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Collectin CL-P1 utilizes C-reactive protein for complement activation

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ABSTRACT

Background: C-reactive protein (CRP) is a plasma pentraxin family protein that is massively induced as part of the innate immune response to infection and tissue injury. CRP and other pentraxin proteins can activate a complement pathway through C1q, collectins, or on microbe surfaces. It has been found that a lectin-like oxidized LDL receptor 1 (LOX-1), which is an endothelial scavenger receptor (SR) having a C-type lectin-like domain, interacts with CRP to activate the complement pathway using C1q. However it remains elusive whether other lectins or SRs are involved in CRP-mediated complement activation and the downstream effect of the complement activation is also unknown.

Methods: We prepared CHO/ldlA7 cells expressing collectin placenta-1 (CL-P1) and studied the interaction of CRP with cells. We further used ELISA for testing binding between proteins. We tested for C3 fragment deposition and terminal complement complex (TCC) formation on HEK293 cells expressing CL-P1.

Results: Here, we demonstrated that CL-P1 bound CRP in a charge dependent manner and the interaction of CRP with CL-P1 mediated a classical complement activation pathway through C1q and additionally drove an amplification pathway using properdin. However, CRP also recruits complement factor H (CFH) on CL-P1 expressing cell surfaces, to inhibit the formation of a terminal complement complex in normal complement serum conditions.

General Significance: The interaction of collectin CL-P1 with CFH might be key for preventing attack on "self" as a result of complement activation induced by the CL-P1 and CRP interaction.

1. Introduction

C-reactive protein (CRP) is an acute phase plasma protein produced by hepatocytes in response to inflammation, tissue damage and trauma [1]. Like other acute phase proteins, CRP is normally present in trace levels (<10 mg/L) in serum but increases rapidly and dramatically in response to a variety of infectious or inflammatory conditions (2). Mild inflammation and viral infections cause elevation of CRP to 10–40 mg/L while bacterial infections produce levels of 40–200 mg/L. Levels higher than 200 mg/L are found in several bacterial infections and burns [2]. CRP is capable of interacting with a variety of ligands such as phosphocholine residues, modified low density lipoprotein (mLDL), and damaged cells as well as activating the complement and opsonizing biological particles [3,4]. The site directed mutagenesis model shows how one globular head group of C1q interacts through the central pore of CRP on the A-face of the pentamer [5]. The classical pathway is mostly activated in an antibody dependent manner, but it is also initiated by C1q directly through recognition of distinct structural moieties on microbial and apoptotic cells or through various soluble pattern recognition molecules, such as CRP and Pentraxin 3 (PTX3) [6,7].

Lectin-like oxidized LDL receptor 1 (LOX-1) was discovered as an endothelial scavenger receptor (SR), having a calcium dependent lectin-like protein [8]. Recent papers demonstrated that CRP bound to Chinese hamster ovary cells (hLOX-1-CHO) express human LOX-1, and that CRP binds to recombinant human LOX-1 in a cell-free system [9,10]. These studies also showed that LOX-1 promotes CRP-induced xenogeneic complement activation by interacting with CRP to develop an inflammatory pathogenic response. Moreover, subsequent research has demonstrated that scavenger receptor type I (SR-AI) binds CRP whereas other SRs, CD36 or SR-B1, do not [11].

Abbreviations: CRP, C-reactive protein; PTX3, pentraxin 3; CRD, carbohydrate recognition domain; Col, collagen; TCC, terminal complement complex; SR, scavenger receptor; CL-P1, collectin placenta 1; SR-AI, scavenger receptor type I; LOX-1, lectin-like oxidized LDL receptor 1; CHO, Chinese hamster ovary; HEK, human embryonic kidney; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; ELISA, enzyme linked immunosorbent assay; poly(I), polyinosinic acid; poly(C), polycytidylic acid; CFH, complement factor H; oxLDL, oxidized low density lipoprotein; LDL, low density lipoprotein; HCS, human complement serum.

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The ligand specificity of the SR family overlaps considerably [12,13]. We previously identified collectin placenta 1 (CL-P1) [14], a type II membrane protein which contains carbohydrate recognition domain (CRD), a long coiled-coil domain, and a transmembrane domain and showed it to be a SR, in addition to its role as a collectin. It is also referred to as a scavenger receptor C-type lectin (SRCL) and collectin 12 [15]. Recent genomic analysis verified CL-P1 as an SR-AI gene of *SCARA4* as well as a collectin gene of *COLEC12* [16,17]. CL-P1 was originally found and identified as an endothelial receptor that can endocytose and phagocytose Gram-negative and -positive bacteria and yeast as well as oxidized low density lipoprotein (OxLDL) in vascular endothelial cells, and it interacts with OxLDL and microbes through the collagen-like domain whereas it utilizes the CRD to bind sugar ligands [18,19]. Furthermore, very recently Ma et al. have hypothesized the existence of a fluid phase molecule of CL-P1 which may initiate complement activation on *Aspergillus fumigatus* [20].

The aim of this study was to investigate the involvement of CL-P1 in CRP-mediated complement activation, as well as to investigate the downstream effects of this complement activation. In the present study, we addressed how CL-P1 interacted with CRP and promoted complement activation through the classical and amplification pathway. At the same time, CRP recruits complement factor H (CFH) depending on CL-P1 to protect the cells from the formation of the terminal complement complex.

2. Materials and methods

2.1. Proteins and reagents

Human CRP purified from pleural fluid (AG723) was purchased from Merck Millipore. Sodium azide in the solution was extensively removed by dialyzing 3 times against a 3000-fold volume of PBS. HAM's F-12 and Dulbecco's minimal essential medium (DMEM)-high glucose, fetal bovine serum (FBS), poly(I), poly(C), phosphocholine, polymyxin B and human complement serum were from Sigma. Anti-myc antibody and Alexa Fluor conjugated antibodies were purchased from Invitrogen. We purchased native C1q, purified human factor H, purified human properdin, rabbit anti-human C5b-9, goat anti-human factor H, goat anti-human properdin, properdin depleted serum and factor H depleted serum from Complement Technology. The MicroVue SC5b-9 plus EIA kit were purchased from Quidel.

2.2. Cell culture and transfection

Chinese hamster ovary (CHO)/ldlA7 cells lacking functional LDL receptors were cultured and transfected as described previously [19]. In brief, cells were grown in HAM's F-12 medium supplemented with 5% heat inactivated FBS at 37 °C, 5% CO_2. 24 h earlier, transfected cells were seeded onto poly-L-lysine-coated 35 mm glass based dishes (Iwaki, Japan). Transfection was carried out by Lipofectamine LTX transfection reagent (Invitrogen) according to the manufacturer's instructions. Lipofectamine LTX /plasmid DNA complex was replaced with fresh HAM's F12 medium with 5% FBS 6 h after transfection. 24 h post transfection cells were immediately used for a ligand binding assay. HEK293 cells were maintained in DMEM-high glucose supplemented with 10% FBS. HEK293 cells were seeded on collagen-coated dishes (Iwaki, Japan) and transfected as described above.

2.3. Construction of expression vectors

Recently, we identified and cloned cDNA sequences of human CL-P1 into pcDNA3.1/myc-HisA expression vectors (18). Constructs for CL-P1 deletion mutants and collagen-like domain positive cluster mutants were prepared as described previously [19]. We cloned LOX-1 from human placental cDNA. The plasmid DNA was purified using an EndoFree maxi kit (Qiagen) according to the manufacture's protocol.

2.4. Recombinant CL-P1 expression and purification

cDNA encoded extracellular domain of human CL-P1 (59–742) was subcloned into pcDNA3.1 vector with insulin leader peptide followed by FLAG tag in the N terminus. The plasmid was transfected into Expi 293-F cells (Invitrogen). After 7 days, the recombinant protein was purified from culture supernatant with anti-FLAG M2 affinity gel (Sigma) according to the manufacturer's instructions.

2.5. Fluorescent labeling and biotinylation of CRP

CRP was fluorescently labeled with an Alexa Fluor 555 antibody-labeling kit (Invitrogen) and dialyzed 3 times (12 h, 4 °C) against a 3000-fold volume of PBS. Biotinylation of CRP was performed using EZ-Link Sulfo-NHS-LC–LC-Biotin (Invitrogen) according to the manufacturer's instructions and dialyzed 3 times (12 h, 4 °C) against a 1000 fold volume of PBS.

2.6. Binding and inhibition assay using the CHO/ldlA7 cell line

The binding of Alexa 555-CRP with full length CL-P1, CL-P1 deletion mutants and LOX-1 was performed as previously described [10]. In brief, 24 h after transfection cells were washed twice with ice-cold HAM's F12 medium with 5% FBS. Then, the medium was replaced with ice-cold Ham's F-12/10 mM HEPES containing 10 μg/ml of Alexa 555-CRP and cells were incubated at 4 °C for 1 h. After being washed with ice-cold PBS, the cells were fixed with 4% phosphate-buffered paraformaldehyde (Wako Pure Chemical Industries). For the inhibition assay, 10 μg/ml poly(I) or poly(C) were pre-incubated with cells before the addition of Alexa 555-CRP. For the phosphocholine inhibition assay, 1 mM phosphocholine was mixed with Alexa 555-CRP and then incubated with cells for 1 h at 4 °C. The expression of CL-P1 and LOX-1 was visualized using anti-myc antibody followed by Alexa 488 anti-mouse IgG. Nuclear counterstaining was performed with Hoechst 33342 obtained from Invitrogen. The images were taken using a fluorescent microscope (BZ-9000, Keyence). Signal intensity was calculated by using the BZ-HIC program (Keyence).

2.7. CRP and CL-P1 interaction ELISA

Recombinant human CL-P1 (0.1 μg) or BSA (0.1 μg) (Thermo Scientific) was immobilized to 96-well immunoplates plates (Maxisorp, Thermo Fisher Scientific) by incubating overnight at 4 °C in a coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05% NaN_3, pH 9.6). After 3 washes with TBSTC, the plates were blocked with BlockAce/PBS (DS Pharma Biomedical) at 37 °C for 1 h. After washing with TBSTC, biotin-CRP in TBSTC (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, 5 mM $CaCl_2$, pH 7.4,) was added to each well, and incubated at 37 °C for 1 h. The plates were then washed 3 times with TBSTC and incubated for 1 h with Elite ABC kit (Vector Laboratories) in TBSTC. After washing, 100 μl SureBlue TMB microwell peroxidase substrate (Kirkegaad & Perry Laboratories) was applied to each well and incubated at room temperature for 15 min. Finally, we added 100 μl of 1 M phosphoric acid to stop the reaction and the absorbance at 450 nm was read with a model 680 microplate reader (Bio-Rad Lab.). For the analyses of the binding of heat-denatured biotin-CRP, CRP solution in PBS was heated in boiling water for 5 min before use. In some experiments a concentration (0–0.1 mM) of phosphocholine or 10 mM EDTA was added to the biotin-CRP in TBSTC and detected as described above.

2.8. Complement activation ELISA

An in vitro complement activation assay was performed as described previously [21]. Recombinant human CL-P1 (5 μg/ml) or heat inactivated BSA (5 μg/ml) was immobilized to each well of a 96-well immunoplate plate by incubating in a coating buffer overnight at 4 °C.

After 3 washes with PBS, the plates were blocked with 2% BSA in PBS at 37 °C for 1 h. After washing, the plates were incubated with 1% human complement serum/PBS (Sigma), C1q-depleted serum (Merck Millipore) or properdin depleted serum at 37 °C for 1 h with or without 20 μg/ml CRP (full serum), inactivated CRP (95 °C, 5 min), or CRP plus polymyxin B (5 μg/ml) in a veronal buffer (0.82 mM $MgCl_2$, 145.45 mM NaCl and 0.25 mM $CaCl_2$, 3.11 mM barbitol, 1.8 mM sodium barbitol) containing 0.1% gelatin. In some assays, C1q depleted serum and properdin depleted serum were supplemented with native C1q (200 μg/ml full serum) or purified properdin (6 μg/ml full serum) respectively. We detected C3d using 3.5 μg/ml rabbit anti-human C3d antibody (Dako) in combination with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:5000, Merck Millipore) diluted in PBS containing 1% BSA, 0.05% Tween 20. Finally we determined peroxidase activity with SureBlue TMB microwell peroxidase substrate as described above.

2.9. Terminal complement complex (TCC) deposition ELISA

For the TCC deposition assay we used the same procedure as described in the complement activation ELISA except that we used 10% CFH depleted serum. For inhibition of TCC formation, CFH depleted serum was supplemented with purified CFH (400 μg/ml full serum). We detected TCC using rabbit polyclonal anti-human C5b-9 antibody.

2.10. Complement activation on cultured cells

We performed the complement activation assay on cultured cells as previously described [13]. In brief, we transfected HEK293 cells seeded in collagen-coated dishes with pcDNA3.1 vector and CL-P1. After washing the cells with PBS, we added 10% human complement serum, C1q depleted serum, and properdin depleted serum in the presence or absence of 20 μg/ml CRP (full serum) in DMEM-high glucose medium. In some assays, C1q depleted serum or properdin depleted serum was replenished with native C1q (200 μg/ml full serum) or native properdin (6 μg/ml full serum). After incubation at 37 °C for 1 h, the cells were washed twice with PBS and fixed with 4% phosphate-buffered paraformaldehyde. We detected the deposition of C3 fragments on the cells by immunostaining with rabbit anti-human C3d antibody (3.5 μg/ml) in combination with Alexa Fluor 594 anti-rabbit IgG antibody. The nuclei of the cells were counterstained with Hoechst 33342. We performed quantitative analysis with the BZ-HIC program.

2.11. TCC formation assay

We performed the formation of the TCC on cell surfaces on cultured cells as previously described with few modifications [22]. Briefly, HEK293 cells were plated on collagen-coated dishes, in DMEM supplemented with 10% FBS. The next day, cells were transfected with pcDNA3.1 vector and CL-P1. 24 h after transfection cells were washed three times with PBS followed by incubation with a final concentration of 10% human complement serum or CFH depleted serum in the presence or absence of 20 μg/ml of CRP (full serum) at 37 °C for 1 h. In the case of recovery experiments, CFH depleted serum was supplemented with (400 μg/ml full serum) purified human CFH. Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min. After three washes in PBS, cells were incubated for 30 min at room temperature with rabbit polyclonal anti-human C5b-9 and anti-myc monoclonal antibody and then for 30 min with goat Alexa Fluor 594 anti-rabbit IgG antibody and goat Alexa Fluor 488 anti-mouse IgG antibody. The nuclei were counterstained with Hoechst 33342. Cells were imaged using a fluorescence microscope.

2.12. CFH and properdin recruitment assay

HEK293 cells were plated on collagen-coated dishes, in growth medium. Cells were transfected with pcDNA3.1 vector and CL-P1. 24 h after transfection cells were incubated with a final concentration of 10% human complement serum in the presence or absence of 20 μg/ml of CRP (full serum) at 37 °C for 1 h. Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min. Cells were then incubated for 30 min at room temperature with goat polyclonal anti-human CFH or goat anti-human properdin with anti-myc monoclonal antibody and then for 30 min with goat Alexa Fluor 488 anti-mouse IgG antibody or donkey Alexa Fluor 594 anti-goat IgG antibody. We imaged the cells using a fluorescence microscope.

2.13. Quantitative measurement of SC5b-9

HEK293 cells transfected as described above were incubated with 10% CFH depleted serum or CFH depleted serum supplemented with purified CFH (400 μg/ml full serum) in the presence or absence of

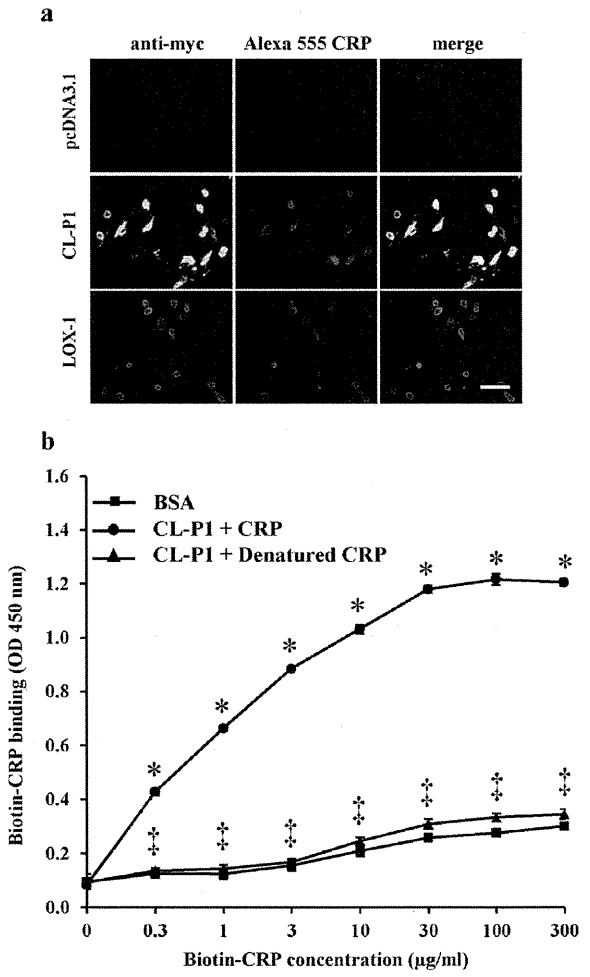

Fig. 1. CRP binds CL-P1 and LOX-1. (a) Alexa 555 labeled CRP binds CL-P1 and LOX-1. CHO/ldlA7 cells transiently transfected with pcDNA3.1 vector, CL-P1, and LOX-1 were incubated with Alexa 555-CRP (red) and then anti-myc antibody followed by Alexa 488 conjugated goat anti-mouse IgG (green), and were counterstained with Hoechst 33342 (blue). The analyses were performed in five independent experiments. (b) Soluble CL-P1 binds CRP. Biotin-CRP interacts with recombinant human CL-P1 dose dependently (●) but not with BSA (■). Denatured biotin-CRP showed no binding with CL-P1 (▲). The asterisks indicate significant differences vs BSA control (*$p < 0.0001$). The daggers indicate significant differences vs active CRP control (*$p < 0.0001$). Data are means ± S.D. ($n = 3$). Scale bars, 20 μm for all images. OD, optical density.

20 μg/ml of CRP (full serum). 1 h after incubation the medium was collected and the levels of SC5b-9 in the serum-containing medium were determined using a commercially available ELISA kit (Quidel Corporation).

2.14. Statistical analysis

Statistical analysis was conducted using the unpaired two-tailed Student's *t* test included in the JMP statistics software package (version 7, SAS). Data are mean ± S.E. $p < 0.0001$ is considered statistically significant.

3. Results

3.1. CRP interacts with CL-P1 and LOX-1

It has been reported that LOX-1 binds CRP in cell experiments and ELISA [10]. To investigate whether CL-P1 interacts with CRP, we inquired into the binding of Alexa Fluor 555-labeled CRP with CL-P1 and LOX-1 using transiently transfected CHO cells. As shown in Fig. 1a, Alexa 555-CRP binds CL-P1 and LOX-1 whereas no CRP binding was observed on pcDNA3.1 control vector transfected cells. The CL-P1 and LOX-1 were co-localized with CRP on the cell surface.

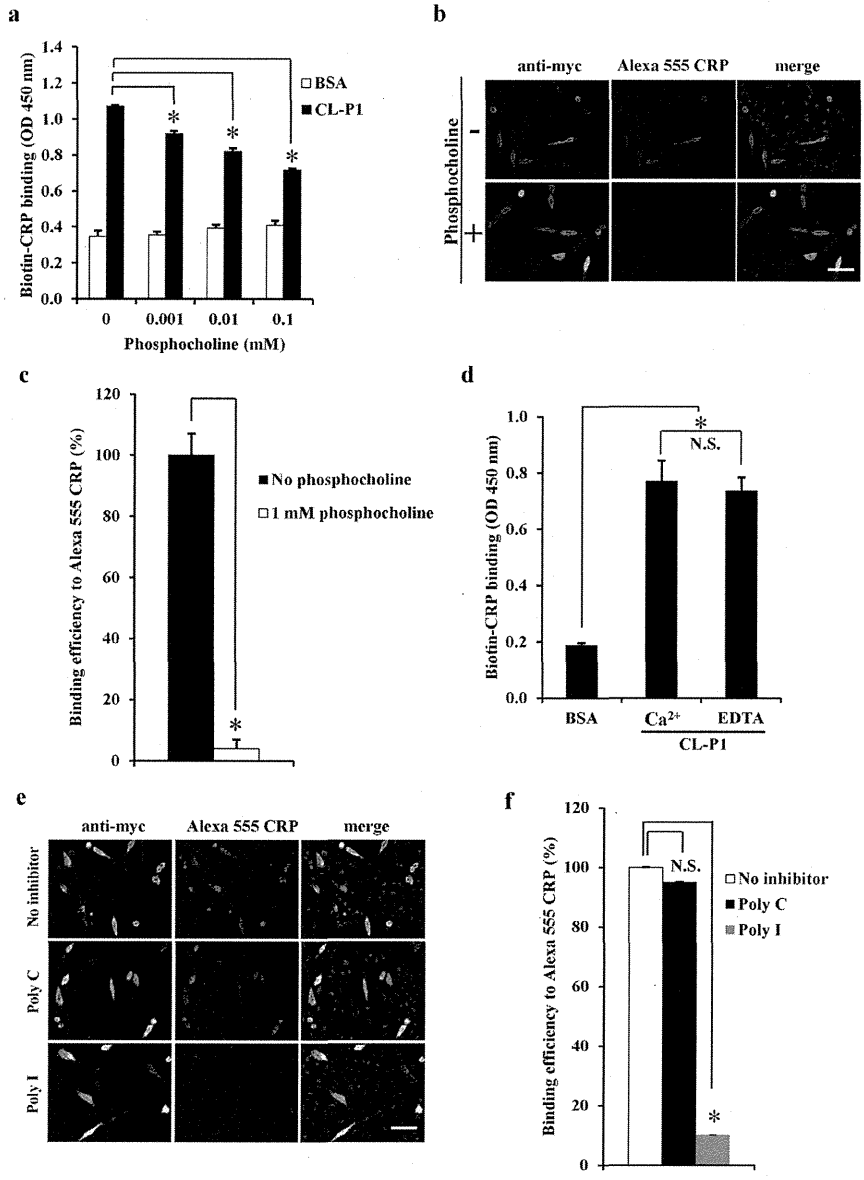

Fig. 2. Phosphocholine and poly(I) inhibit CRP and CL-P1 interaction on ELISA and cultured cells. (a) Competition of phosphocholine with biotin-CRP for the binding to recombinant CL-P1. Phosphocholine dose dependently inhibited biotin-CRP binding to CL-P1 in ELISA. Data are means ± S.D. (n = 3). (b) Phosphocholine inhibition assay using CHO/ldlA7 cells. Phosphocholine almost completely inhibits the interaction of Alexa 555-CRP (red) with CL-P1 (green). (c) Quantification of phosphocholine inhibition efficiency. The analyses were performed in three independent experiments. (d) Calcium independent binding of Biotin-CRP to CL-P1 in ELISA. Data are shown as mean ± S.D. (n = 3 independent repeat). (e) Poly(I) and poly(C) inhibition assay using CHO/ldlA7 cells. (f) Quantification of poly(I) and poly(C) inhibition efficiency. We imaged the cells using a Keyence microscope (KZ9000). Data are means ± S.D. of three independent experiments. *$p < 0.0001$. Scale bars, 20 μm for all images. N.S. — not significant; MFI, mean fluorescence intensity, OD, optical density.

We further characterized the CRP and CL-P1 interaction in the ELISA system. The presence of a soluble form of CL-P1 is still under debate. The soluble CL-P1 used in this study was prepared by forcing the extracellular domain to be a secreted protein and attaching an insulin leader peptide. We observed that biotin-CRP bound CL-P1 in a dose dependent manner (0–300 μg/ml) (Fig. 1b) and the heat-denatured biotin-CRP lost its ability to bind CL-P1.

3.2. Charge dependent interaction of CRP with CL-P1

It is known that CRP interacts with phosphocholine and activates the classical complement pathway [23]. The complement activation site in CRP is located in the A-face, whereas the phosphocholine binding site is in the B-face. We found that biotin-CRP and CL-P1 binding in ELISA was inhibited by phosphocholine in a dose dependent manner (0–0.1 mmol/L) (Fig. 2a). We demonstrated that incubation of a phosphocholine and Alexa 555-CRP mixture with CL-P1 transfected cells reduced the CRP binding to CL-P1 (Fig. 2b). Quantitative analysis indicates that 1 mM phosphocholine can completely inhibit the CRP binding (Fig. 2c). These results suggest that CL-P1 might interact with the B-face in CRP.

To check whether this binding of biotin-CRP and CL-P1 was calcium dependent, we performed an EDTA inhibition assay in ELISA which showed no significant decrease in biotin-CRP and CL-P1 binding (Fig. 2d). These results suggest that the interaction of CL-P1 with CRP might be calcium independent.

We further checked whether the interaction of CL-P1 with CRP is charge dependent. The pre-incubation of Poly(I) with the CL-P1 transfected cells before the addition of Alexa 555-CRP reduced CRP binding to CL-P1, whereas poly(C) did not reduce the binding (Fig. 2e and f). Therefore we concluded that the interaction of CL-P1 with CRP might be mainly charge dependent.

3.3. CL-P1 interacts with CRP mainly through the collagen-like and coiled-coil domain

We prepared several CL-P1 deletion mutants to find out the binding domain with different ligands as previously described [19] (Fig. 3a). We confirmed that MIF and western blotting analysis for the expression of full length and deletion mutants of CL-P1 showed almost similar expression levels and patterns on the cell surface except Δcc-col-CRD [19]. We found that the collagen-like domain and coiled-coil domain were involved in CL-P1 and CRP interaction (Fig. 3b and Supplemental Fig. 1). The deletion of CRD could not inhibit the interaction. There are three positively charged clusters in the collagen-like domain of human CL-P1 [19]. To verify which positive cluster was involved in the CL-P1 and CRP interaction, we prepared the positively charged cluster mutants (Fig. 3c). We then performed the binding experiments of Alexa 555-CRP with CL-P1 positively charged cluster mutants. Our results showed that cluster I and cluster III in the collagen-like domain of CL-P1 were involved in the interaction with CRP (Fig. 3d and Supplemental Fig. 2). These results also show that the interaction between CRP and CL-P1 takes place in a charge dependent manner but does not show Ca^{2+} dependent lectin activity, although the detailed mechanism is still unknown.

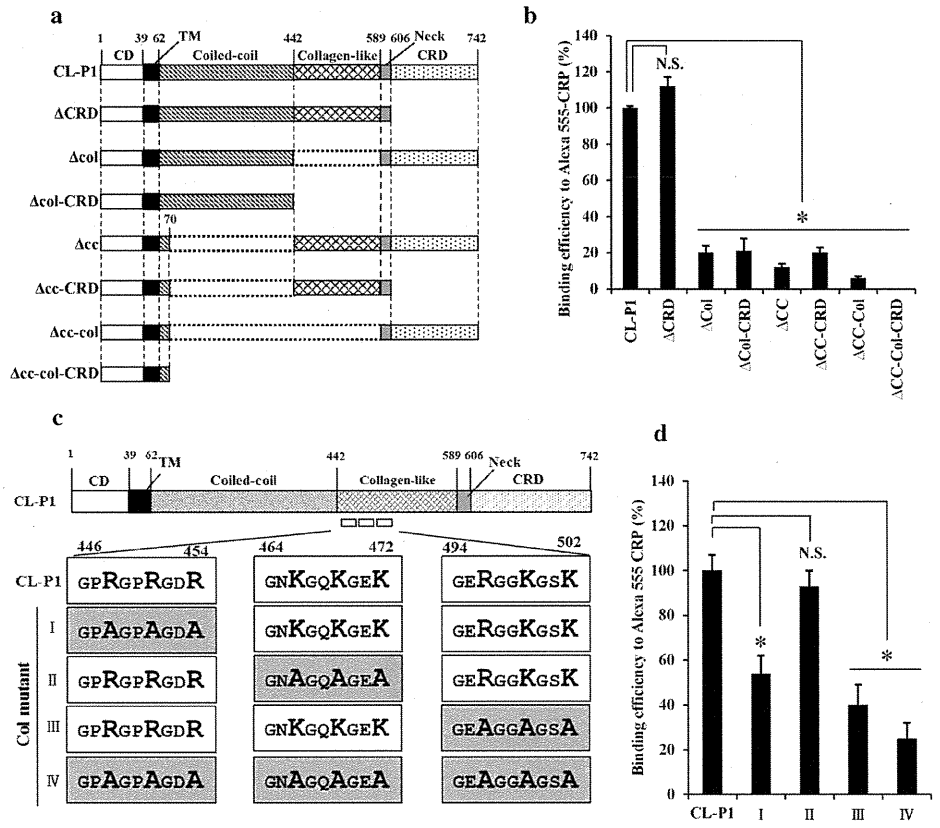

Fig. 3. CL-P1 interacts with CRP mainly through a collagen-like and coiled-coil domain. (a) Schematic diagram showing the structure of CL-P1 deletion mutants. (b) Signal intensity for the binding of CRP with CL-P1 deletion mutants. (c) Schematic diagram showing the structure of CL-P1 positively charged cluster mutants. (d) Signal intensity for the binding of CRP with positively charged cluster mutants. The first and third positive cluster mutants show lower affinity to CRP. The results shown are representative of three independent experiments. *$p < 0.0001$. N.S. — not significant; MFI, mean fluorescence intensity.

3.4. CRP and CL-P1 interaction activates the classical complement pathway

It is reported that the interaction of LOX-1 with CRP mediates complement activation [9]. We tried to detect the interaction of CL-P1 with the CRP activating complement pathway. We examined the complement activation using CRP and recombinant human CL-P1 in an ELISA system. We found that there was complement activation only in the recombinant CL-P1-coated wells but boiled CRP failed to activate the complement system (Fig. 4a). Supplementation of polymyxin B did not affect complement activation (Fig. 4a). These data suggest that the complement activation observed therein is an effect of C-reactive protein in a CRP solution, but not caused by lipopolysaccharide.

Next, we determined the deposition of C3 fragments using CL-P1 expressing HEK293 cells from a human embryonic kidney cell line. The C3 fragments were deposited on CL-P1 in a CRP positive condition (Fig. 4b), although there were some non-specific depositions of C3 fragments. We observed that the degree of specific C3 fragment deposition was 78% on CL-P1 expressing cells and unspecific deposition was 22% in these images. Phase contrast images showed the edges of the cells and that the deposition of C3 fragments occurs all over the surface of CL-P1 expressing cells (Fig. 4b). The signal intensity of the deposition of C3 fragments was increased in CL-P1 expressed cells compared with those of control pcDNA3.1 when human complement serum was used (Fig. 4c). It is known that CRP can interact with the C1q [19]. Next, we tested the possible involvement of C1q in the complement activation in our system. Our ELISA analysis demonstrated that the deposition of C3 fragments occurred in the presence of CRP and C1q (Fig. 5a).

We then analyzed the co-staining of the C3 fragments and C1q. Our results clearly demonstrate the co-staining of C3 fragments and C1q on the cell surface (Fig. 5b). Next, we focused in the deposition of C3 fragments using C1q depleted serum in our HEK293 cell system. In absence of C1q, complement activation was not detected regardless of the presence or absence of CRP (Fig. 5c and d). Supplementation of C1q recovered the deposition of the C3 fragments and it indicates the interaction of C1q and CRP in this system (Fig. 5c and d).

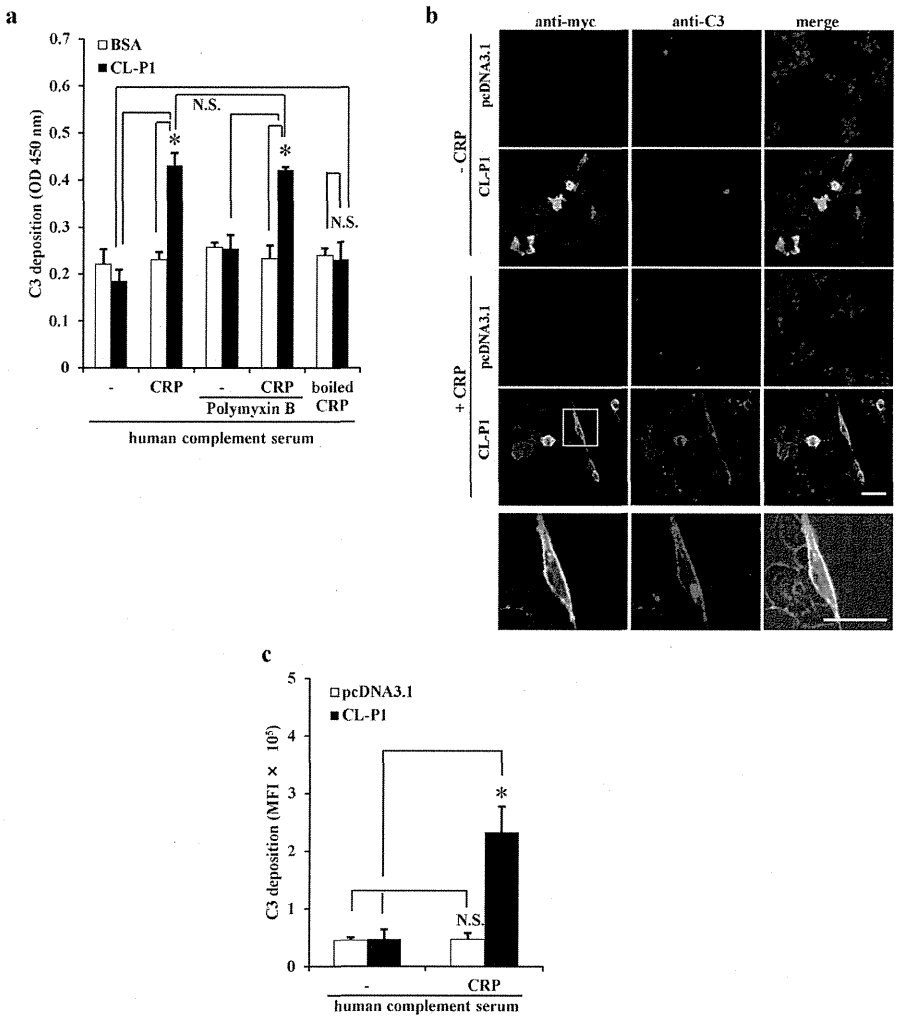

Fig. 4. Interaction of CRP with CL-P1 mediates complement activation on ELISA and cultured cells. (a) Complement activation by CRP (20 μg/ml) on recombinant human CL-P1 or BSA immobilized on ELISA using human complement serum. Data are means ± S.D. (n = 3). (b) Complement activation detected by the deposition of C3 fragments (red) on HEK293 cells. The anti-myc staining showed the CL-P1 expression (green) and cell nuclei were counterstained with Hoechst 33342 (blue). Phase contrast images (×40) clearly show the co-localization of CL-P1 and C3 fragments on the surface of the cells. (c) Fluorescence intensity of the deposition of C3 fragments using human complement serum. Values represent the mean of three independent experiments. *$p < 0.0001$. Scale bars, 20 μm for immunofluorescence and phase contrast images. OD, optical density; N.S. — not significant; MFI, mean fluorescence intensity.

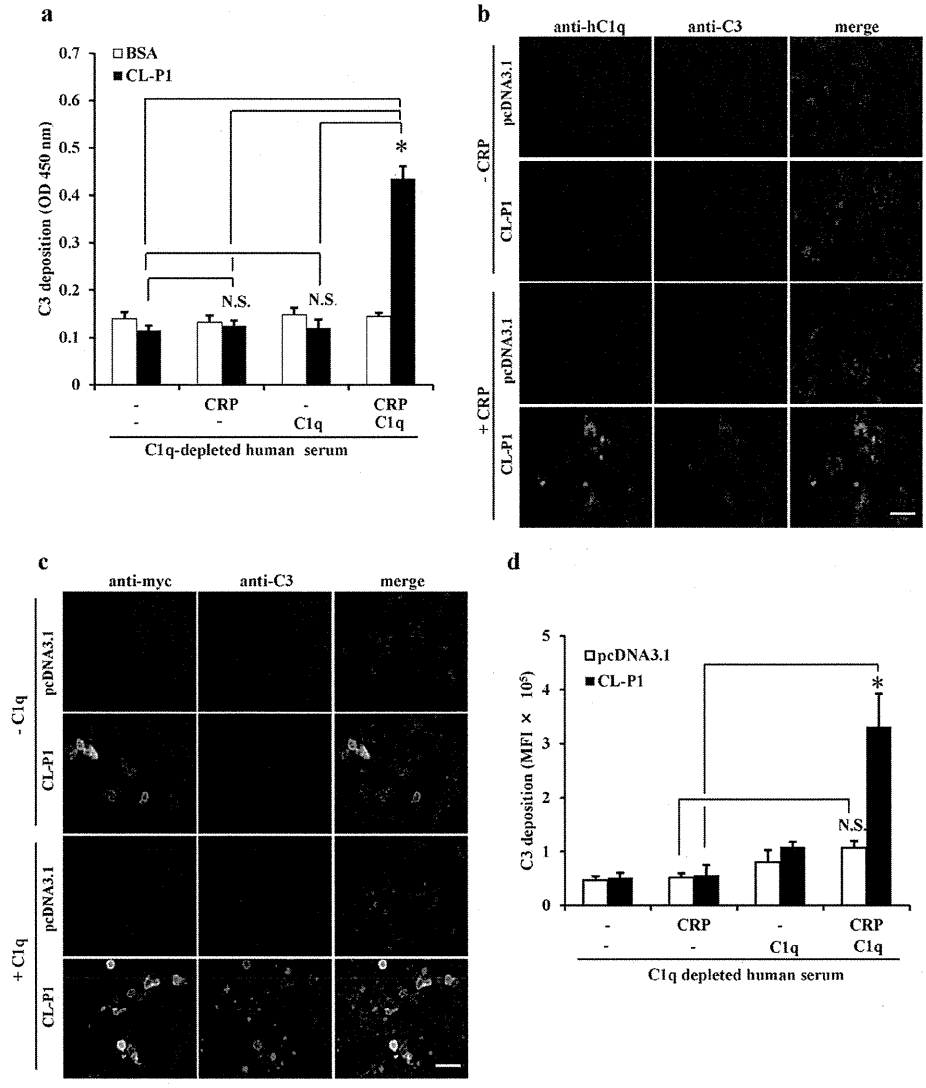

Fig. 5. Classical pathway activation by CRP via CL-P1. (a) CRP (20 μg/ml) induced complement activation on recombinant human CL-P1 or BSA immobilized on an ELISA using C1q depleted human serum with/without the complementation of native C1q. Data are means ± S.D. (n = 3). (b) C3 fragments (red) and C1q (green) co-staining on HEK293 cells. Results are from two independent experiments. (c) Depletion of C1q suppressed the C3 deposition (red) on HEK293 cells depending on CL-P1 (green) and CRP. (d) Mean fluorescence intensity of the deposition of C3 fragments using C1q depleted serum in presence or absence of CRP. Data are means ± S.D. (n = 3). *$p < 0.0001$. Scale bars, 20 μm for all images. OD, optical density; N.S. – not significant; MFI, mean fluorescence intensity.

3.5. CRP induces the additional activation in the amplification pathway via CL-P1

Activation of the classical pathway inevitably initiates the alternative pathway. To determine whether CRP plays a role in classical pathway-triggered alternative pathway complement amplification, we used ELISA and found a significant decrease in the deposition of C3 fragments which was recovered when purified properdin was added back to the properdin depleted serum (Fig. 6a). We then confirmed the recruitment of properdin on the cell surface in the presence of CRP depending on CL-P1 (Fig. 6b). We next determined, to what degree classical pathway-triggered alternative pathway complement activation depends on properdin in our assay system. The deposition of C3 fragments was observed even using properdin depleted serum and the supplementation of purified properdin increased the C3 fragments deposition intensity (Fig. 6c and d). Purified properdin apparently has a propensity to form aggregates. So we took extreme care while using purified properdin. Commercially available properdin forms higher oligomers or aggregates upon repeated freezing and thawing were originally called "activated" properdin due to their ability to promote complement activation and consumption when added to serum. So, we avoided repeated freezing and thawing of purified properdin and used in experiments upon receipt to minimize aggregation of the properdin that can occur with prolonged storage. Thus, the interaction of CRP with CL-P1 basically activates the C1q dependent complement pathway and furthermore performs the additive amplification using the alternative pathway.

3.6. CRP negatively regulates TCC assembly on CL-P1 expressing cells by recruiting CFH

To determine the effect of CRP on downstream complement components, we examined the assembly of the TCC on CL-P1 expressing cells. As shown in Fig. 7a, we found no TCC formation using human complement serum. It is reported that CRP drives the classical pathway of the

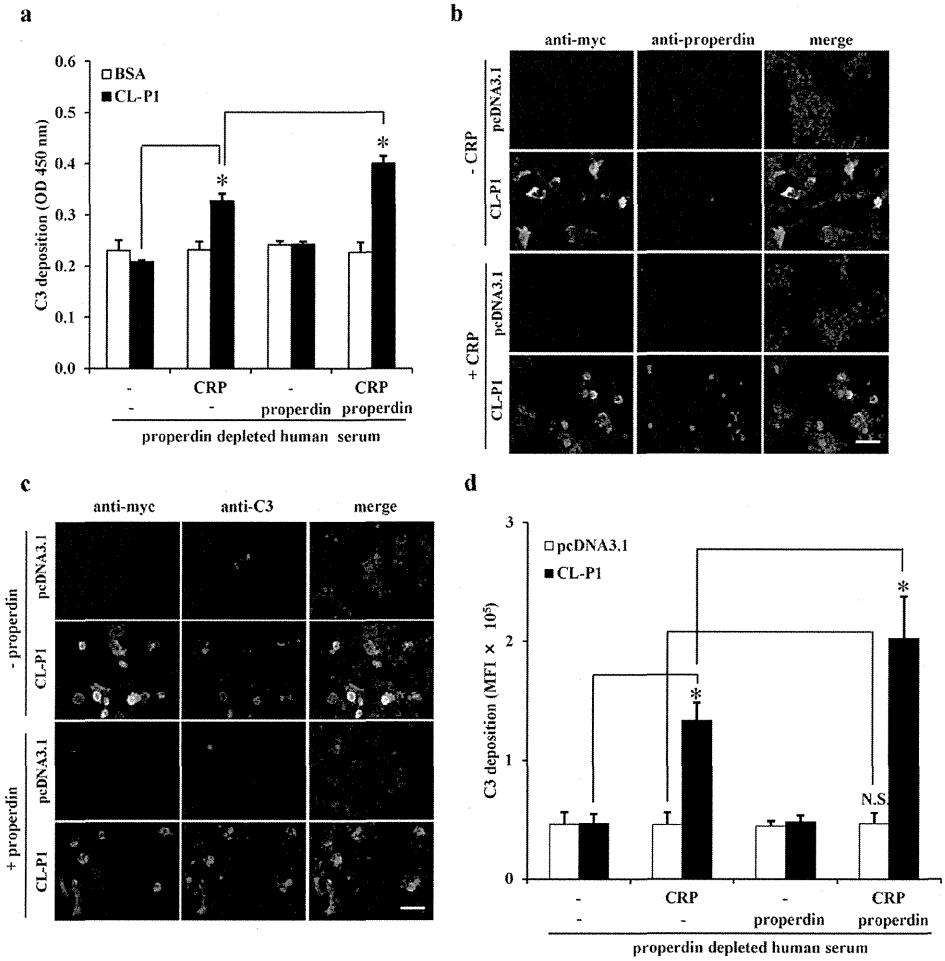

Fig. 6. CRP additionally activates Classical pathway-triggered alternative pathway amplification on CL-P1. (a) Properdin depleted serum shows lower deposition of C3 fragments. Properdin depleted serum replenished with native properdin recovered the deposition of C3 fragments on the cells. (b) Properdin (red) was recruited only in CL-P1 (green) and CRP positive conditions on HEK293 cells. Results are representative images from three independent experiments. (c) Mean fluorescence intensity of the deposition of C3 fragments using properdin depleted serum with/without supplementation of properdin. Cell nuclei were counterstained with Hoechst 33342 (blue). Data are means ± S.D. of three independent experiments. $^{*}p < 0.0001$. Scale bars, 20 μm for all images. N.S. — not significant; MFI, mean fluorescence intensity.

complement on nucleated cells without TCC formation or causing cytolysis [24]. Other studies have shown that CRP bound to CFH [25,26], a complement regulatory protein that accelerates the decay of the C3 and C5 convertases and inhibits the assembly of the terminal complement components [27]. Our results demonstrated the binding of CFH with CRP using human complement serum depended on CL-P1 (Fig. 7b). To determine whether CFH was required for the prevention of the TCC formation, we incubated CL-P1 expressing cells with CFH depleted serum with or without CRP. CFH depleted serum failed to prevent the TCC deposition (Fig. 7c). This inhibitory activity could be restored by the addition of CFH (Fig. 7c). Phase contrast images clearly demonstrated the pattern of TCC deposition on the surface of CL-P1 expressing cells (Fig. 7c). We found that a similar phenomenon occurs in the ELISA system (Fig. 7e). Next, we analyzed the soluble TCC (SC5b-9) formation in the serum-containing culture medium 1 h after TCC formation reaction. The level of SC5b-9 significantly increased in the serum-containing medium after the reaction of CL-P1 transfected cells including CRP compared with no CRP addition (Fig. 7d). Therefore, we concluded that the recruitment of CFH by CRP is required for the prevention of the TCC assembly on CL-P1 expressing cells (Fig. 8).

4. Discussion

CRP is an acute phase protein involved in complement activation through the classical pathway [3]. Recently CRP has shown to bind LOX-1 and induce complement activation [9]. However the mechanism by which CRP induces complement activation is poorly understood. Our study aimed to identify whether collectin CL-P1 can interact with CRP. Here, we demonstrated that CRP might be a novel ligand for CL-P1 and mediate some biological effects through complement activation.

Fujita et al. reported that LOX-1 interacted with CRP in a calcium dependent manner as EDTA completely ended the interaction [9]. In the case of CL-P1, EDTA was unable to inhibit the interaction of recombinant CL-P1 with CRP and this suggests that the interaction of CL-P1 and CRP might be calcium independent. It was proposed that LOX-1 interacted with the B-face of CRP since recombinant LOX-1 and CRP interaction was inhibited by phosphocholine [9]. Two inhibition studies using phosphocholine in ELISA and cell experiments indicate that CL-P1 might also interact with the B-face of CRP.

Next, we focused on the inhibition effects by polycations or polyanions on the interaction of CRP with CL-P1. It was shown that

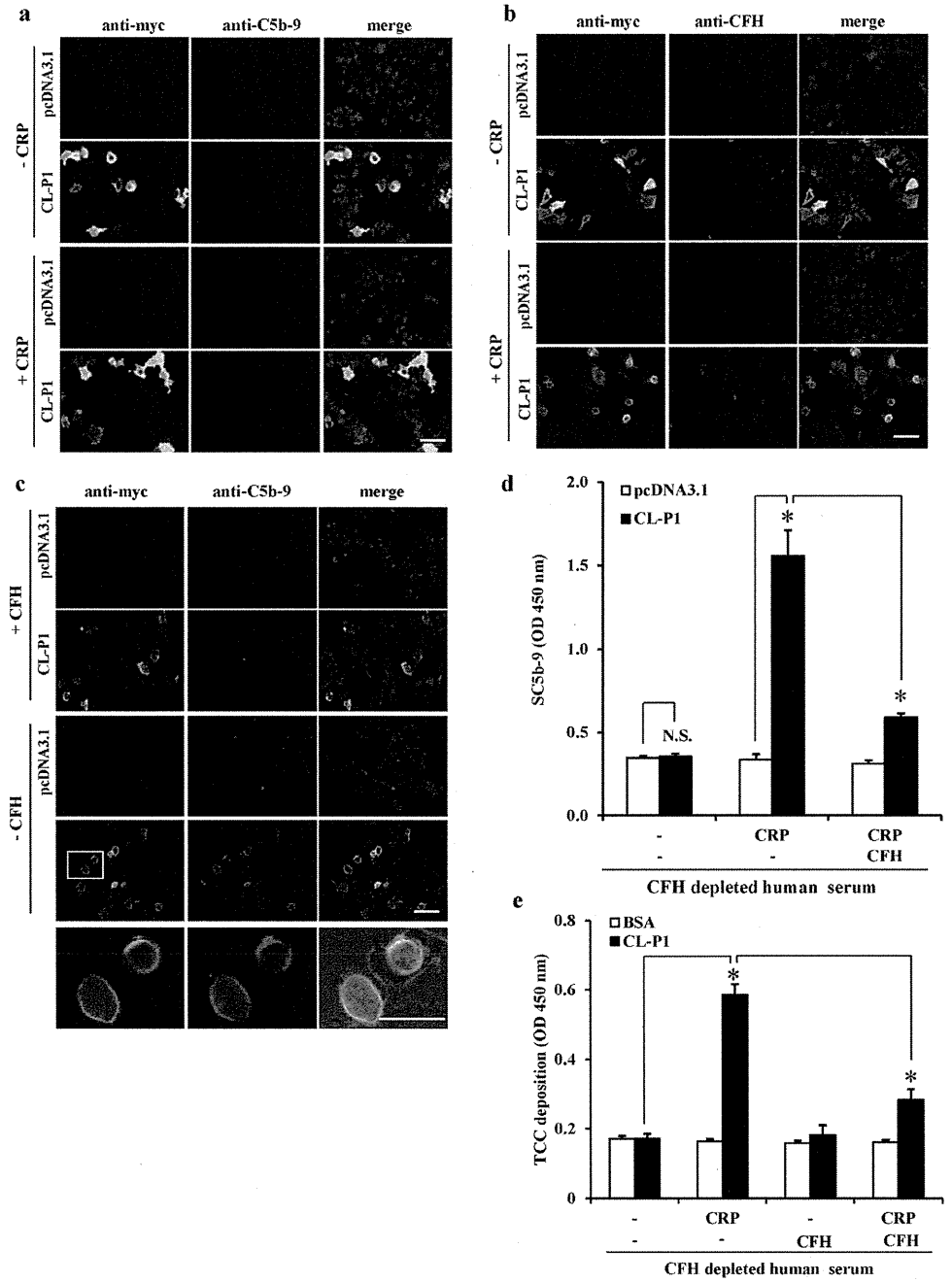

Fig. 7. CFH prevent the TCC assembly on CL-P1 expressing cells through CRP. (a) Complement activation of the TCC assembly was not detected by C5b-9 depositions (red) on HEK293 cells using human complement serum and CRP. The anti-myc staining showed the CL-P1 expression (green) and cell nuclei were counterstained with Hoechst 33342 (blue). Representative images of three independent experiments are shown. (b) CFH (red) was recruited only in CL-P1 (green) and CRP positive conditions on HEK293 cells. The experiment shown is representative of three independent assays. (c) CFH depleted serum failed to prevent TCC formation (red). Phase contrast images (×40) show the TCC formation on the surface of the cells. (d) SC5b-9 formation in medium of Fig. 7c was detected by ELISA. CFH depleted serum induced the SC5b-9 formation which can be inhibited by the addition of CFH. (e) TCC deposition was detected by ELISA. Depletion of CFH failed to prevent CRP dependent TCC formation. The result was reproduced in three independent experiments. Scale bars, 20 μm for immunofluorescence and phase contrast images.

polycations bound CRP on the B-face [28,29]. Our microscope data showed that the interaction was completely inhibited by the pre-incubation of polyanions but not by polycations before the addition of CRP in CL-P1 expressed cells. This could be explained by the neutralization of a positive charge on CL-P1 by polyanions that inhibit the interaction with CRP. Lee et al. has found phosphocholine can apparently bind cationic sites on CRP and the binding site does not overlap with the polycationic binding site [30]. Therefore we hypothesize that CL-P1 might interact with the B-face of CRP in a charge dependent manner.

The site directed mutagenesis study has shown that the OxLDL and CRP binding site on LOX-1 is different but shares some common features because a carrageenan and anti-LOX-1 antibody were able to act as competitors to both [31]. Recently, we have shown that the collagen-like domain of CL-P1 is responsible for the interaction with OxLDL and

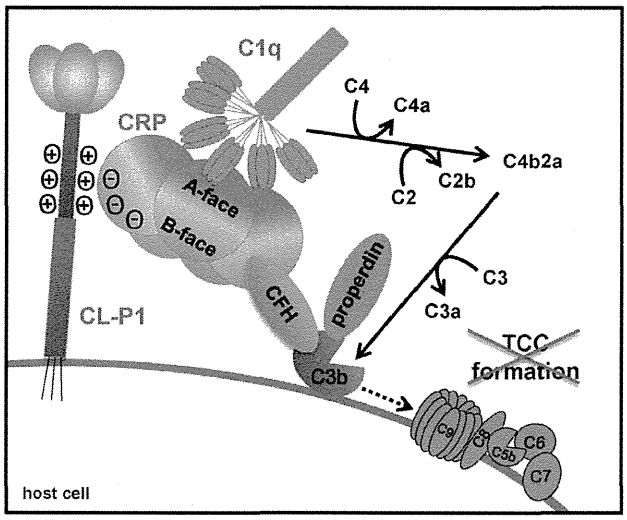

Fig. 8. Proposed mechanism for CRP and CL-P1 mediated complement regulation. CRP is composed of 5 subunits and has an A-face and B-face. First, CL-P1 traps CRP on the B-face. Next, C1q binds to the A-face and activates the complement system through the classical and properdin mediated amplification pathway. The binding of CRP to CL-P1 also recruits CFH, which reduces the downstream activation of the complement and might inhibit the assembly of terminal complement complex (TCC).

microbes using CL-P1 deletion mutants [19]. Our results also suggest that the collagen-like domain and coiled-coil domain of CL-P1 are involved in CRP binding. The study using mutants of the positively charged cluster in the collagen-like domain of CL-P1 revealed that the first and third positively charged clusters are involved in interaction with CRP. However, previous mutant experiments demonstrated that the second and third charged clusters were important for the binding of OxLDL and microbes [19]. These results suggest that the third positively charged cluster of CL-P1 is an important domain for CRP and OxLDL binding.

CRP activates the complement system in human and mouse serum because CRP is known to bind C1q and activate pathways through its A-face [32]. Here, we prepared a complement activation system using human complement serum and human cell line HEK-293 cells. Our Figs. 4 and 5 also demonstrate that the interaction of CRP with CL-P1 mediates complement activation depending on C1q as a classical pathway without antibodies. The deposition of C3 fragments using soluble CL-P1 is low in our ELISA analysis. This is could be due to the randomness of the orientation of the soluble CL-P1 in the ELISA plate.

Activation of the alternative pathway can occur secondary to classical pathway activation or be initiated independently. CRP has been shown to enhance the alternative pathway activation via the C3 convertase generation and function with it as a pre-antibody host defense mechanism [33]. Properdin plays an important role in the activation of the alternative pathway not only by maintaining the stability of C3bBb, but also by providing a platform for the de novo C3 convertase assembly [34]. Our results suggest that the interaction of CRP with CL-P1 on the cells first activates the classical pathway through C1q and next drives the alternative pathway as an amplification loop using properdin (Fig. 6). We believe that this auto-activation of the complement on the body's own cells only takes place if a very high concentration of CRP exists in the serum. Furthermore, Ma et al. have reported that the interaction of soluble CL-P1 and properdin activates the alternative pathway and reacts effectively with *A. fumigatus*. [20]. Usually, CL-P1 is expressed in vascular endothelial cells but a natural soluble form of CL-P1 has been found in human umbilical cord plasma [20]. Although the soluble CL-P1 was not clearly detected in normal adult venous plasma, CRP might collaborate with soluble CL-P1 for pathogen recognition and complement activation.

Fujita et al. could not demonstrate the final stage of the CRP and LOX-1 mediated complement activation. It has been found that CRP activates the classical pathway on nucleated cells without activating the TCC or causing cytolysis [24]. CRP has been shown to bind apoptotic cells and protects the cells from assembly of the TCC by recruiting CFH [35]. Our results also demonstrated that the complement activation by CRP and CL-P1 interaction could not form the TCC. Our data show that depletion of CFH allows TCC formation which was prevented when CFH was added back to the CFH depleted serum (Figs. 7), although the interaction of CL-P1 and CRP initiates complement activation. We suggest that, the complete CFH level in the serum might be able to suppress terminal complement activation even if acute inflammation or injury occurs with up-regulation of CRP. Thus, the interaction of elevated levels of CRP with CL-P1 could be disastrous in a CFH compromised condition. Furthermore, another study showed no enhanced CFH binding or any protective effect of CRP regarding C9 deposition [36]. Why these observations differ from some other reports may relate to the quality of proteins and the semantics regarding the stages of cells.

The role of CRP in cardiovascular disease is controversial [37,38]. For example, reports of atherosclerosis resulting in apolipoprotein E deficient mice overexpressing CRP were conflicting, with one study finding a positive relationship between CRP and cardiovascular disease [39], whereas other studies did not [40,41]. Our study suggests that in CFH dysfunctional individuals, CRP might augment endothelial injury by activating the complement via CL-P1 to initiate cardiovascular disease.

Du Clos et al. have found that local production and activation of the complement has an important role in I/R injury and allograft rejection [42]. By localizing at sites of tissue damage CRP has the potential to contribute to the complement activation at these sites, but CRP may also regulate this activation by its interaction with CFH depending on CL-P1.

In this paper, we found an important role of collectin CL-P1 in the limited activation of the complement system induced by the acute phase level of CRP. These results might provide a novel insight into the involvement of CL-P1, CRP and complement factors in complement related diseases. Of course, future studies are needed to characterize more details of the CFH-CRP-CL-P1 interaction and their relationship to diseases.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2016.02.012.

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Immediate suppression of seizure clusters by corticosteroids in PCDH19 female epilepsy

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ABSTRACT

Purpose: The pathomechanism and treatment of *PCDH19* female epilepsy (*PCDH19*-FE) remain unclear. Here, we report that corticosteroids are effective for control of the seizure clusters or other acute symptoms of *PCDH19*-FE and argue for the possible involvement of a compromised blood-brain barrier (BBB) in its pathogenesis.
Methods: The efficacy of corticosteroids was retrospectively reviewed in five Japanese patients with *PCDH19*-FE. The results of antibody assays against the *N*-methyl-D-aspartate-type glutamate receptor (abs-NR) in serum/cerebrospinal fluid were also compiled.
Results: Corticosteroid treatments significantly improved the acute symptoms, including seizure clusters, in all cases, most often immediately after the initial administration. However, the effect was transient, and some seizures recurred within a few weeks, especially in association with fever. Serum and/or cerebrospinal fluid abs-NR were detected in all patients. Target sequences of the detected antibodies were multiple, and the titers tended to decrease over time. In one patient, immunohistochemical analysis using rat hippocampal slices also revealed serum antibodies targeting an unknown epitope in neuronal cytoplasm.
Conclusion: Our findings imply an involvement of inflammatory processes in the pathogenesis of *PCDH19*-FE and therapeutic utility for corticosteroids as an adjunctive option in acute treatment. *PCDH19* is well expressed in brain microvascular endothelial cells and thus its impairment may cause BBB vulnerability, which may be ameliorated by corticosteroids. The abs-NR detected in our patients may not indicate an autoimmune pathomechanism, but may rather represent non-specific sensitization to degraded neuronal components entering the general circulation, the latter process facilitated by the BBB vulnerability.

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1. Introduction

A heterozygous defect in the gene encoding protocadherin 19 (*PCDH19*) causes early-onset intractable epilepsy in females (*i.e.*, *PCDH19*-related female epilepsy, *PCDH19*-FE, or previously, epilepsy and mental retardation limited to females, EFMR) [1]. PCHD19, an adhesion molecule of the δ2-protocadherin subclass of the cadherin superfamily, is highly expressed in the vertebrate brain. δ-Protocadherins are intimately involved in brain development and neural functions, as well as in many neurological diseases [2]. However, the homophilic adhesion capacity of PCDH19 by itself is low, and its exact function remains unclear.

The hallmark clinical feature of *PCDH19*-FE is recurrent seizure clusters consisting of brief focal seizures and/or generalized convulsions, which can be triggered by febrile or afebrile illnesses [3]. The seizures do not recur regularly, but once they recur, the cluster continues for days to weeks despite multiple treatments. Conventional antiepileptic drugs fail to control or prevent most of these seizures. Ictal symptoms and EEG findings indicate that the seizures mainly involve the limbic system and medial frontal region [4].

The clinical features indicate a possible immune/inflammation involvement in seizure generation, which could be a non-genetic modifier of the disease phenotype. In agreement with this, we have previously reported patients showing excellent efficacy of corticosteroids for seizure clusters [5]. We have also encountered cases having antibodies to the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor (abs-NR) in the serum or cerebrospinal fluid (CSF). Abs-NR cause limbic encephalitis, predominantly in young women (anti-NMDA receptor encephalitis) [6], but may also appear secondarily and non-specifically in various neurologic diseases including epilepsy [7]. In the latter case, abs-NR are not significantly involved in the disease pathogenesis.

This study aims to explore whether corticosteroids have an ability to improve the seizures in *PCDH19*-FE and if any immune mechanism is involved. We retrospectively reviewed and summarized the clinical results of corticosteroid treatments as well as the results of an assay for abs-NR in Japanese patients. The potential significance of these findings with regard to the pathomechanisms of this disorder is also discussed.

2. Methods

2.1. Patients

Japanese patients with *PCDH19*-FE who received corticosteroid treatments and/or underwent the ab-NR assay were retrospectively studied. They were genetically diagnosed at Fukuoka University[5] and clinical details were collected from their doctors in charge. Since the patients were children, the doctors obtained written informed consent from the parents before the blood and/or CSF samples were drawn for genetic analysis of *PCDH19* and/or for assay for ab-NR. Genetic analysis of *PCDH19* was approved by the ethics committee of Fukuoka University. The ab-NR assay was approved by the National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders.

2.2. ab-NR assay

The ab-NR assay was performed at National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders. Experimental details have been described elsewhere [8]. Briefly, serum and CSF samples were examined by enzyme-linked immunosorbent assay (ELISA) or, in one patient, by immunoblot analysis targeting GluN2B. The ELISA target peptides were the extracellular N-terminal (NT) and/or intracellular C-terminal (CT) regions of the GluN2B, GluN1, and GluD2 subunits (Supplementary Table 1). Titers were determined by comparing the optical densities of our patients with those of 35 patients with non-inflammatory focal epilepsy, who served as controls. The results were expressed as number of standard deviations of the controls (SD) from the mean of the controls and were considered positive when $\geq$2 SD.

2.3. Immunohistochemical analysis for anti-neuronal autoantibodies

In two patients, the presence of anti-neuronal autoantibodies was further examined immunohistochemically using rat hippocampal slices exposed to serum or CSF from the patients. Experimental details are described in Supplementary information.

3. Results

Five patients (Patients 1–5) received corticosteroid treatments mainly during the acute phase before and/or after the diagnosis of *PCDH19*-FE. Abs-NR were examined in these patients mostly before corticosteroid administration and in four other patients (Patients 6–9). These tests were performed because autoimmune or inflammatory processes were clinically suspected in the pathogenesis of the epilepsy, although none of these patients except Patient 4 showed pleocytosis or an elevation of CSF protein level. Outlines of Patients 1, 2, and 5–9 have been described previously [3,5].

3.1. Efficacy of corticosteroid treatments

Treatment details and results are summarized in Table 1. The details of the clinical courses are described in Supplementary information. Overall, corticosteroids dramatically improved acute neurological symptoms: ongoing seizure clusters in Patients 1–3 and 5, and an acute encephalopathic episode that developed after a seizure cluster in Patient 4, were controlled. In most cases, the improvement was achieved after the first administration. In the cases of seizure cluster, the initial administration was conducted well in advance of the expected time of spontaneous remission of the cluster. Furthermore, in Patient 3, corticosteroids were initiated on the second day of each cluster to confirm that the cluster continued for more than 1 day despite midazolam administration. Regarding drugs and dosages, four young patients (Patients 1–4) received an intravenous drip infusion of 10–30 mg/kg methylprednisolone once daily, for up to 3 days. Patient 5 received an intravenous infusion of 0.35 mg/kg prednisolone once or twice, depending on the cluster, followed by oral administration of prednisolone at 1 mg/(kg·day) at age 11. However, as observed in Patients 1, 3, and 5, the effect was fundamentally transient; seizure clusters often recurred within a few weeks, especially when fever appeared.

For Patient 4, methylprednisolone was used at age 1 for an encephalopathic episode with decline of consciousness and systemic weakness, which abruptly developed 3 days after the termination of a one-day seizure cluster. EEG showed an increase of δ-waves, but no ictal activity. Mild CSF pleocytosis (85 cells/μL) was identified a week before the episode. These symptoms completely disappeared immediately after the initial administration of methylprednisolone.

For Patient 1, corticosteroids were administered prophylactically after age 3, with 3 days of oral betamethasone or prednisolone administration at times of fever appearance. After starting this treatment, no or only mild recurrences (not requiring hospitalization) were observed, even during fever.

Table 1
Details and efficacy of corticosteroid therapy.

Pt no	*PCDH19* mutation	Age at onset (m)	Age at CS TX	CS	Route & dose	Target symptom	Simultaneous TX	Usual duration of Sz cluster	Result	Present intellect
1	p.L719*	13	2y4m	mPSL	IV, 30 mg/kg, 3d	Sz cluster	MDL CBZ CZP VPA LTG LEV	Days ~2 wk	Disappeared after 1st IV	Normal 5y1m
			2y10m	mPSL	IV, 30 mg/kg, 3d	Sz cluster			Disappeared after 1st IV Recurred in 2 wk w/fever[a]	
			2y11m	mPSL	IV, 30 mg/kg, 3d	Sz cluster			Disappeared after 1st IV Recurred in 1wk w/fever	
			3y0m	mPSL	IV, 10 mg/kg, 3d	Sz cluster			Disappeared after 1st IV	
			3y4m	BET	Oral, 0.01 mg/kg, 3d When fever appeared	Sz prevention			No or mild recurrence	
			4y1m	PSL	Oral, 1–1.5 mg/kg, 3d When fever appeared	Sz prevention			No or mild recurrence	
2	p.K120Rfs*3	10	10m	mPSL	IV, 30 mg/kg, 3d	Sz cluster	MDL PB ACV IVIG EDV	–	Disappeared after 1st IV Recurred in 1wk	Moderate delay 3y
3	p.D417H p.D596Y	5	1y11m	mPSL	IV, 20 mg/kg, 2d	Sz cluster	MDL fPHT CLB LEV KBr DZP	Days ~2wk	Disappeared after 1st IV	Normal 2y8m
			2y1m	mPSL	IV, 20 mg/kg, 3d	Sz cluster			Disappeared after 1st IV	
			2y2m	mPSL	IV, 20 mg/kg, 2d	Sz cluster			Disappeared after 1st IV	
			2y5m	mPSL	IV, 10 mg/kg, 1d *fol. by* 20 mg/kg, 1d	Sz cluster			Disappeared after 2nd IV	
			2y7m	mPSL	IV, 20 mg/kg, 1d	Sz cluster			Disappeared after 1st IV Recurred in 9d w/flu	
			2y7m	mPSL	IV, 20 mg/kg, 2d	Sz cluster			Disappeared after 1st IV	
4	p.D596G	6	1y0m	mPSL	IV, 30 mg/kg, 3d	Encephalopatic symptoms	CBZ fPHT LDC PB	1d	Disappeared after 1st IV	Hyperactive 1y6m
5	p.D45Gfs*43	8	11y5m	PSL	IV, 0.35 mg/kg x1 *fol. by* Oral, 1 mg/kg[b]	Sz cluster	KBr CZP	Half a day	Disappeared after 1st IV	Moderate delay 11y8m
			11y6m	PSL	IV, 0.35 mg/kg x1 *fol. by* Oral, 1 mg/kg	Sz cluster			Disappeared after 1st IV Recurred in 1wk w/fever	
			11y6m	PSL	IV, 0.35 mg/kg x1 *fol. by* Oral, 1 mg/kg	Sz cluster			Disappeared after 1st IV	
			11y8m	PSL	IV, 0.35 mg/kg x2 *fol. by* Oral, 1 mg/kg	Sz cluster			Disappeared after 2nd IV	

[a] Noted when seizures recurred within 3 weeks after corticosteroid administration.
[b] In Patient 5, oral prednisolone was gradually tapered off. Pt no, Patient number; m, month(s); CS, corticosteroid; TX, treatment; Sz, seizure; y, year(s); mPSL, methylprednisolone; BET, betamethasone; PSL, prednisolone; IV, intravenous route; d, day(s); MDL, midazolam; CBZ, carbamazepine; CZP, clonazepam; VPA, valproic acid; LTG, lamotrigine; LEV, levetiracetam; wk, week(s); PB, phenobarbital; ACV, acyclovir; IVIG, intravenous immunoglobulin; EDV, edaravone; *fol. by*, followed by; fPHT, fosphenytoin; CLB, clobazam; KBr, potassium bromide; DZP, diazepam; LDC, lidocaine.

3.2. ab-NR and further anti-neuronal autoantibody assays

Eight of the nine patients who underwent the assay showed positivity to multiple epitopes in the serum or CSF (Patients 1–7 and 9, 88.9%, Table 2). The epitopes included GluN1-NT, which has been reported to be critical for the emergence of neuropsychiatric symptoms in anti-NMDA-receptor encephalitis [6]. Patients 1 and 2 had high CSF titers of antibodies during the acute phase (>10 SD). Patient 6 showed positivity in the CSF at onset and in serum half a year later. Patients 3, 5, and 9 underwent follow-up assays, and their titers were found to decrease over time. In Patient 5, immunohistochemical analysis during seizure recurrence at age 11, using serum drawn before prednisolone administration, revealed autoantibodies to the cytoplasm of hippocampal neurons, as demonstrated in hippocampal slices taken from rats (Supplementary Fig. b). The assay failed to identify the epitope. These results suggest that following seizure clusters, an immune reaction occurs non-specifically to degraded neuronal proteins, including NMDA-type glutamate receptor, inside and subsequently outside the brain. Such reactions appear to be strong at early ages, but do not show a uniform pattern.

4. Discussion

This study revealed the therapeutic potency of corticosteroids for acute symptoms in *PCDH19*-FE. The rapid and efficient response was remarkable and might be a useful indicator for this disease. The cases of Patients 1 and 9 suggested that oral corticosteroids taken during interictal periods might exert some prophylactic effects, but further assessments are necessary to establish this.

Table 2
Results of assay for antibodies to *N*-methyl-D-aspartate-type glutamate receptor.

Pt no	*PCDH19* mutation	Age at onset (m)	Age at assay	Serum					Cerebrospinal fluid				
				GluN2B		GluN1	GluD2		GluN2B		GluN1	GluD2	
				NT	CT	NT	NT	CT	NT	CT	NT	NT	CT
1	p.L719*	13	2y5m	1.61	**3.12**		1.93	0.45	**14.10**	**15.10**		**10.80**	**8.10**
2	p.K120Rfs*3	10	11m						**15.42**				
			4y2m	**2.21**	1.53	**3.58**	**3.63**						
3	p.D417H	5	11m	**2.17**	**2.94**	**3.62**	**2.85**						
	p.D596Y		1y0m						**3.39**	**3.28**	**2.81**	**2.35**	
	Heterozygous		1y7m	**2.65**	**2.21**	**2.17**	1.95						
			1y10m	1.23	0.37	0.72	0.99						
4	p.D596G	6	1y0m						**2.67**	**2.39**	1.51	1.68	
5	p.D45Gfs*43	8	9m	+					–	+			
			3y5m	–					–				
6	Whole del	10	11m	0.57	1.70		0.46	(0.34)	1.61	**2.73**		1.34	0.75
			1y5m	**2.33**	**2.73**		**2.48**	0.92	0.46	1.18		1.13	0.70
7	p.Asn340Ser	9	8y1m	**3.49**	**2.07**	**9.55**	1.87						
8	p.R198L	7	6y5m	1.70	0.90	1.78	1.68						
9	p.Y366Lfs*10	5	6y0m	**9.52**		**12.50**	**9.20**		−0.88		−1.37	−1.08	
			8y5m	0.60	(0.35)	0.25	0.37						

Results are expressed as standard deviation (SD, enzyme-linked immunosorbent assay) or plus/minus sign (immunoblot, Patient 5 only). Bold values denote positivity (≥2 SD or +). Blanks are unexamined. *Patients who received corticosteroid treatments. Pt no, Patient number; m, month(s); NT, N-terminal; CT, C-terminal; y, year(s); Whole del, Whole *PCDH19* deletion.

Despite such efficacy, short-term administration of corticosteroids provided only transient effects and had no potential for preventing further seizure clusters. Therefore, the clinical benefit will be limited for cases with frequent recurrences such as Patients 3 and 5. Moreover, excessive amounts of corticosteroids may have adverse effects, actually increasing seizure-proneness as described below. These findings suggest that the attending physician should consider discontinuing corticosteroid administration for acute treatment soon after seizure disappearance. Indication for treatment should be based on various patient conditions such as age, seizure severity, and comorbid infections.

Corticosteroids may exert primarily excitatory/pro-convulsive actions on brain neurons both *in vitro* and *in vivo*, especially under chronic stress conditions such as epilepsy [9]. Despite this, the therapeutic efficacy of corticosteroids is well established in many intractable epilepsies such as West syndrome, Landau-Kleffner syndrome, and autoimmune epilepsy/encephalitis [10]. In these diseases, clinical improvements after corticosteroid administration are usually delayed and are probably mediated by mechanisms such as immunosuppression and feedback inhibition of corticotropin-releasing hormone secretion. In our patients, however, the clinical effects appeared quickly, usually immediately after the initial administration. Moreover, significant brain inflammation was not found, suggesting a particular pathogenesis and mechanism of action of corticosteroids in *PCDH19*-FE.

Such a mechanism may be restoration of blood-brain barrier (BBB) integrity [11]. This is known to be a crucial action of corticosteroids, the underlying molecular basis of which has been partially elucidated [12]. Seizures are easily triggered by a mild breakdown of brain homeostasis due to a compromised BBB, which can be prevented or alleviated by corticosteroid administration [13]. Although PCDH19 is abundantly expressed in brain neurons, it is unclear how the heterozygous mutation in *PCDH19* leads to epilepsy in females. "Cellular interference," that is, the presence of somatic mosaicism in PCDH19 expression between normal and abnormal neurons, is the currently proposed explanation for such sex-specific pathogenesis, but the concept is very theoretical and has yet to be established. However, *PCDH19* is also expressed in the BBB. In mouse, the BBB-specific transcriptome included *PCDH19*, and *PCDH19* expression in microvascular endothelial cells was significantly higher in the brain than in the liver or lung [14]. Although currently no human evidence exists, this study suggests that PCDH19 plays a role in the BBB, and speculatively, that *PCDH19* mutation leads to a functional BBB vulnerability, that underlies the pathogenesis of *PCDH19*-FE.

Interestingly, *PCDH19* expression may be significantly altered in the BBB during systemic inflammation, which is the predisposing factor for seizure recurrence in *PCDH19*-FE. In cultured mouse brain microvascular endothelial cells, treatment with lupus serum or an activated complement, C5a, significantly down-regulated miR-320a expression [15]. *PCDH19* is one potential target of this microRNA. Although systemic inflammation will impair BBB function to some extent [16], the impairment may be exacerbated in patients with *PCDH19*-FE, probably due to PCDH19 insufficiency. The resulting seizure clusters will further exacerbate the BBB dysfunction by inducing brain inflammation. Corticosteroids may ameliorate such dysfunction and thus the acute neurological symptoms.

Other aspects of *PCDH19*-FE and the findings of this study are also consistent with the BBB hypothesis: The seizures mainly involve the limbic system, which is anatomically close to some of the periventricular regions that lack a BBB due to their endocrine roles; seizure occurrence and remission are strongly age-related, and BBB integrity also develops age-dependently; abs-NR in our patients covered multiple epitopes of various subunits, and Patient 8 had antibodies to some neuronal cytoplasmic component(s) as well. These results indicate that anti-neuronal antibodies may be produced in *PCDH19*-FE non-specifically and commonly. Various neuronal proteins will be degraded by recurrent seizures and the compromised BBB may then facilitate leakage of such degraded proteins into the bloodstream. This may induce non-specific sensitization to them outside the brain, resulting in the high rate of anti-neuronal autoantibody positivity seen in our patients. Thus, the abs-NR found in our patients does not represent an autoimmune pathogenesis.

However, a possible modifying effect of the abs-NR on the neurological phenotype in our patients with *PCDH19*-FE can also be considered. The seroprevalence of abs-NR has been found to be identical between patients with psychiatric diseases, including schizophrenia, and healthy individuals, but the disease phenotypes were more severe in patients with compromised BBBs than in those without [17]. Serum abs-NR could have passed the compromised BBB and worsened the psychiatric symptoms of these patients. In anti-NMDA-receptor encephalitis, a primary involvement of antibodies to GluN1-NT in causing its neuropsychiatric symptoms has been suggested [6]. Therefore if BBB compromise exists in *PCDH19*-FE, the anti-GluN1-NT could be partially responsible for the neuropsychiatric symptoms also seen in this disease.

This study proposes corticosteroid treatment as an efficacious adjunctive treatment for the acute symptoms of *PCDH19*-FE and suggests BBB involvement in this disease. Although *PCDH19*-FE is rare, future multicenter clinical trials should be conducted to verify the acute and long-term efficacy of corticosteroid treatment and to define the therapeutic indications of such treatment, since the present study is only a retrospective review of 5 patients. In addition, reliable animal models should be devised to elucidate the entire molecular pathogenesis of *PCDH19*-FE. Importantly, not only must neurons be studied, but also other actors such as the BBB and the inflammatory system as well.

Conflict of interest

All authors wish to confirm that there are no known conflict of interest associated with this publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seizure.2015.02.006.

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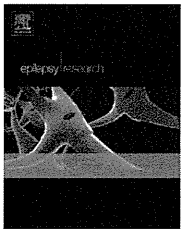

Semi-quantitative analyses of antibodies to *N*-methyl-D-aspartate type glutamate receptor subunits (GluN2B & GluN1) in the clinical course of Rasmussen syndrome

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Summary
Objective: In Rasmussen syndrome (RS), in addition to the predominant involvement of cytotoxic T cells, heterogeneous autoantibodies against neural molecules are also found, but their function has not been elucidated. We examined antibodies to *N*-methyl-D-aspartate (NMDA) type glutamate receptor (GluR) subunits (GluN2B & GluN1) semi-quantitatively in cerebrospinal fluid (CSF) samples from RS patients, and evaluated their changes over time and their roles in immunopathogenesis.
Methods: Autoantibodies against N-terminal and C-terminal of GluN2B and GluN1 were examined in 40 CSF samples collected from 18 RS patients 5 to 180 months after the onset of RS. Epileptic patients without infectious etiology or progressive clinical course served as disease controls ($n = 23$). Synthesized peptides encoding the extracellular and intracellular domains of human GluN2B and GluN1 subunits were used as antigens in ELISA. We defined the cut-off for

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these antibodies as mean +2 standard deviations (optimal density) of the disease controls. MRI were evaluated according to the MRI staging proposed by Bien et al. (2002b, Neurology 58, 250). *Results:* CSF levels of antibodies against N-terminal and C-terminal of GluN2B were higher in RS patients than in disease controls ($p < 0.01$). Likewise, CSF levels of antibodies against N-terminal and C-terminal of GluN1 were also higher in RS patients than in disease controls ($p < 0.01$). All four antibodies tested were below cut-off levels in almost all CSF samples collected within one year from epilepsy onset. The proportions of CSF samples with these antibodies above cut-off levels were highest from 12 to 23 months after epilepsy onset, and declined after 24 months. CSF levels of these antibodies were higher when seizure occurred daily than when seizure occurred less frequently ($p < 0.01$), and were higher at MRI stage 3 than at MRI stages 0, 2 and 4 ($p < 0.05$), except for anti-GluN1-CT antibody at stage 2.
Conclusions: Broad epitope recognition spectrum and delayed production of autoantibodies to NMDA type GluR in CSF of RS patients suggest that the autoantibodies are produced against NMDA type GluR antigens derived from cytotoxic T cell-mediated neuronal damages. These antibodies may impact the pathophysiology of RS in the most active stage, and could be a marker for active inflammation in the clinical course of RS. Further studies including passive transfer of the antibodies to mice may reveal the pivotal roles of the antibodies in RS.

Introduction

Rasmussen syndrome (RS) is the childhood epilepsy characterized by progressive, severe cortical inflammation and destruction, predominantly affecting one cerebral hemisphere. RS shows disabling neurologic deficits, cognitive decline and intractable seizures including epilepsia partialis continua (EPC), evolutionally. This syndrome is usually diagnosed by comprehensive assessment of characteristic clinical symptoms, electroencephalogram (EEG), magnetic resonance imaging (MRI), and histopathologic findings (Anderman and Rasmussen, 1991; Bien et al., 2005; Oguni et al., 1992; Rasmussen et al., 1958; Rasmussen, 1978).

The etiology and pathogenesis of this severely disabling inflammatory disease remain enigmatic. Histopathologic findings in RS include meningeal and perivascular lymphocytic infiltrates, microglial nodules, neuronal cell loss, and gliosis of the affected hemisphere. Active brain inflammatory lesions contain large numbers of T lymphocytes, which are recruited early within the lesions, suggesting that T cell-dependent immune response contributes to the onset and progression of the disease (Farrell et al., 1995). Further immunohistiochemical studies on RS brain specimens provided evidence for a granzyme B-mediated cytotoxic T lymphocyte (CTL) attack against neurons (Bien et al., 2002a,b, 2005). CTL may also be responsible for the degeneration of astrocytes found in RS lesions (Bauer et al., 2007). Granzyme B and IFNγ levels in cerebrospinal fluid (CSF) were elevated in RS patients, especially in the early stage of disease (Takahashi et al., 2009). Immunomodulatory therapies using intravenous immunoglobulin, plasmapheresis and tacrolimus have been reported to improve outcome (Bien et al., 2005; Takahashi et al., 2013). From these data, cellular autoimmunity mediated by CTLs seems to play a primary role in the development of RS and this CTL-mediated mechanisms appear to explain the progressive pathology in the brain.

GluRs in the central nervous system play important roles in brain functions, and they are divided into ionotrophic and metabotrophic receptors. The ionotrophic GluRs are subdivided into three major types: the *N*-methyl-D-aspartate (NMDA) type, α-amino-3-hydroxy-5-methyl- 4-isoxazole propionate (AMPA) type, and kainate type. The NMDA type GluR forms heterotetramer, usually composed of two GluN1 subunits and two GluN2 (A–D) or GluN3 (A–B) subunits. The molecular diversity of NMDA type GluR subunits contributes to their physiological roles in development, synaptic plasticity, learning, and memory. The NMDA type GluR also plays important roles in pathological processes in ischemic brain injury, neurodegenerative disease, and epilepsy. Rogers et al. (1994) were the first to identify the presence of antibodies against GluR3 (GluA3), one of the AMPA type GluRs, in patients with RS. Autoantibodies against GluN2B, which is the essential molecule for synaptic plasticity and development (Mori et al., 1998), were also detected in the serum and CSF of RS patients in our previous studies (Takahashi et al., 2003, 2005). Although many studies supported that activated CTLs were primary factors to cause RS (Bien et al., 2002a; Takahashi et al., 2003, 2009, 2013) and antibodies against GluR3 and GluN2B are neither specific nor restricted to RS (Ganor et al., 2005; Takahashi, 2008; Watson et al., 2004; Wiendl et al., 2001), and the pathophysiological contribution of these autoantibodies in RS are still debatable.

In RS, cognitive deterioration is characteristic and important for prognosis. GluN2B and GluN1 are essential for synaptic plasticity and increase of GluN2B in synapse is needed for recovery of synaptic activity (Lau and Zukin, 2007). In this study, we measured anti-GluN2B and anti-GluN1 antibodies semi-quantitatively in CSF samples from RS patients, and evaluated their changes over the clinical course and the relations with clinical features to reveal their roles in immunopathogenesis.

Methods

Patients and methods

We studied 40 CSF samples from 18 patients (male, 9; female, 9) who were diagnosed with RS based on diagnostic criteria proposed by Bien et al. (2005) at the National

Table 1 Clinical characteristics and antibodies to *N*-methyl-D-aspartate type glutamate receptor subunits (ELISA).

Pt	Sex	Age at onset (years)	Age at surgery and method	Histologic findings	Age at sampling(years)	Sampling stage (months)	Seizure frequency	Immuno logic treatment	MRI stage	Antibodies (OD)			
										GluN2B-NT2	GluN2B-CT	GluN1-NT	GluN1-CT
1	F	0.2	2.8 y: R-FH	MGN + VBS + EP +SD + PVC	0.7	6	D	–	2	0.464	0.715	0.566	0.789
					1.4	14	D	–	3	1.120	1.155	1.495	1.108
					2.5	27	D	–	3	0.563	0.608	0.703	0.735
2	M	0.2			1.2	13	D	–	–	0.774	1.058	1.007	1.039
3	F	0.5			2.0	18	D	–	0	0.155	0.239	0.206	0.201
					3.1	31	D	Pulse	0	0.164	0.167	0.273	0.281
4	M	2.4			3.2	10	D	–	3	0.322	0.373	0.778	0.803
					4.2	22	D	Pulse	3	0.665	0.842	0.899	0.882
					5.3	35	N	Tacrolimus	3	0.939	1.187	1.168	1.182
5	M	3.6	4.8 y: R-FH	MGN + VBS + PVC	11.6	97	D	–	PS	0.134	0.258	0.291	0.260
6	M	3.9			6.8	36	D	–	3	1.775	2.440	2.152	2.383
					9.7	71	D	–	3	1.667	1.398	1.544	1.652
7	M	4.1			18.4	168	D	Tacrolimus	4	0.391	0.415	0.950	0.670
					20.0	180	D	Tacrolimus	4	0.384	0.499	0.478	0.453
8	F	4.7			8.4	45	D	–	0	0.281	0.322	0.610	0.694
					8.8	51	D	Pulse	0	0.259	0.449	0.336	0.4
					9.8	63	D	Pulse	0	0.463	0.375	0.639	0.359
					10.6	72	D	Pulse	0	0.361	0.493	0.444	0.443
					11.1	78	D	Pulse	0	0.203	0.304	0.251	0.36
9	M	5.3			6.5	14	D	–	4	0.698	0.513	0.482	0.535
					7.2	23	D	–	4	0.315	0.338	0.510	0.519
					11.4	73	D	Tacrolimus	4	0.378	0.480	0.493	0.471
10	M	6.1	15 y: R-FD	NE	7.2	12	D	–	3	0.919	1.087	1.003	1.183
11	F	6.5			7.9	17	D	–	3	0.657	0.941	0.812	0.713
					8.3	23	D	–	3	0.788	1.212	1.039	1.155
					9.1	31	D	Pulse	3	0.298	0.387	0.670	0.671
					9.3	34	M	Pulse	3	0.242	0.327	0.422	0.407
12	F	7.1	9 y8 m: R-FH	MGN + VBS + EP + SD + PVC	9.7	31	D	Pulse	3	0.574	0.779	0.651	0.685
13	F	7.4			8.2	9	M	–	0	0.179	0.394	0.448	0.482
					9.5	26	M	Tacrolimus	–	0.337	0.441	0.431	0.575
14	M	8.7	9 y8 m: L-FR	MGN + VBS + EP + PVC	9.2	6	M	Pulse	2	0.150	0.106	0.330	0.309
					9.7	12	M	Pulse	2	0.096	0.076	0.103	0.104
					12.1	40	W	Pulse	PS	0.059	0.155	0.178	0.184
					12.8	50	W	Pulse	PS	0.081	0.092	0.162	0.160
15	F	8.8			19.7	132	D	Tacrolimus	3	0.445	0.623	0.591	0.627
16	F	9.0			12.1	38	D	–	3	2.216	1.702	2.347	1.546
					15.2	74	D	Tacrolimus	3	0.657	0.879	0.920	0.911
17	M	18			25	84	D	–	–	0.568	0.821	0.773	0.821
					28	120	D	–	–	0.625	0.914	0.900	1.317
18	F	42			42	5	M	–	4	0.302	0.364	0.345	0.310

FH, functional hemispherectomy; FD, frontal disconnection; FR, frontal resection; MGN, microglia nodule; VBS, vascular genesis on brain surface; EP, endothelial proliferation; SD, spongy degeneration; PVC, perivascular cuffing; NE, not examined; Sampling stage, duration between epilepsy onset and sampling of cerebrospinal fluid (CSF); D, daily; W, weekly; Y, yearly; N, no seizure; Pulse, methylprednisolone pulse therapy; Pts. 1, 5, 7, 8, 10, 12, 15 and 16 were reported as Pts. 1, 4, 8, 9, 16, 19, 22 and 23, respectively in our previous paper (Takahashi et al., 2009).