CD40/CD40 Ligand Signaling in Mouse Cerebral Microvasculature After Focal Ischemia/Reperfusion

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Background—CD40/CD40 ligand (CD40L) signaling contributes to proinflammatory and prothrombogenic responses in the vasculature. CD40/CD40L expression is elevated in patients after a transient ischemic attack or stroke. The purpose of this study was to investigate the role of CD40/CD40L signaling in cerebral microvascular dysfunction and tissue injury response to middle cerebral artery occlusion (MCAO) and reperfusion.

Methods and Results—Intravital fluorescence microscopy was used to visualize the cerebral microcirculation of wild-type (WT), CD40-deficient, and CD40L-deficient mice subjected to 1-hour MCAO and 4-hour reperfusion. The adhesion of platelets and of leukocytes and vascular permeability were measured in postcapillary venules after 4-hour and 1-hour reperusions, respectively. Cerebral infarct volume was analyzed 24 hours after reperfusion. Platelet and leukocyte adhesion was elevated and blood/brain barrier function was compromised by MCAO in WT mice. Blood cell recruitment and increased permeability were blunted in both CD40-deficient and CD40L-deficient mice. Infarct volume was also reduced in CD40- and CD40L-deficient mice compared with WT mice.

Conclusions—Our findings indicate that CD40/CD40L signaling contributes to inflammatory and prothrombogenic responses and brain infarction induced by MCAO and reperfusion. The CD40/CD40L dyad may play a significant pathogenic role in the acute phase of ischemic stroke.

Key Words: platelets ■ leukocytes ■ cerebral ischemia ■ microcirculation ■ stroke

The pathophysiology of ischemic stroke is known to include both inflammatory and thrombogenic components. Leukocyte recruitment has been reported in models of both global1-4 and focal5-7 cerebral ischemia/reperfusion (I/R). Manipulation of several adhesion molecules that mediate leukocyte-endothelial interactions, in particular, intercellular adhesion molecule-1 and P-selectin, has revealed a role for I/R-induced leukocyte recruitment in the pathogenesis of the resultant organ injury found in the brain.8,9 We have recently reported that platelet recruitment is another important characteristic feature of the cerebral microvascular response to I/R.3,10 Platelets can contribute to an acute ischemic episode in the brain by forming homotypic (platelet–platelet) and heterotypic (platelet–leukocyte) aggregates that lead to cerebral vascular occlusion and by liberating inflammatory mediators and vasoconstrictors, such as platelet-derived growth factor, thrombospondin, and thromboxane A2.11 However, the mechanisms underlying the prothrombogenic phenotype generated by cerebral I/R remain poorly understood.

CD40, a membrane glycoprotein belonging to the tumor necrosis factor receptor superfamily, is expressed on cell populations that include lymphocytes, monocytes/macrophages, platelets, dendritic cells, and endothelial cells, as well as neuronal cells.12 CD40/CD40 ligand (CD40L) signaling plays a key role in inflammation through the induction of cellular adhesion molecules13 and tissue factor in endothelial cells and by enhancement of the production of proinflammatory cytokines.14,15 Recent reports indicate that patients with acute cerebral ischemia exhibit increased expression of CD40L on platelets, whereas its receptor, CD40, is upregulated on monocytes.16,17 Although it was inferred from these observations that the CD40/CD40L dyad might play an important role in creating and/or maintaining the proinflammatory/prothrombotic milieu and promoting tissue injury in patients with transient ischemic attacks or stroke, this possibility has not been directly addressed in an established experimental model of acute ischemic stroke.

The objectives of this study were to (1) define the contributions of CD40 and CD40L to the leukocyte–platelet–vessel wall interactions elicited in cerebral venules by focal I/R and (2) determine whether the consequences of CD40 and CD40L deficiency on these blood cell–vessel wall interactions include an improvement in blood/brain barrier (BBB) permeability and infarct volume.
Methods

Animal Preparation
Male wild-type (WT) C57BL/6J mice, B6.129S2-Tnfsf5tm1Imx (CD40L−/−) mice, and B6.129P2-Tnfsf5tm1Kik (CD40−/−) mice, 6 to 8 weeks old, were obtained from Jackson Laboratories (Bar Harbor, Me; gene-targeted mice were on a C57BL/6J background). All mice were tracheostomized and artificially ventilated with room air during observation by intravital microscopy. In some instances, pancuronium (0.4 mg/kg IV, Sigma Chemicals) was administered to facilitate breathing. The femoral artery and vein were cannulated to monitor mean arterial blood pressure, sample arterial blood for blood gas analysis, and intravenously administer labeled platelets and rhodamine 6G. Rectal temperature was maintained at 36.5°C to 37.5°C. The experimental procedures described here were reviewed and approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee.

Middle Cerebral Artery Occlusion and Reperfusion
Anesthesia was induced with 4% and maintained with 3% isoflurane in air with use of a Vapamatic apparatus (Bickford Inc). Focal I/R was achieved by occluding the middle cerebral artery (MCAO) with the previously described intraluminal filament method (6-0 nylon). In brief, a midline neck incision was made, and the left external carotid artery (ECA) and left pterygopalatine artery were isolated and ligated. The internal carotid artery (ICA) was clipped at the peripheral site of the bifurcation of the ICA and the pterygopalatine artery with a small vascular clip. The blunted tip of a 6-0 nylon monofilament (0.2- to 0.22-mm-diameter tip) was advanced through the ICA to the carotid bifurcation of the ICA and ECA. The nylon thread and ECA were ligated with 6-0 silk sutures, and the ECA was cut and rotated with the nylon thread. The nylon thread was advanced until light resistance was felt, so that the distances from the nylon thread tip to the ICA-terygopalatine artery bifurcation and the ICA-ECA bifurcation were slightly >6 mm and slightly <9 mm, respectively. The nylon thread was removed after 60-minute occlusion. In the sham group, these arteries were visualized but not disturbed.

Brain Preparation for Intravital Microscopy
For monitoring of blood cell recruitment, mice were anesthetized with α-chloralose (60 mg/kg IP) and urethane (600 mg/kg IP): 1% lidocaine was used for local anesthesia) toward the end of 4-hour reperfusion, and the head of each mouse was fixed in a plastic frame in the spinthex position. The left parietal bone was exposed by a midline skin incision, followed by a craniotomy (2.5-mm diameter) 1 mm posterior from the bregma and 4 mm lateral from the midline. The dura mater remained intact. A 12-mm glass coverslip was placed over the exposed brain tissue, which was suffused with artificial cerebrospinal fluid (CSF).

Intravital Fluorescence Microscopy
An upright Nikon microscope with a mercury lamp was used to observe the venules penetrating the cerebral cortex. The microscopic images were received by a silicon intensified tube camera (C2400-08, Hamamatsu Photonics) and recorded on video. Five randomly selected venular segments, 30 to 40 μm in diameter and 100 μm long, were evaluated for 1 minute each in each preparation for platelet recruitment followed by leukocyte recruitment, and the mean for each parameter was calculated. Rolling platelets and leukocytes were defined as cells crossing the 100-μm venular segment at a velocity that was significantly lower than the centerline velocity. Adherent platelets and leukocytes were stationary for ≥2 seconds and ≥30 seconds, respectively. These parameters were expressed as the number of cells per square millimeter of venular surface, calculated from diameter and length and assuming a cylindrical vessel shape.

Observation of Platelets and Leukocytes
Platelets were isolated from donor WT, CD40L−/−, or CD40−/− mice and labeled with carboxyfluorescine diacetate succinimidyl ester (Molecular Probes), as previously described. This platelet isolation procedure does not alter surface P-selectin expression, suggesting the absence of platelet activation. Platelets (108) were infused into recipients over 5 minutes via the femoral vein, yielding ~10% of the total platelet count. The platelets were allowed to circulate for 5 minutes before 1-minute recording in each of 5 randomly selected venular segments, with use of a B-2A filter block for an excitation wavelength of 450 to 490 nm (Nikon). This procedure was followed by intravenous administration of a 50-μL bolus of 0.02% rhodamine 6G (Sigma Chemicals), followed by a continuous infusion (2 mL/h) of the fluorochrome for 5 to 10 minutes, thus allowing monitoring and recording of rolling and adherent endogenous leukocytes in the same venular segments with a G-2A filter block for an excitation wavelength of 510 to 560 nm (Nikon). Platelets and leukocytes could not be observed simultaneously with this approach.

WT, CD40−/−, and CD40L−/− mice were assigned to 6 experimental groups: (1) sham operation group, (2) WT→WT group (WT platelets were infused into WT MCAO mice), (3) CD40−/−→WT group (CD40−/− platelets were infused into WT MCAO mice), (4) CD40L−/−→WT group (CD40L−/− platelets were infused into WT MCAO mice), (5) WT→CD40−/− group (WT platelets were infused into CD40−/− MCAO mice), and (6) WT→CD40L−/− group (WT platelets were infused into CD40L−/− MCAO mice). To avoid any influence of exogenous knockout platelets on the recruitment of endogenous WT leukocytes, WT leukocyte adhesion was measured in group 2 only (not groups 3 and 4).

Permeability to Fluorescein Isothiocyanate–Albumin
Permeability was assessed in 3 groups: (1) sham operation, (2) WT mice with MCAO and 60-minute reperfusion, and (3) CD40−/− mice with MCAO and 60-minute reperfusion by using a closed cranial-window model, as previously described. Fluorescein isothiocyanate–labeled bovine serum albumin (50 mg/kg IV, Sigma Chemicals) was infused at 30 minutes of reperfusion. Before and 30 minutes after FITC-albumin administration, the sulfusate fluid (artificial CSF) was collected, and an arterial blood sample was drawn for determination of FITC-albumin concentration by using a standard curve for FITC-albumin concentration generated with a spectrophotofluorometer (VersaFluor fluorometer system, Bio-Rad). The clearance of FITC-albumin (mL×s×10−3) was calculated by multiplying the sulfusate-plasma concentration ratio by the flow rate of the artificial CSF (0.22 mL/min).

Leakage of FITC-albumin from venules into the interstitium was directly observed by intravital fluorescence microscopy. The cerebral venules in the closed cranial window were recorded before (background), immediately after (IM), and 30 minutes after administration of FITC-albumin. Images were captured onto a computer, and the fluorescence intensity of 2 areas measuring 10×50 μm (1 in the center of the venule [IV-IM and IV-1 hour] and the other 10 μm away from the venular wall in the perivenular interstitium [IV-IM and IV-1 hour] were analyzed for each section with NIH Image 1.62 software, as previously described. The albumin leak index was calculated with the formula ([IV-1 hour]−[IV-IM])/([IV-IM]−[IV-background]).

Infarct Volume
The volume of cerebral infarct was quantified in WT, CD40−/−, and CD40L−/− mice that underwent MCAO and 24-hour reperfusion. At 24-hour reperfusion, each animal was decapitated and the brain was rapidly removed. Coronal sections (1 mm) cut from the frontal pole were incubated for 10 minutes in 2% 2,3,5-triphenyltetrazolium chloride in phosphate-buffered saline at 37°C and fixed in 10% formaldehyde. An image of each slice was analyzed with NIH Image software. The infarct volume of each section was calculated from the infarct area (total area of the stained nonschismic hemisphere—the stained [noninfarcted] area of the ischemic hemisphere) multiplied by 1 mm, and the total infarct volume for each brain was then calculated from the sum of infarct volumes.
Statistics
All data were analyzed by ANOVA with the Fisher post hoc test. Data are reported as mean±SE. Statistical significance was set at \( P<0.05 \).

Results
Platelet– and Leukocyte–Endothelial Cell Interactions
MCAO for 1 hour followed by 4-hour reperfusion in WT mice was associated with significant increases in the numbers of rolling and adherent WT platelets (WT\( \rightarrow\)WT group) when compared with sham-operated controls (Figures 1A and 1B and 2A). However, a deficiency of either CD40 (CD40\( ^{-/-} \)\( \rightarrow\)WT) or CD40L (CD40L\( ^{-/-} \)\( \rightarrow\)WT) on the exogenous platelets led to a reduction of platelet rolling almost to sham levels (Figures 1C and 1D and 2A). Furthermore, there was a \( \approx 50\% \) attenuation of platelet adhesion (\( \geq 2 \) seconds) in these groups. In recipient mice lacking CD40 (WT\( \rightarrow\)CD40\( ^{-/-} \)) or CD40L (WT\( \rightarrow\)CD40L\( ^{-/-} \)) on all cells, the levels of rolling and adhesion of exogenous platelets from a WT donor after MCAO were similar to those in sham-operated WT animals and significantly lower than in the WT\( \rightarrow\)WT-MCAO group (Figure 2A). Rolling and adhesion of leukocytes were also elevated in WT mice (Figures 1F–1H and 2B) when compared with sham controls (Figures 1E and 2B). Leukocyte rolling was significantly reduced in CD40\( ^{-/-} \) compared with WT mice after MCAO, although levels remained significantly greater than in sham-operated controls. The CD40L\( ^{-/-} \) group demonstrated leukocyte rolling that was comparable to that in CD40\( ^{-/-} \) mice. Leukocyte adhesion was significantly reduced to sham levels in both the CD40- and CD40L-deficient mice 4 hours after MCAO when compared with WT mice undergoing the same focal ischemic insult (Figure 2B).

Permeability
There were no significant differences in the plasma concentrations of FITC-albumin between WT sham-operated mice and WT or CD40\( ^{-/-} \) mice exposed to MCAO (Figure 3A). However, there was a significant increase in the amount of FITC-albumin leaking into the CSF 60 minutes after MCAO in WT mice when compared with sham-operated controls (Figure 3B). A similar result was found for albumin clearance, which was significantly elevated in WT animals after MCAO versus the sham group (Figure 3C). Both the concentration of FITC-albumin in the artificial CSF and albumin clearance were attenuated almost to control levels in the CD40\( ^{-/-} \) group after MCAO compared with their WT counterparts (Figure 3B and 3C). Representative images of the brain microcirculation 30 minutes after FITC-albumin administration are shown in Figure 3E–3G, ie, at 60-minute reperfusion. It is clearly shown that FITC-albumin (white) has leaked from the vessels and into the interstitium of WT-MCAO mice (Figure 3F) when compared with sham-operated mice (Figure 3E). Leaky sites occurred primarily in venules, although this was sometimes difficult to elucidate because of fluorescence in the overlying arterioles. When albumin leakage was quantified by intravital microscopy, the ratio of FITC intensity between the interstitium and the vessel was augmented by MCAO in WT mice (Figure 3D).

### Figure 1
Representative images of platelet (A–D) and leukocyte (E–H) interactions in pial microcirculation of WT mice after 1-hour MCAO and 4-hour reperfusion. In WT recipients exposed to MCAO, adhesion of platelets from either CD40\( ^{-/-} \) (C) or CD40L\( ^{-/-} \) (D) donor mice was reduced compared with WT platelets (B). Adhesion of leukocytes in these same WT-MCAO animals was comparable between groups (F–H). Sham animals showed minimal leukocyte adhesion (E). Arrows indicate flowing platelets. Scale bar=50 \( \mu \)m.

### Figure 2
Changes in platelet (A) and leukocyte (B) recruitment in pial venules after 1-hour MCAO and 4-hour reperfusion (n=6 to 8 per group). *\( P<0.05 \) vs sham; #\( P<0.05 \) vs WT-MCAO.
agreement with spectrophotometric results, MCAO-induced FITC-albumin leakage was significantly attenuated in the CD40/CD40L mice compared with the WT group (Figure 3D and 3G).

Infarct Volume
Infarct volume, an indicator of tissue injury, was 63 ± 5.3 mm³ in WT mice at 24 hours after MCAO. Although infarcted areas were evident in the brains of both CD40/CD40L and CD40L-/- mice, these areas were significantly reduced when compared with those of WT animals (Figure 4).

Discussion
Ischemic stroke is the third leading cause of death in the United States. Primary therapy uses a thrombolytic agent, tissue plasminogen activator, which must be administered early during reperfusion to be effective. However, studies have revealed that simultaneous use of this thrombolytic agent and anti–adhesion molecule therapy may increase the therapeutic window available after stroke.²¹ This suggests that an association may exist between platelets and leukocytes in the pathogenesis of ischemic stroke, wherein inhibition of both cell types exerts a synergistic benefit over blocking one or the other cell population alone. One possible link between the prothrombogenic and proinflammatory pathways during stroke is the cytokine CD40L and its receptor CD40. After stroke in mice, platelet- and T cell–associated CD40L and monocyte CD40 expression are increased. Furthermore, circulating levels of soluble CD40L (sCD40L) are elevated in stroke patients.¹⁷

Extracerebral endothelial cells have been demonstrated to constitutively express CD40,²²–²⁵ and more recently, CD40 was shown to be constitutively expressed on brain microvessel endothelial cells, which could be upregulated by cytokines.¹³ Engagement of this receptor by cytokines, such as tumor necrosis factor-α or CD40L, can lead to activation of several inflammatory pathways and result in the formation of reactive oxygen species, production of inflammatory mediators (cytokines, chemokines), and up-regulation of adhesion molecules that support blood cell recruitment.¹³,²⁶–²⁸ Because all of these inflammatory pathways have already been implicated in the pathogenesis of stroke, it appears likely that engagement of CD40 with its ligand CD40L is an important early event that initiates the inflammatory and tissue injury responses in acute ischemic stroke. Despite this evidence, the exact contribution of CD40/CD40L to the pathogenesis of stroke has not been determined to date. The results of this study indicate that a deficiency of either CD40 or CD40L attenuates both leukocyte and platelet recruitment in cerebral venules after focal ischemia. The attenuated blood cell–venular wall adhesive interactions in CD40- or CD40L-deficient mice were accompanied by a corresponding reduction in tissue injury that is normally manifested as increased BBB permeability and ischemic infarction.

Our findings in WT mice are in agreement with previous reports on stroke that the postischemic brain microvasculature assumes an inflammatory phenotype characterized by recruitment of leukocytes in the postcapillary segment (venule) of the microcirculation.¹ We found that mice deficient in either CD40 or CD40L exhibited a significant reduction in rolling and adhering leukocytes when compared with WT mice at 4-hour reperfusion after stroke.
Previous work on murine models of ischemic stroke has indicated that neutrophils represent the predominant leukocyte population that adheres within the brain microvasculature early after reperfusion.8 Because neutrophils do not express either CD40 or CD40L, the CD40/CD40L signaling pathway may elicit the recruitment of neutrophils in an indirect manner that involves (1) enhanced production of inflammatory mediators (cytokines, chemokines) that results from CD40/CD40L interactions between other blood cell populations (eg, platelets and T lymphocytes) and/or (2) engagement of circulating sCD40L with CD40 on endothelial cells, which then assume an inflammatory phenotype and become hyperadhesive to neutrophils.

CD40 is constitutively expressed on the surface of platelets,29,30 whereas CD40L is normally stored in the cytosol, where it can be mobilized to the cell surface almost immediately on platelet activation.15 When platelets from CD40+/− or CD40L−/− mice were infused into WT mice exposed to MCAO, rolling was reduced toward sham levels, whereas the firm adhesion of platelets was decreased by ∼50% compared with WT platelets. These results strongly support the possibility that CD40/CD40L signaling in platelets contributes to the prothrombogenic state that is observed in the postischemic cerebral microvasculature. Platelets are activated and P-selectin expression is induced by ligation of platelet CD40 by CD40L.30 We have previously reported that platelet-associated P-selectin is a critical determinant of platelet recruitment in the postischemic cerebral microvasculature3,10 and that P-selectin−deficient platelets exhibit complete abrogation of these responses. Hence, the results of the present study, coupled with evidence from the literature, suggest that CD40L on platelets may be interacting with CD40 on other platelets and/or endothelial cells to induce P-selectin expression on platelets as well as endothelial cells, thereby resulting in recruitment of both platelets and leukocytes. Another potential role for platelets in these responses is through the release of sCD40L, which is elevated in human stroke.17 Although platelet-derived sCD40L does not appear capable of engaging CD40,29 it does retain many of its biological properties. For example, it can bind platelet glycoprotein IIb/IIIa, thereby promoting thrombus stabilization and platelet activation.31 Inhibition of the glycoprotein IIb/IIIa receptor can reduce platelet infiltration of the brain and infarct size in a murine model of focal cerebral ischemia.32 Thus, platelet-associated CD40L and CD40 as well as platelet-derived sCD40L may be important in the thrombogenic phenotype initiated by stroke.

Although it is well established that BBB disruption is an important component of the tissue injury response to acute ischemic stroke,33 the factors that mediate this response remain poorly understood. We provide novel data that implicate a major role for CD40/CD40L signaling in the BBB dysfunction induced by MCAO. Although a clear role for the CD40/CD40L dyad as a mediator of vascular permeability has not been established for any other vascular bed, it was shown that injection of soluble CD40L-CD8 fusion protein (which engages and activates CD40 receptors) into the murine pulmonary circulation elicited an inflammatory response and led to the formation of interstitial edema, which may have been related to increased vascular permeability.34 In our study, a large and highly significant increase in brain vascular permeability to albumin was demonstrated after MCAO in WT mice, but this response was dramatically attenuated in CD40+/− mice. Although the mechanism(s) underlying the MCAO-induced, CD40-mediated increase in BBB permeability remains unclear, there are several possibilities. For example, engagement of CD40 promotes recruitment of activated adherent leukocytes, which release oxygen radicals, matrix metalloproteinases, and/or other factors that increase BBB permeability. Alternatively, direct activation of CD40 on endothelial cells can result in the production of factors that exert an autocrine effect on endothelial barrier function. Such factors have also been implicated in the pathogenesis of stroke and include reactive oxygen species,28,35 proinflammatory cytokines and chemokines,26,27,36–38 and metalloproteinases.39,40 Although it appears unlikely that platelets can directly elicit the altered BBB permeability observed shortly after reperfusion (platelet adhesion in cerebral venules is not evident during the early reperfusion period),3 these cells may provide the CD40L (either soluble or cell associated) that engages CD40 on circulating leukocytes, thereby activating these cells to liberate their permeability-inducing factors.

The findings of this study also implicate the CD40/CD40L dyad as a contributor to the tissue necrosis observed 24 hours after 60-minute focal ischemia. The magnitude of infarct volume reduction (30% to 40%), though substantial and statistically significant, did not approach the more dramatic (53% to 89%) attenuations in infarct volume previously reported in rodents that were either genetically deficient in specific adhesion molecules or in WT animals that received adhesion molecule–blocking monoclonal antibodies.8,9,41,42 This is somewhat surprising in view of our observation that CD40+/− and CD40L−/− mice were associated with nearly complete inhibition of MCAO-induced leukocyte recruitment. Although the different protective responses noted between the adhesion molecule ablation studies and our CD40/CD40L experiments may be attributed to differences in species (rats versus mice) studied as well as the magnitude (global versus focal ischemia) and duration (30 versus 60 minutes) of the ischemic insult, it is also possible that CD40/CD40L may play a more important role in mediating the early events that occur after acute ischemic stroke. Furthermore, the CD40 and CD40L expressed by microglia and other neuronal cells may be important for the survival of these cells after injury, because CD40 ligation on neuronal cells protects these cells against serum withdrawal–induced injury.12 Similarly, it has been shown that CD40+/− mice exhibit neuronal dysfunction, as evidenced by neuronal morphological changes, apoptosis, and gross brain abnormalities.12 Hence, the net protective effect evidenced by the 25% reduction in infarct volume observed in our study likely represents a balance between the vascular benefits afforded by CD40/CD40L ablation and...
the potential deleterious effects of this ablation on neuronal cells.

In conclusion, our findings indicate that CD40/CD40L signaling plays a major role in inducing the proinflammatory and prothrombogenic phenotype that is assumed by the cerebral microvasculature after transient, focal, cerebral ischemia. This signaling pathway also makes a significant contribution to the endothelial barrier dysfunction and tissue necrosis that accompany brain I/R. These observations lend support to the development of clinical strategies that target the CD40/CD40L dyad for the treatment of cerebrovascular diseases such as ischemic stroke. Blocking the CD40/CD40L pathway may prolong the therapeutic window for (and improve the effectiveness of) thrombolytic agents in the management of stroke patients. Targeting the CD40/CD40L pathway, which mediates both the inflammatory and thrombogenic responses to stroke, may also improve the outcome of stroke patients who are not candidates for thrombolytic therapy.

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