Background and Purpose—Several types of chaperone proteins, such as heat shock proteins, have been reported to be associated with brain ischemia. The purpose of this study was to investigate whether an abnormal expression of 14-3-3 proteins, a novel type of molecular chaperones, occurs in human gray and white matter ischemic lesions.

Methods—We prepared formalin-fixed, paraffin-embedded sections from 33 autopsied brains, consisting of 7 normal controls, 4 cases with cerebral thrombosis, 5 cases with cerebral embolism, 8 cases with multiple lacunar infarctions, and 9 cases withBinswanger disease. Deparaffinized sections from all cases were immunostained with anti–14-3-3 antibodies using the avidin–biotin–peroxidase complex method, and some sections were also double-immunostained for 14-3-3 and glial markers.

Results—In the normal control brains, 14-3-3 immunoreactivity was mainly localized to the neuronal somata and processes. Strongly 14-3-3–immunopositive astrocytes were distributed in the infarct lesions and were particularly abundant in infarcts at the chronic stage. Intensely 14-3-3–immunolabeled astrocytes were also observed in the ischemic white matter lesions, and in the severely affected white matter lesions from patients withBinswanger disease, dense 14-3-3 immunoreactivity was found in clasmatodendritic astroglia as well as in reactive astrocytes.

Conclusions—Our results suggest that 14-3-3 proteins may be induced mainly in astrocytes from human cerebrovascular ischemic lesions, and that the upregulated expression of 14-3-3 proteins in astrocytes may be involved in the formation of astrogliosis. (Stroke. 2006;37:830-835.)

Key Words: astrocytes  Binswanger disease  cerebral infarction  immunohistochemistry

14-3-3 proteins constitute a family of highly conserved molecules expressed in a wide range of eukaryotic cells.\(^1,2\)

The name of 14-3-3 was derived from their fraction number on DEAE-cellulose chromatography and their migration pattern on starch gel electrophoresis.\(^2\) There are 7 mammalian 14-3-3 isoforms named by the Greek letters β, γ, ε, ζ, η, σ, and θ, and the isotypes originally designated as α and δ have been confirmed to be the phosphorylated forms of β and ζ, respectively.\(^2,3\) 14-3-3 proteins are involved in various kinds of signal transduction pathways through phosphorylation-dependent protein–protein interactions.\(^2\)

Several types of chaperone proteins, including heat shock protein 70 (HSP70),\(^4\) HSP27,\(^5\) and 150-kDa oxygen-regulated protein,\(^6\) have been demonstrated to be associated with ischemia-induced brain damage. Because ischemic rat brains have been reported to contain strong immunoreactivity for 14-3-3,\(^7\) we hypothesized that 14-3-3 proteins, a novel type of molecular chaperones, would be expressed abnormally in brains from patients with cerebrovascular disease (CVD). In the present study, we performed immunohistochemical studies on 14-3-3 proteins in autopsied human brain tissues from patients with CVD, including Binswanger disease. We found the increased expression of 14-3-3 immunoreactivity in astrocytes from human cerebrovascular ischemic lesions.

Materials and Methods

Tissue Preparation

Tissue materials were obtained from autopsied brains collected at the Neuropathology Laboratories of Kyoto University and the University of Vienna. We selected a total number of 33 cases consisting of 7 normal controls (5 men, 2 women; age range 68 to 84 years; mean ± SD, 75.9 ± 5.6 years), 4 patients with cerebral thrombosis (3 men, 1 woman; age range 74 to 87 years; mean ± SD, 80.3 ± 6.2 years), 5 patients with cerebral embolism (3 men, 2 women; age range 63 to 87 years; mean ± SD, 76.0 ± 9.4 years), 8 patients with multiple lacunar infarctions (5 men, 3 women; age range 72 to 86 years; mean ± SD, 78.6 ± 4.6 years), and 9 patients with Binswanger disease (6 men, 3 women; age range 64 to 86 years; mean ± SD, 76.1 ± 8.2 years). Binswanger disease is a form of vascular dementia, and diffuse white matter (WM) lesions are the main pathological features of brains with Binswanger disease. All autopsied brains were fixed in 10% neutral formalin for ~2 weeks at room temperature. Several paraffin-embedded tissue blocks from each case were prepared and were cut into 6-μm-thick sections on a microtome. For routine pathological evaluation, deparaffinized sections from all cases were stained with hematoxylin and eosin, Klüver-Barrera, and modified Bielschowsky stains. The stages of the infarcted lesions
were classified into 3 types as follows: acute (within 1 week after onset), subacute (1 to 4 weeks), and chronic (over 4 weeks). The WM lesions were graded as normal (grade 0), low (grade I; reduced meshwork density with scattered, irregularly widened axons), moderate (grade II; further reduction in meshwork density compared with grade I, mainly composed of relatively short axons), and high (grade III; depletion of axon meshwork with a few remaining long axons) according to the modified criteria proposed by Englund and Brun. The areas examined in this study are schematically presented in Figure 1, and the clinicopathological profiles from all cases are summarized in Table 1. All procedures followed were in accordance with institutional guidelines, and informed consent was obtained from relatives of all subjects.

**Primary Antibodies**

To examine the immunohistochemical localization of 14-3-3 proteins in autopsied brains, we used several types of anti–14-3-3 antibodies as follows: a mouse monoclonal anti–14-3-3β antibody (H-8; Santa Cruz Biotechnology [SCB]; diluted 1:1000), a rabbit polyclonal anti–14-3-3β antibody (C-20; SCB; diluted 1:400), a rabbit polyclonal anti–14-3-3γ antibody (C-16; SCB; diluted 1:400), a rabbit polyclonal anti–14-3-3ε antibody (T-16; SCB; diluted 1:400), a rabbit polyclonal anti–14-3-3ζ antibody (C-17; SCB; diluted 1:400), a rabbit polyclonal anti–14-3-3η antibody (Immuno-Biological Laboratories [IBL]; diluted 5 μg/mL), and a rabbit polyclonal anti–14-3-3r antibody (IBL; diluted 2 μg/mL). The anti–14-3-3β antibody (H-8) recognizes all human 14-3-3 isoforms, whereas the other antibodies are 14-3-3 isoform specific.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene, followed by rehydration in a series of ethanol solutions of decreasing concentration. The deparaffinized sections were pretreated with 0.3% H2O2 (Santoku) in 0.1 mol/L PBS for 30 minutes at room temperature to inhibit endogenous peroxidase activity. After washing with 0.1 mol/L PBS, the sections were blocked with 0.1 mol/L PBS plus 3% skimmed milk for 2 hours at room temperature. After rinsing with 0.1 mol/L PBS, the primary antibody diluted in 0.1 mol/L PBS was applied onto the sections, and these sections were maintained at room temperature overnight in a humidified chamber. After washing with 0.1 mol/L PBS, bound antibodies were visualized by the avidin-biotin-peroxidase complex (ABC) method using biotinylated secondary antibodies (Vector Laboratories), Vectastain ABC kits (Vector), and diaminobenzidine tetrahydrochloride (Dojin) as a chromogen. Some sections were incubated with either nonimmune mouse or rabbit serum, and no specific immunopositive staining was detected in these negative control sections (data not shown).

**Double Immunostaining for 14-3-3 and Glial Markers**

To evaluate the relationship between 14-3-3 proteins and glial cells in cerebrovascular ischemic lesions, we selected a total number of 55 areas from both control and CVD groups, consisting of 5 acute infarcted areas, 5 subacute infarcted areas, 5 chronic infarcted areas, 10 WM areas of grade 0, 10 WM areas of grade I, 10 WM areas of grade II, and 10 WM areas of grade III. These area-containing sections were double-immunostained using antibodies directed against 14-3-3 (H-8) and glial markers. The following antibodies were prepared: an anti–glial fibrillary acidic protein (GFAP) antibody (DAKO; rabbit polyclonal; diluted 1:1000) as a major marker for astrocytes, an anti-vimentin antibody (DAKO; mouse monoclonal; diluted 1:1000) as another marker for astrocytes, an anti-vimentin antibody (DAKO; rabbit polyclonal; diluted 1:1000) as a marker for oligodendrocytes, and an anti-CD11b antibody (SCB; goat polyclonal; diluted 1:100) as a marker for microglia. After an incubation with the primary antibodies, the sections were reacted with secondary antibodies consisting of fluorescein isothiocyanate–labeled goat anti-mouse IgG (DAKO) and rhodamine-conjugated swine anti-rabbit IgG (DAKO) or rhodamine-conjugated donkey anti-goat IgG (Chemicon International). After washing with 0.01 mol/L PBS, the slides were coverslipped with Vectashield (Vector) and viewed with the aid of a fluorescence microscope. The 14-3-3-immunolabeled glial density was assessed by calculating the average percentage of 14-3-3–positive glial cells in various types of ischemic lesions and was divided into 5 categories: high (>80%), moderate (from 50% inclusive to 80% exclusive), low (from 10% inclusive to 50% exclusive), very low (<10%), or absent. The density of vimentin–positive astrocytes in the ischemic lesions was evaluated using the same double-immunofluorescence staining for GFAP and vimentin.

**Results**

In the normal control brains, 14-3-3 immunoreactivity was mainly localized to the neuronal somata and processes (Figure 2A). In contrast to this intense neuronal immunolabeling pattern, the glial...
elements generally showed faint or no 14-3-3 immunoreactivity in the normal control cortical and WM areas (Figure 2B).

In the acute infarcted lesions, there were many neurons in which an excessive accumulation of 14-3-3 immunoreactivity was found in the perinuclear areas (Figure 3A), and moderately immunopositive glial cells, including astrocytes, were sparsely scattered (Figure 3B). At the subacute stage, 14-3-3 immunoreactivity was well-preserved in most remaining neurons (Figure 3C). Intensely immunostained reactive astrocytes were distributed around the infarcts (Figure 3D), and immunolabeled macrophages accumulated in the center of these infarcts (Figure 3E). In the chronic infarcted lesions, intensely immunopositive ballooned neurons were scattered (Figure 3F). Numerous reactive astrocytes showed strong 14-3-3 immunoreactivity (Figure 3G), and densely immunopositive gemistocytic astrocytes with hypertrophic cell bodies (Figure 3H) and fibrillary astrocytes (Figure 3I) were observed in some areas of the chronic infarcts.

In the mildly affected WM lesions (grade I), some glial cells and axons exhibited moderate 14-3-3 immunoreactivity was found in the perinuclear areas (Figure 2A), and moderately immunopositive glial cells, including astrocytes, were sparsely scattered (Figure 2B). At the subacute stage, 14-3-3 immunoreactivity was well-preserved in most remaining neurons (Figure 3C). Intensely immunostained reactive astrocytes were distributed around the infarcts (Figure 3D), and immunolabeled macrophages accumulated in the center of these infarcts (Figure 3E). In the chronic infarcted lesions, intensely immunopositive ballooned neurons were scattered (Figure 3F). Numerous reactive astrocytes showed strong 14-3-3 immunoreactivity (Figure 3G), and densely immunopositive gemistocytic astrocytes with hypertrophic cell bodies (Figure 3H) and fibrillary astrocytes (Figure 3I) were observed in some areas of the chronic infarcts.

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In the moderately affected WM lesions (grade II), the number of immunopositive glial cells, some of which were strongly immunostained, had increased (Figure 4B), and immunolabeled glial cells were abundant in some areas of these WM lesions (Figure 4C). In the severely affected WM lesions (grade III) fromBinswanger disease brains (Figure 4G), numerous reactive astrocytes were intensely immunopositive for 14-3-3 (Figure 4D). In addition, in the Binswanger disease cases with severe rarefactions (Figure 4H and 4I), the so-called “clasmatodendritic astroglia,” were also densely immunostained (Figure 4E), and their cell bodies were swollen and vacuolated, with their processes disintegrated (Figure 4F).

Immunohistochemical studies on the 14-3-3 isoforms in several sorts of human cerebrovascular ischemic lesions showed that the astrocytes were immunoreactive for all types of 14-3-3 isoforms. In the severely affected WM lesions, not only reactive astrocytes (Figure 5A through 5E) but also clasmatodendritic astroglia (Figure 5F) were immunolabeled by all of the 14-3-3 isoform-specific antibodies. Further immunohistochemical studies of serial sections immunostained by the different 14-3-3 isoform-specific antibodies demonstrated that most reactive astrocytes contained ≥2 14-3-3 isoform immunoreactivities, suggesting that several kinds of 14-3-3 isoforms may be induced into each reactive astrocyte.

The double-immunostaining studies revealed that the majority of 14-3-3-immunopositive glial cells were astrocytes in the infarcted areas and ischemic WM lesions (Figure 6A through 6C), and some oligodendrocytes (Figure 6D through 6F) and
microglia (Figure 6G through 6I) were also immunoreactive for 14-3-3 in both types of ischemic lesions. The data for the density of 14-3-3–positive glial cells and vimentin-positive astrocytes (figure not shown) is summarized in Table 2.

**Discussion**

In the present study, we performed immunohistochemical studies on 14-3-3 proteins in autopsied human brains with CVD. The most remarkable finding of our results was the upregulated expression of 14-3-3 immunoreactivity in astrocytes from both cerebral infarctions and ischemic WM lesions, suggesting that 14-3-3 proteins may be induced mainly in astrocytes by ischemic stress. The enhanced astroglial immunoreexpression of 14-3-3 has been documented in demyelinated lesions from patients with multiple sclerosis and in cortical and subcortical lesions from patients with Creutzfeldt-Jakob disease. Moreover, similarly to multiple sclerosis and Creutzfeldt-Jakob disease, reactive astrocytes were immunoreactive for all kinds of 14-3-3 isoforms in the cerebrovascular ischemic lesions. These data suggest that the increased expression of 14-3-3 immunoreactivity in astrocytes is not specific for ischemia, and that 14-3-3 proteins may be induced in astrocytes by several kinds of pathological conditions.

GFAP and vimentin, both of which are the markers most often used for identifying astrocytes, increase in reactive astrocytes. Satoh et al found the differential immunoreexpression of 14-3-3 isoforms in reactive astrocytes from demyelinating lesions in patients with multiple sclerosis and demonstrated that the 14-3-3 isoforms, particularly 14-3-3ε, interacted with GFAP and vimentin in cultured human astrocytes. On the basis of the fact that GFAP and vimentin are coexpressed and copolymerized in assembled filaments in astrocytes, they proposed the possibility that 14-3-3 proteins may play an organizing role in the intermediate filament network in reactive astrocytes from the demyelinating lesions of multiple sclerosis. Not only GFAP, but also vimentin are expressed in reactive astrocytes of human cerebral infarcts, and our present study showed the immunohistochemical localization of 14-3-3 isoforms, including 14-3-3ε, in reactive astrocytes from infarcted lesions. Our results suggest that similarly to the demyelinating lesions of multiple sclerosis, 14-3-3 proteins may act as an adaptor that connects GFAP and vimentin in reactive astrocytes of human cerebral infarcts. Although...
neurons contain intense 14-3-3 immunoreactivity, they are generally sensitive to ischemic insults, suggesting that the network formation of GFAP and vimentin by 14-3-3 proteins may be closely related to the survival of astrocytes under ischemic conditions.

In ischemic WM lesions, some astrocytes undergo morphological alterations in which their cell bodies become swollen and vacuolated, and their disintegrated processes are identified as granules of 1 to 3 μm in diameter. These regressive astrocytes are termed “clasmatodendritic astroglia,” and their cytochemical and immunohistochemical features suggest that clasmatodendritic astroglia incorporate edematous fluid and phagocytose cellular debris and eventually degenerate as a result of cerebral edema.18–20 These regressive astrocytes are termed “clasmatodendritic astrocytes,” and their cytochemical and immunohistochemical features suggest that clasmatodendritic astroglia incorporate edematous fluid and phagocytose cellular debris and eventually degenerate as a result of cerebral edema.18–20 Thus, our findings suggest that both GFAP and vimentin may be target proteins for 14-3-3 proteins in clasmatodendritic astroglia as well as reactive astrocytes in the severely affected WM lesions from patients withBinswanger disease. Both reactive and clasmatodendritic astroglia have been reported to be immunoreactive for GFAP and vimentin.18–20 Thus, our findings suggest that both GFAP and vimentin may be target proteins for 14-3-3 proteins in clasmatodendritic astroglia as well as reactive astrocytes in the severely affected WM lesions from patients withBinswanger disease. Both reactive and clasmatodendritic astroglia have been reported to be immunoreactive for GFAP and vimentin.18–20 Thus, our findings suggest that both GFAP and vimentin may be target proteins for 14-3-3 proteins in clasmatodendritic astroglia as well as reactive astrocytes in the ischemic WM lesions, and that 14-3-3 proteins may be associated with the regulation of the intermediate filament network, in even these regressive astrocytes, but 14-3-3 overexpression mechanisms might be insufficient to protect them from ischemic damage under severe ischemic conditions.

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Upregulated Expression of 14-3-3 Proteins in Astrocytes From Human Cerebrovascular Ischemic Lesions
Yasuhiro Kawamoto, Ichiro Akiguchi, Hidekazu Tomimoto, Yoshitomo Shirakashi, Yasuyuki Honjo and Herbert Budka

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