Thromboembolic Events Predispose the Brain to Widespread Cerebral Infarction After Delayed Transient Global Ischemia in Rats

W. Dalton Dietrich, PhD; Gary Danton, BS; Aviva C. Hopkins; Ricardo Prado, MD

Background and Purpose—Transient distal platelet accumulation after common carotid artery thrombosis (CCAT) leads to hemodynamic, metabolic, and molecular events that may influence the response of the postthrombotic brain to secondary insults. We investigated how a thromboembolic insult would affect histopathological outcome when combined with an ischemic insult induced 24 hours later.

Methods—Three groups of rats underwent either (1) CCAT+10 minutes of normothermic 2-vessel occlusion (n=6), (2) CCAT+sham ischemia procedures (n=6), or (3) sham CCAT procedures+10 minutes of 2-vessel occlusion (n=6). At 7 days, rats were perfused for quantitative histopathological and immunocytochemical analysis.

Results—Rats undergoing combined insults (group 1) had significantly larger areas of ischemic injury (P,0.05) within the cerebral cortex, striatum, and thalamus compared with the other, single-injury groups. Increased ischemic damage included selective neuronal necrosis, infarction, and focal hemorrhage. By means of glial fibrillary acidic protein immunocytochemistry and lectin histochemistry, reactive astrocytes and microglia were found to be associated with widespread tissue necrosis. In contrast, infrequent infarction or CA1 hippocampal neuronal necrosis was observed in groups 2 and 3, respectively.

Conclusions—A prior thromboembolic event is a risk factor for widespread cerebral infarction and hemorrhage when combined with a delayed ischemic insult. The understanding of what factors enhance the susceptibility of the postthrombotic brain to secondary insults may aid in the development of neuroprotective strategies to be applied after transient ischemic attacks to prevent the initiation of stroke. (Stroke. 1999;30:855-862.)

Key Words: astrocytes ■ cerebral infarction ■ microglia ■ platelets ■ risk factors ■ rats

A transient ischemic attack (TIA) is characterized by the sudden onset and complete resolution of a focal neurological deficit occurring within a specific cerebrovascular territory.1,2 Its occurrence identifies a patient as being “at increased risk” of developing a stroke.3,4 The risk is not uniformly distributed in time: it is highest immediately after the episode and declines gradually over subsequent years.4 It is widely accepted that TIAs result when an embolus, vascular constriction, or other process causes a transient drop in local cerebral blood flow. Emboli can arise from various sites, including ulcerating atherosclerotic plaques or the heart, where arrhythmias or valvular abnormalities promote formation of clots. The reasons why TIA patients are at increased risk for stroke are unknown. Thus, it is important to understand the pathobiology of embolic events and how these transient episodes can affect the vulnerability of the brain to subsequent insults.

To investigate the pathobiology of TIAs, photochemical models of thromboembolic stroke have been developed.5–8 Nonocclusive common carotid artery thrombosis (CCAT) is a rat model of thromboembolic stroke that causes rapid damage to the carotid vascular endothelium and deposition of platelet emboli in the microvasculature of the brain.5–7 This embolic process produces transient hemodynamic and behavioral abnormalities similar to those seen in TIA patients.9–12 Recent data also indicate that embolic processes subsequent to CCAT lead to repetitive episodes of cortical spreading depression (CSD) and increased expression of several genes, including brain-derived neurotrophic factor, heat shock protein–70, and glial fibrillary acidic protein (GFAP) mRNA.13 Thus, embolic processes after CCAT induce neuronal and glial genes that may affect the vulnerability of the postthrombotic brain to subsequent insults.

A brief, sublethal ischemic period14–18 or CSD19,20 has been reported to protect against subsequent lethal ischemic insults. Ischemic tolerance has been demonstrated in a number of laboratories under a variety of experimental conditions. Although the mechanisms underlying the development of
ischemic tolerance are unknown, a number of investigators have suggested that the synthesis of stress proteins, including heat-shock proteins, plays a role.\textsuperscript{21,22} In addition, the production of neurotrophic factors and specific growth factors may also participate in conferring neuroprotection to the preconditioned brain.\textsuperscript{23–25} Because CCAT induces multiple episodes of CSD and the expression of both stress and neurotrophic genes, we questioned whether CCAT would induce ischemic tolerance.

The purpose of this study was to determine how a prior thromboembolic insult would affect histopathological outcome after transient global ischemia induced 24 hours later. A secondary global ischemic insult was chosen because it produces a consistent pattern of selective neuronal necrosis in selectively vulnerable brain regions, including the CA1 hippocampus, striatum, and cerebral cortex.\textsuperscript{26} We provide quantitative histopathological and immunocytochemical data indicating that a prior thromboembolic event significantly worsens outcome after transient global ischemia.

**Materials and Methods**

**Animal Groups**

Histopathological experiments were performed on 18 male Wistar rats weighing from 250 to 300 g, obtained from Charles River Laboratory (Wilmington, Mass). Rats were randomly assigned to 3 groups. Group 1 underwent right CCAT followed 24 hours later by 10 minutes of transient global ischemia induced by 2-vessel occlusion (2VO). Group 2 underwent CCAT plus sham ischemia procedures 24 hours later. Finally, in group 3, sham CCAT procedures were followed by 24 hours later by 10 minutes of global ischemia. Rats were fasted 24 hours before the global ischemic insult. At 7 days, rats were perfusion-fixed for quantitative histopathological and immunocytochemical analysis.

**Surgery**

Surgical procedures for CCAT with the use of the photochemical technique have previously been described.\textsuperscript{6,11} Briefly, animals were anesthetized with halothane, intubated, and artificially ventilated with a rodent respirator. A tunable argon dye laser, with wavelength of 562 nM and peak power of 325 mW, was focused by a 61-cm focal length spherical lens onto the saline-submerged right common carotid artery for 10 minutes. Simultaneously, the photosensitizing dye rose bengal (15 mg/mL) in 0.9% saline were injected intravenously to circulate over a 90-second period. This photochemical procedure has been shown to produce 50% to 75% stenosis of the common carotid artery.\textsuperscript{27} Body temperature was monitored throughout the procedure and maintained between 36.8°C and 37.2°C with a heating pad.

The methods for producing normothermic (37°C) global ischemia have previously been described in detail.\textsuperscript{28,29} Briefly, rats were initially anesthetized with 3% halothane and were intubated and ventilated mechanically with mixtures of 0.5% halothane, 70% nitrous oxide, and a balance of oxygen. Animals were immobilized with pancuronium bromide (0.75 mg/kg IV). The femoral arteries were cannulated with polyethylene tubing to permit blood pressure measurements and sampling for arterial blood gases and plasma glucose; arterial PCO\textsubscript{2} and PO\textsubscript{2} were maintained in the normal range by ventilatory adjustments. The common carotid arteries were exposed bilaterally, and a loop of close-fitting polyethylene tubing contained within dual-bore silicone elastomer tubing was placed around each carotid artery. Brain temperature was indirectly monitored by means of a thermocouple implanted into the temporalis muscle.

Transient global forebrain ischemia was produced by lowering the mean arterial blood pressure to \(~\text{45 to 50 mm Hg}\) by controlled exsanguination and the tightening of ligatures around the 2 common carotid arteries.

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<tr>
<th>TABLE 1. Physiological Variables in Rats Undergoing 2VO</th>
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<td><strong>Day 1</strong> Pre-CCAT + Sham Ischemia</td>
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Values are mean±SD. MAP indicates mean arterial pressure.

*Significantly different from day 1 values.

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<th>TABLE 2. Physiological Variables in Rats Undergoing CCAT + 2VO</th>
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<td><strong>Day 1</strong> Pre-CCAT</td>
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carotid arteries. After 10 minutes the carotid ligatures were loosened and the blood, kept at 36°C to 37°C, was reinfused to restore mean arterial blood pressure to normal values. The carotid arteries were inspected to ensure patency, and the rats were returned to their cages and kept in a quiet environment. Sham-operated rats underwent all of the surgical procedures but did not undergo carotid artery occlusion or blood removal.

**Histopathological and Immunocytochemical Procedures**

Seven days after the ischemic insult, the rats were reanesthetized and perfused through the ascending aorta with FAM, a mixture of 40% formaldehyde, glacial acetic acid, and methanol (1:1:8 by volume) for 20 minutes after a 1-minute initial perfusion with physiological saline. The brains were removed, and brain sections were prepared at 250-μm intervals. At coronal levels of interest (0.7 and 2.2 mm from bregma), sequential sections were stained with hematoxylin and eosin (H&E), reacted for the immunocytochemical visualization of glial fibrillary acidic protein (GFAP), or reacted for the histochemical visualization of microglia with the BS-1 isoelectin B4 from Bandeiraea simplicifolia. The vectostain ABC method (Vector Labs) and 3,3-diaminobenzidine were used for visualization of primary antibody binding, as previously described.29

**Quantitative Assessment**

In addition to routine histopathological assessment, quantitative histopathological and immunocytochemical analyses were conducted by a researcher blinded to the experimental groups. For these studies, the cerebral cortex, striatum, hippocampus, and thalamus were analyzed. Areas of neuronal necrosis visualized by H&E histopathology, as well as areas of increased GFAP and lectin staining, were drawn with the use of a camera lucida attachment to a light microscope. Once these areas of interest were traced, the areas (expressed in square millimeters) were calculated by a computer program.

To determine whether the thromboembolic insult altered CA1 neuronal outcome after global ischemia, normal neuronal cell counts were conducted. For this aim, numbers of viable neurons were counted within the lateral, middle, and medial CA1 subsectors as previously described.28 Three sections were analyzed for each rat. Neuronal cell counts were obtained from the right and left hemispheres, and an average value was determined for each rat.

**Statistical Analysis**

Histopathological data are expressed as mean±SEM. Data were compared by the Kruskal-Wallis 1-way ANOVA by ranks. Further group comparisons were evaluated with the Mann-Whitney U test.

**Results**

Physiological data are summarized in Tables 1 and 2. Physiological values were generally within normal ranges before
the photochemical and global ischemic insults. However, compared with day 1 values, mean arterial pressure was significantly reduced on day 2 after either sham or thrombotic procedures. PCO₂ values were significantly decreased on day 2 in rats that had undergone a prior CCAT insult. However, these depressed values remained within normal limits and would not be expected to influence ischemic outcome. Although preischemic blood glucose levels were mildly elevated, no significant differences were demonstrated between the various experimental groups. Finally, the physiological data for the CCAT alone group were similar to the day 1 CCAT data. One rat from the combined CCAT+2VO group died before perfusion fixation and was therefore not included in the histopathological analysis.

Rats undergoing combined insults (CCAT+delayed global ischemia) had larger areas of cerebral infarction than the single-injury groups (Figure 1). Areas of neuronal necrosis were observed in the cerebral cortex, hippocampus, striatum, and thalamus (Figure 2). In 4 of 5 rats, regions of infarction were observed bilaterally. In addition to areas of overt neuronal necrosis, increased staining of GFAP and lectin was observed in the combined-injury group (Figure 3).

In contrast to the combined-injury group, CCAT or 2VO alone produced limited histopathological damage. For example, after CCAT alone, infarct areas (cortical and subcortical structures) at bregma levels 0.7 mm and -2.8 mm were 0.15±0.11 and 0.13±0.12 mm², respectively. After 10 minutes of 2VO, only scattered neuronal necrosis was present within the CA1 hippocampus, dorsolateral striatum, and cerebral cortex. In these groups, abnormal GFAP and lectin staining was restricted to brain regions showing focal infarction or selective neuronal necrosis.

Quantitative assessment of areas of tissue injury demonstrated that the combined-injury group (CCAT+2VO) had significantly larger areas of neuronal damage, GFAP immunoreactivity, and lectin staining than animals undergoing 2VO alone (Figure 4). Differences were most apparent within the cerebral cortex and thalamus. However, CCAT+2VO also increased the area of striatal damage compared with 2VO alone.

To determine the consequences of CCAT on CA1 hippocampal neuronal vulnerability after 2VO, normal neuronal cell counts were conducted in the 3 experimental groups. As shown in Figure 5, cell counts in the 3 subsectors of the CA1 hippocampus demonstrated large numbers of normal-appearing neurons after CCAT alone. However, compared with CCAT alone, significant reductions in viable neurons were demonstrated in rats undergoing either 2VO alone or CCAT+2VO. Thus, compared with cell counts from the global ischemia alone group (2VO), no evidence for CA1 neuronal protection was observed in postischemic rats that underwent a previous thromboembolic insult.

**Discussion**

The major finding of this study was that a prior thromboembolic event is a risk factor for widespread cerebral infarction when combined with a delayed global ischemic insult. Aggravated neuronal damage was seen bilaterally in CCAT rats and was associated with increased activation of microglia and astrocytes. In some cases, areas of infarction were hemorrhagic. These data appear to be the first to demonstrate the increased sensitivity of the postembolic brain to secondary insults. The experimental protocol may mimic in some ways the clinical condition of “increased risk” of TIA patients for developing stroke. The understanding of pathomechanisms responsible for this increased sensitivity could promote the development of therapeutic strategies to mitigate the risk of stroke in TIA patients.

The mechanisms underlying this increased sensitivity to a secondary injury are most likely multifactorial. Platelet activation after carotid vascular injury may lead to increased platelet/endothelial interactions in remote vascular beds that could influence the response of the microvasculature to subsequent ischemic events. Transient platelet embolization may also damage the vascular endothelium and thereby affect the way the cerebrovascular bed responds to secondary insults. Mechanical occlusion of the previously damaged carotid segment during the global ischemic insult may also have dislodged remaining thrombus and accounted for the potentiated injury in the combined injury group. Although the status of the common carotid
artery after thrombosis was not assessed, previous ultrastructural and indium-labeled platelet data indicate that the carotid thrombus has dissolved by 24 hours. Thus, a reduction in carotid artery patency or mechanical dislodgement of remaining thrombus most likely does not account for the present findings with secondary ischemia.

Thrombotic processes might also lead to disturbances in the synthesis of vasoactive substances, including endothelin.

**Figure 4.** Areas of infarction (H&E histopathology), microglial activation (lectin histochemistry), and reactive astrocytes (GFAP immunocytochemistry) from bregma level 0.7 mm (left column) and bregma level −2.8 mm (right column). Data are presented as mean±SEM. *Significantly different from 2VO alone (P<0.05). CTX indicates cerebral cortex; STR, striatum; HPC, hippocampus; and THAL, thalamus.
or nitric oxide, resulting in limited vasodilator capacity of collateral vessels. In this regard, treatment with the nitric oxide synthase inhibitor nitro-\(\text{L}\)-arginine methyl ester (L-NAME) immediately after CCAT increases numbers of indium-labeled platelets in the thrombosed hemisphere, significantly depresses local cerebral blood flow, and exacerbates water maze retention deficits compared with nontreated thrombosed rats. In a bilateral carotid occlusion model, L-NAME treatment was reported to limit the normalization of regional cerebral blood flow during vascular occlusion and recirculation. Although 1-arginine administration has been reported not to improve cortical perfusion or histopathological outcome in rats after photothermic occlusion of the distal middle cerebral artery, there may be a therapeutic potential for nitric oxide to ameliorate stroke if administered after an embolic event.

Vascular thrombosis and subsequent platelet embolization damage the blood-brain barrier. Increased blood-brain barrier dysfunction after thrombosis and platelet accumulation could increase brain and/or perivascular glial swelling that might affect microvascular perfusion or collateral vaso-dilator reserve during secondary insults. In this regard, patients with TIA demonstrate abnormalities in cerebral blood flow reactivity, and recent experimental data indicate that a delayed hypovolemic hypotensive period exacerbates the hemodynamic and histopathological consequences of CCAT. Transient platelet accumulation may also increase the sensitivity of the brain to subsequent ischemic insults by the modulation of receptor-mediated neuronal and glial responses. In this regard, it would be important to determine whether the postthrombotic brain demonstrates an increased sensitivity to various neurotoxins, including excitatory amino acids.

An indication that microvascular abnormalities participate in the response of the postthrombotic brain to secondary injury was the presence of hemorrhagic damage. Focal hemorrhage was seen within the ipsilateral cerebral cortex and striatum, regions known to be susceptible to platelet accumulation and embolic stroke after CCAT. Previous ultrastructural findings after CCAT indicate that structural damage to the vessel wall can be associated with occlusive distal platelet emboli. Structural damage caused by platelet emboli may therefore increase the susceptibility of the microvasculature to reperfusion injury and subsequent hemorrhage. Whether the hemodynamic consequences of secondary ischemia/reperfusion are affected by the thromboembolic insult remains to be determined.

Although increased damage with secondary ischemia was most apparent within the thrombosed hemisphere, bilateral infarction after unilateral CCAT was evident in 4 of 5 rats. Previous studies have shown that unilateral CCAT can lead to platelet accumulation within the contralateral hemisphere. In addition, the microvascular consequences of CCAT, including blood-brain barrier disruption and hemodynamic depression, can be produced in intact rats that receive thrombogenically activated blood sampled downstream from a platelet thrombus forming in a donor rat. Thus, the contralateral effects reported in this study may be the result of platelet emboli and blood-borne factors generated after CCAT that produce microvascular consequences bilaterally. The regionality of the vulnerability patterns to secondary ischemia may result from complex interactions between the 2 insults that require further clarification.

Previous studies have reported that CSD protects against subsequent lethal ischemic insults. As previously discussed, CCAT produces repetitive episodes of CSD that lead to the expression of stress and neurotrophic genes that could produce ischemic tolerance. However, in the present study we demonstrated that CCAT actually aggravates the histopathological consequences of a delayed ischemic insult. Thus, although embolic events induce molecular responses that could potentially regulate the ability of the brain to resist injury, other embolic processes, including neuronal injury and microvascular damage, may override these protective responses at 24 hours after CCAT. Because the interval separating repeated ischemic insults is critical in studies of ischemic tolerance, it will be important to determine whether longer time intervals between the 2 insults result in findings that differ from the present investigation.

In summary, we report that the postembolic brain is predisposed to cerebral infarction after delayed transient cerebral ischemia. Because TIA patients are at increased risk for stroke, the present model may help to clarify mechanisms underlying this clinical phenomenon. In addition, the double-insult model may prove useful in testing therapies that may be used to treat TIA patients before a lethal stroke occurs. Potential therapies may target microvascular and thrombotic events or include receptor blockers that have been reported to have limited benefits when given after a lethal ischemic insult. Agents that promote oxygen delivery, improve endothelial function, or enhance cerebral perfusion may also prove beneficial. Brain cooling, which can be produced in stroke and head trauma patients, might also provide protection if administered after the thrombotic event. Thus, continued investigation of this experimental model should clarify the reasons for the increased sensitivity of the postthrombotic brain and provide important information for the continued development of neuroprotective strategies to be applied after TIAs to prevent initiation of stroke.
Acknowledgments

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References

Editorial Comment

In the American population, strokes occur as frequently as once every minute and with highly variable severity, a pattern that reinforces the view that cerebral infarcts result from a complex interplay of numerous mechanisms influenced by multiple independent risk factors. Important among these are episodes of TIA, which can dramatically heighten stroke risk, particularly during the period immediately after a TIA. Paradoxically, in some experimental paradigms, prior exposure to mild ischemia can offer increased resistance to subsequent ischemic insults through the poorly understood phenomenon known as “ischemic preconditioning.” Why TIAS aggravate, and not improve, subsequent stroke risk is unclear, and this deficit in understanding persists largely because of the lack of adequate experimental models for the study of interactions between TIAs and subsequent ischemic insults.

The accompanying article by Dietrich et al introduces a new animal model for the study of interactions between TIA and subsequent stroke risk. Based on the reasonable premise that a significant fraction of TIAs involves some type of coagulopathy or thrombotic event, the primary insult in this rat model is the production of a nonocclusive carotid artery thrombosis using a well-established light-dye technique. Forebrain ischemia is then produced 24 hours later by a combination of bilateral carotid occlusion coupled with hemorrhagic hypotension. Seven days later, individual brain regions can be analyzed by any of a variety of histological or immunocytochemical techniques. Interestingly, the results offered in this study suggest that alone neither carotid artery thrombosis nor hypotensive ischemia produces extensive necrotic damage. In combination, however, these insults produce distinct patchy areas of regionalized infarction, indicating that prior carotid artery thrombosis enhances vulnerability to subsequent ischemic insults and provides no protection through ischemic preconditioning.

Certainly, some caution should be exercised when these results are extrapolated. Ischemia was produced in the presence of anesthesia, which may have influenced regional ischemic vulnerability. In addition, the numbers of animals used were uncertain, which leaves open the possibility that the sizes of the experimental groups were too small to detect significant effects of either thrombosis or hypotensive ischemia alone (a power analysis would have been most useful in this regard). Also, some TIAs may not involve thrombus formation and instead result from pathological vasoconstriction secondary to derangements such as vasospasm, hyperactivity to circulating vasoconstrictors, abnormal endothelial function, or anomalous perivascular nerve activity. Nonetheless, the rat model developed and described by Dietrich et al is highly original, much needed, and offers great promise for better understanding the mechanisms coupling thrombotic events, and thereby perhaps some TIAs, to increased risk for ischemic stroke.

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