Fibrin Content of Carotid Thrombi Alters the Production of Embolic Stroke in the Rat

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**Background and Purpose** Mechanical denudation of the endothelium of the carotid artery in animals produces a nonocclusive thrombus, but the brains of these animals have not been examined for the presence of embolic stroke.

**Methods** The endothelium of the right carotid artery of 16 Wistar rats was denuded using a balloon catheter. Phosphotungstic acid hematoxylin (PTAH) staining and scanning electron micrographs of the nonocclusive thrombi in the carotid arteries were compared with those produced by photochemical methods, and brains were examined for infarcts.

**Results** Although nonocclusive thrombi were present in the carotid arteries of 4 of 4 rats killed at 4 hours and in 8 of 12 killed at 24 hours, neither cerebral infarcts nor emboli were seen in the 14 brains evaluated by light microscopy. PTAH demonstrated a high fibrin content in the thrombus produced by the endothelial denudation, with almost no fibrin seen in photochemically induced thrombi. Scanning electron microscopy confirmed dense networks of fibrin in the thrombi produced by balloon denudation.

**Conclusions** The composition of a nonocclusive thrombus may determine the embolic potential of this thrombus. A low fibrin content in a nonocclusive platelet thrombus may enhance the embolic potential. This suggests that platelet inhibition may also be indicated in patients with carotid artery disease who are being treated with anticoagulant.

**Materials and Methods**

Carotid denudation was performed in 16 male Wistar rats 2 to 4 months of age with the method described by Clowes et al. Each rat was anesthetized with chloral hydrate (4.5 mg%, 10 mg/kg) administered intraperitoneally and supplemented as needed. Animal temperature was monitored by a rectal probe and maintained between 37°C and 38°C by a heating pad.

Balloon catheters were made from a 15-cm length of polyethylene tubing (PE-10). One end of the polyethylene tubing was inserted a distance of 1 cm into a 2-cm piece of silicone elastomer tubing. This connection was secured with silicone elastomer glue, and the tip of the tubing was occluded with the glue. Water was injected into the PE-10 tubing via a 1-mL syringe with a 26-gauge needle, resulting in ballooning of the silicone elastomer tip; catheters were checked for absence of fluid leakage before use.

The right common carotid artery of anesthetized rats was surgically exposed and dissected distally to the bifurcation under the operating microscope. The external carotid artery was dissected and ligated with a 4-0 silk suture approximately 7 mm distal to the bifurcation. The internal carotid artery was dissected, and a 4-0 suture was placed around the common carotid artery, the internal carotid artery just distal to the bifurcation. To prevent bleeding, tension was placed on each of these sutures by clamping a hemostat to the sutures. With microscissors a small incision was made in the external carotid artery between the ligature and the bifurcation. The silicone elastomer tip of the catheter was inserted, and the sutures on the external and common carotid artery were loosened. The catheter was advanced approximately 2 cm proximally into the internal carotid artery. Saline was inserted into the catheter until a slight bulge could be seen in the carotid artery at the level of the silicone elastomer portion of the catheter. The catheter was pulled toward the bifurcation and the balloon deflated. The catheter was advanced proximally, inflated, and the entire procedure was repeated for a total of three passes. As the catheter was removed, the external carotid artery was ligated just distal to the bifurcation, and the sutures were removed from the internal and common carotid arteries. The neck wound was then closed.

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Fra 1. Top left, Phosphotungstic acid hematoxylin (PTAH) staining of cross section of carotid artery of a rat after photochemical production of a nonocclusive thrombus. Lack of blue strands suggests the absence of fibrin. Bar=100 μm. Top right, PTAH staining of a similar cross section after balloon denudation of carotid artery of a rat. Circular strands of fibrin, staining blue, encircle and encase most of the nonocclusive thrombus. Bar=100 μm. Bottom left, Higher power of a PTAH stain on a nonocclusive thrombus produced by balloon denudation. The blue fibrin strands encase platelet aggregates. Bar=50 μm.

Four animals were killed at 4 hours, 2 for light microscopy of the carotid artery and the brain and 2 for electron microscopy of the carotid artery. The other 12 rats were killed at 24 hours. All rats were killed under intraperitoneal chloral hydrate anesthesia by transcardiac perfusion with 300 mL of saline followed by 300 mL of fixative at a pressure of 100 mm Hg controlled by a blood pressure manometer. The fixative for light microscopy was 10% neutral buffered formalin and for electron microscopy was 3% glutaraldehyde cacodylate buffer. A 7- to 8-mm segment of the right carotid artery (including the area of irradiation) was removed and allowed to soak in fixative for 24 hours. For light microscopy the arteries were processed and embedded in paraffin. The heads of the 14 rats perfused with formalin were immersed in the fixative, and the brains were removed at least 24 hours later and placed in fixative. Twenty-four hours later the brains were cut into five 3-mm coronal pieces, processed, and embedded in paraffin.

Brain tissues were prepared for light microscopic evaluation by cutting four coronal sections (two per slide) with a microtome, 7 mm thick, at intervals of 1 mm. Brain sections from each level were stained with hematoxylin and eosin and Nissl stains and evaluated for cerebral infarcts. Cross sections of the carotid artery were cut 4 mm thick, and 24 serial sections (on four slides) were taken at intervals of 0.5 mm. One slide was stained with hematoxylin and eosin and one with phosphotungstic acid hematoxylin (PTAH); the unstained slides were saved.

For electron microscopy the two carotid arteries were opened longitudinally. They were then postfixed in 1% buffered osmium for 2 hours, dehydrated through progressive concentrations of ethanol, and dried using the critical point drying method. The interior of the vessels was sputter coated with metal and examined in the scanning electron microscope operated in its secondary electron imaging mode.

Results

Four hours after the endothelial denudation, a nonocclusive thrombus was seen by light microscopy in both of the carotid arteries stained with hematoxylin and eosin. There were no emboli seen in the brains.

There were no cerebral infarcts seen in any of the 12 brains examined 24 hours after the endothelial denudation, the time at which multiple infarcts are seen with the photochemical model of nonocclusive carotid thrombus production. No emboli were seen in cerebral vessels. Eight of the 12 carotid arteries had residual nonocclusive thrombi, 1 had an occlusive thrombus, 1 had no residual thrombus, and 2 samples were lost during processing. There was also damage to smooth muscle cells and some disruption to the internal elastic laminae, as described by others with this model.

To determine why the nonocclusive thrombi in the carotid artery were not embolizing, we reexamined these vessels. Because the thrombi appeared to have more strandlike structures than we had seen in our photochemically induced thrombi, we performed PTAH stains on contiguous sections of 6 of these arteries and on slides of 6 nonocclusive thrombi from previous photochemical experiments. Five of the 6 photochemical thrombi contained no fibrin by PTAH (Fig 1, top left panel), and 1 contained only a few fine
variability of clinical presentation in patients with un-
was unexpected, particularly since we were successful in
thrombi in coronary arteries has been correlated with
retinal emboli. Variable fibrin content in platelet-rich
et al, who reported a lack of fibrin in documented
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vessel, has been called "platelet-fibrin," but on micro-
scopic examination McBrien et al found a "congluti-
sectioned longitudinally and opened. The nonocclusive nature of the thrombus is apparent. Bar=100 μm. B, Higher power of the thrombus produced by balloon denudation is remarkable for strands of fibrin with red blood cells (large arrow) and few platelets (small arrow). Bar=10 μm. C, An area adjacent to the main thrombus shows exposed endothelium with scattered adherent red blood cells (large arrow) and platelets (small arrow). Bar=10 μm. D, The nonocclusive thrombus produced by photochemistry contains an occasional red blood cell (arrow) and a large aggregate of platelets with no fibrin strands. Bar=10 μm.

PTAH-positive strands. There were prominent strands of fibrin in 5 of the 6 nonocclusive thrombi produced by balloon denudation (Fig 1, top right and bottom left panels).

Two carotid arteries were examined by scanning electron microscopy. The lumen of each vessel contained a nonoccluding thrombus (Fig 2A) with varying quantities of erythrocytes and platelets, frequently enmeshed in fibrin (Fig 2B). In other areas the endothelial surface of this vessel was disrupted (Fig 2B), with a few adherent erythrocytes as well as adherent, activated blood platelets. By comparison, scanning electron micrographs of photochemically induced nonocclusive thrombi were most prominently composed of platelet aggregates (Fig 2C), with rare areas of fine fibrin strands (Fig 2D).

Discussion
The results of this experiment are directly related to embolic stroke in humans. For decades there has been controversy regarding the role of embolism in the pathogenesis of transient ischemic attacks and small ischemic strokes, even though emboly of various types are observed in the retina of patients with carotid artery disease. Study of the pathology of retinal emboli has been limited because of difficulty in getting autopsy specimens of eyes containing emboli. David et al were the first to identify the bright yellow embolus described by Hollenhorst as cholesterol. These emboli are often asymptomatic, since they may not occlude the vessel. The whitish embolus, which generally occludes the vessel, has been called "platelet-fibrin," but on microscopic examination McBrien et al found a "conglutination of platelets" with an occasional leukocyte and a small quantity of lipid, but no fibrin.

The lack of embolic stroke after balloon denudation was unexpected, particularly since we were successful in producing a nonocclusive thrombus. The presence of a high fibrin content in thrombi that did not produce embolic stroke and the propensity of photochemically induced thrombi to embolize suggest that platelet thrombi are more prone to embolize if not stabilized by fibrin. This fits the observations in humans by McBrien et al, who reported a lack of fibrin in documented retinal emboli. Variable fibrin content in platelet-rich thrombi in coronary arteries has been correlated with variability of clinical presentation in patients with unstable angina and myocardial infarction.

The ability of a nonocclusive thrombus or of an aggregate of blood cells to produce embolic cerebral infarcts depends on three factors: (1) the friability of the nonocclusive thrombus, allowing fragments of the thrombus to break loose and embolize; (2) the stability of the fragments, allowing them to remain in a distal vessel for a period long enough to produce cerebral ischemia and/or infarction; and (3) the size of the embolus. With the development of embolus detection with ultrasound by use of transcranial Doppler, cerebral embolism can now be monitored in patients. This technique has revealed a high frequency of asymptomatic embolism, suggesting that either the emboli are so fragile that they do not remain in the vessel or that they are so small that symptoms are not produced.

Experimentally, Fieschi et al produced platelet aggregates and emboli in the rabbit by the infusion of ADP into the carotid artery. Although transient cerebral ischemia was produced, these emboli were not stable enough to produce cerebral infarction. Embolic stroke has been produced by producing mechanical damage to a translucent vessel in the ear of rabbits, producing a platelet thrombus that is removed and injected into the carotid artery. If that thrombus is allowed to "mature" for 24 to 72 hours before removal and injection, embolic stroke is produced in most animals. This maturation time apparently allows for some process of stabilization, since cerebral infarction is not produced if the thrombus is injected into the carotid artery 3 hours after the mechanical damage to the endothelium. In the present experiment we presume that the fibrin stabilized the nonocclusive thrombus to the extent that a significant degree of embolization failed to occur, based on the lack of either infarcts or emboli on careful inspection of the brains.

Specimens of carotid artery plaques removed at endarterectomy reveal mixed thrombi, including platelet aggregates and fibrin-stabilized clot. There are reports of cases of recurrent amaurosis fugax that do not stop with heparin but do stop after carotid endarterectomy. Based on our findings, it is possible that in the face of active platelet aggregation, the inhibition of fibrin formation without concomitant inhibition of platelet aggregation could increase platelet embolization in some patients. The study of combined therapy with platelet inhibitors and anticoagulants would be a rational approach to the prevention of embolization from atherosclerotic plaques.

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References
The composition of arterial thrombus has important implications. First, the most effective mechanism of prevention of the formation of the thrombus may depend on its composition. A thrombus composed mostly of platelets would be prevented most effectively by drugs that inhibit platelet aggregation, whereas a thrombus with high fibrin content might require conventional anticoagulants. Second, a thrombus with high fibrin content might be amenable to dissolution by fibrinolytic agents, whereas a pure platelet thrombus may not be responsive to such intervention. There is a third important implication of the composition of thrombus raised by the study of Halvorsen et al in the accompanying article. These authors found that arterial thrombus formed by mechanical denudation of the carotid artery had a high fibrin content and was not associated with a high incidence of embolization. This is unlike what happens with photocoagulation, which causes pure platelet thrombi that are associated with a high incidence of embolization. It will be interesting to determine whether this experience can be translated to clinical situations. The composition of the arterial thrombus may explain why embolization may occur in some cases and not in others. If this is true, the determination of the composition of the thrombus may provide an important basis for determining the most appropriate intervention.

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