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The roles of a ribosomal protein S19 polymer in a mouse model of carrageenan-induced acute pleurisy

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ABSTRACT

C5-deficient mice usually present moderate neutrophil activation during the initiation phase of acute inflammation. Conversely, C5a receptor (C5aR)-deficient mice show unusually excessive activation of neutrophils. We identified the ribosomal protein S19 (RP S19) polymer, which is cross-linked at Lys122 and Gln137 by transglutaminases in apoptotic neutrophils, as a second C5aR ligand during the resolution phase of acute inflammation. The RP S19 polymer promotes apoptosis via the neutrophil C5aR and phagocytosis via the macrophage C5aR. To confirm the roles of the RP S19 polymer, we employed a carrageenan-induced acute pleurisy mouse model using C57BL/6J mice with a knock-in of the Gln137Glu mutant RP S19 gene and replaced the RP S19 polymer with either an S-tagged C5a/RP S19 recombinant protein or the RP $S19^{122–145}$ peptide monomer and dimer (as functional C5aR agonists/antagonists) and the RP $S19^{122–145}$ peptide trimer (as a functional C5aR antagonist). Neutrophils and macrophages were still present in the thoracic cavities of the knock-in mice at 24 h and 7 days after carrageenan injection, respectively. Knock-in mice showed structural organization and severe hemorrhaging from the surrounding small vessels of the alveolar walls in the lung parenchyma. In contrast to the RP $S19^{122–145}$ peptide monomer and trimer, the simultaneous presence of S-tagged C5a/RP S19 and the RP $S19^{122–145}$ peptide dimer completely improved the physiological and pathological acute inflammatory cues. The RP S19 polymer, especially the dimer, appears to play a role at the resolution phase of carrageenan-induced acute pleurisy in C57BL/6J model mice.

1. Introduction

Acute inflammation is sometimes divided into two phases: initiation and resolution (Henson, 2005). Antibody/antigen complexes, carbohydrates on the surface of microorganisms and specific proteins on the surface of pathogens commonly activate one of the three types of complement pathways (i.e., classical, alternative or lectin) during the initiation phase (Nangaku, 1998). C5a is specifically cleaved from C5 by C5 convertases (C4b2a3b or C3bBb3b) through any of the complement pathways. Conversely, C5-deficient mice showed a low sensitivity of neutrophils in pathogen-induced acute inflammation (Lambrecht, 2006). Therefore, it is believed that C5a receptor (C5aR) expression on neutrophils promotes pro-inflammatory and anti-apoptotic signaling during the initiation phase (de Jong et al., 2010).

To prevent the excessive activation of neutrophils, the neutrophil C5aR recycling system is quickly initiated by phosphorylation of its C-terminus via the extracellular signal–regulated kinase (ERK) 1/2 pathway (Scola et al., 2009). In addition, we have shown de novo synthesis of C5aR in apoptotic cells, including neutrophils (Hunt et al., 2005). Conversely, C5aR-deficient mice showed opposing sensitivities of neutrophils, with increased sensitivity observed in *Pseudomonas aeruginosa*-induced bronchopneumonia and decreased sensitivity observed in an antibody/antigen complex-induced reverse passive Arthus reaction (Hopken et al., 1996; Hopken et al., 1997). However, the roles of the recycling or de novo synthesis of C5aRs on neutrophils as well as the general expression of C5aR on macrophages during the resolution phase of acute inflammation have not been fully discussed.

In contrast to C5a, we originally found a plasma-derived and coagulation factor XIIIa-dependent monocyte-specific chemoat-

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tractant in serum (Okamoto et al., 1992). Similar monocyte-based chemotactic proteins were recently detected in two extracts derived from rheumatoid arthritis-synovial tissues and atherosclerotic lesions and were identified as ribosomal protein (RP) S19 polymer (Shi et al., 2005; Nishiura et al., 1996). The RP S19 dimer, which is cross-linked between Lys122 and Gln137 by activation of tissue transglutaminases during apoptosis, is capable of docking to C5aR based on a molecular design support system driven under Molecular Operating Environment prepared by Chemical Computing Group Inc. (Montreal, Canada) using the crystal structure of *Pyrococcus abyssi* RP S19 (Nishiura et al., 2010a,b). The RP S19 monomer did not bind to the neutrophil C5aR. A distance between the three-dimensional N-terminus and C-terminal L_{72}GR moiety in C5a is important for binding to C5aR by a two-step mechanism (Monk et al., 2007). Therefore, we suggested that the distance between the first and second binding sites of C5aR corresponds to the distance between the K_{38}LAKHK and L_{131}DR regions of one monomer and the corresponding regions of the other molecule in the RP S19 dimer.

We prepared anti-human RP S19 and anti-human C5a rabbit IgGs (NY-4 and NY-5) and detected the RP S19 monomer in neither any types of cells nor in plasma (Nishiura et al., 2005; Nishiura et al., 2013). RP S19 dimers and trimers were mainly detected in apoptotic cells, including neutrophils, and serum, respectively. We showed that the RP S19 C-terminal L_{131}DR, I_{134}AGQVAAAN and K_{143}KH regions are involved in C5aR binding, membrane penetration and switching between the antagonistic and agonistic moieties, respectively (Shibuya et al., 2001; Shrestha et al., 2003). S-tagged C5a/RP S19 was prepared by linking I_{134}AGQVAAANKKH to the C-terminus of a G73D mutant C5a recombinant protein; this linkage served as a functional analogue of the RP S19 dimer (Revollo et al., 2005; Oda et al., 2008). S-tagged C5a/RP S19 functions as a C5aR antagonist through activation of the apoptosis-inducing factor delta lactoferrin in neutrophils; this occurs via suppression of the ERK 1/2 pathway by producing the GTPase activator regulator of G protein signaling 3. S-tagged C5a/RP S19 also functions as a C5aR agonist through an activation of the calcium channel operator annexin A3 in macrophages via the p38 mitogen-activated protein kinase (MAPK) pathway (Nishiura et al., 2014; Nishiura et al., 2015).

The RP S19 gene is responsible for Diamond-Blackfan anemia (Draptchinskaia et al., 1999). Mice with RP S19 gene knockdown showed a high sensitivity of erythrocyte precursor cells against programmed cell death (Hamaguchi et al., 2003; Flygare and Karlsson, 2007). Conversely, we showed that C5aR expression on both erythroblast-like cells differentiated from human erythroleukemia K562 cells by $C_{34}H_{32}CLFeN_4O_4$ (hemin) and macrophage-like cells differentiated from human monocytic THP-1 cells by phorbol-12-myristate-13-acetate (Nishiura et al., 2012). In the *in vitro* cell co-culturing system, erythroblast-like K562 cells differentiated and maturated into erythrocyte-like K562 cells during exposure to macrophage-like THP-1 cells. The ratio of differentiation and maturation to death was decreased by either blocking C5aR with a C5aR antagonistic peptide (PMX-53) or neutralizing the RP S19 polymer with NY-4 and NY-5 (Kohl, 2006). S-tagged C5a/RP S19 can increase the ratio of differentiation and maturation to death.

Programmed cell death is also distinguished as the early and late stages (Trouw et al., 2008). In acute inflammation, apoptotic cells, including neutrophils, release the G protein-coupled receptor (GPCR) agonists ATP/UTP or lysophosphatidylcholine during the early stage of the resolution phase and the GPCR antagonist/agonists RP S19 dimer or annexin A1 during the late stage of the resolution phase (Horino et al., 1998; Vago et al., 2012; Pupjalis et al., 2011; Ravichandran, 2011). We suggested that the RP S19 dimer and trimer can bind to the recycled and de novo synthesized C5aR on neutrophils to promote apoptosis without C5aR internalization and interact with macrophages via C5aRs. To validate the role of the RP S19 polymer in acute inflammation, we prepared Gln(CAG)137Glu(GAG) RP S19 mutant knock-in C57BL/6J mice (knock-in mice) (Chen et al., 2014; Chen et al., 2016).

2. Materials and methods

2.1. Animals

Pathogen-free mice (Charles River, Yokohama, Japan) were maintained at the Center for Animal Resources and Development, Kumamoto University and Hyogo College of Medicine under the control of the Ethical Committee for Animal Experiment (Grant number: B23-165). Similar to other researchers, we did not find any ribosomal dysfunctions in knock-in mice (Supplementary Figs. 1–3) (Chen et al., 2014; Chen et al., 2016).

We mainly employed an acute mouse pleurisy model by a carrageenan injection into the thoracic cavity (TC) (Supplementary Fig. 4). The carrageenan-induced attraction of neutrophils during the initiation phase of acute inflammation in the model mice was not suppressed by C5aR antagonistic peptides (e.g., PMX-53 and L-156,602) (Ting et al., 2008; Tsuji et al., 1992). However, secondary hypernociception and delayed-type hypersensitivity were blocked in neutrophil-depleted rats and in animals treated with PMX-53 and L-156,602, respectively. These data indicated that C5a induced chemical mediators via C5aR expressed on neutrophils, even during the resolution phase of acute inflammation. Under these conditions, we demonstrated that the RP S19 polymer released from apoptotic neutrophils functions as a neutrophil C5aR antagonist but a macrophage C5aR agonist in humans and mice. There was an interesting report that production of C5 and its derivatives in $CD4^+$ T cells contributes to chronic inflammation and autoimmune diseases in humans and mice (Cravedi et al., 2013). Conversely, carrageenan was suggested to activate peripheral blood neutrophils via complement pathways in rats (Camussi et al., 1990). Therefore, there is a species- and cell-dependent sensitivity against carrageenan.

2.2. C5aR ligands

Both the N-terminal Trx-His-S- and C-terminal IAGQVAAANKKH-tagged mouse C5a recombinant protein (Trx-His-S-tagged C5a/RP S19) were prepared by using the pET32a-Rosetta gami (B) Lys-S system and served as functional analogues for the mouse RP S19 dimer (Nishiura et al., 2010a,b). In addition, the C-terminal KLPQGQRDLDRIAGQVAAANKKH peptides were prepared by using Fmoc-solid phase synthesis and an automated peptide synthesizer (Liberty Blue; CEM Corporation, NC, USA) to generate trimer cross-linked at 1K and 15Q by thioether and triazole bonds (Nishiura et al., 2016).

2.3. Western blotting analysis

Proteins separated by 12% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate were transferred to a membrane using a semi-dry Electroblotter (Sartorious, GM) for 90 min at an electric current of 15 V (Laemmli, 1970; Kyhse-Andersen, 1984). After incubating with primary antibody (100 ng/ml) for 1 h at 22 °C, the membrane was treated with HRP-conjugated anti-rabbit IgG (20 ng/ml) (Santa Cruz, California, USA) for 30 min at 22 °C. The ECL Plus Western Blotting Detection System™ was used to observe the antibody signal.

In addition to anti-human RP S19 and anti-human C5a rabbit IgGs (NY-4 and NY-5), anti-mouse C5a antibodies were developed in New Zealand White male rabbits (NY-6) as previously described. Approximately 500 μl of 1 mg/ml recombinant mouse C5a in phosphate-buffered saline (PBS) was mixed with 500 μl of Freund's complete adjuvant (Sigma-Aldrich St., Louis, MO), made