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AJNR Am J Neuroradiol 2009, 30 (3) 469-472

doi: <https://doi.org/10.3174/ajnr.A1410>

<http://www.ajnr.org/content/30/3/469>

This information is current as of August 15, 2025.

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Vulnerable Plaque in a Swine Model of Carotid Atherosclerosis

BACKGROUND AND PURPOSE: Distal embolism and acute thrombosis due to rupture of a vulnerable atherosclerotic plaque are the common mechanisms of stroke in patients with carotid disease. The purpose of this study was to develop the first animal model of vulnerable carotid atherosclerotic plaque.

MATERIALS AND METHODS: Carotid atherosclerotic models were created in 12 Yucatan minipigs by using a combination of partial ligation and high cholesterol diet. Retia mirabilia from these animals were examined histopathologically to identify distal embolism. The association of distal embolism with advanced atherosclerosis and a thin fibrous cap was analyzed by using the Fisher exact test.

RESULTS: Typical features of vulnerable plaques, including a thin fibrous cap, necrotic core, and intraplaque hemorrhage, were observed in this swine model of carotid atherosclerosis. Distal embolism was detected in retia mirabilia supplied by 7 of 10 carotid arteries with advanced atherosclerotic plaques, compared with 3 of 14 carotid arteries without advanced plaque ($P < .05$).

CONCLUSIONS: This swine model of carotid atherosclerosis contains the salient features of vulnerable plaques, including plaque rupture and distal embolism.

Not all symptomatic carotid atherosclerotic plaques have a high grade of stenosis. A lesion with $<70\%$ stenosis may contain unstable plaque, which can rupture, expose the highly thrombogenic necrotic core of the atherosclerotic plaque to the blood stream, cause acute thrombosis, or send distal emboli, resulting in cerebral infarctions. An atherosclerotic plaque that undergoes transformation from a stable and asymptomatic lesion to one that causes ischemic symptoms is considered a vulnerable plaque. Recent studies have shown promise of using morphologic features of vulnerable carotid plaques to identify patients who are at increased risk of ischemic stroke.¹⁻³

Various mouse models of plaque rupture have been created in Apolipoprotein E or low-attenuation lipoprotein-receptor knockout mice, but whether they represent the same type of plaque rupture is still debated.⁴⁻⁷ The advantages of the mouse model include the low cost and the availability of a transgenic technique to dissect the relevant pathologic processes. The size of the vessel and hemodynamic factors are, however, very different from those in humans, making it difficult to generalize the results to patients. In contrast, swine have blood pressure and heart rates similar to those in humans, and their vessel sizes also closely match those in humans. We have recently created a new swine model of carotid atherosclerosis based on

combined dietary hyperlipidemia and partial surgical ligation.⁸ Morphologic features of advanced human atherosclerosis, such as necrosis, ulceration, calcification, and intraplaque hemorrhage, were observed in this model. Because artery-to-artery embolism is one of the major mechanisms of stroke in patients with carotid atherosclerosis,⁹⁻¹¹ we now examined distal embolism in this animal model to confirm the presence of vulnerable carotid atherosclerotic plaques. Because swine do not have cervical internal carotid arteries but derive their cerebral blood supply from the rete mirabile, we looked for distal emboli in this network of 300- to 500- μm diameter arterioles at the end of ascending pharyngeal arteries in the skull base, which serve as effective filters of emboli from carotid arteries.

Materials and Methods

Animal Experiments

All animal experiments were carried out in accordance with policies set by the National Institutes of Health guidelines and the Animal Research Committee at UCLA. Twelve male Yucatan minipigs (S&S Farms, Ranchita, Calif), weighing 20–30 kg, were used to develop carotid atherosclerosis by using the combination of partial surgical ligation and hyperlipidemia, as described previously.⁸ All animals were fed a high fat and high cholesterol diet containing 4% cholesterol, 20% saturated fat, and 1.5% supplemental choline (Test Diet; Purina, St. Louis, Mo) 2 weeks before surgery and were maintained on this diet for at least 3 months to induce hypercholesterolemia.

Seventeen carotid arteries were partially ligated to create approximately 80% stenosis, and sham operations were performed on the other 7 arteries. The surgical details have been previously described.⁸ The animals were allowed to recover after surgery and live for 3 months before sacrifice. 3D rotational angiograms of carotid arteries were obtained to confirm the degree of stenosis and patency of the carotid arteries immediately after the surgery and at the time of sacrifice.

Immediately after euthanasia, a catheter was placed into the brachiocephalic trunk of the animals and bilateral carotid arteries were perfused with 10% formalin at physiologic diastolic pressure for 10

Received July 21, 2008; accepted after revision October 21.

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H.V.V. is supported by the Daljit S. and Elaine Sarkaria Chair in Diagnostic Medicine and National Institutes of Health Grant NS044378.

Paper previously presented in part at: Annual Meeting of Society of NeuroInterventional Surgery, July 30–August 3, 2007; Dana Point, Calif.

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DOI 10.3174/ajnr.A1410

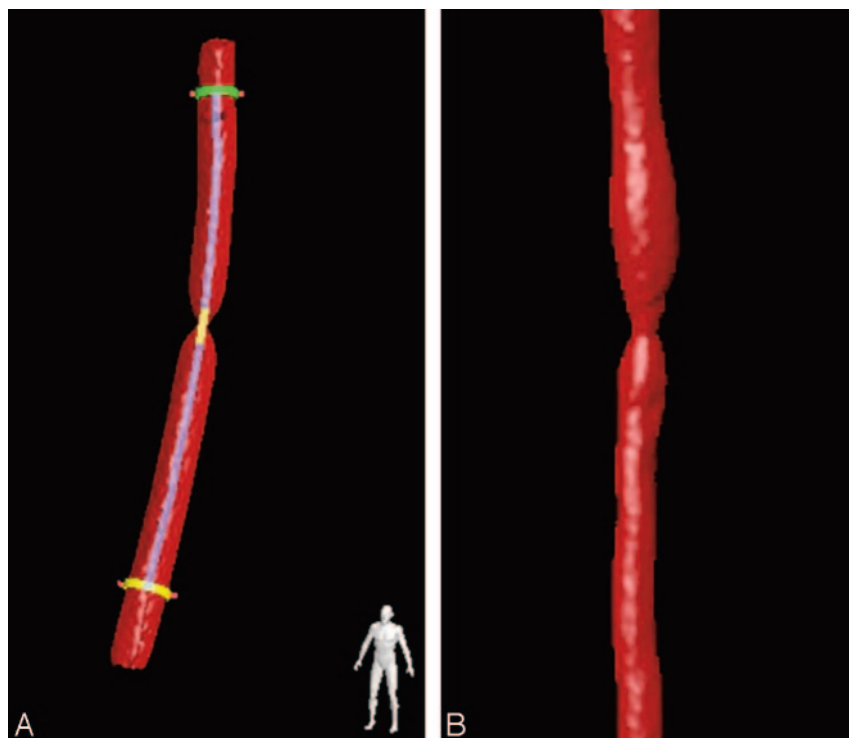


Fig 1. 3D rotational angiograms of partially ligated carotid arteries. *A*, Carotid angiogram demonstrates 82% stenosis at the site of partial surgical ligation in the midcervical carotid artery. *B*, Luminal irregularities are observed 3 months later in the proximal segment, consistent with the development of atherosclerotic plaques. The stenosis at the ligation site remains >70%. Poststenotic dilatation can be seen distal to the ligation, suggesting vessel remodeling.

minutes, to minimize the collapse of these arteries during fixation. Bilateral carotid arteries and retina mirabilia from these animals were collected and fixed in 10% formalin for at least 24 hours.

Histologic and Immunohistochemical Analysis of Carotid Arteries

The carotid arteries were cut into 4-mm segments, embedded in paraffin, sectioned at 5- μ m thickness, and stained with hematoxylin-eosin (HE) to determine the presence of atherosclerotic lesions. An immunohistochemical study using primary antibody to smooth muscle actin (SMA; DakoCytomation Denmark, Glostrup, Denmark) was also used to characterize the atherosclerotic lesions. The atherosclerotic changes of these carotid arteries were classified by using Stary stages from type I to VI set forth by the American Heart Association Committee on Vascular Lesions.¹² In brief, type I lesions are characterized by the presence of smooth muscle proliferation within the intima; type II lesions have intimal proliferation with foamy macrophages; type III lesions contain small pools of extracellular lipid; type IV lesions have extracellular lipid cores; type V lesions have type IV features plus fibrous thickening; and type VI are characterized by intraplaque hemorrhage. The lesions of type IV–VI were defined as advanced plaques. The thickness of the fibrous cap was measured on HE-stained sections at the site of the most severe stage of atherosclerosis.

Identification of Distal Embolism in Rete Mirabile

Evidence for distal embolism from carotid artery plaques was sought in the rete mirabile. After fixation, specimens of rete mirabile were embedded in paraffin, and serial 6- μ m-thick sections were obtained. Sections from 3 different depths of the rete mirabile in the paraffin blocks were stained with HE and van Gieson elastic to identify intraluminal emboli. The adjacent sections were also stained with anti-collagen IV (DakoCytomation Denmark) and anti-smooth muscle actin to further characterize histologic changes within the lumina of arterioles within the rete mirabile.

Statistics

The association of a distal embolism in the rete mirabile with advanced atherosclerotic lesions and a thin fibrous cap in the carotid arteries was analyzed by using the Fisher exact test. All data were expressed as mean $\pm$ standard error of the mean.

Results

Partial surgical ligation created an $84.6 \pm 4.7\%$ stenosis in the midsegment of the carotid artery (Fig 1A, -C). All 24 carotid arteries remained patent at 3-month follow-up carotid angiography, and the degrees of stenosis were well maintained ($77.4 \pm 7.5\%$) (Fig 1B, -D). Irregularities and stenoses were observed angiographically in segments of atherosclerosis, but only 1 vessel with intraplaque hemorrhage caused >70% stenosis at 3 months. Poststenotic dilation was seen in all arteries.

Early atherosclerotic changes of Stary stage I and II were observed in 14 arteries. Ten arteries showed advanced plaques of Stary stage V and VI with a necrotic core, fibrous cap, calcium deposition, and intraplaque hemorrhage (Fig 2). Eight of these 10 carotid arteries were in the surgical ligation group, whereas only 2 vessels in the control group had advanced plaques (Table). Advanced atherosclerotic plaques were found more frequently in the vessel wall proximal to the partial ligation.

Evidence of distal embolism was found in 10 of 24 retina mirabilia. Typically, foamy macrophages and collagen matrix were found in the lumina of occluded arterioles (Fig 3). The internal elastic lamina remained intact, suggesting that the pathologic process was limited to the luminal surface of the vessel. Local atherosclerotic diseases, ranging from intimal hyperplasia to advanced plaques, were observed in a small number of the blood vessels in 20 of 24 retina mirabilia. Most of these local atherosclerotic changes consisted of a hyperplastic intima with a few underlying lipid-laden macrophages. The advanced local atherosclerotic plaques all had disrupted internal

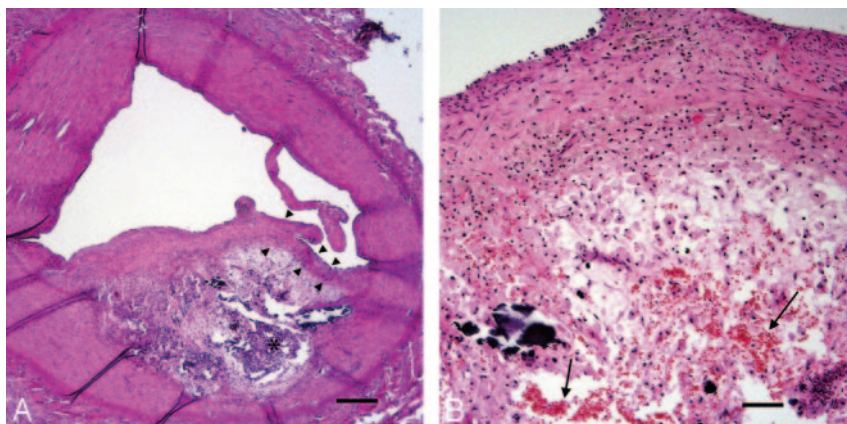


Fig 2. Vulnerable carotid atherosclerotic plaque. *A*, Low-magnification view of HE-stained sections of an atherosclerotic plaque reveals a thin fibrous cap (between arrowheads) and a necrotic center (asterisk). *B*, A magnified view of the same plaque shows small intraplaque hemorrhages (arrows). The necrotic center is to the lower left. Strings of red blood cells can also be seen on the left side of the image, representing neovascularity. The basophilic material to the left side of this panel is calcium. The scale bars are equal to 500 μm in *A* and 200 μm in *B*.

Advanced atherosclerotic plaque and distal embolism				
	Advanced Plaque		Nonadvanced Plaque	
	Embolism	No embolism	Embolism	No embolism
Surgical ($n = 17$)	5	3	2	7
Control ($n = 7$)	2	0	1	4
Total	7	3	3	11

elastic laminae and intact fibrous caps, distinguishing them from emboli. There was a significantly increased frequency of distal embolism in carotid arteries with advanced plaques compared with vessels with early stages of atherosclerosis (70% versus 21%, $P = .02$) (Table). All advanced lesions with distal emboli contained thin fibrous caps of $<200 \mu\text{m}$ in thickness, whereas the other advanced lesions had thick fibrous caps of $>400 \mu\text{m}$ ($P = .012$).

Discussion

Vulnerable plaques are atherosclerotic lesions with a high likelihood of thrombotic complications or rapid progression.¹³ This swine model of carotid atherosclerosis contains many morphologic features of vulnerable carotid atherosclerotic plaques, such as a necrotic core, a thin fibrous cap, and intraplaque hemorrhage. These features are frequently observed in symptomatic carotid atherosclerotic plaques obtained at endarterectomy.¹⁴ MR imaging studies have established the association of these carotid plaque characteristics with ischemic events in initially asymptomatic patients.³ The finding of distal atheroembolism fulfills the functional definition of vulnerable plaque in this animal model. Without the intervening rete mirabile, these emboli would have traveled to the brain and caused infarcts.

This is the first animal model of atherosclerosis that shows distal embolism. The previous swine iliac and carotid atherosclerosis models were created through balloon denudation of the endothelium and subsequent intimal hyperplasia, which resulted in stable and fibrous lesions.^{15,16} Advanced stages of atherosclerotic lesions were not observed in these models. Plaque ruptures have been observed in mouse models of atherosclerosis, which were created to simulate coronary artery diseases.^{4,6} Because distal embolism is not a main mechanism of acute coronary syndrome, this phenomenon has never been reported in these models.

Rupture of a thin fibrous cap in the setting of inflammation and high shear stress is thought to be the main mechanism of

transformation from a stable to a symptomatic plaque in patients with acute coronary syndromes.¹⁷ The association of distal embolism with the presence of advanced atherosclerotic lesions and thin fibrous caps in the ipsilateral carotid arteries suggests that this mechanism may contribute to the atheroembolism in this model of carotid atherosclerosis. The presence of foamy macrophages in the atheroemboli is consistent with the emboli coming from the breakdown of atherosclerotic plaques as a result of plaque rupture. The disruption of a thin fibrous cap has been observed in some of the proximal lesions. Small plaque ruptures may have been missed in other lesions between sections, or the rupture could have occurred many days earlier with the rupture sites having healed by the time of sacrifice. In 3 retia mirabilia with distal emboli, no advanced lesion could be found in the proximal carotid arteries; repetitive angiographies and other mechanisms may be involved in the generation of distal embolism in these animals.

Although the Yucatan miniswines are heavily inbred, they are, by no means, a pure strain. The heterogeneous genetic background may have contributed to the variability of atherogenesis in this animal model. In fact, we have observed variable responses to a high cholesterol diet in recent animals. The lack of response to a high cholesterol diet may explain the slightly lower rate of advanced atherosclerotic plaque in the current study (7/17) compared with our preliminary series (4/6).⁸ Two of the control animals also developed advanced atherosclerotic plaques. Both of them had advanced atherosclerotic plaques on the contralateral surgical side as well. Surgical ligation of 1 carotid artery could have increased flow on the contralateral control side. Alternatively, a high cholesterol diet alone may induce advanced atherosclerosis in a subset of animals as well.

This animal model is created with hyperlipidemia and the alteration of flow dynamics without physical injury to the intima. The molecular and cellular mechanisms of atherosclerosis are thus expected to simulate primary human atherosclerosis closely, instead of restenosis. The exact hemodynamic changes induced by partial ligation of the swine common carotid arteries require further characterization. Although the outflow of the swine common carotid artery is mainly to the external carotid branches, the Doppler waveform in the swine common carotid arteries (unpublished observation, L.F., 2007) is similar to that of human common carotid arteries, suggesting intermediate resistance to flow. Partial ligation in the mid-common carotid arteries disrupts laminar flow in

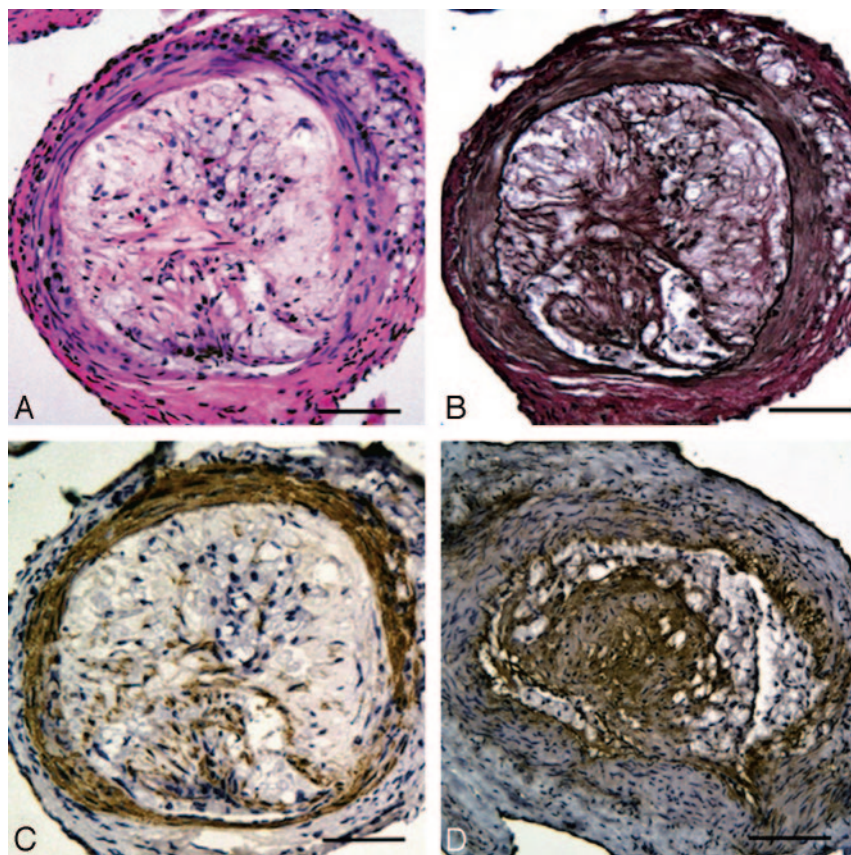


Fig 3. Distal atheroembolism in rete mirabile. A–D, This small arteriole is completely occluded by a lesion containing foamy macrophages (clear areas) and collagen fibers (eosinophilic in A and brown in D). The internal elastic lamina (black line in B) is intact. There are very few smooth muscle cells in the lumen of the vessel (positive immunostaining in C). A, HE staining. B, van Gieson elastic staining. C, Anti-smooth muscle actin staining. D, Collagen type IV staining. The scale bars are equal to 100 μ m.

the artery and may generate reversal of flow and turbulence at sites both proximal and distal to the ligation. Wall shear stress and other flow characteristics around the ligation can be measured with computational flow dynamic simulation and correlated with molecular pathways and pathologic changes in the vessel wall.

The generous size of the swine carotid arteries allows the direct application of imaging techniques developed for the detection of vulnerable plaques in patients. It is possible to correlate the imaging findings with atherogenesis and plaque rupture with time. The interaction between a stent and native atherosclerotic plaques can also be evaluated in this model to provide insight into how to minimize distal embolism.

Conclusions

This swine model of carotid atherosclerosis demonstrates histopathologic features of both vulnerable plaques and distal atheroembolism.

Acknowledgments

We thank the technical staff in the Leo G. Rigler Center, John Roberts, Fernando Viñuela, Jr., and Shawn McGill, for their invaluable assistance in performing the experiments.

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