White Matter Changes in the Gerbil Brain Under Chronic Cerebral Hypoperfusion

Takahiro Kurumatani, MD, PhD; Takashi Kudo, MD, PhD; Yasumitsu Ikura, MD, PhD; Masatoshi Takeda, MD, PhD

Background and Purpose—An animal model of chronic cerebral hypoperfusion was developed with coiled clips applied to both carotid arteries of adult Mongolian gerbils for between 1 week and 2 months. In the brain of this animal model, rarefaction of white matter with dilatation of the ventricles was frequently observed. To better understand the mechanism of white matter alteration under cerebral hypoperfusion, the chronological sequence of molecular changes in the cerebral white matter of the animal model was determined.

Methods—Specially designed coiled clips were placed around both carotid arteries of Mongolian gerbils to create stenosis without occlusion. Changes in levels of myelin basic protein (MBP) as a marker of myelin, neurofilament H (NFH) as a marker of axonal proteins, and glial fibrillary acidic protein (GFAP) in astroglia after 2 months of cerebral hypoperfusion were analyzed with Western blotting and enzyme-linked immunosorbent assay.

Results—Western blotting of the white matter after 2 months of hypoperfusion showed that the levels of MBP and NFH decreased, whereas that of GFAP increased. The time course of MBP and NFH changes determined with enzyme-linked immunosorbent assay revealed that the change of MBP preceded that of NFH.

Conclusions—In the present study it was shown that the damage to myelin precedes that to the axon in the white matter in a chronic cerebral hypoperfusion animal model, suggesting that the change in myelin is the primary pathological event in the cerebral white matter under chronic hypoperfusion. The present study may help in understanding the mechanisms of white matter pathology in leukoaraiosis. (Stroke. 1998;29:1058-1062.)

Key Words: ischemia ■ leukoaraiosis ■ white matter ■ gerbils

It is well known that CBF is reduced in the brains of people suffering from dementia and that there is a correlation between the extent of CBF reduction and the severity of the dementia. However, it is not known whether chronic cerebral hypoperfusion directly causes dementia.

A number of studies on animal brains with experimentally induced ischemia have been reported. However, most of these studies employed methods involving temporal interruption of the blood flow in major cerebral arteries,1,2 and it is not likely that these methods reflect the condition of chronic cerebral hypoperfusion that is seen in some cerebrovascular dementia patients. To reproduce the chronic cerebral hypoperfusion state experimentally, we developed an animal model by applying coiled clips to both of the carotid arteries, which reduced, but did not interrupt, CBF for between 1 week and 2 months.3 With the hydrogen clearance method, it was shown that CBF of this animal model was reduced to approximately 75% of the level in normal controls.5 The learning ability of this animal model as estimated by the passive avoidance paradigm was significantly impaired.1 A reduction in the level of microtubule-associated protein 2 and an increase in that of GFAP were observed in the brain of this animal model, even in areas without apparent neuronal loss, suggesting that chronic cerebral hypoperfusion damages some cytoskeletal proteins and causes neuronal death.3,4 Among the evidence observed in this animal model, the most conspicuous findings are changes in the white matter and dilatations of the ventricles, which are not always accompanied by lesions in the gray matter.3

In the present study, changes in the levels of MBP, NFH, and GFAP, which are markers of the major white matter components myelin, axon, and astroglia, respectively, were investigated in an animal model with Western blotting to advance understanding of the mechanism of white matter changes in chronic cerebral hypoperfusion. The chronological sequence of MBP and NFT changes in the animal model was also determined with an ELISA.

Materials and Methods

Surgical Operation

All experiments were conducted according to the guidelines issued by the Animal Care Committee of Osaka University. Surgery was performed as previously reported.1 In brief, the left carotid artery of adult Mongolian gerbils was exposed under anesthesia with penito-
barbital (70 mg/kg), and a coiled clip (inside diameter, 0.25 mm; one pitch, 0.5 mm; whole length, 2.5 mm) made of stainless steel wire (diameter, 0.1 mm) was applied to the artery. After a recovery period of 1 week, the right carotid artery was operated on in the same way. These animals composed the experimental group. The experimental group was divided into four subgroups that were kept for 1 week (n = 12), 2 weeks (n = 10), 1 month (n = 12), or 2 months (n = 15) (1-week, 2-week, 1-month, and 2-month groups, respectively). Animals in the control group (n = 10) were given a sham operation, in which their carotid arteries were merely touched with the clips. The animals were kept under conditions of controlled temperature and humidity with free access to food and water. The incidence of ventricular dilatation in each group was estimated to check the presence of chronic cerebral hypoperfusion.

Sample of White Matter
Animals were killed with a fatal dose of pentobarbital and decapitated after confirmation of death. White matter was carefully dissected from other tissues and homogenized with four times its volume of homogenate buffer (0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L Tris buffer, pH 7.2) in a cold room (at 4°C).

Western Blotting
The white matter homogenate from the 2-month group (n = 8) and the control group (n = 7) was subjected to 7.5% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane. The membranes were immunostained with a 1:100 dilution of monoclonal anti-MBP antibody (Serotec), a 1:400 dilution of monoclonal anti-NFH antibody (Sigma), or a monoclonal anti-GFAP antibody (Amersham) as the primary antibody, followed by a 1:2500 dilution of alkaline phosphatase–conjugated antibody to mouse IgG (Jackson). The BCIP/NBT kit (Bio Rad) was used to visualize the band pattern. The bands corresponding to the protein were quantified with an optical densitometer (Shimadzu).

Capture ELISA for MBP
The wells of microtiter plates (MaxisorpTM/Nunc) were coated with a 1:100 dilution of monoclonal anti-MBP (Serotec) in 100 μL TBS buffer (10 mmol/L Tris-HCl, pH 7.6, 0.85% NaCl, 0.1% NaN3) for 1 hour at 37°C and 18 hours at 4°C. The remaining protein binding sites were blocked with 200 μL of 10% normal goat serum in TBS, 0.2% Tween-20, 10 μmol/L leupeptin, 2 μg/mL aprotinin, and 1 μg/mL pepstatin A for 30 minutes at 37°C. The plates were washed three times with 200 μL of washing buffer (100 mmol/L Tris-HCl, pH 7.6, 0.05% Tween-20). To make the standard curve, 100 μL of different amounts of purified MBP in dilution buffer (TBS, 1% normal goat serum, 0.2% Tween-20, 10 μmol/L leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin A) was applied to precoated plates. For the brain homogenate assay, 20 μg of white matter homogenate in 100 μL dilution buffer was added to the plates. The samples were incubated in the plates for 2 hours at 37°C. After they were washed, a 1:200 dilution of polyclonal anti-MBP antibody (Dako) in 100 μL dilution buffer was added to each well, and the plates were incubated for 18 hours at 22°C. The plates were washed as above and incubated with 100 μL per well of alkaline phosphatase–conjugated affinity-purified antibody to rabbit IgG (Jackson Immunoresearch Laboratories) at a dilution of 1:3000 in dilution buffer. After 2 hours at 22°C, the plates were again washed as above and incubated with 1 mg/mL p-nitrophenyl phosphate (Sigma) in 10% diethanolamine (Fisher Scientific), pH 9.8, for 0.5 to 1 hour at 22°C. Absorption at 405 nm was read with a microplate reader (Corona). Each sample was assayed in duplicate. Controls without antigen were measured to determine the background.

Capture ELISA for NFH
The assay for NFH was performed in a manner similar to that for MBP, except that monoclonal anti-NFH antibody (1:400; Sigma) was employed as the solid-phase antibody and polyclonal anti-NFH antibody (1:150; Chemicon) as the primary antibody, and purified NFH was used for the standard curve. For this assay, 50 μg of white matter homogenate in 100 μL of the dilution buffer was added to the plates.

Results
Changes in MBP, NFH, and GFAP Detected With Western Blotting
MBP, NFH, and GFAP were chosen as markers for the major white matter components myelin, axon, and astroglia, respectively. Western blotting with anti-MBP antibody showed that the 2-month and control groups had the same band pattern, with 21.5 kD as the main band (Figure 1A). Anti-NFH antibody, which binds to both phosphorylated and unphosphorylated NFH, stained the 200-kD band corresponding to this protein but also cross-reacted with other intermediate filaments, including neurofilaments L and M (Figure 1B). Between the 2-month and control groups, there was no difference in the pattern of bands with anti-NFH antibody. Blotting with anti-GFAP antibody also revealed the same band pattern of 45, 50, and 54 kD in both groups (Figure 1C).

To estimate the levels of these proteins, the main bands were scanned with an optical densitometer. The level of 21.5-kD MBP polypeptide in the 2-month group was reduced to 50% of the control group level (Figure 2A). Two-month hypoperfusion also reduced the level of 200-kD NFH to 80% of the control group level (Figure 2B). In contrast, the total level of 45-, 50-, and 54-kD GFAP polypeptides in the 2-month group was 3.5 times higher than that of the control group (Figure 2C).

Chronological Sequence of MBP and NFH Changes
The study with Western blotting revealed that 2 months of cerebral hypoperfusion decreased the levels of MBP and NFH in the white matter. To investigate these changes in detail, capture ELISA methods for MBP and NFH were established, and the levels of these proteins were assayed in 1-week, 2-week, 1-month, and 2-month groups. A reduction in the MBP level was seen already in the 2-week group, and a significant reduction in the MBP level was seen in the 1-month and 2-month groups compared with the control group level (Figure 3). On the other hand, the NFH level was significantly reduced only in the 2-month group (Figure 3). Therefore, it was observed that the reduction in the MBP level precedes that in the NFH level under chronic cerebral hypoperfusion (Figure 3).
Discussion

We have previously reported various results concerning the effects of chronic cerebral hypoperfusion on the brain with a specially developed animal model. Among the findings obtained with this animal model, one conspicuous result was the rarefaction of the white matter. This change was observed among animals that had suffered reduced CBF for 4 weeks, even those that did not show cortical or hippocampal changes, and the incidence of white matter rarefaction increased until 12 weeks. In the present study we determined the chronological sequence in which molecular changes develop in the cerebral white matter of the same animal model, with a view to advancing our understanding of the mechanisms of the development of white matter changes in the chronically hypoperfused brain.

The major components of white matter are myelin, axon, and glia. MBP, NFH, and GFAP were chosen as respective markers of these components because of their abundance and functional importance. Thus, MBP is the most abundant protein in the myelin sheath, NFH is one of the major cytoskeletal proteins of the axon, and GFAP is a major protein in the astroglia. Western blotting with anti-MBP antibody and anti-NFH antibody showed that the levels of MBP and NFH decreased after 2 months of cerebral hypoperfusion. On the other hand, hypoperfusion for 2 months increased the GFAP level according to blotting with anti-GFAP antibody. These data suggest that the myelin and axon were damaged by cerebral hypoperfusion for 2 months and that the astroglia proliferated as a result of the damage to these white matter components.

To determine the chronological sequence of changes in the white matter, a capture ELISA was performed for MBP and NFH. The reduction in the MBP level started earlier than that in the NFH level and was already significant in the 1-month group. A significant reduction in NFH occurred only in the 2-month group. These data suggest that the myelin damage may precede that in the axon in this animal model. This suggests the possibility that the myelin damage may be the cause of the axonal damage in white matter exposed to chronic cerebral hypoperfusion.

It has been reported that the oligodendrocyte is vulnerable to various kinds of stress. It has also been reported that the oligodendrocyte is more easily impaired by ischemia than other cell types that constitute the white matter tissue.

Figure 1. Western blot analysis of hypoperfused brain with anti-MBP, anti-NFH, and anti-GFAP antibodies. Blot with anti-MBP antibody (A) shows that 2-month and control groups have the same band pattern of 21.5 kD as the main band (arrow). Anti-NFH antibody, which binds to both the phosphorylated and unphosphorylated forms of NFH, stained the 200-kD band (arrow) corresponding to this protein but cross-reacted also with other intermediate filaments including neurofilaments L and M. There was no difference in the band pattern with anti-NFH antibody between the 2-month and control groups (B). Blots with anti-GFAP antibody showed the same band patterns of 45, 50, and 54 kD in both the 2-month and control groups (C). 1 indicates control group; 2, 2-month group. Molecular weight markers are shown on the right of each blot.
recent study with a middle cerebral artery occlusion model showed that pathological changes in oligodendrocytes appear in the early stage and that these changes seem to be primary and simultaneous with, but independent of, neuronal perikaryal injury. MBP is produced by the oligodendrocytes. Oligodendrocytes cultured under hypoxic conditions have been reported to show a reversible reduction of MBP production. One possible reason for the early and increasing reduction in the MBP level in our animal model is a reduction in MBP production in the oligodendrocytes, which seem to be especially damaged by chronic cerebral hypoperfusion.

Another possible explanation for the reduction in the MBP level is that proteolysis, either general or MBP-specific, is increased in this animal model. There are several reports concerning the activation of calcium-dependent protease under ischemic conditions. A study with anoxic optic nerves showed that the axonal cytoskeleton is protected by decreased extracellular calcium, suggesting that anoxia triggers an abnormal influx of calcium into myelinated axons and activates proteolysis. An analogous mechanism may increase MBP proteolysis in our animal model.

A third possible explanation is that an increase in free radical production as a result of chronic cerebral hypoperfusion injures the myelin sheath and decreases the MBP level in our model. It is known that the activated microglia in cerebral hypoperfusion, axonal injury, and demyelinating disease releases cytotoxic oxygen or nitrogen metabolite. It has also been reported that, in transient ischemia, peroxidation of polyunsaturated fatty acids by oxygen free radical production after recirculation leads to impairment of the myelin sheath.

The present study revealed that the change in the MBP level precedes that in the NFH level in the chronic hypoperfused brain. It is suggested that the primary event in cerebral hypoperfusion is change in the oligodendrocyte, which is vulnerable to this condition, and that the change in the neurofilament follows those in the oligodendrocyte. It has been reported that in peripheral nerves the myelin sheath has a direct influence on the structure and function of the neuronal axon through the regulation of phosphorylation in the neurofilament and other substrates. Through a similar mechanism, alterations in the myelin may affect the integrity of the neurofilament or neuronal axon in the white matter of our animal model.

It is possible that the myelin alterations observed in the animal model in the present study reflect the clinical condition of leukoaraiosis, although the causes of this condition are incompletely understood. The recent understanding is that leukoaraiosis is directly caused by ischemic injuries but not by alterations in cerebrospinal fluid circulation or disturbances in the blood-brain barrier, which are also observed in leukoaraiosis. The myelin rarefaction in leukoaraiosis has been interpreted as incomplete infarction or as the result of an ischemic event not severe enough to cause panneerosis. This explanation for the primary cause of leukoaraiosis is supported by the present study, which shows that myelin rarefaction occurs in an animal model of chronic cerebral hypoperfusion. It is thought that in the brains of patients who have diffuse cerebral white matter changes and progressive cognitive impairment, changes in small arterial branches cause minimal parenchymal lesions whose accumulation directly leads to white matter rarefaction and cognitive impairment without any history of strokes or stepwise progression of symptoms. The situation in the animal model in the present study is similar to that of leukoaraiosis in that stroke attacks and a stepwise progression were never observed, although the animals showed degenerated white matter and learning impairment.

In conclusion, the present study suggests that in cerebral white matter under chronic hypoperfusion the change in...
myelin is the primary pathological event and that the alteration in the axon follows that in myelin. This may help to advance our understanding of the mechanisms of white matter changes in cerebrovascular dementia.

References


Editorial Comment

Leukoaraiosis is characterized by patchy decreases in density in the white matter detected in patients by imaging techniques. This condition is frequently associated with dementia. There is increasing evidence that the pathogenesis of leukoaraiosis is related to ischemia.

In the accompanying article, Kurumatani and colleagues continue studies in a model of chronic ischemia in gerbils. In this model, sustained moderate ischemia for several weeks is associated with lesions in the white matter similar to those of human leukoaraiosis. This model, therefore, may be useful in studying the pathogenesis of the white matter lesions due to ischemia.

Kurumatani and his colleagues measured the concentrations of markers of the three main components of white matter, namely, myelin, axons, and astroglia. They concluded that the earliest change (and very likely, therefore, the primary change in the white matter resulting from ischemia) was disturbed metabolism and synthesis of myelin, resulting in its deterioration. These findings should encourage additional studies to identify the pathogenesis of the myelin lesions due to ischemia.

Hermes A. Kontos, MD, PhD
Associate Editor for Basic Science
Medical College of Virginia
Virginia Commonwealth University
Richmond, Virginia
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