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Role of complement receptor 1 (CR1; CD35) on epithelial cells: A model for understanding complement-mediated damage in the kidney

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Abstract

The regulators of complement activation gene cluster encodes a group of proteins that have evolved to control the amplification of complement at the critical step of C3 activation. Complement receptor 1 (CR1) is the most versatile of these inhibitors with both receptor and regulatory functions. While expressed on most peripheral blood cells, the only epithelial site of expression in the kidney is by the podocyte. Its expression by this cell population has aroused considerable speculation as to its biologic function in view of many complement-mediated renal diseases. The goal of this investigation was to assess the role of CR1 on epithelial cells. To this end, we utilized a Chinese hamster ovary cell model system. Among our findings, CR1 reduced C3b deposition by ~ 80% during classical pathway activation; however, it was an even more potent regulator (>95% reduction in C3b deposition) of the alternative pathway. This inhibition was primarily mediated by decay accelerating activity. The deposited C4b and C3b were progressively cleaved with a $t_{1/2}$ of ~ 30 min to C4d and C3d, respectively, by CR1-dependent cofactor activity. CR1 functioned intrinsically (i.e., worked only on the cell on which it was expressed). Moreover, CR1 efficiently and stably bound but didn't internalize C4b/C3b opsonized immune complexes. Our studies underscore the potential importance of CR1 on an epithelial cell population as both an intrinsic complement regulator and an immune adherence receptor. These results provide a framework for understanding how loss of CR1 expression on podocytes may contribute to complement-mediated damage in the kidney.

Keywords

Complement receptor 1; Epithelial cells; Decay accelerating activity; Cofactor activity; Immune complexes; Complement activation

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1. Introduction

CR1 is expressed by neutrophils, monocytes, B lymphocytes and erythrocytes but not platelets or most T cells (Fearon, 1980; Tedder et al., 1983). On neutrophils and monocytes, it mediates adherence of C4b/C3b-bound ligands which is commonly followed by internalization (Lay and Nussenzweig, 1968; Huber et al., 1968; Wright and Silverstein, 1982). CR1 on B lymphocytes facilitates antigen presentation to T cells (Hivroz et al., 1991). On erythrocytes, it serves as the immune adherence (IA) receptor for C3b/C4b coated antigens (Nelson, 1963; Paccaud et al., 1990; Subramanian, 1996) that it transports (taxi-like) to the liver and spleen for clearance. However, its expression on epithelial cells is more limited with the only known sites being the retinal pigment epithelial cells (Fett et al., 2012) in the eye, keratinocytes in the skin (Dovezenski et al., 1992) and podocytes in the kidney (Gelfand et al., 1975; Fischer et al., 1986; Appay et al., 1990). Its function on these epithelial cell populations is an enigma. The common size variant of CR1 possesses three C4b and two C3b binding sites, as well as a binding site for C1q, collectins, ficolins and mannan-binding lectin (Klickstein et al., 1997; Ghiran et al., 2000; Tetteh-Quarcoo et al., 2012; Jacquet et al., 2013) (Fig. 1). Upon binding C4b, CR1 accelerates decay of the classical and lectin pathway convertases (Holers et al., 1986; Iida and Nussenzweig, 1981; Medof and Nussenzweig, 1984; Krych-Goldberg et al., 1999; Hourcade et al., 2002). This is known as decay accelerating activity (DAA). Similarly, through binding C3b, CR1 deactivates the alternative pathway (AP) convertases. CR1 is also a cofactor for the cleavage of C4b and C3b by the plasma serine protease factor I (FI), a property known as cofactor activity (CA) (Ahearn and Fearon, 1989; Liszewski et al., 1996). It is thus a key player in controlling the fate of immune complexes (IC), particularly those that form in blood.

Reports, mostly from several decades ago, described reduced glomerular CR1 expression in a wide variety of glomerulonephritides (Pettersson et al., 1978; Kazatchkine et al., 1982; Nolasco et al., 1987; Moll et al., 2001). However, CR1's participation in modulating glomerular diseases has not been explored. Is CR1 a key player in glomerular diseases featuring IC formation and complement fragment deposition? In this report, we utilized Chinese hamster ovary (CHO) cells expressing CR1 as a model system to explore the biologic function of CR1 on an epithelial cell population.

2. Materials and methods

2.1. Cell lines

Initially, we employed an immortalized human podocyte cell line (Saleem, 2002). However we were unable to detect CR1 expression on the surface of these cells. Nevertheless, we (unpublished data), along with several other groups (Gelfand et al., 1975; Fischer et al., 1986; Appay et al., 1990; Pettersson et al., 1978; Kazatchkine et al., 1982; Nolasco et al., 1987; Moll et al., 2001), have shown the presence of CR1 on human podocytes in kidney tissue by immunohistochemical staining, mRNA analysis and WB. We hypothesize that cross-talk between podocytes, endothelial cells and mesangial cells are key to the maintenance of glomerular capillary wall function and this integrity is disrupted by podocyte isolation leading to loss of protein expression. Therefore, in order to begin functional studies on an epithelial population, we utilized the CHO cell lines.

A stable CHO cell line expressing CR1 comparable to the expression level on renal podocytes (Fischer et al., 1986) was chosen for our studies. Since CHO cells carry no membrane regulatory proteins with activity for human complement components (Barilla-LaBarca et al., 2002), we could address how CR1 functions in inhibiting CP and AP activation and in altering C4b and C3b deposited epithelial cells. We have employed this model system in the past to delineate the functional capabilities of human MCP and DAF (Barilla-LaBarca et al., 2002; Liszewski et al., 2007).

CHO cells were cultured in Ham's F-12 medium with 10% FCS. CR1 cDNA was cloned into the BamHI and XhoII sites of the expression vector (pH β aprl-neo) (Gunning et al., 1987). This was used to transfect the CHO cells using Lipofectamine (Life Technologies, Grand Island, NY) following the manufacturer's recommendations. Stably expressing clones were obtained utilizing selection markers (Geneticin 250 μ g/ml). CR1 expression was established by flow cytometry and Western Blotting (WB) using a rabbit polyclonal anti-CR1 Ab (Affinity purified IgG; a gift from Henry Marsh, Celldex Therapeutics, Needham, MA) (Makrides et al., 1992). Based on quantity and stability of the CR1 expression profile, three CHO cell lines were chosen for further use. RCHO, a clone transfected with CR1 cDNA in reverse orientation, served as a control.

2.2. Flow cytometry

The quantity of CR1 expressed per cell was initially assessed by flow cytometry as described previously (Barilla-LaBarca et al., 2002). Briefly, cells were harvested by trypsinization (0.05% trypsin; 0.53 mM EDTA for 1 min) and washed in 1% FCS-PBS (FACS buffer). Rabbit polyclonal anti-CR1 Ab (100 μ l; 1:25 dilution of 3.8 mg/ml protein A affinity purified IgG) was added and incubated with the cells for 30 min at 4 °C. Following centrifugation (1500 rpm for 5 min; TOMY high speed refrigerated microcentrifuge MX180) and two washes, FITC-donkey anti-rabbit IgG was added (100 μ l of a 1:100 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; catalog # 711-095-152). After 30 min incubation at 4 °C, cells were resuspended in FACS buffer and analyzed by flow cytometry (10,000 events). Rabbit IgG (protein A affinity purified) and secondary Ab alone served as controls. Time and dose-dependent analysis of the effect of varying trypsin concentrations on CR1 expression showed that addition of 1 ml of 0.05% trypsin for 1 min did not affect the CR1 copy number/cell. The clones employed were designated as follows: CR1-2 m (2×10^6 CR1/cell), CR1-200k (2×10^5 CR1/cell) and CR1-10k (1×10^4 CR1/cell).

2.3. Enzyme linked immunosorbent assay

ELISA strips were coated with 3D9 (murine mAb to CR1; 100 μ l at 2.5 μ g/ml in PBS overnight at 4 °C) (Krych et al., 1991). Wells were blocked (1% BSA, 0.1% Tween-20, 0.02% sodium azide in PBS) for 2 h at 37 °C and washed in PBS with 0.05% Tween-20 (wash buffer). Dilution of cell lysates of the CHO transfectants (100 l) was made in sample buffer (PBS with 0.05% Tween-20, 0.25% non-ionic detergent Nonidet P-40, 4% BSA) and incubated for 1 h at 37 °C followed by three 2-min washes in wash buffer. Rabbit polyclonal anti-CR1 Ab (1:20,000 dilution in sample buffer) was incubated for 1 h at 37 °C followed by a similar washing schedule. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit

IgG (100 μ l of a 1:15,000 dilution; GE healthcare, UK) was added and then incubated for 1 h at 37 °C followed by washing. Detection was made using orthophenylenediamine dihydrochloride (100 l of a 1:20 dilution) in a buffer containing 0.02% hydrogen peroxide in citrate phosphate buffer (pH 6.35). The reaction was stopped by adding 100 l of 2N sulfuric acid (2N H₂SO₄). The ELISA plates were read at 490 nm using the Micro Quant reader (Biotek Instruments, Inc). Soluble CR1 was used as the standard (gift from Henry Marsh, Celldex Therapeutics, Needham, MA) (Nickells et al., 1998).

2.4. Western blotting

Cells were trypsinized, washed and then lysed with the cell lysis buffer (1% nonionic detergent Nonidet P-40, 0.05% SDS in PBS, 2 mM phenylmethylsulfonyl fluoride). The solubilized preparation was centrifuged at 11,200 μ g for 10 min in a microcentrifuge at 4 °C and supernatants were analyzed via SDS-PAGE using 6% gels under reducing and non-reducing conditions (Novex Electrophoresis, Invitrogen) (Park et al., 2014). After SDS-PAGE, transfer to a nitrocellulose membrane (Bio-Rad, California) and blocking (5% nonfat dry milk in PBS with 0.05% Tween-20), rabbit polyclonal anti-CR1 Ab was added (3.8 μ g/ml) in blocking buffer for 1 h at 37 °C. Following three washes (PBS with 0.05% Tween-20), an HRP-conjugated donkey anti-rabbit IgG (GE Healthcare, UK) was added at a 1:5000 dilution in sample buffer at room temperature (RT) for 1 h. Detection, using Super Signal Pico Chemiluminescent substrate, was performed according to the manufacturer's directions (Pierce, Rockford, IL).

2.5. Initiation of complement activation

Initiation and assessment of complement activation on CHO cells have been described (Barilla-LaBarca et al., 2002; Liszewski et al., 2008). Briefly, following trypsinization, CHO cells (10 million cells/ml) were harvested and washed in FACS buffer. The cells (100 μ l/well) were sensitized by incubating with rabbit anti-CHO antibody (affinity purified IgG; 1 mg/ml in FACS buffer) for 30 min at 4 °C. Following two washes with FACS buffer, 100 μ l of 10% C7-deficient (C7d) serum (courtesy of P. Densen, University of Iowa, Iowa City, IA) in gelatinveronal buffer (GVB⁺⁺; GVB with calcium and magnesium; Complement Technologies, TX, USA) was added. In order to assess classical pathway activation (CP) alone, Factor B-depleted serum was employed (Quidel, San Diego, CA) (Fig. 4 Supplement).

Separate experiments were performed to evaluate complement activation by the AP. The CP was blocked by utilizing GVB containing 10 mM EGTA and 7 mM magnesium chloride (Mg²⁺-EGTA). After a 60 min incubation at 37 °C on a thermomixer (Eppendorf Thermomixer), cells were harvested and washed twice in FACS buffer before analysis of C4 and C3 fragment deposition. Antigenic levels of FH, C4BP and FI in the C7d serum (measured at National Jewish Medical and Research Center, Denver, CO) were 117, 152, and 119% of normal values, respectively. C4BP functional activity in the C7d serum was comparable to that of normal human serum (NHS) as described previously (Barilla-LaBarca et al., 2002). C8-deficient serum (donated by P. Densen, University of Iowa, Iowa City, IA) was substituted for C7d serum and gave equivalent results. Antigenic levels of FH, FI and

C3 were within normal limits in the C8-deficient serum (FH, 328 µg/ml; FI, 54 µg/ml; C3, 1.45 mg/ml).

2.6. Flow cytometry analysis of complement fragment deposition

Following incubation with a complement source and washing, murine mAbs to the human complement fragments [C4c, C4d, C3c or C3d (Quidel, San Diego, CA)] were added (100 µl at 5 µg/ml) to the cells for 30 min at 4 °C³¹. The cells were next washed twice in FACS buffer before FITC-conjugated goat anti-mouse IgG was added (Sigma–Aldrich; 100 µl of a 1:100 dilution) for 30 min at 4 °C. Cells were washed in FACS buffer, resuspended in 0.5% paraformaldehyde and then analyzed by flow cytometry (10,000 events). Separate controls were used for each time point. Experiments were performed at least three times and each condition was performed in duplicate. Data analysis was performed with Microsoft Excel. The kinetics of C4b and iC3b cleavage were determined by regression analysis to fit an exponential decay curve, $Y = Ae^{-kx}$. Half-life was calculated from: $T_{1/2} = -\ln(1/2)/k$.

2.7. Intrinsic versus extrinsic complement regulation by CR1

To address the question of the intrinsic versus extrinsic inhibitory profile of CR1 (Makrides et al., 1992; Oglesby et al., 1992; Medof et al., 1982a), CR1–200k and RCHO cells were mixed in varying proportions (one part CR1–200k: one part RCHO; one part CR1–200k: four parts RCHO; and four parts CR1–200k: one part RCHO). The cell mixtures were sensitized using the rabbit anti-CHO Ab and then incubated with C7- or C8- deficient human serum at 37°C for 60 min in GVB⁺⁺ or in Mg²⁺-EGTA buffer (for AP activation alone). The complement fragment deposition was analyzed via flow cytometry. The ability of CR1-expressing CHO cells to protect the bystander RCHO by an extrinsic mechanism was assessed by evaluating the deposition of C3b and its degradation into C3c and C3dg on the two cell populations.

2.8. Immune complex processing by CR1

Preformed purified soluble IC (8 µl of 1.25 mg/ml rabbit antiserum against horseradish peroxidase; MP biomedical LLC, OH; catalog #55968) were diluted in 42 l GVB⁺⁺. They were opsonized by the addition of 50 µl of 100% NHS (to obtain a final concentration of 50 g/ml IC in 50% NHS) and incubated in a 37 °C water bath for 30 min. Heat-inactivated serum (HIS; serum exposed to 56 °C for 30 min) was used to prepare the negative control of unopsonized IC. CHO cell populations were incubated with IC at 37 °C for 30 min with constant shaking (Eppendorf Thermomixer). Following centrifugation and two washes in PBS at 4 °C, binding of opsonized IC to CR1 was analyzed using FITC-labeled anti-rabbit IgG Ab (Santa Cruz Biotechnology, USA). To determine the specificity of IC binding to CR1, the receptor sites were blocked by 3D9 (Holers et al., 1986; Nickells et al., 1998; O'Shea et al., 1985) (mAb to CR1 that abrogates binding to C4b and C3b) by incubating the cells with the mAb (100 µl at 75 µg/ml) at 4 °C for 30 min before the addition of complement bearing IC.

We also employed the ¹²⁵I-labeled BSA/anti-BSA IC. These were utilized to compare binding on the surface of RBC to that of CR1-expressing CHO cells and to further compare the CR1–200k and CR1–2m cell lines. The IC were prepared as previously described

(Medof and Oger, 1982). For the first part of the experiment, the IC were opsonized by the addition of 10% NHS at 37 °C for 30 min. The opsonized IC were then incubated with RBC and CR1-expressing CHO cells at 37 °C for varying time periods (2 min to 30 min). The percent of IC binding to the surface of the cells was calculated by counting the radioactivity for cell pellet and the supernatant separately. [The percent of binding = cell counts/(cell counts + supernatant)].

For the second part of this experiment, we compared IC binding between the CR1–200k and CR1–2 m cells at equivalent CR1 amounts. In particular, we used 1×10^6 cells for the CR1–2 m (2×10^6 CR1/cell) and 2×10^7 cells for the CR1–200k (2×10^5 CR1/cell) to obtain a total CR1 quantity of 2×10^{12} for each cell line. The cells were then exposed to serum-opsonized IC at 37 °C for 30 min. The percent of IC binding was calculated by monitoring the radioactivity for the cell pellet and supernatant (as above). We also compared the two cell lines at a total CR1 quantity of 1×10^{12} (Gelfand et al., 1975).

3. Results

3.1. Characterization of CR1 expressing cell lines

Three CHO cell lines stably expressing varying levels of CR1/cell were produced. The expression of CR1 on each line was characterized using flow cytometry, ELISA and WB. The cell lines were designated according to their CR1 expression level: CR1–2m (2×10^6 CR1/cell); CR1–200k (2×10^5 CR1/cell); CR1–10k (1×10^4 CR1/cell) (Fig. 2A; Table 1). Western blotting established that CR1 migrated as anticipated under non-reducing conditions (Fig. 2B) and reducing conditions (not shown). An additional band was detected in CR1–2m, consistent with pro-CR1, a pre-Golgi CR1 precursor possessing high mannose N-linked oligosaccharides (Lublin et al., 1986). RCHO served as a negative control. For most of the experiments that follow, we employed CR1–200k because its expression level is comparable to that of human podocytes (Fischer et al., 1986).

3.2. Classical pathway (CP) activation

Deposition and processing of C4b on CHO cells was determined by flow cytometry using monoclonal antibodies (mAbs) that recognize either the C4c or C4d fragment (Barilla-LaBarca et al., 2002) (Fig. 1 supplement). The mAb to C4c detects uncleaved C4b while the mAb to C4d detects C4b and C4d.

C4b activation and deposition was rapid with similar maximal levels attained on RCHO and CR1–200k in <5 min [mean fluorescence intensity (MFI) = 726] (Fig. 3A and B). Subsequent C4b cleavage occurred only on CR1–200k. Degradation of the covalently attached C4b to C4c (which is released into the fluid phase) and C4d (which remains covalently attached) fit an exponential decay curve with a $t_{1/2} \sim 30$ min (Fig. 3C) and with 87% of the deposited C4b being cleaved by 90 min (Figs. 3A and C; Table 2). No C4b cleavage was observed on RCHO (Fig. 3B). Thus, CR1 and not C4 binding protein (C4BP) served as a cofactor for FI-mediated cleavage of C4b to C4c and C4d.

In the next set of analogous experiments, C3b deposition and its cleavage fragments were monitored (Fig. 2 supplement). Total C3b deposition on CR1–200k (MFI = 726) was

decreased by 78% compared to RCHO (MFI = 2224) which occurred in <5 min (Fig. 4A and B). These results establish that CR1 inhibits even the highly efficient CP driven by Ab-sensitized cells.

Following deposition of C3b, Factor H (FH) in the serum and CR1 could each potentially serve as the cofactor protein for the conversion of C3b to iC3b (Fig. 2 supplement). In this model system, we have previously demonstrated that FH carries out this task (Barilla-LaBarca et al., 2002). Since FH is not a cofactor for the degradation of iC3b into C3c and C3dg, we anticipated this next step would be CR1-dependent. To study this, a mAb to C3c was utilized to detect C3b and iC3b and a mAb to C3d portion of C3dg was employed to detect C3b, iC3b and C3dg. iC3b cleavage to C3c and C3dg began immediately on CR1–200k and followed an exponential decay curve with a $t_{1/2}$ of 24 min (Fig. 4C). Also, similar to C4b cleavage, it was nearly complete by 90 min. (Fig. 4A and C; Table 3). No cleavage of iC3b occurred on RCHO (Fig. 4B).

Western blotting was employed to characterize the C3b fragments generated by CP activation on RCHO versus CR1–200k. We identified cleavage fragments consistent with iC3b on RCHO (due to CA of FH and FI in the serum) (Fig. 3 supplement). However, the majority of iC3b on CR1–200k was cleaved to C3c and C3dg as outlined above.

3.3. Alternative pathway (AP) activation

C3b deposition by AP was rapid with maximal levels attained on RCHO in < 5 min (Fig. 5A). Remarkably, the quantity of C3b deposited decreased >95% on cells expressing CR1 (RCHO MFI = 712 vs CR1–200k MFI = 23) (Fig. 5B). Also, the modest quantity of C3b deposited underwent complete degradation to C3c and C3dg in the presence of CR1 (C3c, 6; C3dg, 20). Serum concentrations ranging from 10 to 50% were utilized with similar results (10% serum: RCHO MFI = 710 vs CR1–200k MFI = 35; 20% serum, RCHO MFI = 714 vs CR1–200k MFI = 40; 50% serum, RCHO MFI = 719 vs CR1–200k MFI = 42). Thus, these studies establish that CR1 is a potent inhibitor of the AP.

3.4. CR1 is not an extrinsically-acting regulator

The prior set of experiments established that CR1 is an intrinsic regulator. To assess if it possesses extrinsic regulatory activity, RCHO and CR1–200k were mixed in varying proportions before addition of the sensitizing Ab. Cells were then treated with serum under conditions which permitted CP activation. We used the anti-C3d mAb (detects C3d portion of C3dg) to determine whether the presence of CR1 influenced total C3b deposition on bystander RCHO: Two peaks were detected (shown as dark green) [Fig. 6A (i)]. The first peak was CR1–200k (MFI = 329) and the second represented RCHO (MFI = 1026). As evident, the total C3b deposition on RCHO was ~ three-fold greater compared to CR1–200k in this cell mixture. This correlated closely with the C3b deposition on the surface of RCHO and CR1–200k alone [Fig. 6A (ii and iii)]. These results demonstrate that CR1 does not inhibit CP-mediated C3b deposition on a neighboring cell.

We next asked if CR1 could cleave the C3b deposited on the surface of an adjacent RCHO. For this purpose, we employed the mAb to C3c and again detected two peaks (shown in

pink [Fig. 6B (i)]. The first peak represents CR1–200k (MFI = 32) and the second RCHO (MFI = 1028). These results resemble C3b cleavage on the surface of an RCHO and CR1–200k if studied separately [Fig. 6B (ii and iii)]. Thus, CR1 does not mediate C3b cleavage on bystander RCHO. We repeated these experiments using varying cell proportions [one part CR1–200k: four parts RCHO; and four parts CR1–200k: one part RCHO (Fig. 6C)] with identical results. Similar experiments were conducted during AP activation and analogous results obtained (C3c MFI: CR1–200k = 36 vs RCHO = 809; C3dg MFI: CR1–200k = 55 vs RCHO = 788). These data indicate that CR1 acts on the surface of cells on which it is expressed and lacks extrinsic regulatory activity in this model system.

3.5. Immune complex processing by CR1-expressing CHO cells and comparison to red blood cells (RBCs)

In primates, CR1 is expressed on RBCs and serves as an IA receptor (Nelson, 1963; Subramanian et al., 1996). Therefore, we compared the efficacy of an epithelial cell versus an RBC in binding and processing IC.

In the first set of experiments, pre-opsonized soluble IC were mixed with CHO cells. No binding was observed on RCHO or in the absence of serum to CR1–200k whereas, with serum exposure, CR1–200k rapidly bound (>80%) complement opsonized complexes (Fig. 7). The complexes were not internalized over a 90-min period. The preincubation of CR1-expressing cells with a function-blocking anti-CR1 mAb (3D9) (Nickells et al., 1998) abrogated IC binding. Serum concentrations ranging from 10 to 70% gave similar results. Comparable findings were also obtained with IC concentrations ranging from 25 to 200 µg/ml. These results demonstrate that CR1 expressed by epithelial cells rapidly and efficiently engages C4band C3b-opsonized IC and that the IC remain attached.

Next, pre-opsonized IC were mixed with RBC or CR1–2 m. It required 5×10^8 RBC or 5×10^6 CHO cells to obtain maximal binding of the IC (Fig. 8A). The RBC donor expressed 333 copies of CR1 per cell which is near the median level of expression for over 100 individuals in our laboratory (unpublished). The experimental conditions were adjusted such that the total number of CR1 was identical in the reaction mixtures. At equivalent CR1 amounts, the kinetics of IC binding were similar, although, initially the RBC tended to be faster (1 min: RBC 26%, CHO 18%; 5 min: RBC 42%, CHO 35%). However, by 15 min, the IC binding for both cell types had equalized at 42%. These results were extended by comparing CR1–2 m to CR1–200 k (Fig. 8B). At comparable CR1 amounts, the IC binding was overall analogous.

4. Discussion

In this report, we characterized a role for CR1 in protecting epithelial cells from complement-mediated attack. We also addressed IC binding by CR1 on epithelial cells.

4.1. Classical pathway inhibition

4.1.1. Effects on C4b—As expected, CR1 did not influence the quantity of C4b deposited; however, it cleaved the deposited C4b to C4c and C4d with a $t_{1/2}$ of ~ 30 min. C4c was released into the fluid phase while C4d remained covalently bound as an

“immunological scar”. Consequently, these experiments indicate that CR1 and not C4BP is the cofactor protein for cleavage of C4b by FI. Moreover, CR1 has comparable cofactor activity in this regard to membrane cofactor protein (MCP) (Barilla-LaBarca et al., 2002). Hence, these two regulators would at least be additive in their ability to serve as a cofactor protein for FI-mediated cleavage of deposited C4b.

4.1.2. Effects on C3b—The quantity of C3b deposited on CR1-expressing cells was decreased by ~80% compared to RCHO. Thereafter, FH and FI carried out the rapid (< 5 min) proteolytic cleavage (Barilla-LaBarca et al., 2002) of the deposited C3b to iC3b on both CR1–200k and RCHO. CR1 though is then the only cofactor for FI capable of mediating the further cleavage of iC3b to C3c and C3dg. However, CR1’s cofactor activity is a relatively slow process (takes 90 min for near completion). Therefore, while CR1 can also serve as a cofactor for the conversion of C3b to iC3b, it is unlikely to have played a role in view of the rapidity of the process. This is also evident from the kinetics of C4b cleavage by CR1 and is similar to the time course for C4b degradation by MCP (Barilla-LaBarca et al., 2002).

In summary, CR1 inhibits the highly efficient CP by limiting the activity of the C3 convertase via DAA, thus deterring further C3 activation. It then processes the deposited fragments by functioning as a cofactor for degradation of the C4b and iC3b. The decrease in C3b deposition occurs in <5 min compared to CR1’s CA which takes 25–30 min to cleave ~50% of the membrane bound fragments. Collectively, these data indicate that DAA must be the mechanism for inhibition of the CP.

4.2. Alternative pathway inhibition

The quantity of C3b deposited on CR1–200 k was dramatically decreased (~50 fold) compared to RCHO. The modest quantity of C3b deposited was cleaved into iC3b and subsequently to C3c and C3dg. C3c is released while iC3b and C3dg remain covalently bound. These two covalently attached fragments (iC3b and C3dg) are hemolytically inactive and do not participate in the AP’s feedback loop. iC3b though binds to complement receptors 3 and 4 to mediate phagocytosis (Gordon et al., 1987; Myones et al., 1988). Similarly, C3dg is recognized by complement receptor 2 which is expressed on B cells and follicular-dendritic cells and facilitates the adaptive immune response (Morikis and Lambris, 2004). However, given the remarkable decrease in C3 fragment deposition mediated by CR1 in our model system, it is not likely that adequate clusters of iC3b or C3dg would be present to serve as ligands for these complement receptors.

DAA is a “temporary fix” as the C3b remains available to bind Factor B and initiate the feedback loop. In contrast, CA is a “permanent fix” as the iC3b or C3dg generated cannot engage this amplification pathway. Moreover, in a previous study by Brodbeck et al (Brodbeck et al., 2000), using a different model system employing limiting amounts of convertases, DAA and CA functioned synergistically to inhibit C3b deposition. In the aforementioned study, DAA was required for CA to catalyze C3b cleavage. Taken together, this denotes that in an inflammatory/immune reaction featuring AP activation, CR1’s DAA provides a rapid initial response that is followed by progressive fragment degradation via

CA. Consequently, CR1 is an exceptionally potent inhibitor of the AP and especially its feedback loop.

4.3. Intrinsic vs extrinsic inhibitory profile

Complement regulatory proteins that have been shown to function intrinsically include MCP, DAF and CD59 (Oglesby et al., 1992; Brodbeck et al., 2000; Lublin and Atkinson, 1989). CR1 is a large linear membrane protein that is a receptor for IC and known to produce degradation of C3b attached to an IC (Medof et al., 1982a). Consequently, extrinsic activity seemed possible, if not likely. Our results establish, however, that on epithelial cells CR1 functions as an intrinsic regulator of CP and AP activation.

4.4. Immune complex binding by CR1

CR1 on RBCs is the IA receptor (Nelson, 1953, 1963; Schifferli et al., 1988, 1989). Binding of IC to CR1 is mediated by a multivalent interaction between clusters of C4b and C3b and CR1 (Medof et al., 1982b; Paccaud et al., 1988; Arnaout et al., 1983). Our studies indicate that CR1 on epithelial cells binds IC as proficiently as CR1 on RBCs. Although these complexes are not internalized, they remain attached via CR1 to the epithelial cells.

The present studies confirm and extend the observations of Makrides and colleagues (Makrides et al., 1992). These investigators demonstrated that CR1 inhibits complement-mediated lysis of CHO cells but not that of untransfected bystander cells establishing that CR1 is an intrinsic regulator of complement activation. We have also corroborated our prior findings that CA is a relatively slow process (compared to DAA) for membrane inhibitors and further point out that C4BP has no regulatory activity in this experimental system (Barilla-LaBarca et al., 2002).

To our knowledge, this is the first direct demonstration of the processing of C4b and C3b by CR1 on epithelial cells. Our data establish that CR1 intrinsically modulates both CP and AP and that DAA is key relative to inhibiting complement activation. CR1 is a particularly potent inhibitor of AP activation. Further, CR1 serves as a cofactor protein for cleavage of the deposited complement fragments. Also, IC binding by CR1 on CHO cells is comparable to that of human erythrocytes.

Our model system is also applicable to the workings of CR1 in human disease featuring an autoantibody such as membranous nephropathy or antibody-initiated graft rejection. In both cases, analogous to our CHO model system, antibodies may bind to the podocyte cell membrane and initiate complement activation.

These data potentially have far-ranging implications since alterations in CR1 expression are observed in many renal diseases, being particularly associated with systemic lupus erythematosus, IC-mediated membranoproliferative glomerulonephritis and other glomeruloproliferative diseases of the kidney (Pettersson et al., 1978; Kazatchkine et al., 1982; Nolasco et al., 1987; Moll et al., 2001). These diseases are characterized by variable degree of immune deposits in the mesangium, subendothelial space, subepithelial space and basement membrane. Despite these established patterns of injury, it has been difficult to conceive as to how circulating IC form intramembranous aggregates or localize in the

subepithelial space. One explanation asserts that the combination of high glomerular intracapillary pressure along with the uniquely fenestrated endothelium facilitates the movement of protein aggregates from the capillary lumen to the basement membrane (Schneeberger, 1974). Our data herein suggest that CR1 could play a critical role in maintaining homeostasis of the renal IC clearance system and that podocytes are significant players in this process. Our hypothesis is that IC that become opsonized with C3b or C4b are trapped within the glomeruli by their binding to CR1 on podocytes, which thereby serves to modulate their inflammatory potential.

Our future studies will utilize human podocytes transfected with CR1. We will systemically define the expression of complement regulators on the surface of these cells and develop experimental systems using these cells to elucidate the role of CR1 in complement regulation and IC handling in normal and disease states. An in-depth understanding of CR1 function will help to further delineate molecular mechanisms underlying the pathogenesis of complement-mediated renal disease and may suggest novel treatment strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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.molimm.2015.07.016>

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Fig. 1.

Structure of CR1.

Diagrammatic representation of the most common size allelic form of CR1 containing 30 complement control repeats (CCPs). There are three C4b binding sites (CCPs 1–3; 8–10 and 15–17) and two C3b binding sites (CCPs 8–10 and 15–17). CCPs 22–28 bind C1q, ficolins and mannose binding lectin (MBL). TM, transmembrane domain; IC, intracytoplasmic domain; the four long homologous repeats (LHR) of this protein are demarcated: 1–7, 8–14, 15–21, 22–28. The functional sites in 8–10 and 15–17 are nearly identical. Repeats in yellow are required for C4b binding and decay accelerating activity while those in purple are required for C3b and C4b binding and cofactor activity. (Modified from: Park et al., 2014).

Fig. 2.

CR1 expression by transfected CHO cells.

(A) Flow cytometry to compare expression of CR1 on transfected CHO cells. RCHO served as a negative control. Rabbit polyclonal anti-CR1 Ab was used as the primary Ab and FITC-labeled donkey anti-rabbit IgG as the secondary Ab. Red line, RCHO; green line, CR1–10 k; blue line, CR1–200k; orange line, CR1–2 m. Representative of four independent experiments (also see Table 1). (B) Western blot of CR1 in lysates of transfected CHO cells. The solubilized cell preparations were analyzed on 6% gel under non-reducing conditions. Following transfer, the blot was developed with the polyclonal anti-CR1 Ab (same Ab as in A). These cell lysates were also analyzed under reducing conditions and the relationship between full length CR1 and Pro-CR1 was the same and additional bands were not detected. Lane 1, RCHO; lanes 2, 3 and 4, sCR1 (15 ng, 20 ng, 25 ng); lanes 5–7, CR1–200 k (1x, 2x, 5x); lanes 8 and 9, CR1–2 m (1x, 2x). 1x equals 20,000 cell equivalents for CR1–200 k and 10,000 cell equivalents for CR1–2 m. Representative experiment of four. (C) Western blot showing a longer exposure of lanes 5–7.

Fig. 3.

Kinetic analysis of C4b cleavage by CR1 following classical pathway activation.

(A) Sensitized CR1–200 k cells were exposed to C7-deficient human serum for 5–90 min and surface C4b (via its C4c epitope) and C4d fragments were detected by flow cytometry. Monoclonal Ab to C4c detects uncleaved C4b [C4b contains the C4c fragment (see Fig. 1 supplement)] while monoclonal anti-C4d Ab detects C4b and the C4d fragment (5 min, anti-C4d MFI = 726). FITC-labeled goat anti-mouse served as a secondary Ab. The solid light line represents unsensitized cells exposed to secondary Ab only. Representative of three

independent experiments. (B) Sensitized RCHO cells were exposed to C7-deficient human serum for 5–90 min and C4 fragments were detected by flow cytometry (5 min, anti-C4d MFI = 726). Representative of three independent experiments. (C) Time course of C4d generation. The kinetics of C4b cleavage, based on the decrease in the anti-C4c signal, closely fit an exponential decay curve with $T_{1/2}$ of ~ 30 min. SEMs were too small to be depicted on the plot. Data points are averages of values obtained from three independent experiments as shown in the representative panel A.

Fig. 4.

Kinetic analysis of C3b cleavage by CR1 following classical pathway activation.

(A) Sensitized CR1–200k cells were exposed to C7-deficient human serum for 5–90 min and surface C3b and its fragments detected by flow cytometry. Monoclonal anti-C3c detects uncleaved C3b and iC3b [C3c is contained in C3b and iC3b (see Fig. 2 supplement)] while monoclonal anti-C3d Ab detects C3b, iC3b and C3d (5 min, anti-C3d MFI = 726). FITC-labeled goat anti-mouse served as the secondary Ab. The solid light line represents unsensitized cells exposed to secondary Ab only. Representative of three independent

experiments. (B) Sensitized RCHO cells were exposed to C7- deficient human serum for 5–90 min and surface C3 fragments were detected by flow cytometry (5 min, anti-C3d MFI = 2224). Representative of three independent experiments. (C) Time course of C3d generation. The steady anti-C3d signal indicates that the number of C3 fragments bound to the cell surface remained constant over the course of the experiment. The kinetics of iC3b cleavage based on the reduction in the anti-C3c signal, closely fit an exponential decay curve with $T_{1/2} \sim 24$ min. SEMs ranged between 0.004 and 0.007 and therefore were too small to be depicted on the plot. Data points are averages of three independent experiments (representative experiment in panel A).

Fig. 5.

CR1 efficiently inhibits alternative pathway activation.

Flow cytometry analysis depicting C3b deposition on sensitized cells exposed to C7-deficient human serum (10%) diluted in AP buffer for 5 min. Murine monoclonal Abs to human C3c and C3d were used as the primary Abs and FITC-labeled goat anti-mouse was the secondary Ab. (A) On RCHO, C3b deposition was substantial but there was no degradation. (B) The C3b deposition on CR1-200 k cells was decreased compared to RCHO. C3b on CR1-200 k is cleaved to C3c (released) and C3dg (remains covalently bound to surface). The solid light line represents unsensitized cells exposed to secondary Ab. Representative of three independent experiments. Similar results were obtained when RCHO and CR1-200 k cells were exposed to C7- deficient human serum for 5–90 min.

Fig. 6.

CR1 protects cells by an intrinsic mechanism.

Flow cytometry analysis demonstrating C3b deposition and degradation after complement activation on CR1-200 k and RCHO cells in a 1:1 ratio. The sensitized cell mixtures were incubated with 10% C7-deficient human serum in GVB or in Mg^{2+} -EGTA buffer for 60 min. Monoclonal Abs to C3c and C3d were used as primary Abs; FITC-labeled goat anti-mouse was the secondary Ab. [A] (i) Monoclonal Ab to C3d detected two peaks in the cell mix, the first represents CR1-200 k and the second represents RCHO. [A] (ii) and (iii) For

comparison, individual populations of RCHO and CR1–200 k are also shown in the middle and right hand panels, respectively. [B] (i) Monoclonal Ab to C3c also detected two peaks in the 1:1 cell mix, the first represents CR1–200 k and the second represents RCHO. [B] (ii) and (iii) For comparison, RCHO and CR1–200 k alone are shown in the middle and right hand panels, respectively. (C) CR1–200 k and RCHO mixed in 4:1 ratio. Monoclonal Ab to C3c used as primary Ab; FITC-labeled goat anti-mouse was the secondary Ab. Light red line represents unsensitized cells exposed to secondary Ab only. Results shown are representative of three independent experiments.

Fig. 7.

CR1 binds opsonized immune complexes.

CR1–200 k cells were incubated with C3b/C4b opsonized or non-opsonized control IC (rabbit peroxidase anti-peroxidase) for 30 min at 37 °C. FITC-labeled anti-rabbit IgG was used to detect IC. To assess specificity of this interaction, cells were pretreated with mAb 3D9, which blocks CR1 interactions with C3b and C4b. Solid line (light), cells plus non-opsonized IC; solid line (dark), cells plus C3b/C4b opsonized IC; dotted line, cells preincubated with 3D9 plus C3b/C4b opsonized IC. Representative of four independent experiments.

Fig. 8.

Comparison of immune complex binding on the surface of red blood cells to CR1-expressing CHO cells.

(A) Red blood cells and CR1-expressing CHO cells were incubated with serum opsonized IC (125 I-labeled BSA/anti-BSA) for 0–30 min. The percent of IC binding to the cells was calculated by counting radioactivity in the cell pellets and supernatants (see Section 2). Grey line, RBC; black line, CR1–2 m (B) CR1–200 k and CR1–2 m cell lines were harvested in amounts that would give equivalent total CR1 numbers. 1×10^7 cells of CR1–200 k (2×10^5

CR1/cell) and 1×10^6 cells of CR1-2m (2×10^6 CR1/cell) were incubated with serum-opsonized IC for 30 min and percent binding calculated.

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Table 1

Analysis of CR1 expression by CHO cell lines*.

Cell Type	CR1Copy Number/Cell	Geo Mean
RCHO	None	3 ± 0.02
CR1-10 k	~5000	6 ± 0.80
CR1-200 k	~200,000	218 ± 7
CR1-2 m	~2,000,000	1893 ± 264

* CR1 copy number/cell as determined by ELISA correlates ($r = 0.9999$, $p < 0.0001$) with the geometric mean obtained by flow cytometry. Values are mean ± SEM for four experiments.

Table 2

Kinetics of C4b cleavage following CP activation.

Time(min)	Anti-C4c(Geo Mean)	Anti-C4d(Geo Mean)	C4b cleavage(%)
5	724 ± 4	726 ± 3	0
15	523 ± 5	728 ± 4	29
30	393 ± 2	726 ± 2	46
45	340 ± 1	726 ± 5	54
60	194 ± 4	733 ± 4	74
90	97 ± 1	740 ± 6	87

Flow cytometry analysis of C4b cleavage. Maximum C4b deposition was achieved in less than 5 min. This was followed by C4b degradation into C4c (which is released into circulation) and C4d (remains covalently bound to cell) by CR1. Anti-C4c Ab detects the uncleaved C4b deposited on the cell surface while the anti-C4d Ab identifies the total C4 on the surface (the uncleaved C4b and the C4d fragment). Hence, the ratio (C4b)/(C4b + C4d) is used to determine the amount of cleaved C4b. 46% of the C4b is cleaved in approximately 30 min and 87% is cleaved by 90 min. Values represent mean ± SEM for three experiments.

Table 3

Kinetics of C3b cleavage following CP activation.

Time(min)	Anti-C3c(Geo Mean)	Anti-C3d(Geo Mean)	C3b cleavage(%)
5	726 ± 1	726 ± 1	0
15	516 ± 1	729 ± 1	29
30	274 ± 1	726 ± 1	62
45	137 ± 2	711 ± 2	81
60	126 ± 2	716 ± 2	83
90	69 ± 2	720 ± 1	91

Flow cytometry analysis of C3b cleavage. Maximum C3b deposition was achieved in less than 5 min. This is followed by C3b degradation initially into iC3b by Factor H and Factor I and then into C3c (which is released into circulation) and C3d (remains covalently bound to cell) by CR1 and Factor I. Anti-C3c Ab detects the uncleaved C3b and iC3b deposited on the cell surface while the anti-C3d Ab identifies the C3dg fragment and its presence in C3b and iC3b. Therefore, the ratio $(C3b + iC3b)/(C3b + iC3b + C3dg)$ is used to determine the amount of C3b cleaved to C3dg. 62% of the C3b is cleaved by 30 min and 91% is cleaved by 90 min. Values represent mean ± SEM for three experiments.