Evidence Supporting a Role for Programmed Cell Death in Focal Cerebral Ischemia in Rats

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Background and Purpose: Cells die by one of two mechanisms, necrosis or programmed cell death. Necrosis has been implicated in stroke and occurs when the cytoplasmic membrane is compromised. Programmed cell death requires protein synthesis and often involves endonucleolytic cleavage of the cellular DNA. We assessed the potential contribution of programmed cell death to ischemia-induced neuronal death.

Methods: Cycloheximide (protein synthesis inhibitor; 1 mg/kg per 24 hours) or vehicle (1 mL/kg per 24 hours) was continuously infused into the right cerebral ventricle of spontaneously hypertensive rats. Neocortical focal ischemia was produced by tandem occlusion of the right common carotid artery and the ipsilateral middle cerebral artery. After 24 hours the brain was stained with 2% 2,3,5-triphenyltetrazolium and the ischemic zone quantitated. Protein synthesis was determined by [3H]methionine incorporation into acid-precipitated protein. DNA integrity was determined in isolated DNA by gel electrophoresis and in whole cells by flow cytometry.

Results: Continuous cycloheximide infusion caused approximately 70% reduction in cortical protein synthesis. Cycloheximide also reduced the size of the infarction produced by focal cerebral ischemia when compared with controls (ischemic brain volume, 147.5±25.9 and 188.7±16.8 mm³ for cycloheximide and saline, respectively; P<.01), suggesting that protein synthesis may contribute to cell death. Purified DNA from the ischemic zone showed evidence of endonucleolytic degradation when fractionated by gel electrophoresis. Flow cytometric analysis demonstrated increased propidium iodide fluorescence in intact cells isolated from ischemic cortex, indicating an increased accessibility of degraded DNA to the intercalating dye.

Conclusions: New protein synthesis appears to contribute to ischemic cell death in which endonucleolytic DNA degradation is apparent. These observations implicate programmed cell death in ischemic injury and may open unique therapeutic approaches for the preservation of neurons in stroke. (Stroke. 1993;24:2002-2009.)

Key Words • apoptosis • cerebral ischemia • neuronal death • protein synthesis • rats

A number of factors that contribute to the loss of neurons following ischemia have been described. A partial list includes excitatory amino acids, calcium, a variety of neurotransmitters, and physical factors such as blood-brain barrier integrity and temperature.1,2 While all these factors contribute to neurotoxicity, there are only two distinct mechanisms that have been described for cell death: necrosis and programmed cell death (PCD).3,4

Necrosis results when the integrity of the cell membrane becomes compromised. Normal ionic gradients are disrupted, mitochondria become dysfunctional, the cell swells, and lysosomal enzymes are activated. In contrast, a primary event in PCD appears to be activation of an endonuclease that cleaves the DNA between nucleosomes, resulting in DNA fragments that are multiples of 180 to 200 base pairs. In PCD, the cytoplasm condenses, but the cell membrane remains intact and functional as the DNA is digested.

Programmed cell death is essential and appropriate in modeling the developing nervous system. In utero, half the neurons that are produced will be terminated, often as a result of the failure to acquire adequate growth factor from the target tissue.5-7 Insufficient exposure to growth factors precipitates PCD, and the degeneration can be blocked by protein and RNA synthesis inhibitors.8-10 Specific growth factors also suppress PCD in cultured primary neurons, where withdrawal of these factors causes cell death by a process that requires macromolecular synthesis.11-14

The developmental models clearly demonstrate that neurons have the capacity to initiate PCD. However, the potential role of PCD in neuronal death after a pathophysiological insult is only beginning to be appreciated.15,16 We examined the possibility that PCD might be involved in the cell loss resulting from focal cerebral ischemia by taking advantage of the fact that PCD in neurons requires the synthesis of new, as yet unidentified, proteins. Thus, a protein synthesis inhibitor should reduce the size of an infarction resulting from focal ischemia if PCD is involved.
We also looked for the characteristic endonucleolytic degradation of the DNA that is a hallmark of PCD. Two approaches were used. The first approach directly examined DNA extracted from ischemic and nonischemic tissue by gel electrophoresis. The second approach used flow cytometry to examine DNA in perfusion-fixed, intact cells isolated from ischemic and nonischemic cortices. The flow cytometer analysis provides an indication of the amount and integrity of the DNA in intact cells. The results implicate PCD in the loss of neurons following focal cerebral ischemia.

Materials and Methods

Spontaneously hypertensive rats (Taconic Farms, Germantown, NY) weighing an average of 275 g were anesthetized with chloral hydrate (500 mg/kg IP) and anesthesia was maintained with supplemental doses as needed. Animals were placed in a stereotaxic frame, and an access hole was drilled through the skull to permit placement of a cannula into the right cerebral ventricle. Brain infusion cannulas (Alzet Brain Infusion Kit, Alza Corporation, Palo Alto, Calif) were stereotaxically placed following the manufacturer's recommendations at the following coordinates relative to bregma: 1 mm posterior, 2 mm lateral, 4.5 mm deep, with the tooth bar fixed –3.8 mm relative to 0 intraaural line.

The cannulas were fixed in place with dental cement and anchored to a screw placed 5 mm away. Cycloheximide (1 mg/kg per 24 hours) or saline (1 mL/kg per 24 hours) was continuously infused into the right cerebral ventricle for 24 hours by Alzet minipumps (model 2001D, 8 μL/h flow rate, Alza Corporation). Immediately before cannula placement in the cerebral ventricle, each animal received an intracerebroventricular infusion (4 μL) of saline or cycloheximide over a 5-minute period starting 30 minutes before middle cerebral artery occlusion.

Neocortical focal ischemia was produced by tandem occlusion of the right common carotid artery and the ipsilateral middle cerebral artery.18,19 The right common carotid was isolated via a midline incision, ligated in two places, and cut between the ligatures. An incision was made midway between the right eye and external auditory canal and the skin and underlying temporalis muscle retracted. The middle cerebral artery was exposed transtemporally without damage to the zygomatic bone through a 3×3-mm craniotomy, raised 0.5 to 1.0 mm above the cortical surface using a microdissecting hook, and severed by electrocautery with minimal thermal trauma to the underlying cortex. Total operating time was approximately 40 minutes.

Animals were maintained at 37.5°C throughout recovery by means of a rectal thermostir connected to a heating lamp directed at the head of the animal until the righting reflex returned. After 24 hours the brain was removed, sliced into 2-mm sections, stained for 15 minutes with 2% 2,3,5-triphenyltetrazolium in phosphate-buffered saline to delineate the ischemic zone, and fixed in 10% phosphate-buffered formalin. The size of the ischemic zone was determined by computer-assisted image analysis.18–20 Comparisons between treatment groups were by analysis of variance followed by Tukey’s procedure for multiple comparisons with P≤.05 considered significant. Data are expressed as mean±SD.

To determine the effect of cycloheximide infusion on the rate of protein synthesis in the rat cortex, six animals were anesthetized with chloral hydrate (500 mg/kg IP) and divided into two groups. A catheter was stereotaxically implanted into the right cerebral ventricle of three rats that were infused with cycloheximide via subcutaneous osmotic pumps as described above. Access holes were drilled through the skull of sham animals (n=3), but catheters were not implanted. An infusion catheter was inserted into the femoral vein of all animals. Ninety minutes after drilling holes through the skull, animals were injected with L-[methyl-3H]-methionine (0.5 mCi, 0.012 μmol/mL) through the femoral catheter. Forty-five minutes after injection of tracer, animals were killed with an overdose of pentobarbital, and the brain was rapidly removed. The hemispheres were then isolated and divided into three equivalent pieces, frozen on dry ice, and stored at –80°C until processing.

The rate of L-[methyl-3H]-methionine incorporation into protein was measured using a modification of the method described by Xie et al.21 Tissue samples were weighed and homogenized with a polytron in 2 mL ice-cold water. After removing an aliquot to determine total tritium levels, 2.5 mL of ice-cold 10% trichloroacetic acid (TCA) was added and incubated on ice for 30 minutes. The precipitate was washed twice in 2.5 mL 5% TCA and collected by centrifugation at 900g for 5 minutes. After the second wash, the pellet was redissolved in 0.2% sodium carbonate, 0.1N sodium hydroxide, and an aliquot was combined with 15 mL scintillation cocktail and counted in a liquid scintillation counter.

To examine cellular DNA for endonucleolytic degradation characteristic of PCD, neocortical focal ischemia was produced by the procedure outlined above except that osmotic pumps were not implanted. The brains were removed 24 hours after occlusion, and the ischemic cortex was dissected and immediately frozen on dry ice along with an equivalent size piece of the contralateral cortex. Tissue was minced, and cells were lysed on ice in 5 mmol/L tris(hydroxymethyl)aminomethane (Tris) containing 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X-100 for 30 minutes. Genomic DNA was pelleted by centrifugation at 13 000g for 20 minutes. DNA that did not sediment during centrifugation was purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation before RNase A digestion (100 μg/mL) for 30 minutes at 37°C. Samples were then reextracted with chloroform/isoamyl alcohol (24:1), reprecipitated in ethanol, and DNA was electrophoresed in 1.8% agarose containing 0.5 μg/mL ethidium bromide at 40 mA for approximately 5 hours.

Total DNA was extracted by digesting the cortex in digestion buffer (100 mmol/L NaCl, 10 mmol/L Tris HCl, 25 mmol/L EDTA, 0.5% sodium dodecyl sulfate, 0.1 mg/mL protease K) at 50°C for 16 hours. Samples were then processed as described above, including extraction, precipitation, RNase A digestion, reextraction, and precipitation before gel electrophoresis.

Flow cytometry was used to compare DNA within the context of the cell from ischemic and nonischemic tissue. Focal cerebral ischemia was induced as described above, and 24 hours later the animals were anesthetized.
with ether and perfusion-fixed by slowly injecting 100 mL of 10% phosphate-buffered formalin into the left ventricle of the heart. Equivalent-sized pieces of ischemic cortex and contralateral nonischemic cortex were dissected from the brain and postfixed in formalin for 2 hours before rehydration through a graded series of ethanol into water. Isolated cells were prepared using a papain dissociation kit (Worthington Biochemical, Freehold, NJ) according to the manufacturer’s recommendation. In brief, tissues were minced, incubated with papain (20 U/mL) for 1 hour at 37°C, and triturated to dissociate cells. Intact cells were purified by centrifugation through a discontinuous density gradient composed of ovomucoid protease inhibitor with bovine serum albumin, stained with propidium iodide containing RNase and NP-40 detergent in citrate buffer, and analyzed with a Coulter EPICS model 752 flow cytometer equipped with an argon ion laser operating at 400 mW and 488 nm (50,000 events per histogram).

**Results**

Tandem occlusion of the right common carotid artery and ipsilateral middle cerebral artery produced well-defined lesions that could be visualized with triphenyltetrazolium staining and quantitated by image analysis. The saline-infused animals exhibited lesions that constituted 15.5% of the total brain mass (Table 1). The size of this lesion compared well with our historic controls that had not been implanted with a constant infusion pump, indicating that the pump did not influence the size of the infarction (nonimplanted control animals: ischemic brain volume, 198.6±39 mm³; percent ischemic brain, 14.4±1.9% [mean±SD, n=14]).

Gross behavioral observation did not reveal any obvious differences between the cycloheximide-treated animals and the vehicle controls, and both groups exhibited similar weight loss from the procedure (Table 2). Quantitation of the ischemic zone from the brains of the cycloheximide-treated animals revealed a significant reduction in the size of the infarction when compared with vehicle-treated controls (Table 1, Fig 1).

The effect of cycloheximide infusion on L-[methyl-3H]-methionine incorporation into TCA-precipitated protein was examined in the cortices of cycloheximide-infused and sham-operated animals (Table 3). L-[Methyl-3H]-methionine was injected 90 minutes after initiation of cycloheximide infusion, and brains were collected 45 minutes later. Thus, the period of [3H]methionine incorporation correlates with the posts ischemic time of 60 to 105 minutes in the middle cerebral artery–occluded animals. Cycloheximide infusion resulted in a 67% and 73% reduction in TCA-precipitated counts (disintegrations per minute per milligram wet weight) from the left and right hemicortices of the control and cycloheximide-treated animals, respectively.

DNA was extracted from the ischemic cortex and the contralateral, nonischemic cortex to determine if DNA degradation had occurred. This experiment permits differentiation between endonucleolytic degradation, as seen by chromatin bands, or lysosomal degradation, which would yield a smear of random-sized DNA, DNA from the nonischemic cortex was largely intact and consequently exhibited little migration in the gel. In contrast, a portion of the DNA from the ischemic zone had undergone endonucleolytic digestion and exhibited the characteristic nucleosome ladder associated with PCD (Fig 2, left and center panels). The nucleosomal ladder is more difficult to distinguish if fragmented DNA is not initially isolated from genomic DNA in the ischemic cortex (Fig 2, right panel).

The structural integrity of the DNA was then determined within the context of the cell by flow cytometry. Equivalent-sized pieces of ischemic and nonischemic cortex were obtained from perfusion-fixed animals. Preliminary experiments had indicated that perfusion-fixation was necessary to prevent ischemic damage to normal tissue during dissection and fixation. To obtain a single cell suspension, the samples were rehydrated and

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**TABLE 1. Effect of Drug Treatment on Total and Ischemic Brain Volume**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Total Brain</th>
<th>Ischemic</th>
<th>% Ischemic Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6</td>
<td>1217±44</td>
<td>188.7±16.8</td>
<td>15.5±1.2</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>6</td>
<td>1241±14</td>
<td>147.5±25.9</td>
<td>11.9±2.0*</td>
</tr>
</tbody>
</table>

*P<.01 vs saline.

**TABLE 2. Effect of Middle Cerebral Artery Occlusion on Animal Weights**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Pre MCAO</th>
<th>Post MCAO</th>
<th>Net Loss (%)</th>
<th>% Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6</td>
<td>274±18</td>
<td>247±15</td>
<td>26.8±5</td>
<td>9.8±1.5</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>6</td>
<td>276±18</td>
<td>254±17</td>
<td>21±3</td>
<td>7.7±0.9</td>
</tr>
</tbody>
</table>

Values are mean±SD weight in grams or percent loss. MCAO indicates middle cerebral artery occlusion.

**TABLE 3. Area of Ischemic Damage**

<table>
<thead>
<tr>
<th>Brain Slice</th>
<th>Area of Ischemic Damage</th>
<th>Brain Slice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.4±1.2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>12.4±1.2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>10.4±1.2</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>8.4±1.2</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>6.4±1.2</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>4.4±1.2</td>
<td>7</td>
</tr>
</tbody>
</table>

*P<.05 vs saline.
TABLE 3. Effect of Cycloheximide Infusion on [\(^{3}H\)]Methionine Incorporation into Rat Cortex

<table>
<thead>
<tr>
<th></th>
<th>Total dpm/mg Cortex (×10^4)</th>
<th>TCA-Precipitated dpm/mg Cortex (×10^4)</th>
<th>% [(^{3}H)] Incorporation into TCA Precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham, left</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17.4</td>
<td>6.9</td>
<td>39.5</td>
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<tr>
<td>2</td>
<td>20.4</td>
<td>8.3</td>
<td>40.5</td>
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<tr>
<td>3</td>
<td>17.3</td>
<td>9.1</td>
<td>52.1</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>18.4±1.4</td>
<td>8.1±0.9</td>
<td>44±5.7</td>
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<tr>
<td>Sham, right</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18.9</td>
<td>6.8</td>
<td>36.1</td>
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<td>20.9</td>
<td>8.2</td>
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<tr>
<td>3</td>
<td>17.4</td>
<td>8.7</td>
<td>49.8</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>19.1±1.4</td>
<td>7.9±0.8</td>
<td>41.7±5.9</td>
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<td>Chex, left</td>
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<td></td>
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<tr>
<td>1</td>
<td>19.2</td>
<td>3.4</td>
<td>17.9</td>
</tr>
<tr>
<td>2</td>
<td>17.0</td>
<td>3.3</td>
<td>19.5</td>
</tr>
<tr>
<td>3</td>
<td>7.1</td>
<td>1.5</td>
<td>20.6</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>14.4±5.3</td>
<td>2.7±0.9*</td>
<td>19.3±1.1*</td>
</tr>
<tr>
<td>Chex, right</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>21.2</td>
<td>2.5</td>
<td>11.7</td>
</tr>
<tr>
<td>2</td>
<td>17.3</td>
<td>2.6</td>
<td>15.3</td>
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<tr>
<td>3</td>
<td>7.5</td>
<td>1.2</td>
<td>16.0</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>15.3±5.8</td>
<td>2.1±0.6*</td>
<td>14.3±1.9*</td>
</tr>
</tbody>
</table>

Cycloheximide (Chex)-treated rats were infused with Chex (1.75 mg/mL, 8 \(\mu\)L/h) into the right lateral ventricle. Left and right cortices were collected 45 minutes after L-[\(^{3}H\)]methionine administration and divided into three equivalent parts. Values shown for each hemicortex represent the mean of those three determinations. dpm indicates disintegrations per minute; TCA, trichloroacetic acid.

\(^*P<.01\) different from sham animals, matched cortex.

Discussion

Most investigations concerning cerebral ischemia have attributed the death of neurons to passive, necrotic processes. In turn, this has directed therapeutic approaches toward the development of compounds that minimize the effect of extracellular toxins generated in the ischemic environment. The present studies were designed to test if cell death was solely necrotic or whether an active process like PCD contributes to the death of neurons in ischemia. If PCD is involved, this might suggest new areas for therapeutic intervention that are directed at those intracellular targets that contribute to the cell death cascade.

A combination of in vivo, cellular, and biochemical experiments have provided data that are consistent with a role for PCD in neuron attrition after focal cerebral ischemia. By demonstrating the protective effect of a protein synthesis inhibitor and the endonucleolytic degradation of the ischemic DNA, two key criteria for PCD have been fulfilled. In addition, the neuroprotective effect of a protein synthesis inhibitor points to the potential utility of designing novel agents that prevent PCD for the treatment of stroke.

The effect of protein synthesis inhibitors on delayed neuronal death in a model of transient ischemia has recently been examined with mixed results. Protective effects were demonstrated after intraperitoneal administration of anisomycin; however, others have suggested that this effect is nonexistent or can be abolished when body temperature is controlled. Both of these studies differed from those presented here in two key aspects. First, they used transient global ischemia and monitored delayed neuronal death in the hippocampus by morphometric techniques. The present study used permanent focal ischemia and monitored ischemic damage by digital quantitation of triphenyltetrazolium chloride staining, which has been demonstrated to be highly correlated with cell counts. Second, the protein synthesis inhibitors were administered by single injections at 24-hour intervals, while our results are based on continuous infusion of the inhibitor directly into the cerebral ventricle.

We were not able to attain adequate intracranial concentrations of cycloheximide by systemic administration without serious toxicity. This could be predicted by applying data derived from PCD investigations in primary neuronal cultures to a simple pharmacokinetic model. In nerve growth factor-deprived neurons, cycloheximide was protective at 18 to 30 \(\mu\)mol/L. Using a one-compartment pharmacokinetic model, it was calculated that a similar concentration could be attained in vivo with a dose of approximately 20 mg/kg, while the LD\(_{50}\) for systemic cycloheximide in rats has been established at 2 to 4 mg/kg. Use of the infusion pump provided a sustained, local, and nonlethal concentration of cycloheximide that was maintained for the duration of the experiment.

Continuous intracerebroventricular administration of cycloheximide was shown to have a rapid and profound inhibitory effect on protein synthesis. Interestingly, ischemia alone can also have a significant effect on protein synthesis. A 90% inhibition of brain protein synthesis was observed within 10 to 30 minutes after 5 minutes of global ischemia in the gerbil.

This apparent dichotomy regarding the attenuation of protein synthesis and the protective effect of cycloheximide also occurs in neurons and neuronal-like cells during PCD. When sympathetic neurons are deprived of nerve growth factor, there is a reduction in protein synthesis that precedes cell death. Likewise, a reduction in protein synthesis is an early observation in neuroblastoma×dorsal root ganglia cells that are driven into PCD by serum deprivation. In these cases, exposure to cycloheximide protects from cell death, even though protein synthesis is already attenuated. Thus, it is possible that cycloheximide may be inhibiting the
Another key consideration in ischemia experimentation is preventing hypothermia. Internal temperature was controlled at 37.5°C during surgery and recovery until the righting reflex was restored and spontaneous motor activity was exhibited (approximately 1.5 hours). This precluded any thermal protection during the “therapeutic window” for postischemic hypothermia, which has been determined to be less than 30 minutes. However, other laboratories have observed reductions in infarct volume when body temperature was reduced to 33°C for 1 hour, starting 1 hour after middle cerebral artery occlusion. This raises the possibility that the therapeutic window for hypothermia might be greater than previously thought. Therefore, even though core temperature was controlled for 90 minutes, we cannot categorically exclude a protective contribution from a late-onset hypothermic response to cycloheximide. It should be noted that the protective effect of hypothermia appears to be much more prominent in ischemia-reperfusion models. In fact, a recent study comparing reperfusion injury with permanent occlusion observed no statistically significant effect on infarct volume after permanent middle cerebral artery occlusion when body temperature was lowered to 30°C for 2 hours after the occlusion.

Obtaining direct evidence for endonucleolytic degradation of DNA from the ischemic cortex provides support for the role of PCD in ischemic cell death in the brain. The 200-base pair bands that correlate with the known size of a mononucleosome are much easier to identify when genomic DNA is first separated from fragmented DNA. It is also apparent from these gels that endonucleolytic degradation appears concurrently with nonspecific DNA degradation, suggesting that PCD occurs concurrently with necrosis. Further studies that describe the time course and specific population of cells exhibiting endonucleolytic DNA degradation should permit one to address the question of whether cycloheximide reduces infarct volume by preventing endonuclease activation.

Most cells undergoing PCD, including lymphocytes, keratinocytes, prostate cells, neurons, and other tissues, demonstrate endonuclease activity as a critical component in the cell death process. Interestingly, glutamate has been shown to trigger endonucleolytic DNA degradation in rat cortical cultures. The nucleosomal fragments appear before glutamate-induced neuronal death, and inhibiting transcription or endonucleases prevents both DNA degradation and cell death. The extent to which other organs activate a PCD cascade in

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**Fig 2.** DNA was isolated from ischemic cortex and the contralateral, nonischemic cortex 24 hours after permanent ipsilateral middle cerebral artery occlusion in three independent preparations and size fractionated using agarose gel electrophoresis. In the left and center panels, fragmented DNA was isolated from genomic DNA before extraction and electrophoresis. The right panel demonstrates the electrophoretic profile after total DNA extraction. Each gel contains a 123-base pair DNA ladder as a size standard.

**Fig 3.** Graphs show intensity of propidium iodide fluorescence from fixed cells isolated from a normal, nonischemic cortex (top panel; mean channel peak, 71) and the contralateral, ischemic cortex (bottom panel; mean channel peak, 83). The x axis represents the integrated red fluorescence channels from 0 to 200.
response to ischemia is not known, but a recent report has identified this phenomenon in kidney, where brief periods of ischemia induce endonuclease activation and the morphological appearance of PCD.34

Our observations on isolated DNA were supported by flow cytometry, which was used to examine the DNA of intact neurons within the context of the cell. Two different patterns of DNA degradation have been described for propidium iodide fluorescence in intact cells that identify PCD (or apoptosis) and distinguish it from necrosis. In whole thymocytes, PCD can be elicited by glucocorticoids.30 The chromatin condenses in these cells, and a hypodiploid peak of diminished fluorescent intensity is revealed by flow cytometer analysis.35-37 This peak is thought to occur because the ability of propidium iodide to intercalate into the DNA is reduced by the chromatin condensation. In contrast, thymocytes undergoing necrotic death show no change in the pattern of DNA fluorescence.

The second pattern of fluorescence can be produced by activating endonucleases in isolated thymocyte nuclei with Ca2+ and Mg2+. In this model, the fluorescent intensity of the nuclei increases in proportion to the extent of DNA degradation.38 In contrast to the intact cells, the chromatin does not condense in these nuclei, and endonuclease digestion of the DNA appears to increase the accessibility of the degraded DNA to the intercalating dye. Thus, when chromatin condensation accompanies endonuclease activity, fluorescent intensity is reduced. In the absence of chromatin condensation, fluorescence intensity increases.

Is chromatin condensation a universal feature of all cells undergoing PCD? Nerve growth factor–deprived superior cervical ganglia neurons undergo PCD that requires macromolecular synthesis.11,14,33 Growth factor removal precipitates extensive neurite degeneration; however, nuclear changes are limited to the appearance of convolutions in the nuclear membrane and a diffuse increase in chromatin density. Interestingly, the late onset of structural changes seen in the nucleus of nerve growth factor–deprived superior cervical ganglia neurons is similar to that described for hippocampal CA1 neurons undergoing delayed neuronal death after transient cerebral ischemic.22,29 In both models, nuclear and mitochondrial elements appear intact until frank neuronal degeneration.

Thus, neurons do not exhibit chromatin condensation during cell death caused by ischemia or growth factor deprivation. In turn, one would predict an increase in fluorescent intensity in neurons undergoing PCD due to the increased accessibility of endonuclease-digested DNA to the intercalating propidium iodide dye. While we favor the hypothesis that the increase in fluorescence is due to increased propidium iodide binding, we have not ruled out the remote possibility that ischemic cells are accumulating additional DNA.

The observation that PCDs do not necessarily require apoptosis-like morphology has recently received strong support from the study of two model PCD systems,7 T cell negative selection and the loss of the intersegmental muscles of the moth Manduca sexta after metamorphosis.40 These two systems were shown to differ in cell-surface morphology, nuclear ultrastructure, DNA fragmentation, and gene expression during PCD. Therefore, the observations of Deshpande et al25 showing a lack of apoptosis-like morphology in hippocampus after transient ischemia does not exclude the possibility that PCD could occur. This is supported by the lack of apoptotic morphology in neurons undergoing PCD after growth factor deprivation.11

In conclusion, three disparate approaches were used to analyze the mechanisms underlying cell death, and each generated data that are consistent with a role for active PCD after focal cerebral ischemia. These data suggest that novel therapeutic approaches directed at disrupting the process of PCD may be useful for preserving functional neuronal tissue after an ischemic insult.

Acknowledgments

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References


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**Editorial Comment**

Cell activation of a suicide gene program causing the cell to die is known as apoptosis or programmed cell death. Linnik et al in the accompanying article discuss this as a potential mechanism in the pathophysiology of ischemic stroke. Apoptosis, first studied by Wyllie in thymocytes, underlies the controlled cell death that is necessary to delete cells during embryonic development, to effect endocrine-regulated atrophy, and to effect T and B cell maturation. In the nervous system, apoptosis has been demonstrated to underlie the neuronal death in sympathetic cultures deprived of nerve growth factor. The conventional features of apoptosis (shrinking death) include (1) intracellular compaction of nuclear chromatin and cytoplasm into sharply circumscribed, uniformly dense masses surrounded by lysosomal membrane (apoptotic bodies); (2) endonuclease digestion, double-strand cleavage of DNA to produce fragments that are multiples of approximately 185 base pairs (DNA ladder); and (3) cell death, which requires activation of a gene program and is therefore inhibited by RNA and protein synthesis inhibitors.

Fascinating “good” and “bad” molecules have been recently discovered to have causally important roles in the apoptotic process. Some genes mediating apoptotic cell death are related to tumor suppressor genes, and their effects oppose those of oncogenes, which promote cell proliferation. In vivo neuronal death in developing *C elegans*, genes that regulate neuronal suicide have been cloned. In thymic cells, a specific protein, p53, has been found to be necessary for radiation-induced apoptosis. Of importance for its therapeutic potential, a variety of molecular strategies prevent forms of apoptotic cell death. Of possible relevance to stroke, one protein with an “antisuicide” action, Bcl-2, is located in the inner mitochondrial membrane, suggesting that genetically programmed cell death may be inherently tied to metabolic function.

Under certain conditions short periods of brain ischemia cause delayed cell death, and neurons, stressed by raised intracellular calcium and the other molecular events that occur in ischemic stroke, make a decision to die. Is that decision executed by activation of a specific gene program, perhaps the same one that causes cell death after raised intracellular calcium in the thymus? If so, it may be prevented by strategies found to prevent apoptosis in other cell types. This important issue for stroke research is opened for scrutiny by the article of Linnik et al. The authors investigated whether there is evidence that a genetic program of cell death may be activated in an animal stroke model. They present evidence for endonuclease activation causing DNA laddering in ischemic brain tissue and show that intrav-
Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats.
M D Linnik, R H Zobrist and M D Hatfield

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