A case study of ligation induced calcification in middle cerebral artery in rat

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Summary. A 90 min ligation of the middle cerebral artery (MCA) followed by 72-hour reperfusion appeared to cause calcium deposition in vascular myocytes of the tunica media and the perivascular tissue of the Sprague Dawley rat. The presence of small ovoid to large irregularly shaped intracellular opaque deposits were demonstrated by light and electron microscopy. Using X-ray elemental analysis the chemical nature of the deposits was found to be calcium phosphate. The functional significance of this first demonstration of acute calcification following transient ligation of the rodent MCA invites further studies.

Key words: Middle cerebral artery, Calcification, Electron microscopy, X-ray elemental analysis

Introduction

Occlusion of the middle cerebral artery (MCA) is a common approach to the experimental induction of cerebral infarction (Fox et al., 1993; Herz et al., 1996). Although infarction can also be produced by placing a monofilament suture at the origin of the MCA, Wang et al. (1995) reported that in aged rats intracranial MCA occlusion was more consistent in producing cerebral infarcts. By varying the duration of ischemia, Du et al. (1996) showed that delayed infarction may involve apoptosis. With the extravascular approach, whether the occlusion is permanent or transitory can be controlled by whether or not the suture is removed. In the latter model, the most common duration of MCA ligation is 90 min. Possible damage to the vessel, however, has not been investigated. This paper reports a case of calcified deposits in the MCA and adjacent tissue 3 days after 90 min ligation and reperfusion.

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Materials and Methods

Adult male Sprague Dawley rats weighing 250-300 g were used in this study. Animal care and anesthesia were in accordance with the PHS Guide for the Care and Use of Laboratory Animals and our institutional animal use guidelines. Rats were allowed free access to food and water until surgery. They were anesthetized with ketamine, placed in a stereotaxic apparatus, and the surgical area cleaned with 2% iodine. Between the left orbit and the left external auditory canal a 2.5 cm incision was made to expose and split the temporalis muscle. Under an operating microscope a section of the exposed temporal bone was removed with a dental drill. The dura covering the middle cerebral artery was removed with watchmaker's forceps. The MCA was ligated for 90 min with an 11-0 suture just proximal to the crossing of the internal cerebral vein. The complete interruption of blood flow was confirmed under the microscope. During these procedures, body temperature was monitored with a rectal probe and maintained at 35 °C with a heating lamp. After 90 min the ligature was released and the restoration of blood flow confirmed visually. After wound closure, rats were returned to their cages (ambient temperature 21 to 23 "C) and allowed free access to food and water for 72 hours. At the end of this time, the rats were anesthetized, perfused intracardially with saline and then with 10% phosphate buffered formalin, and their brains removed for histological examination.

From three rats that showed signs of ipsilateral cortical necrosis, tissue containing the ligated MCA was removed from the cerebral cortex under a dissecting microscope. Samples were cut into approximately 2 mm square cubes, postfixed in 2% osmium tetroxide, dehydrated in ascending concentrations of ethanol and embedded in Araldite 502. The polymerized blocks were sectioned at 1 μm and stained with toluidine blue and examined by light microscopy. Selected blocks were further sectioned at 800 Å, stained with uranyl acetate and lead citrate before examined by transmission electron microscopy.

Microglia were demonstrated by the procedure of Wu et al. (1992) using horseradish peroxidase conjugated IB4 lectin from Griffonia simplicifolia (Sigma, St. Louis, L5391). Free floating vibratomed 40 μm coronal sections of areas showing cortical necrosis were incubated over night at 4 $^\circ$ C in 25μg/ml of lectin in 0.1M phosphate buffer pH7.4, containing 1% Triton X100. Sections were washed and developed in a diaminobenzidine/H_2O_2 solution before processed for plastic embedding. Thin sections (800 Å) were cut and examined without staining using a Phillips 201C transmission electron microscope. For elemental analysis, unstained sections were collected on 200 mesh copper grids and placed in a Phillips CM12 electron microscope with an EDAX 9900 X-ray detection system. The microscope was operated at 100 keV and with a probe diameter of 1μm.

Results

During surgery the rat MCA was located by its relatively consistent relation to the inferior cerebral vein (Fox et al., 1993). After the animals were terminated and their brains removed, blocks of tissue cut transverse to the MCA were selected and examined. In all cases the endothelial layer appeared intact and was separated from the tunica media by a prominent internal elastic membrane. The 2-3 layers of circumferentially arranged myocytes were separated from the adjacent neuronal tissues by a fibrous partition. No external elastic membrane was detected. All ligated vessels appeared to be patent and without endothelial defects. The remaining layers of the MCA also appeared unaffected by the 90 min occlusion. A frequent feature not found in the contralateral MCA was the appearance of perivascular phagocytic cells. The latter accumulated lipid droplets and contained numerous lysosomes. These lectin-positive cells were either microglia or blood-borne monocytes (Fig. 1).

Perivascular electron-opaque deposits were found in one ligated MCA sample. The irregularly shaped deposits, stainable with toluidine blue and visible in the light microscope, were eccentrically located (Fig. 2). The deposits were found mainly in the perivascular tissues but parts of the MCA vessel wall also contained such deposits. However, none were found in the adjacent smaller vessels.

By electron microscopy, intracellular floccular deposits were found in some perivascular cells as well as in a few vascular myocytes in a part of the MCA. In the former, the opaque intracellular deposits ranged from small concretions not much larger than a mitochondrion to others several microns in diameter. The MCA was lined by normal-appearing endothelium. The internal elastic membrane immediately adjacent to the endothelium appeared uneven and granular; the matrix of the membrane became more homogeneous toward the smooth muscle cells of the tunica media. Most of the vascular smooth muscle cells were unremarkable, showing varying numbers of myofilaments, prominent nuclei and a few endocytic vesicles (Fig. 3). In the portion of the MCA nearest the extravascular deposits of electron-opaque material, spherical to oblong electron-opaque bodies appeared in the vascular myocytes (Fig. 4). Some of the opaque bodies resembled clusters of enlarged mitochondria with a fuzzy coating; some appeared to be coalescing with others.
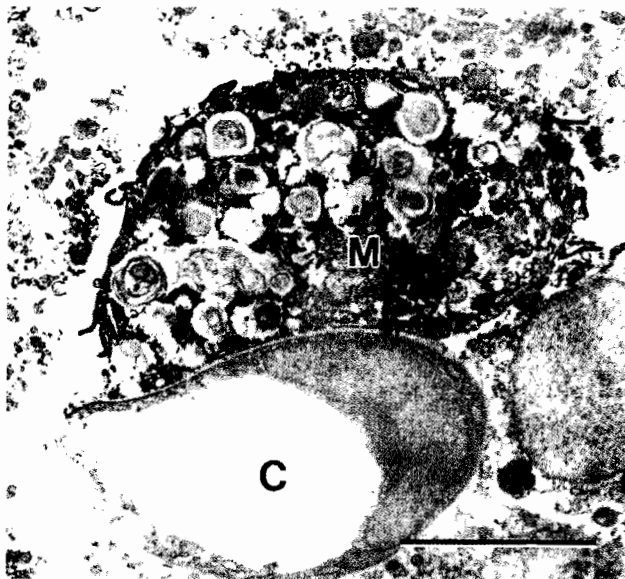

Fig. 1. A pericapillary lectin labeled cell near a capillary (C). Note the abundance of concentric membrane whorls and lipid droplets in the monocytic cell (M). Bar: 5 μm.
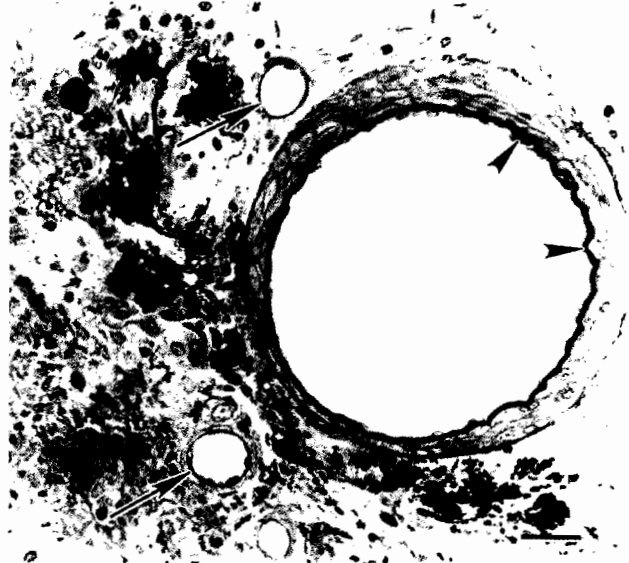

Fig. 2. A transverse section of a transiently ligated MCA showing perivascular deposits. Note the prominent staining of the internal elastic membrane (arrow heads) and the adjacent patent small vessels (arrows). Bar: 10 μm.

The elemental composition of the deposits was determined by placing the X-ray probe on the opaque material in the vascular myocyte or in the perivascular cells. X-ray spectra thus derived showed conclusively the presence of calcium (Fig. 5), this in contrast to the trace amount of calcium present in other parts of the cell cytoplasm (Fig. 6). The elemental spectra also indicated a substantial amount of phosphate in the deposits. The chemical composition of the deposits is therefore considered likely to be calcium phosphate.

Discussion

Diffused and widespread calcification of the arteries is associated with chronic systemic diseases and metabolic disorders, but they are rarely localized (Peterson, 1978; Kossard and Winkelmann, 1979).

Localized calcification of unknown etiology in the tunica media of a terminal branch of the MCA in an aged individual has been reported (Stehbens and Mustapha, 1973). Calcification of cerebral arteriovenous aneurysms is more frequently associated with the venous component (Stehbens, 1972). Other studies of vascular calcification are documented in arterial anastomoses with different types of sutures (Gersak, 1993) as well as in children with HIV infection (Bode and Rudin, 1995). Calcification is also a known complication of vascular stenosis. Fibrous cap and plaque surface morphology and the fibrous intimal tissue including calcification in vascular plaques can be sonographically and histologically identified (O'Donnell et al., 1985; Hatukami et al., 1997). Even though a highly stenotic carotid plaque may be clinically silent, a near 20% 5-

Fig. 3. Low power electron micrograph of the portion of MCA showing the electron-opaque deposit. Note their presence in the vascular smooth muscle cells (arrows) and in the perivascular tissue (arrow heads). Bar: 5 μm.
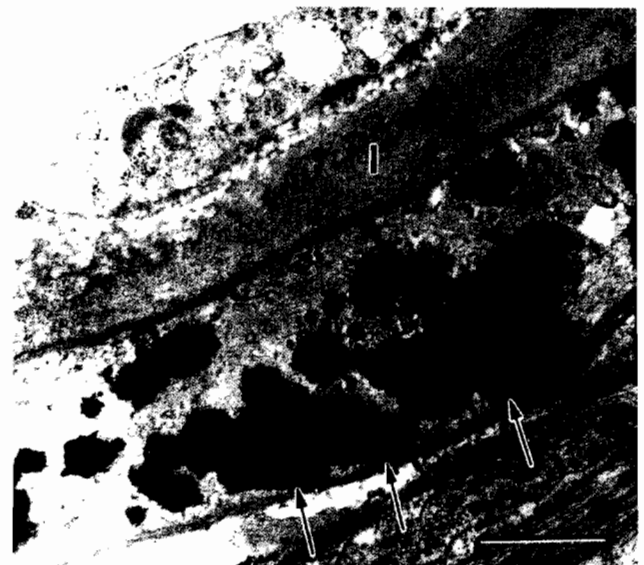

Fig. 4. A close-up view of the myocyte containing electron-opaque bodies. Note the heterogeneous matrix of the internal elastic membrane (I) interposed between the endothelial cell and the myocyte and the spherical to oblong opaque bodies (arrows). Bar: 100 nm.
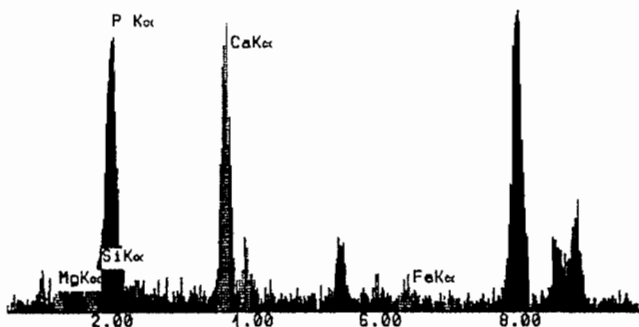

Fig. 5. X-ray elemental analysis of an opaque deposit in a vascular myocyte. Note the presence of calcium and phosphate peaks. Other peaks represent material present in the copper grid.
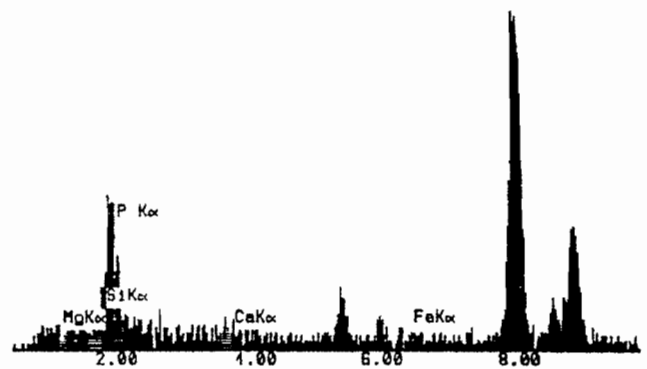

Fig. 6. X-ray elemental analysis result shows the elemental profile of the cell cytoplasm free of the opaque matter. Elemental calcium is barely detectable while phosphate is clearly present.

year risk for transient ischemic attack or stroke has been assigned by the Asymptomatic Carotid Atherosclerosis Study (Executive Committee for Asymptomatic Carotid Atherosclerosis Study, 1995). Calcium deposition in chronic settings may thus be of clinical relevance.

Consistent with the previous report of Lee (1995), this study found the MCA to lack an external elastic membrane and only a thin tunica media. Unlike the arteries of chronic hypertension that exhibits reduced internal and overall diameters (Lee and Smeda, 1985; Baumbach and Heistad, 1989), after physical trauma this study found an otherwise normal MCA with an acute calcium deposit. The perivascular calcium deposits in the adjacent tissue suggest an injury caused by the insertion of the suture. The location of MCA injury was diagonally opposite to the knot of the suture. Although sterile technique was used during animal surgery and in the post-operative care, bacterial infection could still contribute to the calcium deposit. A recent report showed that nanobacteria, the smallest cell-walled bacteria, form distinct colonies in 7 to 60 days and can cause calcium deposit in a variety of biological substrate. After heavy infestation of nanobacteria, 3T6 fibroblast exhibited nuclear abnormalities and formed cytoplasmic needle-like calcium apatite in 48 h (Kajander and Ciftcioglu, 1998). However, the distinct morphology of the calcium deposits and the absence of nuclear abnormality observed in our in vivo study make the bacterial origin of the reported lesion less likely. It is our opinion that the calcification of the perivascular tissue is a direct consequence of the MCA ligation and the myocyte involvement may be secondary. Our finding on calcium deposition in the MCA represent the first documentation of such abnormality occurs acutely. The frequency as well as the permanence of such calcification needs to be determined before the full functional significance of this observation can be realized.

References

Baumbach G.L. and Heistad D.D. (1989). Remodeling of cerebral arterioles in chronic hypertension. Hypertension 13, 968-972.

Bode H. and Rudin C. (1995). Calcifying arteriopathy in the basal ganglia in human immunodeficiency virus infection. Pediatr. Radiol. 25, 72-73.

Du C., Hu R., Csernansky C.A., Hsu C.Y. and Choi D.W. (1996). Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis. J. Cereb. Blood Flow Metab. 16, 195-201.

Executive Committee for Asymptomatic Carotid Atherosclerosis Study. (1995). Endarterctomy for asymptomatic carotid artery stenosis. JAMA 273, 1421-1428.

Fox G., Gallacher D., Shevde S., Loftus J. and Swayne G. (1993). Anatomic variation of the middle cerebral artery in the Sprague Dawley rat. Storke 24, 2087-2093.

Gersak B. (1993). Presence of calcium in the vessel wall after end-to-end arterial anastomoses with polydioxanone and polypropylene sutures in growing dogs. J. Thorac. Cardiovasc. Surg. 106, 587-591.

Hatukami T.S., Ferguson M.S., Beach K.W., Gordon D., Detmer P., Burns D., Alpers C. and Strandness E. (1997). Carotid plaque morphology and clinical events. Stroke 28, 95-100.

Herz R.C.G., Wildt D.J.D. and Versteeg D.H.G. (1996). The effects of γ2 melanocyte-stimulating hormone and ninmodipine on cortical blood flow and infraction volume in two rat models of middle cerebral artery occlusion. Eur. J. Pharmacol. 306, 113-121.

Kajander E.O. and Ciftcioglu N. (1998). Nanobacteria: An alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. Proc. Natl. Acad. Sci. USA 95, 8274-8279.

Kossard S. and Winkelmann R.K. (1979). Vascular calcification in dermatopathology. Am. J. Dermatopathol. 1, 27-34.

Lee R.M.K.W. (1995). Morphology of cerebral arteries. Pharmacol. Ther. 66, 149-173.

Lee R.M.K.W. and Smeda J.S. (1985). Primary versus secondary structural changes of the blood vessels in hypertension. Can. J. Physiol. Pharmacol. 63, 392-401.

O'Donnell T.F.Jr., Erodoes L., Mackey W.C., McCullogh J., Spheard A., Heggerick P., Isner J. and Callow A.D. (1985). Correlation of B-mode ultrasound imaging and arteriography with pathologic findings at carotid Endarterctomy. Arch. Surg. 120, 443-449.

Peterson R. (1978). Small vessel calcification and its relationship to secondary hyperparathyrodism in the renal homotransplant patient. Radiology 126, 627-633.

Stehbens W.E. (1972). Pathology of the cerebral blood vessels. C.V. Mosby. St. Louis.

Stehbens W.E. and Mustapha R.M. (1973). A typical calcification of enlarged terminal branches of the middle cerebral artery. Arch. Neurol. 29, 282-284.

Wang L.J., Futrell N., Wang D.Z., Chen F.J., Zhai Q.H. and Schultz L.R. (1995). A reproducible model of middle cerebral infarcts, compatible with long-term survival in aged rats. Stroke 26, 2087-2090.

Wu C.H., Wen C.Y., Shieh J.Y. and Ling E.A. (1992). A quantitative and morphometric study of the transformation of amoeboid microglia into ramified microglia in the developing corpus callosum in rats. J. Anat. 181, 423-430.