Anti-C5a complementary peptide ameliorates acute peritoneal injury induced by neutralization of Crry and CD59

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1Nephrology, Nagoya University Graduate School of Medicine, Nagoya, Japan; 2Clinical Sciences and Neuropsychopharmacology, Meijo University Graduate School of Pharmaceutical Sciences, Nagoya, Japan; 3Renal Replacement Therapy, Nagoya University Graduate School of Medicine, Nagoya, Japan; 4Immunology, Nagoya City University Graduate School of Medicine, Nagoya, Japan; and 5Choju Medical Institute, Fukushimura Hospital, Toyohashi, Japan

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Mizuno T, Mizuno M, Imai M, Suzuki Y, Kushida M, Noda Y, Maruyama S, Okada H, Okada N, Matsuo S, Ito Y. Anti-C5a complementary peptide ameliorates acute peritoneal injury induced by neutralization of Crry and CD59. Am J Physiol Renal Physiol 305: F1603–F1616, 2013. First published July 31, 2013; doi:10.1152/ajprenal.00681.2012.—In peritoneal dialysis (PD) therapy, physical stresses such as exposure to peritoneal dialysate, catheter trauma, and peritonitis may induce peritoneal injury that can prevent continued long-term PD therapy. Therefore, protection of the peritoneum is an important target to enable long-term PD therapy in patients with end-stage renal disease. We previously showed that neutralization of the membrane complement regulators (CRegs) Crry and CD59 in rat peritoneum provokes development of acute peritoneal injury due to uncontrolled complement activation. C5a is a key effector molecule of the complement system released during acute inflammation. Control of C5a has been proposed as a strategy to suppress inflammatory reactions and, because peritoneal injury is accompanied by inflammation, we hypothesized that C5a targeted therapy might be an effective way to suppress peritoneal injury. In the present study we used an established acute peritonitis model induced by neutralization of CRegs to investigate the effects on acute peritoneal injury of inhibiting C5a. Intravenous administration of an anti-C5a complementary peptide (AcPepA) up to 4 h after induction of injury significantly and dose-dependently prevented accumulation of inflammatory cells and reduced tissue damage in the model, accompanied by decreased C3b deposition. We show that C5a contributed to the development of peritoneal injury. Our results suggest that C5a is a target for preventing or treating peritoneal injury in patients undergoing prolonged PD therapy or with infectious complications.

C5a; complement; peritoneal dialysis; peritoneal injury; peritonitis

IN PATIENTS WITH END-STAGE renal disease (ESRD) undergoing peritoneal dialysis (PD), physical stresses such as exposure to peritoneal dialysate, catheter trauma, and peritonitis are associated with peritoneal injury that can prevent continued long-term PD therapy. Peritonitis is a particularly serious problem for patients undergoing PD, and often prevents adherence to long-term PD therapy in Japan and around the world (18, 19). Therefore, protection of the peritoneum is an important target to enable long-term PD therapy in patients with ESRD.

The complement (C) system plays important roles in innate immunity, protecting the host from pathogens, and regulating acquired immunity (32). However, uncontrolled activation of C is harmful to the host. To control unwanted C activation in disease, several anti-C agents have been developed, including soluble CR1, which has been reported to show efficacy in various animal disease models (21–23). Recently, administration of anti-C agents such as C1 inhibitor and anti-C5 antibody (Eculizumab, Alexion Pharma, Cheshire, CT) have been established as treatments in clinical fields, targeting specific C-associated pathologies such as hereditary angioedema and C-dependent hemolysis, respectively, in patients with paroxysmal nocturnal hemoglobinuria (4, 17). The C system may contribute to maintenance of homeostasis in the peritoneum as it does in other tissues. Uncontrolled C activation can induce peritoneal damage, and membrane C regulators (CRegs) play important roles in controlling activation of C as shown in our previous reports (24–26). We previously showed that neutralization of the CRegs Crry and CD59 in rat peritoneum caused development of acute peritoneal injury (25), and in relevant models it also triggered arthritis and nephritis (20, 39). The animal model described here can be used to develop anti-Complement agents for prevention of acute peritoneal injury associated with C activation in the peritoneum.

C5a, an anaphylatoxin generated during C activation, is also a potent chemotactic factor for neutrophils and other inflammatory cells that express C5a receptors (C5aRs). It was reported that C5a contributed to the initiation and/or amplification of various pathological conditions such as ischemia-reperfusion injury, inflammatory bowel disease, arthritis, antiglomerular basement membrane nephritis, septic shock, Alzheimer’s disease, and hemodialysis in animal models and humans (1, 5, 7, 14, 16, 27, 40, 41, 44). Therefore, C5a might be a target as an anti-C therapy as a way to prevent inflammatory tissue injury. There are numerous agents for inhibition of C5a, such as C5aR antagonists (C5aRA) and monoclonal anti-C5a IgG (10, 28, 42). However, several of these have been difficult to develop because of their high molecular mass, cost of industrial production, and/or antigenicity (22). A 17-amino acid complementary peptide for C5a, PepA, directly bound to C5a and blocked the effects of C5a, and showed protective effects in a lipopoly saccharide-associated lethal shock model in rat (8, 27). Recently, an acetylated form of PepA (AcPepA) was generated to improve the inhibition capacity of PepA (30). AcPepA prevented acute skin inflammation (30) and acute rejection of islet transplantations in models (38).

A few reports have investigated C5a related to infected peritonitis, such as bacterial diffuse peritonitis as a septic

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complication, and spontaneous bacterial peritonitis in patients with liver cirrhosis (6, 13). The roles of C5a in acute peritoneal injury induced by C activation. We also investigated whether an anti-C5a complementary peptide, AcPepA, had the potential to control acute peritoneal injury in our model.

MATERIAL AND METHODS

Animals. Male Sprague-Dawley (SD) rats weighing ~250 g (Chubu Kagaku Shizai, Nagoya, Japan) were used. All animal experiments were carried out according to the Animal Experimentation Guide of Nagoya University School of Medicine. Rats were operated on, injected intraperitoneally (i.p.) and intravenously (i.v.), and killed under ether anesthesia.

Agents and antibodies. Mouse monoclonal antibodies (mAbs) against rat Crry (mAb 5I2) and CD59 [6D1; a kind gift of Prof. B. Paul Morgan (Cardiff, UK)] were characterized in previous reports by Konteatis et al. (15) and has been confirmed to inhibit C5a activation in animal models (14, 27). Optimal cutting temperature compound was purchased from Sakura Finetechical (Tokyo, Japan). Peritoneal dialysis fluids (PDF1, DIaneal PD-4 4.25% pH ~5 and PDF2, DIaneal-N PD-4 2.5% pH ~7) were purchased from Baxter (Tokyo, Japan).

Table 1. Experimental protocol to investigate effects of AcPepA or C5aRA pretreatment before peritoneal injury induction

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Rats</th>
<th>Pretreatment,* mg/animal</th>
<th>Administration Route†</th>
<th>mAb Injection‡</th>
<th>Timing of Observation, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>None</td>
<td>None</td>
<td>–</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Saline</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>AcPepA, 0.33</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>AcPepA, 0.66</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>AcPepA, 1.33</td>
<td>i.v.</td>
<td>+</td>
<td>24, 12, 24</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Saline</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>AcPepA, 1.33</td>
<td>i.v.</td>
<td>+</td>
<td>6, 12, 24</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>Saline</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>C5aRA, 0.75</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>AcPepA, 1.33</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>Saline</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>AcPepA, 1.33</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>AcPepA, 1.33</td>
<td>i.v.</td>
<td>+</td>
<td>24</td>
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</tbody>
</table>

*Intravenous injection of an acetylated form of antisense peptide of C5a (AcPepA) or C5a receptor antagonist (C5aRA) 10 min before administration of blocking mAb against the CRegs Crry and CD59. †Intraperitoneal administration of blocking mAb against Crry and CD59. ‡Intravenous injection of blocking mAb against membrane the CRegs Crry and CD59.

Experimental protocol. In the present study, we used an acute peritonitis model induced by neutralizing the CRegs Crry and CD59 as described previously (25). First, to determine the dose-dependent effects of AcPepA, rats were divided into five groups: untreated (group 1); and intravenous (i.v.) injection of vehicle (group 2), AcPepA 0.33 mg (group 3), AcPepA 0.66 mg (group 4), or AcPepA 1.33 mg/animal (group 5) in 0.5 ml of isotonic saline. At 10 min after injection of AcPepA, each rat was injected intraperitoneally (i.p.) with 0.5 mg/animal of neutralizing mAbs 5I2 and 6D1 against Crry and CD59, respectively, diluted in 10 ml of PDF1. Twenty-four hours after injection of mAbs, all rats were killed and parietal peritoneums were collected for examination. In addition, to investigate the time-dependence of effects after AcPepA pretreatment, rats were i.v. injected with 1.33 mg/animal of AcPepA (group 7) or vehicle (group 6) at 10 min before administration of the mAbs, and then killed at 6, 12, and 24 h after the mAbs had been administered.

Second, to compare the protective effects of AcPepA, C5aRA, and vehicle on peritoneal injury, rats were divided into three groups with i.v. injections as follows: vehicle (0.5 ml isotonic saline; group 8); 0.75 mg/animal C5aRA (group 9); or 1.33 mg/animal AcPepA (group 10), respectively. Injections occurred 10 min before blocking both Crry and CD59, and all rats were killed at 24 h after blocking CRegs.

Third, to compare the effects of i.v. and i.p. administration routes of AcPepA, 1.33 mg/0.5 ml of AcPepA were injected i.p. or i.v. in isotonic saline/animal at 10 min before blocking both Crry and CD59 (group 12 and group 13, respectively). All rats were killed at 24 h after blocking CRegs.

The detailed experimental protocol described above and the number of rats in each group is shown in Table 1.

Separately, to evaluate the potential of AcPepA for treatment of acute peritoneal injury, AcPepA 1.33 mg/animal (group 15) or vehicle (group 14) was i.v. administered; after 2 h, 4 h, or 6 h mAbs were administered i.p. as described above to block the function of Crry and CD59. All rats were killed at 24 h after blocking CRegs.

The experimental protocol and number of rats in each group appears in Table 2.

Histological analysis. The parietal peritoneum was dissected in strips (four strips, ~5 × 30 mm, from each side of parietal peritoneum). Two strips from each side were randomly selected, fixed in 10% buffered formalin, and embedded in paraffin. Sections 4.5 μm thick were stained with hematoxylin and eosin (H&E) for histological analysis. The other strips were used as snap-frozen sections. Peritoneal damage was defined as subperitoneal tissue edema, accumulation of inflammatory cells, necrotic changes in peritoneal fatty tissues, and/or muscle destruction under light microscopic (LM) findings. Twenty independent fields were randomly captured at ×200 magnification using a Zeiss Z1 microscope and Axiosview Windows soft-

Table 2. Experimental protocol to investigate the potential of AcPepA after peritoneal injury induction

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Rats</th>
<th>Treatment*</th>
<th>Timing of Treatment, h</th>
<th>mAb Injection‡</th>
<th>Timing of Observation, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>6</td>
<td>Vehicle</td>
<td>2, 4, 6</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>AcPepA</td>
<td>2, 4, 6</td>
<td>+</td>
<td>24</td>
</tr>
</tbody>
</table>

*Intravenous injection of 1.33 mg/animal of an acetylated form of antisense peptide of C5a (AcPepA) or vehicle after administration of blocking mAbs against Crry and CD59. †Intraperitoneal administration of blocking mAb against Crry and CD59. Each mAb was injected with 0.5 mg/body in peritoneal dialysis fluid.
ware version 4.4 (Carl Zeiss, Oberkochen, Germany). In each captured field, the area with tissue injury such as accumulation of inflammatory cells, subperitoneal edema, and damaged subperitoneal muscular cells in the subperitoneum was measured using MetaMorph 6.3 image analysis software (Universal Imaging, West Chester, PA) and the mean value of 20 fields was used as the area of peritoneal injury in each rat.

**Immunohistochemical analysis.** Peritoneal strips obtained as described above were snap-frozen with a cryostat in sections 4.5 μm thick and fixed in acetone according to our previous report (24).

To detect C3b deposition, FITC-labeled polyclonal goat anti-rat C3b was incubated on the frozen sections. Normal rabbit serum was used as a negative control for polyclonal antibody, and the peritoneum of untreated rats was also used as a negative control tissue. To estimate deposition of C3b around injured peritoneum, 20 sequential fields in each rat were assessed under 200 magnification using a Zeiss Z1 microscope and Axiovision Windows software version 4.4. Areas with C3b depositions were measured using MetaMorph 6.3 image analysis software and the average area of the 20 sequential fields was taken for each rat.

For analysis of accumulation of inflammatory cells such as total white blood cells or macrophages, frozen sections were incubated with FITC-labeled mouse anti-rat LCA mAb or with FITC-labeled mouse anti-rat monocyte mAb (ED1). To detect neutrophils, frozen sections were incubated with mouse anti-rat neutrophil mAb (RP3), followed by incubation with FITC-labeled anti-mouse IgM as the second antibody. Furthermore, to observe T cells or B cells, frozen sections were incubated with anti-CD3 or anti-rat CD45R antibody, followed with rhodamine-labeled anti-rabbit IgG or FITC-labeled anti-mouse IgG. The number of LCA-, ED1-, RP3-, CD3-, or CD45R-positive cells per field was calculated by counting positive cells in 20 sequential fields under ×200 magnification and taking the average.

For CD31 staining, endogenous peroxidase activity in the frozen sections was first blocked using 0.1% NaN₃ and 0.3% hydrogen peroxide in PBS, and nonspecific protein-binding sites were blocked with normal goat serum. After that, the sections were incubated with anti-CD31, followed by a conjugate of polyclonal goat anti-mouse IgG antibody, and horseradish peroxidase-labeled polymer (Histofine Simple Stain, Nichirei, Tokyo, Japan) as a secondary reagent. Finally, enzyme activity was detected using 3-amino-9-ethylcarbazole (Dako). The number of CD31-positive vessels was counted in 20 random submesothelial areas under ×200 magnification and the average was taken.

**Primary cell cultures of rat mesothelial cells.** Primary culture of rat mesothelial cells was performed from SD rat omentum as previously reported (25). Briefly, dissected omentum from an SD rat (7 wk old, male) was digested in 10 ml of 0.25% trypsin with 1 mM EDTA·4 Na (Gibco) during 30 min at 37°C, followed by a 1-h incubation
another 0.25% trypsin with 1 mM EDTA at 37°C. After that, the remaining omentum fragment was removed from the solution. M199 medium (Invitrogen, Carlsbad, CA) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin was added into the above trypsin solution at 1:1 (vol/vol) and centrifuged at 1,000 g for 5 min. The cell pellet was resuspended in 4 ml of M199 medium with 10% FBS (Invitrogen), separated into a type 1 collagen-coated 60-mm dish (IWAKI, Tokyo, Japan), and incubated at 37°C for 4 days. After that, the semiconfluent cells were separated into three dishes. We characterized the primary culture of rat mesothelial cells according to our previous report (25).

**Flow cytometry analysis by fluorescent-activated cell sorting (FACS) analysis and expression of CRegs on peritoneal mesothelial cells under peritoneal dialysate.** To observe expression of Crry or CD59 in mesothelial cells, the primary culture cells were first incubated with mAb S12 or D1, which were mouse IgG1 class, or mouse IgG1 as an isotype-matched control, following incubation with FITC-anti mouse IgG.

To investigate the change in expression of CRegs, mesothelial cells were incubated for 1 h in M199 medium (control medium), PDF1, or PDF2. After exposure under different mediums described above, mesothelial cells were harvested in suspension by treatment with EDTA, washed in PBS, and resuspended at 0.30 × 10⁶ cell/ml. A 1-ml cell suspension was incubated with 25 μg/ml of mAb S12 or D1 on ice for 60 min, washed with PBS, and incubated for an additional 50 min with Alexa Fluor 647 goat anti-mouse IgG (Invitrogen, Camarillo, CA) as the second antibody. To eliminate the effects of dead cells, cells were stained with 5 μl of 7-AAD and incubated for 10 min at room temperature in the dark. The geometric mean titer of CRegs was evaluated via FACS analysis using a BD FACS-Canto flow cytometer II (Becton Dickinson, San Jose, CA) to detect expression changes on cell surfaces. The experiments were repeated six times.

**Functional assay of C activation in rat mesothelial cells affected by peritoneal dialysate.** To confirm a functional decrease in CRegs in the experiments described above we observed the degree of C activation by normal rat serum on rat mesothelial cells that were incubated with M119 medium (control), PDF1, or PDF2. Rat mesothelial cells were plated at 2.0 × 10⁶ cells/well and incubated in M199 medium (control), PDF1, or PDF2 for 1 h. After that, incubated mesothelial cells were harvested, washed with M199 medium three times, and then incubated in M199 medium with normal rat serum (RS) or with heat-inactivated RS for 1 h at 37°C, following detection of C3 deposition on mesothelial cells incubated with FITC-rabbit anti-rat C3. Heat-inactivated serum was obtained by incubation at 56°C for 30 min. The concentration of RS was adjusted from 0 to 10%; the same amount of heat-inactive RS as a negative control for the in vitro experiments described above.

After incubation, the cells were washed with PBS three times, and C3b deposition was detected with FITC-labeled anti-rat C3. Cells were double-stained with DAPI (Sigma-Aldrich) to facilitate cell counting. According to a previous report (25), total and C3b-deposited cells were counted randomly in 20 fields, and the C3b deposition was calculated according to the following formula:

\[
\text{deposition of C3b (％) } = \frac{\text{number of C3b-deposited cells}}{\text{total number of cells}} \times 100
\]

The experiments described above were repeated six times.

**Statistical analysis.** Data are displayed as means ± SE. Comparisons among multiple groups were performed using ANOVA (with a Tukey’s test). Analyses between two groups were performed using an unpaired t-test. In these tests, a two-sided value of \( P < 0.05 \) was considered significant. SPSS v19.0 software (SPSS, Chicago, IL) was used for statistical analysis.
RESULTS

A C5a complementary peptide blocker, AcPepA, suppressed acute peritoneal injury in a dose-dependent manner. In an acute peritonitis model induced by neutralizing CRegs in rat peritoneum, pretreatment with 1.33 mg/animal of AcPepA (group 5) decreased accumulation of inflammatory cells in the peritoneum, and ameliorated injury in subperitoneal fatty tissues and subperitoneal muscles (arrowheads in Fig. 1E) compared with vehicle-injected rats (Fig. 1B) under LM, and clearly decreased the area of injury (Fig. 1F). Although a significant decrease in injured area was not observed in the peritoneum of rats treated with 0.33 or 0.66 mg/animal of AcPepA, suppression of peritoneal injury was observed, depending on the amount of AcPepA (Fig. 1F).

When we investigated C3b deposition in the peritoneum of rats treated with 0.33, 0.66, or 1.33 mg/animal of AcPepA (groups 3, 4, and 5, respectively) and vehicle control rats (group 2), deposition of C3b in rats in groups 4 and 5 was significantly decreased compared with that of control rats (group 2) (Fig. 2).

Assessment of inflammatory cell accumulations in injured peritoneum showed abundant LCA-, RP3-, and ED1-positive cells in the peritoneum of control rats treated with vehicle (group 2; Fig. 3, D–F). The number of LCA-, RP3-, and ED1-positive cells decreased in a dose-dependent manner upon administration of AcPepA, and the number of these cells was significantly decreased in rats pretreated with 1.33 mg/animal of AcPepA (group 5) compared with rats without AcPepA.

Fig. 4. CD31-positive vessels, and CD3- and CD45R-positive cells in the peritoneum 24 h after induction of acute peritoneal injury in rats pretreated with AcPepA. A, B, and C: number of CD31-positive vessels, CD3- and CD45R-positive cells, respectively, in peritoneum. Nonsignificance (n.s.) for groups 3, 4, and 5 vs. group 2.

Fig. 5. Light microscopy changes in parietal peritoneum at 6, 12, and 24 h after induction of acute peritoneal injury in rats pretreated with 1.33 mg/animal of AcPepA. A, B, and C: typical images from group 6 (vehicle); D, E, and F: group 7 (pretreatment with 1.33 mg/animal of AcPepA). The timing of death is displayed at the top of each set. Arrowheads indicate site of peritoneal injury. Original magnification ×200. Scale bar at upper right in A is 100 μm. G: area of tissue injury in parietal peritoneum in groups 6 and 7. *P < 0.05; **P < 0.01 vs. vehicle group.
pretreatment (group 2) (Fig. 3). Among the groups, the number of CD3- and CD45R-positive cells was few and not significantly different. There were few CD31-positive vessels, and there was not a significant increase in group 2 rats compared with untreated rats (group 1) (Fig. 4).

Of note, in untreated rats (group 1), peritoneal injury was not observed and there were few LCA-, RP3-, and ED1-positive cells in the peritoneum (Fig. 1, A and F; Fig. 3). From the results described above, we chose a dose of 1.33 mg/animal of AcPepA for subsequent experiments.

**Time course of peritoneal injury after pretreatment with AcPepA in the acute peritonitis model.** In rats pretreated with 1.33 mg/animal of AcPepA, using LM we estimated peritoneal injury at 6, 12, and 24 h after induction of acute peritoneal injury by

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**Time course of peritoneal injury after pretreatment with AcPepA in the acute peritonitis model.** In rats pretreated with 1.33 mg/animal of AcPepA, using LM we estimated peritoneal injury at 6, 12, and 24 h after induction of acute peritoneal injury by
blocking CRgs. At 6 h after induction of peritoneal injury, the suppressive effects of AcPepA were already observed (group 7; Fig. 5). Up to 24 h after peritonitis induction, significant suppressive effects of pretreatment with AcPepA were clearly observed (group 7; Fig. 5).

When we compared the peritoneal area that was positive for C3b deposition, it was decreased in rats pretreated with AcPepA at each time point (6, 12, or 24 h) after induction of acute peritonitis (Fig. 6). Along with decreased C3b deposition, the number of LCA-, RP3-, and ED1-positive cells in the peritoneum was also significantly suppressed by pretreatment with 1.33 mg/animal of AcPepA at each time point after induction of acute peritonitis (Fig. 7).

Comparison of the effects of AcPepA and C5aRA in acute peritoneal injury after blocking Crry and CD59. According to previous reports (14, 27), we chose an effective dose of 0.75 mg/animal of AcPepA for pretreatment (group 10; Fig. 5). Table 1 shows the number of C3b deposits in each group. The number of C3b deposits in group 10 was significantly lower than in group 8. The number of LCA-, RP3-, and ED1-positive cells was also significantly lower in group 10 than in group 8. These results suggest that AcPepA has a suppressive effect on peritoneal injury after blocking Crry and CD59.
**Fig. 9.** Statistical analysis of effects of C5a inhibition by either C5aRA or AcPepA in prevention of peritoneal injury. Graphs show pooled data from rats in group 8 (vehicle), group 9 (treatment of C5aRA), and group 10 (treatment of AcPepA). Areas with peritoneal tissue injury (A), C3b deposition (B), LCA-positive cell infiltration (C), RP3-positive cell infiltration (D), and ED1-positive cell infiltration (E) were all significantly decreased in groups 9 and 10 compared with group 8. ∗∗∗P < 0.001; ∗∗P < 0.01 vs. vehicle-injected rats (group 8).

**Fig. 10.** Effective comparison of AcPepA with different administration routes (i.p. and i.v.) in peritoneal injury and accumulation of inflammatory cells in peritoneum. Graphs show pooled data from rats in group 11 (vehicle), group 12 (i.p. injection of AcPepA), and group 13 (i.v. injection of AcPepA). Areas with peritoneal tissue injury (A), C3b deposition (B), LCA-positive cell infiltration (C), RP3-positive cell infiltration (D), and ED1-positive cell infiltration (E) were all significantly decreased in groups 12 and 13 compared with group 11. White bars, hatched bars, and black bars show groups 11, 12, and 13, respectively. ∗∗P < 0.01 vs. vehicle-injected rats (group 11).
mg/animal of C5aRA (i.v.). When rats were administered C5aRA instead of AcPepA (group 9), the areas of peritoneal injury were clearly suppressed and to a similar degree as with injection of AcPepA (group 10; Figs. 8 and 9). In the peritoneum in C5aRA-treated rats (group 9), deposits of C3b were decreased to a similar degree as in rats that had been pretreated with AcPepA (group 10) (Figs. 8 and 9). Pretreatment with C5aRA also inhibited accumulation of LCA-, RP3-, and ED1-positive cells to a similar degree as that in rats treated with AcPepA (Fig. 10). Pretreatment with AcPepA at 2 h or 4 h after induction of acute peritonitis with C5aRA instead of AcPepA (group 15) was also clearly decreased compared with that of vehicle-injected rats (Fig. 12). At 2 h or 4 h after induction of acute peritonitis, C3b deposition in AcPepA-treated rats (group 15) was also clearly decreased compared with that of vehicle injection (group 14; Figs. 11 and 12). Accumulation of inflammatory cells was also assessed; the number of LCA-, RP3-, and ED1-positive cells in the peritoneum of rats in group 15 was also clearly reduced compared with that treated with control medium (group 14) that had been posttreated with AcPepA at 2 h or 4 h, but not at 6 h, after induction of acute peritonitis (Fig. 12).

Incubation of peritoneal dialysate decreased expression of CRegs on rat mesothelial cells and the decrease impaired protection of complement activation on rat mesothelial cells. Expression of the CRegs Crry and CD59 on rat mesothelial cells after 1 h of incubation with the peritoneal dialysate Dianeal PD-4 4.25% (PDF1) was significantly decreased compared with that treated with control medium or Dianeal-N PD-4 2.5% (PDF2) (Fig. 13, A and B).

Larger C3b deposition was achieved by incubating mesothelial cells with 2% and 5% RS, leading CReg expression to decrease via preincubation with PDF1 for 1 h, compared with control medium and PDF2 (Fig. 13C), supporting the concept that impairment of CRegs occurs upon exposure to PDF1.
DISCUSSION

Although the C system plays important roles in preserving homeostasis in the host, exaggerated activation of the C system initiates and amplifies various diseases (22, 23). We previously showed that peritoneal CRegs controlled the C activation system in rat peritoneum and that dysregulation of them induced acute peritonitis (25). We also showed that uncontrolled activation of the C system caused acute inflammation in the peritoneum and enhanced peritoneal injury in a zymosan-induced peritonitis model (24, 26). We suggested that it was important to maintain homeostasis of the C system in the peritoneum. However, there was no attempt to identify pathogenic roles for C5a in the peritoneum until now.

In the acute peritonitis model induced by blocking the CRegs Cry and CD59, we showed here that directly blocking C5a by a C5a inhibitor, AcPepA, or that blocking C5aR by C5aRA, suppresses acute peritoneal injury and is accompanied by a decrease in infiltration of inflammatory cells such as neutrophils and ED1-positive cells. Inhibition of C5a or of C5aR also decreased C3b deposition in the peritoneum. These data suggest that C5a enhanced acute peritoneal injury. Moreover, with the timing of AcPepA administration, the protective effects against peritoneal injury were also observed by AcPepA injection even if inflammation had already been induced in the peritoneum.

In many pathogenic conditions such as septic shock and systemic inflammatory response syndrome, C5a plays chemotactic roles in initiating and amplifying inflammation (28, 42). Receptors for C5a were reported on various cells, predominantly white blood cells (11, 28). In acute peritoneal injury induced by blocking CRegs in rats, accumulation of RP3-positive neutrophils and ED1-positive mononuclear cells were observed in the early stage of acute peritoneal inflammation that was dependent on activation of the C system (25). Although the contributions of C5a had remained unclear in peritoneal inflammation, our results showed that C5a amplified the inflammation and that control of C5a in early stages of inflammation was important in preventing progression of peritoneal injury. As a result, it was considered that further complement activation might be suppressed and that C3b deposi-
with control medium, PDF2 (Dianeal-N PD-4 2.5% pH –7), or PDF1 (Dianeal PD-4 4.25% pH –5). For statistical analysis of data, geometric mean titers were evaluated \((n=6)\). To observe a functional change in expression of Crry and CD59 after stimulation of control medium with PDF2 or PDF1, C3b deposition incubated with untreated rat serum was also evaluated in rat mesothelial cells \((C)\). \(P<0.01\) vs. control medium.

Fig. 13. Exposure of peritoneal dialysate decreases membrane complement regulators Crry and CD59 in mesothelial cells of rat peritoneum and C3b deposition caused by C activation increases in mesothelial cells. \(A\) and \(B\): flow cytometric analysis of expression of Crry and CD59 in rat mesothelial cells after stimulation with control medium, PDF2 (Dianeal-N PD-4 2.5% pH –7), or PDF1 (Dianeal PD-4 4.25% pH –5). For statistical analysis of data, geometric mean titers were evaluated \((n=6)\). To observe a functional change in expression of Crry and CD59 after stimulation of control medium with PDF2 or PDF1, C3b deposition incubated with untreated rat serum was also evaluated in rat mesothelial cells \((C)\). \(P<0.01\) vs. control medium.

From recent reports related to C5a ligands, two different receptors, one G protein-coupled, C5aR (CD88) and the other not G protein-coupled, C5L2 (GPR77), were expressed as receptors for C5a \((42)\). Although C5a is well known as a chemotactic factor involved in various diseases, C5a also plays important physiological roles; for example, in liver regeneration \((34)\) and neurorepair \((3)\). The two receptors C5aR and C5L2 collaborated in some pathological conditions such as experimental sepsis and allergic asthma \((33, 45)\). In other conditions the two receptors showed opposite behavior, with C5L2 acting as a decoy receptor and negative modulator of C5aR-mediated signal transduction \((2, 9)\). In our study, it remains unclear how these receptors collaborate in the peritoneum. In future, it might be possible to clarify their roles.

In patients with ESRD on PD therapy, the peritoneum is always exposed to physical stresses including peritonitis. These physical stresses can cause tissue injuries in the peritoneum that are both functionally and histopathologically obvious. As a result, prolongation of PD therapy might be prevented because of impairment of peritoneal function and risk of induction or progression of encapsulating peritoneal sclerosis (EPS), which is a potentially lethal complication associated with PD therapy. Because of its role as a physical PD-related stress for the peritoneum, peritonitis is an important complication \((18, 19)\). Until now, there have been few reports to show the relationship between the C activation system and PD therapy in humans and roles of the C activation system as a defense against infective peritonitis \((31, 37, 43)\). Our recent reports showed that severe peritoneal injuries caused by fungal infections might be related to C activation and that dysregulation of the C system might be a trigger to initiate EPS under persistent peritoneal injury \((24, 26)\). When we search for possibilities of anti-C therapies against peritoneal injury, we also have to consider the double-edged nature of the C activation system. The complete and long-term prevention of activation of the C system would prevent normal immune responses and induce pathologies such as severe infection, autoimmune diseases, and carcinogenesis in the host. Therefore, anti-C agents expected to cause minimal systemic inhibition of C activation through titration of dose, timing of administration, and targeting strategies may be more beneficial, retaining physiological roles while causing sufficient inhibition at target sites to inhibit the development of pathology \((22)\). Because suppression of C5a carries a lower risk for infection than inhibiting the C pathway entirely, anti-C5a therapy could be more useful for inflammatory diseases; targeting C5a might be a particularly useful strategy for preventing the development of peritoneal injuries in patients undergoing PD.

From the present results, we showed that C5a was a mediator in the development of inflammation in the peritoneum and that blocking C5a was useful in reducing acute peritoneal injury. Our results suggest that anti-C5a therapy using the C5a blocker AcPepA as a targeted therapeutic strategy has the potential to decrease and/or prevent peritoneal injury in pa-
tients undergoing PD and may prolong the duration of PD therapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


