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Degenerating and regenerating changes in remote areas from the caudo-putaminal mass lesion in rats

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Abstract The degree of intracerebral damage after hemorrhage usually depends on the localization and size of the hematoma. However, unexpected worsening of symptoms can occur in some patients with intracerebral hematoma, partly due to reduction of cerebral blood flow (CBF) or metabolism in areas remote from the primary lesion. In order to examine the features of neuronal degeneration and synaptic reorganization in such remote areas, we investigated neuropathological changes using a modified hemorrhagic rat brain model. Intracerebral mass lesions were created by inflating a microballoon in the caudo-putamen for 10 or 120 min. Physiological parameters, including mean arterial blood pressure and blood gas and pH, were measured at three time points (before, 60 min after microballoon inflation, and after microballoon deflation). Neuropathological changes were assessed in animals sacrificed 1, 2 and 7 days after microballoon deflation, using the suppressive Nauta silver method and immunostaining for growth-associated protein (GAP)-43. There were no significant differences in any of the physiological variables. The focal CBF in the contralateral primary somatosensory cortex decreased 5 min after microballoon inflation, then returned to the baseline 30 min after inflation. After microballoon deflation, the focal cortical CBF increased, being greater than the baseline level 30 min later. In this model, neuronal damage was not observed histopathologically in spite of the reduction in cortical CBF. Argyrophilic degenerated neurons were observed widely in laminae 3 and 5 of the bilateral cerebral cortex (more so ipsilaterally) and in the substantia nigra one day after microballoon deflation. The difference in the number of degenerated neurons between rats subjected to microballoon inflation for 10 and 120 min did not reach statistical significance. GAP-43 immunoreactivity became apparent 2 days after deflation and lasted for 7 days. Inhibition of neuronal degeneration and promotion of synaptic reorganization in remote areas could improve the outcome of patients with intracerebral hemorrhage.
(303 words)

Introduction

The prognosis of spontaneous intracerebral hemorrhage (ICH) generally depends on its location and size. In some patients, however, the clinical symptoms and neurological outcome seem to be worse than those predicted by the location and size of the ICH, even if there are no other clinical complications.

Several studies using magnetoencephalography[1,2] have revealed that neurons in the neighborhood of a lesion gain new receptive fields after creation of cortical lesions in the adult monkey or human. Baron et all.[3] used positron emission tomography to measure cerebral metabolism in patients 4 days after the onset of thalamic hemorrhage, and observed that cortical metabolism was reduced in the contralateral as well as the lesioned side. Likewise, using xenon- enhanced computed tomography, Tanaka et al.[4] demonstrated that contralateral hemispheric cerebral blood flow (CBF) was still reduced two months after the onset of putaminal or thalamic hemorrhage. These reductions in areas remote from the primary lesion are explained by a transneural depression ("diaschisis") originating from the dysfunctional thalamus or putamen.[3,4]

However, such neuropathological changes have never been fully investigated using a hemorrhagic animal brain model.

In order to gain further insight into such remote effects, we used a microballoon technique[5] to investigate physiological changes including mean arterial blood pressure and gas and pH, and the focal cortical cerebral blood flow (CBF) in the contralateral primary somatosensory cortex. We then studied neuronal degeneration and synaptic reorganization in areas remote from the caudo-putaminal mass lesion using the suppressive Nauta method[6,7] and immunostaining for growth-associated protein (GAP)-43, which is produced during the axonal sprouting that leads to synaptic reorganization.[8]

Materials and Methods

Animal preparation

The experimental protocols used in this study were approved by the Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine, and were carried out according to the Guidelines for Animal Experimentation of Yamaguchi University School of Medicine, and Japanese Law (No.105) and Notification No.6 of the Japanese Government.

The experiments were performed on 33 male Wistar rats weighing 280 to 320g. The animals were anesthetized in a chamber using 4% (v/v) halothane in a mixture of 30% oxygen and 70% nitrous oxide. Anesthesia was maintained under spontaneous respiration with 0.8-1.0% (v/v) halothane in the same oxygen and nitrous oxide mixture, administered using a face mask. Subsequently, a tail artery was cannulated for continuous measurement of mean arterial blood pressure (MABP) and blood gas analysis. The core body temperature was maintained between 36.8 and 37.2℃ with a heating pad. In all experimental groups, the MABP was maintained above 90 mmHg, $PaCO_2$ at 45-50 mmHg, and PaO_2 above 100 mmHg.

Intracerebral mass model

Intracerebral mass model was made according to the method by Kingman et al.[5,9,10] This method consistently produces an identical solid mass lesion using a microballoon. Deflating the balloon can also simulate surgical treatment of ICH lesions. Briefly, each animal was placed in a stereotaxic head frame and the skull was exposed through a 10 mm midsagittal skin incision. A single burr hole was made above the right caudo-putamen with a saline-cooled dental drill, based on stereotaxic coordinates (1.0 mm anterior to and 3.2 mm lateral to the bregma). The dura mater was opened, and a No. 15 Goldvalve balloon (Ingenor Laboratories, Paris, France), mounted on a 25-gauge needle, was inserted stereotaxically to a depth of 5.5 mm from the brain surface into the center of the caudo-putamen. The burr hole was sealed with bone wax. After a 30-min stabilization period, the microballoon was inflated over 30 s with 50 μl normal saline delivered from a microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). The microballoon remained inflated for 120 min and was then deflated. The needle and microballoon were withdrawn 30 min after deflation. Following recovery from anesthesia, all the animals returned to normal feeding and activities.

The rats were sacrificed at various times after deflation of the microballoon (1, 2 and 7 days ; n = 6 for each time point). The animals were randomly allocated to each group, and were perfusion-fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline

(PBS) under deep general anesthesia. The brains were removed carefully, fixed for 1 day in 4% (v/v) paraformaldehyde in PBS, and embedded in paraffin. The brains were cut, and 5-μm-thick coronal sections were made every 0.5 mm between 2.0 mm anterior and 6.0 mm posterior to the bregma.

In rats subjected to a sham operation (n = 3 for each time point), the microballoon and the needle were inserted, as described above, but the microballoon was not inflated. Survival times of these rats after were 1, 2 and 7 days. To investigate whether the time for which the microballoon is inflated affects the degree of neuronal damage in remote regions, the microballoon was inflated for 10 min in another 6 rats. These rats were sacrificed 1 day after deflation of the microballoon.

Histopathological examination for degenerative changes

In the Nauta silver impregnation technique, the binding sites for metallic silver are cytoplasmic protein fractions, produced by the disassembly of microtubules and microfilaments.[6,7,11] When neurites become nonviable , they can be detected with a high degree of selectivity and sensitivity.[12] In brief, the sections were treated with 15% (v/v) ethanol, then with 0.5% (v/v) phosphomolybdic acid. They were then oxidized in a solution of 0.5% (w/v) potassium permanganate to suppress the argyrophilia of normal neuronal cells and fibers. After rinsing, the sections were bleached in a mixture of equal parts 1% (w/v) oxalic acid and 1% (w/v) hydroquinone, after which they were impregnated with a 1.5% (w/v) solution of silver nitrate, then with ammoniacal silver nitrate solution. The sections were then transferred to a reducing fluid, until a brown color developed. Changes in neuronal morphology and its distribution were examined by light microscopy.

One day after microballoon deflation, we compared the number of argyrophilic neurons between rats subjected to inflation for 10 and 120 min. The numbers of argyrophilic neurons in the following 10 regions were counted at two representative coronal levels[13]: primary somatosensory cortex on the lesioned and contralateral sides, secondary somatosensory cortex on both sides at 1.0 mm anterior to the bregma, and visual cortex, auditory cortex and substantia nigra on both sides at 5.2 mm posterior to the bregma.[14]

Immunohistochemistry for GAP-43

The labelled streptavidin-biotin (LSAB) method was employed for GAP-43 immunohistochemistry. This was performed using an antibody against GAP-43 (0.5 μg/ml, mouse monoclonal, Boehringer Mannheim, Germany). Sections were placed in a plastic Coplin jar containing a sufficient quantity of Target Retrieval Solution (DAKO, CA, USA), and microwaved at 98°C for 10 min with a microwave processor (BIO RAD H-2500, BIO RAD, England).[15,16] Sections were incubated with the primary antibody against GAP-43 for 48 h at 4°C in a humidified chamber. All sections were counterstained with hematoxylin. The negative control sections were incubated with PBS and normal mouse serum instead of the primary antibody.

Cerebral blood flow measurement

Cortical cerebral blood flow (CBF) in the contralateral cortex was measured in six animals subjected to inflation of the microballoon for 120 min using laser Doppler flowmetry (LDF) (ALF 21, Advance, Japan), which allowed continuous monitoring of focal CBF during the experiments. For these focal cortical CBF measurements, another burr hole was made above the left primary somatosensory cortex, that is, 2.0 mm posterior to and 4.5 mm lateral to the bregma. The LDF probe was then placed gently on the dura mater.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Differences in physiologic variables were tested for statistical significance using one-way analysis of variance (ANOVA) followed by Scheffé's test for multiple comparisons. Differences in the number of degenerated neurons between rats subjected to microballoon inflation for 10 and 120 min were analyzed using the unpaired t-test. A p value <0.05 was considered to indicate a statistically significant difference.

Table 1. Physiological variables before, 60 minutes after microballoon inflation, and after microballoon deflation.

	before inflation	60min. after inflation	after deflation
MABP (mmhg)	116.5± 5.0	118.7±4.9	118.5± 3.4
pH	7.39± 0.02	7.38±0.02	7.38± 0.02
PO_2 (mmHg)	129.1±11.6	140.8±9.4	143.2±10.5
PCO_2 (mmHg)	47.5± 5.0	47.7±1.7	48.2± 1.2

Results

Physiological Parameters

As shown in Table 1, there were no significant differences in any of the physiological variables, such as mean arterial blood pressure and blood gas or pH, between the three time points (before and 60 min after microballoon inflation, and after microballoon deflation).

Focal cortical CBF

The changes in focal cortical CBF in the contralateral primary somatosensory cortex are shown in Fig. 1. The focal cortical CBF decreased by 12.2±7.0% from baseline (100%) 5 min after microballoon inflation, then returned to baseline 30 min after inflation. After microballoon deflation, the focal cortical CBF increased, becoming 22.8± 19.7% greater than the baseline level 30 min later. In this model, neuronal damage was not observed histopathologically in spite of the

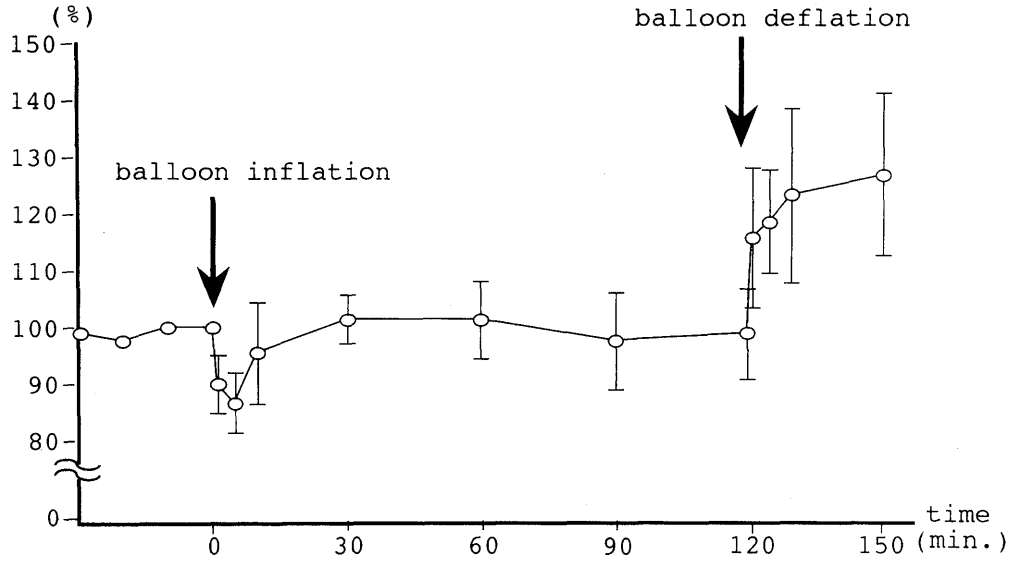

Fig. 1 The changes of focal cerebral blood flow (CBF) in the contralateral primary somatosensory cortex in rats with 120 min microballoon inflation. The baseline of focal cortical CBF is that just before microballoon inflation. The focal cortical CBF temporarily, but not critically, decreased between 5 and 30 min after microballoon inflation.

Table 2. The number of degenerated neurons at 1 day after microballoon deflation

area No.	1	2	3	4	5	6	7	8	9	10
10min	56±12	30±6	51±5	31±3	83±6	53±6	81±16	51±13	71±3	16±8
120min	50±10	50±10	48±5	29±3	54±7	54±7	82±13	58±13	70±6	25±8

Area No. 1-4 is 1.0 mm anterior to the bregma, and 5-10 is 5.2 mm posterior to the bregma. 1 and 5 : medial part of the lesioned side, 2 and 6 : medial part of the contralateral side, 3 and 7 : lateral part of the lesioned side, 4 and 8 : lateral part of the contralateral side, 9 : substantia nigra of the lesioned side, 10 : substantia nigra of the contralateral side.
Values are mean ± standard deviation (SD).

reduction in cortical CBF.

Degenerative changes by the suppressive Nauta method

The suppressive Nauta method gave highly reproducible and selective argyrophilic staining of degenerated neurons (Fig. 2a). In rats given a sham operation, only the regions along the tract of microballoon insertion were destroyed, and very few argyrophilic neuronal changes were scattered in the cerebral cortex. There was no statistically significant difference in the number of degenerated neurons between the rats subjected to 10 and 120 min of microballoon inflation (Table 2).

In rats with microballoon inflation, degenerated neurons were detected in laminae 3 and 5 of the motor, sensory, sensorimotor, visual, and auditory cortex, and were medium to large pyramidal neurons, 10±25 μm in size. These degenerative changes occurred on both sides, but were predominant on the lesioned side, except for the rostral medial part of the cerebral cortex.

In the substantia nigra pars reticulata (SN pr), the degenerated neurons were multipolar or triangular, and 20±35 μm in size (Fig. 2b and c). These degenerated neurons were predominantly located in the SNpr on the lesioned side, and were observed less frequently on the contralateral side. In other remote areas, such as the hippocampus and thalamus, no significant numbers of degenerated neurons were observed. The distribution of degenerated neurons is shown in Fig. 3.

Degenerated neurons with argyrophilic changes were detectable as early as 1 day after microballoon deflation, without any

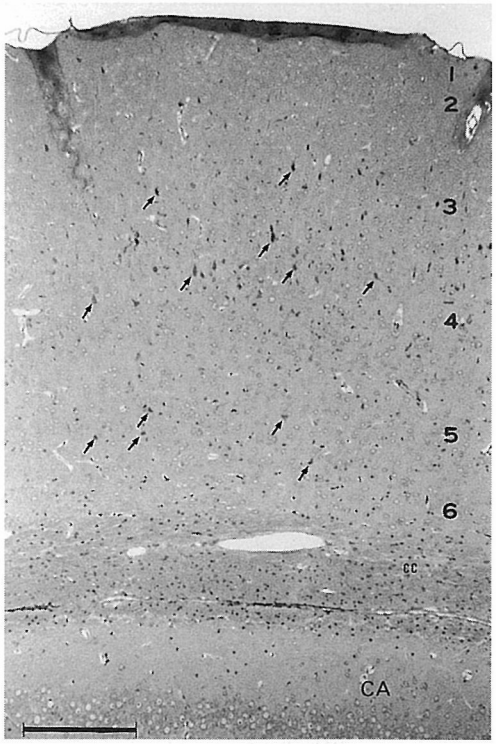

Fig. 2a Degenerated neurons in the somatosensory cortex on the lesioned side. Degenerated neurons are argyrophilic (arrows). cc: corpus callosum, CA: hippocampus. Lamina is indicated as 1 to 6. Scale bar is 400 μm.

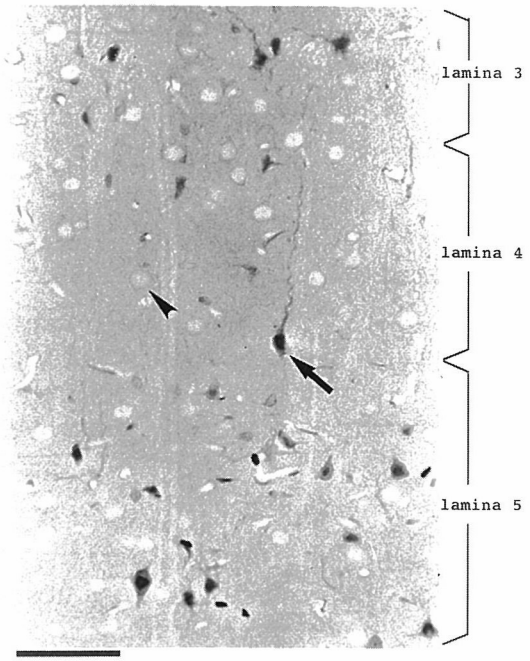

Fig. 2b Higher magnification of fig. 2a. Degenerated neurons are heavily argyrophilic (arrow), whereas non-degenerated neurons are unstained (arrowhead). Degenerated pyramidal neurons 10±25 μm in size are detectable in laminae 3 and 5. Scale bar is 100 μm.

noticeable structural changes in such neuron. In both the cortex and SNpr, there was no obvious change in the number or distribution of degenerated neurons during the 7 days after microballoon deflation. However, the nucleus and nucleolus of each argyrophilic neuron became vague at 7 days.

Immunohistochemistry for GAP-43

In rats given a sham operation, the GAP-43 immunoreactivity was increased in the cytoplasm of the pyramidal cell layer of the hippocampus. In other areas such as the cerebral cortex, no such increase was detected.

In rats subjected to microballoon inflation, except for the pyramidal cell layer of the hippocampus, the immunoreactivity of GAP-43 was increased in the deep lamina of the cerebral cortex at 2 days after microballoon deflation. Such immunoreactivity increased gradually until 7 days after deflation in the perikarya and growth cones (Fig. 4a and b), and was detected in the deep lamina of the cerebral cortex on both sides at 7 days. GAP-43 immunoreactivity was not observed in the white matter. These cortical areas showing GAP-43 immunoreactivity were larger than the areas in which neuronal degeneration was observed. GAP-43 was

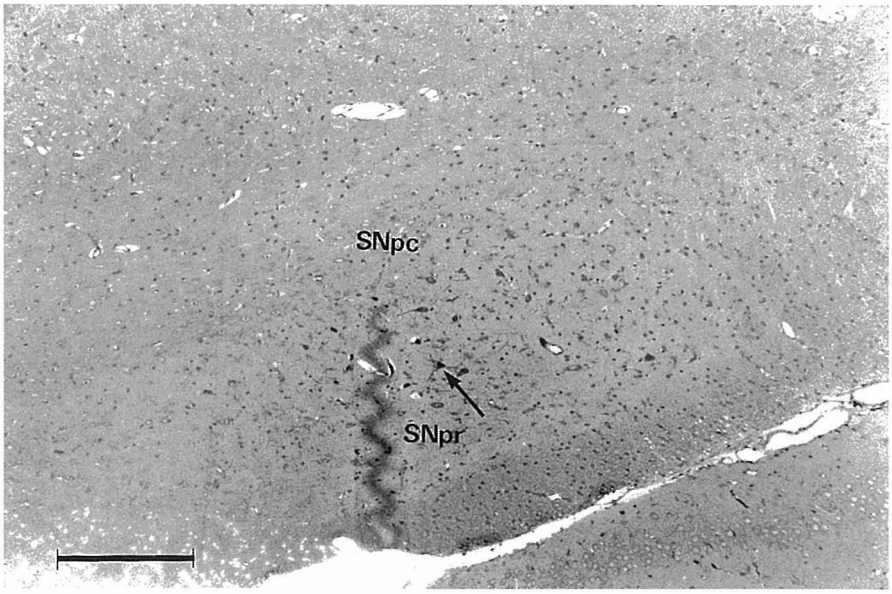

Fig. 2c Degenerated neurons in the substantia nigra pars reticulata on the lesioned side. Degenerated neurons are multipolar or triangular, measuring 20±5 μm (arrow). SNpr : substantia nigra pars reticulata, SNpc : substantia nigra pars compacta. Scale bar is 400 μm.

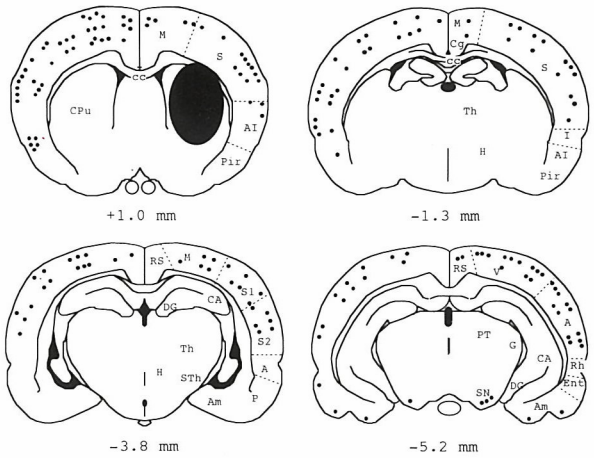

Fig. 3 The distribution of neuronal degeneration by suppressive Nauta method. One dot shows 10 degenerated neurons. (+ : anterior to the bregma, − : posterior to the bregma) Degenerated neurons were distributed in laminae 3 and 5 of the cortex and in substantia nigra (pars reticulata) of the contralateral as well as the lesioned hemisphere. Black area shows the lesion by microballoon inflation.

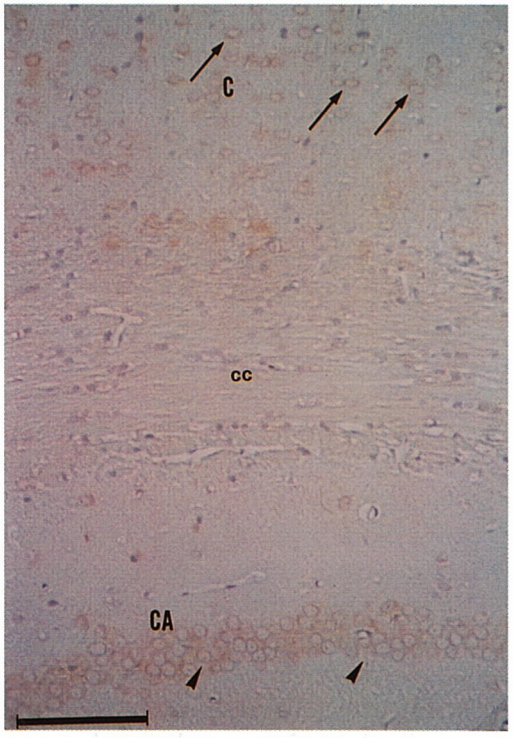

Fig. 4a GAP-43 immunostaining in the deep lamina of the contralateral somatosensory cortex at 7 days after microballoon deflation. GAP-43 immunoreactivity was positive diffusely in the cerebral cortex (arrows) and pyramidal cell layer of the hippocampus (arrowheads). C : cerebral cortex, cc : corpus callosum, CA : hippocampus. Scale bar is 400 μm.

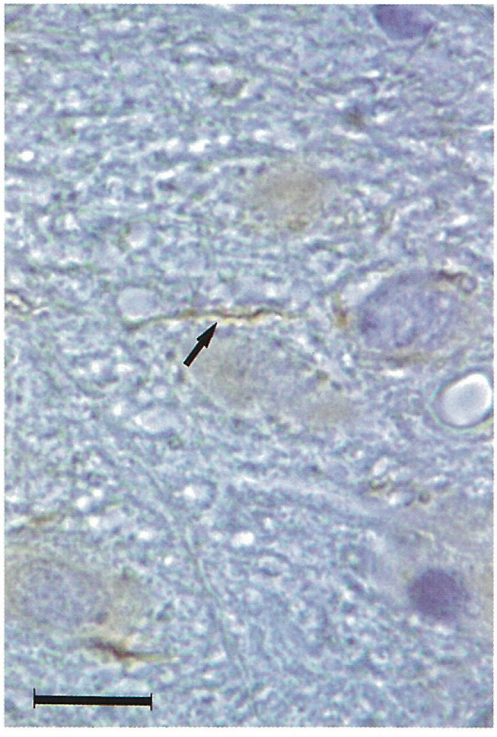

Fig. 4b Higher magnification of fig. 4a. GAP-43 immunoreactivity was also positive in the growth cone (arrow). Scale bar is 25 μm

immunohistochemically positive in the pyramidal cells of the hippocampus, as well as in rats subjected to a sham operation. GAP-43 immunoreactivity was not detected in the

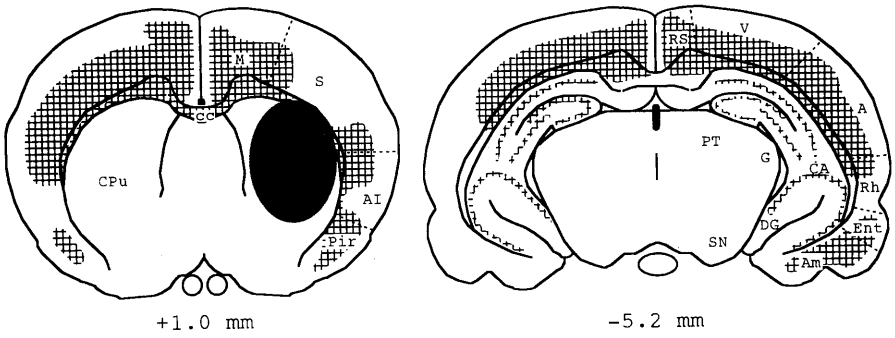

Fig. 5 The distribution of GAP-43 immunoreactivity at 7 days after microballoon deflation (+ : anterior to the bregma, − : posterior to the bregma, Black area shows the lesion by microballoon inflation.). The increased immunoreactivity of GAP-43 is detected in the deep lamina of bilateral motor, sensory, somatosensory, visual, auditory, and piriform cortices, and in granule cells in the hippocampus. The lattice area shows the distribution of GAP-43 immunoreactivity.

substantia nigra. The distribution of the increased immunoreactivity for GAP-43 at 7 days after microballoon deflation is shown in Fig. 5.

Discussion

Remote effects of intracerebral mass lesions

In these experiments, microballoon inflation did not affect any physiological variables such as mean arterial blood pressure and blood gas or pH. Argyrophilic neuronal changes were observed in all major regions of the cerebral cortex and SNpr 1 day after microballoon deflation. These argyrophilic changes were detected on the contralateral as well as in the ipsilateral side (more so ipsilaterally). Regarding the neuronal degeneration in the contralateral cerebral cortex, two plausible factors can be considered. One is ischemic changes caused by the mass effect resulting from cortical compression and distortion. It is unclear whether or not CBF is reduced in the cerebral hemisphere. Kingman et al.[5] reported no significant reductions in CBF in the cerebral hemisphere when a 50-μl microballoon was used in a rat model similar to ours, whereas Tanaka et al.[4] reported that CBF was reduced in the whole hemisphere using xenon-enhanced computed tomography. There has been no detailed study investigating CBF in the cerebral cortex. Although we did not investigate CBF in the whole hemisphere, no marked reduction of CBF was observed in the somatosensory cortex where argyrophilic neurons were detected. Therefore, we concluded that the argyrophilic neuronal changes in the cerebral cortex were not induced by cerebral ischemia in this experiment. Another possibility is transneuronal degeneration resulting from caudoputaminal destruction. In a detailed investigation by McGeorge and Faull,[17] all major regions of the cerebral cortex of normal rats were shown to project bilaterally onto the striatum, but predominantly ipsilaterally. Donoghue and Wise[18] reported the existence of a strong bilateral projection to the striatum from the rostral medial part (i.e. the motor cortex). Likewise, neuronal cells projecting to the striatum were observed to lie mainly in laminae 3 and 5 of the neocortex and mesocortex, and in the deep lamina of the allocortex.[19] In other studies, these cells were also observed to a small extent in lamina 6.[20,21] Our present results are compatible with the findings of McGeorge and Faull,[17] and Donoghue and Wise.[18]

In the present study, neuronal degeneration also occurred in the SNpr. Stefanis and Burke[22] observed that excitotoxic injury to the striatum of rats produced secondary neuronal degeneration in the SNpr within a few days, but not in the globus palli-

dus or substantia nigra pars compacta. The striatonigral gamma-aminobutyric-acid (GABA)-ergic pathway is well known to project from the caudo-putamen to the SNpr, and a long-lasting decrease in the GABA levels of the ipsilateral SNpr has been recognized to result from destruction of the striatum.[23,24] Accordingly, we speculate that neuronal degeneration in the SNpr, as observed in the present study, may well result from excessive excitation due to loss of inhibitory GABA-ergic input caused by the destruction of the striatum. The reason why neuronal degeneration was not observed in other areas except for cerebral cortex or SNpr remains undetermined in this study. Although plausible factors including types of neurotransmitter or neuronal fibers can be considered, further investigations are required.

Effects of removal of intracerebral mass lesions

The therapeutic strategy for spontaneous ICH remains controversial.[25,26,27,28] In our experiment, there was no difference in the number of degenerated neurons between the rats subjected to microballoon inflation for 10 and 120 min. Of course, other factors, such as blood-induced toxicity, which presumably play a role in producing such neuronal degeneration, can affect patients' outcomes. Our results, however, did not support the idea that relief of the mass effect at an early stage can minimize the number of degenerated neurons.

Synaptic reorganization

GAP-43, which is a component of axonal growth cones, is synthesized predominantly in the neuronal cell body and then conducted by fast axonal transport to the growth cone.[29,30] The immunohistochemical expression of GAP-43 increases in the early stage of synaptic organization, but certain "plastic" areas such as hippocampus and limbic areas have been shown to continue to maintain considerable levels of GAP-43 even in the normal adult.[31,32]

In the present study, immunoreactivity for GAP-43 increased in the deep lamina of the bilateral cerebral cortex 2 to 7 days after the production of a lesion. These areas were larger than those in which neuronal degeneration was observed. Thus, the neurons around the degenerated ones might be prompted to sprout axons to support the interrupted neural network.

In other regions such as the substantia nigra or those adjacent to the mass lesion, we did not detect any increase in GAP-43 immunoreactivity. In the substantia nigra, Goto et al.[33] reported that GAP-43 expression was increased transiently in the SNpr between 3 and 4 days after transient occlusion of the middle cerebral artery. In our study, the increase in GAP-43 immunoreactivity in the substantia nigra might have been very subtle ; otherwise, we might not have been able to detect the transient increase in immunoreactivity. Although it remains undetermined why we did not detect any increase in GAP-43 immunoreactivity in areas adjacent to the mass lesion, the destruction of lesions or interruption of the neuronal network might have been irreversible in these areas.

Conclusions

Neuronal degeneration occurs in areas remote from the primary lesion after creation of a caudo-putaminal mass lesion, and neuronal sprouting occurs in these areas of neuronal degeneration. Widespread neuronal degeneration, which was not ameliorated by shortening the period of microballoon inflation, could be an important factor that worsens the clinical symptoms of patients with ICH. Taken together, we believe that the inhibition of neuronal degeneration and promotion of neuronal sprouting in remote areas might also improve the outcome of patients with ICH.

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