Short Communication

Bacterial Chaperone Protein, Skp, Induces Leukocyte Chemotaxis via C5a Receptor

Arjun Shrestha,* Lei Shi,[†] Sumio Tanase,[‡]
Makiko Tsukamoto,[§] Norikazu Nishino,[§]
Kazutaka Tokita,* and Tetsuro Yamamoto*

From the Departments of Molecular Pathology* and Medical Biochemistry,[‡] Faculty of Medical and Pharmaceutical Sciences, and the Department of Molecular Pathology,[†] Graduate School of Medical Sciences, Kumamoto University, Kumamoto; and the Department of Biological Functions and Engineering,[§] Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Kitakyushu, Japan

C5a receptor has been identified as a leukocyte chemotactic receptor to two intrinsic chemical mediators, C5a and the S19 ribosomal protein dimer, so far. We found an *Escherichia coli* protein that also induced the chemotactic responses of monocytes and polymorphonuclear leukocytes via the C5a receptor. We identified the *E. coli*-derived chemoattractant to be Skp by the molecular size and the N-terminal amino acid sequence. Skp is a periplasmic chaperone protein widely present in gram-negative bacterial species. Immunoabsorption experiments indicated that Skp was the major leukocyte chemotactic factor in the *E. coli* extract. Receptor-antagonizing experiments with analogue peptides of S19 ribosomal protein and of C5a suggested that Skp induced the receptor activation by the two-step binding mechanism as in the cases of the intrinsic mediators, sharing the ligand-binding site of the receptor among them at each binding step. The C5a receptor would play a role in the host defense directly recognizing the bacteria-derived protein, besides identifying the signals of the intrinsic chemical mediators. (*Am J Pathol* 2004, 164:763–772)

C5a receptor is one of the most important leukocyte receptors involving in the inflammatory reaction. C5a receptor was initially identified as the receptor of C5a, which is a 74 amino acid peptide liberated from the α -chain of complement C5 by C5 convertases during the complement activation. C5a attracts polymorphonuclear leukocytes (PMNs), monocytes, and many other leuko-

cytes via the C5a receptor. In addition to this, C5a stimulates PMNs via the C5a receptor to release granule contents and to produce radical oxygen species.¹ C5a also promotes monocytes/macrophages via the C5a receptor to synthesize interleukin (IL)-1, IL-6, and several other cytokines.² The C5a receptor gene, which is located on chromosome 19 q13.3-13.4 (from the Genome Database), is also expressed in nonmyeloid cells such as astrocytes and hepatocytes at least under inflamed conditions. Hepatocyte C5a receptor would participate in the acute phase protein synthesis.²

Besides, we have realized that the S19 ribosomal protein (RP S19) dimer attracts monocytes via the C5a receptor as in the case of C5a.³ RP S19 is a component of the small subunit of ribosome, being composed of 145 amino acid residues. RP S19 is intermolecularly cross-linked by a transglutaminase-catalyzed reaction^{4,5} during apoptotic process and the dimer is liberated from the apoptotic cells.^{6,7} The RP S19 dimer attracts monocytes/macrophages and promotes them to phagocytically clear the apoptotic cells from which the chemoattractant molecule has been originated. In contrast to this, the RP S19 dimer antagonizes the C5a receptor-mediated chemotaxis of PMNs.³

Whereas a calculated homology in the amino acid sequence between C5a and RP S19 is only 4%, C5a and the RP S19 dimer activate monocyte C5a receptor by the same interaction mechanism. The C5a receptor, composed of 350 amino acid residues, is a member of the G-protein-coupled 7 transmembrane protein receptor family. The ligand-receptor interaction between C5a or the RP S19 dimer and C5a receptor is a two-step binding process.⁸ In the first binding, a basic cluster of the ligand molecules; a cluster three-dimensionally formed by His15, Arg46, and Lys49 of C5a, or a cluster with the Lys41-His42-Lys43 tandem of RP S19, seems to bind to the amino-terminal acidic moiety of C5a receptor, which is composed of several Asp residues and two sulfated

A.S. and L.S. contributed equally to this work.

Accepted for publication November 21, 2003.

Address reprint requests to Tetsuro Yamamoto, Department of Molecular Pathology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. E-mail: tetsu@gpo.kumamoto-u.ac.jp.

Tyr residues. The high-affinity first binding does not activate the receptor, but effectively raises the local concentration of C5a or the RP S19 dimer and thereby promotes the second binding. In the second binding, the carboxyl-terminal -Leu72-Gly73-Arg74-COOH of C5a, or -Leu131-Asp132-Arg133- of RP S19, interacts with the transmembranous helical regions of the receptor. The second binding initiates the chemotactic signaling via a trimeric G-protein complex.^{8,9}

In our research on the monocyte chemotactic RP S19 dimer, we have prepared various mutants as well as a wild-type of recombinant RP S19.^{5,7,9} During the preparation of the recombinant proteins, we accidentally found that an *Escherichia coli* protein attracted monocytes via the C5a receptor. It surprised us much, because we had believed that the C5a receptor was a receptor to recognize the intrinsic chemical mediator. Therefore, we purified the *E. coli*-derived chemoattractant and identified it by amino acid sequencing and molecular weight analyses. The chemoattractant was a periplasmic chaperone protein, Skp, which stands for 17-kd protein.¹⁰

Skp was initially reported to be a DNA-binding protein of *E. coli*,¹¹ then predicted as an outer membrane protein named OmpH,¹² and finally identified to be a periplasmic protein present between the inner and outer membranes. Skp is now thought to be a molecular chaperone required for the formation of soluble periplasmic intermediates of outer membrane proteins in gram-negative bacteria.¹⁰ It is totally unclear whether a common molecular mechanism is present between the two functions of Skp, which aids the protein folding and activates the C5a receptor.

In the current study, we then examined whether the third ligand of C5a receptor, Skp, shares the ligand-binding sites of C5a receptor with the intrinsic factors, C5a and the RP S19 dimer. We also attempted to estimate the functional significance of Skp in the total leukocyte chemotactic capacity of the *E. coli* extract by means of immunoabsorption with anti-Skp antibody beads because this seems to be the first report on the leukocyte chemotactic capacity of Skp.

Materials and Methods

Animals

Albino-Hartley strain guinea pigs of both sexes (200 to 250 g body weight) and New Zealand White male rabbits (~2.5 kg body weight) were used. The animal experiments were performed under the control of the Animal Experiment Committee of Kumamoto University.

Reagents and Others

RPMI 1640 medium and Hanks' balanced salt solution were purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum was a product of GIBCO BRC (Paisley, Scotland). Mono-Poly resolving medium was a product of Flow Laboratory (Herts, UK). Bovine serum albumin, formyl-Met-Leu-Phe (f-MLF), and C5a were purchased from Sigma Chemical Co. (St. Louis, MO). [¹²⁵I]-

Bolton-Hunter-labeled C5a was a product of New England Nuclear (Boston, MA). A multiwell chamber for chemotaxis assay was obtained from Neuro Probe (Bethesda, MD). Nuclepore filters were purchased from Nuclepore (Pleasant, CA). *E. coli* strain JM109 competent cells were purchased from TaKaRa Biomedicals (Otsu, Japan). NMePhe-Lys-Pro-dCha-dCha-dArg, which had been synthesized as described previously,¹³ was a kind gift from Dr. M. Mizuno of the Third Department of Internal Medicine, Nagoya University, Nagoya, Japan. Ac-Val-Lys-Leu-Ala-Lys-His-Lys-Glu-Leu-Ala-Pro-Tyr-Asp-Glu (Ac-VKLAKHKELAPYDE) and Ac-Leu-Lys-Lys-Ser-Gly-Lys-Leu-Lys-Val-Pro-Glu-Trp-Val-Glu (Ac-LKKS-GKLKVPEWVD) were synthesized as described previously.⁹ Freund's complete adjuvant was purchased from Difco Laboratories (Detroit, MI). Immobilon transfer membrane was a product of Millipore (Bedford, MA). Horse-radish peroxidase-conjugated anti-rabbit IgG goat IgG were a product of Molecular Probes (Eugene, OR). Block-ace was a product of Dainippon Pharmaceutical (Osaka, Japan). The ECL Plus Western blotting detection system was purchased from Amersham Bioscience (Piscataway, NJ). All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) or from Wako Pure Chemicals (Osaka, Japan) unless otherwise specified.

Preparation of RP S19 Dimer

A recombinant RP S19 was prepared in an *E. coli* transgene expression system as described previously.³ RP S19 was dimerized by treatment with type II transglutaminase, and the RP S19 dimer was then separated by immunoaffinity column chromatography with anti-isopeptide bond monoclonal antibody beads as described previously.⁷ In the current study, the protein concentration was determined by the absorbance at 280 nm under the assumption that the absorbance unit 1.0 was equivalent to 1 mg/ml.

Preparation of *E. coli* Extracts

DE3 *E. coli* cells were precultured in 40 ml of Lenox broth (LB) medium (10 g/L bacto tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) overnight at 37°C. The precultured *E. coli* was suspended into 1 L of LB medium, and further cultured overnight at 37°C. After washing by centrifugation for 10 minutes at 5,000 × *g* at 4°C, the DE3 cells were resuspended in 50 mmol/L of Tris-HCl buffer containing 2 mmol/L of ethylenediaminetetraacetic acid (pH 8.0). The DE3 cells were lysed with 100 mg/ml of lysozyme and 0.1% Triton X-100 for 15 minutes at 30°C followed by sonication with 10 15-second pulses at a high-output setting. The bacterial cell lysate was centrifuged at 18,000 × *g* for 30 minutes, and the supernatant was used as *E. coli* extract. The *E. coli* extract was usually used for the purification of the bacterial leukocyte chemotactic factor. Aliquots of four different batches of the *E. coli* extract were dialyzed against phosphate-buffered saline (PBS, pH 7.4) with a molecular cut 3500 membrane, and

were used for Western blotting and immunoadsorption analyses described below.

Purification of Bacterial Leukocyte Chemotactic Factor

The bacterial extract was dialyzed against 10 mmol/L of phosphate buffer (pH 6.0) containing 100 mmol/L NaCl overnight at 4°C, and was centrifuged at $20,000 \times g$ for 30 minutes at 4°C. The supernatant was applied to a CM-Toyopearl 650 mol/L column ($\Phi 16 \times 50$ mm; bed volume, 10 ml) equilibrated with the dialysis buffer. After extensive washing of the column with the equilibration buffer, the leukocyte chemotactic factor was eluted by a stepwise change of NaCl concentration to 1 mol/L in the same buffer. The eluate containing the chemotactic factor was diluted to adjust the NaCl concentration to 100 mmol/L with 10 mmol/L of phosphate buffer (pH 6.0), and applied to high performance liquid chromatography (HPLC) with a SP-5PW column (TSK gel; Tosoh, Tokyo, Japan) ($\Phi 7.5 \times 75$ mm; bed volume, 3.3 ml) equilibrated with 10 mmol/L of phosphate buffer (pH 6.0) containing 100 mmol/L of NaCl. After washing the column with the equilibration buffer, binding proteins were eluted from the column by a gradient change of NaCl concentration up to 1 mol/L. The fraction containing the chemotactic factor was mixed with 10% trifluoroacetic acid and neat acetonitrile at the final concentrations of 0.1% and 5%, respectively, and applied to reverse-phase HPLC with a C4 column (Cosmosil 5C4-AR-300, Nacalai) ($\Phi 4.6 \times 150$ mm; bed volume, 2.5 ml) equilibrated with 0.1% trifluoroacetic acid containing 5% acetonitrile. After washing the column with the equilibration buffer, binding proteins were eluted from the column by a gradient change of acetonitrile from 5 to 95% in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/minute. The protein concentration was determined by the absorbance at 280 nm under the assumption that 1.0 absorbance unit was equivalent to 1 mg protein/ml.

NH₂-Terminal Amino Acid Sequence Analysis

Limited NH₂-terminal amino acid sequencing was performed in duplicate for the initial 20 cycles with a protein sequencer (477A, Applied Biosystems, Tokyo, Japan), equipped with a phenylthiohydantoin-derivative analyzer (120A, Applied Biosystems), according to the instrument manual as described previously.⁴ The homology search for the amino acid sequence was performed using the DBGET integrated data base retrieval system, Swiss-Prot.

Mass Spectrometric Analysis

Mass spectrometric analysis was performed by the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) method with a reflex type mass spectrometer (Bruker Reflex, Billerica, MA). As a molecular mass control, horse myoglobin was used.

Preparations of Rabbit Anti-Skp IgG Antibodies and Immunoabsorbent Gel (Antibody Beads)

Antiserum to Skp was raised in rabbits by multiple intradermal injections of the purified Skp emulsified with Freund's complete adjuvant. At the initial sensitization, 90 μ g of the protein were injected per rabbit, and then 45 μ g were, respectively, given at second and third sensitization in a week intervals. The IgG fraction was prepared from the antiserum by ammonium sulfate precipitation at 40% saturation followed by gel permeation HPLC with a preparative 3000 SWG column ($\Phi 21.5$ mm $\times$ 60 cm; bed volume, 220 ml). An aliquot of the IgG antibodies was used for the immunoblotting analysis.

An aliquot of the anti-Skp IgG antibodies was covalently coupled by succinylation to *N*-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) in a 0.2-mol/L NaHCO₃ buffer containing 0.5 mol/L NaCl (pH 8.3). The remaining uncoupled *N*-hydroxysuccinimide ester groups on the gel beads were blocked with monoethanolamine. Control rabbit IgG beads were prepared in the same process. These IgG beads were used for the immunoadsorption experiments.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

Electrophoresis was performed on a vertical slab gel of 15% polyacrylamide according to the method of Laemmli.¹⁴ The sample was boiled for 2 to 5 minutes in the presence of SDS and then applied to the gel. After electrophoresis at 20 mA for 40 minutes, the gel was stained with Coomassie brilliant blue or was used for immunoblotting.

Transfer of proteins from the SDS-PAGE gel to a membrane was performed electrophoretically according to the method of Kyhse-Andersen¹⁵ with some modifications using a semi-dry electroblotter (Sartorius) for 90 minutes at 22°C with an electric current of 1 mA/cm² slab gel. As the transfer membrane and the transfer buffer, Immobilon transfer membrane (polyvinylidene difluoride membrane) and Tris-glycine buffer (pH 9.3) containing 5% methanol were used, respectively. After the transfer, the membrane was treated with a blocking reagent, Blockace (4%), for 30 minutes at 22°C. The membrane was subjected to immunoreactions. The first reaction was performed with the anti-Skp rabbit IgG in PBS containing Tween 20 (PBS/Tween) at 0.03% for 1 hour at 22°C. After washing with 0.3% PBS/Tween, the second reaction was performed with the horseradish peroxidase-conjugated anti-rabbit IgG goat IgG in 0.03% PBS/Tween for 1 hour at 22°C. After washing with 0.3% PBS/Tween, the enhanced chemiluminescence (ECL) reaction was performed on the membrane with the ECL Plus Western blotting detection system.

Immunoabsorption with Antibody Beads

For an immunoabsorption experiment with the anti-Skp rabbit IgG beads, a batch-wise method was used. Briefly, the Skp-rich SP-5PW column chromatography fraction or the *E. coli* extract was incubated with the anti-Skp IgG beads at an equal volume for 60 minutes at 4°C with continuous shaking. After centrifugation at $10,000 \times g$ for 20 minutes at 4°C, the supernatant was recovered and monocyte chemotactic activity, polarization activity, and leukocyte infiltration induction capacity remaining in it was measured as described below.

Leukocyte Chemotaxis Assay and Morphological Polarization Assay

Mononuclear cells and PMNs were isolated from heparinized human venous blood of healthy donors according to the method of Fernandez and colleagues¹⁶ as described previously.¹⁷ The mononuclear cell fraction contained monocytes at a ratio of ~20%, and the PMN fraction was composed of PMNs more than 95%. The mononuclear cells and PMNs were, respectively, suspended at a cell density of 1×10^6 cells/ml in RPMI 1640 containing 10% fetal bovine serum, and in Hanks' balanced salt solution containing 2% bovine serum albumin for the multiwell chamber assay or for the morphological polarization assay.

The multiwell chamber assay was performed according to the method of Falk and colleagues¹⁸ using a Nucleopore filter with a pore size of 5 μ m for monocytes and of 3 μ m for PMNs as described previously.¹⁷ After incubation for 90 minutes, each membrane was separated, fixed with methanol, and stained with Giemsa solution. The total number of monocytes or of PMNs migrated beyond the lower surface of the membrane was counted in five microscopic high-power fields. The results are expressed as the number of migrated monocytes or PMNs.

The morphological polarization assay was performed according to the method of Cianciolo and Snyderman¹⁹ as described previously.¹⁷ Briefly, the mononuclear cell suspension was incubated with each sample for 10 minutes at 37°C in a polypropylene tube. Immediately after the incubation, 1 ml of cold paraformaldehyde (8% w/v) buffered with 0.1 mol/L of phosphate (pH 7.2) was added to fix the cells. The fixed cells were washed twice with PBS and were stained cytochemically for the nonspecific esterase with α -naphthyl acetate as the substrate for 50 minutes at 37°C to identify monocytes in the mononuclear cells. The number of polarized and nonpolarized cells of monocytes was counted at least until 100 using an inverted microscope (IMT-2; Olympus Optical Co., Tokyo, Japan). The activity of the sample was expressed as the percentage of the monocyte number with polarized morphology against the total number of monocytes counted. Checkerboard analysis of Skp was performed by the method of Zigmond and Hirsch²⁰ using the multiwell chamber assay as described previously.⁴ The samples used for these assays had been equilibrated with PBS by dialysis or by dilution.

Histological Examination

Samples equilibrated with PBS were injected intradermally into guinea pigs at 0.1 ml/site with 27-gauge needles. In each guinea pig, 12-hour and 6-hour lesions were evoked with each sample at several different concentrations. The animals were exsanguinated under ether anesthesia, and then the skin lesions were immediately resected and fixed in 10% formalin. Paraffin sections were prepared for the center area of the lesions at 4 μ m thickness and stained with hematoxylin and eosin in the usual way.

Competitive Binding Assay with Radiolabeled C5a

A PMN (10^7 cells/ml) suspension in Hanks' balanced salt solution containing 10 mmol/L HEPES buffer and 2% bovine serum albumin was divided into 100- μ l volume aliquots in a 96-well plate and mixed with 100 μ l of various concentrations of unlabeled C5a, unlabeled Skp, or unlabeled f-MLF in the same buffer as used for the PMN suspension, respectively. After being kept for 30 minutes at 4°C, [¹²⁵I]-C5a (10 μ l) was then added to each mixture well at a final concentration of 2×10^{-10} mol/L, and the microplate was further kept for 60 minutes at 4°C. After centrifugation of the microplate at 1000 rpm for 10 minutes at 4°C, the supernatant of each well was discarded. The cell pellet of each well was resuspended into 200 μ l of PBS, and the remaining radioactivity of [¹²⁵I]-C5a was counted with a γ -counter (Hitachi Software Engineering, Yokohama, Japan) for 1 minute. Each experiment was performed by a triplicate manner.

Results

Purification and Amino-Terminal Amino Acid Sequence Analysis of E. coli-Derived Leukocyte Chemotactic Factor

Initially, we recognized the presence of an *E. coli*-derived leukocyte chemotactic factor in a lysate of *E. coli* transformed for the recombinant RP S19 production. Different from RP S19, the bacterial factor exhibited the chemoattracting activity without the treatment with transglutaminase. In the purification steps, therefore, the monocyte chemotactic activity was measured by the multiwell chamber assay to identify the chemotactic factor-rich fraction.

The bacterial chemotactic factor had a basic nature. It was initially separated by a conventional cation-exchange column chromatography using a CM-Toyopearl column. The chemotactic factor was then purified by means of HPLC with a SP-5PW column and a C4-reverse-phase column, in this order. The bacterial chemotactic factor was clearly separated from the recombinant wild-type RP S19 in the SP-5PW-HPLC. We also prepared the bacterial chemotactic factor from lysates of nontransformed *E. coli* in the same way. The column chromato-

Figure 1. SDS-PAGE of purified *E. coli*-derived leukocyte chemotactic factor. The major peak fraction of the C4 reverse-phase HPLC was subjected to SDS-PAGE with a 15% polyacrylamide slab gel (**lane 3**), and the protein was stained with Coomassie brilliant blue. The positions corresponding to six marker proteins are shown in **lane 1** (94 K, phosphorylase b; 67 K, bovine serum albumin; 43 K, ovalbumin; 30 K, carbonic anhydrase; 20 K, soybean trypsin inhibitor; 14 K, α -lactalbumin). As an additional marker, S19 ribosomal protein (RP S19) was run in the same gel (**lane 2**).

graphic behaviors of the bacterial factor were the same as in the case of the preparation from the transfected *E. coli*.

The chromatographic pattern of reverse-phase HPLC with the C4 column demonstrated a single major peak of the absorbance at 210 nm at the acetonitrile concentration of 38% (data not shown). When the major peak fraction was analyzed by SDS-PAGE, it demonstrated a single band stained by Coomassie blue dye with an apparent molecular size of 17,000, which is the same size as RP S19 as shown in Figure 1.

Aliquots of the C4-HPLC fraction were also analyzed for the initial 20 amino-terminal amino acids. The amino acid sequence obtained was Ala-Asp-Lys-Ile-Ala-Ile-Val-Asn-Met-Gly-Ser-Leu-Phe-Gln-Gln-Val-Ala-Gln-Lys-Thr. When the database analysis was performed using Swiss-Plot, the 20 amino acid residues were completely overlapped with those of *E. coli* Skp.

An aliquot of the C4-HPLC fraction was further analyzed by the MALDI-TOF mass spectrometric method. As shown in Figure 2, the mass spectrometric pattern demonstrated a major signal with a molecular mass 15,679.86 and a minor signal with a molecular mass 15,888.74. From the heights of these peaks, the ratio of the minor component was only one-twentieth of the major component. From the mass sizes, another small signal with a molecular mass 7833.80 was thought to be the same molecule as that of 15,679.86 but doubly ionized, and a

Figure 2. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of C4 reverse-phase HPLC fraction. Mass sizes 7833.80 and 31,394.84 of a small and a tiny signal are one half and double of the mass size 15,679.86 of the major signal, respectively.

tiny signal with a molecular mass 31,394.84 was thought to be dimer of the 15,679.86 molecule. No other component was seen in the sample. The molecular size of *E. coli* Skp with 141 amino acid residues was calculated to be 15,691.44 by our hand. The difference between 15,691.44 and 15,679.86 is 11.58 (0.074%) that is within the error commonly observed, 0.15%. Therefore, the major component with the apparent molecular mass 15,679.86 was thought to be Skp. From the molecular size and the amino-terminal amino acid sequence, we determined the major purified protein to be *E. coli* Skp.

Dose-Related Leukocyte Chemotactic Capacities of Skp in Vitro

Skp in the C4-HPLC fraction was subjected to the *in vitro* chemotaxis assays with PMNs and monocytes at various concentrations from 10^{-11} mol/L to 10^{-6} mol/L. As shown in Figure 3, Skp attracted PMNs and monocytes making

Figure 3. Leukocyte chemotactic capacities of Skp. Various concentrations from 10^{-6} to 10^{-11} of Skp were subjected to chemotaxis assays using a multiwell chamber and a Nuclepore filter with a pore size of 3 μ m or 5 μ m for PMNs or monocytes, respectively. After a 90-minute incubation, the membrane was stained with Giemsa solution and the number of monocytes (**open circles**) or PMNs (**closed circles**) that migrated beyond the membrane was counted in five high-power fields. The experiments were performed with triplicate samples. Values are expressed as mean $\pm$ SD. The optimum concentration was present at 10^{-9} mol/L in this batch of purified Skp, and in some other batches, it was at 10^{-8} mol/L.

Table 1. Checkerboard Analysis of Monocyte Chemoattraction Induced by Skp*

Upper well	Lower well			
	0	10^{-10} mol/L	10^{-9} mol/L	10^{-8} mol/L
0	14 ± 5	97 ± 2	142 ± 5	165 ± 7
10^{-10} mol/L	15 ± 3	40 ± 5	70 ± 4	95 ± 4
10^{-9} mol/L	26 ± 4	35 ± 3	27 ± 3	66 ± 2
10^{-8} mol/L	29 ± 5	36 ± 5	26 ± 7	21 ± 8

*Skp obtained by the SP-5PW HPLC column chromatography were present in lower and/or upper wells of the multiwell chemotaxis chamber at various concentrations (mol/L). The number of migrated monocytes was shown as mean ± SD. The experiments were performed in triplicate samples.

bell-shape dose-activity relation patterns with the optimum concentration at 10^{-9} mol/L or 10^{-8} mol/L as in the cases of the other chemotactic factors. In the case of the *in vitro* chemotaxis assay, there was no significant difference in terms of the dose dependency and the migrated number of monocytes at the optimum concentration between Skp in the SP-5PW-HPLC fraction and that in the C4-HPLC fraction (data not shown).

Checkerboard Analysis of Leukocyte Chemoattraction Induced by Skp

To examine whether the leukocyte migration caused by Skp was chemotaxis or random locomotion, the checkerboard analysis was performed with the multiwell chamber. A typical result is shown in Table 1. Skp attracted monocytes from the upper chamber to the lower chamber only when the concentration of Skp in the lower chamber was higher than that in the upper chamber. This indicates that the effect of Skp to leukocytes is chemotaxis.

Preparation of Monospecific Antibodies against Skp

Anti-Skp antibodies were raised in rabbits, and the IgG fraction was prepared as described in Materials and Methods. Monospecificity of the antibodies was examined with three different batches of the *E. coli* extract. As shown in Figure 4, the antibodies recognized only a single molecule in these *E. coli* extracts on the immunoblotting membrane. This indicates the monospecificity of the antibodies to Skp.

Immunoabsorption of Leukocyte Chemoattraction Capacity of *E. coli* Extracts

The absorbing capacity of the anti-Skp IgG beads was initially examined with Skp in the SP-5PW HPLC fraction. In this experiment, the change of monocyte chemotactic capacity in the fraction by the immunoabsorption was examined with the multiwell chamber assay. As shown in Figure 5, the antibody beads but not the control beads absorbed almost all of the monocyte chemotactic capacity in the Skp-rich fraction.

Figure 4. Monospecific recognition of Skp in *E. coli* extract with anti-Skp rabbit IgG antibodies in immunoblotting analysis. Two sets of three different batches of the *E. coli* extract (I, II, and III) were subjected at a volume of 10 μ l to SDS-PAGE with 15% polyacrylamide slab gels. **A:** One of the gels was stained with Coomassie brilliant blue. **Lane M** demonstrates molecular size marker proteins pre-conjugated with a blue dye (214 K, myosin; 118 K, α -galactosidase; 92.0 K, bovine serum albumin; 52.2 K, ovalbumin; 35.7 K, carbonic anhydrase; 28.9 K, soybean trypsin inhibitor; 20.8 K, lysozyme). **B:** The proteins of the other gel were electrophoretically transferred to a polyvinylidene difluoride membrane. Two-step immunoreactions were then performed with anti-Skp rabbit IgG for the first reaction and with horseradish peroxidase-conjugated anti-rabbit IgG goat IgG for the second reaction. The horseradish peroxidase activity was finally visualized by an enhanced chemiluminescence reaction.

In the next experiment, three different batches of the *E. coli* extract were subjected to the immunoabsorption experiment to estimate the contribution of Skp in the whole leukocyte chemotactic capacity of the extract to monocytes and to PMNs. In this experiment, the morphological polarization assay was used instead of the multiwell chamber assay to measure the chemotactic capacity in these extracts. It is because that in a crude sample,

Figure 5. Immunoabsorption of monocyte chemotactic activity of Skp with anti-Skp antibody beads. A 45- μ l aliquot of the Skp-rich fraction in the SP-5PW column chromatography, which had been adjusted to 3×10^{-6} mol/L Skp in phosphate-buffered saline (PBS), was treated with 45 μ l of anti-Skp IgG beads (open circles) or with 45 μ l of control rabbit IgG beads (closed circles) for 60 minutes at 4°C, and the supernatant was recovered. Remaining monocyte chemotactic activity in the supernatant was then measured in a serial dilution by the multiwell chamber assay. The filled squares denote the chemotactic activity of the original Skp-rich fraction. The open column denotes the migrated monocyte number to PBS. The experiments were performed in triplicate samples. Values are expressed as mean ± SD.

Figure 6. Immunological estimation of functional significance of Skp in total leukocyte chemotactic capacity of *E. coli* extract. A 200- μ l aliquot of an *E. coli* extract was treated with 200 μ l of anti-Skp IgG beads (open circles) or with 200 μ l of control rabbit IgG beads (filled circles) for 60 minutes at 4°C, and the supernatant was recovered. Remaining chemotactic activity to monocytes (A) or to PMNs (B) in the supernatant was then measured in a serial dilution by the morphological polarization assay. The filled squares denote the chemotactic activity of the original Skp-rich fraction. The open columns denote the migrated monocyte number to PBS. The experiments were performed in triplicate samples. Values are expressed as mean $\pm$ SD

chemotactic or chemokinetic factors might be generated during the assay period and that the polarization assay is quick enough to prevent such a secondary reaction. The experimental results were reproducible both in the monocyte and PMN chemotaxis assays, and typical results are shown in Figure 6. Both of the polarization capacities for monocytes and for PMNs in these extracts were significantly reduced by the incubation with the anti-Skp IgG beads. Judging from the dose-response curves after the treatment with the antibody beads or with the control IgG beads, more than 90% of the leukocyte chemotactic capacity in the extracts was removed by the anti-Skp IgG beads. This indicates that Skp encompasses the majority of the leukocyte chemotactic capacity in the *E. coli* extract.

Leukocyte Infiltration Induction Capacity of Skp in Guinea Pig Skin

We next examined the capacity of Skp to induce leukocyte infiltration *in vivo*. In this experiment, Skp in the SP-5PW HPLC fraction was used because the leukocyte infiltration induced with the C4 HPLC fraction was significantly weaker (data not shown), although the chemoattraction capacities *in vitro* were not significantly different between them. To confirm the responsibility of Skp in the SP-5PW HPLC fraction on the leukocyte infiltration caused, we combined the immunoabsorption method in this experiment. The Skp fraction pretreated with anti-Skp beads or with control IgG beads was respectively injected into the guinea pig skin. The injected samples had contained 10^{-6} mol/L Skp before the treatment with the immunobeads, respectively. Figure 7 shows typical histological pictures obtained 12 hours after the intradermal injection. A severe leukocyte infiltration with mixed popularity between PMNs and monocytes is observed in the papillary dermis, deep dermis, and suprapanniculus carnosus muscle layer when the Skp fraction was injected after the pretreatment with the control IgG beads. In contrast to this, the same Skp fraction but pretreated with the anti-Skp beads did not induce the leukocyte infiltration. These results indicated that Skp possesses the capacity to recruit PMNs and monocytes to extravascular tissue space from the blood circulation. On the other hand, the f-MLF injection (0.1 ml of 10^{-6} mol/L) did not induce a significant leukocyte infiltration in a wide time period from 6 to 24 hours after the injection (data not shown), while the f-MLF lot injected demonstrated a leukocyte chemotactic activity *in vitro* as strong as the Skp used.

Figure 7. Skp-induced leukocyte infiltration in the guinea pig skin. The Skp-rich fraction of SP-5PW column was adjusted to 10^{-5} mol/L in PBS. Aliquots of 100 μ l were, respectively, incubated with 100 μ l of the anti-Skp antibody beads (B) or of the control IgG (A) overnight at 4°C. After centrifugation at 10,000 rpm for 20 minutes at 4°C, the supernatants were recovered, 10-fold diluted with PBS, and intradermally injected into guinea pigs. Skin specimens were obtained 12 hours after the intradermal injection. Paraffin sections of the specimen were stained with H&E.

Figure 8. Cross desensitization between C5a and Skp. **A:** Preventive effect of pretreatment of indicator cells with Skp on C5a-induced leukocyte chemotaxis. The monocytes were pretreated with various concentrations of Skp for 10 minutes at 37°C, and used for the chemotaxis assay with C5a or with f-MLF (final concentration, 10^{-9} mol/L) as the chemoattractant. The **filled** and **grayed columns** denote the chemotactic responses to C5a and to f-MLF, respectively. **B:** Vice versa, preventive effect of pretreatment of indicator cells with C5a on Skp-induced monocyte chemotaxis was examined in the same way as **A**. The **filled** and **grayed columns** denote the chemotactic responses to Skp and to f-MLF, respectively. Three sets of experiments were performed. The number of migrated monocytes is expressed as mean $\pm$ SD.

Skp-Induced Chemoattraction via C5a Receptor

In the next series of experiments, participation of C5a receptor in the chemotactic response of leukocytes to Skp was studied *in vitro* by the use of monocytes as the indicator cells. In the experiments, the prophylactic inhibition or antagonizing method was used.

Cross Desensitization between C5a and Skp

Inhibition of C5a-Induced Chemotaxis by Pretreatment of Monocytes with Skp or Vice Versa

To briefly examine whether Skp shares the ligand-binding receptor regions with the intrinsic chemical mediators, the indicator monocytes were treated with Skp or with C5a just before the chemotaxis assay to C5a or to Skp, respectively. As shown in Figure 8A, the chemotaxis of the monocytes pretreated with Skp to C5a significantly decreased depending on the concentration of the pretreated Skp. The same result was observed in the reverse combination as shown in Figure 8B. The chemotaxis of the monocytes pretreated with C5a to Skp significantly decreased depending on the concentration of the pretreated C5a. In contrast to these, the chemotaxis to f-MLF was not affected by the pretreatment with either Skp or C5a (Figure 8, A and B). The cross desensitization between C5a and Skp suggested that Skp would attract leukocytes via C5a receptor.

Competition of Receptor Binding between Radiolabeled C5a and Skp on PMNs

The C5a receptor-dependent leukocyte chemoattraction by Skp also was examined by the competitive binding assay using [^{125} I]-C5a unlabeled (cold) C5a and unlabeled Skp. As shown in Figure 9, the binding of the radiolabeled C5a to PMNs was inhibited by the presence of Skp in a dose-dependent manner similar to the cold C5a. f-MLF did not interrupt the binding of radiolabeled C5a (data not shown). These results demonstrated that

Figure 9. Competition of receptor binding between radiolabeled C5a and Skp on PMNs. Cells (10^6 cells/200 μ L) were incubated with various concentrations of unlabeled C5a (**filled circles**), unlabeled Skp (**open circles**), or the vehicle PBS (**open column**) for 30 minutes at 4°C, then [^{125} I]-C5a (10 μ L) was added at a final concentration of 2×10^{-10} mol/L. After another incubation for 60 minutes at 4°C, radioactivity of [^{125} I]-C5a that bound to PMNs was measured. Each experiment was performed by a triplicate manner.

Skp binds to the C5a receptor on PMNs. This joins the cross desensitization between C5a and Skp in indicating that Skp attracts leukocytes via C5a receptor.

Inhibition of Skp-Induced Chemotaxis by Pretreatment of Monocytes with Synthetic Peptides Mimicking First Ligand Region of RP S19 Dimer

The RP S19 dimer as well as C5a activates C5a receptor by the two-step binding mechanism, and C5a and the RP S19 dimer share two receptor regions for the ligand binding.^{8,9} Pretreatment of monocytes with the analogue peptide having the same amino acid sequence as the first ligand moiety of the RP S19 dimer prevents the chemotactic migrations to the RP S19 dimer and to C5a but not to f-MLF.⁹ This is an evidence for sharing the first ligand-binding region of C5a receptor between C5a and the RP S19 dimer.

Skp was subjected to the same experiments using the first ligand analogue peptide, Ac-VKLAKHKELAPYDE, of the RP S19 dimer. As shown in Figure 10, the pretreatment with this peptide prevented the monocyte migration to Skp. In contrast to this, Ac-LKKSGKLVPEWVD, an analogue peptide of another region of the RP S19 dimer with the basic nature as the first ligand analogue peptide did not affect the monocyte migration to Skp.

Inhibition of Skp-Induced Chemotaxis with Second Ligand-Binding Site Antagonist

NMePhe-Lys-Pro-dCha-dCha-dArg is an authentic peptide antagonist of C5a receptor. This antagonist inhibits the second binding between C5a receptor and C5a or the RP S19 dimer.⁹ The indicator monocytes were pretreated with this peptide at various concentrations just before the chemotaxis assay for C5a and for Skp. As shown in Figure 11, both of the chemotactic responses to C5a and to Skp were inhibited by this pretreatment. This indicates that Skp attracts monocytes via the same sec-

Figure 10. Inhibition of Skp induced chemotaxis with first ligand analogue peptide, Ac-VKLAKHKELAPYDE, of the S19 ribosomal protein (RP S19) dimer. The indicator monocytes were pretreated with the first ligand analogue peptide at various concentrations from 10^{-6} to 10^{-4} mol/L (dark gray columns), or with an analogue peptide of RP S19 at another region (Ac-LKSGKLVPEWVD), as basic as the first ligand, at a concentration of 10^{-4} mol/L (light gray column), or with the vehicle buffer (filled column) for 10 minutes at 37°C, and used for the chemotaxis assay attracted with Skp at the concentration of 10^{-9} mol/L. The experiments were performed in triplicate samples. Values are expressed as mean $\pm$ SD.

ond ligand-binding site of C5a receptor as in the case of C5a. These results indicate that Skp shares the first and second ligand-binding sites of C5a receptor with C5a and the RP S19 dimer.

Discussion

In the current study, the Skp preparations all demonstrated the optimum leukocyte chemotactic activity at concentrations between 10^{-9} mol/L and 10^{-8} mol/L. A possibility of contamination of a small molecular size chemotactic factor such as f-MLF in the preparations was neglected by the MALDI-TOF mass spectrometric analysis. The mass spectrometric analysis revealed a minor contamination of a macromolecule with the molecular mass 15,888.74. The concentration of the contaminant was approximately one-twentieth of that of Skp in the preparation. Although we have not identified the contaminated molecule, we assumed that it could not contribute to the chemotactic capacity of the Skp preparations. The final concentrations of the contaminant in the chemotaxis assay samples were calculated to be 5×10^{-10} mol/L and 5×10^{-11} mol/L that must be too low to cause the maximum leukocyte chemotaxis.

The present study demonstrates that Skp is a chemotactic ligand of leukocyte C5a receptor. Skp is the third ligand of C5a receptor discovered so far. It surprises us, because the other two ligands are intrinsic chemical mediators; C5a is a well-known chemical mediator of acute inflammation,¹ and the RP S19 dimer has been demonstrated to be a chemical mediator involved in the phagocytic clearance of apoptotic cells.^{6,7} Because Skp is widely distributed in gram-negative bacterial species, the recognition of Skp by leukocytes would be of benefit to the innate immunity against gram-negative bacterial pathogens. In terms of the biological significance, the recognition of three different ligands by a single leukocytic receptor, C5a receptor, would be understood from

Figure 11. Prevention of Skp-induced chemotaxis with second ligand-binding site antagonist of C5a receptor. The indicator monocytes were pretreated with various concentrations of an authentic C5a receptor antagonist (NMePhe-Lys-Pro-dCha-dArg), which binds to the second ligand-binding site, for 10 minutes at 37°C, and used for the chemotaxis assay with C5a or with Skp (final concentrations, 10^{-9} mol/L) as the chemoattractant. As the source of Skp, the SP-5PW HPLC fraction was used. The filled and open circles denote the chemotactic responses to C5a and to Skp, respectively. The experiments were performed with triplicate samples. The number of migrated monocytes is expressed as mean $\pm$ SD.

the aspect of host defense, because all of these ligands indicate the presence and location of foreign or abnormal cells that should be cleared by phagocytic leukocytes as soon as possible. Because Skp is a periplasmic protein, it would not be spontaneously liberated from the bacterial cell wall, but Skp would leak out when the outer membrane is injured. It is known that bactericidal/permeability-increasing protein²¹ and lysozyme in the leukocytic granules are capable of destroying the outer membrane of gram-negative bacteria.

Involvement of bacteria-derived N-formylpeptides such as f-MLF to initiate the leukocyte infiltration against invading bacteria is occasionally stated. On the other hand, Skp was the major leukocyte chemotactic factor in the extract of *E. coli* in our current study (Figure 6). In the preparation process of the extract, we used the dialysis to remove Triton X-100 and ethylenediaminetetraacetic acid that affect the chemotactic response of leukocytes. It is a possibility that N-formylpeptides with small molecular sizes were lost from the extract during the dialysis with a membrane of molecular cut at 3500. However, it was our current observation that the capacity of f-MLF to induce leukocyte infiltration *in vivo* was much weaker than that to attract leukocytes *in vitro*. It is probably because of a rapid disappearance of f-MLF without formation of a stable concentration gradient in the interstitial tissue because of the small molecular size and lack of the cationic moiety that binds to glycosaminoglycans, whereas these characteristics of f-MLF would be an advantage to leukocytes to receive a rapid signal because of a high diffusion rate of f-MLF. It is, therefore, our assumption that N-formylpeptides mainly play a role at early stage in the leukocyte response to the bacterial invasion. In a later phase, Skp leaked from the bacteria and C5a generated in the complement activation on the bacterial cell wall would form more stable concentration gradients binding to negatively charged glycosaminoglycans, which result in a steadier leukocyte chemotactic response. This is

consistent with our current observation that Skp induced a strong leukocyte infiltration when injected into the guinea pig skin (Figure 7). A combined use of N-formylpeptide receptors (FPRs) and C5a receptor to recognize the different bacterial products would be important for phagocytic leukocytes playing roles in the innate immunity, at least against gram-negative bacteria. This may explain, at least partly, why the C5a receptor knockout mouse exhibits an impaired mucosal defense against gram-negative bacteria such as *Pseudomonas aeruginosa*.²²

Among C5a, RP S19, and Skp, there is no relationship in terms of the molecular family, and homologies in the overall amino acid sequence are indeed low. Therefore, we should assume the local structures that bind to C5a receptor would be resembled among these molecules. The RP S19 dimer as well as C5a activates C5a receptor by the two-step binding mechanism.^{8,9} We have identified the ligand sites of the RP S19 dimer to C5a receptor; the first ligand site is a basic cluster region including -Lys41-His42-Lys43- and second ligand site is the -Leu131-Asp132-Arg133- moiety.⁹ The analogue peptide of the first ligand site of RP S19, Ac-VKLAKHKELAPYDE, competed with Skp in the chemotactic C5a receptor activation (Figure 10), in addition to this, the authentic antagonist to the second ligand-binding site of C5a receptor inhibited the monocyte chemotactic response to Skp as in the case the response to C5a (Figure 11). For those reasons, we assume that Skp activates C5a receptor by the two-step binding mechanism as in the cases of C5a and the RP S19 dimer. To identify the first and second ligand sites on the Skp molecule, chemotaxis experiments with mutated recombinant Skp proteins and with synthetic analogue peptides, as were done for the RP S19 dimer,⁹ are needed. This study is now in progress.

Acknowledgments

We thank the members of our laboratory especially Miss T. Kubo and Miss T. Matsuda for their technical assistance with the histological preparations and Dr. H. Nonaka for his kind advice about our computer work; and Dr. N. Takamune of the Department of Pharmaceutical Biochemistry, Kumamoto University, for his mass spectrometric analysis of the Skp preparation.

References

- Vogt W: Anaphylatoxins: possible roles in disease. *Complement* 1986, 3:177-188
- Schieferdecker HL, Schlaf G, Jungermann K, Gotze O: Functions of anaphylatoxin C5a in rat liver: direct and indirect actions on non-parenchymal and parenchymal cells. *Int Immunopharmacol* 2001, 1:469-481
- Nishiura H, Shibuya Y, Yamamoto T: S19 ribosomal protein cross-linked dimer causes monocyte-predominant infiltration by means of molecular mimicry to complement C5a. *Lab Invest* 1998, 78:1615-1623
- Nishiura H, Shibuya Y, Matsubara S, Tanase S, Kambara T, Yamamoto T: Monocyte chemotactic factor in rheumatoid arthritis synovial tissue: probably a cross-linked derivative of S19 ribosomal protein. *J Biol Chem* 1996, 271:878-882
- Nishiura H, Tanase S, Shibuya Y, Nishimura T, Yamamoto T: Determination of the cross-linked residues in homo-dimerization of S19 ribosomal protein concomitant with exhibition of monocyte chemotactic activity. *Lab Invest* 1999, 79:915-923
- Horino K, Nishiura H, Ohsako T, Shibuya Y, Hiraoka T, Kitamura N, Yamamoto T: A monocyte chemotactic factor, S19 ribosomal protein dimer, in phagocytic clearance of apoptotic cells. *Lab Invest* 1998, 78:603-617
- Nishimura T, Horino K, Nishiura H, Shibuya Y, Hiraoka T, Tanase S, Yamamoto T: Apoptotic cells of an epithelial cell line, AsPC-1, release monocyte chemotactic S19 ribosomal protein dimer. *J Biochem* 2001, 129:445-454
- Siciliano SJ, Rollins TE, DeMartino J, Konteatis ZD, Malkowitz L, Van Riper G, Bondy S, Rosen H, Springer MS: Two-site binding of C5a by its receptor: an alternative binding paradigm for G protein-coupled receptors. *Proc Natl Acad Sci USA* 1994, 91:1214-1218
- Shibuya Y, Shiokawa M, Nishiura H, Nishimura T, Nishimura T, Nishino N, Okabe H, Takagi K, Yamamoto T: Identification of receptor binding sites of monocyte chemotactic S19 ribosomal protein dimer. *Am J Pathol* 2001, 159:2293-2301
- Schafer U, Beck K, Muller M: Skp, a molecular chaperone of Gram-negative bacteria, is required for the formation of soluble periplasmic intermediates of outer membrane proteins. *J Biol Chem* 1999, 274:24567-24574
- Holck A, Kleppe K: Cloning and sequencing of the gene for the DNA-binding 17K protein of *Escherichia coli*. *Gene* 1988, 67:117-124
- Koski P, Rhen M, Kantele J, Vaara M: Isolation, cloning, and primary structure of a cationic 16-kDa outer membrane protein of *Salmonella typhimurium*. *J Biol Chem* 1989, 264:18973-18980
- Konteatis ZD, Siciliano SJ, Van Riper G, Molineaux CJ, Pandya S, Fischer P, Rosen H, Mumford RA, Springer MS: Development of C5a receptor antagonists: differential loss of functional responses. *J Immunol* 1994, 153:4200-4205
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227:680-685
- Kyhse-Andersen J: Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 1984, 10:203-209
- Fernandez HN, Henson PM, Otani A, Hugli TE: Chemotactic response to human C3a and C5a anaphylatoxins. I. Evaluation of C3a and C5a leukotaxis in vitro and under stimulated in vivo conditions. *J Immunol* 1978, 120:109-115
- Matsubara S, Yamamoto T, Tsuruta T, Takagi K, Kambara T: Complement C4-derived monocyte-directed chemotaxis-inhibitory factor: a molecular mechanism to cause polymorphonuclear leukocyte-predominant infiltration in rheumatoid arthritis synovial cavities. *Am J Pathol* 1991, 138:1279-1291
- Falk W, Goldwin RH, Leonard EJ: A 48 well micro-chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J Immunol Methods* 1980, 33:239-247
- Cianciolo GJ, Snyderman R: Monocyte responsiveness to chemotactic stimuli in a property of subpopulation of cells that can respond to multiple chemoattractants. *J Clin Invest* 1981, 67:60-68
- Zigmond SH, Hirsch JG: Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. *J Exp Med* 1973, 137:387-410
- Elsbach P: The bactericidal/permeability-increasing protein (BPI) in antibacterial host defense. *J Leukoc Biol* 1998, 64:14-18
- Hopken UE, Lu B, Gerard NP, Gerard C: The C5a chemoattractant receptor mediates mucosal defence to infection. *Nature* 1996, 383:86-89