Apoptosis in a Neonatal Rat Model of Cerebral Hypoxia-Ischemia

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Background and Purpose—The mechanisms of excitotoxic cell death in cerebral ischemia are poorly understood. In addition to necrosis, apoptotic cell death may occur. The purpose of this study was to determine whether an established model of cerebral hypoxia-ischemia in the neonatal rat demonstrates any features of apoptosis.

Methods—Seven-day-old neonatal rats underwent bilateral, permanent carotid ligation followed by 1 hour of hypoxia, and their brains were examined 1, 3, and 4 days after hypoxia-ischemia. The severity of ischemic damage was assessed in the dentate gyrus and frontotemporal cortex by light microscopy. Immunocytochemistry was performed to detect the cleavage of actin by caspases, a family of enzymes activated in apoptosis. Terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) reactivity was examined in the cortical infarction bed and dentate gyrus. Neonatal rat brain DNA was run on agarose gel electrophoresis to detect DNA fragmentation. Ethidium bromide–staining and electron microscopy were used to determine whether apoptotic bodies, 1 of the hallmarks of apoptosis, were present.

Results—The frontotemporal cortex displayed evidence of infarction, and in most rats the dentate gyrus showed selective, delayed neuronal death. Immunocytochemistry demonstrated caspase-related cleavage of actin. TUNEL and DNA electrophoresis provided evidence of DNA fragmentation. Ethidium bromide–staining and electron microscopy confirmed the presence of chromatin condensation and apoptotic bodies.

Conclusions—Features of apoptosis are present in the described model of cerebral hypoxia-ischemia. Apoptosis may represent a mode of ischemic cell death that could be the target of novel treatments that could potentially expand the therapeutic window for stroke. (***Stroke***. 1998;29:2622-2630.)

Key Words: apoptosis ■ cerebral ischemia ■ DNA damage ■ rats

Controversy exists as to the precise mode of cell death after cerebral ischemia. However, evidence has been mounting that neuronal death, including ischemia-induced death, occurs via apoptosis as well as necrosis.[1–7] Apoptosis may represent a different mode of cell death in cerebral ischemia, which may have implications for stroke therapies. Necrosis may predominate in more intense forms of ischemic damage, whereas apoptosis may occur in milder forms of ischemic damage.[5] In addition, unlike the acute damage of necrosis, apoptotic injury may take time to develop.[5,8,9] Therefore, demonstrating the presence of apoptosis as the result of hypoxia-ischemia might expand the therapeutic window for stroke by potentially making available delayed neuroprotective interventions aimed at blocking the apoptotic cascade.

Although no universal definition of apoptosis currently exists, characteristics of apoptosis have been described that are distinctly different from necrosis. Recent evidence demonstrates that apoptosis involves the activation of caspases, a

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unique family of structurally related, highly conserved, aspartate-specific, cysteine proteases that are necessary to carry out the signal for apoptotic cell death.[10–12] Two members of the caspase family, caspase-1 and caspase-3, are known to cleave the most abundant caspase target substrate, actin.[13,14] The 45-kDa actin is cleaved by caspase activation between Asp11 and Asn12 and between Asp244 and Gly245 to produce N-terminal $\approx$32-kDa fragments and C-terminal $\approx$15-kDa fragments.[13,15] A polyclonal antibody to the last 5 amino acids of the C-terminus of the 32-kDa fragment of actin generated by caspase cleavage of intact actin has been developed and named "fractin" for "fragment of actin."[14] Fractin labeling provides indirect evidence of caspase activation and demonstrates initiation of an apoptotic pathway, but does not rule out secondary necrosis. Other markers for apoptosis include biochemical evidence of oligointernucleosomal DNA fragmentation into approximately 180-bp multi-

ples resulting from endonuclease activation that can be demonstrated with a typical "laddering" appearance on agarose gel electrophoresis. In addition, the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) technique, which identifies 3′-OH ends of DNA-strand breaks, has been widely used as a marker of DNA damage or repair.[16] However, the lack of specificity of TUNEL in detecting oligointernucleosomal DNA fragmentation precludes its use as a defining feature of apoptosis. Rather, TUNEL may be most appropriately used in conjunction with more reliable techniques for detection of apoptosis such as the demonstration of apoptotic bodies by electron microscopy (EM). The electron microscopic appearance of apoptotic bodies has been well characterized.[8,17,18] Compaction of the cytoplasm with plasma membrane blebbing can be seen with nuclear condensation and fragmentation in the presence of generally well-preserved cytoplasmic organelles and little surrounding inflammation. Apoptotic bodies can be observed as membrane-bound spherical entities consisting of nuclear or cytoplasmic remnants that may be engulfed by phagocytes and undergo secondary necrosis. In addition to EM, investigators such as Ellerby et al[19] have demonstrated the presence of apoptotic bodies by high-power magnification fluorescence microscopy with ethidium bromide–staining in vitro. Despite the existence of several potential markers of apoptosis, an unequivocal definition of apoptosis remains elusive. The combination of evidence of caspase activation, demonstration of apoptotic bodies by EM and ethidium bromide–staining, TUNEL reactivity, and the presence of oligointernucleosomal DNA fragmentation as detected by electrophoresis may be sufficient to define apoptosis. Requiring several lines of morphological and biochemical evidence to define apoptosis is a concept previously reported by other investigators.[20]

A model of cerebral hypoxia-ischemia has been developed in the neonatal rat that reliably produces infarction primarily involving the cortex in the territory of the middle cerebral artery and selective neuronal death of the inner granule cell layers of the dentate gyrus.[21–23] The purpose of this study was to determine whether this model of ischemic damage in the cerebral cortex and inner granule cell layers of the dentate gyrus demonstrates evidence of apoptosis.

Materials and Methods

Hypoxia-Ischemia

All experiments were performed in accordance with approved institutional animal care guidelines. Wistar rat pups of either sex (Simonsen Labs, Gilroy, Calif) were caged with the dam and kept on a 12-hour light/dark schedule. Seven-day-old rat pups underwent permanent bilateral carotid ligation. The animals were anesthetized in a small jar containing cotton soaked with methoxyflurane, and deep anesthesia was maintained throughout the surgery with a small plastic tube placed over the nose containing cotton soaked with methoxyflurane. The neck was incised in the midline. Then, the common carotid arteries were isolated and doubly ligated with 4-0 silk suture and severed between sutures bilaterally. Total time of surgery never exceeded 7 minutes. Approximately 4 to 6 hours after surgery, the animals were exposed to a 1-hour period of hypoxia (93.5% N_2, 6.5% O_2) by placing them in an airtight container partially submerged in a 37°C water bath. The pups were left in the water bath overnight, then returned to the dam. Animals that undergo
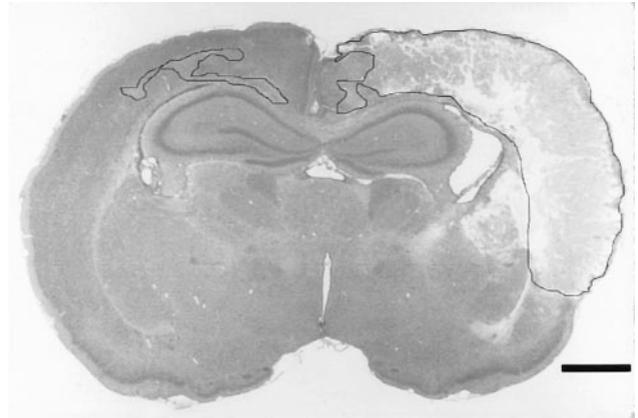

Figure 1. Example of cortical infarction area, outlined in black (bar=1 mm).

hypoxia-ischemia generally show global neurological impairment that includes severe motor and feeding dysfunction as compared with control animals.

The rat pups were sacrificed 3 or 4 days after hypoxia-ischemia under deep pentobarbital anesthesia (60 mg/kg, IP) and perfused with buffered 10% formalin. The brains were either embedded in paraffin, sampled for biochemical studies, or cryoprotected with 30% sucrose before freezing for cryostat sectioning. Macroscopically, ischemic brains consistently showed evidence of infarction, whereas those of control animals did not. Overall mortality from the procedure was approximately 10%.

Infarction Area

Eight-micrometer paraffin-embedded sections of neonatal rat brain at stereotaxic standard levels of 0.4 (0.4 mm anterior to the interaural line per Sherwood and Timiras[24]), 2.0, and 2.6 A from 11 ischemic animals sacrificed 4 days after hypoxia-ischemia and from 10 nonischemic control animals of the same age were stained with hematoxylin and eosin. Hypoxic-ischemic sections consistently showed signs of ischemic injury by light microscopy, such as eosinophilic cytoplasm and pyknotic nuclei, but none of the control animals displayed any evidence of ischemic injury by light microscopy. To quantify the extent of infarction in the hypoxic-ischemic sections, microscope slides were scanned with SprintScan (Polaroid). The area of infarction was defined by the loss of normal hematoxylin and eosin–staining pattern (Figure 1). The cortical infarction area and the total cortical area from the rhinal sulcus to the interhemispheric fissure of the left and right hemispheres were outlined using Image-Pro Plus (Media Cybernetics). The cortical infarction area was calculated as a percent of the total cortical area. The mean cortical infarction area±SE of hypoxic-ischemic sections (n=11) at stereotaxic levels 0.4, 2.0, and 2.6 A was determined.

In separate experiments, the early time course of ischemic damage seen in the hippocampus was investigated by a fluorescence microscopy technique essentially as previously described by our group.[25] Briefly, formalin-fixed, hematoxylin and eosin–stained sections of neonatal rat brain from nonischemic control animals (n=12) and animals sacrificed 4 hours (n=6), 24 hours (n=6), and 72 hours (n=12) after hypoxia-ischemia were viewed under fluorescence microscopy. The strong fluorescence of eosin in the cytoplasm of injured neurons facilitates the identification of ischemic cell change. The mean±SE of ischemic neurons in dentate gyrus and CA1 was determined at the respective time points.

Fractin Antibody Immunolabeling

Cryostat sections (10 μm) of neonatal rat brain taken 3 days after hypoxia-ischemia were immunolabeled with primary fractin antibody using a peroxidase-coupled secondary antibody and visualized with diaminobenzidine essentially as previously described.[14,26] The fractin antibody was generated by injecting into the rabbit a synthetic
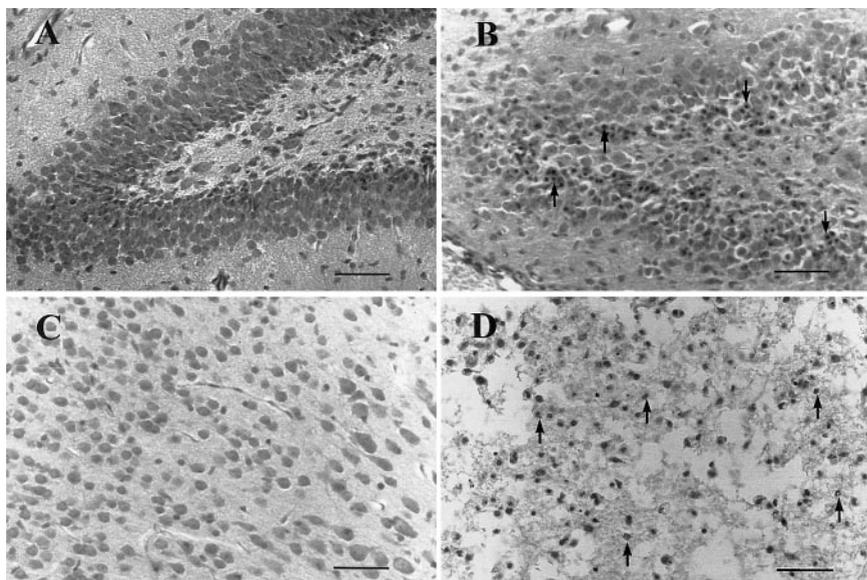
Figure 2. Hematoxylin and eosin–stained sections of neonatal rat brain 4 days after cerebral hypoxia-ischemia (magnification ×40). Control, nonischemic dorsal hippocampal dentate gyrus (A). Hypoxic-ischemic dorsal dentate gyrus showing multiple damaged cells in the inner granule layers with pyknotic, condensed nuclei (B; arrows). Control, nonischemic frontotemporal cortex (C). Ischemic frontotemporal cortex displaying marked tissue damage (D). Condensed, irregularly shaped nuclei can be seen (arrows). (Bars=40 μm.)

peptide representing the last 5 amino acids of the C-terminus of the 32-kDa product of caspase-cleaved actin. Sections were incubated with fractin antibody (1:400) at 37°C for 1 hour and then with the secondary antibody. After immunolabeling with fractin, the sections were counterstained with hematoxylin. To demonstrate the specificity of fractin labeling, sections were also treated with primary fractin antibody preabsorbed with free–5 amino acid actin antigen.

TUNEL

Eight-micrometer paraffin-embedded sections (at 2.0 A) from rat pups sacrificed 4 days after hypoxia-ischemia were examined for TUNEL reactivity using an ApopTag In situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD), which identifies digoxigenin-labeled nucleotides catalytically added to 3′-OH ends of DNA-strand breaks by terminal deoxynucleotidyl transferase (TdT). Briefly, sections were treated with 20 μg/mL proteinase K in PBS (phosphate-buffered saline [PBS]; 50 mmol/L sodium phosphate, 200 mmol/L sodium chloride, pH 7.4) for 12 minutes and immersed in 6% H_2O_2 in methanol for 15 minutes. After a 5-minute treatment with equilibration buffer, the sections were incubated at 37°C in a humidified chamber for 1 hour in TdT enzyme and then placed in stop/wash buffer. Next, slides were treated with anti-digoxigenin antibodies tagged with horseradish peroxidase for 0.5 hour in a humidified chamber. Visualization of labeled, incorporated nucleo-

tides was carried out with diaminobenzidine. Slides were then counterstained with 1% methyl green, and sections were placed on coverslips.

DNA Electrophoresis

Immediately upon sacrifice 4 days after hypoxia-ischemia, brains were quickly removed, and samples of hippocampal dentate gyrus and cerebral cortex were microdissected and fresh-frozen in dry ice. DNA was extracted from dentate gyrus and frontotemporal cerebral cortex using a QIAamp tissue kit (Qiagen, Chatsworth, CA), which isolates DNA by a column elution method, according to the manufacturer's specifications. The samples were first treated with Qiagen lysis buffer and protease and incubated at 55°C until the tissue was completely lysed. Then, RNase (20 mg/mL) was added to each sample and incubated at room temperature for 2 minutes. Afterward, the samples were incubated at 70°C for 10 minutes. Next, 210 μL of ethanol was added to each sample, and the mixture was transferred to a spin column. The columns were centrifuged at 8000 rpm for 1 minute and washed twice. The DNA was then eluted from the spin column by centrifugation. DNA was pooled from a minimum of 8 animals per treatment group. Spectrophotometry of resulting samples revealed $_{260/280}$absorbance ratios of 1.8 to 2.0, indicating relative DNA purity. Approximately 7 μg of DNA was loaded in each lane and run at 70 V on a 1.25% agarose gel stained with ethidium bromide (0.5 μg/mL). One microgram of a 1-kb DNA standard (GIBCO-BRL, Gaithersburg, MD) was run in a separate lane with each gel.

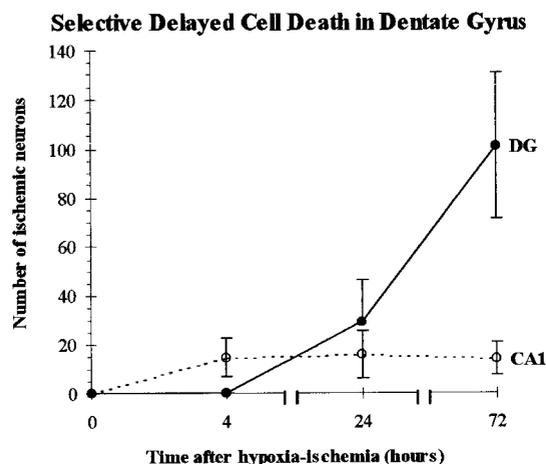## Ethidium Bromide–Staining

Sections at 2.0 A taken from rat pups sacrificed 4 days after cerebral hypoxia-ischemia were also stained with ethidium bromide (2.5 μg/mL) in H_2O for 5 minutes and examined under rhodamine fluorescence microscopy (magnification ×100; oil immersion). Apoptotic bodies were counted in sections from 10 control and 11 ischemic animals in a blinded manner. For the purposes of this study, apoptotic bodies were defined using strict criteria of clear evidence of nuclear fragmentation into 2 or more distinct entities. Extreme care was taken to only count actual fluorescent apoptotic bodies and not other nonspecific fluorescent structures like blood vessels and nucleoli. This method of counting apoptotic bodies is likely to be quite specific but not maximally sensitive. Apoptotic bodies in both dentate gyri were counted for each section using the criteria for nuclear fragmentation described above. In addition, the maximal density of apoptotic bodies per high-powered field was determined for the left and right frontotemporal cortices of each section. The total number of apoptotic bodies per section was calculated by
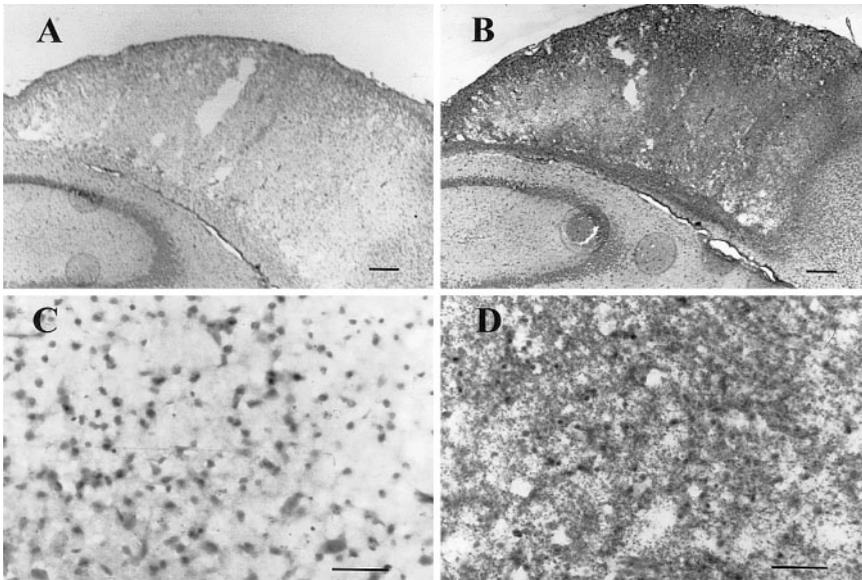

Figure 3. Time course of ischemic cell death in hippocampal CA1 region (CA1) and dentate gyrus (DG).

Figure 4. Regional fractin antibody immunolabeling pattern in hypoxic-ischemic frontotemporal cortex 3 days after hypoxia-ischemia (magnification ×4). Hypoxic-ischemic frontotemporal cortex treated with fractin antibody preabsorbed with actin antigen displaying no immunolabeling (A). Fractin antibody immunolabeling of hypoxic-ischemic frontotemporal cortex treated with unbound fractin antibody (B). (A and B, bars=0.2 mm.) High-power magnification of hypoxic-ischemic frontotemporal cortex (magnification ×40). Hypoxic-ischemic frontotemporal cortex treated with fractin antibody preabsorbed with actin antigen displaying no immunolabeling (C). Hypoxic-ischemic frontotemporal cortex treated with unbound fractin antibody demonstrating marked immunostaining(D). (C and D, bars=40 μm.)

adding the values from the left and right dentate gyri and the left and right high-powered fields of frontotemporal cortex. The median number of apoptotic bodies in the dentate gyrus and frontotemporal cortex of the control group (n=10) and the hypoxic-ischemic group (n=11) were compared using a Mann-Whitney rank sum test.

Electron Microscopy

For EM, upon sacrifice 1 or 3 days after hypoxia-ischemia, the rat pups were perfused through the ascending aorta with 20 mL of 0.1 molfl PBS (pH=7.4) followed by 0.1 molfl sodium phosphate–buffered fixative containing 2% formaldehyde and 3% glutaraldehyde. After perfusion, the brains were removed and postfixed for 1 day in the same solution. Coronal sections 200-μm-thick through the hippocampus and frontotemporal cortex were cut on a vibratome and collected in Tris-PBS. Sections were postfixed for 1 hour in 1% OsO_4 and stained en block in 2% aqueous uranyl acetate for 30 minutes. Sections were then dehydrated in ascending concentrations of ethanol, passed into propylene oxide, and embedded in durcupan (Fluka) resin. Finally, ultrathin sections through dentate gyrus and cerebral cortex were cut on the ultracute and placed on single-hole grids covered by butvar and stained with uranyl acetate and lead

citrate. Thin sections were examined by EM, and photographs of the dentate gyrus and frontotemporal cerebral cortex were obtained.

Results

Infarction Area

The inner granule cell layers of the dentate gyrus and the frontotemporal cerebral cortex displayed significant evidence of infarction, whereas those of nonischemic control animals did not (Figure 2). The mean area of cortical infarction of day 4 hypoxic-ischemic cortex was determined. At the levels 0.4, 2.0, and 2.6 A, the mean cortical infarction areas expressed as a percentage of entire cortical area above the rhinal fissure were 36.9±8.6%, 38.2±6.4%, and 58.1±9.7%, respectively. The ischemic dentate gyrus also showed evidence of damage in the inner granule cell layers and to a lesser extent in CA1. The mean numbers of ischemic neurons with fluorescent cytoplasm and pyknotic nuclei per section in CA1 and dentate gyrus at 4 hours were 14.8±8.0 and 0±0, respectively, at 24
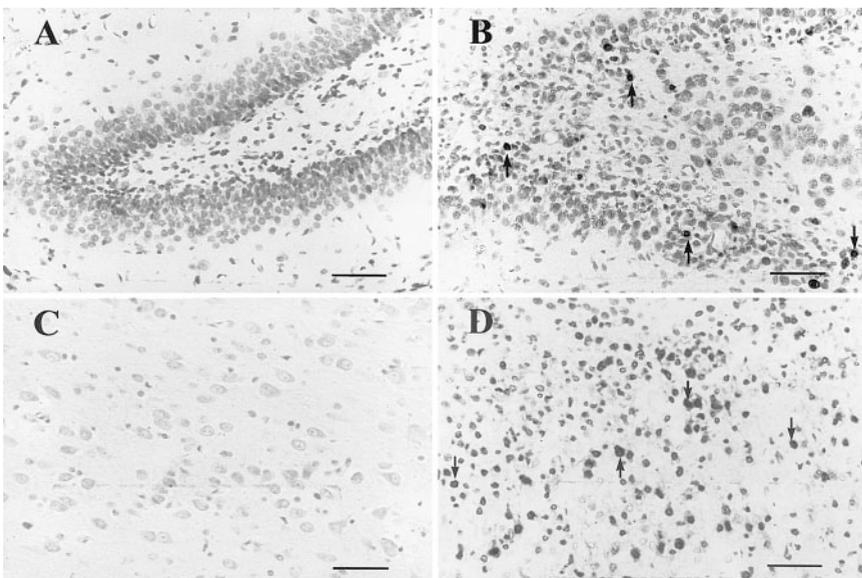
Figure 5. TUNEL of neonatal rat brain 4 days after cerebral hypoxia-ischemia (magnification ×40). Control, nonischemic dorsal hippocampus displaying no TUNEL reactivity (A). Ischemic dorsal hippocampus showing TUNEL reactivity in the inner granule cell layer and hilar areas (B; arrows). Control, nonischemic frontotemporal cortex showing no TUNEL reactivity (C). Ischemic frontotemporal cortex with marked TUNEL reactivity (D; arrows). (Bars=40 μm.)
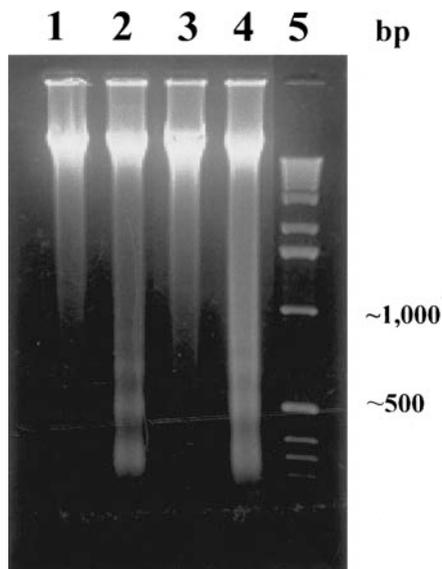
Figure 6. Agarose gel electrophoresis of neonatal rat brain DNA 4 days after cerebral hypoxia-ischemia. Lane 1, Nonischemic dentate gyrus with no evidence of DNA fragmentation; lane 2, ischemic dentate gyrus displaying a prominent laddering pattern indicative of oligointernucleosomal DNA fragmentation; lane 3, nonischemic frontotemporal cortex without a laddering pattern; lane 4, ischemic frontotemporal cortex showing a similar DNA laddering pattern; and lane 5, 1-kb DNA standard.

hours were 15.9 ± 9.7 and 29.5 ± 17.0, and at 72 hours were 14.2 ± 6.8 and 101 ± 30.0 (Figure 3). The mean number of ischemic neurons in dentate gyrus and CA1 of nonischemic control animals (represented by time point "0" in Figure 3) was 0 ± 0. In areas of ischemic injury in the frontotemporal cortex and dentate gyrus, polymorphonuclear leukocytes were identified occasionally but not included in the cell counts.

Fractin Antibody Immunolabeling
Hypoxic-ischemic sections treated with fractin antibody preabsorbed with actin antigen did not display immunolabeling.

However, sections treated with unbound fractin antibody showed marked immunolabeling of the frontotemporal cortex (Figure 4).

TUNEL
The control cortex and dentate gyrus displayed no positive TUNEL reactivity, whereas the hypoxic-ischemic cortex had strikingly positive TUNEL reactivity (Figure 5). The dentate gyrus also showed TUNEL staining 4 days after hypoxia-ischemia (Figure 5).

DNA Electrophoresis
In agarose gel electrophoresis, nonischemic tissue displayed no evidence of DNA fragmentation. Day 4 hypoxic-ischemic dentate gyrus and frontotemporal cortex samples exhibited a DNA laddering pattern in approximately 200-bp multiples, characteristic of oligointernucleosomal DNA fragmentation. In both the dentate gyrus and cortex the DNA laddering pattern extended below the 500-bp range (Figure 6).

Ethidium Bromide–Staining
In the day 4 ethidium bromide–stained, hypoxic-ischemic dentate gyrus, apoptotic bodies were detectable in small numbers under high-power magnification fluorescence microscopy (Figure 7). However, no significant differences were found in the number of apoptotic bodies counted between ischemic and control dentate gyri (Table).

Day 4 ethidium bromide–stained, ischemic frontotemporal cortex consistently showed neurons with nuclear condensation, clumping, and fragmentation into multiple spherical entities. Apoptotic bodies were clearly present without detectable signs of surrounding inflammation (Figure 7). There was a significant difference between the median number of apoptotic bodies in the control group and that in the hypoxic-ischemic group (1 versus 48; $P < 0.0001$; Table).

Electron Microscopy
Approximately 15% of granule cells in the inner layers of the dentate gyrus and 10% of neurons in the frontotemporal
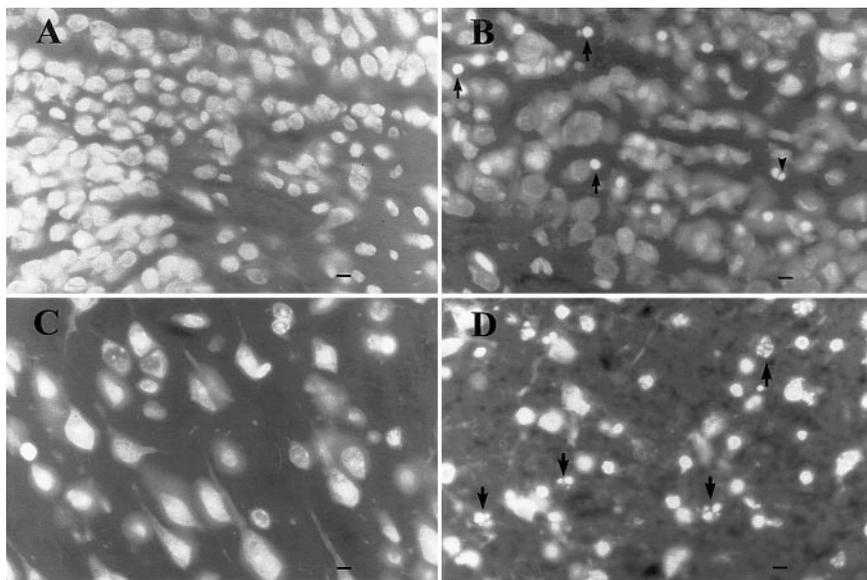
Figure 7. Ethidium bromide–staining of neonatal rat brain 4 days after cerebral hypoxia-ischemia viewed under fluorescence microscopy (magnification $\times 100$; oil immersion). Control, nonischemic dorsal dentate gyrus without evidence of nuclear fragmentation (A). Ischemic dorsal dentate gyrus with evidence of pyknotic nuclei (arrows) and nuclear fragmentation (arrowhead) consistent with the formation of apoptotic bodies (B). Control, nonischemic frontotemporal cortex without evidence of nuclear fragmentation (C). Ischemic cortex with multiple, condensed, fragmented nuclei (D; arrows). (Bars=4 μm.)

Number of Apoptotic Bodies in Ethidium Bromide–Stained Sections

	Animal Number											Median	P
	1	2	3	4	5	6	7	8	9	10	11		
Dentate gyrus													0.2420
Control	0	1	0	0	0	0	0	1	0	0		0	
Hypoxic-ischemic	0	12	4	29	1	1	0	0	0	0	0	0	
Frontotemporal cortex													<0.0001
Control	0	2	1	1	2	0	1	1	0	0		1	
Hypoxic-ischemic	3	49	54	79	54	48	32	19	23	48	53	48	

cortex exhibited prominent characteristics of apoptotic bodies by EM (Figure 8): rounding up of cell bodies, cytoplasmic and nuclear compaction, and chromatin clumping and fragmentation into multiple spherical bodies typical of apoptosis. In apoptotic cells, the cytoplasmic organelles appeared relatively intact in the presence of condensation and fragmentation of the nucleus. In the cortex, evidence of cytoplasmic membrane blebbing was seen 1 day after hypoxia-ischemia. Membrane-bound apoptotic bodies in various states of degradation, some engulfed by phagocytes, were clearly present. Control dentate and cortex samples did not display any features of apoptotic bodies (Figure 8). No polymorphonuclear leukocytes were observed by EM.

Discussion

This study provides multiple lines of evidence that in a neonatal rat model, cerebral hypoxia-ischemia produces apoptotic cell death. In this model, cortical infarction and selective delayed cell death were present in the granule cells of the inner layers of the dentate gyrus. In situ immunolabeling with fractin antibody demonstrated caspase-related cleavage of actin. TUNEL reactivity was present but lacked specificity for defining DNA fragmentation. A DNA laddering pattern consistent with oligointernucleosomal DNA fragmentation was observed on agarose gel electrophoresis. Ethidium bromide–staining and EM indicated that cerebral ischemia results in the formation of apoptotic bodies, a hallmark of apoptosis. These findings support the notion that cerebral hypoxia-ischemia produces apoptosis in this neonatal rat model.

In the detection of apoptosis in cerebral hypoxia-ischemia, several experimental variables may confound the results. The method of hypoxia-ischemia including its severity and duration may determine whether apoptosis occurs. The arterial blood pressure and brain temperature of the rat pups were not monitored during these experiments. Therefore, a significant difference in blood pressure or brain temperature between animal groups could have influenced the results of this study. In addition, the timing of tissue examination after the insult may be critical. Apoptosis likely occurs for a few days after an insult, but a precise time frame has not been established. In this study, evidence of apoptosis was found 1, 3, and 4 days after cerebral hypoxia-ischemia but may be present at other time points.

The sensitivity and specificity of experimental methods may determine whether apoptosis is detected. Oligointernucleosomal DNA fragmentation can be demonstrated bio-chemically with electrophoresis when apoptotic bodies are detectable in only a small fraction of cells.[20] DNA electrophoresis may provide biochemical evidence of oligointernucleosomal DNA fragmentation but is subject to problems of sensitivity. EM is the most definitive method for demonstrating the presence of apoptotic bodies but can only examine small areas of tissue. Other techniques that can be used to examine larger samples of tissue such as ethidium bromide–staining or TUNEL may lack specificity. In particular, TUNEL reactivity alone is not adequate to demonstrate apoptotic bodies. However, in this model, TUNEL reactivity was present in the same population of cells displaying prominent features of apoptosis by EM and evidence of caspase activation and oligointernucleosomal DNA fragmentation. These multiple lines of biochemical and morphological evidence are sufficient to establish the presence of apoptosis.

A number of factors may affect the results of studies of apoptosis. Recent evidence shows that genetic differences among strains of animals alter the susceptibility to excitotoxic insult.[27] In addition, in vitro evidence indicates an increased susceptibility to apoptosis in immature cortical neurons.[28] One possibility is that younger animals display apoptosis more readily than mature animals. This may be a feature of the developing brain because many components of the cell cycle that are highly expressed in developing organs participate in apoptosis. In fact, experimental evidence suggests that the stage of the cell cycle may influence apoptosis.[19] Furthermore, the nature of a specific cell type such as a granule cell may determine its susceptibility to undergo apoptosis. The granule cells lack calbindin-D28 k and, therefore, may be more vulnerable to calcium influx triggering apoptosis.[29] Because very small numbers of polymorphonuclear leukocytes were detected in this study, it is unlikely that their presence significantly affected the results. Any of these factors could influence the production of apoptosis after cerebral hypoxia-ischemia. In the present study, we have attempted to control for these variables and to demonstrate that apoptosis occurs following cerebral hypoxia-ischemia.

Although necrotic mechanisms may dominate in severe ischemic insults, apoptosis may represent a target that may potentially expand the therapeutic window for stroke treatment because it appears to involve different mechanisms than necrosis and may occur in areas of milder ischemic injury for days after the initial insult. Previous studies[22,30] have shown that granule cells of the dentate gyrus that undergo apoptosis
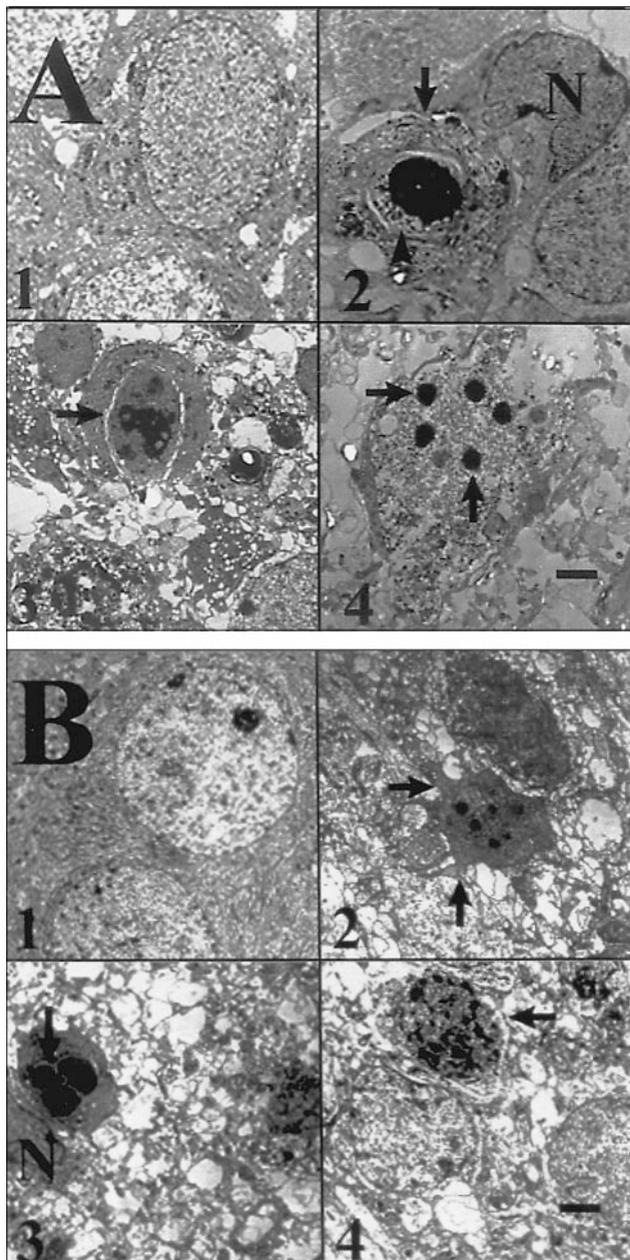
Figure 8. EM of neonatal rat brain (magnification ×2000). A, The inner granule cell layer of dorsal dentate gyrus 3 days after cerebral hypoxia-ischemia: 1, nonischemic inner granule cell layer of dorsal dentate gyrus; 2, apoptotic body surrounded by a membrane (arrowhead) with evidence of condensation of the cytoplasm and electron-dense nucleus. The cytoplasm (arrow) and nucleus (N) of a phagocyte engulfing the apoptotic body can be seen; 3, ovoid-shaped, membrane-bound apoptotic body (arrow) possessing a shrunken, crenated nucleus with evidence of fragmentation; 4, multiple, spherical apoptotic bodies containing condensed nuclear remnants (arrows). (Bar=4 μm.) B, Frontotemporal cerebral cortex: 1, nonischemic frontotemporal cortex of the 10-day-old neonatal rat; 2, cortical neuron 1 day after hypoxia-ischemia showing blebbing of cytoplasmic membrane (arrows) and nuclear condensation and fragmentation into spherical bodies; 3, multiple, rounded, membrane-bound apoptotic bodies (arrow) containing condensed nuclear remnants present 3 days after hypoxia-ischemia. The nucleus (N) of a surrounding phagocyte can be seen; 4, partially degraded apoptotic body (arrow), which may lie within a neighboring phagocyte. (Bar=4 μm.)

can be rescued post hoc by *N*-methyl-D-aspartate blockers (MK801, felbamate) given after hypoxia-ischemia. More recent reports have indicated a potential neuroprotective effect in hypoxia-ischemia of caspase inhibition[31,32] and in caspase-deficient mice.[33] Further investigation of excitotoxicity in cerebral ischemia may lead to a better understanding of modes of cell death such as apoptosis and necrosis and to more effective stroke treatments in the future.

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Editorial Comment

Ischemic injury to the central nervous system (CNS) can have devastating lifelong effects to the developing fetus or to the neonate. The possibility that transient or sustained hypoxia in utero or at parturition may affect vulnerable cells of the developing brain is highly relevant to the course of CNS development as seen from the disabilities of cerebral palsy. The nature of the injury to the cell populations at risk is highlighted by the possibility that apoptosis may be triggered in neurons and glial cells in models of neonatal hypoxia. Apoptotic mechanisms have been identified in the development of the rodent CNS.[1] The identification of apoptotic processes among specific cell populations at risk of ischemic injury might offer potential targets for limiting these types of early CNS injury.

In their work with the neonatal rat model of hypoxia-ischemia, Pulera et al describe the presence of apoptotic changes by identifying cells incorporating labeled nucleotides at exposed 3′-OH breaks in DNA by TdT, evidence of DNA fragmentation by gel electrophoresis, morphological alterations in neurons by electron microscopy, and evidence of actin cleavage as an indirect measure of enhanced caspase activity. They demonstrate evidence of delayed neuronal injury in the hippocampal dentate gyrus between 1 and 4 days after the hypoxic-ischemic insults, but earlier evidence of necrotic injury in cells of the frontotemporal cortex. These findings are in line with the recently published work of Renolleau et al who identified in situ DNA single-strand nicks in cells as early as 6 hours after an hypoxic-ischemic insult in a reperfusion model.[2] The particular vulnerability of rat dentate gyrus cells has been emphasized by their susceptibility to adrenalectomy.[3,4] This speaks again to the differential susceptibility of neurons (and perhaps other cells) in different territories of the brain to external factors. These differential susceptibilities are also reminiscent of the responses of the cortex and striatum to injected interleukin-1β in the adult rat.[5]

Focusing on the features of apoptotic injury, several points may be made. First, the evidence in favor of an apoptotic response of cells in the dentate gyrus to ischemia in these experiments, while strong, needs further support. For instance, although actin cleavage was taken as an indirect measure of caspase activation, it has been recently noted that caspase-mediated cell death may not have all the features of apoptosis in the CNS.[6,7] Also, actin degradation may result from several proteolytic processes,[8,9] and other measures of caspase activity, including poly(ADP-ribose) polymerase (PARP) proteolysis, may be more helpful.[10] Protection of neuron morphology and function by inhibitors of caspase-1 and caspase-3 may prove a more helpful strategy. Indeed, Cheng et al recently demonstrated that an inhibitor of caspase-3 in the same model of neonatal hypoxic-ischemic brain injury results in neuron protection.[11] Furthermore, Parsadanian et al reported that Bcl-xl overexpression in a murine neonatal hypoxia-ischemia model was "neuroprotective."[12] It will be enormously important for the present experiments to be extended in these directions.

Second, the other measures of nuclear DNA injury are subject to alternative interpretations. For instance, DNA nick labeling techniques may suggest processes of DNA scission, but also of DNA repair. This is one potential explanation for the much more rapid appearance of dUTP incorporation in the adult primate than in the Wistar rat following focal cerebral ischemia.[13] Evidence of DNA segmentation by gel electrophoretic methods is extremely useful in demonstrating nuclear DNA scission; however, the specific nature of the fragments may be very relevant. MacManus et al[14] have clearly demonstrated that the ends of fragmented DNA appearing in injured brain tissue of rats undergoing focal

cerebral ischemia differ from those seen in isolated cells or in cardiac myocytes, which may undergo apoptosis. Finally, the morphological criteria for apoptotic injury in the CNS cannot be so readily borrowed from the injuries seen in cells or organs in which apoptosis is common (eg, thymocytes).[15]

The techniques used here to detect DNA fragments suggest an injury process with lethal consequences to the neuron. Whether the injury to the dentate gyrus neurons in this model results from apoptosis (as defined in other systems), caspase-mediated injury, other yet-to-be defined processes, or a combination, this descriptive work indicates both differential vulnerability of the developing brain to hypoxic-ischemic injury and the possibility that at least some of these processes may afford targets for future neuron protective interventions.

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