Transient Platelet Accumulation in the Rat Brain After Common Carotid Artery Thrombosis

An \(^{111}\text{In}\)-Labeled Platelet Study

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**Background and Purpose:** Thromboembolic events are a major cause of ischemic stroke. To obtain evidence for platelet embolization after cerebrovascular injury, the accumulation of indium-labeled platelets was documented after photothermolysis of the rat common carotid artery.

**Methods:** Heterologous blood was collected from donor rats, and the isolated platelets were labeled with \(^{111}\text{In}\)-tropolone. Labeled platelets were then infused into Wistar rats 30 minutes before right carotid artery thrombosis. Nonocclusive common carotid artery thrombosis was induced by a laser-driven rose bengal-mediated photochemical insult to the vascular endothelium, and the rats were killed 15 minutes or 3 hours later. Carotid arteries and brains were immediately removed and dissected for regional radioactivity assessment or sectioned for the autoradiographic visualization of platelet emboli.

**Results:** At 15 minutes after thrombosis, the ratio of right-to-left common carotid artery radioactivity was significantly elevated compared with control (33±12 [mean±SEM] versus 0.97±0.2). Within individual brain regions, including the frontal and frontoparietal cortices and hippocampus, significant elevations in right-to-left radioactivity ratios were also documented. Autoradiographic images revealed multiple foci of \(^{111}\text{In}\)-labeled platelets throughout the thrombosed hemisphere. At the level of the frontal cortex, bilateral platelet accumulation was seen. Regional counts demonstrated significantly increased platelet density within select cortical and subcortical regions. In contrast to the 15-minute findings, right-to-left ratios of carotid arteries or brain regional radioactivities were not significantly elevated at 3 hours after injury. In addition, the areal densities of autoradiographically visualized platelets in the 3-hour group were not different from control except in the right frontal cortex.

**Conclusions:** These data demonstrate (1) the acute accumulation of labeled platelets in downstream vessels after nonocclusive common carotid artery thrombosis, (2) that platelet accumulation is widespread and also involves contralateral areas, and (3) that platelet accumulation within the thrombosed carotid artery and brain is largely transient. (Stroke. 1993;24:1534-1540.)

**KEY WORDS** • embolism • platelet aggregates • thrombosis • rats

Transient ischemic attacks are generally attributed to emboli arising from lesions in the heart, aorta, or large cerebral arteries.\(^1\)-\(^6\) In an attempt to investigate the acute pathomechanisms and consequences of cerebrovascular injury, animal models of photochemically induced vascular thrombosis have been developed.\(^7\)-\(^10\) In these thrombotic models, widespread morphological, hemodynamic, and biochemical abnormalities have been described that are generally not seen in conventional models of focal and/or global ischemia.\(^9\)-\(^14\) For example, acute blood-brain barrier and local cerebral blood flow (ICBF) abnormalities have been reported in recipient rats after the intracarotid infusion of blood sampled downstream from a forming carotid thrombus.\(^11\),\(^12\) In another study, downstream blood levels of serotonin were shown to increase 15-fold during and after common carotid artery (CCA) thrombosis.\(^14\) These investigations indicate that important consequences of vascular thrombosis include the generation of humoral substances and the activation of blood elements that could participate in the pathogenesis of transient ischemic attacks and acute stroke.

Platelet emboli originating at the site of a diseased vessel may occlude small cerebral vessels and cause regional cerebral infarction. In patients with transient ischemic attacks, small emboli have been observed in retinal vessels as well as in the cerebral circulation.\(^15\)-\(^18\) Experimentally, luminal platelets and small cerebral infarcts have been documented morphologically after nonocclusive thrombosis of the middle cerebral artery (MCA) and CCA.\(^9\),\(^10\) In an ultrastructural study, platelet aggregates were detected in pial and parenchymal vessels 15 minutes...
after MCA thrombosis.\textsuperscript{9} In another study, cerebral infarcts consistent with embolic mechanisms were documented histopathologically 1 to 4 days after CCA thrombosis.\textsuperscript{10} Morphological approaches for the detection of intravascular platelets are limited due to sampling problems.\textsuperscript{19} In addition, the use of parenchymal necrosis as a guide for the location of platelet emboli is problematic because necrotic tissue may itself cause secondary vascular complications including vascular stasis and platelet aggregation.\textsuperscript{20}

An alternative method for identifying luminal platelets is the use of \textsuperscript{111}In-labeled platelets.\textsuperscript{19,21-23} Owing to their radioactivity, \textsuperscript{111}In-labeled platelets provide a sensitive technique for the identification of a platelet thrombus and the distribution of cerebral emboli. Although labeled platelets have been detected in the postischemic brain,\textsuperscript{19} the regional distribution of acute platelet embolization after cerebrovascular thrombosis has not been investigated. Thus, the major aim of this study was to obtain evidence for acute \textsuperscript{111}In-labeled platelet embolization after photochemically induced CCA thrombosis. In addition, we sought to determine whether evidence for spontaneous recanalization at longer postirradiation periods could be observed.

### Methods

**Platelet Labeling with \textsuperscript{111}In-Tropolone**

Sterility was maintained throughout the indium labeling procedures discussed in detail previously.\textsuperscript{21-23} For each study, a total of 30 mL blood was first withdrawn from the femoral arteries of two donor rats (250 to 400 g) for platelet labeling. Blood was collected into two 30-mL syringes containing a total of 6 mL acid citrate dextrose (ACD) solution (maintaining a 6:1 ratio of whole blood to ACD). The whole blood was next centrifuged for 10 minutes at 200g, and the supernatant of platelet-enriched plasma was centrifuged for 10 minutes at 1600g. The platelet-poor supernatant was discarded, and the remaining platelet pellet was resuspended in 1 mL ACD/saline. Next, 25 \( \mu \)g tropolone was added to 200 \( \mu \)Ci \textsuperscript{111}In chloride in a conical centrifuge tube and vortexed. The resuspended platelets were added to \textsuperscript{111}In-tropolone and allowed to incubate for 30 minutes at room temperature after being resuspended in an additional 1 mL ACD/saline solution. The suspension of labeled platelets was then centrifuged at 1600g for 10 minutes. The labeled platelets were resuspended in 2 mL ACD/saline solution. For each rat, a 1-mL fraction of the \textsuperscript{111}In-labeled platelets was withdrawn into a 10-mL syringe. The remaining fraction of labeled platelets was saved to determine harvesting efficiency by counting platelets with a Coulter counter. The radioactivity of labeled platelets and washings were measured with a gamma counter.

**Common Carotid Artery Thrombosis**

Male Wistar rats weighing between 250 and 300 g were initially anesthetized with 3% halothane for 3 to 5 minutes during intubation procedures. Rats were then mechanically ventilated and maintained on 1.5% halothane and a mixture of 70% nitrous oxide/30% oxygen. Femoral artery and venous catheters were inserted for the measurement of arterial blood pressure and blood gases and for fluid administration. The rats were placed on their backs, and the right CCA was exposed using a Zeiss operating microscope. The beam of a tunable argon dye laser (562 nm; peak power, 325 mW) was next focused to an intensity of approximately 40 W/cm\(^2\) onto the CCA via a 48-cm focal length spherical lens. The exposed CCA segment was submerged in a saline-filled surgical cavity. The \textsuperscript{111}In-tropolone–labeled platelets were next injected and allowed to circulate for 30 minutes before vascular injury. The photosensitizing dye rose bengal (40 mg/kg in 0.9% saline) was injected and the CCA irradiated for 10 minutes. Published data indicate that this protocol leads to a 50% to 75% stenosis of the irradiated carotid segment.\textsuperscript{14} Control rats (n=11) underwent all surgical procedures, were injected with labeled platelets, and were administered rose bengal but not irradiated. Five minutes before the rats were killed, 5 mL blood was withdrawn and saved for the determination of platelet counts and free iodine levels. Rats were then heparinized to prevent postmortem clot formation and were killed with an overdose of potassium chloride injected into the femoral artery at either 15 minutes or 3 hours.

### Measurement of Regional Radioactivity

Surfaces of carotid artery segments and brains were first sponged to remove adherent blood. Five-millimeter CCA segments were dissected from the left (contralateral) and right (irradiated) arterial beds and placed in gamma counter tubes. Brains were next divided into right (ipsilateral) and left (contralateral) hemispheres. Each hemisphere was then dissected into eight portions, including the frontal, frontoparietal, and parietal occipital cortices; striatum; hippocampus; thalamus; cerebellum; and brain stem. Tissue samples from other organs, including the heart, lungs, liver, spleen, kidneys, and leg muscle, were also harvested. Each specimen was immediately placed in a preweighed tube, weighed in a microbalance, and measured for radioactivity in a gamma counter. The spectrometer window of the gamma counter was adjusted to include the 171, 245, and 426 keV (sum) photopeaks of the \textsuperscript{111}In radioisotope. Ratios of radioactivity (normalized to tissue weight) between the right (R) and left (L) hemispheres were calculated. This R/L ratio was important because platelet emboli generated at the carotid thrombus should be most concentrated within the ipsilateral (R) hemisphere.

### Autoradiographic Visualization of Labeled Platelets

For these studies, brains were removed from the skull and frozen over liquid nitrogen. Frozen sections (10 \( \mu \)m) were cut in a cryostat and exposed to Kodak SB-5 x-ray film for 5 days. To determine the regional frequency of occurrence of platelet emboli, labeled platelet foci were counted using a dissecting microscope, and actual positions were noted on line drawings of the corresponding coronal brain sections. In this way, the representative regional areal density of emboli was determined for the ipsilateral and contralateral frontal, frontoparietal, and parietal occipital cortices; striatum; hippocampus; thalamus; cerebellum; and brain stem. For each rat, five brain levels corresponding to 12.2, 9.7, 6.2, and 2.7 mm anterior and 1.8 mm posterior to the interaural line were analyzed.\textsuperscript{24}

Physiological data, regional radioactivity ratios, and embolus counts were compared by one- or two-way
analysis of variance (ANOVA), and statistical significance was assessed by Scheffé's and Dunn's multiple comparison procedures. Radioactivity ratios among the three animal groups were also analyzed by the Kruskal-Wallis one-way analysis of variance by ranks.

Results

Physiological data including $P_{CO_2}$, $P_O_2$, and pH from control and thrombosed rats were within normal ranges (Table 1). No significant differences were documented between the individual animal groups. Blood pressure values were normal, and arterial hypotension was not observed during the study period.

Radioactivity Ratios

The R/L radioactivity ratios of carotid artery segments obtained from the 15-minute (n=3) and 3-hour (n=3) sham-operated control groups were not significantly different (P>.05); these control groups were therefore combined. Compared with control, the R/L carotid artery ratio was significantly elevated in the 15-minute (n=7) thrombosed rats (ANOVA, P<.05) (0.96±0.21 versus 33±12 [mean±SEM]). Although the R/L carotid ratio in the 3-hour rats (n=5) was also elevated (4.9±1.9), this value was not significantly different from control.

Fig 1 summarizes the regional radioactivity findings from control, 15-minute, and 3-hour thrombosed rats. Kruskal-Wallis analysis of variance by ranks of the different brain regions demonstrated significant differences among the three animal groups (P<.01). In control and 3-hour experimental rats, individual brain regions commonly demonstrated a R/L value of approximately 1, indicating no side-to-side differences in radioactivity. In contrast, the 15-minute thrombosed rats displayed regional R/L ratios which were significantly elevated compared to control (ANOVA, P<.05). Regions that displayed elevated ratios included the frontal cortex (1.62±0.22), frontoparietal cortex (1.72±0.24), and the hippocampus (2.32±0.68).

Autoradiographic Findings

In a second series of animals which underwent autoradiographic assessment, brain images from sham-operated 15-minute (n=3) and 3-hour (n=2) control rats demonstrated low areal densities of $^{111}$In-labeled platelets. When present, platelet foci were observed on the brain surfaces and appeared to correspond to large cerebral arteries or pial vessels. In contrast to control findings, the 15-minute thrombosed rats (n=11) demonstrated multiple foci of $^{111}$In-labeled platelets throughout the thrombosed hemisphere (Fig 2a through 2d). Focal regions of dense radioactivity appeared overlying the pial surface and brain parenchyma. At the level of the frontal cortex, platelet emboli appeared bilaterally (Fig 2a). In more posterior brain sections, platelet emboli were seen primarily ipsilateral to carotid thrombosis or restricted to the contralateral anterior cerebral artery territory (Fig 2b through 2d). Platelet emboli were not detected commonly within the cerebellum or brain stem. In the 3-hour thrombosed rats (n=6), the overall regional areal density of platelet emboli appeared to be reduced compared with the 15-minute rats. When present, labeled platelets were mainly associated with surface vessels.

Regional counts of platelet aggregates from autoradiographic images demonstrated significant increases in platelet density within specific brain regions from the 15-minute experimental rats compared with control (Fig 3). In the frontal cortex, increases in embolus density were detected bilaterally. Overall, the highest areal density of platelet emboli was detected in the ipsilateral cerebral cortex. By autoradiographic counts, the regional accumulation of labeled platelets subcortically was less than in the cortex. Platelet accumulation within the striatum and hippocampus was, however, significantly elevated compared to control (P<.01). In the cerebellum and brain stem, platelet density was commonly low. In contrast to these acute findings, the areal density of platelet emboli was dramatically reduced in the 3-hour thrombosed group. Significant elevations in platelet foci were detected only in the right frontal cortex at the 3-hour postirradiation period.

Platelet Distribution in Other Organs

Table 2 summarizes the distribution of $^{111}$In-labeled platelets in organs other than the brain. In control animals injected with rose bengal (15 minutes), radioactivity was highest in the blood, followed by the liver

**Table 1. Physiological Variables From Control and Thrombosed Rats**

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>$P_{CO_2}$ (mm Hg)</th>
<th>$P_O_2$ (mm Hg)</th>
<th>pH</th>
<th>MAP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=11)</td>
<td>38±1</td>
<td>137±8</td>
<td>7.40±0.01</td>
<td>105±4</td>
</tr>
<tr>
<td>Thrombosed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-minute (n=18)</td>
<td>37±1</td>
<td>140±3</td>
<td>7.42±0.01</td>
<td>98±2</td>
</tr>
<tr>
<td>3-hour (n=11)</td>
<td>39±1</td>
<td>136±8</td>
<td>7.40±0.01</td>
<td>102±3</td>
</tr>
</tbody>
</table>

MAP indicates mean arterial pressure. Values recorded immediately before the infusion of labeled platelets and are mean±SEM.
and spleen. In the 15-minute thrombosed rats, blood and liver contained the highest percentages of radioactivity. By 3 hours after thrombosis, levels of radioactivity had decreased in the blood and increased in the liver and spleen.

Discussion

This study provides direct evidence for the acute accumulation of $^{111}$In-labeled platelets in the brain after nonocclusive CCA thrombosis. The rose bengal photochemical method of producing large vessel thrombosis has been discussed in previous publications. Light-dye interactions featuring singlet molecular oxygen production lead to acute endothelial injury with subsequent platelet recruitment and aggregation. Although vascular injury and platelet aggregation in this model are induced by a light-dye insult, similar platelet responses to vascular injury may occur clinically. For example, atherosclerotic plaque hemorrhage and ulceration can lead to subsequent platelet thrombosis and embolization.

In addition, cerebral emboli of cardiac origin are reported to be almost exclusively platelet aggregates or thrombotic material from mural thrombi. In a recent experimental study of coronary artery luminal injury, reocclusion after successful thrombolysis with human tissue-type plasminogen activator was associated with a new thrombus composed mainly of aggregated platelets. Thus, the present findings of CCA thrombosis and subsequent platelet accumulation appear to incorporate thromboembolic processes relevant to clinical stroke.

Elevated levels of radioactivity were detected within the CCA segment 15 minutes after irradiation. These data are consistent with morphological findings describing a platelet-rich thrombus immediately after CCA irradiation. At 3 hours after CCA thrombosis, the degree of $^{111}$In-labeled platelet deposition within the carotid artery was significantly reduced compared to the 15-minute value. This finding is also consistent with histopathological data showing clearing of the platelet thrombus as early as 24 hours after nonocclusive CCA thrombosis. Spontaneous recanalization may be due to a relative absence of fibrin to stabilize the clot and the fact that carotid stenosis and complete occlusion was investigated. Alternatively, intraplatelet membrane binding via GP IIb-IIIa cross-links (mediated by fibrinogen and stabilized by thrombospondin) may not have proceeded to completion.

Examination of the 15-minute thrombosed rats demonstrated evidence for widespread platelet accumulation, primarily within the ipsilateral hemisphere. At the level of the frontal cortex, bilateral elevations in the areal density of platelet emboli were also detected. In earlier histopathological studies, a low frequency of contralateral infarction was reported after unilateral CCA thrombosis. Contralateral ischemic foci have
also been documented autoradiographically following CCA thrombosis.11 Taken together, these results indicate that platelet emboli from a carotid source may reach the contralateral hemisphere. Because contralateral platelet accumulation in this study was most intense within the territory of the anterior cerebral artery, emboli may have crossed via the anterior communicating artery.

In addition to the frontal cortex, other brain regions showed regional vulnerability to platelet accumulation. For example, significant increases in R/L radioactivity ratios were seen in the frontoparietal cortex and hippocampus. In the rat, the posterior cerebral artery is a branch of the internal carotid artery,31 a fact that explains the hippocampal vulnerability seen in this study. Differences in the magnitude of regional platelet accumulation presently reported using radioactivity ratios and autoradiographic images may be due to the fact that the two approaches are measuring different end points. Platelet aggregates as well as individual residual platelets from both hemispheres would contribute to the radioactivity ratios. However, regional counts of platelet emboli only involved platelet aggregates of sufficient size to generate a focus of radioactivity large enough to be differentiated from background.

Acute platelet aggregation and subsequent embolization after vascular thrombosis may participate in some of the previously reported neuronal and microvascular consequences of large cerebral artery thrombosis.9-14 Platelet emboli may occlude small vessels and cause focal ischemia and infarction.10,11,29 Platelet aggregates could also mechanically injure the vascular endothelium

FIG 3. Areal density of 111In-labeled platelet aggregates within selected brain regions from control (open bars), 15-minute thrombosed (hatched bars), and 3-hour thrombosed (solid bars) rats. Mean±SEM. *Significantly different from control values (ANOVA, P<.05).
TABLE 2. Distribution of Labeled Platelets in Other Organs

<table>
<thead>
<tr>
<th>Organs</th>
<th>Control (15-Minute) (n=3-7)</th>
<th>Experimental 15-Minute (n=7-8)</th>
<th>3-Hour (n=2-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>29.7±20.5</td>
<td>40.2±13.1</td>
<td>28.4±18.8</td>
</tr>
<tr>
<td>Heart</td>
<td>0.3±0.2</td>
<td>1.0±1.4</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>2.8±1.7</td>
<td>3.7±2.3</td>
<td>4.4±1.7</td>
</tr>
<tr>
<td>Liver</td>
<td>17.5±7.9</td>
<td>18.9±7.7</td>
<td>23.1±16.9</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.6±2.7</td>
<td>8.2±4.6</td>
<td>17.7±16.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.9±0.4</td>
<td>0.9±0.3</td>
<td>2.1±1.2</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.4±1.2</td>
<td>3.2±1.6</td>
<td>9.2±7.6</td>
</tr>
</tbody>
</table>

Values are mean±SEM and represent percentages of the total injected dose of 111In. Blood samples were taken immediately before potassium chloride injection.

by “dynamic stress,” leading to local blood-brain barrier disruption.32 As previously mentioned, plasma serotonin levels are significantly increased immediately after CCA thrombosis.14 Thus, blood serotonin or other vasoactive substances33-39 released at the thrombotic site and/or from platelet emboli would also be expected to contribute to the permeability and hemodynamic consequences of CCA thrombosis.11,12

In animal models of mechanically induced global and focal ischemia, platelet accumulation is a relatively late event and usually associated with severe parenchymal injury.19,20,40 In contrast, based on the present data, the first insult that the brain experiences after photochemically induced CCA thrombosis is exposure to platelet emboli that in some cases are of sufficient size to occlude distal vessels. The conclusion that platelet accumulation is a consequence of thromboembolic processes originating within the injured carotid artery is consistent with the acute nature and regional distribution within both hemispheres of the 111In-labeled platelet aggregates. It should also be mentioned that recent ultrastructural studies following CCA thrombosis have failed to provide evidence for overt structural damage to microvascular endothelium at sites of platelet accumulation.41 Thus, platelet aggregates appear to accumulate distally because of their size and inability to successfully pass through the microvascular circulation. Finally, because CCA thrombosis leads to microfocal regions of severe ischemia11 and acute neuronal injury,41 it is also possible that active substances released from microregions initially rendered ischemic by an embolus could participate in the pathogenesis of this injury. The functional integrity of the vascular endothelium or the release of local mediators may be very important in determining whether increased collateral circulation is facilitated or, on the other hand, whether spontaneous recanalization or infarction eventually occurs within the vicinity of the originally occluded site.

At 3 hours after CCA thrombosis, regional R/L ratios were not significantly elevated compared with control. Additionally, regional counts of individual emboli demonstrated only moderate increases restricted to the ipsilateral frontal cortex. Thus, in young healthy rats, the majority of trapped platelet emboli are spontaneously washed away by 3 hours. Transient platelet accumulation has also been recently documented in the baboon brain after the intracarotid infusion of 111In-labeled platelets.42 In situations where platelet emboli remain for extended periods, more permanent consequences, including severe perfusion deficits and tissue necrosis, would be expected.10,11,13

In summary, evidence for acute platelet accumulation within the rat brain has been presented using 111In-labeled platelets in conjunction with photochemically induced CCA thrombosis. Platelet accumulation within the injured carotid artery and thrombosed brain was largely transient. Whether similar degrees of spontaneous recanalization occur in thrombotic models that incorporate stroke risk factors, including age and chronic hypertension, merits future investigation. Although the pathophysiology of cerebrovascular disease is multifactorial, acute platelet embolization appears to be an important determinant of irreversible as well as reversible brain injury after photochemically induced CCA thrombosis.

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References

The authors have demonstrated several important findings: emboli from a single source may be bilateral, and emboli lodge only transiently in the distal circulation. These features of an experimental model of nonocclusive carotid thrombosis are, of course, highly relevant to transient ischemic attacks. The authors assume that emboli are caught in the distal arterial tree by a sieving mechanism and that emboli simply lodge in vessels smaller than the emboli themselves. This is certainly a valid hypothesis. However, one other possibility should be considered. Materials released by the platelets in the primary thrombosis may injure distal endothelium, which then becomes selectively adhesive for the passing emboli. Several articles in the literature suggest that one or more substances released by aggregating platelets are, in fact, toxic to endothelium. 1,3 Dietrich et al rely on work showing “normal” endothelial structure to rule out damage distal to the thrombosis and prior to the lodging of emboli. However, normal endothelial ultrastructure does not rule out functional abnormalities, such as reduction of local endothelium-derived relaxing factor levels that render the affected endothelium more able to “capture” passing activated platelets. 4 This possibility cannot be ruled out in the study of Dietrich et al. However, whether or not sieving is the sole explanation of the temporary lodging of platelets in the distal vessels, the bilateral embolization demonstrated by Dietrich et al is an important experimental finding.

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