Preclinical evaluation of the neuroprotective effect of soluble complement receptor Type 1 in a nonhuman primate model of reperfused stroke


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Object. Postischemic cerebral inflammatory injury has been extensively investigated in an effort to develop effective neuroprotective agents. The complement cascade has emerged as an important contributor to postischemic neuronal injury. Soluble complement receptor Type 1 (sCR1), a potent inhibitor of complement activation, has been shown to reduce infarct volume and improve functional outcome after murine stroke. Given numerous high-profile failures to translate promising antiinflammatory strategies from the laboratory to the clinic and given the known species-specificity of the complement cascade, the authors sought to evaluate the neuroprotective effect of sCR1 in a nonhuman primate model of stroke.

Methods. A total of 48 adult male baboons (Papio anubis) were randomly assigned to receive 15 mg/kg of sCR1 or vehicle. The animals were subjected to 75 minutes of middle cerebral artery occlusion/reperfusion. Perioperative blood samples were analyzed for total complement activity by using a CH50 assay. Infarct volume and neurological scores were assessed at the time the animals were killed, and immunohistochemistry was used to determine cerebral drug penetration and C1q deposition. An interim futility analysis led to termination of the trial after study of 12 animals. Total serum complement activity was significantly depressed in the sCR1-treated animals compared with the controls. Immunostaining also demonstrated sCR1 deposition in the ischemic hemispheres of treated animals. Despite these findings, there were no significant differences in infarct volume or neurological score between the sCR1- and vehicle-treated cohorts.

Conclusions. A preischemic bolus infusion of sCR1, the most effective means of administration in mice, was not neuroprotective in a primate model. This study illustrates the utility of a translational primate model of stroke in the assessment of promising antiischemic agents prior to implementation of large-scale clinical trials.

Key Words • complement system • neuroprotection • stroke • Papio anubis

With the realization over the last 10 years that timely pharmacological and mechanical revascularization is capable of improving outcome after ischemic stroke, it is acknowledged that patients with ischemic stroke are at risk for varying degrees of reperfusion injury that can lead to progressive neurological damage and impaired recovery. Several potential mechanisms for this damage are recognized, including infiltration and accumulation of neutrophils, monocytes, and platelets; adhesion molecule upregulation; and the production of reactive oxygen species and cytokines. Targeting these postischemic inflammatory processes has resulted in improved outcomes in several rodent models of cerebral ischemia.

The complement cascade, an important contributor to the postischemic inflammatory response, has been implicated in the pathogenesis of cerebral ischemia-reperfusion injury. Early studies have demonstrated the effect of complement component upregulation on neurons and microglia after transient cerebral ischemia, as well as ischemic neuroprotection by targeting complement with agents such as C1 inhibitor and cobra venom factor. Although C1 inhibitor and cobra venom factor may exhibit some of their protective effect by interfering with processes that are not dependent on complement, more specific complement inhibitors, such as sCR1, have also proved effective in limiting cerebral ischemia/reperfusion injury in rodents, with treated animals demonstrating a 40% reduction in infarct volume and significantly improved neurological function. These studies have definitively established targeted anti-complement therapy as a viable neuroprotective option among antiinflammatory strategies, but efforts to translate other promising antiinflammatory strategies from the laboratory to the clinical arena have failed. These shortcom-
ings highlight the difficulty of ushering therapeutic advances from rodent studies to human stroke trials.\cite{8,16} This difficulty may be at least partly explained by the extensive physiological and anatomical differences between humans and rodents. As a result, nonhuman primate models of disease have become an important tool in translational stroke research, providing a large brain with white-to-gray matter ratios, cerebral vasculature, and immune and coagulation systems that more closely resemble those of humans.\cite{12,17} Furthermore, functional neurological deficits are easily assessed in nonhuman primates and represent more clinically relevant outcomes than the rudimentary evaluations devised for rodents, whose ability to recover and regenerate appears to be different from that of humans.\cite{31}

In an attempt to develop our successful murine anticomplement strategy into a relevant clinical therapy, we undertook a randomized, blinded trial of sCR1 administration in a nonhuman primate model of reperfused stroke. In considering all the promising stroke therapies that have proved successful in rodent models, we believed that sCR1 was an ideal candidate for this trial for several reasons. First, sCR1 had already successfully advanced to Phase I and Phase II human clinical trials in acute respiratory distress syndrome, cardiopulmonary bypass, and lung transplantation.\cite{14,18,27} Therefore, the dose required for complement inhibition, as well as the means and safety of administration, had already been rigorously established in primates and in humans. Thus, if a primate study yielded positive findings, a human clinical trial could be rapidly undertaken on the basis of both preclinical data and clinical data from other human disease states. Second, given the well-documented interspecies differences in the complement cascade, as well as the potentially beneficial role complement appears to play in cell clearance and organ regeneration,\cite{2} we felt obligated to evaluate sCR1 in a species with a greater phylogenetic similarity to humans prior to clinical translation. This paper describes our efforts to employ a primate model of stroke to bring a compelling anticomplement neuroprotective strategy to the brink of human trials.

Materials and Methods

Soluble Complement Receptor Type 1

This investigation was approved by the Institutional Animal Care and Use Committee at Columbia University. Forty-eight adult male baboons (Papio anubis) (Buckshire Farms, Perkasie, PA) were randomly assigned to receive either recombinant sCR1 (TP10) or a dose–volume equivalent vehicle (Avant Immunotherapeutics, Inc., Needham, MA). All personnel remained blinded to the identity of the experimental agent throughout the experiment. Recombinant sCR1 was supplied as a lyophilized agent reconstituted with 10 ml of sterile water at room temperature, diluted with 0.9% sodium chloride solution to a total volume of 100 ml, and administered within 8 hours of reconstitution. The infusate was administered over the 45 minutes immediately prior to vessel occlusion. The dose was determined according to each baboon’s weight, with each animal receiving 15 mg/kg, the same dose that demonstrated neuroprotection in our previous murine studies.\cite{21} This dose was also felt to be safe and effective on the basis of Phase I studies in human acute respiratory distress syndrome, where a single 30-minute infusion of 10 mg/kg resulted in a maximum plasma concentration of 150 ± 30 g/ml, a half-life of 67 ± 30 hours, a 70–90% reduction in baseline CH4 levels over the first 48 hours, and a 50% reduction in circulating C3a and C5b–C9 levels over a similar period.\cite{21} Similar blood clearance and complement inhibition were also observed in a recent study of high-risk cardiac surgery patients undergoing cardiopulmonary bypass.\cite{18}

Baboon Model of Focal Cerebral Ischemia

Drug infusion was followed by a transorbital craniectomy and exposure of the circle of Willis using a high-speed pneumumatic drill (Medtronic, Minneapolis, MN) as previously described in detail.\cite{13} Micro-Yaşargil aneurysm clips were placed on the cerebral arteries, and vascular occlusion was confirmed by laser Doppler flowmetry.\cite{11} Strict control of arterial blood pressure, central venous pressure, intracranial pressure, PCO2, and core body temperature was maintained throughout the experiment. The ischemic duration (75 minutes) was chosen because it provided the optimal balance between the consistency of major infarction and the ability of the animal to survive to the study endpoint. Postoperatively, the animals remained intubated with continuous monitoring for 18 hours by a trained member of the neurosurgical team. The morning after surgery the animals were extubated and monitored in the intensive care unit for the duration of the experiment.

Animal welfare was closely supervised by a trained veterinarian who was blinded to the animals’ experimental group. Immediately prior to the MR imaging scan on the third postoperative day, animal viability was assessed on the basis of the animal’s ability to sit upright, maintain an airway passage, and effectively self-feed. Those animals meeting these criteria were deemed self-caring and were allowed to awaken from the MR imaging scan on the third postoperative day and to survive until the 10th postoperative day. The animals that were not deemed self-caring were killed immediately after MR imaging.

Neurological Examination and Radiographic Imaging

Prior to surgery, all of the animals were neurologically intact, with a neurological function score of 100. Blinded neurological examinations were conducted daily beginning on the second postoperative day using a validated primate task-oriented neurological outcome scale (Mack modified score), which has been shown to correlate more closely with infarct volume in our experimental model than previously published measures.\cite{8} This 100-point scale is used to assess behavior, by grading the animal’s levels of awareness and ability to self-care, as well as the level of motor function, by evaluating both tone/posture and distal strength/co-ordination. The neurological score at the time the animals were killed was used in the final data analysis, with higher scores reflecting better functional status.

Imaging was performed to assess stroke volume on the 3rd and 10th postoperative days.\cite{17,22} Animals were sedated with 5 mg/kg of ketamine followed by an intravenous propofol infusion titrated to light sedation. A 1.5-tesla imaging unit (Sigma Advantage; GE Medical Systems, Milwaukee, WI) was used to obtain T2-weighted images (3-mm slice thickness). Infarct volume was calculated by two independent observers and averaged using digital planimetric analysis (Adobe Photoshop 4.0; Adobe Systems, Inc., San Jose, CA; NIH Image; http://rsb.info.nih.gov/nih-image/). Infarct volumes are reported as a percentage of the volume of the ipsilateral hemisphere.

Brain Harvest and Preservation

Immediately after the animals were killed, they were perfused via aortic catheterization with 1 L of ice-cold heparinized saline solution, followed by 1 L of heparinized paraformaldehyde; the descending aorta was clamped during this process to ensure maximal delivery of preservative to the head. The brain was then rapidly removed en bloc and preserved in formalin until it was sectioned for immunohistochemical analysis.

Immunostaining Procedure

Forty-micrometer brain sections were obtained using a standard vibrating-blade microtome (Leica VT1000S; Leica Microsystems GmbH, Wetzlar, Germany) through ischemic and contralateral equivalent nonischemic tissue in both the sCR1-treated animals and the controls. Blocking serum and primary and secondary antibodies were applied in 0.2% Triton X-100 in PBS. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 70% methanol. Next,
the sections were washed for 30 minutes at room temperature in a
10% normal donkey serum solution followed by incubation with the
primary antibody for 1.5 hours at room temperature. The following
antibodies were used: goat anti-human C1q (1:150) (a gift from
Andrea Tenner) and rabbit affinity-purified anti-human sCR1 (1:
100) (Avant Immunotherapeutics, Inc.). The sections were washed
in PBS and incubated with the appropriate biotinylated secondary
antibody (1: 600) (Jackson Immunoresearch, Inc., West Grove, PA)
for 1 hour at room temperature. This was followed by incubation
in ABC complex (Elite ABC Kit; Vector Laboratories, Burlingame,
CA) for 1 hour at room temperature and subsequent washing in PBS.
The product of the reaction was visualized with 3,3’-diaminobenzen-
idine using nickel ammonium sulfate. Negative controls were pre-
pared using the same protocol but omitting the primary antibody.

Total Serum Complement Activity

Fifteen-milliliter peripheral blood samples were collected at nine
time points: prior to intubation; at agent administration; at ischemia;
at 30 minutes, 2 hours, 6 hours, and 12 hours after arterial occlusion;
and on the 3rd postoperative day; and (if available) on the 10th post-
operative day. Each sample was collected into glass blood collection
tubes (Vacutainer; BD, Franklin Lakes, NJ) and allowed to clot at
room temperature for 30 minutes. Plasma was isolated by centrifu-
gation at 2000 rpm for 17 minutes and was stored at –70°C. Total
plasma complement activity was measured by CH50 assay using a
commercially available kit (Diamedix, Miami, FL).

Statistical Analysis

Infarct volume, CH50, and physiological parameter data were ana-
lyzed using two-tailed, unpaired Student t-tests. Neurological scores
were analyzed using the unpaired Mann–Whitney test. All values are
expressed as the means ± the standard error of the mean. A proba-
bility value less than 0.05 indicated statistical significance.

An initial sample size determination was performed on the basis
of the historic infarct volume and neurological deficit score in this
model. Using this procedure we determined that 24 animals in each
arm would permit the detection of a 50% reduction in the percentage
of ipsilateral ischemic cerebral tissue, as defined on T2-weighted MR
imaging at the time the animals were killed, as well as a 50% rela-
tive improvement in the degree of neurological impairment, as de-
efined by the Mack modified score. This sample size would provide
a statistical power of 80% and a two-sided alpha value of 0.05 in our
model. A series of quartile interim analyses by a statistician were
planned a priori.

Results

Physiological Findings

There were no significant differences in animal weight, peripro-
tative mean arterial blood pressure, central venous pressure, exponential CO2, core body temperature, serum glucose, or hematocrit between the sCR1- and vehicle-treated cohorts (data not shown).

Infarct Volume and Functional Neurological Outcome

As we have previously demonstrated, the animals with larger strokes as shown on MR imaging scans exhibited more profound neurological deficits and poorer neurologi-
cal scores (r = −0.9, p < 0.0004).22 No animals were ex-
cluded from analysis based on incomplete data on cerebral
blood flow as assessed by laser Doppler flowmetry. Among
the 12 animals enrolled before the planned interim data
analysis (six in each group), there were no significant dif-
fferences between the two groups in mean infarct volume,
either on the 3rd postoperative day (sCR1-treated group = 42 ± 9% of ipsilateral hemisphere, vehicle-treated group = 24 ± 7%, p = 0.15) or at the time the animals were killed
(sCR1-treated group = 31 ± 10%, vehicle-treated group = 23 ± 8%, p = 0.55) (Fig. 1 upper and center) and no sig-
nificant differences between groups in neurological func-
tion score at the time the animals were killed (sCR1-treated
group = 46 ± 12%, vehicle-treated group = 56 ± 13%, p = 0.58) (Fig. 1 lower). In fact, the interim data analysis
demonstrated trends suggesting that the treated animals did
worse.

Total Serum Complement Activity (CH50)

Total serum complement activity levels (CH50 values) were signifi-
cantly greater in the vehicle-treated cohort than in the sCR1-treated group (before operation: 264 ± 21
compared with 238 ± 32; agent administration: 235 ± 20
compared with 192 ± 30; 0 minutes: 136 ± 40 compared with 6 ± 2; 30 minutes: 155 ± 37 compared with 7 ± 2; 2
hours: 152 ± 36 compared with 7 ± 2; 6 hours: 150 ± 34
compared with 12 ± 4; 12 hours: 199 ± 42 compared with
25 ± 10; 3rd postoperative day: 311 ± 27 compared with 187 ± 63; and 10th postoperative day: 307 compared with
265 ± 35). Each group included six animals at every time
point except at 0 minutes, when there were five animals
in the vehicle-treated cohort; on the 3rd postoperative day,
when there were three animals in the vehicle-treated group
and four animals in the sCR1-treated group; and on the 10th
postoperative day, when there was one animal in the vehi-
cle-treated group and four animals in the sCR1-treated
small. Total complement activity remained depressed for at
least 12 hours after agent administration (p < 0.05 at 0 min-
utes, 30 minutes, 2 hours, 6 hours, and 12 hours after isch-
emia) (Fig. 2).

Immunohistochemical Analysis

Results of immunostaining showed that C1q immunore-
activity was limited to ischemic brain tissue in brain tissue
harvested at 72 hours (Fig. 3). Soluble complement recep-
tor Type I immunoreactivity was observed in the brains of
the sCR1-treated animals but not in the brains of the vehi-
cle-treated animals (Fig. 4).

Discussion

In the current study, we employed our well-established nonhuman primate model of transient cerebral ischemia12 to
further investigate the translational potential of a promising
neuroprotective agent, sCR1. The membrane-bound glyco-
protein CR1 is a potent inhibitor of both the classic and al-
ternative complement pathways.9,13 Complement receptor 1
displaces the catalytic subunits from the C3 and C5 conver-
tases and serves as a cofactor for degradation of C3b and
C4b. This interaction effectively inhibits the generation of
anaphylatoxins (C3a and C5a) and the assembly of the ter-
ninal membrane attack complex.29 Removal of the trans-
membrane and cytoplasmic domains of CR1 allows synthe-
sis of a soluble protein, sCR1, which was administered in
our studies.

Nonhuman primate models provide several advantages
over rodent models in investigation of stroke. However,
as with human clinical trials, ethically responsible primate
experimentation requires an interim data analysis, with
mandatory interruption of an experiment if the analysis sug-
gests that the treatment will not succeed within the designs
of the study. Also, the use of a preischemic dosing schedule,
which affords the greatest neuroprotection in the murine ex-

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The sample size in the current trial (a total of 24 animals in the treatment group and 24 control animals) was determined using data obtained in an identical model by our team over a 5-year period. A planned quartile interim data analysis and statistical futility measure by a statistician not otherwise involved in the study suggested that the administration of sCR1 would not yield a neuroprotective effect relative to placebo. Power calculations based on the actual observed variability indicated that a total of 350 animals would be required to yield an 80% power to detect a clinically meaningful relative decrease in infarct volume of 20% (absolute decrease of 6%) with sCR1 (alpha = 0.05). Similar analysis performed for neurological scores revealed that a total of 294 animals would be required for the same power. Furthermore, given the 14% absolute increase in infarct volume observed with sCR1, a statistical futility analysis indicated that there was only a 16% chance of ultimately achieving the desired 20% relative reduction in infarct volume. This statistical analysis indicated that establishing the efficacy of sCR1 was not realistic in this experiment. The study was thus terminated prematurely, which accounts for the small sample size for which results are reported.

Our group’s prior work had demonstrated improvements in infarct volume and neurological outcome scores in mice treated with sCR1. The persuasive results drawn from our interim analysis, therefore, warranted interpretation of our data to verify some criteria. In the present study, we utilized immunohistochemistry and complement activity analysis to demonstrate that preischemic dosing of sCR1 (15 mg/kg) results in brain penetration and successful inhibition of the complement system during the critical postischemic period. Given these facts, it is not clear why we did not observe a neuroprotective effect in our primate model. One explanation may lie in the fundamental pathophysiological difference between primates and mice, a hypothesis supported by recent literature identifying significant disparity among the complement cascades of different species. Moreover, although our finding of postischemic neuronal C1q immunoreactivity supports our previous results in mice, the significance of this C1q immunostaining remains to be elucidated. It is possible that viable neurons express C1q and are then targeted for killing by the reticuloendothelial system. Alternatively, complement-tagged neurons may be beyond salvage despite any amount of complement system inhibition or C1q blockade.

The results of the current study demonstrate that C1q immunoreactivity is increased in the ischemic primate brain, that sCR1 suppresses total complement activity in peripheral blood, and that sCR1 penetrates the brain parenchyma. Despite these findings, we were not able to demonstrate a neuroprotective effect with a dosing regimen shown to be effective in blocking complement in both humans and primates. These results mandate further preclinical scrutiny of anticomplement strategies prior to any acute stroke trials. Furthermore, these results demonstrate the utility of a nonhuman primate model as an important preclinical screening paradigm in translational stroke research. Rigorous assessment using multiple diverse species may be critical in assessing even the most promising neuroprotective drugs.

**Conclusions**

A preischemic bolus infusion of sCR1 at the same dose...
Preclinical evaluation using a primate model of stroke

Fig. 2. Graph depicting mean total complement activity (CH50 values) in sCR1-treated and vehicle-treated adult male baboons subjected to middle cerebral artery occlusion/reperfusion. The animals treated with sCR1 had significantly depressed total complement activity for at least 12 hours after agent administration. Data are presented for six sCR1-treated and six vehicle-treated animals for each time point, except for 0 minutes postischemia, when there were five vehicle-treated animals; the 3rd postoperative day, when there were four sCR1-treated and three vehicle-treated animals; and the 10th postoperative day, when there were four sCR1-treated animals and one vehicle-treated animal.

Fig. 3. Light micrographs demonstrating positive neuronal C1q immunostaining in the ischemic hemisphere (a) and nonischemic hemisphere (b) of a vehicle-treated male baboon subjected to middle cerebral artery occlusion/reperfusion. Scale bar = 50 μm.
that has been demonstrated to provide suppression of complement activation in previous animal and human trials did not provide a neuroprotective benefit in our primate stroke model. Our results illustrate the utility of a translational primate model of stroke in the assessment of promising anti-ischemic agents prior to the implementation of large-scale clinical trials.

Acknowledgment

Drs. Mocco and Mack contributed equally to this work. We thank Daniel Batista for technical assistance.

Disclosure

Dr. Marsh is a full-time employee of Avant Immunotherapeutics, Inc.

References


Fig. 4. Light micrographs demonstrating (a) positive sCR1 immunostaining in the ipsilateral hemisphere of an sCR1-treated male baboon subjected to middle cerebral artery occlusion/reperfusion and (b) negative sCR1 immunostaining in the ipsilateral hemisphere of a vehicle-treated male baboon subjected to middle cerebral artery occlusion/reperfusion. Scale bar = 100 μm.
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