Base of molecular mimicry between human ribosomal protein S19 dimer and human C5a anaphylatoxin

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Short running head: Molecular mimicry between receptor ligands

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Abstract

The crosslinked homodimer of human ribosomal protein S19 (hRP S19) but not hRP S19 monomer shares the hC5a receptor ligation capacity with anaphylatoxin hC5a. The hRP S19 dimer engages hC5a receptor-bearing monocytes in chemotactic movement and secretion as does hC5a. Two submolecular regions essential for the receptor ligation were already identified in hRP S19 as well as in hC5a. Using the tertiary structure data base of an archaeobacterial RP S19 as template, we made a tertiary structure model of hRP S19. The obtained structure was almost entirely α-helical with two short β-sheet regions, and folds a five α-helix bundle organized around a central amphipathic α-helix. While the secondary structure components were similar to those of hC5a, the gross tertiary structure of hRP S19 was loose and the distance between the two receptor binding regions was rather big in comparison to that of hC5a. Anti-recombinant hC5a rabbit antibodies cross-recognized not only the crosslinked hRP S19 dimer but also the guinea pig (gp) RP S19 dimer, however, these antibodies reacted hRP S19 monomer and cross-linked Gln137Asn-hRP S19 mutant dimer at significantly less extents. These antibodies neutralized the monocyte attracting capacity of the hRP S19 dimer *in vitro* and that of the gpRP S19 dimer *in vivo*. We assume that the crosslinkage between Lys122 of one hRP S19 molecule and Gln137 of the other one would assemble the

hC5a-like structure probably providing one of two receptor binding regions by each hRP S19 subunit.

1. Introduction

Ribosomal protein S19 (RP S19) is a component of the small subunit of eukaryotic and archaeobacterial ribosomes. Human (h) RP S19 is a simple protein synthesized with 145 amino acid residues in which no cysteine residue is present. Accumulating evidence indicates an essential role at least of mouse (m) RP S19 and hRP S19 in the ribosomal biogenesis [1-4]. Role of RP S19 in the translation of mRNA by ribosomes seems unclear. When mouse or human cells undergo apoptosis, RP S19 molecules seems to be intermolecularly crosslinked by cytoplasmic transglutaminase (type 2 transglutaminase) in the apoptosis-initiated cells and extracellularly released; and the RP S19 dimer (and oligomers as well) promotes the cells to undergo apoptosis execution on one hand and recruits monocytes/macrophages on the other which in turn scavenges the apoptotic cells [5-9]. Thus the RP S19 dimer synchronizes the apoptosis execution and apoptotic cell scavenge. Interestingly enough, hRP S19 is also present in normal blood as a plasma protein [10]. In an analogical fashion observed in apoptotic cell scavenge, when human blood is coagulated, hRP S19 molecules in plasma is cross-linked by plasma transglutaminase (coagulation factor XIIIa); and the hRP S19 dimer recruits monocytes/macrophages, which infiltrate into the coagulum on one hand and cover the coagulum surface on the other, then the infiltrated

monocytes/macrophages scavenge the coagulum (Ota et al. unpublished data). The intermolecular crosslink that is formed between Lys122 and Gln137 confers on the hRP S19 dimer ligand capacity to the hC5a receptor.[11] Ligation of the C5a receptor by the hRP S19 dimer is the common mechanism in the chemoattraction of mononuclear phagocytes and the apoptosis promotion caused by the RP S19 dimer [8, 9, 12, 13]. The C5a receptor was initially identified as a pan-leukocyte chemotactic receptor [14]. The cells which usually do not express the C5a receptor commonly *de novo* synthesize it after apoptosis initiation [8, 9]. The classical ligand of the C5a receptor is C5a that is liberated from complement C5 by the C5a convertase formed during the complement activation. hC5a is composed of 74 amino acid residues with 3 disulfide bridges.

In this manner, there is no molecular family relationship between RP S19 and C5a, and a calculated homology in the primary structure between these molecules in human is only 4%. In spite of the difference in the overall primary structure, hC5a and the hRP S19 dimer ligate the hC5a receptor in the same two step binding mechanism. At the first step, a basic cluster composed of His15, Arg46 and Lys49 in hC5a or the Lys41-His42-Lys43 sequence in hRP S19 binds to the amino-terminal extracellular portion with acidic nature of the hC5a receptor [14, 15]. The high-affinity first binding does not activate the receptor, but effectively raises the local concentration of these

ligands and thereby promotes second binding. The second step binding to the cylindrical transmembrane portion of the hC5a receptor induces the receptor activation [14, 16]. The second binding site of hC5a is -Leu72-Gly73-Arg74-COOH where amino acid residues of Leu72 and Arg74, and the terminal α-carboxyl group of Arg74 are essential [29, 30]. The second binding site of the hRP S19 dimer is -Leu131-Asp132-Arg133- in hRP S19 where the amino acid residues of –Leu-Xxx-Arg- are present as in the case of hC5a and the β-carboxyl group of Asp132 is thought to play equivalently to the α-carboxyl group of Arg74 in hC5a [13]. These localized homologies explain the molecular mimicry between these molecules in part [17].

The tertiary structure of hC5a has been solved by means of nuclear magnetic resonance spectroscopy [15, 18, 19]. However, the structure of hRP S19 has not yet been reported, although the crystal structure of *Pyrococcus abyssi* RP S19 was recently solved by Gregory et al. [20]. They also reported that *Pyrococcus abyssi* RP S19 shares 36% identity and 57% similarity with hRP S19 in the amino acid sequence. We, therefore, currently predicted the tertiary structure of hRP S19 by means of the template-based protein structure modeling. Although the secondary structure components quite resembled between hC5a and hRP S19, the overall tertiary structure of hRP S19 was much looser than that of hC5a as shown below. Concerning the different

capabilities between hRP S19 monomer and the hRP S19 dimer in ligating the hC5a receptor, we raised antibodies against a recombinant hC5a and against a recombinant hRP S19 in rabbits, and we compared the immunoreactivity to these antibodies between hRP S19 monomer and the hRP S19 dimer. The anti-hC5a antibodies cross-recognized the hRP S19 dimer but practically not hRP S19 monomer *in vitro*, and neutralized the monocyte chemotactic capacity of the guinea pig (gp) RP S19 dimer *in vivo*. It is because that the RP S19 sequence between the human and the guinea pig is completely over-lapped [21]. We now speculate that the presumed hC5a-like tertiary structure would be assembled around the interface of two hRP S19 molecules in association to the inter-molecular single isopeptide bond formation between Lys122 and Gln 137.

2. Materials and Methods

2.1. Animals

Male albino-Hartley strain guinea pigs (body weights of around 400 g) and New Zealand White male rabbits (2kg body weight) were purchased from Kyudo Co. (Kumamoto, Japan). The animal experiments were performed under the control of the Animal Experiment Committee of Kumamoto University.

2.2. Recombinant proteins

A recombinant hRP S19 and a recombinant hC5a were prepared using an *E. coli*

expression system with pET32a vector and Rosseta gami(B) Lys-S as the host bacteria as described previously [22]. The Trx-tag-His-tag-S-tag was proteolytically removed on demand. The crosslinked dimer of the recombinant hRP S19 was prepared with factor XIIIa as described previously [11].

2.3. Template-based protein structure modeling

As the template we used Protein data base (PDB) number 2v7f for *Pyrococcus abyssi* RP S19 reported by Gregory et al. [20]. In the sequence alignment between the archaeobacterial RP S19 and hRP S19, we also utilized the data of them. We performed the modeling using homology modeling function in a molecular design support system driven under Molecular Operating Environment (MOE) prepared by Chemical Computing Group Inc. (Montreal, Canada). For molecular mechanics we used a soft ware package Assisted Model Building and Energy Refinement (AMBER). There are two submolecular regions lacking coordinate data in 2v7f which would be disordered loops. We modeled these parts in two different patterns. In one pattern, we applied the segment match method restricting to the loop regions. In the other, we applied the segment match method including several amino acid residues neighboring these loops which showed large values of B factor. Thus the regions are expanded to 35Val-36Lys-37Thr-disordered loop-44Leu-45Pro-46Glu-47Gln-48Glu, and

78Gly-79Gly-disordered loop-84Gly. In this case, we did not use the coordinate data in 2vf7 on these neighboring residues.

For the modeling of hC5a, we utilized PDB number 1KJS reported by Zhang et al. in the milieu of MOE [15]. We also modeled hRP S19 dimer intermolecularly crosslinked by an isopeptide bond between Lys122 and Gln137 in the milieu of MOE under the assumptions that the gross structure of each hRP S19 subunit was not affected by the isopeptide bond formation but side chains of both hRP S19 subunits at the interface could be shifted according to the least energy condition.

2.4. Preparation of rabbit anti-hC5a antibodies and anti-hRP S19 antibodies

The anti-hC5a antibodies were raised in a New Zealand White mail rabbit. We mixed 500 μl of 1 mg/ml recombinant hC5a in phosphate-buffered saline (PBS) and 500 μl of Freund's complete adjuvant (Sigma-Aldrich St., Louis, MO), made water-in-oil emulsion, and intradermally injected at multiple sites. We boosted 3 times at 2-weeks intervals with 300 μg of hC5a and the complete adjuvant at first time or the incomplete adjuvant at 2nd and 3rd times. One week after the final boosting we took out blood of the animal, and then prepared serum in a usual way. We prepared anti-recombinant hRP S19 rabbit IgG in a similar way as described previously [8]. We separated IgG from the sera using a HiTrap™ Protein-G HP column (Amersham Biosciences KK, Tokyo, Japan) as described

previously [8], and stored them at 4 mg/ml in PBS at -80°C.

2.5. Transformation of HL-60 cells to over-produce either Qln137Asn-hRP S19 or wild type hRP S19

HL-60 cells were maintained in RPMI 1640 medium (Nissui Pharm. Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin (GIBCO BRC, Paisley, Scotland). pCAGGS-IRES neomycin resistant (pCAIN) vector, which bears either wild-type or Gln137Asn mutant haemaglutinin-hRP S19 cDNA was prepared as described previously [8]. Either of these pCAIN vectors was introduced into HL-60 cells by means of electroporation, and transformed HL-60 cells were clones as described previously.

2.6. Induction of apoptosis

Apoptosis of these HL-60 transformed clones was induced with Mn (II) according to the method of Oubrahim et al.[23] as described previously [8]. In brief, the transformed clones of HL-60 (5 x 10^6 cells/10 ml) were respectively cultured in Ø100 mm culture dishes, and treated with 0.5 mM $MnCl_2$. After 18 hours, cells were re-suspended in 100 μl of 10 mM Tris-HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol, and 9M urea (the electrophoresis sample buffer) (Nacalai Tesque, Kyoto, Japan).

2.7. Preparation of plasma and serum samples

Human blood was taken from the peripheral veins of healthy individuals with 1/9 volume of 3.2% citrate-3Na-2H_2O or without any reagent to prepare human plasma or serum, respectively. Guinea pig blood was taken from an ether-anesthetized guinea pig without anti-coagulant by cardiac puncture using a disposable plastic syringe with a 21 gauge needle to prepare guinea pig serum, respectively. For western blotting analysis, the plasma and serum samples were pretreated with dextran sulfate and with acetone (Wako Pure Chemicals, Osaka, Japan), in this order, to remove extra-large molecules and small ions in the samples prepared as described previously [10]. The acetone precipitates were recovered, dissolved into the initial volume of electrophoresis sample buffer, and heat-treated for 20 minutes at 42°C. For monocyte chemotaxis assay, the serum sample was decomplemented by the heat-treatment for 30 minutes at 56°C, and used.

2.8. Double immunodiffusion and western blotting

Immunodiffusion was carried out according to the method of Ouchterlony [24] for 18 hours at 4°C with the use of the rabbit anti-hC5a antiserum and the recombinant hC5a (500 μg/ml). Western blotting analysis was performed as described previously [8]. In brief, the samples were subjected to 12.5% SDS–polyacrylamide gel electrophoresis

(PAGE), and then transferred to Immobilon-P^{SQ} membranes (Millipore, Bedford, MA). After washing, the membrane was treated with 1% BlockAce (Dainippon Pharmaceutical, Suita, Japan), and then reacted with the anti-hRP S19 rabbit IgG (100 ng/ml) or with the anti-hC5a IgG (100 ng/ml) for 1 hours at 22°C. After incubation with HRP-conjugated anti-rabbit IgG goat IgG (5 ng/ml) (Santa Cruz Biotechnology, CA, USA) for 30 minutes at 22°C, the bound HRP was detected using the ECL Plus Western blotting detection system (Amersham Biosciences KK, Tokyo, Japan).

2.9. Chemotaxis assay

Peritoneal exudate macrophages were harvested from guinea pigs 5 days after an injection of liquid paraffin (15 ml) (Kokusan Kagaku, Tokyo, Japan). The peritoneal cells were washed with PBS, resuspended in RPMI1640 medium supplemented with 0.1% bovine serum albumin at a cell density of 1 x 10^6 cells/ml as described previously [25]. Mononuclear cells were isolated from heparinized human venous blood of healthy donors according to the method of Fernandez et al. [26] as described previously [27]. The mononuclear cell fraction contained monocytes at about 20% (data not shown). The mononuclear cells were suspended at a cell density of 1 x 10^6 cells/ml in RPMI 1640 containing 10% FBS for the multiwell chamber assay (Neuro Probe, Bethesda, MD). The multiwell chamber assay was performed according to the method of Falk et al.[28] using

a Nucleopore filter (Nucleopore, Pleasant, CA) with a pore size of 5 μm as described previously [27]. After incubation for 90 minutes, each membrane was separated, fixed with methanol, and stained with Giemsa solution. The total number of monocytes 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.

2.10. Intraperitoneal coagulum resorption model

Guinea pig bloods were mixed either the anti-hC5a rabbit IgG or the FITC-conjugated control rabbit IgG (Sigma-Aldrich St., Louis, MO) at the final concentration of 100 μg/ml, poured a 1 ml aliquot of blood into a sterilized glass cylinder (inside Ø 7 mm and length 7.2 cm), and stood it on the shielded end for 60 minutes at 22°C. We performed a 2.5 cm midline laparotomy in guinea pigs under general anesthesia using pentobarbital. Then we removed the parafilm sheet from the bottom side of the coagulum-contained glass cylinder and inserted two coagula by pushing from the other end with a sterilized glass stick that fit the cylinder inside-caliber. We sutured the abdominal incision and kept the animal at liberty for water and food uptake until the next surgical operation to recover the coagulum.

2.11. Histological examination

Recovered coagula were fixed in 4% paraformaldehyde in PBS, pH7.4 overnight at 4°C.

Paraffin sections were prepared at 4 μm thickness and stained with hematoxylin and eosin.

2.12. Statistical analysis

Statistical analyses were performed using paired or unpaired Student's *t*-tests with Stat-View software (ver. 5.0; SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1. Tertiary structure models of human hRP S19 and comparison with hC5a

Two predicted structures of hRP S19 are shown in Figure 1. In either model, the structure is almost entirely α-helical with two short β-sheet regions, and folds a five α-helix bundle organized around a central amphipathic α-helix. Differences between two models are lengths of the disordered loops. By means of functional analyses, we have previously identified submolecular regions such as heparin-binding region, first step binding site and the second step one to the hC5a receptor, and the switch moiety that confers the antagonist function on the hRP S19 dimer to the neutrophil hC5a receptor [29]. In either model, the heparin-binding region and the first ligation site to the hC5a receptor locate on the first disordered loop, and the second ligation site to the hC5a receptor and the switch moiety locate on the helix 6. Lys122 and Gln137 that are

cross-linked residues locate on β-sheet 2 and on α-helix 6, respectively. At this moment we do not know which model is more reliable.

According to the solution structure of hC5a reported by Zhang et al. [15], we re-modeled it utilizing the same soft ware as used for the hRP S19 modeling. The hC5a model and one of the hRP S19 model (model 1) are comparatively shown in Figure 2. In the case of hC5a, an antiparallel 4-helix bundle (residues 1–63), the four different helical segments (4–12, 18–26, 32–39, 46–63) being stabilized by three disulfide bonds (Cys21-Cys47, Cys22-Cys54, Cys34-Cys55) and connected by loop segments 13–17, 27–33 and 40–45. The 63-residue helix bundle fragment is highly cationic and confers high affinity for the cell surface. The C-terminal residues 69–74 also form a bulky helical turn connected to the 4-helix bundle by a short loop.

Apart from two short β-sheets in hRP S19, the secondary structure components are similar between hC5a and hRP S19. However, the overall tertiary structure of hRP S19 seems loose, which must be a reflection of no disulfide bond within the molecule. The hRP S19 dimer as well as hC5a ligates the hC5a receptor by the two-step binding mechanism as described above. The N-terminal acidic region from Asp10 to Asn21 of the hC5a receptor involves in the first interaction, and extracellular face of helix 5 including Arg206 of the receptor involves in the second interaction [30, 31]. Therefore, the

distance of the first binding site and the second one of the ligand molecule must be limited. From this aspect, the distance between the first binding site and the second one in a single hRP S19 molecule seems too much wide by reference to the hC5a molecule. The first receptor binding site of hC5a was reported to be a basic cluster tertiary assembled by His15, Arg46 and Lys49 residues, and the second binding site of it is the C-terminal region composed of Leu72-Gly73-Arg74 [14, 32, 33]. We measured the distance between Arg46Cα and Gly73Cα to be 22.66 Å. On the other hand, we have identified the first binding site and the second of the hRP S19 dimer to be Lys51-His52-Lys53 and Leu131-Asp132-Arg133 [13]. The distance between His52Cα and Asp132Cα in the present hRP S19 model was 35.71 Å. Therefore, if one assumed that both of the first and second binding sites were provided by a single hRP S19 monomer subset of the crosslinked dimer, the distance between these binding sites would become more than 10 Å bigger than in the case of hC5a. We then modeled the hRP S19 dimer crosslinked by the single isopeptide bond between Lys122 and Gln137 under the assumption that the gross structure of each hRP S19 subunit was not affected by the crosslinkage. We attempted to twist one subunit on the isopeptide bond as the center to minimize the distance between the first receptor binding site on one subunit and the second receptor binding site on the other subunit under the limitation by steric

hindrance among side chains of amino acid residues located at the interface of these subunits. We could arrange the same distance between these receptor binding sites as shown in hC5a (data not shown). We, therefore, speculate that a hC5a-like tertiary structure including the proper topology between the first and second binding sites would be assembled in association to the intermolecular isopeptide bond formation between Lys122 and Gln137. In this case, the first binding site and the second one to the hC5a receptor would be separately provided by each hRP S19 subunit.

The second hC5a receptor binding sites of hC5a and the hRP S19 both form α-helices. It is consistent with information from the hC5a receptor antagonist design mimicking the second binding site of hC5a; antagonism is related to a turn conformation, which can be stabilized in cyclic molecules that are preorganized for receptor binding [34].

When we modeled the hRP S19 dimer crosslinked a single isopeptide bond between Lys122 and Gln137 as described above, we concomitantly observed the locations of the other Lys122 and Gln137. These molecules were separately present at opposite peripheries of the dimer; it was difficult to make the other isopeptide bond between the remained Lys122 and Gln137 without severe bending at several portions of the main chains of hRP S19 subunits. These submolecular locations of the remained Lys122 and Gln137 would explain the oligomer formation of hRP S19 instead of the double bridged

dimer formation.

3.2. Cross immunoreactivity of chemotactic hRP S19 dimer to anti-hC5a polyclonal antibodies

To examine the above assumption we prepared rabbit antibodies against a recombinant hC5a and compared the immunoreactivity to the anti-hC5a antibodies between hRP S19 monomer and the monocyte attracting hRP S19 dimer. We also examined the immunoreactivity to the anti-hC5a antibodies of Gln137Asn-hRP S19 dimer which possesses an incorrect isopeptide bond without the chemoattracting capacity.

As shown in Figure 3A, the antibodies demonstrate a precipitation line against the recombinant hC5a in Ouchterlony's double immunodiffusion. In western blotting, the anti-hC5a rabbit IgG bound to the cross-linked hRP S19 dimer but not to hRP S19 monomer, whereas the anti-hRP S19 rabbit IgG bound to both of the dimer and the monomer (Figure 3B).

When cells are initiated to undergo apoptosis, they generate the chemotactic RP S19 dimer and oligomers, and extracellularly release these molecules [5, 7, 8]. When human or mouse cells had been transformed to over synthesize Gln137Asn-hRP S19 and were initiated to undergo apoptosis, they generate incorrectly crosslinked RP S19 dimer which are incapable of monocyte chemoattraction [9]. In this experiment, we used

HL-60 transformants which over produced either Gln137Asn-hRP S19 or wild type hRP S19. We extracted apoptotic transformed cells and performed the western blotting analysis. As shown in Figure 3C, the anti-hC5a rabbit IgG recognized the wild type hRP S19 dimer but neither the Gln137Asn-hRP S19 dimer nor these monomers practically, whereas the anti-hRP S19 rabbit IgG recognized both of the dimmers and both of the monomers.

hRP S19 is also present in normal blood as a plasma protein, and is cross-linked during blood coagulation by factor XIIIa; and the dimer recruits monocytes to the blood coagulum formed [10]. The recruited monocytes cover the coagulum surface on one hand, penetrate into the coagulum on the other, and eventually clear the coagula phagocytically. We subjected human plasma and serum to the western blotting (Figure 4). The anti-hC5a rabbit IgG recognized only the dimer in serum, whereas the anti-hRP S19 antibodies recognized both of the monomer and the dimer.

In the series of western blotting analyses using the anti-hC5a rabbit IgG and the anti-hRP S19 rabbit IgG, the latter antibodies recognized all of the molecules derived from hRP S19 including Gln137Asn-hRP S19. In contrast to this, the anti-hC5a rabbit IgG recognized only the hRP S19 dimer authentically cross-linked between Lys122 and Gln137 but not the other molecules, indicating that in accompanying to the authentic

crosslinkage the conformation of hRP S19 with the antigen epitopes mimicking hC5a emerges.

Importantly, only the authentically crosslinked hRP S19 dimer is capable of attracting monocytes due to ligating the hC5a receptor as is hC5a. The immunological data indicate that the molecular mimicry of hRP S19 to hC5a emerges only when intermolecularly crosslinked between Lys122 and Gln137.

From the immunological point of view on the above data, we would be capable of expecting two things. One is that the discriminative recognition of the anti-hC5a antibodies to the hRP S19 dimer would be useful to measure the dimer separately from the monomer by means of immunochemistry in future. The other is that the recognition of the anti-hC5a antibodies specific to the authentically cross-linked hRP S19 dimer suggests the neutralizing capacity of the antibodies to the function of the hRP S19 dimer. We currently examined it as follows.

3.3. Neutralization of monocyte chemotactic capacity of gpRP S19 dimer by anti-hC5a antibodies in vitro

We examined the functional neutralization capacity of the anti-hC5a antibodies targeting the RP S19 dimer in human serum or guinea pig serum, respectively. In this experiment, we utilized the monocyte migration assay toward serum in the micro-well

chamber. As shown in Figure 5, the monocyte chemoattracting capacity of human serum or guinea pig serum was significantly suppressed by the presence of the anti-hC5a rabbit IgG. The neutralization capacity of the rabbit IgG is equivalent to that of anti-hRP S19 rabbit IgG.

3.4. Neutralization of monocyte chemotactic capacity of gpRP S19 dimer by anti-hC5a antibodies in vivo

We finally examined the neutralizing capacity of the anti-hC5a rabbit IgG against the gpRP S19 dimer *in vivo*. In this examination, we utilized the coagula resorption model in the guinea pig peritoneal cavity. Guinea pig blood was coagulated in the presence of the anti-hC5a rabbit IgG or FITC-conjugated normal rabbit IgG *in vitro*, and these coagula were inserted intraperitoneally as a set into each guinea pig. Two days later, the green fluorescence in each section of the recovered coagulum was observed by a fluorescence microscopy for negatively selecting the pretreated coagulum with anti-hC5a rabbit IgG (data not shown). The coagulum pretreated with anti-hC5a rabbit IgG was significantly bigger than that pretreated with control rabbit IgG (Figure 6A). Moreover, the number of monocytes/macrophages on the coagulum pretreated with the anti-hC5a rabbit IgG seemed to be smaller than that pretreated with the control rabbit IgG (Figure 6B). We interpret these results to be due to the neutralization of the gpRP

S19 dimer released from the coagulum with the anti-hC5a antibodies (Figure 5).

In plasma and serum, gpC5, the precursor of gpC5a, is present. We do not know the structural homology of C5 or C5a between the human and the guinea pig, because the primary structures of the guinea pig molecules have not yet reported. The maintained neutralization capacity of the anti-hC5a antibodies to the gpRP S19 dimer in serum or released from the coagulum as currently shown suggests a negligible cross-reactivity of the antibodies to gpC5. Indeed, we could not observe an immuneprecipitation line between guinea pig plasma and the anti-hC5a antibodies in double immunodiffusion (data not shown).

The above experiments *in vitro* and *in vivo* substantiated the neutralization capacity of the anti-hC5a antibodies to the function of the gpRP S19 dimer. We now believe that the anti-hC5a antibodies would be a useful experimental tool to examine the extra-ribosomal role of the gpRP S19 dimer in the physiological as well as pathological context *in vivo*.

From the above protein structure modeling and immunological analyzes, we like to conclude that in association to an intermolecular single isopeptide bond formation between Lys122 and Gln137 of RP S19 molecules, a molecular structure that resemble the hC5a receptor binding moiety of hC5a would be assembled and that because of the

submolecular locations of remained Lys122 and Gln137 in the hRP S19 dimer (far from each other), the following transglutaminase-catalyzed reaction forms crosslinked oligomers instead of double bonded hRP S19 dimer.

In the present study, we have focused on the molecular base to explain the similar functions *via* the C5a receptor between hC5a and the hRP S19 dimer; both chemical mediators engage monocytes/macrophages in chemotactic movement, granule release and superoxide generation [12, 35]. On the other hand, there are different or even opposite functions *via* the C5a receptor between hC5a and the hRP S19 dimer; the hRP S19 dimer causes hC5a receptor antagonist effect on neutrophils, and the antagonist effect at low concentrations but the agonist effect at high concentrations on mast cells [12, 35, 36]. Even in the chemotactic and secretary responses of monocytes/macrophages *via* the hC5a receptor, hC5a guides the classical extracellular signal-regulated kinase 1/2 pathway but the hRP S19 dimer guided the alternative p38 mitogen-activated protein kinase pathway [36]. Furthermore, to the apoptosis-initiated cells, the hRP S19 dimer and hC5a promotes and delays the apoptosis execution, respectively, *via* the hC5a receptor [8, 9]. We have functionally demonstrated that the different functions between these hC5a receptor ligands depend on the C-terminal region of hRP S19 with IAGQVAAANKKH sequence, reproducing the functions of the hRP S19 dimer with

hC5a/RPS19 chimera bearing the IAGQVAAANKKH sequence at the end of hC5a body [9, 22, 29, 35, 36]. However, the molecular mechanism how the IAGQVAAANKKH sequence converts the intracellular signal pathways like a molecular switch is still unclear.

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Figure legends

Figure 1. Tertiary structure models of human RP S19. Protein data base number 2v7f for *Pyrococcus abyssi* RP S19 was used as the template for the sequence alignment between the archaeobacterial RP S19 and hRP S19. We performed the modeling using homology modeling function in a molecular design support system driven under Molecular Operating Environment. For molecular mechanics we used a soft ware package Assisted Model Building and Energy Refinement.

Figure 2. Comparative demonstration between human C5a structure and human RP S19 structure. The solution structure of hC5a (MMDB ID: 73492, PDB ID: 1CFA) was re-modeled utilizing Molecular Operating Environment and compared the position of the second binding moiety between hC5a (Leu-Gly-Arg) and hRP S19 (Leu-Asp-Arg).

Figure 3. Selective cross-reaction of anti-human C5a rabbit IgG to cross-linked human RP S19 dimer. (A) 20 μl of the recombinant hC5a and 20 μl of serial dilutions of rabbit anti-hC5a antiserum were apply to Ø 4 mm of center and 6 surrounding wells in 1% agar gel. (B) 10 ng of recombinant hRP S19 monomer and dimer in 12.5% SDS-PAGE were transferred to membrane. The membrane was incubated with the anti-hRP S19 rabbit IgG or the anti-hC5a rabbit IgG, respectively. (C) Cell aliquots (15 μl) of apoptotic wild type and mutant HL-60 cells applied to 12.5% SDS-PAGE were transferred to membrane, and anti-hRP S19 rabbit IgG or the anti-hC5a IgG were incubated,

respectively.

Figure 4. Selective demonstration of human RP S19 dimer in serum with anti-human C5a antibodies in western blotting. Human plasma and serum samples subjected to 12.5% SDS-PAGE were electrophoretically transferred to a membrane and reacted with either anti-hRP S19 rabbit IgG or anti-hC5a rabbit IgG. After incubation with HRP-conjugated anti-rabbit IgG goat IgG, the bound HRP was detected using the ECL Plus Western blotting detection system.

Figure 5. Neutralization of monocyte attracting capacity of human or guinea pig serum with anti-human C5a antibodies. Human (open columns) or guinea pig serum (closed columns) pretreated with either anti-hRP S19 IgG, anti-hC5a IgG or normal rabbit IgG at the final concentration of 5 μg/ml for 15 minutes at 37°C, subjected to the monocyte chemotaxis assay in a multiwell chamber (n=4).

Figure 6. Effect of anti-human C5a antibodies on fate of intraperitoneally inserted guinea pig coagula. (**A**) A coagulum containing the anti-hC5a rabbit IgG and a coagulum containing fluorescent-labeled normal rabbit IgG were inserted as a set into the guinea pig peritoneum. Two days later, the coagula were recovered, checked for the fluorescent activity, and weighed (n=3). (**B**) Paraffin sections of a representative set of recovered coagula were stained with hematoxylin and eosin.