Original Contribution

Effect of a Broad-Specificity Chemokine-Binding Protein on Brain Leukocyte Infiltration and Infarct Development

Seyoung Lee, BSc(Hons); Hannah X. Chu, BSc(Hons); Hyun Ah Kim, PhD; Nicola C. Real, BSc(Hons); Saeed Sharif, DVM, MSc; Stephen B. Fleming, PhD; Andrew A. Mercer, PhD; Lyn M. Wise, PhD; Grant R. Drummond, PhD; Christopher G. Sobey, PhD

Stroke accounts for >10% of all deaths, as well as prolonged disability of one third of survivors.1 Approximately 80% of all adult strokes are ischemic in origin.2 After a disruption of blood supply to the brain, an infarct develops, comprising a central core of dead neurons surrounded by a penumbra of surviving but compromised tissue that is vulnerable to further inflammation-related injury or death caused by infiltrating immune cells.3,4

Chemokines are a family of chemotactic cytokines that attract leukocytes toward a site of injury.5,6 Thus, together with their receptors expressed on leukocytes, chemokines play a crucial role in the extravasation and migration of leukocytes under inflammatory conditions.7 In the highly inflammatory setting of acute ischemic stroke, chemokines are generated by brain-resident microglial cells and infiltrating immune cells, resulting in further leukocyte attraction and activation.8,9 Chemokines and their cognate receptors regulate both physiological and pathological processes in the central nervous system, and it is currently postulated that chemokines play a generally deleterious role by contributing to brain injury after cerebral ischemia–reperfusion.10

We have shown that expression of numerous chemokine-related genes is upregulated in the mouse brain from 4 hours for ≥3 days after ischemia–reperfusion,11 raising the possibility that certain key chemokines/chemokine receptors could represent targets for acute stroke therapy. For example, we...
found that expression of neutrophil-associated CXCR2 and its ligands, CXCL1 and CXCL2, were substantially upregulated in the brain after cerebral ischemia and that treatment with an antagonist of CXCR2 could markedly reduce both expression of CXCR2 and neutrophil infiltration after stroke. However, those effects of the antagonist were not associated with any reduction in either functional impairment or infarct volume, raising the possibility that such an approach may need to target a broader suite of chemokines/chemokine receptors to be efficacious.

Many viruses secrete proteins that bind chemokines as a means of evading the host immune system and ensuring their own survival. Recently, a novel type of chemokine-binding protein (CBP) has been identified from orf virus, and fellow parapoxviruses, bovine papular stomatitis virus (BPSV), and pseudocowpox virus. This CBP shows high-affinity binding for both human and mouse CC, CXC, and C chemokines and has been demonstrated to prevent inflammatory monocyte recruitment to the skin and to block dendritic cell trafficking to peripheral lymph nodes. Because of its capacity to bind 3 classes of chemokine and to impair leukocyte trafficking in vivo, the parapoxvirus CBP represents a powerful tool with which to target a broad range of chemokines and dissect their role in the pathogenesis of diseases driven by inflammation. Thus, here we have tested the hypothesis that acute systemic administration of CBP after cerebral ischemia–reperfusion in mice may reduce plasma chemokine levels, leukocyte infiltration, and infarct development.

Materials and Methods

Animals

This study was conducted in accordance with the National Health and Medical Research Council of Australia guidelines for the care and use of animals in research. A total of 96 male C57Bl/6 mice (6–10 weeks old) were studied. The mice had free access to water and food pellets before and after surgery. Data were included from 73 mice, and 23 mice were excluded from the study because during the surgical procedure to induce cerebral ischemia–reperfusion, (1) there was inadequate (<60%) or excessive (>90%) reduction in regional cerebral blood flow (n=11); (2) no measured increase in blood flow at reperfusion after 1 hour ischemia (n=2); (3) death occurred before the designated period of 23 or 47 hours of reperfusion had elapsed (n=4); (4) there were technical problems with intravenous injections (n=4); or (5) data were excluded as statistical outliers based on infarct volume (n=2; Grubbs' test; GraphPad Prism version 6; GraphPad Software Inc., San Diego, CA).

Cloning, Expression, and Purification of CBP

The CBP open reading frame was amplified by PCR using a plasmid containing the 20.7 kb BamHI A fragment from BPSV strain V660 as a template with the following primers: 112BPSV_F (5′-GGCGCCGGGCTGGGAAATCATCGATTTATCC-3′) and 112BPSV_R (5′-AAGGGCCGGGCTGGGAAATCATCGATTTATCC-3′). The PCR product was digested with AscI and ligated into a pAPEX-3-derived vector. The recombinant CBP, tagged with the FLAG octapeptide at the C terminus, was expressed in 293-EBNA cells, then purified and quantified, as previously described.

Assay of CBP Activity

Chemokine binding activity can be measured indirectly by the ability of a protein to interfere with the detection of the chemokine by specific ELISA. CCL2 and CXCL8 OPTI EIA ELISA kits (BD Biosciences, Franklin Lakes, NJ) and CCL3, CCL5, CCL19, XCL1, CXCL2, CXCL4, CXCL10, and CXCL12 DuoSet ELISA Development kits (R&D Systems Inc, Minneapolis, MN) were used in accordance with the manufacturer’s instructions unless stated otherwise. MaxiSorp 96-well immunoplates (Nalgene Nunc, Penfield, NY) were coated overnight at 4°C with capture antibody, then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour at room temperature. In nonabsorbent 96-well plates, chemokines (murine (m)CCL2, 720 pmol/L; mCCL3, 400 pmol/L; human (h)CCL5, 200 pmol/L; mCCL19, 730 pmol/L; hXCL1, 640 pmol/L; mCXCL2, 700 pmol/L; hCXCL4, 370 pmol/L; hCXCL8, 360 pmol/L; hCXCL10, 370 pmol/L; and hCXCL12, 375 pmol/L) were mixed with increasing amounts of CBP diluted in PBS. The chemokine/CBP mixes were incubated for 45 minutes at 37°C and then transferred with chemokine standards to the coated plates for a further 15 minutes incubation at 37°C. The plates were washed with PBS/0.05% Tween-20, then incubated for 1 hour at room temperature with biotinylated detection antibody and streptavidin-conjugated horseradish-peroxidase. After a final wash, captured chemokine was detected with 3,3′,5,5′-tetramethylbenzidine (BD Biosciences), and the reaction was stopped with H2SO4, and the absorbance at 450 nm was measured.

Binding Analysis Using Surface Plasmon Resonance

The surface of a CM5 sensor chip was immobilized with α5β3 integrin response units (pg/mm²) of CBP in 10 mmol/L CH3H2O2 (pH 4.5) by standard amine coupling using a Biacore X100 instrument. All experiments were performed at 25°C with HBS-EP buffer (20 mmol/L HEPes, 150 mmol/L NaCl 3.4 mmol/L EDTA, 0.005% polyorbate 20, pH 7.4). The coupled CBP was exposed to chemokines at a flow rate of 30 μL/min at concentrations ranging from 0.195 mmol/L to 100 mmol/L for a duration of 180 seconds. Chemokines were allowed to dissociate in 600 seconds before the surface was regenerated using 10 mmol/L glycine-HCl (pH 2.0). The data were analyzed using BIAevaluation software version 2.0.1 (Biacore, Stockholm, Sweden) using the 1:1 protocol for binding with mass transfer.

Cerebral Ischemia–Reperfusion

Focal cerebral ischemia–reperfusion was induced as described previously. Briefly, mice were anesthetized with a mixture of ketamine (80 mg/kg IP) and xylazine (10 mg/kg IP). Rectal temperature was monitored and maintained at 37.0±0.5°C throughout the procedure with a heat lamp until animals regained consciousness. After a midline neck incision, the right external carotid and pterygopalatine arteries were isolated and cauterized. The internal carotid artery was lifted and occluded at the peripheral site of its bifurcation as soon as the distal common carotid artery was clamped. Focal cerebral ischemia was induced by intraluminal filament occlusion of the right middle cerebral artery (MCA) for 1 hour using a 6-0 nylon monofilament with a silicone-coated tip (0.20–0.22 mm; Doccoll Co., Redlands, CA). Reduction in regional cerebral blood flow was confirmed using transcranial laser-Doppler flowmetry (PFS010 LDLPM Unit; Perimed; Järfalla, Sweden) in the area of cerebral cortex supplied by the MCA (=2 mm posterior and =5 mm lateral to bregma). Mice were treated intravenously with 0.1 mL of either BSA (control, 10 μg; n=32) or CBP (1 μg; n=9; 10 μg; n=22) at the commencement of reperfusion. Based on the incomplete reduction in post-stroke plasma chemokine levels after administration of 1 μg CBP (see below), our study focused on the effects of 10 μg CBP only. Sham-operated mice (n=18) were anesthetized, and the common carotid artery was dissected free from surrounding connective tissue, but the MCA was not occluded. When animals regained consciousness, they were injected subcutaneously with 1 mL saline to prevent postoperative dehydration, before returning them to their cage for recovery.

Neurological Assessment and Quantification of Infarct Volume

Neurological deficit was evaluated in all mice at 24 hours, and in some mice at 48 hours, in a blinded fashion using a 5-point scoring system.
Flow Cytometry

Quantitative analysis of immune cell subsets was conducted as previously described. In brief, 35 mice (12 sham-operated, 12 BSA-treated post-stroke; 11 CBP-treated post-stroke) were deeply anesthetized with isoflurane, blood was collected into a syringe containing 10% (wt/vol) 400 IU clexane (enoxaparin sodium; Sanofi, Macquarie Park, NSW, Australia) via cardiac puncture, and the animals were then perfused with PBS and euthanized by decapitation. Brains and spleens were also collected for cell analysis. Right brain hemispheres were enzymatically digested with collagenase type XI (125 U/mL), hyaluronidase (60 U/mL), and collagenase type I-S (450 U/mL) in Ca2+/Mg2+-supplemented PBS (Sigma, St Louis, MO) at 37°C for 30 minutes and then passed through a 70 μm filter (BD Falcon, Bedford, MA) to obtain single cell suspensions. Cells were washed and resuspended in 30% percoll (GE Healthcare, Uppsala, Sweden), underlaid with 70% percoll, and centrifuged for 20 minutes at 2400 rpm in the absence of a brake. The cells at the interphase of the density gradients were collected for antibody staining. For blood samples, leukocytes were purified with red blood cell lysis buffer (155 mmol/L NH4Cl, 10 mmol/L KHCO3, and 3 mmol/L EDTA). Spleens were mechanically dissociated and passed through 70 μm filters to obtain a single-cell suspension before incubating with red blood cell lysis buffer. All cells were then resuspended in buffer (1% BSA in PBS) containing a mixture of antibodies as listed in Table I in the online-only Data Supplement. After antibody staining, cell populations were analyzed using an LSRII Flow Cytometer run with DIVA software (BD Biosciences, San Jose, CA). For blood and spleen analyses, 5x10^5 or 2x10^5 events were captured, respectively. Data were analyzed with FlowJo software (Tree star Inc, Ashland, OR).

Chemokine ELISAs

The concentrations of CCL2/MCP-1 and CXCL2/MIP-2 in plasma from separate mice (not used for flow cytometric analyses) were assessed by ELISA using OPTI EIA or DuoSet kits following the manufacturer’s instructions. Specifically, plates were coated with capture antibody, blocked, then incubated with serial dilutions of plasma and chemokine standards for 1 hour at room temperature. After antibody staining, cell populations were analyzed using an LSRII Flow Cytometer run with DIVA software (BD Biosciences, San Jose, CA). For blood and spleen analyses, 5x10^5 or 2x10^5 events were captured, respectively. Data were analyzed with FlowJo software (Tree star Inc, Ashland, OR).

Results

CBP Binds a Broad Spectrum of CC, CXC, and C Chemokines With High Affinity

Certain characteristics of the parapoxvirus CBPs have previously been described. The CBP from orf virus, the type species of the parapoxvirus genus, binds a range of CC, CXC, and C chemokines with high affinity and can neutralize the activity of CC chemokines in vitro and in vivo. Publication of the complete genome sequence of another parapoxvirus, BPSV strain AR-02 revealed an equivalent CBP (64 kb from the right terminus of the genome). We therefore sequenced the corresponding region in the BamHI A fragment of the genome from BPSV strain V660 and identified an equivalent CBP. The sequence of the BPSV V660 CBP has been deposited in GenBank under accession no. KM400588. The CBP from BPSV V660 is predicted to be 298 aa in length with a 19 aa signal peptide. The predicted proteins from the 2 BPSV strains share 95% identity, whereas both share only 42% identity with the orf virus CBP.

The CBP from BPSV V660 was expressed with a C-terminal FLAG octapeptide and then purified. Chemokine-binding activity of the purified protein was then assessed using an ELISA. The CBP was found to bind all CC and C chemokines tested, including CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, CCL19/MIP-3α, and XCL1/lymphotactin (Figure 1A and 1B). The CBP also interacted with CXCL2/MIP-2 and CXCL4/IP10 but did not bind CXCL8/IL-8, CXCL10/IP10, or CXCL12/SDF-1 (Figure 1C). To confirm its broad spectrum binding across different classes of chemokines, we investigated the binding affinity of the CBP for murine CCL2/MCP-1, XCL1/lymphotactin, and CXCL2/MIP-2 using surface plasmon resonance (Table II in the

Data Analysis

Unless otherwise stated, data are presented as means± SEM. Statistical significance (P<0.05) was determined by 1-way analysis of variance with Dunnett’s post hoc test (for plasma ELISA data), Bonferroni’s multiple comparison tests (for flow cytometry data), unpaired t test (infarct and edema volume data), or Mann–Whitney test (neurological deficit score data), as appropriate, using GraphPad Prism version 6 (GraphPad Software Inc, La Jolla, CA).

Chemokine-Binding Protein in Acute Stroke

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Figure 1. Chemokine-binding activity measured by ELISA. CC chemokines (A), C chemokine (B), and CXC chemokines (C) were incubated with increasing amounts of the chemokine-binding protein (CBP). Unbound chemokine was then detected using a capture ELISA. Results are presented as a percentage of total chemokine detected in the absence of an inhibitor. A high percentage indicates no interference in the detection of the chemokine (no chemokine binding), whereas a low percentage indicates interference with detection (positive chemokine binding). Values are means±standard deviation (n=6).
online-only Data Supplement). The CBP showed highest affinity for CCL2/MCP-1 (KD value of 0.186 nmol/L), whereas it bound XCL1/lymphotactin and CXCL2/MIP-2 with slightly lower affinity (0.725 and 1.358 nmol/L, respectively). Each chemokine bound the CBP with fast association kinetics, but the rate of dissociation was much slower for CCL2/MCP-1.

Cerebral Blood Flow and Neurological Deficit

To test the effect of CBP on various outcome measures after stroke, mice were intravenously injected with 10 μg CBP or BSA (as a control protein) at the commencement of reperfusion after 1 hour MCA occlusion. Cerebral ischemia was confirmed in all animals by ≈80% reduction in regional cerebral blood flow during 1 hour MCA occlusion (Figure 2A). Blood flow transiently returned to ≈100% of the preischemic level after injection of BSA or CBP at the commencement of reperfusion, although mice treated with CBP tended to have a slightly higher mean level of flow for the remaining 20 minutes recording period (Figure 2A). At 24 hours, CBP-treated mice had a lower median neurological deficit score than those treated with BSA (Figure 2B). Moreover, 7 of 26 (27%) BSA-treated mice and only 1 of 19 (5%) CBP-treated mice received the most severe score (score of 4). Further, 2 of 26 (8%) BSA-treated mice compared with 7 of 19 (37%) CBP-treated mice received a score of 0, indicating no deficit. However, by 48 hours after stroke in control mice, the numbers of infiltrating neutrophils, macrophages, and monocytes further increased by ≈2-fold, whereas numbers of T cells and microglia declined (Figure 4B and 4C).

Plasma Chemokines

Compared with sham-operated mice, plasma levels of both CCL2/MCP-1 and CXCL2/MIP-2 were increased by 25-fold at 24 hours in control mice subjected to cerebral ischemia–reperfusion (Figure 3A and 3B). Between 24 and 48 hours, plasma chemokine levels in control mice declined by ≈50%, but nonetheless remained elevated compared with the sham control group (Figure 3). By contrast, in CBP-treated mice, plasma levels of both CCL2/MCP-1 and CXCL2/MIP-2 were similar to levels in sham-operated mice at both the 24 and 48 hours time points (Figure 3). In preliminary experiments, we also tested the effect of a 10-fold lower dose of CBP (1 μg, n=9) on plasma chemokine levels at 24 hours after stroke and found it to cause reductions of only ≈20% versus BSA control treatment (CBP 1 μg versus BSA for CCL2/MCP-1: 950±278 pg/mL versus 1113±466 pg/mL; CXCL2/MIP-2: 921±332 pg/mL versus 1205±566 pg/mL), and so in subsequent experiments, only the 10 μg dose of CBP was studied.

Flow Cytometric Analysis of Leukocytes in Brain, Blood, and Spleen

There was a 3-fold increase in the total number of leukocytes present in the brains of control mice at 24 hours after cerebral ischemia compared with sham-operated mice (P<0.05, n=6–7; Figure 4A). However, in mice treated with CBP after stroke, leukocyte numbers in the brain at 24 hours were reduced almost to sham levels (Figure 4A). This profile of effect was also seen for individual cell types, including neutrophils, T cells, and monocytes, whereas the increased number of macrophages in the brain after stroke tended to be unaffected by CBP treatment (Figure 4B). Furthermore, in control mice, stroke increased the number of microglia in the brain by ≈3-fold at 24 hours, but this effect was virtually abolished by CBP treatment (Figure 4C). From 24 to 48 hours after stroke in control mice, the numbers of infiltrating neutrophils, macrophages, and monocytes further increased by ≈2-fold, whereas numbers of T cells and microglia declined (Figure 4B and 4C). Moreover, by 48 hours in CBP-treated
mice, the numbers of total leukocytes, neutrophils, macrophages, and monocytes all tended to be even higher than those measured in the brains of control mice, whereas numbers of T cells and microglia were no longer elevated above sham levels (Figure 4A–4C).

Cerebral ischemia–reperfusion resulted in 2- to 3-fold greater numbers of total leukocytes, neutrophils, T cells, and monocytes in blood at 24 hours (Figure 5A–5D), but no change in cell numbers in the spleen at 24 hours (Figure 1A–1F in the online-only Data Supplement). In contrast to its effects in the post-stroke brain, CBP had no effect on immune cell numbers in either the blood (Figure 5A–5D) or spleen (Figure 1A–1F in the online-only Data Supplement).

**Infarct Analysis**

Total and cortical infarct volumes and also edema volume were all smaller in CBP-treated versus control mice at 24 hours (Figure 6A–6C; Figure II A and IIB in the online-only Data Supplement). Subcortical infarct volume also tended to be ≈40% smaller at 24 hours in mice treated with CBP compared with control mice (Figure 6D; Figure IIC in the online-only Data Supplement). Infarct and edema volumes were
similar in control mice at 24 and 48 hours post-stroke; however, at 48 hours, the volume of infarcted tissue and swelling had increased in CBP-treated mice such that there was no longer any difference from control mice (Figure 6A–6D; Figure IID–IIF in the online-only Data Supplement).

Discussion
This is the first study to investigate the use of a broad spectrum viral CBP to assess the effects of chemokine-induced cell migration on stroke outcome in mice. After the identification and generation of a recombinant CBP from the parapoxvirus, BPSV, we confirmed its interaction with a broad spectrum of CC, C, and CXC chemokines and its ability to bind CCL2/MCP-1, XCL1/lymphotactin, and CXCL2/MIP-2 with high affinity. The chemokine-binding spectrum and affinities of the BPSV CBP were consistent with those reported for the CBP from its fellow parapoxvirus, orf virus.13–15,18 After establishing the ability of the CBP to inhibit a broad spectrum of chemokines, we investigated its efficacy in vivo. We found that intravenous administration of the CBP after transient cerebral ischemia largely prevented stroke-induced increases in circulating levels of key chemokines in association with milder neurological deficit at 24 hours. Flow cytometric analysis revealed that treatment with CBP also attenuated leukocyte infiltration in the brain after stroke. In addition, early development of brain infarct and swelling were markedly reduced in mice treated with CBP compared with control mice injected with BSA. Our data therefore support the concept that microglia in the ischemic brain is to phagocytose debris in damaged tissue, neutrophils, and apoptotic cells that have the potential to release damaging molecules.27 Indeed, CCL2/MCP-1 overexpression in the brain is reported to result in increased microglial activation, whereas CCL2/MCP-1-deficient mice exhibit impaired microglial accumulation and reduced leukocyte infiltration in numerous neuroinflammatory diseases.28,29

In association with delayed leukocyte infiltration in the post-ischemic brain, we found that infarct development was retarded in CBP-treated mice. Such a profound effect from a single post-stroke administration of CBP is noteworthy and is conceptually consistent with data from mice with genetically modified levels of chemokine expression. For example, infarct volume is larger in CCL2/MCP-1-overexpressing mice,30 whereas global

![Figure 6. Cerebral infarct and edema volumes at 24 and 48 h after cerebral ischemia–reperfusion in mice treated with bovine serum albumin (BSA; control) or chemokine-binding protein (CBP). A, Total infarct volume; B, edema volume; C, cortical infarct volume; and D, subcortical infarct volume (control, n=11 at 24 h and n=9 at 48 h; CBP, n=6 at 24 h and n=5 at 48 h). Data are mean±SEM (*P<0.05).](http://Stroke.ahajournals.org/content/6/February/2015/figure資格がない)
CCL2/MCP-1 deficiency results in delayed infarct development associated with less severe blood–brain barrier disruption. With reference to this latter study, our data thus indicate that systemic administration of a CBP immediately after cerebral ischemia–reperfusion can achieve therapeutic neuroprotection that is comparable to that seen in animals that are globally deficient in chemokines. It is also interesting to note that CBP treatment effectively prevented T cell infiltration into the ischemic brain for 48 hours, during which time neurological deficits and infarct volume developed to similar levels as in control mice. This observation would tend to argue against a major overall role of T cells in causing post-stroke brain injury, although it is plausible that the lack of T regulatory cells in the brain was detrimental. In addition to directly interacting with chemokines, CBP could decrease chemokine synthesis, but we think this to be unlikely. We have found that CBP administration has no immediate effects on inflammatory chemokine or cytokine expression in skin, but levels do eventually decrease because of the reduced influx of inflammatory cells expressing the same proteins. Moreover, we have observed no inhibition of chemokine expression by CBPs when administered to cultured monocytes (data not shown).

An emerging paradigm is that there are temporal changes in the hierarchy of chemotactic molecules in injured or infected tissue, such that end target chemottractants (e.g., complement component C5a or bacterial products) ultimately override the signals delivered by chemokines (i.e., “intermediate” chemottractants) released at intermediary sites, such as the endothelium. It is noteworthy that neutrophils—which preferentially respond to end target chemottractants—compared >50% of the leukocytes infiltrating the ischemic brain at 48 hours. We therefore predict that sustained protection from leukocyte-mediated brain injury after stroke will require effective targeting of chemokines together with other important chemottractants. Indeed, delaying infarct development and neurological deficits by targeting early chemokine-dependent mechanisms of injury after stroke could be beneficial, perhaps by extending the window of opportunity for other treatments to be administered.

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Disclosures

None.

References

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### Supplementary Table I: Antibody used for flow cytometry.

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### Supplementary Table II: Binding affinity of BPSV CBP to murine chemokines.

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Supplementary Figure I

Populations of leukocytes in the spleen measured by flow cytometric analysis. (A) Total CD45⁺ leukocytes, (B) Ly6G⁺ neutrophils, (C) CD49b⁺ CD90⁺, NK1.1⁺, B220⁺ lymphoid cells, (D) CD3⁺ T cells, (E) F4/80⁺ macrophages, (F) Ly6C⁺ monocytes (Sham, n=6; Control, n=7; CBP, n=5). Data are mean ± SEM cells per 2x10⁵ cell events.

Spleen Leukocytes: 24 h
Supplementary Figure II
Total (A, D), cortical (B, E), and subcortical (C, F) brain infarct distribution in Control and CBP-treated mice at 24 h (Control, n=11; CBP, n=6) or 48 h (Control, n=9; CBP, n=5).