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Therapeutic uses of recombinant complement protein inhibitors

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Introduction

Complement acts in parallel and in concert with the immune system to protect the individual from microbial infection by mediating a variety of biologic reactions: increased vascular permeability, chemotaxis of leukocytes, opsonization, cytolysis of target organisms, and enhancement of the immune response. Activation of the complement system occurs via the alternative pathway in response to bacteria, yeast, virally infected cells [76, 84], or damaged tissue [10, 45], and via the classical pathway by particles or tissues recognized by classical pathway-activating antibodies [76, 84]. Both classical and alternative pathway activation serve to coat the offending surface with C3b and/or C4b by way of the metastable thiolester in these proteins. C3b and C4b are structural subunits of the C3 and C5 convertases, which proteolytically cleave C3 and C5. When unregulated, the convertases amplify the initial deposition of C3b, and allow the cascade to proceed to the assembly of fragments C5b through C9, the membrane attack complex (MAC), creating channels through the membrane which can cause cell death. Besides leading to cytolysis of activating cells, byproducts of C3 and C5 breakdown have additional inflammatory roles. The anaphylotoxins, C3a and C5a, induce histamine release by mast cells which leads to vasodilation and increased vascular permeability. C5a is a potent chemotactic factor which recruits and activates neutrophils, and both C3a and C5a induce rapid and enhanced production of leukotrienes from IL-3-primed basophils [11, 60]. Opsonization, or the deposition of C3b and C4b on an activating surface, marks the foreign particle with ligands for complement receptors involved in phagocytosis, and can lead to recognition by signaling receptors on phagocytes and lymphocytes specific for breakdown fragments of C3b.

While complement activation is a valuable first-line defense against potential pathogens, the activities of complement which promote a protective inflammato-

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ry reaction also carry the potential for harming the host by both indirect and direct mechanisms. For example, activated neutrophils are indiscriminate in their release of destructive enzymes, and complement components may be deposited on nearby cells as well as on targets. There is substantial evidence for the activation of the complement system in human disease, and for the dependency of tissue damage in animal models of disease on an intact complement pathway [18, 20, 23, 37, 61, 105].

Several proteins have evolved to control the extent of complement activation. The classical pathway C1 inhibitor will be discussed elsewhere in this volume, as will proteins which interfere with the insertion of the terminal components of the MAC, such as CD59 and homologous restriction factor. We will concentrate on proteins which have evolved to regulate complement activation at the level of C3 and C5 convertases, namely, members of the regulators of complement activation (RCA) gene family, and how they may be used to inhibit tissue damage due to complement in both antibody-independent and -dependent systems.

RCA family

The RCA gene family, summarized in Table 1 [14, 49], is a genetically linked group of proteins found in a 900-kb region on human chromosome 1, band q32 [12, 16, 66, 93, 95, 96, 106]. Two of the RCA members, factor H and C4 binding protein (C4bp) are serum proteins, while decay-accelerating factor (DAF) [65], membrane cofactor protein (MCP) [62, 65], and complement receptors type 1 and

Table 1. Characteristics of the proteins of the regulators of complement activation

RCA proteins	Size (kDa)[a]	Ligands	Binding SCRs	Decay acceleration		Cofactor activity	
				C3bBb	C4b2a	C3b	C4b
Factor H	160	C3b	1–6 [4,5]	+	–	+	–
C4bp	590	C4b	1–3 [b] [80]	–	+	–	+
DAF	70	C3b C4b	2–4 [22] 2–4	+	+	–	–
MCP	58–63	C3b C4b	2–4 [2] 1–4	–	–	+	+
CR1	190[c]	C3b C4b	8–11, 15–18 1–4 [55, 57–59]	+	+	+	+
CR2	140	C3dg iC3b EBV	1–2 [15, 63] 1–2 [54] 1–2 [15, 63]	–	–	[d]	–

SCRs, Short concensus repeats; DAF, decay-acelerating factor; MCP, membrane cofactor protein; EBV, Epstein-Barr virus

[a] Apparent size on SDS-PAGE of nonreduced samples

[b] Murine C4bp

[c] Most common allotype

[d] iC3b

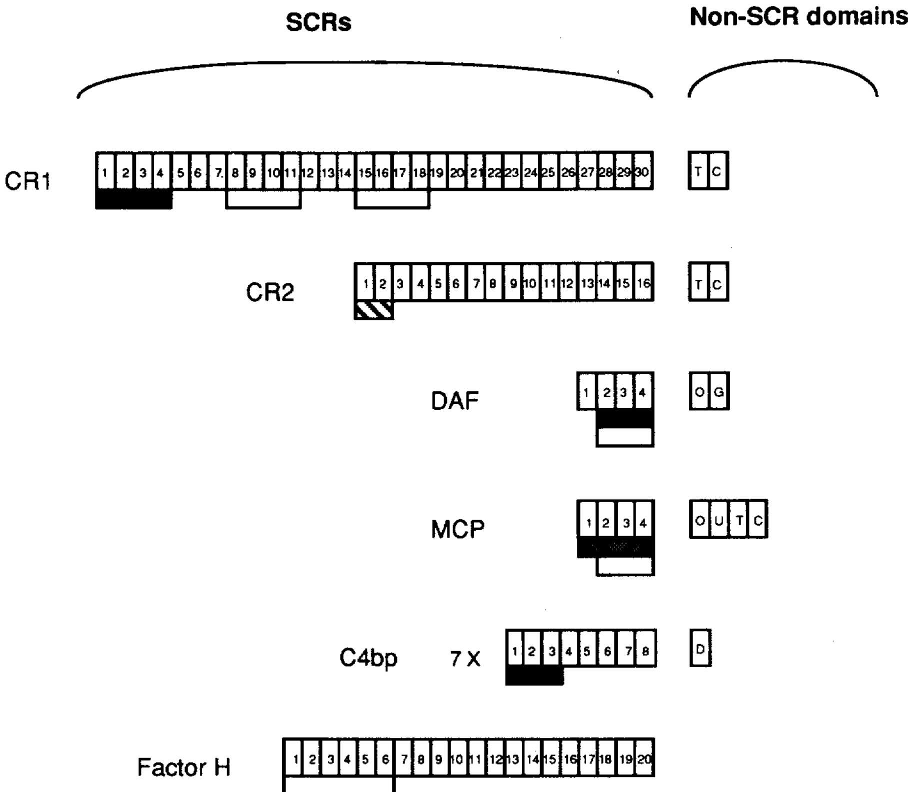

Fig. 1. Schematic representations of members of the RCA gene family, with *closed boxes* representing C4b-binding regions; *open boxes*, C3b-binding regions; *cross-hatched boxes*, iC3b-/C3dg-binding regions; *O*, site of O-linked glycosylation; *G*, glycolipid anchor; U, domain with unknown function; *D*, disulfide bridge-containing domain; *T*, transmembrane domain; and *C*, cytoplasmic domain

2 (CR1, CR2) [3] are membrane bound. They are structurally related proteins composed of 4 to 30 of the globular domains termed short consensus repeats (SCRs) [92] (Fig. 1). Each SCR is approximately 60 amino acids long, and has several conserved residues, including 4 cysteines which are disulfide linked within each SCR in a 1 to 3, 2 to 4 manner [53, 64]. Finally, the RCA proteins are functionally similar in that they recognize activation products of C3 or C4. Factor H [109] regulates alternative pathway complement activation in the fluid phase and on nonactivating surfaces, while C4bp inactivates classical pathway-activating particles containing C4b [35, 41, 98]. CR2, which has been reported to have weak cofactor activity for the proteolytic degradation of iC3b by the serum protease factor I [74], is more interesting for its signaling role on B lymphocytes [17, 43, 44, 68, 102].

DAF, MCP, and CR1 interact with C3b and C4b and regulate their ability to form C3 and C5 convertases. DAF accelerates the dissociation of the catalytic subunits of alternative and classical pathway C3 convertases, Bb, and C2a, respectively [36], and prevents association with new catalytic subunits. MCP irre-

versibly inactivates C3 or C5 convertases by acting as a cofactor for the proteolytic digestion of C3b or C4b. CR1 has all of these functions. It has been postulated that MCP and DAF have evolved cooperatively to protect autologous cells from complement-mediated damage, and that the function of CR1 is to inactivate C3b and C4b found on complement-activating complexes [6, 99].

The structure and function of DAF, MCP (and CR1) are discussed by Morgan and Meri elsewhere in this volume and a summary of their characteristics is shown in Table 1. However, CR1 is considered in detail here because it is the molecule which has principally been used for therapy.

CR1 (CD35). The most common allotype of CR1 is composed of 30 SCRs, 28 of which are arranged in tandemly repeating units of 7 SCRs termed long homologous repeats, or LHRs-A through -D, followed by two additional SCRs, a hydrophobic transmembrane domain, and a 43 amino acid cytoplasmic domain [57, 58, 48]. Deletion mutant analysis showed that the first two SCRs of LHR-A were responsible for C4b binding specificity, while the first two SCRs in LHRs-B and -C specified for C3b binding [58]. Thus, the most common allotype of CR1 has a total of three active binding sites, and is bivalent for C3b. Other more rare allotypes of CR1 [26–29, 113] are generally caused by the duplication or elimination of an intact LHR with C3b-binding function, leading to allotypes with more or fewer C3b-binding sites. This alteration can affect CR1 function, as assessed by the loss of affinity in binding C3b dimers, and a reduction in the effectiveness in inhibiting the C3 and C5 convertases [112]. The genomic structure of CR1 suggests that it evolved by duplication of segments of the gene encoding LHRs, perhaps by homologous recombination with unequal crossover [50, 114]. The extensive internal homologies between LHRs would facilitate their insertion or deletion to create the different alleles encoding the polymorphic allotypes of CR1, which differ by 30–40 kDa, the predicted size of an LHR. An additional CR1-like region [48, 50, 115] is found in the RCA locus, evidence of a non-functional rearrangement of the CR1 genetic elements.

Krych and colleagues [59] have mapped the amino acids in SCRs-8 and -9 which confer C3b-binding ability when transferred to the C4b-binding site in SCRs-1 and -2. Interestingly, these changes did not affect C4b binding. The limits of the intact binding site were mapped with additional deletion mutants [55], showing that the first four SCRs of either LHR-B (SCRs-8 through -11) or -C (SCRs-15 through -18) were necessary and sufficient to reconstitute the binding affinity for polymerzed C3b and the cofactor activity of full-length CR1. As the third and fourth SCRs in LHR-A are virtually identical to those in the C3b-binding LHRs, it is postulated that these SCRs play a similar role in C4b binding. When DNA encoding SCRs-8 through -11 is inserted at the amino terminus of the secreted form of an immunoglobulin heavy chain gene and expressed in a cell with an endogenous light chain, the purified chimeric protein retains all C3b-binding and cofactor functions of CR1, and has been shown in vitro to inhibit alternative pathway activation induced in human serum by zymosan [55].

CR1 is present on erythrocytes, B lymphocytes, neutrophils, monocytes, macrophages [31], and eosinophils, as well as on some T lymphocytes [111], follicular dendritic cells [94], glomerular podocytes [28, 56], Kupffer cells [46], and Schwann cells [116]. It was purified on the basis of its factor H-like ability to dissociate the alternative pathway C3 convertase and to act as a cofactor for the

factor I-mediated cleavage of C3b [30]. On a weight basis, it was eight-fold more active than factor H, and was not restricted by alternative pathway activating surfaces. Further studies showed that CR1 could act as a cofactor for C4b cleavage [51], thus inactivating the classical convertases as well as alternative pathway convertases. CR1 also accelerates the decay of each convertase [40, 51]. Unlike cells expressing MCP or DAF, CR1 expression on a cell allows it to rosette with C3b-bearing particles, or to bind soluble polymerized C3b, and to act as a cofactor for the cleavage of C3b and C4b in these particles [69, 70, 97]. Although CR1 has intrinsic activity as well as extrinsic activity [70], it is reasonable to postulate that the 90-nm extension of CR1 from the cell membrane [107] allows control of complement activation on adjacent complexes. CR1 has been implicated in controlling the solubility and clearance of immune complexes [21], participating in endocytosis of complexes and particles by neutrophils and macrophages [1], as well as having other putative immunoregulatory roles [3, 24, 108].

Potential clinical applications of RCA proteins

Complement has been shown to play a role in the pathogenesis of some types of inflammatory tissue damage initiated by both non-immunologic [39, 45] and immunologic [37, 61, 91, 105] conditions. In animal models, removal of classical pathway-activating antibodies [33, 87], depletion of complement by cobra venom factor [19, 32, 39, 61], or testing the models in animals genetically deficient in specific complement components [39, 105] abrogates or delays pathogenesis. Tissue injury can be caused by complement either directly or indirectly. The formation of the MAC on diseased or healthy cells may lead to cell lysis or even to activation, as for example occurs with endothelial cells in which MAC insertion results in calcium flux and expression of binding sites for various thrombolytic mediators [42], as well as release of heparin sulfate from endothelial surfaces to contribute to a hypercoagulable state [86]. Indirectly, the byproducts of C3 and C5 degradation recruit and activate neutrophils, and may synergize with interleukins to cause synthesis of inflammatory mediators such as leukotrienes [11, 60]. Neutrophil activation caused by the complement system has been implicated in damage to organs distinct from those in which activation has occurred, such as in adult respiratory distress syndrome (ARDS) [100].

For a complement inhibitor to be clinically useful, several requirements must be met. First, the molecule should be able to inhibit complement activation at the level of the C5 convertases of both alternative and classical pathways, not only to protect a cell from lysis, as may be achieved by CD59, but also to block the generation of C5a which would induce neutrophil influx and attendant inflammation. Secondly, an inhibitor should have a high affinity for the multivalent complexes of C3b and C4b found in the convertases. Third, it should irreversibly inactivate the convertases, and fourth, the inhibitor should be able to recycle and inhibit multiple convertases.

The members of the RCA family represent potential therapeutic inhibitors that can be evaluated according to these requirements. C4bp and factor H cause irreversible inactivation of C4b and C3b, but they are unlikely to be useful. The former acts only to prevent spontaneous, but not immune complex-induced classical pathway activation. As its major ligand is C4b, damage caused by primary

alternative pathway activation or that recruited by classical pathway activation would be unaffected. The latter serves only to prevent spontaneous but not induced activation of the alternative pathway.

Except for DAF found in the fluid phase, which functions similarly to C4bp in C4b2a decay acceleration [72], DAF and MCP are intrinsic regulators of complement, which function only when incorporated into a cell membrane. DAF does not induce irreversible inactivation of C3b and C4b, is restricted by alternative pathway activating surfaces [83], and is not associated with a clinical phenotype in its absence on Inab blood group erythrocytes [47, 73, 101], all of which suggests a modest inhibitory potential. MCP meets several requirements by acting in both pathways, by irreversibly inhibiting the function of C3b and C4b, and by being able to recycle with additional substrate. These characteristics may indicate that MCP is a suitable candidate for a cell-bound inhibitor, but its monovalency and relatively low affinity suggest that it could not serve as an effective soluble inhibitor.

CR1 meets these requirements by being capable of inhibiting both the classical and alternative pathway C3 and C5 convertases, by interacting multivalently with these complexes through its three binding sites, by irreversibly inactivating C3b and C4b through promoting their proteolysis by factor I, and by recycling for inhibition of additional C3/C5 convertases after release from degraded C3b and C4b.

Applications of soluble CR1

A soluble form of CR1 (sCR1) was made by genetically engineering a stop codon in CR1 cDNA before the transmembrane and cytoplasmic domains [107]. When purified from the media of cells transfected with the altered cDNA, this molecule was able to bind C3b and serve as a cofactor for C3b cleavage. The classical pathway-mediated lysis of sensitized sheep erythrocytes and the alternative pathway activation induced by zymosan were efficiently inhibited by sCR1 at concentrations 100-fold lower than normal serum concentrations of factor H and C4bp [107]. This molecule has been tested for its ability to inhibit damage caused by complement in several models of disease (Table 2).

Non-antibody-dependent complement activation

Reperfusion injury. The complement system was suggested to have a deleterious effect on ischemic myocardium by the finding that administration of cobra venom factor to dogs prior to coronary artery occlusion decreased the size of the myocardial infarction [67]. The maintenance of viable myocardium was correlated to reduced neutrophil infiltration. To determine whether complement activation contributed to reperfusion injury of ischemic myocardium, 1 mg sCR1 was administered as a single bolus injection to rats immediately before temporary occlusion of the left coronary artery for 35 min, after which perfusion was reestablished. Seven days later, the animals were killed and sCR1 was found to have reduced the sizes of the infarcts by 44%. In a second group of rats subjected to myocardial ischemia and reperfusion, the mechanism of the protective effect of sCR1 was shown to be inhibition of MAC deposition on endothelial cells in the

Table 2. Complement-dependent disease models in which soluble CR1 reduces pathology

Complement-dependent disease models	sCR1 effects
Antibody mediated:	
Hyperacute rejection:	
Cardiac allografts in Lewis rats [88]	Allograft heart survival increased from 3.25−/+0.81 h to 32 −/+4.47 h
Cardiac xenograft (guinea pigs to rats) [89, 117]	Xenograft survival increased from 17−23 min to 64−747 min in a dose-dependent manner
Immune complex-induced inflammation:	
Reversed passive Arthus reaction [119]	Reduces vasculitis
Immune complex-induced alveolitis [77]	Reduces alveolitis by 68−72%
Non-antibody mediated:	
Reperfusion injury:	
Reperfusion injury of ischemic myocardium in rats [107]	Reduces myocardial infarction size by 44%
Reperfusion injury of intestine in rats [45a]	Reduces intestinal and pulmonary injury
Thermal trauma:	
Acute skin and lung injury by thermal trauma [77]	Reduces damage in lung by 45−46% and in skin by 25% (1 hr), 44% (4 h)

sCR1, soluble CR1

ischemic zones, and suppression of neutrophil accumulation at these sites [107]. This study demonstrated for the first time the potential utility of sCR1 as a therapeutic inhibitor of complement, and has been extended by another study showing reduction of local and remote injury after ischemia-reperfusion of the intestine [45a].

Thermal trauma. In a rat model of thermal injury to 25−30% of body surface area, sCR1 reduced dermal vascular permeability by 44%, and pulmonary vascular permeability and hemorrhage by 45% [77]. Thus, sCR1 is protective both locally at the primary site of non-immune injury, and distally in the lung in this model of ARDS.

Antibody-dependent complement activation

Hyperacute rejection. The current shortage of human organs available for transplantation is an incentive to develop systems in which xenogeneic organs could be used [7, 9]. However, the occurrence of natural antibodies directed to endothelial antigens in discordant xenografts [87], or of an alternative pathway activating capability of the xenograft [34, 75, 104] causes rapid rejection by a thrombotic process induced by MAC assembly on endothelial cells [13, 75, 85]. A similar process may account for hyperacute graft rejection that occurs in recipients having alloantibodies specific for donor antigens. The role of complement in this process was confirmed and a potential inhibitor described when human sCR1 was shown to suppress hyperacute rejection of allo- or xenografts. Hyperacute rejec-

tion of cardiac allografts in sensitized Lewis rats was delayed from 3.25 ± 0.81 h in untreated rats to 32.00 ± 4.47 h in animals that received 3 mg/kg sCR1 [88]. When administered to rats immediately prior to reperfusion of guinea pig cardiac xenografts, sCR1 extended graft survival from 17 min in untreated rats to 64 to 747 min, with the time of survival correlating with the dose of CR1 administered, ranging from 3 to 60 mg/kg in this trial [89]. Both treated and untreated animals showed significant IgM deposition in the xenografts, indicating that sCR1 inhibited damage at the level of complement activation, rather than affecting the levels of natural antibody [89, 90]. The use of recombinant RCA proteins expressed in endothelial cells of xenografts is discussed below.

Immune complex-induced inflammation. In a rat model of immune complex-induced alveolitis, sCR1 treatment reduced vascular permeability, hemorrhage and neutrophil accumulation by 68–72% [77]. sCR1 also inhibited the reversed passive Arthus reaction in rats, in which intravenous injection of ovalbumin (OVA) is followed by intradermal injection of anti-OVA antibodies [119].

Other forms of complement inhibitors

Systemic complement inhibition may be undesirable because of suppressed host defense; the targeting of an inhibitor to a particular tissue may overcome this drawback. In the mapping of the CR1-binding site [55], it was shown that the C3b-binding SCRs could be transferred to the amino termini of antibody heavy chains and retain function. This expression of SCRs near the antigen-binding site had no effect on the ability of the antibody to bind its hapten, 4-hydroxy-3-nitrophenacetyl, and allowed purification of the chimera on hapten-sepharose. Thus, if a monoclonal antibody specific for a particular tissue antigen unique to the site of complement activation were identified, a chimeric CR1/antibody inhibitor could be created that might yield effective local, rather than systemic complement inhibition.

Long-lasting suppression of complement activation by particular cells may be achieved through introduction into such cells of genes encoding complement regulatory proteins. This strategy may be applicable to the creation of endothelial cells having low complement-activating potential, the best example of potential utility being in xenografts because of the incompatibility between DAF, MCP, and CD59 expressed by xenografts and the heterologous source of complement [118].

To provide an experimental rationale for this approach, DAF was incorporated into porcine endothelial cells in a dose-dependent manner, and was shown to confer protection from lysis by 10% human serum [25], a result anticipated by earlier findings on the effects of DAF incorporated into PNH erythrocytes [71, 78], or expressed in murine/human cell hybrids [103]. A more prolonged effect of DAF was achieved in vitro by transfection of murine NIH-3T3 cells with human DAF cDNA, which suppressed their lysis by antibody and human complement [81, 110]. Similarly, high levels of expression of MCP cDNA in NIH-3T3 cells also diminished their sensitivity to classical pathway-mediated lysis [81, 110]. It is hoped that the protection in vitro of cells from lysis by heterologous complement may predict the successful creation of a transgenic donor animal for xenogeneic transplantation [8, 25, 82, 85, 103]. However, the occurrence of hyperacute allo-

graft rejection, in which species-specific DAF, MCP, and CD59 [79] are incapable of suppressing complement activation induced by antibodies directed to alloantigens on graft endothelial cells suggests that the expression of these regulatory proteins in xenografts derived from transgenic animals will not avoid hyperacute rejection.

The capacity of sCR1 to suppress hyperacute rejection in a dose-dependent manner for several hours may indicate that this RCA protein should be evaluated for its potential role as a membrane inhibitor of complement activation. Accordingly, preliminary studies have demonstrated that expression of wild-type CR1 in transformed human endothelial cells confers relative resistance to lysis mediated by human complement and rabbit antibody (Braverman, Seok-Yong Kim, and Fearon, unpublished).

Conclusion

In conclusion, it is apparent that researchers are poised at the threshold of developing inhibitors of complement activation from the molecules in the RCA family. By creating soluble forms of these protective proteins for in vivo administration, or by making transgenic animals expressing these proteins or their derivatives, it may be possible to inhibit complement-mediated pathology stemming from autoimmune disease, reperfusion injuries, and physical trauma. This technology combined with current attempts to protect allografts from cellular rejection with monoclonal antibodies against members of the integrin family of adhesion molecules [52] makes it possible that the excessive mortality due to the severe shortage of human donor organs could be overcome by the use of xenografts.

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