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Development of complement therapeutics for inhibition of immune-mediated red cell destruction

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Abstract

A major objective of my National Blood Foundation (NBF)-funded proposal was to produce recombinant soluble forms of a complement regulatory protein called complement receptor 1 (CR1) that carries the Knops blood group system antigens to perform antibody neutralization studies. By generating these recombinant proteins, we were able to inhibit several Knops antibodies in patient serum samples, thereby demonstrating their usefulness for clinical use. Interestingly, the recombinant CR1 proteins generated through NBF funding were also found to strongly reduce complement-mediated red cell destruction in a mouse hemolytic transfusion model. In this review, I will outline our NBF-funded studies, give an overview of recent advances from our group and others in the development of complement therapeutics, and highlight their potential use in the transfusion medicine setting.

THE COMPLEMENT SYSTEM IN TRANSFUSION MEDICINE

The complement system is an important part of the innate immune system that responds to challenges by microorganisms before an adaptive response has developed.¹ It is also an important regulator of B-cell, and possibly T-cell, immunity.² In addition to its essential role in immune defense, the complement system contributes to tissue damage in many clinical conditions, including ischemia-reperfusion injuries after myocardial infarction, and in patients undergoing cardiopulmonary bypass.^{3,4} In the transfusion medicine setting, complement-mediated red cell (RBC) destruction plays a critical role, being involved in both intravascular and extravascular hemolysis.⁵ Generally, in the presence of a potent, complement-binding antibody and large numbers of closely situated RBC antigens, complement activation can proceed to completion, resulting in intravascular hemolysis, which can be fatal.⁶ The majority of blood group antibodies (including both allo- and autoantibodies) that can fix complement, however, only activate complement up to the C3 stage but do not go on to act as hemolysins. Although antibody-coated RBCs can be destroyed extravascularly without complement activation, RBC removal by tissue macrophages in the spleen and liver is enhanced considerably when C3 is present on RBCs in addition to immunoglobulin G (IgG). Indeed, as many as 50 percent of patients with autoimmune hemolytic anemia have both IgG and complement on their RBCs.⁷

Complement-mediated intravascular and extravascular destruction of RBCs has been implicated as a contributing cause of anemia associated with sickle cell disease (SCD)^{8–18} and in severe malarial infection.^{19–25} In addition, in patients with paroxysmal nocturnal hemoglobinuria (PNH) syndrome, the observed intravascular hemolysis is due to increased sensitivity of RBCs to autologous complement-mediated lysis.^{26,27} It is generally believed that the lack of CD59, which inhibits the assembly of the membrane-attack complex C5b-C9 by interacting with C8 and C9,^{28,29} is most likely responsible for the increased sensitivity of PNH RBCs and platelets to complement.^{28,30–34}

Complement regulatory proteins and complement receptor 1

Depending on the type of activators, the complement system can be divided into three separate pathways (classical, alternative, and lectin) and nearly 30 different serum and membrane proteins, which following activation interact in a highly regulated enzymatic cascade to generate reaction products that mediate inflammation and host protection (Fig. 1).¹ Because of the direct and indirect powerful cytolytic activity of complement, there exists a family of structurally and functionally related proteins known as regulators of complement activation (RCA) that prevent potential host cell damage from complement activation.³⁵ Complement receptor 1 (CR1), also known as CD35, is the most versatile of the RCA family because it possesses decay-accelerating and cofactor activities that can inactivate the two critical enzymes of the complement activation pathways.^{36–39} Specifically, by binding to C4b, it can displace the catalytic subunits of the C3 convertase of the classical pathway, thereby inhibiting the enzyme. In addition, by acting as a cofactor for plasma protease factor I, it is responsible for the degradation of C4b and thus complete inactivation of this C3 convertase. Similarly, CR1 inhibits and inactivates C3b-containing convertases. By binding to C3b, it can displace the catalytic subunits C3 convertase of the alternative pathway and C5 convertases of both classical and alternative pathways. Moreover, through its cofactor activity, it can cleave C3b to iC3b and then to C3dg plus C3c. Indeed, CR1 is the sole known cofactor for the second cleavage reaction by factor I of iC3b to C3dg and C3c.

CR1 structure and expression pattern

In humans, four allotypes of CR1 varying in size are known.⁴⁰ The predicted amino acid sequence of the common allotype (CR1*1) is 2039 residues, including a 47-residue signal peptide, an extracellular domain of 1930 residues, a 25-amino-acid transmembrane domain, and a 43-amino-acid cytoplasmic region (Fig. 2).^{41–43} The extracellular domain of CR1 with 25 potential N-glycosylation sites can be divided into 30 short consensus repeats (SCRs), each having 60 to 70 amino acids with sequence homology between SCRs ranging from 60 to 90 percent.⁴² The first 28 SCRs are further arranged into four longer regions of similarity termed long homologous repeats (LHRs) consisting of seven SCRs each.^{41,43} The number of LHRs among CR1 allotypes varies from three to six, thereby explaining the size differences between the allotypes.⁴⁰

CR1 is expressed on a number of cell types in the blood and at low levels in soluble form in the plasma.¹ Of the RCA family members, CR1 has the widest range of activities and functions including immune complex clearance, regulation of complement activation,

phagocytosis, and the antibody response. On RBCs, CR1 has the important function of transporting immune complexes or particles bearing C3b clusters, for example, parasites and bacteria, to the spleen and liver for their removal.^{44–48} CR1 on macrophages and neutrophils mediates phagocytosis of pathogens or immune complexes carrying C3 and C4 split products.^{49,50} CR1 is also present on B cells, modulating the threshold for B-cell activation.^{51,52} Moreover, CR1 is expressed on 10 to 15 percent of a subpopulation of human T lymphocytes,^{53–56} although its function on such cells is not clear. Finally, CR1 is present on antigen-presenting cells and reservoirs of antigen such as follicular dendritic cells where it is thought to be important for trapping immune complexes within germinal centers in lymphoid organs, thereby improving the immune response to antigen.^{57–65} In this regard, it is conceivable that CR1 plays a role in deletion of autoreactive B cells and possibly T cells. In addition, CR1 was shown to be a ligand in the rosetting of malarial-infected RBCs with noninfected cells, a phenomenon that occurs in severe forms of malarial infections.⁶⁶

CR1-associated Knops system antigens

RBC CR1 carries blood group antigens in the Knops system.^{67,68} Antibodies to Knops system antigens belong to a group of antibodies referred to as high-titer, low-avidity antibodies. These antibodies are characteristically weak and have variable reactivity, but are not clinically significant. Nevertheless, their presence in the patient sera interferes with identification of underlying clinically significant antibodies as well as with cross-matching. In 1996, Moulds and Rowe⁶⁹ demonstrated that Knops antibodies can be neutralized with a recombinant soluble form of CR1, thereby providing a tool to identify and remove Knops system antibodies from sera. Based on this report, I proposed in my National Blood Foundation (NBF) grant to further characterize the location of Knops antigens with the goal of producing smaller and more cost-effective recombinant proteins that neutralized Knops system antibodies. To this end, several truncations of the CR1 protein were expressed as soluble forms in a mammalian expression system. We found that for the most part, LHRD of the CR1 protein was able to neutralize most of the antibodies to the Knops system antigens, indicating that Knops antigens were located in LHRD.⁷⁰ In addition, some of the antibodies to Kn^a antigens were neutralized by LHR-C, and we therefore concluded that a mixture of LHRC and LHRD recombinant proteins would be sufficient for removing Knops system antibodies from sera.

Extensive population studies by Moulds and her colleagues have led to a more complex picture of antigenic determinants of the Knops system antigens (see below). Thus far, eight Knops blood group antigens have been described (KN1 through KN8).⁷¹ To date no individuals completely lacking the CR1 protein have been identified. RBCs that express low levels of CR1 (10% of normal values) and its associated Knops blood group antigens known as the Helgeson phenotype have been described, however.⁷² A detailed characterization of the molecular basis of most of the Knops system antigens was performed by Moulds and coworkers.^{73–75} The authors confirmed their data by performing inhibition studies with recombinant CR1 truncations. They found that single-nucleotide changes in SCR25 of LHRD result in the KN1/KN2 (Kn^a/Kn^b), KN3/KN6 (McC^a/McC^b), KN4/KN7 (SI^a/Vil), and Kn^a/Kn^b antigens. The knowledge of the molecular basis of these antigens will enable

accurate typing of Knops system antigens as well as construction of smaller recombinant proteins for antibody neutralization studies.

CR1 DERIVATIVES AS COMPLEMENT THERAPEUTICS

Because CR1 is the most versatile complement regulatory protein among the RCA proteins, it was hypothesized that it could be manipulated for development of a complement inhibitor for abrogation of complement-mediated tissue damage. Indeed, a soluble form of CR1 (sCR1), lacking the transmembrane and cytoplasmic tail that retains all the functional properties of a cell-bound CR1 was generated⁷⁶ and successfully shown to prevent complement-mediated tissue injury in several animal models.^{77,78} More importantly, sCR1 has been in human clinical trials for the treatment of acute respiratory distress syndrome and to reduce tissue damage in myocardial infarction and lung transplantation^{79–81} with possible favorable outcomes.⁸⁰ Despite the role of CR1 as a global inhibitor of complement activation, the antibacterial defenses of the patients undergoing a single treatment were not compromised, underscoring the usefulness of sCR1 as a therapeutic agent.⁸¹

The potential of sCR1 for inhibiting complement-mediated RBC destruction after transfusion immunization events has important clinical implications that we decided to explore with the recombinant sCR1 proteins produced with NBF funding. These studies used a mouse model of transfusion reactions in which human RBCs were injected intravenously to maximally activate the complement system.⁸² By injecting the mice with recombinant full-length sCR1 at 10 mg per kg, a dose well tolerated in human subjects, at the time of transfusion, we were able to demonstrate that deposition of complement components was drastically reduced and the survival of transfused RBCs was significantly prolonged.⁸² These data strongly indicated that sCR1 is a potential antihemolytic therapeutic agent. For our NBF studies, we had also prepared several recombinant CR1 truncations of the entire extracellular domain of CR1. In a short period of time we were able to test the effectiveness of these constructs in our mouse xenotransfusion model.⁸² Based on the results, we went on to define a 250-amino-acid-long region at the N-terminal domain of CR1 that contains a complement inhibitory domain and can significantly prolong survival of the transfused RBCs in mice.⁸³ We are currently performing more detailed structure-activity studies of this 250-amino-acid domain for the future design of CR1-based small molecule inhibitors.

OTHER COMPLEMENT THERAPEUTICS

Other membrane-bound RCA proteins have been exploited as therapeutic agents, although, unlike CR1, they only possess one complement inhibitory activity (either cofactor activity or decay accelerating activity) and are therefore by themselves not as effective as CR1-based derivatives.^{84,85}

Besides the use of complement receptors, different strategies for limiting complement activation are currently being explored.⁸⁴ Some of the approaches and their potential for prevention of complement-mediated RBC destruction are discussed below.

Small peptides

Another promising anticomplement agent that inhibits the C3 step is compstatin, a cyclic 13-residue peptide that binds naïve C3 and prevents its cleavage by convertase enzymes.⁸⁶ It has been tested in several animal models and shown to be an effective complement inhibitor.⁸⁷ Its ability to inhibit complement-mediated immune hemolysis in vivo remains to be tested, however. In addition, C1q-binding peptides that specifically inhibit the classical pathway were recently described,⁸⁸ although they have not yet been tested in vivo.

Small organic molecules

Among synthetic small-molecular-weight compounds, the protease inhibitor FUT-175 has been shown to be a potent complement inhibitor for shutdown of both the classical and alternative pathways in animal models and in human clinical trials.^{89,90} We recently showed that FUT-175 reduced complement-mediated RBC destruction in our mouse model of transfusion reactions.⁹¹ Another effective small molecule is C1s-INH-248, which acts as a reversible and competitive inhibitor of the first complement components C1s and has been shown to inhibit tissue injury in a rabbit myocardial infarct model.⁹² Its effectiveness in inhibiting complement-mediated RBC destruction remains to be tested.

Antibodies

A humanized C5 monoclonal antibody (MoAb; eculizumab) and modified forms of it that inhibit the cleavage of C5, thereby blocking the activation of terminal complement components, have been shown to effectively reduce complement-mediated injury both in animal models of disease and in humans.^{93–95} Hillmen and colleagues⁹⁶ recently described the effectiveness of eculizumab in reducing the incidence of intravascular hemolysis, hemoglobinuria, and transfusion requirements in patients with PNH. In a small study of RBCs from patients with SCD, defective regulation of the membrane attack complex formation in sickle RBCs has been observed.¹⁸ Such patients can potentially be treated with eculizumab to reduce complement-mediated hemolysis. Another antibody molecule that may have potential use in the transfusion medicine setting is antifactor D. MoAbs to factor D have been shown to reduce the alternative pathway activation during extracorporeal circulation of human whole blood used as a simulated model of cardiopulmonary bypass.⁹⁷

Targeting complement inhibitors to cell surfaces

Different strategies have been taken for targeting of complement inhibitors to specific sites to avoid the problems of nonselective complement blockade. Several complement regulatory molecules expressed in recombinant soluble forms have been modified posttranslationally by addition of a membrane-targeting tag (addressin) composed of a peptide sequence with affinity for phospholipid headgroups as well as for the membrane bilayer interior.^{98–100} A fragment of CR1, previously shown to be an active complement inhibitor,¹⁰¹ was fused to such addressins, and the hybrid molecule termed APT070 is currently in clinical trials in the United Kingdom in patients with rheumatoid arthritis.⁹⁸ The usefulness of APT070 for inhibiting immune hemolysis remains to be tested. Nevertheless, it is interesting to note that the functional homolog of human CR1 in mice was found to be the principal regulator of

spontaneous C3 activation,¹⁰² implying that increasing the levels of CR1 on RBCs by agents such as APT070 may render RBCs resistant to lysis.

Addition of sialyl Lewis^x moiety, known to bind selectins, onto sCR1 has resulted in an agent that can be targeted to sites of inflammation and inhibit selectin-dependent white blood cell adhesion.^{103,104} For targeting of complement inhibitors specifically to RBCs, a single-chain antibody fragment (scFv) specific for TER-119, an RBC-specific mouse antigen, was recently fused to DAF, resulting in large numbers of DAF molecules targeted to the mouse RBC membrane and protection against lysis.¹⁰⁵ This strategy can potentially be used to restore complement inhibitory properties to RBCs of patients with PNH¹⁰⁵ or to patients with malarial anemia who have been found to have reduced levels of complement regulatory proteins on their RBCs.²⁵

CONCLUSIONS

Different strategies for limiting complement activation are currently being explored including the use of soluble forms of complement receptors, antibodies to specific components of the complement pathway, and small-compound inhibitors.⁸⁴ In the transfusion medicine setting, the successful application of these inhibitors will ultimately depend on our understanding of the role of complement in a particular disease state. For example, in hemolytic transfusion reactions, we know that complement is strongly activated with the release of the large amounts of both C3a and C5a anaphylatoxins.⁵ Because activation of one complement pathway leads to the recruitment of another, blocking the C3 step may be the best way to totally shut off the complement cascade. Thus, agents that inhibit the C3 step such as CR1 derivatives, compstatin, and even FUT-175 are likely to be the most effective anticomplement inhibitors for acute treatment of hemolytic transfusion reactions. In addition, prophylactic treatment with such C3 inhibitors may permit mismatched RBC transfusions to certain patients such as trauma patients for whom there is no time to type and cross-match blood. It is important to note that total complement blockade should be short-term as chronic treatment with C3 inhibitors is likely to result in infection because opsonization of microbes is blocked¹⁰⁶ as well as in induction of autoimmune diseases due to inhibition of immune complex clearance.¹⁰⁷

In treating PNH patients, where the complement defect is at later stages of the complement cascade, the use of agents that block C5 such as anti-C5 (eculizumab) is likely to be sufficient to correct the complement-mediated RBC destruction. By inhibiting the C5 step, the early complement components are left intact for opsonization and immune complex clearance. In contrast, the exact mechanism(s) of complement deregulation in SCD and malarial anemia needs to be elucidated such that specific anticomplement therapeutics can be tailored to treat these patients. For example, if defects in specific complement regulatory proteins are indeed found to be a mechanism that contributes to anemia in SCD and malarial infections, the approach of selective inhibition of specific steps in the complement cascade can be taken. By this targeted approach of selective inhibition, partial complement system function can be retained to exert some of its important biologic activities. Finally, given that long-term systemic inhibition of the complement system can be detrimental to the patient's

health, the strategy of targeting the inhibitors with addressins or hybrid antibodies to the site of pathology may be an alternative to systemic complement inhibition.

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ABBREVIATIONS

CR1	complement receptor 1
LHR(s)	long homologous repeat(s)
NBF	National Blood Foundation
PNH	paroxysmal nocturnal hemoglobinuria
RCA	regulators of complement activation
SCD	sickle cell disease
SCR(s)	short consensus repeat(s)
sCR1	soluble form of CR1

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Fig. 1.

The complement system. The classical pathway is activated by antigen-antibody complex and the alternative and lectin pathways by microbial surfaces. Activation of these pathways results in the generation of the key enzymes C3 and C5 convertases, which in turn results in the release of C3a, C4a, and C5a anaphylatoxins (inflammatory response) and C3b (opsonization of target cells) and the generation of the membrane attack complex in the target cell (lysis). The different steps of the cascade where some of the complement regulatory proteins inhibit complement activation are shown. MBL = mannose-binding lectin; MASP = MBL-associated serine protease; C4bp = C4-binding protein, CR1; DAF = decay-accelerating factor; MCP = membrane cofactor protein. Adapted from Makrides.⁷⁷

Fig. 2. Schematic representation of the common isotype of CR1. The 30 SCRs are represented by square blocks and are numbered according to Klickstein and colleagues.⁴¹ Groups of seven SCRs are further subdivided into four LHRs. TM = transmembrane; cyto = cytoplasmic; aa = amino acids.