Cyclin D1 and Cdk4 Protein Induction in Motor Neurons After Transient Spinal Cord Ischemia in Rabbits

Masahiro Sakurai, MD; Takeshi Hayashi, MD; Koji Abe, MD, PhD; Yasuto Itoyama, MD, PhD; Koichi Tabayashi, MD, PhD

Background and Purpose—The mechanism of spinal cord injury has been thought to be related to the vulnerability of spinal motor neuron cells against ischemia. However, the mechanisms of such vulnerability are not fully understood. We hypothesized that spinal motor neurons might be lost by programmed cell death and investigated a possible mechanism of neuronal death by detection of double-strand breaks in genomic DNA and immunohistochemical analysis for cyclin D1 and cyclin-dependent kinases (Cdk) 4.

Methods—We used a rabbit spinal cord ischemia model with a balloon catheter. Spinal cord was removed at 8 hours and 1, 2, and 7 days after 15 minutes of transient ischemia, and histological changes were studied with hematoxylin-eosin staining. In situ terminal deoxynucleotidyl transferase (TdT)–mediated dUTP-biotin nick-end labeling (TUNEL), DNA fragment with gel electrophoresis, Western blot analysis for cyclin D1 and Cdk4, and temporal profiles of cyclin D1 and Cdk4 immunoreactivity were investigated.

Results—Most motor neurons were preserved until 2 days but were selectively lost at 7 days of reperfusion. Immunocytochemistry showed positive TUNEL selectively at 2 days of reperfusion in spinal motor neuron nuclei. Typical ladders of oligonucleosomal DNA fragments were detected at 2 days of reperfusion. Immunoreactivity of cyclin D1 and Cdk4 proteins was induced selectively at 8 hours in motor neuron nuclei, which eventually died.

Conclusions—These results indicate that induction of cyclin D1 and Cdk4 may be implicated in programmed cell death change after transient spinal cord ischemia in rabbits. (Stroke. 2000;31:200-207.)

Key Words: cyclin-dependent kinases n cyclins n ischemia n motor neurons n spinal cord

Motor neuron dysfunction due to spinal cord injury after successful operation on thoracic aorta is a disastrous complication in humans. Reported incidences of paraplegia in such surgery range from 2.9% to 23%.1 Acute spinal cord dysfunction is believed to be caused by ischemic damage during cross-clamping. Ischemia can occur because of permanent exclusion of the essential intercostal arterial blood supply to the spinal cord or temporary interruption of blood flow to the spinal cord.2,3 However, patients who undergo thoracic aneurysm repair and awaken with no neurologic deficit immediately after operation sometimes eventually may develop paraplegia.4,5 However, the exact mechanism of such vulnerability is not fully understood. In the rabbit spinal cord ischemia model, we have reported delayed and selective motor neuron death after transient ischemia.6–9 To evaluate the mechanism of such selective vulnerability in motor neurons, we attempted to create a reproducible model for spinal cord ischemia and statistically analyzed cell damage.

In normal cells, cellular proliferation follows an orderly progression controlled by protein complexes that are composed of cyclins and cyclin-dependent kinases (Cdks). The cyclin family of proteins comprises the regulatory proteins for the Cdk family members, and cyclins are differentially synthesized and degraded at specific points during the cell cycle.10–15 Cyclin D1 is upregulated early in the G1 phase and subsequently peaks by mid-G1, interacts with its kinase Cdk4, and usually decreases as cells approach S phase.13 Cyclin D1 is selectively induced in vitro in postmitotic sympathetic neurons undergoing programmed cell death,16 and cyclin D1 is associated with apoptosis. Cdk4 also promotes neuronal apoptosis.17 Thus, cyclin D1 and Cdk4 have multiple functions: they control cell proliferation, death, and survival in various cell types. Therefore, we examined immunoreactivities of cyclin D1 and Cdk4 proteins after spinal cord ischemia for possible involvement of apoptosis in this type of neuronal death.

Materials and Methods

Animal Models

During the experiment, animals were treated in accordance with the Declaration of Helsinki and guiding principles in the care and use of animals. The animal studies were approved by the institutional animal care and use committee. The experimental protocol was designed to preserve cord function as much as possible.

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animals. The experimental and animal care protocol was approved by the Animal Care Committee of Tohoku University School of Medicine.

Twenty-four Japanese domesticated white rabbits (weight, 2 to 3 kg each; Funakoshi, Japan) were used in this study and divided into 2 subgroups: a sham control group and a 15-minute ischemia group. Anesthesia was induced by intramuscular administration of ketamine at a dosage of 50 mg/kg and maintained with 2% halothane inhalation. A 5F pediatric thermolodination catheter (model 405, B. Braun Melsungen A.G.) was inserted through a femoral artery and advanced 15 cm forward into the abdominal aorta. Preliminary investigations confirmed that the balloon in the distal end of the thermolodination catheter was positioned 0.5 to 1.5 cm just distal to the left renal artery. During the experiment, aortic pressures were continuously monitored both at the proximal and distal positions of the balloon. Body temperature was monitored with a rectal thermometer and was maintained at 37°C with the aid of a heating pad during surgery and subsequent ischemia. Animals were then allowed free access to water and food at ambient temperature.

The animals treated with 15 minutes of ischemia were divided into 2 experimental groups: group A, to be investigated by use of histological, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL), and immunohistochemical studies (n=15); and group B, to be investigated by Western blot study (n=9).

Group A

Animals were allowed to recover at ambient temperature and were euthanatized by use of deep anesthesia with sodium pentobarbital (100 mg/kg IV) at 8 hours and 1, 2, and 7 days after reperfusion (n=3 at each time). The 3 sham-operated control animals were euthanatized just after the insertion of catheter into the abdominal aorta without inflation of the balloon. After euthanatization, spinal cords were quickly removed with the plunger of a 1-mL syringe. Samples for TUNEL and immunohistochemical studies were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer and then stored at 4°C for 1 week; they were then cut by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer and then stored at 4°C for 1 week; they were then cut transversely at the L2 or L3 level and embedded in paraffin.

Group B

For Western blot analysis and analysis of DNA fragmentation, animals were euthanatized at 8 hours and 1 and 2 days after blood flow restoration (n=3 at each time point), and the samples were obtained as noted for group A. Samples were kept at −80°C until use. Sham control samples (n=3) were also obtained.

Histological Study

To see the pathological changes of the spinal cord after ischemia, we performed hematoxylin-eosin (HE) staining on a set of sections and examined them with light microscopy. An observer unaware of animal group and neurological outcome examined each slide. With HE staining, cells were considered “dead” if cytoplasm was diffusely eosinophilic and “viable” if cells demonstrated basophilic stippling (ie, contained Nissl substance).14 For quantitative measurement of the number of motor neurons that underwent apoptosis, the number of intact large motor neuron cells in the ventral gray matter region was counted. Results were expressed as mean±SD.

TUNEL Reaction

To detect DNA fragmentation in cell nuclei, modified TUNEL reaction was applied to the cryosections according to a previously reported method18 by use of a kit (4810-30-K; Trevigen Inc). After deparaffinizing was complete, nuclei of tissue sections were stripped of proteins by incubation in 20 µg/mL proteinase K for 10 minutes. After treatment with 0.3% H2O2 in distilled water for 5 minutes, nuclei were incubated with TdT and biotinylated dUTP in TdT buffer in a humidified chamber at 37°C for 120 minutes. Further incubation with peroxidase-conjugated streptavidin was performed for 30 min-
utes at room temperature. Slices were stained with diaminobenzidine/H2O2 solution and then counterstained with methyl green.

For quantitative measurement of the number of motor neurons that underwent apoptosis, we counted motor neurons that were positive or negative with TUNEL. Results were expressed as mean±SD.

Analysis of DNA Fragmentation

DNA was prepared according to the method of Gavrilie et al.20 Spinal cords (n=3 for each time point) were minced, and cells were lysed on ice in 3 mmol/L Tris-HCl (pH 8.0) that contained 5 mmol/L EDTA and 0.5% Triton-X 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 extracted again with chloroform/isoamyl alcohol and precipitated again in ethanol; DNA was separated on 2% agarose gel, visualized with ethidium bromide, and photographed under UV illumination.

Western Blot Analysis

To investigate changes in cyclin D1 and Cdk4 expression, we performed Western blot analysis. Tissue samples were homogenized in a lysis buffer (0.1 mol/L NaCl; 0.01 mol/L Tris-HCl, pH 7.5; 1 mmol/L EDTA; and 1 µg/mL aprotinin), and then the homogenates were centrifuged at 7000g for 15 minutes at 4°C. Supernatants were used as protein samples. Assays to determine the protein concentration of the supernatants were subsequently performed by comparison with a known concentration of BSA by use of a kit (BCA protein assay reagent kit No. 23225). SDS-PAGE was performed in a 10% polyacrylamide gel under nonreducing conditions. In brief, protein samples were boiled at 100°C in 2.5% SDS and 5% β-mercaptoethanol, and lysates equivalent to 20 µg of protein from each sample were run on the gel for 90 minutes at 20 mA together with a size marker (rainbow-colored protein, Amersham). The electrophoresis running buffer contained 25 mmol/L Tris base, 250 mmol/L glycine, and 0.1% SDS. Proteins on the gel were then transferred to a polyvinylidene fluoride membrane (Micron Separations Incorporation) with a transfer buffer that consisted of 48 mmol/L Tris base, 39 mmol/L glycine, 0.4% SDS, and 20% methanol.

After the transfer, membranes were placed in 1% powdered milk in PBS to block nonspecific binding. They then were incubated with primary antibodies at 1:400 dilution for 20 hours at 4°C. Primary antibodies used were as follows: monoclonal mouse anti-cyclin D1 antibody (SC-6281; Santa Cruz Biotechnology Inc) and goat polyclonal anti-Cdk4 antibody (SC-826; Santa Cruz Biotechnology). After they were washed in PBS, the membranes were incubated with biotinylated anti-mouse IgG (PK-6102; Vector Laboratories) and biotinylated anti-goat IgG (PK-6105; Vector Laboratories) at 1:200 dilution in PBS for 90 minutes, respectively.Membranes were washed in PBS and incubated with avidin-biotin–horseradish peroxidase complex (PK-6102; Vector Laboratories) for 60 minutes. Membranes were then developed with diaminobenzidine as a color substrate. The reaction was stopped by washing each membrane in distilled water. To ascertain specific binding of the antibody for the protein, another membrane was stained in a similar way without the primary antibody.

Cyclin D1 and Cdk4 Immunocytochemistry

We also performed a immunohistochemical study to investigate changes in expression of cyclin D1 and Cdk4. After they were deparaffinized, spinal cord sections were rinsed in 0.1 mol/L PBS for 20 minutes and blocked in 2% normal horse serum for 2 hours at room temperature. The sections then were incubated with primary antibodies in 10% normal horse serum or 10% normal rabbit serum and 0.3% Triton-X 100 for 20 hours at 4°C. The primary antibodies used were the same as those used for the Western blot analysis noted above, and each dilution was at 1:200 for antibody against cyclin D1 and against Cdk4.
After endogenous peroxidase activity was quenched by exposure of the slides to 0.3% H₂O₂ and 10% methanol for 20 minutes, the slides were washed in PBS and incubated for 3 hours with biotinylated anti-mouse IgG (PK-6102; Vector Laboratories) or biotinylated anti-goat IgG (PK-6105; Vector Laboratories) at a 1:200 dilution in PBS that contained 0.018% normal horse or rabbit serum, respectively. Subsequently, the slides were incubated with avidin-biotin–horseradish peroxidase complex (PK-6102; Vector Laboratories). Slices were stained with diaminobenzidine/H₂O₂ solution, and cytoplasm was counterstained with hematoxylin. To ascertain specific binding of antibody for the protein, a set of sections was stained in a similar way without the primary antibody.

**Results**

When the balloon of the thermodilution catheter was inflated in the abdominal aorta, systemic blood pressure of the rabbit did not change. Arterial pressure distal to the inflated balloon fell to near zero, and no pulsation was recorded. Upon deflation of the balloon, systemic blood pressure of this portion decreased for 15 minutes and then returned to the normal level (data not shown). Spinal cord ischemia was achieved by inflation of the balloon to stop blood flow to the spinal cord.²¹,²²

**Histological Study**

Representative photographs of sections stained with HE are shown in Figure 1. In sham-operated animals, the spinal cord was intact, with many large motor neurons in the anterior horn (Figure 1A). Spinal cord at 1 (not shown) and 2 days after blood flow restoration (Figure 1B) also exhibited no pathological changes. However, in spinal cord at 7 days after blood flow restoration (Figure 1C), approximately 70% of motor neurons was lost, without involvement of other neurons or glial cells. Apoptotic bodies were verified (Figure 1D, black arrowheads), and some neurons exhibited cell shrinkage at 7 days after blood flow restoration. On the other hand, no neurons had eosinophilic structureless cytoplasm that would indicate necrosis. No apparent gliosis or cellular infiltration was observed. Results of cell counting in the ventral gray matter region on the paraffin sections from animals are shown in Table 1. The 15-minute ischemia at 7 days after the procedure affected the number of motor neuronal loss cells, in contrast to results in sham controls. Thus, selective loss of motor neurons was confirmed, in accordance with our previous reports.⁶⁻⁹

<table>
<thead>
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<th>Animal Group</th>
<th>Time, d</th>
<th>Cell Nos.</th>
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<tbody>
<tr>
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<td>21.2±3.6</td>
</tr>
<tr>
<td>15-min ischemia</td>
<td>2</td>
<td>20.7±3.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.4±3.4</td>
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Values are mean±SD; n=3 at each time point.

>TABLE 1. Number of Large Motor Neurons in Ventral Gray Matter at 2 and 7 Days After Ischemia

![Figure 1. Histological findings of the spinal cord after 15 minutes of ischemia stained with HE. Spinal cord of sham control (A) and at 2 days after ischemia (B) showed no histological changes. However, at 7 days after ischemia (C), motor neurons were selectively lost, without apparent gliosis or cellular infiltration. Apoptotic bodies (black arrowhead) and shrunken neuronal cells are indicated (D). Bar=100 μm.](http://stroke.ahajournals.org/)

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The integrity of DNA was preserved after gel electrophoresis. After 15-minute ischemia, several patterns of DNA were observed: no DNA degradation at 8 hours and 1 day of reperfusion; a typical DNA ladder pattern with oligonucleosome-sized $\approx$180-bp fragments at 2 days of reperfusion; and a smear pattern that suggests random DNA degradation at 4 and 7 days of reperfusion (data not shown).

Western Blot Analysis
Representative results of Western blot analysis are shown in Figure 4. With antibody against cyclin D1, weak banding was detectable in samples of sham control, but those at 8 hours...
Molecular weight of this band was 34 kDa, and corresponded to the p34 subunit of cyclin D1. This band became scarcely detectable at 1 day after reperfusion. With an antibody against Cdk4, no band was detectable in samples of sham control, but those at 8 hours after blood flow restoration revealed 1 band (Figure 4, bottom). Molecular weight of this band was 34 kDa and corresponded to the p34 subunit of Cdk4. This band became scarcely detectable at 1 day after reperfusion. The membrane without the primary antibody revealed no band (data not shown).

Histochemical Study

Immunoreactive cyclin D1 and Cdk4 in sections from spinal cords are shown in Figure 5. Spinal cords of sham-operated animals did not show cyclin D1 (Figure 5A) and Cdk4 (Figure 5D) antigen immunoreactivity in any cell. Nuclei of motor neurons selectively showed strong immunoreactivity for cyclin D1 (Figure 5B) and Cdk4 (Figure 5E) antigen at 8 hours of reperfusion after 15 minutes of ischemia. Immunoreactivity of motor neuron cells for cyclin D1 (Figure 5C) and Cdk4 (Figure 5F) antigen was almost undetectable in the motor neurons at 1 day.

Discussion

We have demonstrated delayed and selective motor neuron death in lumbar regions of the rabbit spinal cord with a reproducible model. The histological patterns of 15 minutes of ischemia in our model are easily reproducible. Analysis of TUNEL and immunohistochemical studies to detect cyclin D1 and Cdk4 were also reproducible at each time point.

We have previously demonstrated delayed and selective motor neuron death in lumbar regions of the rabbit spinal cord with the same reproducible model. Fifteen minutes of ischemia is a relatively short ischemic period compared with those of previously reported models, and after ischemia, delayed and selective motor neuron damage was observed only after 7 days of reperfusion. This phenomenon is known as selective neuronal death in motor neuron cells after spinal cord ischemia, and is similar to the delayed selective neuronal death in hippocampal CA1 cells after cerebral ischemia. Despite restoration of blood flow, motor neurons, which initially appear to have survived ischemic insult, die days later. This result shows that motor neuron cells are vulnerable to spinal ischemic injury.

Recent studies have suggested that delayed neuronal death after transient ischemic injury in rat and gerbil brains has some apoptotic features. Apoptosis is associated with the activation of a genetic program in which apoptosis effector genes promote cell death. Apoptosis is characterized by compaction of the cell body and internucleosomal DNA fragments. TUNEL reaction is based on the specific binding of TdT to 3'-hydroxy termini of DNA, from which synthesis of a biotinylated polydeoxynucleotide polymer ensues. Obviously, fragmentation of nuclear DNA also occurs in necrosis. Because DNA is degraded by nonspecific lysosomal DNases in necrotic cells, these cells are supposed to be stained as well. However, because of nonspecific DNA cleavage, necrotic nuclei might not exhibit a stainable concentration of 3'-hydroxy termini of DNA. Nuclei of neurons undergoing apoptosis are known to show positivity to TUNEL in a granular pattern, although those cells in necrosis show slight staining with a homogenous pattern. In this study, the nuclei at 2 days after ischemia were densely stained, with a granular pattern (Figure 2B); the motor neurons were suggested to be undergoing programmed cell death. A DNA ladder pattern with oligonucleosome-sized fragments of ~180 bp as shown by gel electrophoresis is commonly considered to be a useful biochemical hallmark of apoptosis. Selective detection of DNA fragmentation in motor neuron cells at a stage of absent neuronal loss at 2 days may indicate that the apoptotic change is occurring in the spinal cord after 15 minutes of ischemia, subsequent to which approximately 70% of these motor neuron cells were selectively damaged after 7 days of reperfusion.

Apoptosis, a form of programmed cell death, plays a critical role in the regulation of development and maintenance of many tissues, including those of the central nervous system. Apoptosis is associated with activation of several genes that mediate the transition from quiescence to prolif-
This activation frequently leads to an abortive cell cycle that fails to enter S phase, although cases exist that demonstrate induction of apoptosis in the S or G2 phase. Because neurons in adult brain and spinal cord are terminally differentiated and postmitotic cells, they do not replicate genomic DNA. Furthermore, forced expression of cell cycle–related proteins in these cells has been reported not to cause mitosis nor DNA replication but to cause apoptotic cell death. Apoptosis also has been hypothesized to be the result of aberrant cell-cycle control. Thus, activation of cell-cycle proteins in terminally differentiated neurons may be important for the induction of programmed cell death.

In proliferating cells, cyclin D1 functions during the G1 phase of the cell cycle. Initial support for a G1 function of cyclin D1 came from its ability to complement a G1 defect in yeast and from its upregulation during G1 in cells stimulated to divide. More direct evidence was recently provided by the demonstration that serum-stimulated fibroblasts failed to enter S phase if cyclin D1 expression or function was blocked. In a complementary experiment, cells engineered to overexpress cyclin D1 modestly moved through G1 at an accelerated rate, had a reduced cell-cycle period, and, consequently, were smaller in size than control cells. Interestingly, Quelle et al also noted that attempts to generate cells that overexpressed higher amounts of cyclin D1 failed, possibly due to a toxic effect of high levels of cyclin D1.

Cyclin D1 regulates progression through the G1 phase of the cell cycle by stimulation of the activity of cyclin D–dependent kinase Cdk4 or Cdk6. An important substrate for these kinase is the product of retinoblastoma susceptibility gene pRb. In G1, underphosphorylated pRb suppresses initiation of S phase, but during mid-to-late G1, pRb is progressively phosphorylated by G1 Cdns and thereby loses its growth-suppressive effect. The cyclin D1–Cdk4 complex is unusual because it forms for only a short period in the cell cycle, at R through early S phase. R point is a critical point at which cells decide whether to enter cell cycle or remain quiescent. Cdk4 plays a critical role in helping the cell move past R point. Thus, part of the regulation of R point is through regulation of the association between cyclin D1 and Cdk4. Microinjection experiments with anti—cyclin D1 antibodies have suggested that cyclin D1–Cdk complexes are important for cell-cycle

![Figure 5. Immunostaining against cyclin D1 and Cdk4 in motor neuron nuclei in a sham spinal cord (A and D) and at 8 hours (B and E) and 1 day (C and F) of reperfusion after 15 minutes of ischemia. Arrowheads show motor neuron nuclei that express immunoreactive cyclin D1 (B) and Cdk4 (E), respectively. Bar=100 μm.](http://stroke.ahajournals.org/Downloadedfrom)
progression only in mid- to late G1 phase. In vitro data suggest that cyclin D1 and Cdk4 regulate the physiological apoptotic process. In the present study, the increase in immunoreactivity of cell cycle–regulated proteins cyclin D1 and Cdk4 was demonstrated in the motor neuron nuclei in the spinal cord after transient ischemia. Furthermore, the peak of immunostaining intensity of cyclin D1 and Cdk4 after ischemic insult preceded DNA fragmentation in the spinal cord. This finding suggests that overexpression of cyclin D1 and Cdk4 may play an important role in inducing DNA fragmentation in the spinal cord. On the other hand, cyclin D1 and Cdk4 were expressed in nonapoptotic neurons after transient focal cerebral ischemia in rat. Therefore, the mechanisms of cell injury in motor neurons of the spinal cord and in hippocampal cells of the brain after ischemia may differ. In summary, selective induction of cyclin D1 and Cdk4 in motor neuron cells at a stage of absent neuronal loss at 8 hours may indicate that the lethal change occurs in the spinal cord after 15 minutes of ischemia and indicates that eventually approximately 70% of motor neurons was selectively damaged after 7 days of reperfusion. Induction of cyclin D1 and Cdk4 is implicated in programmed cell death after transient spinal cord ischemia in rabbits.

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References

11. Deleted in proof.
Programmed cell death may be an important pathway to delayed neuronal death in selected neuronal areas after hypoxia or ischemia and even within areas of infarction, thereby increasing the numbers of dead neurons ultimately that occur in an infarct.1,2 Because of this, the transcription or translation of mRNA and the proteins that control such cell death have become putative targets of drug therapies designed to limit the extent of infarction. Such agents might possibly be useful even if given after the onset of stroke or after the beginning of the ischemic or hypoxic event.

The authors of this article have demonstrated that cyclin D1 and cyclin-dependent kinases can be added to the list of proteins whose transcription and translation may determine whether programmed cell death occurs in neurons. Their text indicates that this may not be so in all areas of the central nervous system. Their demonstration concerns motor neurons of the spinal cord after transient ischemia. The use of the spinal cord is particularly welcome because experimentalists have thus far paid more attention to the brain.

In most of the article, the authors label their findings “programmed cell death.” But occasionally they use instead the word “apoptosis.” This reflects what has been until recently2 a failure to recognize that the death may be programmed (ie, mediated by genes that control the cycle of life and death in otherwise healthy cells; genes not involved in classic necrosis) but that such death may not necessarily display the morphology that, strictly speaking, defines apoptosis. The purist will demand that the latter term be used only when, at least, apoptotic bodies are demonstrated with the light microscope and ideally when electron microscopy is performed and demonstrates typical clumped chromatin and an absence of swollen intracytoplasmic organelles. The authors did not perform electron microscopic studies and have not provided a quantitative statement that concerns the prevalence of apoptotic bodies. However, the TUNEL positivity, the pattern of TUNEL staining, and the presence of a DNA ladder support their assertion that the neuronal death in this case is apoptosis-like, and their findings of cyclin and cyclin kinase activation further suggest that the death is indeed due to activation of factors normally part of the genetically determined “program” of life and death. I believe that such findings are important and that debates about the correct definition of apoptosis should not be permitted to deflect attention from the importance of the activation of a “program” or “programs” as potential targets for stroke therapy.

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