LABORATORY INVESTIGATION

J Korean Neurosurg Soc 38 : 293-298, 2005

p53 Protein Expression Area as a Molecular Penumbra of Focal Cerebral Infarction in Rats

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Objective : The authors investigate the spatial characteristics of apoptotic genes expressed around the focal cerebral infarction, and attempted to explain the penumbra with them.

Methods : A delayed focal cerebral infarction was created in twelve adult Sprague-Dawley rats. We performed the immunohistochemical staining for the apoptosis, bcl-2 and p53 proteins and measured the local cerebral blood flow (CBF) at the infarction core area and peri-infarct area pre- and intra-operatively. The peri-infarct area was divided into six sectors by distance from the infarction border.

Results : The size (mm^2) of apoptosis, bcl-2, and p53 areas were 3.1 ± 1.2, 4.7 ± 2.1, and 6.8 ± 2.4, respectively. Apoptosis, bcl-2 or p53 positive cells were concentrated at the peri-infarct area adjacent to the infarction core. Their numbers reduced peripherally, which was inversely proportional to the local CBF. The p53 area seems to overlap with and larger than the ischemic penumbra.

Conclusion : The p53 positive area provides a substitutive method defining the penumbra under the molecular base of knowledge.

KEY WORDS : Infarction · Penumbra · Cerebral blood flow · Apoptosis · p53.

Introduction

The cell death process in cerebral infarction is related to underlying biochemical and electrophysiological mechanisms[35,38], with apoptosis being a recently-stressed concept[10,14]. The most representative apoptotic gene is p53, which induces apoptotic cell death through modulation of pro-apoptotic secondary genes such as Bax and p21, but can also upregulate anti-apoptotic bcl-2 gene[29-31]. The extent of tissue injury from cerebral infarction seems to be determined by an interaction between the pro- and anti-apoptotic genes in the penumbric area[20].

Although the penumbra is generally defined in relation to local CBF, there have been many trials to redefine[19,22]. Recently, "molecular penumbra" in contradiction to the ischemic penumbra was proposed as a peri-infarction area containing various apoptotic genes and cytokines[36]. But, actually, the correlation between the gene expression and the level of local CBF had not been investigated. The authors hypothesized that the area containing early apoptotic genes could represent the so-called molecular penumbra, and it would overlap with the area of ischemic penumbra. In this study, we analyzed the spatial characteristics of the apoptotic gene expression along with their relationship to the local CBF.

Materials and Methods

Delayed focal cerebral infarction model in rats

The Clinical Experimental Studies Committee of Chung-Ang University School of Medicine approved this study. Twelve adult Sprague-Dawley rats (6males and 6females), each weighing 250~300gm, were allowed free access to food and water during the peri-operative period. The rats were anesthetized initially with ketamine hydrochloride (15mg/kg, IM), and maintained by 2% halothane mixed with oxygen and compressed air. The right femoral artery was cannulated for the measurement of arterial blood pressure. Core body temperature was monitored with a rectal probe and maintained at 38°C with a heating pad. Bilateral common carotid arteries(CCA)

• Received : April 29, 2005 • Accepted : May 24, 2005
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were exposed through a midline anterior neck incision. Then, the rat was placed in a prone position. Under the operating microscope, the right middle cerebral artery(MCA) was exposed through a subtemporal craniectomy, leaving the zygomatic arch intact. The olfactory tract and MCA were exposed by gentle retraction of the brain. The MCA was occluded for 30minutes with a 10-0 nylon tie at the olfactory tract level. At the same time, the ipsilateral CCA was coagulated and the contralateral CCA was occluded for 30minutes with a microclip. After releasing the right MCA and left CCA, the restoration of blood flow through the right MCA was verified. The wounds were closed in layers, and the rat was allowed to recover from anesthesia. The rat showed mild transient left hemiparesis and circular movement for about 2hours after the operation. Additional six rats were underwent sham operation, in which right MCA and bilateral CCA were exposed for 45minutes with the right frontal base of the brain slightly compressed for 5minute. Seventy-two hours after the operation, the rat was again anesthetized with ketamine hydrochloride and halothane. The brain was stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, Inc., St. Louis, MO, USA) by cardiac perfusion through the left ventricle for about 15minutes. The stained whole brain was removed from the calvarium and the cerebellum excised. A focal cerebral infarction was visibly well defined at the right frontal area.

The brain was fixed with 4% buffered paraformaldehyde for more than 2weeks, and then was cut into 1mm-thick slices with a rat brain matrix (Harvard Bioscience, Inc., Holliston, MA, USA). The area (mm^2) of the infarction core was measured with the digital image analysis system (a digital CCD camera (VK-C370, Hitachi, Inc., Tokyo, Japan), a video capture board (Oculus TCX board, Coreco, Inc., St. Laurent, Quebec, Canada), and image analyzer program (OPTIMAS 5.2, Optimas, Inc., Bothell, WA, USA) using the TTC-stained 1mm-thick brain slice showing the largest infarction area. Brain slices were then paraffin-embedded. The prepared brain section showing the largest infarction area was cut into 5㎛-thick slices for TUNEL staining for apoptosis (ApopTag Plus Kit, S7101-KIT, Oncor, Inc., Norcross, GA, USA), and immunohistochemical staining of p53 protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and bcl-2 protein (Phamingen, Inc., San Diego, CA, USA).

Local CBF

Another six adult male Sprague-Dawley rats were prepared for measuring local CBF with laser tissue blood flowmeter (FLO-C1, Omegawave, Inc., Tokyo, Japan). Preoperative CBF (ml/100g/min) (CBF-pre) was measured at the point 2mm distal to the presumed MCA occlusion site. Intraoperative CBF was measured at two sites, 2mm (CBF-2) and 8mm (CBF-8) distal, vertically, to the MCA occlusion, before releasing the vessel. The tissue blood flowmeter can measure CBF continuously and non-invasively by just contacting the probe on the brain cortex with the measuring depth of about 1mm, which has been designed based on the dynamic light scattering method.

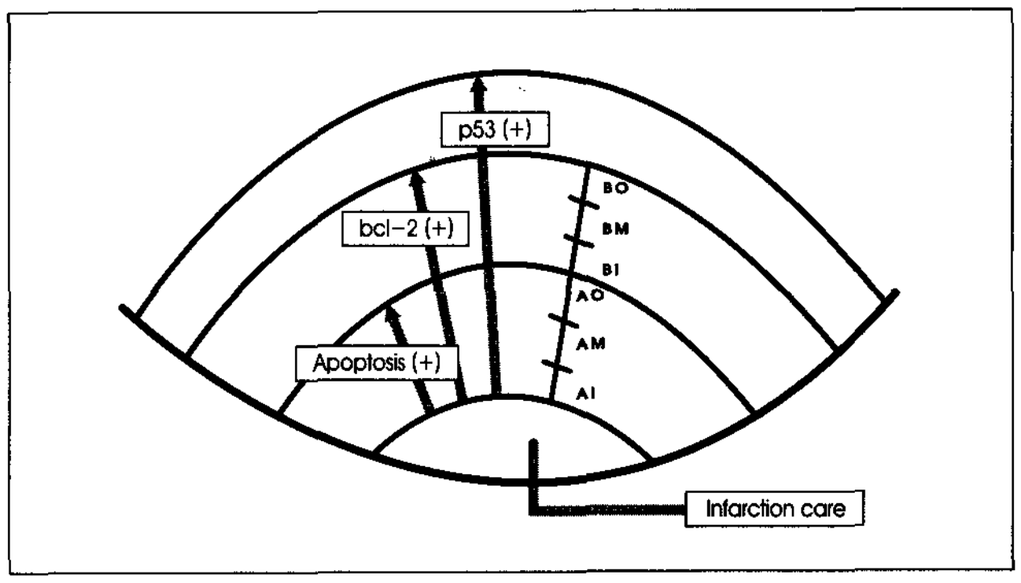

Fig. 1. Schematic figure showing the infarction core and sectors of peri-infarct area. The TUNEL-positive and bcl-2 protein positive areas were divided into 6 sectors (AI, AM, and AO for the TUNEL-positive area; BI, BM, and BO for the bcl-2protein positive area without TUNEL-positive cell) by their distance from the border of the infarction core.

Immunohistochemical staing

The TUNEL-positive peri-infarct area was divided into 3 sectors; inner(AI), middle(AM), and outer TUNEL-positive sector(AO), by the distance from the border of the infarction core (Fig. 1). The three sectors were applied to the bcl-2 protein positive area where it overlapped with the TUNEL-positive area. The remaining bcl-2 protein positive area without TUNEL-positive cell was also divided into 3 sectors; inner(BI), middle(BM), and outer bcl-2 sector(BO), by the distance from the border of the TUNEL-positive area (Fig. 1). Because the p53 protein positive area largely overlapped with the bcl-2 area, all of the above six sectors were applied to the p53 area (Fig. 1).

The size (mm^2) of each area containing TUNEL-positive cell, bcl-2, or p53 protein positive cells, and the width (㎛) from the border of the infarction core were measured with micro-ruler under magnification (×100). The area and width were determined with the digital image analysis system using the section slice showing the largest infarction. The number of apoptotic cells, bcl-2, and p53 protein positive cells were counted 3times at the sectors under a high power (×400) light microscope, and their means were calculated and recorded as the number of cells per high-power field(cells/HPF). The target of cell count was glial cell.

Statistical methods

Data presentations are means±S.D. Significance was tested with an unpaired t-test, using Bonferroni corrections. Differences were considered statistically significant if a p value was less than 0.05.

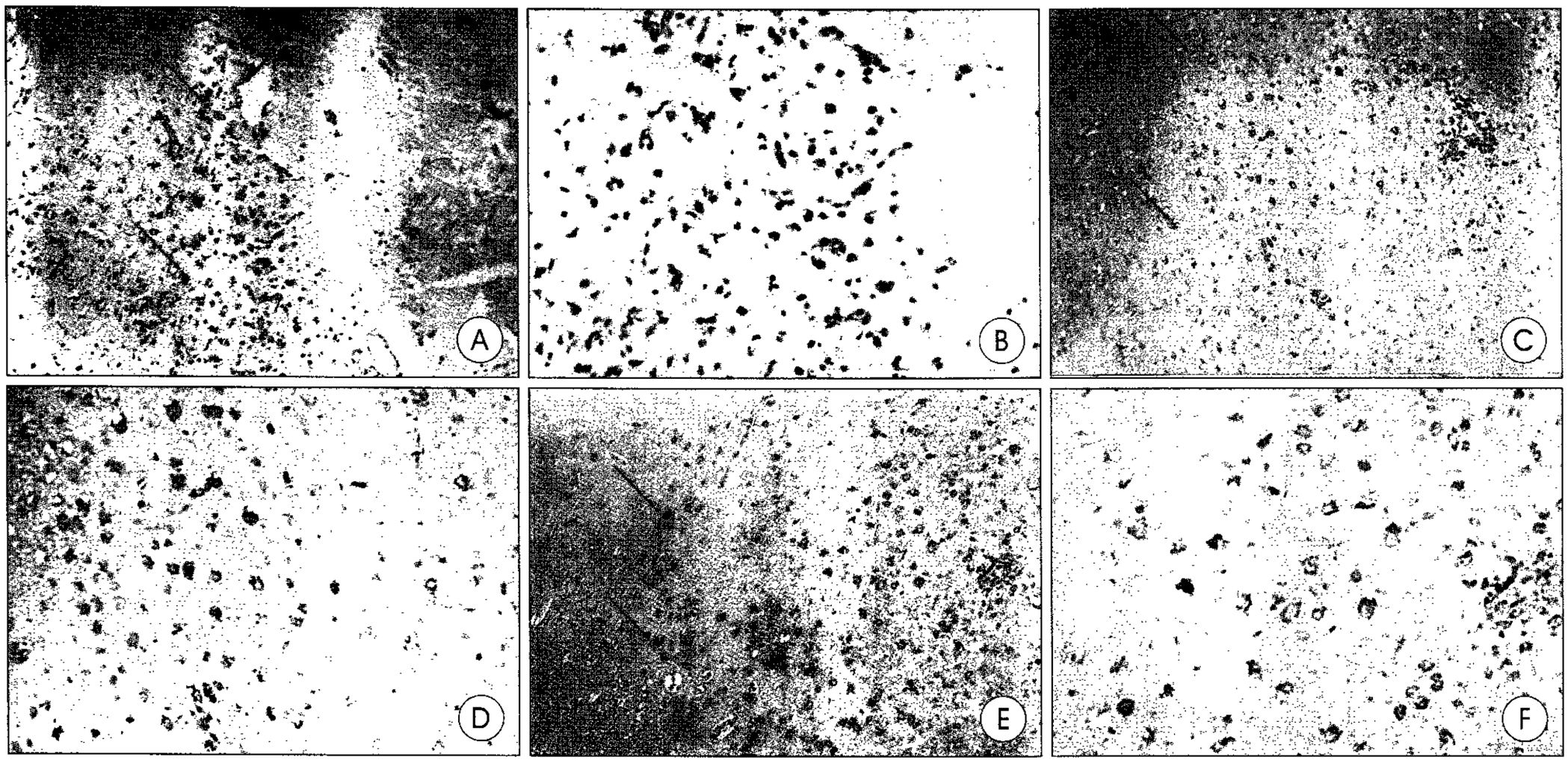

Fig. 2. Pictures of immunohistochemical staining in the peri-infarct area. (original magnification X100 and X400.) (A) and (B); TUNEL staining for apoptosis, (C) and (D); staining for bcl-2 protein, (E) and (F); staining for p53 protein. The numbers of TUNEL-positive, bcl-2 protein and p53 protein (black arrows) positive cells become higher when it comescloser to the infarction core (white arrows). The p53 protein positive area is the largest among the three areas.

Table 1. Size (mm^2) and width (μm) of the infarction core, TUNEL-positive, bcl-2 protein and p53 protein positive areas

	Core	TUNEL	bcl-2	p53
Size of area (mm^2)	3.0±1.0	3.1±1.2	4.7±2.1*	6.8±2.4**
Width of area (μm)	-	35.8±15.1	77.1±41.9***	91.5±54.1***,†

Values represent mean±SD. n=12 *p<0.05 vs. TUNEL; **p<0.01 vs. bcl-2; ***p<0.01 vs. TUNEL; †p<0.01 vs. bcl-2 Core : infarction core, TUNEL : TUNEL-positive area, bcl-2 : bcl-2 protein positive area, p53 : p53 protein positive area

Table 2. Number of TUNEL-positive cells, bcl-2 protein positive cells, and p53 protein positive cells in high power field (X400) at the infarction core and sectors of peri-infarct area (cells/HPF)

	Core	AI	AM	AO	BI	BM	BO
TUNEL	6.1±3.7	4.3±2.7	2.4±1.4	1.0±0.5*	-	-	-
bcl-2	3.8±3.0	9.7±5.4**	6.6±4.2	3.4±3.3	2.8±2.9	2.3±2.2	1.6±1.3***
p53	6.6±3.6	26.6±8.0†	16.2±4.8	7.5±3.6	5.0±2.0	3.6±2.0	1.7±1.3††

Values represent mean± SD. n = 12 *p<0.001 vs. Infarction core; **p<0.01 vs. Infarction core; ***p<0.001 vs. AI; †p<0.001 vs. Infarction core; ††p<0.001 vs. AI. Core means infarction core. AI, AM, and AO mean inner, middle, and outer one-third of the TUNEL-positive area, respectively. BI, BM, and BO mean inner, middle, and outer one-third of the bcl-2 protein positive area outside the AO sector, respectively. TUNEL : TUNEL-positive cells, bcl-2 : bcl-2 protein positive cells, p53 : p53 protein positive cells

Results

Local CBF

The CBF-pre, CBF-2, and CBF-8 were 40.5±1.6ml/100g/min, 9.6±0.5ml/100g/min, and 39.2±1.1ml/100g/min, respectively. The CBF-2 and CBF-8 were 23.7±1.2% and 96.9±2.1% of CBF-pre, respectively. The CBF-2 was significantly lower than CBF-pre ($p<0.001$).

Infarction core

A small focal cerebral infarction was established at the right frontal lobe including both cortex and white matter. It was distinguishable as a zone of pallor within the TTC staining. Its mean size was 3.0±1.0mm^2, which was smaller than other areas (Table 1). TUNEL-positive cells, bcl-2, and p53protein positive cells were found in a narrow around the infarction core (Fig. 2). There was no brain injury in the sham operation group.

TUNEL staining

The size of the TUNEL-positive area was 3.1±1.2mm^2, which was smaller than that of the bcl-2 protein positive areas ($p<0.05$) (Table 1). The width of the TUNEL-positive area from the border of infarction core was 35.8±15.1 m, which was narrower than that of the bcl-2 protein positive area ($p<0.01$) (Table 1). The numbers of TUNEL-positive cells were 6.1±3.7cells/HPF, 4.3±2.7cells/HPF, 2.4±1.4cells/HPF, and 1.0±0.5cells/HPF in the infarction core, AI, AM, and AO sectors, respectively (Table 2). The number of TUNEL-positive cells was greater at the area adjacent to the infarction core and became lesser when it went farther from the infarction core (Table 2), (Fig. 1). There was no TUNEL-positive cell in the sham operation group.

bcl-2 protein

The size of the bcl-2 protein positive area was 7.7±2.1mm^2, which was larger than that of the TUNEL-positive area ($p<0.05$) but smaller than that of the p53 protein positive area ($p<0.01$) (Table 1). The area encompassed with the margin of the bcl-2 protein positive area, composed of the bcl-2 protein positive area and infarction core, was 2.6±0.3 times as large as the infarction core. The width of the bcl-2 protein positive area was 77.1±41.9㎛, which was wider than that of the TUNEL-positive area ($p<0.01$) but smaller than that of the p53 protein positive area ($p<0.01$) (Table 1). The numbers of the bcl-2 protein positive cells were 3.8±3.0cells/HPF, 9.7±5.4cells/HPF, 6.6±4.2cells/HPF, 3.4±2.9cells/HPF, 2.8±3.3cells/HPF, 2.3±2.2cells/HPF, and 1.6±1.3cells/HPF in the infarction core, AI, AM, AO, BI, BM, and BO sectors, respectively (Table 2). The number of bcl-2 protein positive cells was significantly greater in the AI sector, the sector adjacent to the infarction core, than that in the BO sector ($p<0.001$) (Table 2), (Fig. 2). The number of bcl-2 protein positive cells became lesser when it went farther from infarction core. There was no bcl-2 protein positive cell in the sham operation group.

p53 protein

The size of the p53 protein positive area was 6.8±2.4mm^2 which was larger than that of the bcl-2 protein positive area ($p<0.01$) (Table 1). The area encompassed with the margin of the p53 protein positive area, composed of the p53 protein positive area and infarction core, was 3.3±0.2 times as large as the infarction core. The width of the p53 protein positive area was 91.5±54.1㎛, which was wider than those of the TUNEL-positive and bcl-2 protein positive areas ($p<0.01$) (Table 1). The numbers of p53 protein positive cells were 6.6±3.6cells/HPF, 26.6±8.0cells/HPF, 16.2±4.8cells/HPF, 7.5±3.6cells/HPF, 5.0±2.0cells/HPF, 3.6±2.0cells/HPF, and 1.7±1.3cells/HPF in the infarction core, AI, AM, AO, BI, BM, and BO sectors, respectively (Table 2). The number of p53 protein positive cells was significantly greater in the AI sector, adjacent to the infarction core, than that in the BO sector ($p<0.001$) (Table 2), (Fig. 2). The number of p53 protein positive cells became lesser when it went farther from infarction core. There was no p53 protein positive cell in the sham operation group.

Discussion

Apoptosis is an important cell death mechanism and related to the activation of several genes[15]. Apoptosis had been found to correlate with cerebral infarction in several models such as complete focal cerebral infarction[25,32], transient focal ischemia[24], transient global ischemia[26], and very delayed focal cerebral infarction[10]. The spatial and temporal patterns of apoptosis in cerebral infarction have also been studied[24,26]. It is known that temporary cerebral ischemia is more helpful to see the gene expression than permanent cerebral infarction[5,6,21]. Our infarction model creates a delayed focal cerebral infarction by transient occlusion of the middle cerebral and bilateral common carotid arteries, and can reduce local CBF up to 92% in the distribution of the middle cerebral artery[1]. 76.2% of preoperative level at 2mm distant to the MCA occlusion site, presumed infarction core area, but it increased to the preoperative level at 8mm distant to the MCA occlusion site. When the duration of vascular occlusion is 90minutes, it will create a complete focal infarction after 24hours. If the occlusion time is 30minutes, a small delayed focal cerebral infarction will appear after 3days and progressively expand over 14days until its size approximates that of the infarction created by a 90-minute occlusion[10]. The expression of apoptotic genes were detected frequently at the penumbra adjacent to the infarction core in the very delayed cerebral infarction[10], and it appears that this kind of infarction model is suitable for studying the expression pattern of apoptotic genes in the peri-infarct area.

The authors tried to detect apoptosis with the TUNEL method (modified TdT-mediated dUTP-biotin nick-end labeling), which utilizes terminal deoxynucleotidyl transferase (TdT)[12]. The apoptotic nuclei should have fragments of double-stranded DNA with single-base 3 overhang. But the TdT can extend not only the double-stranded DNA with single-base 3 overhang but also the single-stranded DNA with 3 base and the double-stranded DNA with blunt or recessed 3 base[8,13]. Because of its low specificity, the TUNEL method should not be considered as a specific marker of apoptosis but can also indicate non-apoptotic DNA damage. It seems like that the TUNEL staining positive cells in this study contain both apoptotic and non-apoptotic DNA damages. But apoptosis had been revealed as an important cell death mechanism in the very delayed cerebral infarction. And the major role of the TUNEL staining in this study is to show weather the DNA's of the cells in the p53 protein positive area are damaged or not. With this viewpoint, the TUNEL staining is useful method to demarcate the apoptosis positive area in this study.

The bcl-2 gene shows an anti-apoptotic effect, which can prolong cell survival and protect against cell death in some conditions[7,37,39]. We checked the number of cells positive for TUNEL staining, p53 and bcl-2 proteins in the peri-infarct area. According to our results, bcl-2 protein expression was highest at the peri-infarct area adjacent to the infarction core and gradually decreased as the distance from the infarction core increased, which was similar to the expression pattern of

the p53 protein. This suggests that bcl-2 as well as p53 protein expression is sensitive to, and inversely proportional to, the local CBF. According to other experimental work, the number of viable cells in the penumbra gradually falls as the distance to the margin of infarction core decreases[11], and the number of normal cells in the penumbra is proportionate to the local CBF[27]. This can be compared with our findings that the numbers of TUNEL-positive and p53 protein positive cells increased as the margin of the infarction core became closer.

In regard to the pro-apoptotic p53 gene function, apoptosis follows a p53-independent pathway in the peripheral nervous system[9,34] and a p53-dependent pathway in the central nervous system[40]. However, the expression of the p53 gene does not by itself dictate cell death[4,17] because the balance among the secondarily expressed apoptotic genes determines the fate of the affected cell. An increased level of intracellular p53 protein leads to transcriptional upregulation of specific proteins such as bcl-2, bax, p21, GADD45 and mdm2, which are critical in the apoptotic process[29,30]. This can help explain the existence of p53 protein positive viable cells in the peri-infarct area in this study. From this viewpoint, we can propose to explain the penumbra with the area containing viable cells with p53 protein expression. Although the factors inducing p53 gene expression have not been well defined yet, DNA damage is thought to be the most possible factor[23]. The fact that the degree of p53 protein expression was in inverse relation to the distance from the infarction core in this study implicates the local CBF as a presumptive inducing factor for p53 expression. In addition, p53 expression was in proportion to the number of TUNEL-positive cells in the TUNEL-positive area. These findings may provide evidences from which we can regard the p53 protein positive area as the penumbra.

Multi-parametric imaging for the developing infarct in rats after permanent MCA occlusion revealed that the infarction core gradually expanded into the penumbric area[28]. As part of recent effort to redefine the penumbra[22], Hossmann introduced a method, which could image the penumbra by subtracting an ATP-depletion area from a tissue acidosis area[19]. He used a diffusion-weighted MRI imaging(DWI)[16,33] to detect tissue acidosis, and [^{14}C]-iodoantipyrine autoradiography[18,28] to detect ATP depletion. After 30minutes of infarction, the penumbric area visualized by DWI is more than twice as large as the area of ATP depletion[19]. After 2hours of infarction, the ratio declined to 1 : 1.4, and the two areas became congruent after 7 hours[3]. In our study, the ratio of the area including the p53 protein positive area and infarction core to the infarction core area was 3.3 ± 0.2 : 1. This reveals that the penumbric area of the Hossmann's study could be included in the p53 protein positive area of this study. Therefore, p53 protein expression seems like to indicate notionally the penumbra because it is correlated with local CBF, it alone does not mandate cell death although can induce cell death, and its area seems to contain the ischemic penumbra.

Conclusion

These observations lead us to conclude that the p53 protein positive area can provide a substitutive method defining the penumbra under the molecular base of knowledge. The penumbra defined by the apoptotic gene expression may provide a useful appraisal method in the field of therapeutic trial for the cerebrovascular disease. But, it would be necessary to investigate molecular penumbra in other cerebral infarction models and study geometric patterns of other early apoptotic genes expressing in the peri-infarct area.

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Commentary

It is of great interest that this study approaches the penumbra in focal ischemic infarction from the aspect of apoptosis and proposes it as a molecular penumbra, focusing on the p53. As we know, recently the ischemic penumbra, which has been classically described on the basis of blood flow and physiological parameters in this condition, is interpreted in the molecular terms. Apoptosis-related genes, which are induced immediately after focal ischemia and may contribute to cell death as a main mechanism, appear to be reliably involved in this molecular penumbra. In this study, the authors demonstrated the penumbra very nicely with the immunohistochemical staining of TUNEL, bcl-2, and p53, which was well correlated with regional cerebral blood flow, using the ideal rat model of delayed focal infarction by transient MCA occlusion. Furthermore, in my opinion, it is very fascinating that this area was distinguished apparently among these three aspects, though in a very tiny scale of μm; the largest with p53, and with bcl-2 and TUNEL in order. Therefore, the authors provide the p53-positive area as an alternative penumbra for assessing the therapeutic effects. The penumbra is very important in the therapeutic aspect of cerebral infarction, as pointed here. From this view point, however, it is still hard to define this area precisely even in animal experiment. In recent study, where the effect of the p53 inhibitor pifithrin α is proven to be markedly effective with histological, motor, and behavior evaluation, it is noted that this compound does not reduce the apoptotic cells (p53), but reduces the apoptotic cells (TUNEL, p53-related p21WAF, caspase 3) in the ischemic brain, especially in the cortical penumbra zone[1]. Therefore, from these results it seems to be certain that the p53 positive area represents the penumbra. However, it may be deduced that it can not be used to assess the effects of therapeutic trials. To my best knowledge, both the constitutive existence and the pleiotropic character of the p53 might contribute to these inappropriate findings. Since there have been few studies on this point, this study gives valuable guidance, and further adds to our knowledge the significance of these findings when further study is performed with regard to the concept of a molecular penumbra.

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