Temporal and Spatial Profile of Apoptotic Cells after Focal Cerebral Ischemia in Rats

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Abstract
The significance of apoptosis in focal ischemia was investigated in the spatial and temporal profiles of apoptotic cells caused by permanent and transient focal ischemia induced in male Wistar rats by intraluminal vascular occlusion. Animals were sacrificed at various times and coronal sections of the brain at the level of the optic chiasm were examined histologically by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling. Animals in both groups showed apoptotic cells in the infarcted area, particularly in the border zone. Animals with permanent ischemia showed more extensive infarct and more rapid appearance of apoptotic cells. Activation of apoptosis might depend on the severity of the ischemic insult. Apoptotic cells were observed at 7 days after the ischemic insult in animals with transient ischemia, suggesting apoptosis is involved in the developments of delayed infarct.

Key words: apoptosis, focal cerebral ischemia, middle cerebral artery, brain edema

Introduction
Apoptosis is a different type of cell death from necrosis, which is characterized by cell shrinkage, chromatin condensation, and the formation of apoptotic bodies, and requires the expression of specific genes. Apoptosis occurs in the developmental nervous system and in normal tissues, as well as in various pathological conditions. Recent investigations have suggested that apoptosis is involved in neuronal death following cerebral ischemia. Apoptosis is believed to be important in delayed neuronal death occurring in the hippocampus after global cerebral ischemia, as well as in focal cerebral ischemia, such as that associated with middle cerebral artery (MCA) occlusion.

However, the true significance of apoptosis remains unknown, particularly in focal ischemia, and the factors influencing the activation of apoptosis have not been determined. Reperfusion may activate apoptosis, but this has not been proved thoroughly. In contrast to necrosis, apoptosis might be controlled by certain genes or factors, so this process could be controlled. Therefore, knowledge of the mechanism of apoptosis might provide new therapeutic approaches to ischemic brain disease.

The present experimental study examined whether there are differences in the localization and the temporal profile of apoptotic cells between permanent and transient focal ischemia in a rat model to investigate the involvement of apoptosis in focal cerebral ischemia.

Materials and Methods

I. Focal ischemia model
The study used 48 male Wistar rats weighing 220 to 300 g (Seiwa Inc., Fukuoka) maintained according to the Guidelines for Animal Experimentation of Oita Medical University and housed in groups of two or three with free access to food and water, and maintained under a 12 hour light/dark cycle.

Focal cerebral ischemia was induced using the method of intraluminal vascular occlusion. In brief, animals were anesthetized with 3.0% halothane and maintained with 1.0% halothane in
70% N₂O/30% O₂ using a face mask. The rectal temperature was maintained at 37°C with a heating pad during the surgery. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were isolated through a ventral midline incision under the operating microscope. A 5-cm length of 4-0 surgical nylon suture, with the distal tip coated with silicon rubber (Xantopren; Bayer Dental Co., Osaka), was inserted from the ECA and gently advanced into the ICA to occlude the origin of the right MCA. Restoration of blood flow in the MCA was accomplished by pulling the nylon suture back to the extracranial ICA.

The rats were divided into three groups: animals with permanent MCA occlusion (Group P), animals with reperfusion of the MCA after 1 hour of occlusion (Group R), and sham-operated animals in which a 15-mm-long nylon suture was inserted into the ICA. This suture was too short to occlude the MCA.

II. Histological examination

Animals in Group P (n = 16) could survive no longer than 24 hours after MCA occlusion due to massive brain edema. Therefore, animals in this group were sacrificed at 3, 6, 12, and 24 hours (n = 4 per time point) after MCA occlusion. In contrast, animals in Group R (n = 28) survived in good condition for more than one week and were sacrificed at 3, 6, 12, and 24 hours and 2, 4, and 7 days after MCA occlusion (n = 4 per time point). Control animals were sacrificed at 24 and 48 hours after the sham operation (n = 2 per time point).

Animals were deeply anesthetized with pentobarbital and the brains were removed following transcardiac perfusion with saline and 4% paraformaldehyde in 0.1 M phosphate-buffered saline. After 2 hours of postfixation, coronal sections were cut at the level of the optic chiasm, embedded in paraffin, and sectioned in the frontal plane at a thickness of 4 µm. Besides routine staining (HE), in situ detection of deoxyribonucleic acid (DNA) fragmentation was investigated using the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) method according to Gavrieli et al. Briefly, after deparafinization, sections were incubated with 20 µg/ml proteinase K for 15 minutes and incubated with 3% H₂O₂ for 5 minutes. Sections were then incubated with TdT [0.3 enzyme unit/µl; Takara Shuzo Co., Ltd., Otsu] and biotin-16-deoxyuridine triphosphate (1 nmol/25 µl; Boehringer Mannheim, Mannheim, Germany) in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM CoCl₂) for 60 minutes at 37°C and then incubated with 2 × SSC (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes. The sections were then processed with the labeled streptavidin-biotin procedure using the DAKO LSAB kit (DAKO Japan, Kyoto). Finally, sections were counterstained with hematoxylin.

TUNEL-positive cells containing apparent apoptotic bodies were regarded as apoptotic cells. Some necrotic cells were also TUNEL-positive, but lacked apoptotic bodies and showed relatively uneven TUNEL staining with cytoplasmic staining (Fig. 1). These cells were not considered as apoptotic cells. Some cells exhibiting cell shrinkage or nuclear condensation mimicked apoptotic cells, but were also excluded unless they contained apoptotic bodies.

Quantification of TUNEL staining used three regions of interest according to the extent of the infarction: the core of the striatum, the medial border zone of the infarct in the striatum, and the deep cortex (Fig. 2). In each region, the total number of apoptotic cells as well as TUNEL-positive cells were counted in five consecutive fields at strong magnification (×400). Statistical analysis using the paired t test was performed to detect differences in the number of cells in the corresponding regions of Group P and Group R at time points up to 24 hours. Scheffe's F tests were also performed to detect differences between the three regions at the same observation time, and between all time segments.

Results

I. Distribution of the infarcted area

All animals in Group P had extensive and well-

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**Fig. 1 Photomicrograph of the cortex 24 hours after permanent occlusion of the ipsilateral middle cerebral artery showing apoptotic cells (arrows) and necrotic cells (arrowheads). TUNEL staining with hematoxylin, × 250.**
demarcated infarction. Soon after the occlusion, the infarction involved most of the striatum and the ipsilateral cortex and the size peaked at 3–6 hours after MCA occlusion (Fig. 2 upper). Necrotic changes such as diffuse pallor of the eosinophilic background, vacuolation of the neuropile, and alterations in the shape of nuclei in the infarction progressed with the duration of MCA occlusion. In contrast, 15 of 28 rats in Group R had infarction localized in the striatum (Fig. 2 middle). In the other 13 rats, infarction extended outside the striatum to the cortex, but the infarcted areas tended to be smaller than those in Group P (Fig. 2 lower).

No evidence of infarction was found in sham-operated rats.

II. Distribution of apoptotic cells

No TUNEL-positive cell was detected in the sham-operated rats. TUNEL-positive cells were observed only in localized areas within the striatum at 3 hours after MCA occlusion in Group P. However, the area containing TUNEL-positive cells extended to the whole striatum including the medial border zone and to the deep cortex after 6 hours and to the whole of infarcted area including the superficial cortex after 12 hours. Up to 24 hours after ischemia, the number of TUNEL-positive cells in each area gradually increased. At every observation time, the number of TUNEL-positive cells was greatest in the core of striatum and smallest in the cortex (Fig. 3 upper).

Apoptotic cells were usually not observed in the early stage after MCA occlusion and became apparent in each area usually after 12 hours. However, the distribution of the apoptotic cells was uneven and there were regional differences. In the core of the striatum, the number of apoptotic cells significantly increased after 12 hours, but remained relatively low throughout the whole observation period. In contrast, the number of apoptotic cells in the medial border zone was several times greater than in the core of the striatum. The cortex was characterized by the delayed appearance of apoptotic cells. A few apoptotic cells were observed in only one of four animals after 12 hours. Thereafter, apoptotic cells became apparent and many more apoptotic cells were observed than in the core of the striatum after 24 hours (Figs. 3 lower and 4).

No TUNEL-positive cells were seen in the corresponding areas of the contralateral hemisphere. However, TUNEL-positive cells appeared in the corpus callosum at 12 hours after MCA occlusion and became apparent at 24 hours. Some of these cells were apoptotic cells (Fig. 5).

Group R showed some differences in the distribution of TUNEL-positive cells compared to Group P. The appearance of TUNEL-positive cells delayed and the smaller number of cells were found in the core of the striatum until 12 hours after MCA occlusion. Thereafter, the number of TUNEL-positive cells sharply increased in the core of striatum and in the medial border zone. At 24 hours, there was no

Fig. 2 Coronal sections at the level of the optic chiasm illustrating the distribution of the infarcted area (shaded area) after permanent middle cerebral artery (MCA) occlusion (upper) and transient MCA occlusion (middle, lower). Three regions of interest (open square) were chosen for quantification of TUNEL staining: the core of the striatum (1), the medial border zone of the infarct (2), and the deep cortex (3).
significant differences in the number of TUNEL-positive cells in those areas compared to Group P. The number of TUNEL-positive cells peaked at 24 hours in the core of the striatum, at 48 hours in the medial border zone, and then rapidly decreased by 7 days in all regions. Animals with infarct only in the striatum had no TUNEL-positive cells in the cortex throughout the observation period, and even animals with extended infarct in the cortex had relatively few TUNEL-positive cells (Fig. 6).

The distribution of the apoptotic cells until 24 hours was similar to that in Group P, but the numbers tended to be smaller in each region. Thereafter,
the number of apoptotic cells increased up to 7 days in the core of the striatum, up to 48 hours in the medial border zone and then gradually decreased up to 7 days. In the cortex the number remained unchanged until 4 days, then decreased at 7 days. When the infarct extended from the striatum to the cortex, the greater number of apoptotic cells were observed in the medial border zone after 24 hours. Apoptotic cells were not observed in the cortex when the infarct was localized in the striatum (Fig. 7).

In contrast to the findings in animals of Group P, no TUNEL-positive cells were found in the contralateral hemisphere including the corpus callosum of Group R.

**Fig. 4 Photomicrographs of the hemisphere 24 hours after permanent occlusion of the ipsilateral middle cerebral artery. upper: Core of the striatum. Most TUNEL-positive cells are necrotic cells and few apoptotic cells are found. TUNEL staining with hematoxylin, ×100. lower: Medial border zone. Apoptotic cells (arrows) are numerous. TUNEL staining with hematoxylin, ×250.**

**Fig. 5 Photomicrograph of the contralateral corpus callosum 24 hours after permanent occlusion of the ipsilateral middle cerebral artery showing apoptotic cells (arrows). TUNEL staining with hematoxylin, ×250.**

**Discussion**

There are several methods to prove the presence of apoptosis under pathological conditions. Apoptosis was originally defined morphologically on the basis of electron microscopic findings. This method still provides the most reliable findings, but is not suitable for quantitative analysis or in situ use. Electron microscopic findings are not always necessary to prove apoptosis if typical apoptotic bodies can be identified using a light microscope.

The TUNEL method is based on the specific binding of TdT to free 3'-OH ends of DNA at the single cell level. This method enables both resolution of the individual cells and in situ use with preservation of the tissue architecture. Therefore, we can examine the quantitative anatomical localization of DNA fragmentation. Since we strictly defined the criteria for apoptotic cells, underestimation of numbers of apoptotic cells is more likely than overestimation.

In our study, TUNEL-positive cells first appeared after 3 hours in the striatum of Group P where the severest ischemic insult occurred in this model and gradually extended to the medial border zone and cortex according to the extension of the infarct. Since these TUNEL-positive cells in the early stage usually did not have apoptotic bodies and were considered necrotic cells, this observation reflects the distribution of necrotic cells after the ischemic insult. Group P showed greater numbers of necrotic cells earlier in the observation period compared to Group R. Therefore, the appearance of necrotic cells could depend on the severity of the ischemic insult,
Fig. 6 Temporal distribution of TUNEL-positive cells in animals with (open circles) and without (filled circles) extended infarct in the cortex after transient middle cerebral artery occlusion (Group R). Only few TUNEL-positive cells were found in the core of the striatum until 12 hours after MCA occlusion. Thereafter, the number sharply increased and peaked at 24 hours in the core of the striatum, at 48 hours in the medial border zone, and at 4 days in the cortex, and then rapidly decreased by 7 days in all regions. Animals with infarct only in the striatum had no TUNEL-positive cells in the cortex and even animals with extended infarct in the cortex had relatively few TUNEL-positive cells. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. the number of cells in Group P.

Fig. 7 Temporal distribution of apoptotic cells in animals with (open circles) and without (filled circles) extended infarct in the cortex after the transient middle cerebral artery occlusion (Group R). In contrast to that in Group P, the numbers of apoptotic cells tended to be smaller in each region until 24 hours, but thereafter increased up to 7 days in the core of the striatum, up to 48 hours in the medial border zone and then gradually decreased up to 7 days. In the cortex the number remained unchanged until 4 days, then decreased at 7 days. When the infarct extended from the striatum to the cortex, the greater number of apoptotic cells were observed in the medial border zone after 24 hours. *p < 0.05 vs. the number of cells in Group P.
as previously reported.\textsuperscript{5,20}

Several previous studies indicated evidence of apoptosis in focal ischemia based on various findings.\textsuperscript{12–15,17,22} The temporal and/or spatial profile of apoptotic cells in focal cerebral ischemia has been investigated in rats using the TUNEL method.\textsuperscript{13–14} Investigation in the cerebral cortex after permanent MCA occlusion found no differences in numbers of "TUNEL-positive cells" between the core of the infarction and the transitional zone.\textsuperscript{14} However, this study did not distinguish between necrotic cells and apoptotic cells among the TUNEL-positive cells. Rats subjected to 2 hours of transient MCA occlusion showed progressive increase of apoptotic cells, primarily in the boundary zone of the infarction, and this increase occurred up to 48 hours after the reperfusion.\textsuperscript{12} Various durations of occlusion, from 10 to 120 minutes, indicated that the effect of reperfusion causes an increase in apoptotic cells after more than 90 minutes of ischemic insult.\textsuperscript{12,13} However, this speculation have not been thoroughly proved, because no animals had permanent MCA occlusion.

The spatial profiles of apoptotic cells in Group R were similar to those in animals with transient ischemia in the previous reports.\textsuperscript{12,13} In contrast to necrotic cells, the appearance of apoptotic cells was delayed and the location was mainly in the medial border zone and rarer in the core of the striatum. The temporal profile in previous reports only described the sequential changes in total numbers of apoptotic cells. Although our data were similar, we showed the regional differences in the temporal profile in animals with transient MCA occlusion.

Comparison of the spatial and temporal profiles of apoptotic cells between animals with permanent and transient focal ischemia showed that animals with transient MCA occlusion had delayed appearance of apoptotic cells, and significantly fewer apoptotic cells. These data suggest that reperfusion does not affect the activation of apoptosis, and that the severity of the ischemic insult mainly contributes to the apoptotic process. However, we could not investigate with different durations of ischemic insult. With shorter durations of occlusion such as 15 or 30 minutes, the histological findings were quite uneven and we could not obtain significant findings (data not shown). With longer duration of occlusion such as 120 minutes, animals could survive no longer than 24 hours as animals with permanent occlusion. Since the pathophysiological conditions should be affected by the duration of ischemic insult, further investigation with various condition should be required to prove factors influencing the apoptotic process in focal ischemia.

The present and previous studies suggest several possibilities for the pathophysiology of apoptosis in focal cerebral ischemia. Apoptotic cells might appear at the penumbra border and cells in this area might not be so rapidly and severely damaged and, as the result, could undergo appropriate cell death rather than undergo necrosis.\textsuperscript{12} Different genes and proteins may be responsible for necrosis after severe ischemia and apoptosis.\textsuperscript{3} Necrotic cell death is accompanied by rapid expression of c-Fos, Fos B, Jun B, c-Jun, and Jun D, whereas apoptotic cell death is associated with prolonged expression of c-Jun.\textsuperscript{3} These findings might support the different distributions of apoptotic cells and necrotic cells.

Apoptosis is very probably involved in delayed neuronal death after global ischemia.\textsuperscript{7,8,18,21,22,29} Recently, a similar phenomenon has been observed in focal ischemia, suggesting that apoptosis might contribute to very delayed infarction in focal ischemia.\textsuperscript{4} The prolonged existence of apoptotic cells found in our study seems to be relevant to this problem. Since severe necrotic changes were already prominent in the entire core of the striatum in the early stage, delayed infarction was not likely in this area. Thus apoptotic cells observed in this area in the chronic stage might have been macrophages or inflammatory cells rather than neuronal or glial cells. On the other hand, apoptotic cells in the medial border zone and the cortex might have been neuronal or glial cells contributing to the very delayed infarct. However, further investigation with the double immunostaining method is necessary to determine the type of apoptotic cells.

Previously, apoptotic cells have never been found in areas away from the primary ischemic lesion. We found some apoptotic cells in the contralateral corpus callosum in animals with permanent MCA occlusion, but histological examination detected no definite ischemic changes. Magnetic resonance imaging of animals with the same model as ours indicated interstitial edematous changes in rats with permanent MCA occlusion and those changes were not obvious in animals with reperfusion as in the animals in Group R.\textsuperscript{26} Apoptotic cells were found in the white matter away from the primary lesion at the early stage in animal models of spinal cord injury.\textsuperscript{2} These apoptotic cells may be oligodendrocytes and possibly were associated with secondary degeneration. These considerations might apply to our observations. However, we did not find apoptotic cells in the contralateral corpus callosum in Group R even 7 days after the insult. Our findings indicate apoptotic cells could be found in edematous areas away from the primary ischemic lesion. Further investigation should be required to prove the sig-

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nificance of these apoptotic cells.

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Commentary

Yamada et al. have reported the results of an innovative experiment in which they investigated the spatial and temporal profiles of apoptotic cells caused by permanent and transient focal ischemia in a rat model. Their findings support other evidence that apoptosis is probably involved in delayed neuronal death after global ischemia. The results further suggest that apoptosis may contribute to delayed infarction in focal cerebral ischemia. This latter finding may be of significant importance in developing novel therapeutic approaches to the treatment of focal cerebral ischemia. By elucidating the spatial and temporal profiles of apoptotic cells, further investigation may lend insight into the specific genes expressed during the apoptotic process. I look forward to additional work from the laboratory at Oita Medical University.

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Apoptosis is believed to be important in delayed neuronal death occurring in the hippocampus after global cerebral ischemia, as well as in focal cerebral ischemia. However, the true significance and pathophysiology of apoptosis remains unknown particularly in focal ischemia. Yamada et al. studied the spatial and temporal profiles of apoptotic cells appearance in a rat model with permanent or transient focal ischemia induced by intraluminal middle cerebral artery occlusion. Animals in both ischemic groups showed TUNEL-positive cells in the infarcted area, particularly in the border zone, not in the edematous areas. Furthermore, more rapid and extended apoptotic changes were observed in permanent ischemia group. In conclusion, they suggested that reperfusion does not affect the activation of apoptosis, and the severity of the ischemic insult mainly contributes to the apoptotic process. Although these results and authors' suggestion are meaningful, their experimental investigations were performed under limited ischemic conditions and observed by only TUNEL method. I would like their further studies to prove the significance of these apoptotic cells more clearly.

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Although the presence of apoptosis in cerebral ischemia has become more widely recognized, the balance between necrosis and apoptosis is not completely understood. This is particularly true with regard to timing and localization of cell death as well as the influence of reperfusion. In the current article, the authors offer insight into the localization of apoptosis after permanent and temporary focal ischemia. While several authors have localized apoptosis to the ischemic penumbra, the current article provides a direct comparison of apoptosis in permanent and temporary ischemia.

It is interesting to note that, while TUNEL positive cells appeared earlier after permanent ischemia, the appearance of apoptotic bodies was similar after permanent and temporary ischemia. This is consistent with the concept of delayed cell death through the activation of a complex cascade of events leading to programmed cell death. The tendency of apoptotic bodies to cluster in the ischemic border zone also supports the theory that this region may receive adequate perfusion to support the energy-consuming process of apoptosis while the core succumbs to necrosis from energy failure. As the authors suggest, the earlier appearance of TUNEL positive cells after permanent ischemia may indicate nonspecific binding to necrotic cells, not an apoptotic response to more severe ischemia. Accordingly, their assertion in the discussion that "reperfusion does not affect the activation of apoptosis, and that the severity of the ischemic insult mainly contributes to the apoptotic process" is not clear from the data. The difference in the number of apoptotic cells counted at 24 hours after permanent or focal ischemia was not large and neither group reached statistical significance over baseline earlier than 24 hours. From Figures 3 and 7, the appearance of apoptotic bodies seems to be similar after permanent or focal ischemia. Similarly, the
presence of a few TUNEL positive cells in the corpus callosum after permanent ischemia may be a spurious finding.

The delayed appearance of apoptosis after cerebral ischemia and its prolonged existence raise several questions. What triggers apoptosis in the ischemic penumbra? Why does the problem persist after reperfusion? If reperfusion does not necessarily protect against apoptosis as it may against necrosis, what mechanisms prevent apoptosis from becoming more widespread after focal ischemia? These and many other concerns remain the crux of the problem.

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The authors depicted the time course and distribution of TUNEL-positive cells precisely and compared transient and permanent ischemia. As one might expect, transient ischemia induced more apoptotic cells than permanent ischemia did. This might be related to reperfusion injury. The authors differentiated necrotic or apoptotic cells with its appearance. As was indicated previously, TUNEL-positive cells are not all apoptotic but necrotic cells are included. It was indeed found in this study that the majority of the TUNEL-positive cells in the permanent occlusion were necrotic but not apoptotic. When reviewing photographs, some apoptotic cells have neuron-like appearance and others are glia-like. Double staining with neuronal or glial markers will obtain further information. Another point of interest is the appearance of apoptotic cells in the contralateral corpus callosum 24 hours after ischemia. The authors suggested this finding as a result of edema fluid transmission through the corpus callosum. Indeed, edema fluid contains toxic substances that might cause apoptotic change in cells.

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