The Ultrastructure of Photochemically Induced Thrombi With Embolization in a Rat Model

Nancy Futrell, MD; and Jeanne M. Riddle, PhD

Background and Purpose: Photochemical techniques, currently used in stroke and cancer research, produce endothelial damage and thrombosis. To further characterize these thrombi and to determine whether they embolize, we studied the ultrastructure of photochemically damaged carotid arteries and small vessels distal to the irradiated carotid.

Methods: The right carotid artery of 9 Wistar rats was irradiated with a laser (632 nm, 200 mW/cm², 15 minutes) after the injection of the photosensitizing dye Photofrin II, 12.5 mg/kg. There were 6 additional control rats: laser only, 2 rats; dye only, 2; carrier only (5% dextrose), 1; and normal, 1. The carotid artery and cerebral arterioles were studied using scanning and transmission electron microscopy.

Results: Endothelial damage was present in all irradiated carotid arteries, and consisted of exposure of the subendothelium and the formation of a nonocclusive thrombus. Although most cerebral arterioles were normal, 32 of these vessels contained peripheral blood elements, with platelet or red blood cell aggregates present in 15. The endothelium adjacent to the aggregates was intact. A few scattered endothelial cells had been lost in the carotid artery of control animals (compatible with normal cell turnover), with a few platelets adhering to the exposed subendothelium.

Conclusions: Aggregates of blood cells and platelets in cerebral vessels in the absence of endothelial denudation verifies embolism as the mechanism for cerebral vascular occlusion in this experimental model. The possibility of embolization distal to the site of photochemical irradiation has implications for potential applications of photochemistry for cancer treatment and the atherosclerosis of vascular malformations and/or aneurysms. (Stroke. 1993;24:1983-1992.)

Key Words • embolism • endothelium • erythrocytes • platelet aggregation • rats

Laser irradiation of blood vessels has been used to produce endothelial damage with resultant vascular occlusion. Addition of photosensitizing dyes (inducing photochemical reactions) facilitated the endothelial damage and vascular occlusion, making it possible to use lower laser intensity and thereby to decrease thermal damage to the vessel. During photochemical irradiation, one can visualize (in translucent vessels) emboli detaching from the developing thrombus and moving downstream.

Photochemistry is used to treat neoplasms. The mechanism of tumor destruction is probably related to occlusion of the tumors' vasculature. Photochemical occlusion or photoagulation also has been suggested as a treatment for vascular malformations and aneurysms. Experimentally, photochemistry has been used to induce platelet aggregation in pial arterioles and to produce cerebral necrosis by direct irradiation of the brain (either by irradiating through the intact skull or in conjunction with a craniotomy). Photochemically induced damage to the carotid artery of the rat has been used to produce a nonocclusive thrombus, which appears to embolize spontaneously, causing multiple cerebral infarcts. Although there is a significant body of evidence that points to embolism as the mechanism of infarct formation in this model, other mechanisms of tissue damage in the distal vasculature have been proposed.

To further characterize the local and distal vascular damage after photochemical irradiation of major vessels, with particular attention to evidence for embolization, we studied the surface features and ultrastructure of photochemically induced thrombi in the common carotid arteries of rats and of small vessels in the brain ipsilateral to the irradiated carotid artery, using both scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Materials and Methods

Fifteen male Wistar rats 2 to 4 months of age were used in this study. Anesthesia for all procedures was chloral hydrate (4.5 mg%, 10 mg/kg) administered intraperitoneally and supplemented as needed. All intravenous injections were given via the internal jugular vein. The right common carotid artery was surgically exposed in 9 rats and a 5-mm length of vessel was irradiated for 20 minutes with a red laser (632 nm, intensity 200 mW/cm²) 30 minutes after an intravenous injection of a photosensitizing agent (Photofrin II, a mixture of hematoporphyrin ethers, 12.5 mg/kg). The

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laser (Coherent, Innova 70, tandem argon-dye laser system) beam was delivered to the tissue through a fiberoptic cable, with the output placed 5 cm from the tissue. The stability of the laser intensity was documented by measurements taken immediately before and after the irradiation. Protocols for the experimental and control animals are summarized (Table 1). Anesthetized animals were killed by transthoracic perfusion of 400 cc of normal saline followed by 800 cc of a fixative (3% glutaraldehyde in 0.1 mol/L cacodylate buffer22), with a perfusion pressure of 100 mm Hg that was monitored with a blood pressure manometer. The brains were removed from the skull after 2 additional hours of immersion fixation, and the whole brain was placed into fresh fixative for at least 24 more hours. The right common carotid artery was removed immediately and immersed in fixative for 24 hours.

The right carotid artery of each experimental and control rat, along with the left carotid artery from 2 experimental rats, was cut longitudinally and postfixed with cacodylate-buffered 1% osmium for 2 hours at room temperature. The samples were then pinned onto a plastic plug with their interiors exposed, dehydrated with progressive concentrations of ethanol, and dried using the CO₂ critical point drying method.23 The dried samples were removed from the plastic plug and each specimen was mounted onto a separate aluminum stub using Duco Cement (Devon Corp, Wooddale, Ill). Each sample was sputter-coated with a thin layer of gold-palladium metal and viewed with the scanning electron microscope operated in the secondary electron mode (20 kV, 0° tilt).

Brain sections studied by TEM were fixed in 0.1 mol/L cacodylate-glutaraldehyde, postfixed with 0.1 mol/L cacodylate-buffered 1% osmium, dehydrated in progressing concentrations of ethanol and embedded in Araldite. Ultrathin sections of the area of interest were produced, mounted on grids, double stained with uranyl acetate as well as lead citrate and viewed with a Philips 201 transmission electron microscope operated at 50 Kv.

Results
Control Animals: Carotid Arteries Viewed by SEM

The common carotid arteries of the control rats (examined after perfusion fixation of a normal artery, dextrose infusion, or exposure to Photofrin II only or irradiation only) as well as the left carotid arteries from two experimental animals (which would be exposed to any substances released into the systemic circulation during irradiation) showed a similar endothelial morphology (Table 2). The endothelial lining was mostly intact, with distinct borders present between adjacent endothelial cells. Individual endothelial cells had typical surface microvilli, with only a rare cell showing either a rounded, bulging nucleus or a small cytoplasmic bleb (Fig 1). Evidence of normal cell turnover was observed, with the occasional finding of either a single endothelial cell or several endothelial cells being lost, thereby exposing the underlying subendothelial fibers. Activated platelets frequently adhered at these sites. Leukocytes, however, were absent.

Experimental Animals: Carotid Arteries Viewed by SEM

The carotid arteries of the 9 experimental animals killed at 0.5 hour to 24 hours after irradiation uniformly showed extensively damaged endothelium, particularly
in the area of the thrombus. Some endothelial cells were only partially detached, so that the borders between neighboring cells were disrupted, while many other endothelial cells were completely lost, thereby exposing large amounts of subendothelium. Activated and aggregated platelets frequently adhered to the exposed subendothelial fibers (Fig 2). A thrombus was present in the lumen of all 9 of these vessels (Table 2). The length of each thrombus based on time after irradiation (0.5, 4.0, and 24 hours) was not significantly different ($P>0.05$). The majority of the thrombi (8 of 9) were nonocclusive, sheet-like, and spread over all or most of the interior of the vessel (Fig 3). A single 1 of the 9 thrombi was shaped like a cylinder, suggesting the possibility of an occlusive thrombus. Each thrombus was composed mostly of activated and aggregated blood platelets (Fig 4). In some of the thrombi, however, there was an admixture of varying numbers of erythrocytes, leukocytes, and occasional areas containing fibrin (Fig 5). In some instances the erythrocytes were collapsed and showed a ruptured membrane (Fig 6). The only alteration with time was an increase in the number of leukocytes.

**Experimental Animals: Cerebral Vessels Examined by SEM**

The interior of a total of 37 cerebral vessels cut in various planes in the brain slices were examined by SEM (Table 3). Peripheral blood elements were present in all but 5 of these blood vessels. Platelets, single or aggregated (Fig 7), were seen in 8 of the 37 vessels (22%); erythrocytes were present in 20 of 37 vessels (54%), and activated leukocytes were adjacent to the endothelium of 4 of these 37 vessels (11%) (Fig 8). In all instances, the endothelial lining next to the peripheral blood elements or occluding thrombus appeared to be intact, with no gaps visible between adjacent endothelial cells.

**Carotid and Cerebral Vessels Examined by TEM**

Two carotid arteries containing thrombi were studied using TEM.

**Carotid endothelium.** At 0.5 hours after irradiation, the cytoplasmic border of endothelial cells was identifiable and contacted by hemolyzing erythrocytes and activated, degranulating platelets (Fig 9). In contrast, at 24 hours after irradiation, the endothelium was destroyed so that no cell features (cytoplasmic boundaries or nuclei) could be recognized. In addition, the und-

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**TABLE 2. Characteristics of Thrombi Present in the Lumen of the Carotid Arteries (SEM)**

<table>
<thead>
<tr>
<th></th>
<th>0.5 Hour</th>
<th>4 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length, mm</td>
<td>2.4±0.3</td>
<td>2.3±0.5</td>
<td>1.5±1.0</td>
</tr>
<tr>
<td>Form</td>
<td>Cylindrical</td>
<td>1 of 3</td>
<td>0 of 3</td>
</tr>
<tr>
<td></td>
<td>Sheet-like</td>
<td>2 of 3</td>
<td>3 of 3</td>
</tr>
<tr>
<td>Components</td>
<td>Platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Many</td>
<td>3 of 3</td>
<td>3 of 3</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Many</td>
<td>0 of 3</td>
<td>0 of 3</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>3 of 3</td>
<td>0 of 3</td>
</tr>
<tr>
<td></td>
<td>Few</td>
<td>0 of 3</td>
<td>3 of 3</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0 of 3</td>
<td>0 of 3</td>
</tr>
<tr>
<td>Leukocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Many</td>
<td>0 of 3</td>
<td>1 of 3</td>
</tr>
<tr>
<td></td>
<td>Few</td>
<td>0 of 3</td>
<td>2 of 3</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>3 of 3</td>
<td>0 of 3</td>
</tr>
<tr>
<td>Fibrin</td>
<td>Small areas</td>
<td>2 of 3</td>
<td>1 of 3</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>1 of 3</td>
<td>2 of 3</td>
</tr>
</tbody>
</table>

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**Fig 1.** Scanning electron micrograph of a carotid artery from a control rat, dye only. Distinct borders were present between endothelial cells, which showed surface microvilli, an occasional bulging nucleus, and rare cytoplasmic blebs. Bar=10 μm.
lying smooth muscle cells were altered. They showed a decrease in size and an increase in electron denseness.

Composition of thrombus. The thrombus that formed 0.5 hours after irradiation was mainly composed of platelets, but also contained numerous erythrocytes, a rare neutrophil, and a small amount of fibrous material, probably fibrin. These findings corroborate our SEM observations (Table 2). At the ultrastructural level, we also observed erythrocytes in various stages of hemolysis. In these cases, the background was filled with a moderately electron-dense material, possibly released hemoglobin (Fig 9). The mass of aggregated platelets showed platelets in various stages of degranulation and disintegration. The rare neutrophils present contained pyknotic nuclear lobes and cytoplasmic lipid droplets.

The thrombus present 24 hours after irradiation was composed again mostly of an aggregated platelet mass, but it contained more neutrophils than were present at 0.5 hour after irradiation. Many of the platelets had degranulated and some were disintegrating. The admixed neutrophils showed cytoplasmic lipid droplets and a decreased number of cytoplasmic granules. Some of the neutrophils were disintegrating and had breaks in their plasma membrane.

Cerebral vessels. Semiserial sections through two pial vessels occluded by thrombotic material (emboli) were also examined by TEM 4 hours after irradiation. In both of these vessels, the endothelium was normal. The mass of aggregated platelets in the lumen of these vessels was not adherent to the endothelium or subendothelium as one would expect if a thrombus had formed in response to damaged endothelium. The aggregated platelet mass contained activated granulated platelets, partially de-
granulated platelets with an intact plasma membrane, and degranulated disintegrating platelets (Fig 10).

Discussion

Although "platelet-fibrin" emboli have been observed with direct ophthalmoscopy in the retina of humans with amaurosis fugax or retinal infarction, there is a paucity of pathological data on the composition of these emboli. In rare instances where retinal emboli were carefully examined, they were composed of platelet aggregates without fibrin. The photochemically induced thrombi in the carotid artery in our study often contained platelet aggregates that were loosely adherent to adjacent thrombotic material, without fibrin stabilization of the nonocclusive thrombus. Fibrin content was present at all sacrifice intervals (4 of 9 thrombi), and did not increase as the thrombus matured. "Platelet-fibrin" emboli may be a misnomer, as less stable platelet aggregates without fibrin may be more prone to embolization.

The severity of endothelial and smooth muscle cell damage produced by the low laser intensity used in this experiment is remarkable and probably results from the high tissue penetration of red light. The low laser intensity required for this experiment makes it possible to use a 5.9-mW helium-neon laser (CR Laser, Auburn, Calif), reducing the cost of the experiment (N. Futrell, unpublished observations). Carotid thrombi induced by this low laser intensity consisted of an admixture of platelets (activated, aggregated, and disintegrating), erythrocytes, and leukocytes that were often (44%) contained within a fibrous network, presumably fibrin.
In contrast, nonocclusive thrombi produced in response to irradiation with an argon laser and Rose bengal dye were composed of platelet aggregates and groups of erythrocytes interspersed within a matrix of "coagulum" (probably caused by the intense heat used in this model) with no identifiable fibrin strands.10

Our study produced significant evidence that the blood deposits identified in cerebral vessels in this model were the result of embolization, as there was no endothelial denudation in cerebral vessels, including sites where peripheral blood elements and platelet aggregates were adjacent to the vessel's surface. The finding of occluded vessels with normal endothelium has long been considered definitive evidence of embolism.30 Although there have been examples of platelets adhering to nondisrupted endothelium, in these studies the platelets did adhere to the endothelium.31,32 While in our study the platelet aggregates did not adhere to the endothelium.

Povlishock and Rosenblum31 have documented platelet aggregation preceding photochemically induced endothelial denudation, but this occurs only at vascular sites directly exposed to laser illumination after the injection of photosensitizing dyes. This was associated with endothelial vacuolation in the absence of endothelial denudation. If the irradiation persists, platelet aggregates could be released from the main thrombus and produce distal emboli, but their experimentdocuments clearly that platelets that were directly injured were never seen to "escape from the vicinity of the laser beam and deposit themselves elsewhere," suggesting that vascular occlusion distal to photochemical irradiation is produced by embolization rather than by direct damage to platelets. Their study also demonstrates that fixation occasionally produces small numbers of discoid platelets with no degranulation in control vessels, but never significant platelet aggregates with degranulated platelets as shown in Fig 10. The platelet aggregates in areas of vacuolated, not denuded, endothelium were not significantly degranulated; degranulation occurred only when the laser irradiation was continued for 60 to 120 seconds after the onset of the aggregation.31 Thus, in the absence of disruption of the endothelium and the absence of direct laser illumination, the presence of platelet aggregates with degranulation is evidence of embolization from the carotid artery, the site of irradiation. This is further substantiated by the similarity of the ultrastructure of the blood deposits in the carotid and the distal brain vessels, suggesting that some severely damaged hematologic cells detected in the brain vasculature were exposed to laser irradiation, originating as part of the nonocclusive carotid thrombus.

Although the initial studies of cerebral infarction after photochemical induction of a nonocclusive carotid thrombus produced strong evidence that the mechanism of infarction was embolism,15,17 a variation of this model demonstrated a bilateral increase in vesicular transport of horseradish peroxidase by cerebral endothelial cells, postulated to be caused by "thrombogenically induced" vasoactive substances.18,19 Since no measures were taken to remove platelet emboli from that experimental system, and since other experiments have documented

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### Table 3. Peripheral Blood Elements Present in the Lumen of the Cerebral Vessels

<table>
<thead>
<tr>
<th></th>
<th>0.5 Hour</th>
<th>4 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>0 of 6 (0%)</td>
<td>1 of 3 (33%)</td>
<td>4 of 28 (14%)</td>
</tr>
<tr>
<td>Aggregated</td>
<td>1 of 6 (17%)</td>
<td>0 of 3 (0%)</td>
<td>0 of 28 (0%)</td>
</tr>
<tr>
<td><strong>Erythrocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>2 of 6 (33%)</td>
<td>0 of 3 (0%)</td>
<td>8 of 28 (29%)</td>
</tr>
<tr>
<td>Aggregated</td>
<td>1 of 6 (17%)</td>
<td>0 of 3 (0%)</td>
<td>9 of 28 (32%)</td>
</tr>
<tr>
<td><strong>Leukocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>1 of 6 (17%)</td>
<td>0 of 3 (0%)</td>
<td>0 of 28 (0%)</td>
</tr>
<tr>
<td>Aggregated</td>
<td>0 of 6 (0%)</td>
<td>0 of 3 (0%)</td>
<td>3 of 28 (11%)</td>
</tr>
</tbody>
</table>

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Fig 6. Scanning electron micrograph of the carotid artery from an experimental rat killed at 30 to 40 minutes. Some erythrocytes were intact in the irradiated area (open arrow); others had a ruptured membrane (solid arrow). Bar=10 µm.
bilateral decreases of the blood brain barrier with bilateral cerebral edema after the unilateral injection of artificial emboli (microspheres). Platelet emboli could be postulated as the etiology of the reported changes.

Our ultrastructural findings contribute to the evidence that platelet emboli are produced by photochemical techniques.

The observation of activated white blood cells (WBCs) in contact with normal endothelium in six cerebral blood vessels 24 hours after irradiation demonstrates that these cells have not damaged the surface of the vessel. Lymphocytes are known to traverse normal cerebral vessels and enter brain tissue, possibly part of an immune surveillance mechanism. WBCs enter cerebral infarcts within 12 hours, and are most frequent in the periphery of infarcts. One of these events may explain the presence of occasional WBCs in contact with normal endothelium in cerebral vessels.

Individual and aggregated red blood cells (RBCs) were seen in the carotid thrombus and were common in cerebral vessels in experimental animals. These were not associated with fibrin. Since the RBCs were not occluding cerebral vessels, we found no evidence that these aggregates might produce cerebral infarction. Some of the RBCs were hemolyzed, suggesting ADP could have been released. Although ADP can include platelet aggregation, direct injection of ADP in the carotid artery does not produce cerebral infarction.

Minimal detached endothelial cells exposing subendothelium with subsequent platelet adhesion and aggregation were seen in the common carotid artery with similar frequency and severity in all control rats, suggesting that...
this condition was probably related to normal turnover of the vascular endothelium. Apparently these areas of endothelial damage are generally too small to produce platelet aggregates that may embolize, as small cerebral infarcts have not been seen in our control animals.\(^{15-17}\) Spontaneous cerebral embolism occurs extremely rarely, if ever, in young rats. In our entire experience with over 200 young (2 to 4 months of age) Wistar and Fisher rats used as controls or in acute stroke (30 minutes to 7 days) experiments, we have found only a single lesion in the hippocampus that was consistent with an old (4 week or more) small cerebral infarct, which could have represented spontaneous embolism (N. Futrell, unpublished observations). The techniques used in this study did not reveal any endothelial damage attributable to the injection of Photofrin II.

In conclusion, the surface features and ultrastructure of the carotid and brain thrombi and the integrity of the endothelium of cerebral arterioles after photochemical irradiation of the carotid artery in the rat provide additional evidence that the mechanism of stroke production in this model is embolism. The possibility of a chemical mediator being released from the carotid thrombus and affecting the reactivity of cerebral vessels is yet to be demonstrated. Since emboli may be produced if large vessels are included in the field of irradiation with photochemical therapy for neoplasms or vascular malformations, the possibility of embolic infarction of tissue distal to such vessels should be considered as part of the risk-to-benefit ratio of such treatment.

Acknowledgments

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References

Futrell and Riddle

Ultrastructure of Emboli 1991

Editorial Comment

Futrell and Riddle have provided morphological evidence for the production of emboli after photodynamic injury of the carotid artery in the rat. This demonstration is part of the documentation required to prove that the infarcts produced by the model are embolic in origin. As the authors point out, regional hypoxia—and I would add regional injury to endothelium—can result from emboli, so emboli rather than toxic substances might account for blood-brain barrier breakdown reported after proximal production of thrombi by the photodynamic technique.

Futrell and Riddle refer to prior studies by Rosenblum and coworkers. Only two of these studies are cited. Unfortunately, reproducible discussion of these papers is somewhat confusing. Contrary to the impression given by Futrell and Riddle, Rosenblum et al frequently see emboli leaving photodynamically induced thrombi. Moreover, using our own laser/dye model, we have reported that after production of a thrombus there is often preferential “capture” of platelets at a minimally injured downstream site. The captured platelets represent either emboli or an accumulation of platelets activated at the upstream thrombus. It was in the latter setting that we failed to see emboli actually leaving the upstream site and adhering downstream. This does not prove that individual activated platelets rather than emboli are captured at the downstream site. The failure to see the emboli could simply be because these emboli were too small to see. Large emboli can be seen, but they pass out of the field entirely. Contrary to the impression given by Futrell and Riddle, our failure to see small emboli was not used to prove that platelets...
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