streptavidin (1:1,000 dilution) at room temperature for 1 h each. Finally, the cover glass was washed with PBS-T for 5 times, and then mounted on a glass slide with Gelmount (CosmoBio, Tokyo, Japan). The cells were examined and photographed with a Zeiss LSM5 PASCAL confocal laser microscope.

RNA Extraction and Reverse Transcription-PCR—Total RNA was prepared from cells using TRIzol® reagent (Invitrogen) and quantified by measuring the UV absorbance. The first strand cDNAs were synthesized from 4 μg of total RNA using Superscript II RNase H⁻ reverse transcriptase and oligo(dT)₁₂–₁₈ primer. The CD44 transcripts were amplified with AmpliTaq DNA polymerase and the primer set CTGGCGCACAGTC-
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GATTGAA (forward, corresponding to the boundary of exon 1 and 2) and GCCGTACGTATAGGACCAGAGGGTTGT (reverse, corresponding to exon 17, with a SplI site). The cycling conditions were: 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 56 °C for 45 s, and 72 °C for 90 s, and finally 72 °C for 5 min.

Cloning of CD44 Variants and Cell Transfection—The full-length cDNA of human standard form CD44 (sCD44 or CD44H), including ~50 bp of the 5'-untranslated region as well as 15 bp of the 3'-untranslated region, was cloned into the EcoRI site of pCDNA3.1/Myc-His(–) B vector (Invitrogen). The stop codon TAA was mutated to GAA, which, together with the 15-bp 3'-untranslated region, generates an extra peptide sequence EHLHHY between the CD44 polypeptides and the vector-derived C-terminal tags. The above sCD44 construct was used to generate the CD44 variant form (CD44V) constructs by inserting proper variable exons (exon 6 to exon 15, or exon V1 to V10) between exons 5 and 16. The Hpal site (GTTAAC) present in exon 5 and a SplI site generated by silent nucleotide mutation (from AGGACA to CGTACG) in exon 17 were selected for cloning, to which PCR-amplified CD44v transcripts were inserted. All CD44 constructs were verified by sequencing with ABI 310 sequencer (PerkinElmer Life Sciences) for transfection of Jurkat cells with Bio-Rad Gene Pulsetransfectants obtained by G418 selection were cultured in a serum-free medium, Cosmedium 001. The recombinant TSG6 protein was then purified from the pooled conditioned medium with the anti-human I antibody, anti-UTI antibody, and biotinylated HABP, and then the respective HRP-conjugated second antibodies (Dako, Carpinteria, CA). Finally, the Liquid DAB Substrate-chromogen system was used to develop a color (Dako).

FIGURE 1. Effects of alkali treatment (NaOH) and hyaluronidase-digestion (Hase) on the adhesion of Hut78 cells to the SHAP HAase complex. HABP-coated plastic or glass surfaces were incubated with PBS (as control) or the SHAP-HA complex (200 μg/ml, treated or not as indicated) and then subjected to cell adhesion assay as described under "Materials and Methods." Both substrata gave the similar results. The images shown are the representatives of two independent experiments on plastic dishes. The cell counting result is shown beneath.
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RESULTS

CD44-positive Cells Adhere More Strongly to the SHAP-HA Complex than to HA—We isolated the SHAP-HA complex from synovial fluids of RA patients by sequential isopycnic centrifugation. The final preparation was a mixture of the SHAP-HA complex and HA immobilized on HABP-coated surfaces. The concentrations used for immobilization are indicated above each image. B, the numbers of adhered cells were counted, and the averages and the standard variations are shown in the curves. Higher concentrations of HA, ~20- to 30-fold than that of the SHAP-HA complex, were required to achieve a comparable strength of cell adhesion. C, negligible effect of SHAP on the immobilizing efficiency of HA on the HABP-coated surface in a sandwich enzyme-linked immunosorbent assay system.

Although there were firm adherent cells at 1.13 dyne/cm² to the SHAP-HA substratum with a coating concentration of 20 μg/ml, those to HA substratum with a coating concentration of 1 mg/ml were barely observed even at 0.28 dyne/cm². The maximum cell rolling was observed at 0.42 dyne/cm² or below on both the SHAP-HA complex and HA substrata, with 4.9 × 10³ cells/mm²/min on the SHAP-HA substratum (coating concentration at 100 μg/ml) and 1.3 × 10³ cells/mm²/min on HA substratum (coating concentration at 1 mg/ml) in average.

CD44-HA Interaction Exclusively Mediates the Strong Adhesion of Hut78 Cells to the SHAP-HA Complex—We next tried to define the mechanism for the enhancing effect of SHAP. First we examined the effect of the presence of SHAP on the immobilization of HA on the HABP-coated surface using an enzyme-linked immunosorbent assay system. The results shown in Fig. 2C indicate that the immobilization of the SHAP-HA complex, HA dissociated from the SHAP-HA complex by alkali treatment, and free HA on the HABP-coated surface are comparable. Therefore, we concluded that the enhancing effect of SHAP on cell adhesion is not a result of the increased immobilizing efficiency of HA.

We then examined the possibility of a direct interaction between SHAP and cell-surface molecules, including CD44 that shares a conserved link module domain with HABP (1). Hyaluronan, but not purified I, significantly inhibited the adhesion of Hut78 cells to the SHAP-HA complex, suggesting that SHAP did not mediate the adhesion of cells directly (Fig. 4). Consistently, Hut78 cells did not adhere to I1L-coated surface, even with a high

solution, ~20–30 times higher, was required for HA to trap as many cells as that on the SHAP-HA substratum (Fig. 2B), although the amounts of immobilized HA were comparable between both substrata (Fig. 2C). The results clearly demonstrate an enhancing effect of SHAP on HA-mediated cell adhesion. Because the SHAP-HA complex at a concentration of 20–50 μg/ml was enough to adhere a large number of cells, the condition was adopted in the following experiments.

In addition to the static adhesion assay, we also performed the adhesion assay under flow shearing conditions (Fig. 3). Similarly, the cells showed a stronger avidity to the SHAP-HA complex than to HA, as manifested by the significantly increased number of cells either firmly adhering to or rolling on the SHAP-HA substratum. Intriguingly, a considerable population of cells exhibited a very low rolling speed, even stopping, on the SHAP-HA complex, in sharp contrast to those mediated by the selectin-ligand interaction that we described previously (49).
coating concentration (0.2 mg/ml, data not shown). On the other hand, pretreatment of cells with anti-CD44 monoclonal antibody (Ab-4 or IM7) completely abrogated the cell adhesion, whereas neither the unrelated anti-I/H9251 nor another anti-CD44 monoclonal antibody, KM81, exhibited any inhibitory effect (Fig. 5). Flow cytometry analysis showed that KM81 did not recognize human CD44 on the surface of Hut78 cell (Fig. 5). These data provide convincing evidence for the exclusive role of CD44-HA interaction in mediating the strong adhesion of Hut78 cells, implying an indirect role of SHAP in enhancing the cell adhesion, i.e. by increasing the HA avidity to CD44.

To further validate the conclusion, we transfected the non-adhesive Jurkat cells with CD44 cDNAs and examined their adhesion to the SHAP-HA complex. Reverse transcription-PCR study revealed transcripts with various lengths (Fig. 6), indicating the expression of both sCD44 and CD44v in Hut78 cells. Cloning and sequencing of the PCR products indicated that the major bands were derived from CD44H, and CD44E, the epithelial form containing variable exons V8–10, whereas the minor one was a mixture of PCR products derived from two variant forms containing V8–9 and V10, respectively. All these CD44 variant forms have been reported (3). All four CD44 transcripts were cloned and introduced into Jurkat cells. The CD44-transfected Jurkat cells exhibited no adhesion to HA substratum (data not shown) until they were activated by culturing in the presence of phorbol 12-myristate 13-acetate, which was reported to induce the dimerization of CD44 and thus increase the avidity of CD44 to HA (30). Again, the activated Jurkat CD44 transfectants exhibited stronger adhesion to the SHAP-HA complex than to HA (Fig. 7). The results provide further evidence for the above conclusion.

Since it has been reported that the RA synovial fluid contains TSG6 complex, and that TSG6 modulates the CD44-HA interaction (39), it is important to examine whether the adhesion-enhancing effect of the purified SHAP-HA complex is due to a contaminating TSG6. We, therefore, generated the recombinant human TSG6 protein in cultured human hepatoma cells. The recombinant protein was efficiently purified via its C-terminal 3×FLAG tag, as demonstrated by the single band in a silver-stained SDS-PAGE gel (Fig. 8). An anti-human TSG6 polyclonal antibody, which was able to detect nanogram amounts of the recombinant TSG6 protein, failed to show any TSG6 immunoreactivity at 38 kDa in the SHAP-HA preparation containing 1/20 protein (Fig. 8). The faint bands with higher molecular weights in lane 4 are background reaction of anti-TSG6 antibody with SHAP proteins, because TSG-Iol complex is known to dissociate after an alkali treatment. The results rule out the possibility of a contaminating activity of TSG6.

Immunofluorescence Study on the Cell Adhesion-mediating CD44 and HA Molecules—We further investigated the molecular interactions upon cell adhesion using immunofluores-
cence microscopy. Hut78 cells attaching to the substratum after 20-min incubation were strongly positive for CD44 staining on their surfaces (Fig. 9, A and B, the flank surface and bottom of the cells, respectively). No apparent difference in the distribution of CD44 was found on the bottom surfaces between the cells adhering to the SHAP/H18528 HA substratum and to the HA substratum, but more diffused distributions were observed on the flank cell surfaces. Although both SHAP/H18528 HA and HA substrata exhibited an even distribution of fluorescent signals of HA staining, stronger signals were found on the surfaces of adhered cells, indicating that HA molecules condensed on the cell surface upon CD44-mediated cell adhesion. The substratum origin of HA was also supported by the negative result in staining cell-surface HA by flow cytometry analysis (data not shown). Interestingly, the condensation was more frequently found on the SHAP-HA substratum than on the HA substratum, especially on the flank surfaces (Fig. 9A), suggesting that the enhancing effect of SHAP may be related to the accelerated condensation of HA on the surfaces of CD44-bearing cells. The cells incubated for 1 h exhibited similar patterns, which excludes the possibility that the difference was due to some metabolic reactions, such as the uptake and degradation of HA.

**Formation of the SHAP-HA Complex in the RA Hyperplastic Synovium**—We further examined the formation and distribution of the SHAP-HA complex in the inflammatory hyperplastic synovium of RA patients. Glycosaminoglycans were extracted from hyperplastic synovium with 4 M guanidine hydrochloride and separated from proteins by cesium chloride isopycnic centrifugation (Fig. 10A). Western blot assay demonstrated the release of SHAP from the synovium membrane-derived glyco-

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**FIGURE 5.** Blockage of cell-surface CD44 with anti-CD44 antibody abrogates the cell adhesion to the immobilized SHAP-HA complex. A, flow cytometry analysis of Hut78 cells with three anti-CD44 antibodies. Dashed line, PBS only; solid line, antibody treated. Anti-human CD44 antibody Ab4 and anti-mouse CD44 antibody IM7, but not anti-mouse CD44 antibody KM81, recognize the cell surface CD44. B, pretreatment of Hut78 cells with the first two antibodies completely abrogated the cell adhesion, whereas neither KM81 nor the unrelated anti-Igα antibody showed any effect.
saminoglycan by alkali treatment and *Streptomyces* hyaluronidase digestion, which specifically degrades hyaluronan (Fig. 10B). The larger molecular weights of hyaluronidase digestion-derived SHAPs are attributed to HA remnants due to the steric hindrance of SHAP proteins and probably alkali treatment-induced loss of O-glycan as well. The results indicated clearly the formation of the SHAP-HA complex in the hyperplastic synovium of RA patients. Histological study showed strong staining of hyaluronan by HABP (Fig. 11), consistent with the previous findings that the hyperplastic synovium extensively produces hyaluronan. Anti-Iol antibody also gave strong staining results, whereas anti-bikunin antibody gave negative results, suggesting that the immunoreactivity corresponds to the SHAP-HA complex rather than Iol. The distribution of SHAP is patchier than the smearing pattern of HA. Interestingly, the CD44-positive lymphocytes in the tissues were strongly positive for both SHAP and HA, implying an interaction of these cells with the SHAP-HA complex in hyperplastic synovium.

**DISCUSSION**

We showed in this study that the covalent association of SHAP with HA greatly enhanced its avidity to the cell-surface CD44 and thus provided a new insight into the complex regulatory mechanism for the CD44-HA interaction-mediated cell adhesion; that is, the regulation can also occur on the HA side. Consistently, Lesley *et al.* (39) showed that TSG6 modulates the interaction between HA and CD44 on the cell surface. However, there are some differences in the regulation pattern between SHAP and TSG6. For example, TSG6-HA complex is capable of mediating adhesion of cells without pre-activation of CD44, whereas the SHAP-HA complex is not.

Many studies have indicated that the CD44-HA interaction could support the rolling and initial adhesion of leukocytes onto the vascular endothelium under physiological shearing force, and could be a new mechanism other than the selectin-glycan interaction for the capture of circulating leukocytes. The present results support the previous findings, and further imply an enhancing role of SHAP in the molecular interaction. Once the endothelial cells deposit HA on their luminal surfaces, the association of SHAP to these HA molecules is very likely, because the blood contains a large amount of Iol and the enzymatic activity, and in fact, the simple incubation of plasma with HA could result in the complex formation (45). Although it is not yet clear what events are triggered by such a mechanism at present, we noticed an interesting observation made by Milinkovic *et al.* (17) in acute graft-versus-host disease, one of the major limitations for the curative therapy of aplastic anemia and hematological malignancy with allogeneic hematopoietic stem cell transplantation. Acute graft-versus-host disease is characterized by dermal lymphocytic infiltration following hematopoietic stem cell transplantation. A lymphocyte adhesion study revealed that the papillary dermal vessels in acute graft-versus-host disease, but not those in most other post-hematopoietic stem cell transplantation skin eruptions, supported the shear-resistant adherence of lymphocytes via a CD44-HA interaction (17, 50). Only the HA deposits on the vascular endothelium, but neither the comparable HA deposits at the dermal-epidermal border and within glandular elements of the reticular dermis, nor HA immobilized on plastic, supported the lymphocyte adhesion, suggesting that the endothelial HA deposits have special properties (17). It would be interesting to examine the formation of the SHAP-HA complex under this condition.

It is noteworthy that the range of shear force where an enhancing effect of SHAP was observed (<1 dyne/mm²) is lower than the physiological levels (at 1–2 dynes/mm²) (Fig. 3), making the physiological relevance of the present finding in a microcirculation environment uncertain. However, as discussed below, many other molecules may participate in the regulation of CD44-HA interaction in vivo, making SHAP effective at higher ranges of shear force as well.

On the other hand, CD44-HA interaction also occurs in extravascular tissues such as the joint cavity and various stromal extracellular matrices with low shear forces. HA is one of the most important components of extracellular matrix supporting in general the active cell propagation, migration, and differentiation. Processes such as inflammation, wound healing, and tumor malignancy are well known to be associated with up-regulated HA production and deposition and the CD44-HA interaction between extravasated leukocytes/metastatic cancer cells and the stromal extracellular matrix. Participation of SHAP in these interactions has been suggested by de la Motte *et al.* (8, 51) in inflammatory bowel diseases. The main pathological changes of IBD include an increase in intestinal mucosal mononuclear leukocytes and a dramatic hyperplasia of the muscularis mucosae. The interaction between recruited leukocytes and mesenchymal smooth muscle cells is thought to be important in the development and propaga-
tion of inflammatory bowel disease. By infecting the cultured colon smooth muscle cells with virus, an etiological factor of inflammatory bowel disease, the authors observed an up-regulation of HA production and an extracellular deposition of these HA molecules into the pericellular “coat” structure and the “cable” structure spanning several cell lengths, in contrast to the small patchy structure in unstimulated cells. The formation of the special HA structures was accompanied by a dramatically increased CD44-mediated adhesion of mononuclear leukocytes. Interestingly, the study indicated that Iod heavy chains are required for the formation of the HA structures (51). In the present study, an immunofluorescence staining study failed to reveal a different microstructure of the immobilized SHAP-HA complex from that of free HA in the absence of cells. The most likely reason was that the SHAP-HA complex used in this study was highly purified, lacking the other components found in de la Motte’s cable structure. In

FIGURE 7. Activated CD44-transfected Jurkat cells adhere more firmly to the SHAP-HA substratum than to the HA substratum. To the left is shown the flow cytometry analysis results of CD44-transfected Jurkat cells using Ab4 antibody. The cells were stimulated with phorbol ester to induce the HA avidity before the adhesion assay. The cells adhered more firmly to the immobilized SHAP-HA complex than to the immobilized HA, a behavior resembling that of CD44-positive Hut78 cells.
FIGURE 8. The synovial SHAP-HA preparation is free of TSG6. The SHAP-HA complex containing 1 μg of protein was treated with NaOH and then subjected to a reducing SDS-PAGE (lane 4) together with purified recombinant human TSG6 protein (1.6, 8, and 40 ng in lanes 1–3, respectively). The gel was either silver-stained (left panel) or subjected to Western blot analysis with anti-TSG6 antibody (right panel).

relation to this, our previous study showed that versican/PG-M interacts with both HA and the heavy chains of IgI to facilitate the complex formation (52); mesenchymal smooth muscle cells synthesize a large amount of versican/PG-M and deposit it around them (53). In addition, several recent studies have shown that TSG6 is able to replace the chondroitin sulfate of IgI to form a covalent complex with the heavy chain and then transfer it to HA (54, 55). The TSG6/HC complex has been found in synovial fluids from RA patients (56) and the extracellular matrix of expanded cumulus oophorus (57). The absence of either TGS6 or SHAP in the cumulus extracellular matrix causes severe functional impairment (45, 59). However, it is still likely that a novel type of fine microstructure of HA was formed in the presence of SHAP because of the aggregating property of the SHAP-HA complex (47) and the increased avidity to CD44 observed in this study. Relating to this possibility, the confocal microscopic observations on the cell-surface distributions of HA and CD44 revealed somewhat different distributions of both molecules: their co-distributed population on the flank surfaces on the SHAP-HA substratum (see the yellow color of the merged photos in Fig. 9). The question remains to be answered using more powerful techniques, for example, the rotary shadowing technique (60, 61) or atomic force microscopy (62), both having been applied to the study of HA microstructure and/or conformational change. Taken together, the present results argue strongly for a role of SHAP modification of HA in the pathogenesis of related diseases, although the presence of other HA-binding molecules, for instance, TSG6 and PG-M/versican, may be further required to form the special HA structures exhibiting high avidity to CD44-bearing leukocytes.

We also examined immunohistologically the involvement of these molecular interactions in synovitis of RA patients. It is known that the synovial fluid of RA patients includes massively the SHAP-HA complex, which was speculated to largely derive from the inflammatory synovium, a site with active HA production and serum protein efflux. As predicted, the results revealed the presence of a large amount of the SHAP-HA complex in the hyperplasic synovium. Notably, most of the infiltrated leukocytes were positive on their surface for the complex, in addition to CD44, suggesting molecular interactions among these molecules on the surfaces of synovial infiltrating leukocytes. Studies in mice with CD44-blocking antibodies (10, 11) or with CD44 knock-out mice (9) have revealed a positive role of CD44 in the development of collagen-induced arthritis, a murine model for human RA. Consistently, we observed that the mice incapable of SHAP-HA complex formation were more resistant to the collagen-induced arthritis than their normal littermates (46). Therefore, it is likely that SHAP, by potentiating the CD44-HA interaction, plays a positive role in the leukocyte infiltration and activation in the hyperplasic synovium of RA joints.
The present results suggest that the enhancing effect of SHAP is indirect. Although further studies are required to define the exact mechanism, it is most likely related to the aggregating property of the SHAP/H18528 HA complex that we observed recently (47). The huge linear HA molecule adopts a random coil configuration with a certain degree of stiffness. The size of the random coils is so large that at concentrations higher than 1 mg/ml the HA coils entangle to form a continuous flexible molecular network and exhibit unique physicochemical and biological features (63). Given the oligomerization of CD44 upon activation (29, 30) and the higher avidity of multivalent HA to CD44 (38), we hypothesize that the CD44-HA interaction-enhancing effect of SHAP is due to the accelerated aggregation of HA as a consequence of the self-association of SHAP molecules. In contrast, we found no such effect on the HABP-HA interaction in this study, although the HABP and CD44 share the conserved HA-binding link module domain. The exact molecular mechanism remains to be further investigated. A noteworthy fact is that both aggrecan and link protein included in the HABP consist of two tandem link module domains forming an extremely stable ternary complex with HA, whereas CD44 has only one, of which the dimerization/oligomerization has been shown to be an important mechanism of the regulation of HA avidity.

The signaling cascades initiated by HA-CD44 interaction have been extensively studied in recent years (64, 65). In leukocytes, the mitogen-activated protein kinase and NF-κB-mediated pathways were found to be important (58). In general, cell response to HA varies depending on the HA amount, HA chain length, and cell background. Although experimental evidence is lacking, it may be expected that the HA-binding proteins (e.g. SHAP and TSG6) become an additional factor modifying HA signaling. The question of how SHAP alters HA signaling, quantitatively or qualitatively, also remains to be answered. However, considering the results of this study, it is likely that SHAP strengthens the signal intensity of HA but does not evoke a novel pathway unresponsive to free HA.

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FIGURE 10. RA hyperplastic synovium contains the SHAP-HA complex. A, fresh hyperplastic synovium (6 g of wet weight) was homogenized and extracted in the presence of 4 M guanidine hydrochloride. The resulting solution was subjected to cesium chloride isopycnic centrifugation with an initial density of 1.38 g/ml and then partitioned into 20 fractions. The fractions were ethanol-precipitated and redissolved in 0.4 ml of MilliQ water. The protein and uronate contents in each fraction were measured by MicroBCA assay and carbazole reaction, respectively. B, fractions 2–5 were pooled, treated if necessary (10 μl each), and subjected to Western blot analysis with anti-I antibody. Lane 1, non-treated control; lane 2, treated with NaOH; lane 3, digested with Streptomyces hyaluronidase (47).

FIGURE 11. Representative immunohistology of the hyperplastic synovium of RA patients (Steinbrocker’s stage III). The synovium is extensively stained by HABP and anti-I antibody (counterstained with methyl green). The negative staining by anti-bikunin antibody (counterstained with hematoxylin) indicates that the immunoreactivity corresponds to the SHAP-HA complex rather than I. Compared with the distribution pattern of HA, that of SHAP is patchier. The infiltrated CD44-positive leukocytes (counterstained with methyl green) are strongly positive on their surfaces for HA and SHAP staining. The arrow and arrowhead indicate the leukocyte and synoviocyte, respectively. Similar staining patterns were obtained with the samples at stage IV. (Magnification: ×400.)
