Experimental Model of Lacunar Infarction in the Gyrencephalic Brain of the Miniature Pig

Neurological Assessment and Histological, Immunohistochemical, and Physiological Evaluation of Dynamic Corticospinal Tract Deformation

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Methods
—Thirty-five miniature pigs underwent transcranial surgery for permanent anterior choroidal artery occlusion. Animals recovered for 24 hours (n = 7), 2 (n = 5), 3 (n = 2), 4 (n = 2), 6 (n = 1), 7 (n = 7), 8 (n = 2), and 9 days (n = 1), 2 weeks (n = 2), 4 weeks (n = 3), and more than 4 weeks (n = 3). Neurology, electrophysiology, histology, and MRI were performed. Seven additional miniature pigs underwent transient anterior choroidal artery occlusion to study muscle motor-evoked potentials and evaluate corticospinal tract function during transient anterior choroidal artery occlusion.

Results
—The protocol had a 91.4% success rate in induction of internal capsule infarction 286 ± 153 mm³ (mean ± SD). Motor-evoked potentials revealed the presence of penumbral tissue in the internal capsule after 6 to 15 minutes anterior choroidal artery occlusion. Total neurological deficit scores of 15.0 (95% CI, 13.5 to 16.4) and 3.4 (0.3 to 6.4) were recorded for permanent anterior choroidal artery occlusion and sham groups, respectively (P < 0.001, maximum score 25) with motor deficit scores of 3.4 (95% CI, 2.9 to 4.0) and 0.0 (CI, 0.0 to 0.0), respectively (P < 0.001, maximum score 9). Histology revealed that the internal capsule lesion expands gradually from acute to chronic phases.

Conclusions
—This new model of lacunar infarction induces a reproducible infarct in subcortical white matter with a measurable functional deficit and evidence of penumbral tissue acutely. (Stroke. 2008;39:205-212.)

Key Words: axonal damage ■ gyrencephalic brain ■ lacunar infarction ■ penumbra ■ reproducibility
dynamic corticospinal tract deformation to clarify the characteristic features of white matter ischemia and to identify the critical period for penumbra formation caused by perforating artery occlusion.

Materials and Methods
Mexican hairless miniature pigs (6 to 8 months old, 19 to 42 kg) were obtained from the National Livestock Breeding Center (Ibaraki, Japan). All procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Gunma University of Medicine.

General Surgical Preparation
Miniature pigs underwent AchA occlusion (AchAO) using a modification of the permanent middle cerebral artery occlusion method (Figure 1). Briefly, pigs were initially sedated with intramuscular injection of 60 mg/kg ketamine hydrochloride (Sankyou Chemi, Tokyo, Japan) and 2.0 mg/kg xylazine (Bayer, Tokyo, Japan) and then intubated and artificially ventilated (Kimura Ventilator, Tokyo, Japan) with 1.2% to 1.9% isoflurane in 2:1 nitrous oxide:oxygen. The right femoral artery was cannulated for continuous physiological monitoring. Arterial pressure was monitored and arterial blood samples were taken at regular intervals for assessment of respiratory status. The pigs were maintained normotensive (mean arterial blood pressure > 80 mm Hg), normocapnic (36 < Paco2 < 44 mm Hg), and adequately oxygenated (pO2 > 100 mm Hg) under anesthesia. Rectal temperature was maintained at 38°C with a heating blanket. A frontotemporal craniectomy with orbital rim osteotomy was performed. AchAO was induced by electrocoagulation for permanent occlusion (n=35) and clipping for transient occlusion (n=7). Sham-operated animals were treated in the same way without occlusion (n=5). Animals undergoing permanent AchAO were allowed to recover for 24 hours (n=7), 2 days (n=5), 3 days (n=2), 4 days (n=2), 6 days (n=1), 7 days (n=6), 8 days (n=2), and 9 days (n=1), 2 weeks (n=2), 4 weeks (n=3), and more than 4 weeks (n=3) before perfusion fixation. The mortality rate was zero in this study.

Neurological Examination
The neurological status of each animal was evaluated according to the neurological grading score with some modifications (supplemental Table I, available online at http://stroke.ahajournals.org). Total neurological deficit included scores generated from a 9-point scale by the extraction of the motor functional assessment from the total scores.

Muscle Motor-Evoked Potentials
Muscle motor-evoked potentials (MMEPs) were monitored to evaluate the corticospinal tract function during 15 minutes of transient AchAO and additionally up to 15 minutes after recirculation (n=7). Direct electrical stimulation (a train of 5; 10.2 to 25 mA in intensity; 0.2 ms in duration; 10-ms intervals every 15 seconds) was applied through 2 plate electrodes placed on the ipsilesional motor cortex. MMEPs were recorded through stainless steel needle electrodes inserted into the forelimb extensor and flexor muscles contralateral to the stimulation side using a NEURO PACK (NIHON KOHDEN Corp).

Evaluation of Ischemic White Matter Damage
The ischemic damage was evaluated by coronal T1- and T2-weighted, fluid-attenuated inversion recovery and diffusion-weighted MRI (DWI) using a 1.5-T MR scanner (GE Medical Systems) at 24 hours after permanent AchAO. Imaging parameters were: T1-weighted, 400 ms/11 ms/1 (repetition time/echo time/excitations); T2-weighted, 3900 ms/100 ms/1; fluid-attenuated inversion recovery, 8002 ms/147 ms/1; and DWI, 4999 ms/100 ms/1, slice thickness 3 mm.

Histological Analysis
Brain sections were analyzed at 24 hours, 1 week, and 4 weeks after ischemia. Preparation of sections and hematoxylin and eosin staining, Luxol fast blue staining, and immunohistochemical study using antibodies to amyloid precursor protein (APP; Chemicon International; diluted 1:2500), glial fibrillary acidic protein (GFAP; Sigma-Aldrich Co; 1:20000), Ki-67 (DakoCytomation; 1:500), and neurofilament 1D (gift from Prof. Y. Nakazato, Department of Human Pathology, Gunma University Graduate School of Medicine14; 1:50) were performed as previously reported. Sections were observed by light microscopy (Olympus BX51) and images captured through a 20 or 40× objective and 10× eyepiece using a high-resolution CCD camera (Olympus DP50). Images were saved for subsequent analysis and quantification. Analysis was conducted by an investigator unaware of the surgical protocol of each animal. The areas of the ischemic lesion were defined as the ischemic core, irreversibly damaged area, perifluent area, and marginal zone according to previous reports with some modification.

Internal capsule (IC) infarction was measured as the diameters of the lesion and IC after Luxol fast blue staining of axial sections to obtain an index (lesion width/IC width). Cells immunopositive for Ki-67 were counted in 0.3-mm² microscope fields (at 200× magnifications) within the lesion. The counting threshold was set at the optical density of the most intensely stained cells in the contralateral IC. The GFAP-immunoreactive area was quantified by image software (Image J 1.36b; National Institutes of Health). Blood vessels were defined morphologically and counted in 0.075-mm² microscope...
fields (at 400× magnification) within the irreversible ischemic damage zone. The area and number of immunoreactive cells were quantified as the average of 9 randomly selected regions of interest in each lesion.

**Electron Microscopy**
Sections were refixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer at pH 7.4, washed with water, and covered with 1% osmium tetroxide in 0.1 mol/L cacodylate buffer at pH 7.4. Ultrathin sections were cut and observed with a JEM-100CX electron microscope (JEOL Ltd, Tokyo, Japan).

**Statistical Analyses**
Data are presented as box plots. Statistical analysis was performed with JMP-IN 5.1 software (SAS Institute Inc). The Wilcoxon rank sum test and Tukey-Kramer’s honest significant difference test were used for comparison of grouped data. Differences were considered significant at *P* < 0.05.

**Results**

**Muscle Motor-Evoked Potentials**
MMEPs faded away at 6.2±1.1 minutes (n=7) after AchAO, but the loss was reversible if the duration of ischemia was less than 15 minutes. A representative MMEP trace is shown in Figure 2A.

**MRI Findings**
Typical DWI, 24 hours after permanent AchAO, is shown in Figure 2B. The sham-operated group showed no changes in signal intensity. Thirty-two of 35 animals in the AchAO group (91.4%) demonstrated hyperintense areas predominantly in the IC area. Axial fluid-attenuated inversion recovery imaging confirmed the lesions (Figure 2C). Some animals also had ischemic damage in the amygdala, crus cerebri, and hippocampus in addition to the IC. These IC lesions were confirmed by histological examination (Figures 2D–F). The other 3 of 35 animals showed no significant abnormal intensity in the IC on DWI. The infarct volume quantified by the DWI was 286±153 mm³ (mean±SD).

**Neurological Deficit Assessment**
Postoperative neurological scores for permanent AchAO and sham-operated groups are presented in Figure 3A. AchAO group scores reached maximum values as early as 12 hours after ischemia and did not change during the first 24 hours. The AchAO group’s total deficit scores were significantly higher compared with the sham-operated group during the first 7 days after ischemia (Figure 3A). During the first 12 days (AchAO group) and 5 days (sham-operated group), the total score gradually decreased to zero. On the other hand, the motor functional scores of the AchAO group were significantly higher than those of the sham-operated group during the first 7 days after ischemia (Figure 3B). The scores of the sham-operated group were near zero at all times, whereas the AchAO group recovered to normal scores by day 10 (Figure 3B).

**Internal Capsule Damage**
Sections double-stained with APP and GFAP clearly delineated the boundary of the IC lesion (Figure 3C). The index at 1 and 4 weeks was significantly higher than that obtained at 24 hours after ischemia (Figure 3D). However, the index was not significantly different between 1 and 4 weeks. In general, the infarction in the permanent AchAO group gradually increased with time, which was particularly attributed to expansion of the infarct during the first week after onset.

**Histological Findings**
Luxol fast blue staining of intact myelin sheaths provided clear contrast between intact and damaged white matter as well as white and gray matter. Damaged myelin sheaths appeared as empty spaces (vacuoles) separating myelin sheaths in the lesion zone of white matter (Figures 2E–F).

Hematoxylin and eosin staining 24 hours after ischemia showed small infarct zones as poorly stained areas involving the IC. The tissue surrounding the ischemic core also showed signs of vacuolation and interstitial edema, or the so-called
irreversible ischemic area (Figure 4A, upper). One week after ischemia, vacuolation and edema had expanded into the periinfarct area surrounding the irreversible damaged area associated with inflammatory cell infiltration (Figure 4A/H11032).

At 4 weeks after ischemia, the core and irreversible ischemic area appeared to form a cavity with cellular aggregation mainly consisting of macrophages and remaining small vessels. Glial scar formation surrounded the lesion (Figure 4A/H11033).

APP immunostaining at 24 hours after ischemia clearly delineated the ischemic area boundary to the naked eye (Figure 4B). One week after ischemia, the APP-immunopositive area had expanded into the periinfarct area and appeared to spread to the surrounding area (Figure 4B/H11032). At 4 weeks, the staining was faint with some positive staining in damaged axons (Figure 4B/H11033). These findings indicate that axonal damage can spread to the periinfarct area during the first week after ischemia.

At 24 hours after ischemia, neurofilament 1D immunostaining was positive mainly in the ischemic core and weak in the periinfarct area (Figure 4C). At 1 week after ischemia, the neurofilament 1D-positive area had shifted from the core into the periinfarct area (Figure 4C/H11032). At 4 weeks, the staining had become fainter in the lesion (Figure 4C/H11033) and some positive staining appeared in a remote area related to the proximal site of the corticospinal tract (data not shown). These findings indicate that axonal damage expanded into the surrounding area during the first 4 weeks after ischemia.

GFAP-positive astrocytes appeared predominantly in the irreversible damaged area and less in the periinfarct area at 24 hours after ischemia (Figure 4D). One week later, GFAP-positive astrocytes were commonly found in the periinfarct zone (Figure 4D/H11033). Increases in GFAP-positive astrocytes number and astrocytic hypertrophic processes accounted for this increased expression from 24 hours to 4 weeks after ischemia (Figure 4D/H11034). The GFAP-positive area in the ischemic core showed no increase compared with the control group, but increased significantly in the periinfarct area at 1 and 4 weeks after AchAO (Figure 5A).

A few Ki-67-immunopositive cells indicated cellular proliferation surrounded the small vessels in the periinfarct area at 24 hours after ischemia (Figure 4E). The number of Ki-67-positive cells increased significantly at 24 hours to 1 week after ischemia and were mostly distributed in the periinfarct area (Figures 4E/H11032 and 5B) and then decreased at 4 weeks after ischemia in the ischemic core and the periinfarct area (Figures 4E/H11033 and 5B). Most Ki-67-positive cells were located in the perivascular lesion or preserved vessels, suggesting that cells with proliferative activity were activated macrophages and angiogenic cells.

Microvasculature Findings

The capillary beds were still preserved in the lesion at 24 hours after ischemia (Figure 5C). Small vessels such as arterioles and venules could be identified in the center of the

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**Figure 3.** Total neurological scores (A) and motor functional score (B) (box plot and mean) in the AchAO and sham-operated groups during the 4-week observation period. (n numbers in the AchAO group are presented under the time points on the x-axis). C. Photographs with APP immunostaining showing the internal capsule deformation visible to the naked eye and revealing the anatomical enlargement of the ischemic zone at the internal capsule level. D. Damage ratio of the internal capsule assessed from histological findings. Dot represents mean. *P<0.05, **P<0.001 by Wilcoxon rank sum test (A, B) or Tukey-Kramer’s honest significant difference test (D). n.s. indicates not significant.
infarct. At 1 week after ischemia, the ischemic core contained mostly necrotic astrocytes and oligodendrocytes with nuclei, but the capillary beds could still be identified. Ki-67-immunoreactive cells were observed, predominantly surrounding the vessel, and some walls were thickened by cellular proliferation. At 4 weeks after ischemia, the ischemic lesion and cerebral softening were associated with extensive necrotic parenchyma infiltration by macrophages, but the small vessels could be identified in nearly the same numbers as before (Figure 5D).

Ultrastructural Analysis of the Internal Capsule Ischemic Lesion

The sham-operated animals revealed normal findings of oligodendrocytes, astrocytes, axons, and myelin in the IC with the characteristic density around the axonal cylinder (Figure 6A). In the ischemic core at 24 hours after ischemia, both axons and myelin were morphologically destroyed and astrocytes were present around vessels, including many glycogen granules and some oligodendrocytes (Figure 6B). One week later, myelin sheath debris and cellular components such as small vessels were observed (Figure 6B'). In the irreversibly damaged area at 24 hours after ischemia, prominent exudates in the extracellular and periaxonal space, disrupted axonal alignment, and lost myelin sheath function were observed (Figure 6C). One week later, the appearance was identical to that of the ischemic core (Figure 6C'). In the perinuclear area at 24 hours after ischemia, many swollen axons with myelin sheaths were present mixed with some morphologically normal areas (Figure 6D). One week after ischemia, macrophages had infiltrated and phagocytized the myelin debris (Figure 6D'). In the marginal area at 24 hours
after ischemia, no significant abnormal findings were observed with few swollen axons and little exudate (Figure 6E). One week later, swollen axons included many organelles with fragile myelin sheaths, indicating progressive expansion of ischemic damage to the marginal area (Figure 6E').

**Discussion**

The present model of isolated deep subcortical white matter infarction mainly located in the internal capsule provided a success rate of 91.4%. This high reproducibility is possible because the individual anatomical variations of the AchA can be appraised, thus allowing selective occlusion of the perforating brain vessels such as the functional end artery. In contrast, the middle cerebral artery occlusion model involves leptomeningeal anastomoses, which are undoubtedly responsible for the repeated observations of significant distal blood flow and for the variations in the size of the resulting infarction.

**Figure 5.** A, Quantification of the GFAP-immunopositive area after permanent AchAO in the core and perifarct area. B, Number of Ki-67-immunopositive cells per field (0.3 mm²) in the ischemic core and perifarct area. C, Photomicrographs of small vessels in the control lesion. Insets show Ki-67 staining to confirm the endothelial viability (mitosis activity). Scale bar=25 μm. D, Number of small vessels per field (0.075 mm²). Control represents contralateral region. Dot represents mean. *P<0.05 by Tukey-Kramer’s honest significant difference test (A, B, D).

**Figure 6.** Electron micrographs showing the dynamic change in the lesion after AchAO. Left lower illustrates the areas of the ischemic lesion from which the sections were taken. A, Contralateral site of the lesion; B and B', ischemic core; C and C', irreversibly damaged area; D and D', perifarct area; E and E', marginal area. Olig indicates oligodendrocyte; Vess, small vessel; Ax, swollen axon; Mac, macrophage. Scale bar=4 μm.
Neurophysiological changes were assessed by MMEP monitoring, which can record pyramidal tract function in real time\textsuperscript{17} based on the concept that the AchA provides blood flow to the IC region. Therefore, AchAO would compromise the function of the pyramidal tract. MMEP monitoring is an excellent modality with high sensitivity and specificity to detect IC ischemia. Moreover, MMEP monitoring during the 6- to 15-minute period of transient ischemia provided interesting insights into the penumbra where blood flow is reduced to the point of electrophysiological silence and losses of membrane ion gradients and energy metabolites,\textsuperscript{18} but energy metabolism is sufficiently maintained to allow morphological tissue preservation. Continuation of the ischemia through permanent AchAO will exhaust this limited capacity and transform the penumbra zone into irreversibly damaged tissue. The initial penumbra is based on IC white matter exposed to AchAO of 6 to 15 minutes’ duration.

White and gray matter show different initial responses to ischemia\textsuperscript{19} and have structural, functional, and metabolic differences. White matter consists of axons and glia but does not include synapses. The highly specialized architecture of myelinated axons is uniquely designed to support rapid and efficient saltatory impulse propagation\textsuperscript{20,21} and contains a highly segregated distribution of ion channels and transporters located on the axon membranes such as several adenosine triphosphate-dependent pumps as well as the Na\textsuperscript{+}/K\textsuperscript{+} antiporter.\textsuperscript{22} Failure of the blood supply to the white matter results in attenuation of Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity, which in turn results in K\textsuperscript{+} cell outflow and accumulation in the extracellular space, leading to a rapid decline in the action potential magnitude with concomitant large membrane depolarization within minutes.\textsuperscript{23} Loss of electrical excitability as a result of the collapse of the Na\textsuperscript{+} and K\textsuperscript{+} gradients will not necessarily induce irreversible damage, because the axons can fully recover with reoxygenation after anoxia of less than 15 minutes,\textsuperscript{24} but longer periods result in irreversible functional damage resulting from the excessive accumulation of intracellular Ca\textsuperscript{2+}.\textsuperscript{25} In our study, AchAO for approximately 6 minutes induced functional impairment of the corticospinal tract.

Morphological damage in white matter was detected as axonal swellings or bulbs immunopositive for APP and neurofilament protein antibody. In the present study, APP immunostaining revealed the ischemic area to the naked eye (Figures 2 and 3). Histology revealed that the lesion expanded gradually from the acute to the chronic phases (Figure 3). The molecular mechanisms responsible for this expansion to the marginal area are currently unclear but appear to be specific for white matter. In gray matter, glutamate is central to the formation of ischemic damage.\textsuperscript{26} In white matter, damage such as lacunar infarction initially results from axonal damage with edema formation. Ultrastructural analysis revealed that axonal damage results in the instability of the myelin sheath and subsequent demyelination (Figure 5). Proteolysis of structural axonal proteins such as neurofilament and APP is probably important in the occurrence of white matter damage.\textsuperscript{27,28}

In the present study, the neurological findings in animals with lacunar infarction were not always correlated with the morphological findings. Obvious signs of neurological deficit were seen with the maximum score at 12 hours after the ischemic insult and were maintained during the first 24 hours. We found significant recovery at 3 days after ischemia with slower recovery of most signs during the following days. The neurological deficit score showed no significant difference between ischemia and sham-operated groups at 12 days. The lack of a measurable neurological deficit beyond 12 days may be due to the sensitivity of the scoring system. Alternatively, methods such as a digital video-based tracking system for automatically tracking the spontaneous locomotor behavior of pigs\textsuperscript{29} could provide a more sensitive assessment and possibly detect a significant difference between permanent and sham-operated groups beyond 12 days. Alternatively, it is possible that the miniature pig has an ability to spontaneous recovery over time. This might be mediated by compensatory behavioral strategies to maximize the use of spared systems and the mechanisms of axonal regeneration such as sprouting and remyelination.

Another notable finding of this study was that small vessels are highly resistant to ischemic stress. The number of small vessels in the IC remained constant before and after ischemia, even in the chronic phase (Figure 5C), probably because of the difference in cellular susceptibility.\textsuperscript{9} The tolerance to hypoxia is critical to cell survival but varies greatly between different cell types. Vascular endothelial cells are highly glycolytic and consume relatively low amounts oxygen compared with other cells, indicating that oxidative phosphorylation is not the main source of adenosine triphosphate generation in this cell type.\textsuperscript{30}

Conclusion

The present new model of white matter ischemia in the miniature pig is extremely useful for the investigation of specific cellular mechanisms of white matter damage and to provide evidence about the ischemic penumbra in white matter. The present study suggests that the perihifacrt zone must be a dynamic and responsive area for a long time after stroke even in the small subcortical white matter, thus allowing salvage by appropriate treatment. Although the present model has some limitation to the relevance to human stroke in terms of spontaneous recovery after ischemia, it may additionally be useful in translational research such as subclinical trials for axonal regeneration in the development of advanced stroke therapy. Further modifications such as the use of aged miniature pigs with/without hypertension may also provide a more severe and longer-lasting neurological deficit.

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Disclosures

None.
References


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