

The Time Evolution of Cerebral Apoptosis in the Permanent Middle Cerebral Artery Occlusion Model in Rats

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Objective : The purpose of this study is to determine the time evolution and distribution of cerebral apoptosis using the middle cerebral artery occlusion model in rats.

Methods : A total of twenty four male rats - with 2, 3, 4, 6, 8, 12, 24 and 48 hours of middle cerebral artery occlusion respectively - were studied. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling(TUNEL) method was used for the observation of the apoptotic cells. The apoptotic ratio was calculated and the distribution of apoptosis was inspected in the pyriform cortex, basal ganglia and middle cerebral artery territory cortex. The rats were divided into three groups(Group I : 2~4 hours of occlusion, Group II : 6~12 hours of occlusion, Group III : 24~48 hours of occlusion).

Results : In this study, the proportion of apoptosis increased with the duration of middle cerebral artery occlusion and reached a maximum after about 12 hours of middle cerebral artery occlusion. The mean values of the apoptotic ratio were $30.7 \pm 11.3\%$ in group I, $60.8 \pm 2.6\%$ in group II and $48.7 \pm 0.7\%$ in group III. The distribution of apoptosis differed in the pyriform cortex, basal ganglia and middle cerebral artery territory cortex according to the duration of time of the middle cerebral artery occlusion.

Conclusion : In the middle cerebral artery occlusion model of the rats, apoptosis is found to increase according to the occlusion time, reaching a peak after 6 hours, and the distribution of apoptosis changed from the pyriform cortex to the basal ganglia and middle cerebral artery territory cortex.

KEY WORDS : · Apoptosis · TUNEL method · middle cerebral artery occlusion · Ischemia.

Introduction

Despite the existence of many other theories, there are thought to be two main processes of cell death - necrosis and apoptosis^{2,12,28}. Necrosis results from cell injuries such as ischemia, hypoxia, chemical toxin, infection and trauma⁶. Apoptosis occurs physiologically in normal embryogenesis and differentiation, and in pathologic conditions such as viral infections, tumor cells, immune disorders, and experimental ischemic models of the rat^{21,30}.

Necrosis is a form of cell death which mainly results from damage to the cellular membrane, whereas apoptosis is characterized by the induction and activation of endonuclease, DNA cleavage and fragmentation by endonuclease, and the phagocytosis of DNA fragments without any inflammatory

reaction². Generally, the induction and activation of endonuclease requires the activation of many other genes and some programmed process, which is why apoptosis is referred to as "programmed cell death"^{7,9,21}. There have been many studies of apoptosis in the focal ischemic model, but very few in the infarction model, even though the latter has more clinical correspondence¹⁵⁻¹⁸.

The purpose of this study was to determine the time evolution and distribution of cerebral apoptosis using the middle cerebral artery occlusion model in rats.

Materials and Methods

Cerebral infarction model

Experiments were performed on a total of 24 male rats(SPF, Sprague Dawley rats) weighing 250~350grams, in which the middle cerebral artery was occluded for 2, 3, 4, 6, 8, 12, 24 and 48 hours, respectively.

Rats were allowed free access to food and water before and after all procedures. The rats were anesthetized with chloral hydrate(400mg/kg IP) and anesthesia was maintained with

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supplemental doses as needed. The rats were placed in the supine position and were shaved on the anterior neck and right inguinal area. After making an incision in the inguinal area, the femoral artery was exposed and a polyethylene tube (SP35, ID 0.5mm, OD 0.9mm) was inserted into the lumen of the femoral artery. The tube was connected to a Biopac (Model MP 100, Biopac System Inc., U.S.A.) in order to verify the arterial pressure. A blood sample was obtained and the pH, PaCO₂, PaO₂, and hematocrit levels and rectal temperature were checked.

Infarction was induced by the intraluminal occlusion method, which was introduced by Nagasawa and Kogure²⁴ and Yip et al.³⁴. After making a median incision in the anterior neck, the digastric muscle and sternocleidomastoid muscle were retracted and the left common carotid artery, external carotid artery and internal carotid artery were exposed under the view of a surgical microscope (Topcon OMS-75). The common carotid artery was clamped with a temporary clip, the distal external carotid artery was tied with black silk, and a 1mm length small incision was made at the proximal portion, and then an 18mm long 4-0 nylon surgical thread coated with paraffin at one end was introduced into the lumen of the external carotid artery. It was advanced toward the anterior cerebral artery through the internal carotid artery until a 2mm length remained. The external carotid artery and the nylon surgical thread were tied together with black silk and the surgical wound was repaired. The rats were exposed

to the preoperative condition and sacrificed after the predetermined time.

Under the view of a surgical microscope, a median incision was made on the head, the skull was removed with a drill and the brain was obtained by transection of the optic nerve, olfactory nerve and medulla. The location of the tip of the nylon thread was identified as being between the origin of the middle cerebral artery and the anterior communicating artery, and those rats for whom the nylon thread was in the wrong position were excluded. The brain was cut coronally from the frontal pole into slices with a thickness of 2mm and fixed in 10% phosphate-buffered formalin.

Observation of apoptosis

The slice made at the level of the optic chiasm was stained with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) method, which was first proposed by Gavrieli for the observation of DNA fragments⁷, and the number of TUNEL-positive cells was counted with a light microscope (magnification $\times 400$). The degree of apoptosis was defined as the ratio of the

Table 1. Physiologic parameters of the experimental animals

Physiologic parameters	Groups		
	I	II	III
MSAP (mmHg)			
Pre-occlusion	102 \pm 3	108 \pm 4	98 \pm 4
Post-occlusion	101 \pm 3	105 \pm 4	104 \pm 3
pH			
Pre-occlusion	7.36 \pm 0.01	7.37 \pm 0.02	7.36 \pm 0.02
Post-occlusion	7.35 \pm 0.02	7.38 \pm 0.01	7.38 \pm 0.01
PaCO ₂ (mmHg)			
Pre-occlusion	40.7 \pm 0.3	41.2 \pm 0.2	42.5 \pm 0.2
Post-occlusion	41.2 \pm 0.2	40.8 \pm 0.1	41.6 \pm 0.3
PaO ₂ (mmHg)			
Pre-occlusion	99.7 \pm 0.2	97.4 \pm 0.2	98.6 \pm 0.3
Post-occlusion	98.7 \pm 0.2	96.7 \pm 0.4	99.4 \pm 0.3
Hematocrit (%)			
Pre-occlusion	43.4 \pm 0.3	42.3 \pm 0.2	41.8 \pm 0.2
Post-occlusion	42.7 \pm 0.3	41.8 \pm 0.2	42.6 \pm 0.3
Temperature (°C)			
Pre-occlusion	36.4 \pm 0.2	36.6 \pm 0.2	36.4 \pm 0.2
Post-occlusion	36.2 \pm 0.2	36.6 \pm 0.2	36.5 \pm 0.2

Values are expressed in mean \pm standard deviation MSAP : Mean systolic arterial pressure

Table 2. Amount of apoptosis according to the occlusion time

Groups	Occlusion time (hours)	Apoptosis (%)
I	2	—
	2	—
	2	41.3
	3	22.9
	3	35.5
	3	35.9
	3	43.2
	4	26.4
	4	33.3
	4	39.7
	4	59.3
	II	6
6		53.5
6		68.8
8		50.7
8		67.7
12		59.4
III	12	67.4
	12	65.9
	24	37.3
	24	41.7
	24	67.9
	48	43.0
48	53.6	

TUNEL-positive cells to all visible cells in the microscopic field. The distribution of apoptosis was observed in the pyriform cortex, basal ganglia and the MCA territory cortex.

For the evaluation of the statistical significance of the differences, the rats were divided into three groups, namely group I(2, 3 and 4 hours of occlusion), group II(6, 8 and 12 hours of occlusion) and group III(24 and 48 hours occlusion). The data was analyzed using the Kruskal- Wallis test.

Results

Table 1 shows the mean arterial blood pressure, pH, blood gas tension(PaO₂, PaCO₂), hematocrit level and body temperature. There were no significant differences between the groups.

The ratios of apoptosis according to the occlusion time were 13.8 ± 19.5, 34.4 ± 4.8, 39.7 ± 12.3, 58.3 ± 13.7, 59.2 ± 8.5 and 64.2 ± 1.7% at 2, 3, 4, 6, 8 and 12 hours respectively, showing a gradual increase, but then decreased to 49.0 ± 13.5

and 48.3 ± 5.3% at 24 and 48 hours, respectively. There was a significant increase between 4 and 6 hours(Table 2, Fig. 1).

The ratios of apoptosis according to the groups were 30.7 ± 11.3% in group I, 60.8 ± 2.6% in group II and 48.7 ± 0.7% in group III. There was a significant difference between group I and group II, but not between group II and group III. Conclusively,

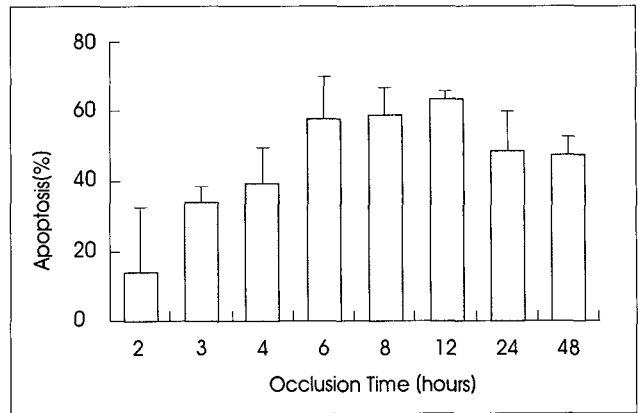


Fig. 1. Amount of apoptosis according to the occlusion time.

Table 3. Distribution of apoptotic cells according to the occlusion time

Groups	Occlusion time(hours)	Location of apoptotic cell		
		Pyriform cortex	Basal ganglia	MCA territory cortex
I	2	-	-	-
	2	-	-	-
	2	+	-	-
	3	+	-	-
	3	+	-	-
	3	+	-	-
	3	+	+	-
	4	-	+	-
	4	+	+	-
	4	-	+	+
II	6	-	+	-
	6	-	+	-
	6	-	+	-
	8	-	+	-
	8	+	-	+
	12	-	+	+
	12	-	+	+
III	12	-	-	+
	24	-	+	+
	24	-	+	+
	24	-	+	+
	48	-	+	+
	48	-	+	+

+: presence of apoptotic cell -: absence of apoptotic cell

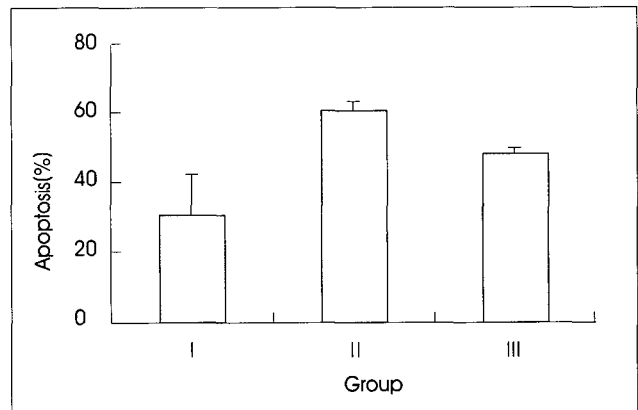


Fig. 2. Amount of apoptosis according to the groups. Group I : 2-4 hours occlusion. Group II : 6-12 hours occlusion. Group III : 24-48 hours occlusion.

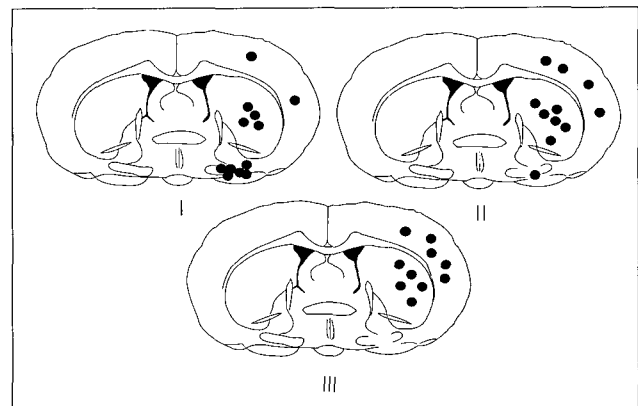


Fig. 3. Distribution of apoptotic cells in group I, II and III. ● : presense of apoptotic cell.

apoptosis reached a maximum at between 6~12 hours after middle cerebral artery occlusion (Table 2, Fig. 2).

Table 3 and Figure 3 show the distribution of apoptosis according to the groups. In the pyriform cortex, 54.5% of the group I, 12.5% of the group II and none of the group III showed apoptosis. In the basal ganglia, 36.4% of the group I, 75.0% of the group II and all of group III showed apoptosis. In the MCA territory cortex, 18.2% of the group I, 50.0% of the group II and all of the group III showed apoptosis (Table 3).

Discussion

There are two fundamental types of cell death, namely, apoptosis and necrosis. Necrosis involves passive cell death caused by cell injuries, whereas apoptosis is an active process that is triggered by embryologic, genetic and environmental information^{1,17,21,23,32}. The term apoptosis was first described by Kerr in 1972, when from among the two types of cell death in the ischemic liver model, delayed peripheral shrinkage necrosis was called apoptosis¹⁰. Morphologically, apoptosis is characterized by the nuclear and cytoplasmic condensation of single parenchymal cells, followed by the loss of the nuclear membrane, the fragmentation of the nuclear chromatin, and the subsequent formation of multiple fragments of condensed nuclear material and cytoplasm. These apoptotic bodies are then engulfed by adjacent cells. However, an inflammatory reaction with infiltrates of leukocytes is typically absent^{1,3,8,10-12,14,19,20,26,29}. Yamada et al³² insisted that apoptosis is regulated by certain factors and genes, and suggested the possibility of new therapeutic methods being developed based on an understanding of the mechanisms of apoptosis.

Apoptosis occurs in various physiological events, such as embryonic tissue remodeling and differentiation, as well as in certain pathologic states, including viral hepatitis, the death of tumor cells, graft-vs-host disease and the ischemic rat model¹¹. Rink et al²⁶ suggested that apoptosis also occurs in traumatic brain injury, Chin et al³ reported that apoptosis plays an important role in secondary injury after acute spinal cord damage.

There have been some reports about biochemical and structural changes in apoptotic cells^{7,19,30}. Generally, the existence of apoptosis is proved by the identification of DNA fragments^{4,8,33}, and there are several methods of identifying DNA fragmentation, such as electrophoresis, flow cytometry and the TUNEL method^{5,16,17,20}. The TUNEL method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-OH ends of DNA, which are abundantly present in apoptotic cells. The TUNEL method has some merits in comparison to other methods, such as in situ visualization and quantitative

analysis^{7,11,16,20,32}, so we used the TUNEL method in this study.

There have been several studies on the time evolution of apoptosis, using the transient ischemic-reperfusion model. In these studies, after 30 minutes of reperfusion, apoptosis was observed and neurons were the predominant cells (90~95%) exhibiting DNA fragmentation^{15,16}. In the permanent infarction model, apoptosis was first observed after 60 minutes of MCA occlusion and reached a peak at between 12 and 48 hours¹³. It seems that apoptosis occurs more rapidly in the transient ischemic-reperfusion model than in the permanent infarction model, but there have been no studies involving the simultaneous observation of these two models under the same conditions. There is more variability in the transient ischemic-reperfusion model, because of the differences of occlusion time in each study. In this study, apoptosis was exhibited after 2 hours of MCA occlusion and gradually increased to reach a peak value at 12 hours, before decreasing slightly thereafter at 24 and 48 hours.

In spite of many studies having been performed about the distribution of infarction in the rat model, none of these concerned apoptosis. Wahl et al³¹ reported that the putamen always showed infarction in the MCA occlusion rat model, and Rubino and Young²⁷ reported that the extensive collateral connections of the frontal and parietal MCA branches with other arterial systems protect the anterior and posterior cortical regions, but that the pyriform branch is an end-artery and the cortical region it supplies is prone to ischemic damage resulting from any reduction in blood flow through the main MCA trunk.

In this study, apoptotic cells were first detected in the pyriform cortex, and then in the basal ganglia and MCA territory cortex, in that order. In group I, apoptotic cells were most abundant in the pyriform cortex. In group II, apoptotic cells were seen in the pyriform cortex in only one case, but were most abundant in the basal ganglia. In group III, no apoptotic cells were seen in the pyriform cortex, whereas they were always found in the basal ganglia and MCA territory cortex. It is thought that this change in the distribution of the apoptotic cells according to the occlusion time is caused by hemodynamic differences, which are in turn influenced by the collateral circulation.

We wondered why we could not see apoptotic cells in the pyriform cortex of the group III. Lee et al¹³ reported that in the cerebral infarction model of the rat, the number of apoptotic cells was diminished by phagocytosis within 30~60 minutes. Also, in a cell culture experiment, apoptosis and phagocytosis were completed within 4 hours^{18,19}. In our study, in which the apoptotic cells disappeared from the pyriform cortex of the rats after more than 12 hours of MCA occlusion, it was thought that the apoptotic cells were diminished by phagocytosis. Magnus et al²² suggested that the uptake of apoptotic cells by

microglia is tolerogenic and results in reduced proinflammatory cytokine production and the reduced activation of encephalotogenic T cells. This might help to restrict inflammation and minimize the amount of damage to the inflamed brain.

There are two aspects of apoptosis which have clinical application, one is the induction of apoptosis, especially in tumor cells, in that some chemotherapeutic agents induce tumor cells to undergo apoptosis. The other clinical application is the reduction of apoptosis, in order to limit the amount of brain injury resulting from various pathologic processes, including cerebral infarction. The therapeutic possibilities in cerebral infarction have been suggested in numerous studies^{4,18,20,25,33} and further studies should be performed to elucidate the mechanism of apoptosis. This suggests the possibility of an extended window of therapeutic opportunity in cerebral infarction.

Conclusion

Apoptosis increased according to the occlusion time, especially after 6 hours, in the middle cerebral artery occlusion model of the rat and the distribution of apoptosis changed from the pyriform cortex to the basal ganglia and middle cerebral artery territory cortex. This suggests that appropriate management for apoptosis can reduce cell death in cases of cerebral infarction.

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