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Correlation Between Neuronal Apoptosis and Expression of Inducible Nitric Oxide Synthase after Transient Focal Cerebral Ischemia

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Background : Neuronal death in acute-phase cerebral ischemic injury is caused by necrosis. However, neuronal injury after reperfusion can be associated with apoptosis. **Methods :** We used Sprague-Dawley rats whose brains were reperfused after middle cerebral artery occlusion for either 30 min or 2 h. We examined a relationship between apoptosis and the expression of inducible nitric oxide synthase (iNOS) in the brain tissue from 3 h to 14 days after reperfusion in both groups. **Results :** TUNEL and iNOS positivity were closely related in both groups. The 2-h ischemia group exhibited increases in the amount of TUNEL and iNOS-positive cells for up to 3 days after reperfusion, at which the TUNEL and iNOS-positive cells decreased. The 30-min ischemia group exhibited peak positivity 24 h after reperfusion, followed by a similar decrease. iNOS mRNA expression peaked 3 h after reperfusion in the 30-min ischemia group, at which time it decreased. In the 2-h ischemia group, iNOS mRNA increased 3 h after reperfusion, peaked 24 h after reperfusion, and then decreased. **Conclusions :** These results indicated the occurrence of delayed apoptosis in transient cerebral ischemia. Increased expression of iNOS is closely associated with this apoptosis, and oxygen free radical-producing materials, such as nitric oxide, may play an important role in the induction of this apoptosis.

Key Words : Focal ischemia; Reperfusion; Brain; Apoptosis; Inducible nitric oxide synthase

In humans, ischemic injuries of the brain are due largely to the impairment of blood flow, cardiovascular abnormalities, or head trauma. Depending on the location and severity of these injuries, fatal results can occur in the acute phase. However, in transient ischemic attacks (TIA) or microinfarctions, patients experience a transient loss of consciousness, but do not exhibit permanent functional impairment, and normally live with only mild symptoms. Although the patients tend to recover from the brain injury, delayed brain injury sometimes follows this phenomenon, resulting in significant morbidity and mortality.

NO is a soluble gas which has been determined to have an important function as a signal molecule, acting as both an intercellular and intracellular neurotransmitter in brain tissue. Brain ischemia triggers a cascade of events, possibly mediated by excitatory amino acids, which culminates in the activation of the two isoforms of calcium-dependent nitric oxide synthase (NOS). Deficiency in neuronal NO production retards the development of apoptotic cell death after ischemic injury, and is associated with preserved Bcl-2 levels and the delayed activation of effector caspases.[1] Up-regulation of endothelial NO activity can be a central strategy in the prevention of ischemic stroke.[2] Inducible NOS (iNOS) is independent of the calcium-calmodulin pathway, but is expressed by various cytokines, bacterial endotoxins, and exotoxins. iNOS, when activated, continuously produces large amounts of NO, which then functions as a cytotoxic substance.[3] After transient focal ischemia, early NO production exerts a neuroprotec-

tive effect, via the modulation of neutrophilic infiltration.[4] Thus, NO exerts an opposite cytotoxic effect and vasodilative cytoprotective function in cerebral ischemia. Whether NO is useful or harmful depends on the rate and location of its production,[5] as well as on the evolutionary stage of the ischemic process and the cellular source of NO.[6] However, the precise mechanisms underlying the action of NO, depending on the timing and degree of vascular flow obstruction, have yet to be adequately clarified.

Although apoptotic cell death has been tentatively implicated in ischemic brain injury, the precise mechanisms underlying ischemic neuronal cell death remain unknown. Here, we considered that neuronal apoptosis might be related to delayed cerebral infarction following the reperfusion of focal ischemic cerebral injury, and that NO in tissue is an apoptosis-inducing factor. We studied delayed neuronal death by analyzing apoptotic patterns and the levels of iNOS expression in the brain tissue of rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 300-400 g (Dae-Han Laboratory Animal Research Center, Eumseong, Korea) were used. Rats were anesthetized with 10% chloral hydrate (2.5 mL/kg body weight, intraperitoneally). Rectal temperature was maintained at 36-37℃ throughout the experiment. A total of 92 rats were divided into three groups: (1) control (sham) group (n=12); (2) 30-min ischemia group (n=40); and (3) 2-h ischemia group (n=40).

Middle cerebral artery (MCA) occlusion

Focal cerebral ischemia was produced in the anesthetized rats via intraluminal filament occlusion of the left MCA. Under the dissecting microscope, a skin incision was made at the midline of the neck, and the left common carotid artery (CCA) was exposed. External carotid artery (ECA) tributaries were then cut by coagulation, and the internal carotid artery (ICA) was carefully separated from the vagus nerve. The distal phenopalatine artery was ligated, and the ICA alone remained. Then, we loosely ligated the proximal part of the ECA at the branching point with 6-0 silk suture, and two microclips were applied at the CCA and ICA. After blunting one end of a 5 cm-length 4-0 nylon suture with the heat from a flame, a small incision was made with a fine needle at the ECA distal to the 6-0 silk-occlusion. At this time, the blunted nylon suture was inserted through the incision site to the ICA, and the loosely ligated silk suture was fastened. The two microclips were then released, and the suture was advanced gently from the ECA into the ICA until resistance was felt and a slight curving of the advancing suture was observed, indicating complete MCA occlusion. For reperfusion, the suture was withdrawn to restore the blood flow, and the blood flow was confirmed under the dissecting microscope. Free access to food and water was allowed after recovery from anesthesia. All of the rats were kept in air-ventilated incubators, with the temperature maintained at 24±0.5℃ until the end of the experiment. The animals were sacrificed at 3 h, 24 h, and 3, 7, and 14 days after reperfusion,

Evaluation of infarct area by TTC stain

Infarct areas were defined by triphenyl tetrazolium chloride (TTC, Sigma, St. Louis, MO). Animals (n=2 in each group) were anesthesized with intraperitoneal injections of 10% chloral hydrate (2.5 mL/kg), followed by intracardiac perfusion with 200 mL of 0.9% NaCl. Brains were cooled in ice-cold saline for 5 min, and then sliced into 2 mm coronal slices. The brain slices were incubated in phosphate-buffered saline (PBS, pH 7.4) containing 2% TTC at 37℃ for 20 min, and then stored in 10% neutral-buffered formalin. Each brain slice was scanned with an ArtixScan 4000tf scanner (Microtex, Hsinchu 300, Taiwan) and the cross-sectional area of the TTC-stained region was determined with an image analysis program (MetaVeu™, Universal Imaging Co., PA, USA).

RT-PCR of iNOS mRNA

In order to identify the iNOS mRNA from control and ischemia-reperfusion brains (n=3 at each group), we conducted reverse transcriptase-polymerase chain reactions (RT-PCR), according to the methods of Iadecola *et al*.[7] and Rosa *et al*.[8] Total RNA was extracted by Chomczynski and Sacchi's method.[9] The integrity of the RNA was verified in denaturing formaldehyde gel. The First-strand cDNA was synthesized with 0.25, 0.5, or 1.0 μg total RNA, oligo (dT) primer, and AMV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 2 μL of reverse transcription product was used for PCR amplification. β-actin was used as a control. NOS primer was chosen differently from other NOS isoforms, and was designed to include genomic iNOS intron-exon boundary that was segregated as a DNA contaminant in the RNA. The iNOS primers

used in this study were: 5′-ACA ACG TGG AGA AAC CCC AGG TG (sense) and 5′-ACA GCT CCG GGC ATC GAA GAC C (anti-sense). iNOS and β-actin were amplified in the same tube. The PCR reaction was performed in a Perkin-Elmer 2400 PCR machine with the GeneAmp PCR reagent kit (Perkin Elmer, Boston, MA, USA). A mixture of 10X PCR buffer 5 μL, 25 mM $MgCl_2$ 3 μL, 1.25 mM dNTP mixture 9 μL, 25 pM each primer 2 μL, and cDNA 2 μL was set at 95℃ for 5 min, and then we conducted a hot-start by the addition of 1.25 units of Taq polymerase. After 5 cycles of denauration (94℃, 30 s), annealing (69℃, 30 s), and polymerization (92℃, 30 s). We conducted a repeated 35 cycles of denaturation (94℃, 30 s), annealing (62℃, 30 s), and polymerization (92℃, 30 s). Electrophoresis was then performed with the PCR reaction products in 1.8% agarose gel, which was then stained with 0.5% ethidium bromide, and a Polaroid picture was taken.

Histopathology

Animals were anesthetized with an overdose of chloral hydrate, and were perfused intracardially with 200 mL of 0.9% cold saline followed by 4% paraformaldehyde in PBS (pH 7.4). Brains were removed and placed in the same fixative overnight. For histological evaluation, each brain was dissected into 2 mm coronal slices and embedded in paraffin. Serial 10 μm cross-sections were cut and stained with hematoxylin-eosin.

Immunohistochemistry

iNOS, GFAP, and OX42 immunostaining

Paraffin-embedded sections from the paraformaldehyde-fixed brain tissues were treated with 0.6% H_2O_2-containing methanol for 30 min in order to remove endogenous peroxidase, and were washed with distilled water and 0.1M PBS (pH 7.4). In order to prevent nonspecific antibody reactions, the slides were treated with normal goat serum for 30 min. Sectoins were allowed to react with anti-iNOS primary antibody (1:1,000, monoclonal, Transduction Lab., KY, USA), anti-GFAP antibody (1:100, monoclonal, Dako, Glostrup, Denmark), and anti-OX42 antibody (1:50, monoclonal, Serotec, NC, USA) in a humidity-controlled acryl box overnight at 4℃, and then washed with PBS. Sections were allowed to react with biotinylated anti-mouse IgG (Vector ABC Elite Kit; Vector Laboratories, CA, USA) for 30 min, then washed with PBS. After 5 min of immersion in Tris-HCL buffer (0.1M, pH7.6), tissue sections were allowed to react with 0.05% 3,3′-DAB (Sigma, MO, USA), and washed with distilled water and counterstained with methylgreen solution. Slides were dehydrated with a graded series of alcohol, mounted in Permount, and coverslipped. Three representative coronal sections of each group were chosen, in which the central infarct area, transitional area (penumbra), and peripheral spared area were all present, and images of 10 high power fields ($\times$400) were captured from each section, in order to count the numbers of iNOS positive cells.

Double-labeling using the anti-iNOS antibody and other cell markers

For the immunofluorescent double labeling, sections were initially incubated with FITC-labelled, anti-iNOS antibody (1:150, monoclonal, Transduction Lab., KY, USA) for 1 h at room temperature. Then, they were incubated with anti-GFAP (1:200, polyclonal, DAKO, Glostrup, Denmark), -OX-42 (1:200, monoclonal, Serotec, NC, USA), or -NF (1:200, polyclonal, Chemicon, CA, USA) antibodies. After incubation with the secondary antibodies, the sections were rinsed with TBS and incubated at room temperature for 30 min with goat anti-mouse AlexFluor 568 for OX-42 labeling, and for 40 min with goat anti-rabbit AlexFluor 568 for GFAP and NF labeling. The slides were dehydrated and mounted with water-soluble mounting media, then observed under fluorescence microscope.

TUNEL assay

Apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay in brains which had undergone ischemia-reperfusion injuries. Tissue sections were deparaffinized and dehydrated with xylene and graded alcohol, and washed in PBS. Sections were incubated with 20 g/mL protein kinase K (Sigma, MO, USA) for 5 min in order to remove intranuclear proteins. The Trevigen apoptotic cell system (TACS) *in situ* apoptosis detection kit (Trevigen Corp. MD, USA) was used according to the manufacturer's instructions for the detection of apoptosis. In summary, after treatment with protein kinase, the tissue sections reacted with a 2% H_2O_2 solution for 5 min and were washed in labeling buffer. Sections were allowed to react with a mixture of TdT and dNTP, and a mixture of divalent cation and TdT, for 60 min at 37℃. The reaction was halted by incubating the setions with stop buffer at 37℃ for 30 min. After washing in PBS, the sections were allowed to react with Streptavidin-HPR solution. The slides were colorized with DAB/H_2O_2 solution (0.05% DAB and 0.02% H_2O_2 in 50 mM Tris-HCl buffer), and counterstained with methyl green. The method used to evaluate the TUNEL positive cells was same

as that used for iNOS immunostaining.

DNA fragmentation analysis

The infarct area of each brain was immediately homogenized and treated with proteinase K (200 μg/mL) to digest intracellular proteins, and the proteins were precipitated with 1M NaCl. Isoproprenol was added to precipitate the DNA, and the sample was washed in ethanol. It was rehydrated in Tris-EDTA solution. DNA (5 μg/lane) was electrophoresed in the presence of RNase A on 1.5% agarose gel containing ethidium bromide (0.3 μg/mL) at 50 volts for 5 h. The DNA was visualized with ultraviolet photography.

RESULTS

The group which was reperfused after 2 h of occlusion exhibited infarction, which did not stain with TTC 3 h after reperfusion. The area of infarction was 5.6% of the total cut surface area. The infarction widened within the 24 h after reperfusion, growing to 11.9% of the total area (Fig. 1). Three days after reperfusion, the infarction reached 25% of the total area. Up to 2 weeks thereafter, about 20 to 29% of the total area was infarcted. In the 30-min ischemia group, the cut surface of the brain exhibited mild edema after 3 h of reperfusion, but infarction was not observed on the TTC stain. After 24 h of reperfusion, a focal infarction occupying about 3 to 8% of the total area was observed. This area widened to 14% within 3 days after reperfusion. Up to 2 weeks thereafter, between 13 and 19% of the total area was found to be infrarcted.

Under the light microscope, edema was observed after 3 h of reperfusion in the 30-min ischemia group. Nuclear and cytoplasmic pyknosis were noted in some neurons (red neuron), and some surrounding glial cells also exhibited pyknosis. One day after reperfusion, we noted neuronal and glial liquefaction necrosis. Mild inflammatory cell infiltration was also noted at the periphery of the necrosis (penumbral region) (Fig. 2), and red neurons were frequently observed in this region. Inflammation gradually subsided with time. Glial cell hyperplasia and cystic change were also noted in the infarct area after 3 days of reperfusion. The 2-h ischemia group exhibited similar histological findings to those of the 30-min ischemia group, although the area of infarction was larger.

In the sham-operated control, no TUNEL positive profiles were observed in the cerebrum. In the ischemia group, however, a great deal were were observed after reperfusion, particularly in the 2-h ischemia group. TUNEL-positive profiles were noted after 3-h of reperfusion, and the scores peaked 3 days after reperfusion, followed by a decline (average score per high power field (×400), reperfusion of 3 h, 11.3; 24 h, 12.3; 3 days, 30; 7 days, 12.6; and 14 days, 18.2). In the 30-min ischemia group, TUNEL positive cells appeared 3 h after reperfusion, peaking at 24 h of reperfusion. These scores remained high until 7 days after initial reperfusion, followed by a decline (average score per high power field (×400), reperfusion of 3 h, 15.0; 24 h, 24.9; 3 days, 10.7; 7 days, 23.4; and 14 days, 12).

The sham operated control group exhibited no iNOS immunopositive cells in the cerebrum. In the 2-h ischemia group, many iNOS positive cells were observed at 3 h reperfusion, and the positive cell score peaked at 3 days of reperfusion, followed by a decline (average score per high power field (×400), reperfusion of 3 h, 31.2; 24 h, 32.1; 3 days, 36.0; 7 days, 29.9; and 14 days, 19.1). In the 30-min ischemia group, iNOS positive cells appeared

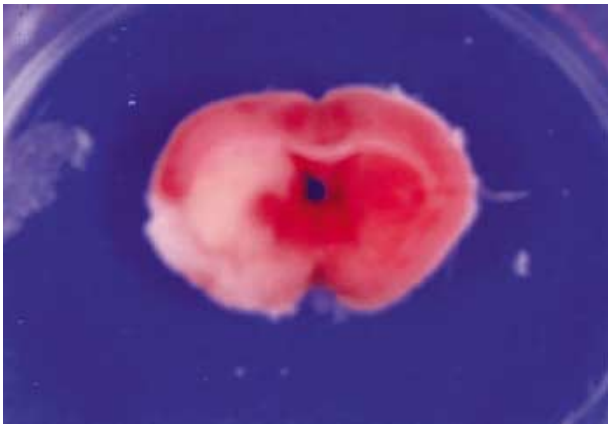

Fig. 1. The group reperfused after 2 h of occlusion shows infarction stained white.

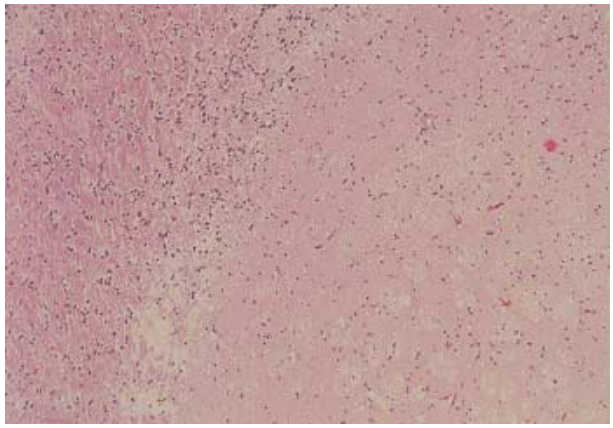

Fig. 2. Mild inflammatory cell infiltration is noted at the periphery of the necrosis (so called penumbral region).

at 3 h reperfusion, and the score peaked at 24 h reperfusion, follwed by a decline (average score per high power field (×400),

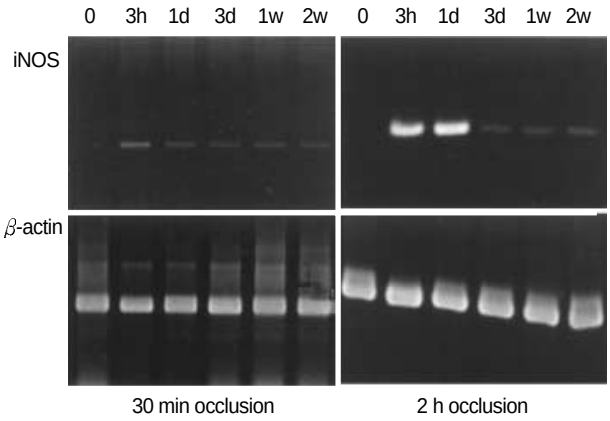

Fig. 3. iNOS mRNA RT-PCR, the expression is most increased after 3 h of reperfusion and decreased subsequently in 30 min ischemia group, the expression is increased after 3 h of reperfusion, arrived peak after 24 h of reperfusion, and decreased subsequently in 2 h ischemia group.

reperfusion of 3 h, 16.0; 24 h, 39.4; 7 days, 28.6; 14 days, 28.6). To summarize TUNEL and iNOS immunohistochemical staining, in the 2-h ischemia group, TUNEL and iNOS-postive cell score increased proportionally until 3 days of reperfusion, and decreased gradually until 2 weeks after reperfusion. In the 30-min ischemia group, the scores of both TUNEL and iNOS positive cells peaked at 24 h reperfusion, and then decreased. The scores of iNOS-positive cells and TUNEL-positive cells were relatively directly proportional in all groups.

In the 30-min ischemia group, iNOS mRNA expression peaked after 3 h of reperfusion and subsequently decreased. In the 2-h ischemia group, iNOS mRNA expression increased after 3 h of reperfusion, arriving at a peak after 24 h of reperfusion, and subsequently decreased (Fig. 3).

DNA fragmentation analysis revealed that a DNA ladder formed in the 30-min and 2-h ischemia groups. In the 30-min ischemia group, a DNA ladder was observed 24 h to 7 days after reperfusion, and the 2-h ischemia group exhibited DNA ladder

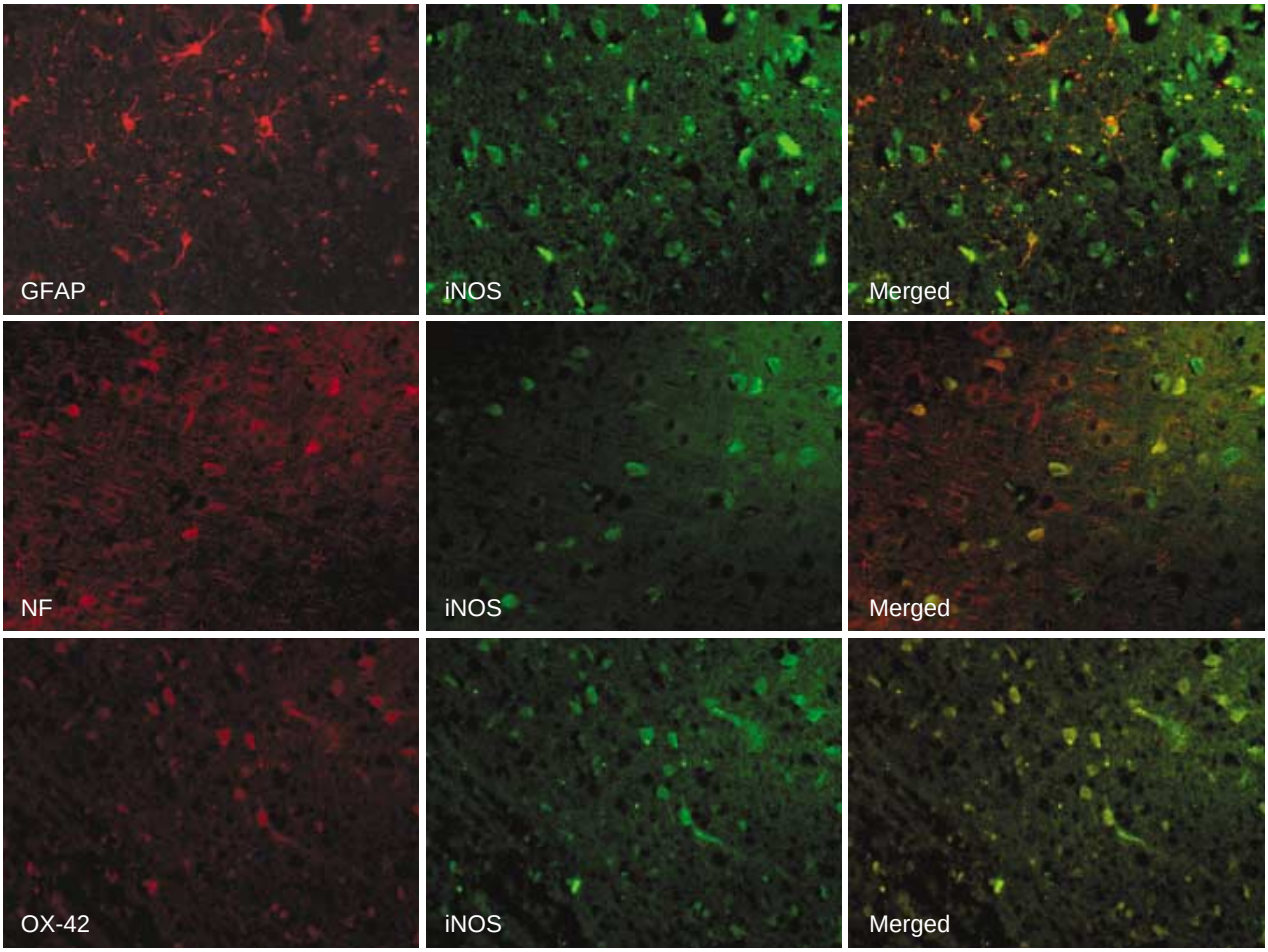

Fig. 4. Double immuohistochemical stain of iNOS/GFAP, iNOS/NF and iNOS/OX42 shows iNOS producing cells are astrocytes, neuron and macrophages. (Red color: GFAP, NF, OX-42 descending order, green color: iNOS).

at all time points after reperfusion.

Double labeling of iNOS and cell markers, GFAP for astrocyte, OX-42 for microglia and macrophages, and neurofilaments for neurons revealed that most of the microglia and macrophages expressed iNOS in their cytoplasm and astrocytes were also expressed as a part of the neurons (Fig. 4).

DISCUSSION

In 1972 Kerr *et al*.[10] divided cellular death into necrosis and apoptosis, and they considered apoptosis to be a pattern of physiological cell death. It has been established that apoptosis is a physiological response in the development of the central and peripheral nervous system.[11] Cerebral ischemia is accompanied by a marked inflammatory reaction, initiated by the ischemia-induced expression of cytokines, adhesion molecules, and other inflammatory mediators, such as prostaglandins and NO. Polymorphonuclear leukocytes in the blood infiltrate brain tissue through the blood-brain barrier in the early stage (within 24 h), followed by an infiltration of the macrophages, which continues for 7 days.[12] These inflammatory responses play a major role in neuronal injury in the initial stages of ischemia. Anti-inflammatory therapies can, thus, be effective in the treatment of cerebral ischemia.[13] The influence of inflammation on brain infarction may also be related to a deficiency of blood flow due to the intravascular aggregation of blood cells, and the toxic effects of various mediators of inflammation. However, the exact mechanism underlying this phenomenon has yet to be clarified. Proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) are up-regulated within an hour in ischemic brain lesions. Cytokines may aslo contribute to the progression of infarcted zones in the post-ischemic prriod, either directly or via the induction of neurotoxic mediators such as nitric oxide. Microglia and astrocytes in the brain tissue also exhibit reactive activational changes.[14] In the infarct region, the microglia are activated within an hour, transforming into phagocytes. Astrocytes upregulate the production of intermediate filaments, synthesize neurotrophins, and form glial scars. Local microglia and infiltrating macrophages demarcate the infarcts and rapidly remove debris. At present, it remains unclear as to whether the detrimental effects of inflammation outweigh the neuroprotective mechanisms, or vice versa.

Among mediators of acute inflammation, NO has recently been found to exhibit strong cytotoxic action when secreted in large quantities. Castillo *et al*.[15] analyzed the relationship between NO metabolite concentrations in cerebrospinal fluid (CSF), and the clinical and neuroimaging parameters of brain injury in patients with acute ischemic stroke. They suggested that NO generation plays an important role in acute ischemic stroke. Increased NO metabolites in CSF were associated with more severe brain injury and early neurological deterioration. NO is a soluble gas molecule, and acts within seconds. It requires no specific receptors and is freely permeable. NO can function as either an intracelluar signal transducer or a cytotoxic substance. It is secreted by activated neutrophils or macrophages, and exerts its cytotoxic effects on the surrounding cells.[16] The secreted NO, in this situation, is produced by inducible NO synthase (iNOS). iNOS is expressed irrespective of intracellular calcium concentration, and when activated, it continuously produces large amounts of NO.[17] NO is produced mostly by infiltrated blood cells, but also to some degree by activated microglia, oligodendrocytes,[18] endothelial cells,[19] and a discrete population of neurons in the neocortex.[20] Astrocytes may also protect neurons from NO toxicity, employing a glutathione-dependent mechanism.[21] Astrocytes can undergo apoptosis independently of the presence of other cell types, including neurons. Ischemia can also induce apoptosis in the astrocytes, contributing to the pathogenesis of ischemic injury in the central nervous system.[22] When pretreated with aminoguanidine, an iNOS suppressor, the size of the infarction decreased.[23] This indicates the cytotoxic effect of NO when it is generated by iNOS. When macrophages are activated with IL-1, iNOS is expressed in the cell, and the generated NO results in the apoptosis of implanted tumor cells.[24] NO produced by iNOS is also considered to play an important role in the apoptosis of parenchymal cells in various inflammations, including gastritis[25] and myocardial infarction.[26] There have, to date, been no mainstream studies regarding the intracellular induction of iNOS and production of NO, or neuronal apoptosis in ischemic cerebral injury. This may be attributable to the absence of a well-established concept of apoptosis as neuronal death, as well as the absence of a confirmed relationship between necrosis and neuronal apoptosis, according to the degree of vascular obstruction in ischemic cerebral injury.

In this study, we considered that the relation of neuronal apoptosis to delayed cerebral infarction following reperfusion, and that NO in tissues is an apoptosis-inducing factor. After the transient occlusion of the unilateral middle cerebral artery of Sprague-Dawley rats for 30 min or 2 h, we observed the patterns of apoptosis, and analyzed the expression of iNOS in the brain tissue for a period 3 h to 14 days. Grossly, the group undergoing 2 h of MCA occlusion (severe ischemia) exhibited infarction 3 h after reperfusion, and the size of the infarction zone increased gradu-

ally for 3 days. In the 30-min ischemia group (mild ischemia), focal infarction was observed from the day after reperfusion, and the area of infarction was increased for 3 days. These findings suggest that infarction after mild focal ischemia develops in a more delayed fashion than in cases of severe ischemia, although the infarction zones were smaller. In both groups, the size of the infarction zones increased with time and reached plateaus, which also suggests delayed infarction. Microscopically, both groups exhibited liquefaction necrosis of the neuron and glial cells in the infarcted areas. Mild inflammatory cell infiltration was noted at the periphery of the necrosis, and red neurons were frequently observed in these regions. Upon TUNEL and iNOS immunohistochemical stains in the infarcted areas, the 2-h ischemia group exhibited increases in TUNEL and iNOS-positive cells until 3 days after reperfusion, and subsequently decreased. The 30-min ischemia group exhibited peak TUNEL and iNOS positivity after one day of reperfusion, at which time it began to decrease. iNOS positivity and TUNEL positivity were closely related in all groups. This suggests that the NO produced by iNOS is a causative factor in apoptosis. The peak of apoptosis (TUNEL positivity) was noted at 1 day and 3 days of reperfusion in the 30-min and 2-h occlusion groups, respectively. In the 30-min occlusion group, peak apoptosis levels were noted when the beginning of necrosis were observed. In the 2-h occlusion group, peak apoptosis levels were noted concurrently with peak necrosis. These results suggest that apoptosis may be the predominant pattern of cell death in milder ischemic injury. Double staining with GFAP/iNOS and OX42/iNOS revealed that activated microglia and macrophages, rather than astrocytes, constituted the source of the iNOS. iNOS mRNA RT-PCR revealed maximal expression levels at 3 h of reperfusion in the 30-min ischemia group, followed immediately by a decrease. In the 2-h ischemia group, iNOS mRNA increased after 3 h of reperfusion and reached maximum after one day, with a decrease immediately afterwards. DNA ladder examinations in infarct tissue revealed DNA ladders occruring from 24 h to one week after reperfusion in the 30-min ischemia group. All groups in the 2-h ischemia group exhibited DNA laddering. A diffusely stained necrosis pattern was admixed with the ladder. This may be attributable to the incomplete occlusion of middle cerebral artery in this study, in contrast with the direct ligation used in the study of Du *et al*.[27] In this study, total infarct tissue was homogenized, but according to Yao *et al*.,[28] DNA fragmentation was noted in the penumbral zone, but not in the core regions. The above results clearly demonstrate the occurrence of delayed cellular apoptosis in reperfused transient ischemic cerebral injury, and the increased expression of iNOS is closely related to this delayed cellular apoptosis.

Neuronal death was considered to be necrosis, but not apoptosis, in cases of focal or global cerebral ischemia.[29] However, when a study of experimental transient or global ischemic state was undertaken, it revealed the selective loss of neurons, and that pretreatment with protein synthesis suppressants such as cyclohexmide or dextraphan under the hypothesis of neuronal apoptosis ameliorated the damage to cerebral ischemia. This, then, implicated apoptosis in cases of ischemic cerebral injury. Acording to Charriaut-Malangue *et al*.,[30] in experimental models of focal ischemic cerebral injury, neuronal apoptosis was observed and increased with time when occluded vessels were reperfused. The centers of ischemic injury exhibited necrosis, but apoptosis was noted at the peripheries of the lesions. Therefore, it can be concluded that neuronal death in ischemic lesions of the brain cannot be explained simply by either necrosis or apoptosis, and that many factors, including miscellaneous differences between individuals, duration of vascular obstruction, metabolic state, ionic imbalance in blood, and duration of reperfusion may have great influence on this. According to Du *et al*.,[27] infarction was not observed until 24 h after reperfusion following a 30-min obstruction of the middle cerebral artery. However, they observed a great degree of neuronal apoptosis at the periphery of infarction after 3 days. They reported that when the vessel was obstructed for 90 min, most neurons exhibited necrotic cell death. These results are slightly different from the results of the present experiment in which we observed a great deal of apoptosis in the 2-h occlusion group. This may be caused by differences in the occlusion procedure: they direct ligation of the MCA, but in our model, the MCA was indirectly occluded, and perhaps was never completely occluded. This suggests that neuronal death can be provoked by transient vascular obstruction and that neuronal apoptosis constitutes a major mechanism in delayed infarction. Between our review of the literature and the results of our experiment, it is clear that apoptosis is an relevant cell death modality in delayed cerebral infarction after cerebral ischemia, and oxygen free radical-producing materials, such as NO, may play important roles in the induction of apoptosis.

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