further transinserted into DA2050A to enrich for peptamers that conferred resistance as opposed to genomic mutations that caused resistance. Cell populations harboring active peptamers typically grew as lawns on selection plates, and individual plasmids were isolated from these plates and restested to confirm that they gave plasmid-dependent resistance.

17. T. C. Norman and A. W. Murray, unpublished data.
25. T. C. Norman, S. M. O’Rourke, A. W. Murray, unpublished results.
31. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
32. A yeast ORF-Gal4 activation domain fusion array has been assembled that expresses about 85 to 90% of the predicted ORFs of S. cerevisiae in strain pJ69-4a [P. James, J. Halladay, E. Craig Genetics 144, 1425 (1996)]. In this strain, GAL4 is absent and the reporters H2E and ADE2 are under GAL4 control. To probe the GAL4-AD fusion array for protein-protein interactions, we mated the array to a strain expressing a Gal-4 DNA binding domain fusion. After selecting for diploids, we identified two-hybrid positives by testing diploids on plates containing different concentrations of 3-aminoanthracene.
35. We performed the following competitive hybridizations: wild-type cells with or without peptamer S-1, with or without peptamer S-5, and with or without expression of SrcR4W. Each comparison was done on four arrays composed of two pairs of hybridizations done with reversal of the fluorophore labelings to eliminate biases of fluorophore incorporation. We used two different sources of mRNAs for labeled cDNA production, including a total RNA protocol (http://www.ncbi.nlm.nih.gov/DRD/CCG/15K/HTML) and twice-purified polyadenylated mRNAs [M. J. Marton et al. Nature Med. 4, 1293 (1998)].
36. Supported by grants from NIH, Human Frontier Science Program, and Chiron (A.W.M.); an NIH senior fellowship (T.C.N.); an NIH grant and the Herbert W. Boyer Fund (S.M.O.); and NIH grant P41-RR11823 (B.L.D. and S.F.). S.F. is an Investigator of the Howard Hughes Medical Institute. We thank L. Hartwell, I. Herskowitz, L. Huang, and J. Rine for yeast strains and plasmids; T. Geyer, Alejandro Colman-Lerner, and R. Brent for communicating unpublished results; and D. Gottschling, I. Herskowitz, L. Pillus, J. Simon, and members of the Seattle Project and the Murray laboratory for valuable discussions.
20 April 1999; accepted 16 June 1999

Neuronal Protection in Stroke by an sLex⁺-Glycosylated Complement Inhibitory Protein

Judy Huang,¹ Louis J. Kim,² Richard Mealey,² Henry C. Marsh Jr.,² Yuan Zhang,³ Andrea J. Tenner,³ E. Sander Connolly Jr.,¹ David J. Pinsky*²

Glycoprotein adhesion receptors such as selectins contribute to tissue injury in stroke. Ischemic neurons strongly expressed C1q, which may target them for complement-mediated attack or C1qR-mediated clearance. A hybrid molecule was used to simultaneously inhibit both complement activation and selectin-mediated adhesion. The extracellular domain of soluble complement receptor-1 (sCR1) was sialyl Lewis x glycosylated (sCR1sLex) to inhibit complement activation and endothelial-platelet-leukocyte interactions. sCR1 and sCR1sLex colocalized to ischemic cerebral microvessels and C1q-expressing neurons, inhibited neutrophil and platelet accumulation, and reduced cerebral infarct volumes. Additional benefit was conferred by sialyl Lewis x glycosylation of the unmodified parent sCR1 molecule.

1. Columbia University, College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032, USA.
2. Avant Immunotherapeutics, Inc., 119 South Avenue, Needham, MA 02494, USA.
3. University of California, Department of Molecular Biology and Biochemistry, Irvine, CA 92697, USA.

*To whom correspondence should be addressed. E-mail: dpj5@columbia.edu

- Neuronal Protection in Stroke by an sLex⁺-Glycosylated Complement Inhibitory Protein
- Glycoprotein adhesion receptors such as selectins contribute to tissue injury in stroke. Ischemic neurons strongly expressed C1q, which may target them for complement-mediated attack or C1qR-mediated clearance. A hybrid molecule was used to simultaneously inhibit both complement activation and selectin-mediated adhesion. The extracellular domain of soluble complement receptor-1 (sCR1) was sialyl Lewis x glycosylated (sCR1sLex) to inhibit complement activation and endothelial-platelet-leukocyte interactions. sCR1 and sCR1sLex colocalized to ischemic cerebral microvessels and C1q-expressing neurons, inhibited neutrophil and platelet accumulation, and reduced cerebral infarct volumes. Additional benefit was conferred by sialyl Lewis x glycosylation of the unmodified parent sCR1 molecule.

- Neuronal Protection in Stroke by an sLex⁺-Glycosylated Complement Inhibitory Protein
- Glycoprotein adhesion receptors such as selectins contribute to tissue injury in stroke. Ischemic neurons strongly expressed C1q, which may target them for complement-mediated attack or C1qR-mediated clearance. A hybrid molecule was used to simultaneously inhibit both complement activation and selectin-mediated adhesion. The extracellular domain of soluble complement receptor-1 (sCR1) was sialyl Lewis x glycosylated (sCR1sLex) to inhibit complement activation and endothelial-platelet-leukocyte interactions. sCR1 and sCR1sLex colocalized to ischemic cerebral microvessels and C1q-expressing neurons, inhibited neutrophil and platelet accumulation, and reduced cerebral infarct volumes. Additional benefit was conferred by sialyl Lewis x glycosylation of the unmodified parent sCR1 molecule.
reduced leukocyte recruitment and improved postischemic perfusion and are moderately protected from cerebral ischemic injury in stroke. Microvascular thrombosis, which occurs in situ in cerebral microvessels downstream from the site of primary occlusion, can be inhibited by the blockade of platelet glycoprotein IIb/IIIa receptor (3).

Although neuronal blood supply may be diminished by microvascular thrombosis and leukostasis, and neurons may be damaged by their proximity to inflammatory foci, it is also possible that inflammatory mechanisms may subject neurons to direct attack. Although neurons do not normally express complement proteins, in Alzheimer’s disease, neurons synthesize and express C1q (4), a critical initiator of the classical pathway of complement activation and a ligand for C1qRp, a receptor on myeloid cells (including microglia) that enhances phagocytosis (5). A role for complement activation in stroke has not been elucidated, though it has been shown to play a role in ischemia-induced damage in other tissues (6, 7).

Brain tissue sections obtained from mice subjected to 45 min of middle cerebral artery (MCA) occlusion followed by 23 hours of reperfusion showed prominent neuronal staining for C1q in the ipsilateral cerebral cortex (Fig. 1A). Neurons in the contralateral cerebral cortex expressed C1q at barely detectable levels (Fig. 1B), and neurons from nonischemic control brain did not express any detectable C1q (Fig. 1D). As a specificity control for these experiments, murine C1q was purified (8) and then mixed with the primary antibody to C1q (anti-C1q) before staining. These experiments confirmed a specific increase in C1q expression in neurons in the ischemic cerebral cortex but not in the nonischemic cerebral cortex (Fig. 2A). Protein immunoblot analysis demonstrated increased density of a band that was immunoreactive for murine C1q in ipsilateral but not contralateral hemispheric tissue extracts 24 hours after stroke (amounts of C1q protein in nonischemic tissue were similar to that observed in nonischemic control brain). Not only was this band immunoreactive for C1q, but it comigrated with a purified C1q-positive control at the expected relative molecular mass of ~28 kD (Fig. 2B).

To examine the functional effects of complement activation in the context of ischemia-reperfusion–driven expression of neuronal C1q, we studied the effects of a potent inhibitor of complement activation, soluble complement receptor–1 (sCR1) (7). In nonstroke (control) brain, an affinity-purified antibody to human sCR1 failed to stain any structures (Fig. 1C). However, we used this same antibody to localize sites of sCR1 binding in stroke. When sCR1 was infused into mice just before stroke and brain tissue was then studied after reperfusion, sCR1 was found to colocalize to neurons that express C1q (Fig. 1, E and F, arrowheads), as well as intraluminally in the cerebral microvessels where C1q was not expressed (Fig. 1, E and F, arrows). Both neuronal bodies and their axonal processes in the ischemic region bound sCR1 (Fig. 1G). In contrast, neurons examined in the contralateral cortex from the same mouse bound little if any sCR1 (Fig. 1H).

Infusion of sCR1 into mice immediately before stroke caused a modest reduction in cerebral tissue injury (9) (Fig. 3, A and B). However, because leukocyte adhesion receptor expression (1, 2) and thrombosis (3) in the postischemic cerebral microvasculature may occur independent of complement activation yet still contribute to cerebral tissue injury in stroke, we sought to modify sCR1 to inhibit these additional deleterious inflammatory and thrombotic events. Because selectin expression is up-regulated in stroke (1, 10), we hypothesized that a single molecule that inhibits both complement activation and selectin-mediated adhesive events might simultaneously limit both inflammatory and coagulant reactions in stroke. We therefore covalently modified sCR1 by sialyl Lewis x (sLe^x) glycosylation (11). We expressed sCR1 in CHO LEC11 cells, which are capable of incorporating the sLe^x tetrasaccharide in the posttranslational glycosylation of

FIG. 1. Ischemia-driven neuronal C1q expression, and localization of sites of sCR1 and sCR1sLex binding. Adjacent tissue sections were incubated with either a primary rabbit antibody to mouse C1q (62 μg/ml, 1:150 dilution in phosphate-buffered saline (PBS); this antibody is a purified immunoglobulin G (IgG) with murine C1q as the rabbit immunogen), or with rabbit affinity-purified anti-sCR1 (19) (42 μg/ml, 1:50 dilution in PBS) at 37°C for 60 min. (A and B) Sections of brain from a control mouse subjected to 45 min of cerebral ischemia and 23 hours of reperfusion. Immunostaining with anti-C1q demonstrates immunoreactivity on neuronal surfaces in the ipsilateral (A) cerebral cortex, with minimal staining in the contralateral (nonischemic) cortex (B). (C and D) Section of brain from an untreated (nonoperated) mouse. Neither sCR1 (C) nor C1q (D) immunoreactivity can be detected. (E and F) Adjacent brain sections from the ipsilateral hemisphere of an sCR1-treated mouse that underwent stroke immunostained with either anti-sCR1 (E) or anti-C1q (F). Arrowheads indicate neurons that exhibit immunoreactivity for both sCR1 and C1q in adjacent sections. Arrows point to a cerebral microvesSEL, which stains intraluminally for sCR1 but not C1q. (G and H) Ipsilateral and contralateral brain sections of an sCR1-treated mouse that underwent stroke. The contralateral section (G) demonstrates detailing of neuronal bodies and axonal processes from immunoreactivity to anti-sCR1. The contralateral section (H) taken from the same mouse shows minimal immunoreactivity for sCR1. (I and J) Ipsilateral sections of a brain from an sCR1sLex^x-treated mouse that underwent stroke. Neuronal immunoreactivity for sCR1 is shown (arrowheads) in the ipsilateral cerebral cortex (I), as well as some immunoreactivity within a cerebral microvesSEL (arrow). Contralateral cerebral cortex from the same mouse (J) shows minimal immunoreactivity for sCR1.
Fig. 2. Effect of cerebral ischemia on neuronal C1q expression. (A) The minimum concentration of IgG that gave positive immunostaining was determined (1:400 dilution), then mixing experiments were performed with an excess of purified murine C1q to block the primary antibody to demonstrate specificity of the neuronal immunostaining. The contralateral (nonischemic) region did not stain with primary antibody alone (23 μg/ml; 1:400 dilution), although the ipsilateral (ischemic) region exhibited neuronal immunoreactivity under the identical staining conditions. When antigen (120 μg/ml, fivefold excess) was mixed with the primary antibody (overnight incubation at 4°C) before experiments, ipsilateral staining was completely blocked. Similarly, no positive immunostaining was detected in the contralateral region in this blocking experiment (not shown). (B) Protein immunoblot analysis for murine C1q. (Reducing conditions were used, 12% SDS-PAGE, 140 μg of total protein per lane, and a dilution of primary antibody of 1:1000). Sites of primary antibody binding were detected with a donkey horseradish-peroxidase–conjugated secondary anti-rabbit IgG (1:2000) and the enhanced chemiluminescent method of immunodetection. Purified murine C1q (250 ng) was used as a positive control (8). Tissue samples from two normal, untreated mice (lanes marked control brain) showed only slight immunoreactivity, similar to that seen in the contralateral hemispheric extracts from two separate mice (mouse A and mouse B) taken 24 hours after stroke. In contrast, a strongly immunoreactive band was seen for both ipsilateral (ischemic) hemispheres from these same mice.
proteins. This glycoprotein product, sCR1sLe^x, includes at least 10 sLe^x moieties per sCR1 molecule (17). In vitro experiments demonstrated that sCR1sLe^x, but not sCR1, binds cell surface E-selectin and also blocks P-selectin-mediated cellular adhesion (17).

sCR1sLe^x given before stroke could be detected bound to neurons and within vascular lumina 24 hours after stroke (Fig. 1, I and J). In its ability to minimize the volume of infarcted cerebral tissue and reduce neurological deficit, sCR1sLe^x was more potent than sCR1 (Fig. 3, A and B). Mice treated with sCR1 before middle cerebral artery occlusion demonstrated less neurological deficit at 24 hours than vehicle-treated controls (Fig. 3A). sCR1sLe^x treatment diminished neurological impairment even further. Mean infarct volumes were lower in the sCR1-treated animals, although large standard errors precluded ascribing statistical significance to these values. However, sCR1sLe^x markedly diminished infarct volume in a dose-dependent fashion, with up to an 11-fold reduction in cerebral infarct volume observed at the highest dose tested (15 mg/kg per kilogram of body weight) (Fig. 3B) (12).

Because sCR1 and sCR1sLe^x localize not only to C1q-expressing neurons but to ischemic cerebral microvessels, we examined vascular mechanisms by which these compounds may confer protection to cerebral tissue in stroke. We administered 111In-labeled neutrophils immediately before surgery and then quantified their accumulation in ischemic cerebral tissue. Compared with animals undergoing sham surgery, animals subjected to middle cerebral artery occlusion and reperfusion exhibited a nearly threefold increase in ipsilateral leukocyte accumulation (Fig. 3C). Pretreatment with sCR1 (15 mg/kg) caused a 1.7-fold reduction in leukocyte accumulation. However, sCR1 treatment did not reduce neutrophil accumulation to baseline, implicating additional noncomplement-dependent mechanisms of leukocyte recruitment in stroke. sCR1sLe^x further reduced polymorphonuclear leukocyte accumulation in the ipsilateral cerebral cortex, to a level just slightly above baseline.

When the accumulation of 111In-labeled platelets was similarly tracked, sCR1sLe^x caused even greater inhibition of platelet accumulation than sCR1 alone (Fig. 3D). These data support the observation that leukocytes and platelets demonstrate substantial adhesion-receptor-dependent interactions (13), which may amplify microvascular thrombosis. Postischemic cerebral hyperperfusion, which is dependent upon the severity of the cerebral ischemic insult and which may be caused by microvascular thrombosis, leukocyte capillary plugging, vascular damage, or abnormal vasoreactivity (14, 15), is also a feature of the stroke model used in these studies. Laser Doppler measurements showed equivalent blood flows between ipsilateral and contralateral cerebral cortices at baseline, upon occlusion of the middle cerebral artery, and immediately upon withdrawal of the occluding suture (Fig. 3E). However, by the time the mice were killed, ipsilateral blood flow was significantly higher in sCR1sLe^x-treated animals, and at an intermediate level in the sCR1-treated animals. The failure of blood flow to return completely back to baseline suggests the recruitment of additional effector mechanisms beyond those that can be inhibited by complement and selectin blockade. Because of concerns that an antiselectin agent, which inhibits platelet recruitment, may increase intracerebral hemorrhage, we performed experiments to examine this possibility. We saw no increase in intracerebral hemorrhage after therapy; there was even a trend toward a decrease with the antiselectin agent (Fig. 3F). Other physiological variables examined did not suggest that the tested compounds had any other adverse effects in mice (16). Even after longer observation periods (72 hours), the beneficial effects of sCR1sLe^x administration were sustained (Fig. 4A).

In clinical stroke, patients present after the onset of the ischemic event. Any therapeutic intervention must therefore be effective after the onset of ischemia. Both compounds (15 mg/kg) were administered 45 min after ischemic onset immediately after removal of the occlusion. sCR1sLe^x maintained cerebroprotective efficacy, as reflected in diminished infarct volumes, albeit less than that seen during preischemic administration (Fig. 4B). Blockade of complement activation after the ischemic event with sCR1 was of intermediate efficacy between that of vehicle and that of sCR1sLe^x. Neurological function and relative cerebral blood flow at 23 hours of reperfusion were also improved by administration of sCR1sLe^x after occlusion. For control versus sCR1sLe^x-treated animals, respectively, the data were as follows: neurological deficit score 2.8 ± 0.3 versus 1.5 ± 0.3, P = 0.02; cerebral blood flow of the ipsilateral hemisphere relative to that of the contralateral hemisphere was 41 ± 15% versus 75 ± 7%, P < 0.05. Although administration 45 min after ischemia in mice cannot be directly translated into clinical relevance without corroborative human data, a recent clinical trial showed that the average time from stroke onset to emergency room presentation was 57 min (although an additional 100 min were required for a CT scan before administration of tissue plasminogen activator) (17). If an "innocuous" agent can be given at presentation without the delay of an intervening CT scan, then an opportunity will open up for therapeutic intervention in stroke.

These studies demonstrate that ischemic neurons express C1q, presumably to flag the complement system to bring about their own demise or to aid in removing damaged cells, or both. It is not surprising that sCR1 targets and binds to C1q-expressing neurons, because CR1 has been shown to be a ligand for C1q (18). sCR1, a soluble, truncated form of the human complement receptor protein CR1, binds to proteins of the multisubunit C3 or C5 convertases formed as a result of complement activation to promote dissociation of the catalytic subunits (7). sCR1 is moderately cerebroprotective in the setting of reperfused stroke, reducing neutrophil and platelet recruitment. However, when this agent is glycosylated with sLe^x to additionally inhibit selectin-mediated events, it significantly protects the reperfused brain against leukocyte and platelet recruitment and neuronal injury.
Correlative Structure of Spontaneous Neuronal Activity in the Developing Lateral Geniculate Nucleus in Vivo

Michael Weliky* and Lawrence C. Katz

The properties of spontaneous activity in the developing visual pathway beyond the retina are unknown. Multielectrode recordings in the lateral geniculate nucleus (LGN) of awake behaving ferrets, before eye opening, revealed patterns of spontaneous activity that reflect a reshaping of retinal drive within higher visual stages. Significant binocular correlations were present only when corticothalamic feedback was intact. In the absence of retinal drive, corticothalamic feedback was required to sustain correlated LGN bursting. Activity originating from the contralateral eye drove thalamic activity far more strongly than that originating from the ipsilateral eye. Thus, in vivo patterns of LGN spontaneous activity emerge from interactions between retina, thalamus, and cortex.

Although visual experience affects visual cortical plasticity during specific critical periods of postnatal development (1), many aspects of visual cortical functional organization emerge before visual experience (2–4). Thus, visual experience is not required for the initial development of cortical functional organization but is necessary for its maintenance and stability.

Before the onset of visual experience, spontaneous activity within the visual pathway has been proposed to provide instructive cues for guiding orientation and ocular dominance column formation within visual cortex (7). Correlated patterns of spontaneous activity have been observed in the developing retina (5, 6); however, very little is known about the in vivo patterns of spontaneous activity within higher stages of the visual pathway such as the lateral geniculate nucleus (LGN). It is unlikely that the LGN simply relays patterns of retinal spontaneous activity to the cortex; rather, it reshapes and transforms these patterns. Mechanisms that could underlie these transformations include nonretinal sources of input such as cholinergic brainstem afferents (7), massive feedback projections from layer 6 of primary visual cortex (8), and LGN and perigeniculate nucleus (PGN) circuitry, which generates endogenous synchronized network oscillations in vitro (9, 10). The eventual patterns of spontaneous activity that underlie the development of visual cortical functional architecture may be quite different from those predicted by retinal activity alone. To investigate

Howard Hughes Medical Institute and Department of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA.

*Present address: Department of Brain and Cognitive Sciences, Meliora Hall, University of Rochester, Rochester, N.Y. 14627, USA.
†To whom correspondence should be addressed. E-mail: weliky@cvs.rochester.edu

8. Murine C1q was purified from 50 ml of mouse sera originating from the ipsilateral eye. Thus, in vivo patterns of LGN spontaneous activity emerge from interactions between retina, thalamus, and cortex.

References and Notes