Temporal Profile of Enhanced Vulnerability of the Postthrombotic Brain to Secondary Embolic Events

Gary H. Danton, BS; Ricardo Prado, MD; Brant D. Watson, PhD; W. Dalton Dietrich, PhD

Background and Purpose—Patients with vascular or cardiac disease may experience recurrent thrombosis and embolization to the cerebral vasculature. 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 emboli. We investigated the effect of repeated embolic episodes on histopathological outcome at various time intervals using a clinically relevant model of embolic stroke.

Methods—Six groups of rats underwent either photochemically induced CCAT followed by sham surgery or 2 episodes of CCAT separated by 10 minutes or 1, 3, 5, or 7 days. Outcome measures included routine histopathological analysis and determination of the number of infarct loci and their total volume.

Results—Rats that underwent a second CCAT at 1, 3, or 5 days after the first insult had 20 to 30 times larger infarct volumes than rats in the single-CCAT group (P<0.05). In addition, rats in the 10-minute and 1-, 3-, and 5-day groups had 2 to 3 times as many infarcts as those in the single-CCAT group (P<0.05). Infarcts produced by double insults commonly extended through the neocortex and were necrotic, edematous, and sometimes hemorrhagic.

Conclusions—A prior thromboembolic event puts the brain at risk for severe infarction after a second embolic event. These findings cannot be explained solely by a greater number of infarcts. Elucidating pathomechanisms responsible for the vulnerability of the postthromboembolic brain may provide targets for new treatment strategies to prevent the severe consequences of embolic stroke. (Stroke. 2002;33:1113-1119.)

Key Words: cerebral infarction • ischemic attack, transient • ischemic preconditioning • platelet aggregation • rats

Clinical intuition, logic, and numerous studies have concluded that transient ischemic attacks (TIAs) categorize a patient as having an increased probability of experiencing a stroke.1–3 The diagnosis of either TIA or stroke is made on the basis of clinical observations, often independent of radiological or pathological evaluation. Therefore, while some argue that patients with any detectable pathology should be given preventative therapies need to be taken to avert subsequent stroke (decrease risk) in TIA patients.7

An important difference between TIA and the preconditioning stimulus in experimental models is the pathological consequences of the 2 insults. Ischemic preconditioning does not cause any detectable pathology, while TIA patients may have deficits in cerebral autoregulation11 or show evidence of infarction on CT or MRI.3,12 TIA patients with radiological findings appear at higher risk of subsequent stroke than TIA patients without radiological findings, but this association is no longer significant when adjusted for other risk factors such as plaque ulceration, age, hypertension, and number of TIAs.9,13 These other factors may lead to subtle cerebrovascular or parenchymal damage that may elude radiological detection after TIA and increase a patient’s risk of having a severe stroke.

The mechanisms of experimental ischemic preconditioning are still under investigation but are thought to involve the upregulation of many neuroprotective genes.4,5 Some of these

Received August 9, 2001; final revision received December 10, 2001; accepted December 14, 2001.

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genes are upregulated immediately after photochemically induced, nonocclusive common carotid artery thrombosis (CCAT) in rats. CCAT is a stroke model of carotid artery–generated emboli that consistently produces hemodynamic deficits and usually (but not always) produces infarction. Nonocclusive CCAT in rats is not considered a model of TIA. However, the embolic nature of CCAT and resulting small infarcts are most relevant to patients currently diagnosed with minor strokes, reversible ischemic neurological deficits, or TIA. Sensorimotor and cognitive deficits after CCAT are transient, and mortality is rare.

Despite upregulation of neuroprotective genes, CCAT followed by either hypotension to a mean arterial pressure of 60 mm Hg or global ischemia led to large infarctions throughout the brain that were often hemorrhagic. Rats with CCAT not only failed to show neuroprotection, but the pathology of subsequent ischemia was more severe. Recent clinical studies found that after TIA, patients are extremely susceptible to severe strokes within 2 days after diagnosis. Repeated embolization due to recurrent thrombosis of a carotid plaque over hours, days, or weeks may account for some cases of repeated TIA and severe infarction. To evaluate the temporal profile of risk and outcome after repeated embolization in an experimental rat model, we produced 2 episodes of CCAT at different time intervals. We tested the hypothesis that repeated episodes of CCAT would increase infarction volume up to 1 week after the initial insult.

Materials and Methods

Animal Groups

Histopathological experiments were performed on 52 male Wistar rats weighing 300 to 380 g obtained from Charles River breeders (Wilmington, Mass). The Animal Care and Use Committee at the University of Miami School of Medicine approved this protocol. Rats were randomly assigned to 6 groups. Rats in the single-CCAT group (n=7) received CCAT followed by a sham surgery 1 day after the initial insult. The 5 groups of experimental animals received CCAT plus a second CCAT at 10 minutes (n=9), 1 day (n=9), 3 days (n=8), 5 days (n=8), or 7 days (n=11) after the initial insult. Rats were subjected to fasting 24 hours before the second surgery.

Surgery

CCAT was produced via the photochemical method. Briefly, rats were anesthetized with 4% halothane and maintained on 0.5% halothane. Erythrosin B (190449, ICN Biomedicals Inc) was injected (35 mg/kg) via the femoral venous catheter at a rate of 17.5 mg/kg per minute with an infusion pump (PHD2000, Harvard). Simultaneously, a tunable argon laser (Innova 70-4, Coherent) (wavelength, 514.5 nm; peak power, 375 mW) was focused via a spherical lens (focal length, 61 cm) on the common carotid artery for 10 minutes. Carotid blood flow and the generation of emboli were monitored with a Doppler ultrasound probe (T206, Transonic Systems) placed distal to the site of thrombosis. After irradiation, the catheters were removed, and incisions were sutured. After surgery, rats were returned to their cages until they were killed. Sham surgeries were performed exactly as described without irradiation. Second insults were performed just as the first except that the common carotid artery was irradiated 2 mm distal to the first irradiation. Consistency between first and second insults was verified by monitoring embolus emission with carotid Doppler.

Histopathological Procedures

Three days after the second surgery, rats were reanesthetized and perfusion-fixed with saline for 30 seconds followed by FAM, a mixture of 40% formaldehyde, glacial acetic acid, and methanol (1:1:8 by volume) for 20 minutes. Brains were removed, paraffin embedded, and cut into 10-μm sections at 300-μm intervals. Sections were stained with hematoxylin and eosin for histopathological examination and for the determination of infarct cross-sectional areas and integrated volumes.

Quantitative Assessment

Areas of infarction were identified by the presence of tissue necrosis consisting of pyknotic nuclei and hypodense neuropil. Areas of each infarct were determined with the use of a rat brain stereotaxic atlas. Volumes were calculated for each infarct with a computer algorithm of numeric integration on the basis of distance between bregma levels.

Statistical Analysis

Histopathological data were expressed as mean±SD (mm³). Volume and frequency data were compared with an ANOVA by ranks followed by Dunn’s post hoc comparison or a 1-way ANOVA followed by Fisher’s least significant difference post hoc test, respectively.

Results

Physiology

Physiological variables were measured and maintained within normal limits during and after the first and second insults (Table 1).

Temporal Profile of Infarction

Infarct volumes were significantly increased (P<0.05) in animals receiving secondary thrombotic insults at 1, 3, and 5 days but not at 10 minutes or 7 days (Table 2). Peak infarction volume was seen in 3-day animals and declined to near single-CCAT levels in rats in the 7-day group. Infarcts were found throughout the brain and occasionally in contralateral structures, likely because of the bilateral distribution of the azygous artery that arises from the confluence of the anterior cerebral arteries. The number of infarcts also increased in the animals in the double-insult group to peak at 3 days and returned to slightly above the value in the single-CCAT group by 7 days (Table 2). While infarct number correlated with volume (Figure 1B), animals with double insults at 1, 3, and 5 days had 2 to 3 times as many infarcts with 20 to 30 times more volume than did the single-CCAT group (Figure 1A).

Infarct variability was notable in animals in the double-insult group, as shown by the minimum and maximum values in Table 2. Six of 7 rats in the single-CCAT group exhibited a total of 14 infarcts with an average total volume of 0.6±0.8 mm³ (Table 2). Infarction in rats receiving CCAT+CCAT with a 10-minute interval ranged between 0.35 and 6.8 mm³, with 1 rat having a total volume of 21 mm³. Animals in the 10-minute group tended to have a greater number of infarcts than those in the single-CCAT group, but infarct volume was not significantly different. Infarcts in rats receiving dual CCAT 1, 3, and 5 days apart were consistently larger and occurred more often than in rats in the single-CCAT and 7-day groups. Most infarcts in the 7-day group...
neuropil (Figure 2D). Lesions in the hippocampus (Figure 2E could often be seen adjacent to infarcts with clearly disrupted necrosis with an intact neuropil. Selective neuronal necrosis exhibited apoptotic bodies consistent with apoptotic cell death (Figure 2B and 2C). Some neurons exhibited spongiotic changes or severe disruption of the neuropil (Figure 2A and 2F) frequently contained selective neuronal necrosis of the CA1, CA2, CA3, or dentate gyrus accompanied by infarction of surrounding tissues. The neuropil in these infarcts usually did not exhibit profound spongiotic or necrotic changes.

Histopathology

Infarctions in rats in the single-CCAT group were small and focal. Cortical infarcts often followed a particular vascular territory that extended partially or entirely through the cortical layers (Figure 2A) but was limited to approximately 500 μm in width. Many of these larger infarcts exhibited eosinophilic cell bodies and pyknotic nuclei scattered among the necrotic neuropil (Figure 2B and 2C). Some neurons exhibited apoptotic bodies consistent with apoptotic cell death (Figure 2B, insert). It was common to observe lesions characterized by the pyknotic nuclei of selective neuronal necrosis with an intact neuropil. Selective neuronal necrosis could often be seen adjacent to infarcts with clearly disrupted neuropil (Figure 2D). Lesions in the hippocampus (Figure 2E and 2F) frequently contained selective neuronal necrosis of the CA1, CA2, CA3, or dentate gyrus accompanied by infarction of surrounding tissues. The neuropil in these infarcts usually did not exhibit profound spongiotic or necrotic changes.

Rats receiving 2 episodes of CCAT with intervals between 10 minutes and 5 days shared some common histopathological features. After dual insults, infarcts were variably sized but often large and extended through the brain rostrocaudally (Figure 3A and 3B), while single-CCAT infarcts were usually restricted to within 1 mm. Infarcts were clearly necrotic, but often large and extended through the brain rostrocaudally.
Endothelial hyperplasia and capillary proliferation were noted in many larger infarcts (Figure 3D). In some cases, segments of large structures such as the anterior hippocampus were completely necrosed (Figure 3E). Edema was conspicuous in many animals as brain structures were distorted, impinged on the ventricles, or developed a midline shift (Figure 3F). In some infarcts, tissue loss was obvious as the ipsilateral ventricle was expanded (Figure 3G). Hemorrhagic infarctions were noted in 2 animals in the 10-minute group and 4 animals in the 1-day group (Figure 3H).

**Discussion**

The major finding of this study is that the postembolized brain is vulnerable to severe infarction on exposure to a second embolic event. The window of greatest vulnerability occurred between 1 and 5 days after the initial insult. In general, double-CCAT infarcts were larger and more edematous and distorted the neuropil substratum more than single-CCAT infarcts. While animals in the dual-insult group typically had a greater number of infarcts, the increase in infarct number did not match the extent of increased infarct volume (Figure 1). In a previous investigation, long-term studies to 4 weeks after single CCAT reported that most infarcts were small, and while their features changed over time, their area did not increase. Thus, the large infarct volumes seen in our model are not due to aging infarcts in the dual-insult group. The extent of infarct severity in that group was surprising given the limited histopathology previously seen after CCAT.

Cerebrovascular consequences of CCAT include transient hemodynamic disturbances, embolic occlusion of small vessels, and microvascular pathology. Transient global and long-term focal blood-brain barrier (BBB) permeability after single CCAT suggest the presence of widespread but subtle vascular disturbances. While vascular damage may not result in obvious histopathology, it may predispose the brain to severe damage after a second insult. Two previous studies demonstrated exacerbated CCAT pathology after delayed hypotension and ischemia. In these studies and the present study, 2 relatively minor insults yielding small, focal damage were combined, resulting in extensive histopathological lesions. This finding has important clinical consequences. Severe strokes may be the result of a previous minor stroke, TIA, or subclinical embolic event exacerbated by second insults. In support of this idea, patients are more susceptible to severe infarction shortly after a TIA, infarcts are more likely to occur in the same territory as a TIA, and microembolic signals on transcranial Doppler predict future stroke.

Mechanisms for the increased vulnerability are under ongoing investigation. Potential mechanisms include damage to the cerebrovasculature or to the brain parenchyma itself.

**TABLE 2. Infarct Volume and Frequency by Group**

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Total Volume, mm$^3$</th>
<th>Mean $\pm$ SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Striatum</td>
<td>Hippocampus</td>
<td>Thalamus</td>
</tr>
<tr>
<td>Single CCAT (7)</td>
<td>0.6 $\pm$ 0.8</td>
<td>0</td>
<td>2.3</td>
<td>9</td>
</tr>
<tr>
<td>10 min (9)</td>
<td>5.3 $\pm$ 7.2</td>
<td>0.35</td>
<td>21.4</td>
<td>23, 3C</td>
</tr>
<tr>
<td>1 d (9)</td>
<td>14.5 $\pm$ 16.4*</td>
<td>2.6</td>
<td>55.4</td>
<td>22, 4C</td>
</tr>
<tr>
<td>3 d (8)</td>
<td>22.0 $\pm$ 13.3†</td>
<td>1.5</td>
<td>37.5</td>
<td>33, 1C</td>
</tr>
<tr>
<td>5 d (8)</td>
<td>16.9 $\pm$ 15.1†</td>
<td>2.5</td>
<td>40.5</td>
<td>22, 3C</td>
</tr>
<tr>
<td>7 d (11)</td>
<td>3.0 $\pm$ 5.0</td>
<td>0</td>
<td>15.8</td>
<td>16</td>
</tr>
</tbody>
</table>

*All infarcts are ipsilateral to the site of thrombosis unless followed by a C (contralateral).

*Significantly different from single-CCAT group ($P<0.05$).

†Significantly different from single-CCAT and 7-day groups ($P<0.05$).
While subtle injury to neurons or glia may help to explain this phenomenon, CCAT results in the upregulation of immediate early and stress-related genes that are thought to be neuroprotective, as seen in models of ischemic preconditioning. An important difference between ischemic preconditioning and dual CCAT is that the first CCAT results in pathology (embolic infarcts), while the preconditioning ischemic exposure does not. However, dual CCAT is clinically relevant because some patients have subtle pathology after TIA. Interestingly, when evidence of infarction on CT was used as an exclusion criterion, TIA patients fared better after stroke than patients without prior TIA. By including only TIA patients without pathology, the authors may have unmasked
neuroprotective effects from the detrimental consequences of TIA or minor stroke.

Alternatively, hemodynamic or vascular mechanisms are more likely to play a role in increasing the brain’s vulnerability to secondary injury. While vascular pathology occurs after ischemia, ischemic preconditioning does not seem to be affected by these changes. The brief preconditioning ischemia induced in the brain during these experiments may protect the microvasculature. In contrast, severe ischemia may lead to infarction and damage the vasculature, making the brain more vulnerable to a second insult. Hemodynamic stresses such as hypotension worsen pathology after CCAT, suggesting that the cerebrovasculature is unable to autoregulate blood flow after injury. Autoregulation deficits are a common clinical finding in patients with carotid stenosis and TIA and may be used as a prognostic indicator in patients who are vulnerable to stroke. In the present study mild hypotension was observed in some of the experimental groups and may have contributed, in part, to the observed increased vulnerability to subsequent emboli.

Endothelial-derived vasodilators and platelet inhibitors govern vascular reactivity and inhibit thrombosis. Damaged endothelial cells may no longer promote vasodilation or inhibit platelet activation, making the vasculature vulnerable to embolic stroke. Alternatively, some clinical and experimental data suggest that the vasculature may become over-responsive after stroke. Luxury perfusion, in which blood flow exceeds the metabolic needs of a tissue, and hyperemia are seen in patients after stroke, carotid endarterectomy, and in our model of CCAT. Thus, disturbances in autoregulation may decrease or increase blood flow to dangerous levels.

A combination of hyperemia, endothelial damage, and BBB permeability likely leads to edema, swelling, and distortion of brain structures in animals subjected to double insult. Mechanisms leading toward loss in endothelial integrity have begun to be elucidated. Permeability factors described by various stroke models, including CCAT, involve pinocytotic transport across endothelial cells and extravasation through necrotic vessels. Necrotic vessels were sometimes seen in large infarcts after multiple insults and may account for rats with severe edema. Reports of ischemia models also describe specific barrier deficits, such as disruptions in tight junctions between endothelial cells and reduced astrocyte-endothelial contact from loss of integrins. Platelet-endothelial interactions and inflammatory mechanisms may participate in these vascular perturbations. BBB permeability is seen both ipsilateral and contralateral to the site of thrombosis in models of photothrombotic middle cerebral artery occlusion and CCAT, suggesting that products released from the thrombus are resulting in downstream consequences in regard to the BBB. Increased levels of activated platelets are found in stroke patients, especially those with vascular thrombosis. Activated platelets have also been found to release matrix metalloproteinases that are implicated in BBB permeability and pathology after focal ischemia. Matrix metalloproteinases are a class of enzymes capable of degrading proteins in the extracellular matrix and are important for restructuring blood vessels during angio-

genesis. Because cerebral ischemia leads to the increased expression of matrix metalloproteinases (2 and 9), the role of these enzymes in causing the postembolic blood vessels to be more vulnerable to secondary injury merits consideration and further study. Endothelial hyperplasia and capillary branching are frequently seen within infarcted regions after CCAT, which may be interpreted as a sign of vascular remodeling. When vascular integrity is compromised, exposure to secondary emboli may cause vascular rupture and hemorrhage into the parenchyma.

Hemorrhagic transformation was seen when CCAT was combined with ischemia and was also noted in 4 animals in the 1-day group and 2 animals in the 10-minute group (Figure 3H). Vascular remodeling may be protective in response to hypoxia-ischemia but may make the brain vulnerable to hemorrhage or edema after additional emboli. Hemorrhage was seen only when second insults were administered within 24 hours.

In summary, secondary exposure to emboli 1, 3, and 5 but not 7 days after an initial episode of CCAT results in significantly greater infarct volumes. While increases in the number of infarcted regions and volume are correlated, the magnitude of increased infarct volume is far greater than can be accounted for by a greater number of infarcts alone. Vascular injury and dysfunction play a large role in the pathophysiology of CCAT. These detrimental changes may contribute to the vulnerability of the postembolic brain to severe infarction. Identifying these mechanisms may provide new therapeutic strategies for preventing or mitigating the devastating consequences of severe stroke. Future studies will attempt to elucidate the pathophysiology and to test prospective treatments.

Acknowledgments

This study was supported by National Institutes of Health grants NS27127-11 and NS23244-16, American Heart Association Predoctoral Fellowship 001010BB, National Institutes of Health predoctoral training grant NS07459-01, and the Lois Pope Life Fellowship Program. The authors thank Dr. Helen Bramlett for critical comments on the manuscript and Susan Kraydieh for technical assistance.

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Stroke. 2002;33:1113-1119
doi: 10.1161/hs0402.105554
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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