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Ischemia-Induced Apoptosis in Primary Cultures of Astrocytes

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ABSTRACT Astrocytes participate in a wide variety of important physiological processes and pathological insults, including ischemia. Information on the mechanism of astroglial injury and death during ischemic insult, however, is scarce. In this study, we investigated the mode of astrocytic cell death using an in vitro ischemic model. Cultured astrocytes exhibited several distinct morphological and biochemical features of apoptosis under ischemia. At 4 h of ischemia, Annexin V staining demonstrated an early commitment of some astrocytes to apoptosis. Condensed nuclei became visible from 4 h and the number increased with ischemic incubation time. Electron microscopy showed compacted and segregated chromatin along the edges of nuclear membranes. The number of TUNEL-positive nuclei and the degree of DNA laddering increased with ischemic incubation. Caspase-3, but not caspase-1, activity was increased in ischemia-injured astrocytes. Swollen mitochondria and vacuoles found in some cells with chromatin condensation indicated that these apoptotic-like cells might die of necrosis. The results imply that astrocytes are capable of undergoing apoptosis without the presence of other cell types, such as neurons. Ischemia can induce apoptosis in astrocytes contributing to the pathogenesis of ischemic injury in the CNS. *GLIA 35:121–130, 2001.*

INTRODUCTION

Astrocytes, the most abundant glial cell type in the brain, have physiological and pathological roles in neuronal activities, in addition to their diverse functions in maintaining CNS homeostasis (Aschner, 1998). The finding that astrocytes are active integrated participants of neurotransmission (Vesce et al., 1999) has opened new horizons for our understanding of astrocyte function. Astrocytes are relatively more resistant to ischemic damage than neurons (Petito et al., 1998). Furthermore, their end feet surround capillaries, and thus, they are the first cells to suffer ischemic insult among all types of neural cells (Garcia et al., 1993). Their reaction to and dysfunction from the insult influences the responses of other neural cells to the ischemia. Evidence that dying glia killed neighboring cells that would otherwise have escaped injury through gap junctions further indicates the important role of astrocytes in brain injury (Lin et al., 1998). It is widely believed that ischemic astrocytes die from necrosis, and experimental evidence for apoptotic death in these cells has been scarce. Astrocytes contribute 5–10% of apoptotic cells in transient focal cerebral ischemia (Li et al., 1995), while DNA single-strand breaks and double-strand breaks in astrocytes in middle cerebral artery occlusion ischemia have been detected (Chen et al., 1997; Davies et al., 1998).

We employed an anaerobic chamber to study ischemia-induced proinflammatory cytokine release (Yu

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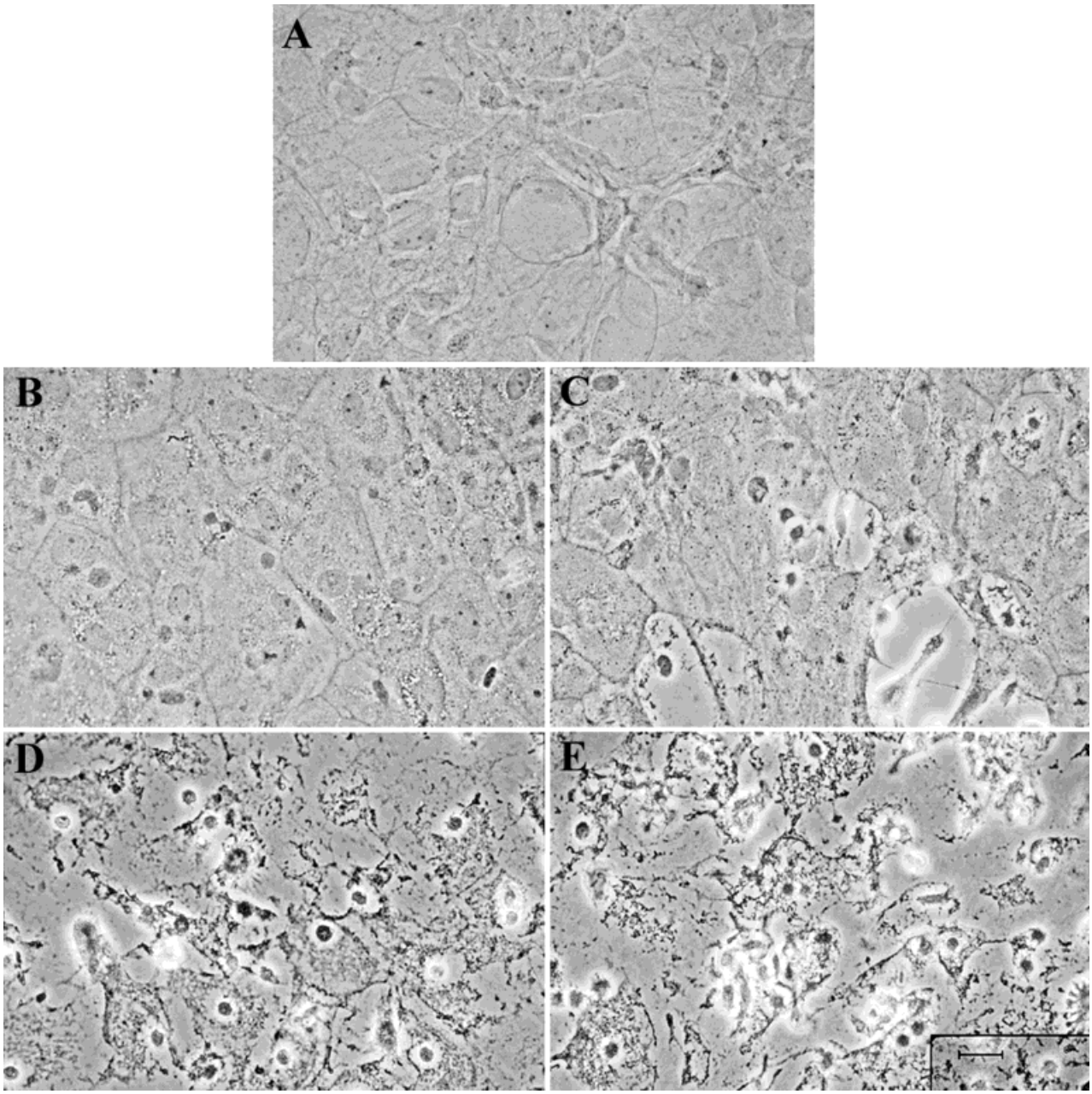

Fig. 1. Phase-contrast micrographs showing morphological changes in astrocytes during ischemia. Astrocytes appeared as a confluent layer of cobblestones before ischemic insult (**A**, 0 h). The cytoplasm became granulated at 2 h of ischemia (**B**, 2 h). Nuclei became condensed and appeared as dark spheres or oval-shaped bodies from 4 h of ischemia (**C**, 4 h; **D**, 6 h; **E**, 8 h). Scale bar = 20 μm.

and Lau, 2000; Lau and Yu, 2001) and the protective role of endothelin (Ho et al., 2001) in astrocytes. In this study, we used a similar model to investigate the mode of astrocytic cell death during ischemia. Apoptotic cells have distinct morphological phenotypes (Kerr et al., 1995; Portera-Cailliau et al., 1997; Lipton, 1999; Love, 1999), and can be detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) and DNA laddering (Arends et al., 1990; Gold et al., 1994). These morphological and biochemical changes were examined in primary culture of astrocyte during ischemic incubation. Results should clarify observations seen in vivo that the pathogenesis of ischemic injury in the CNS includes induction of apoptosis in astrocytes.

MATERIALS AND METHODS

Preparation of Cerebral Astrocytic Cultures

Primary cultures of cerebral cortical astrocytes were prepared from newborn ICR mice (Animal Care Facility, Hong Kong University of Science and Technology) as described by Yu et al. (1982), Yu and Lau (2000), and

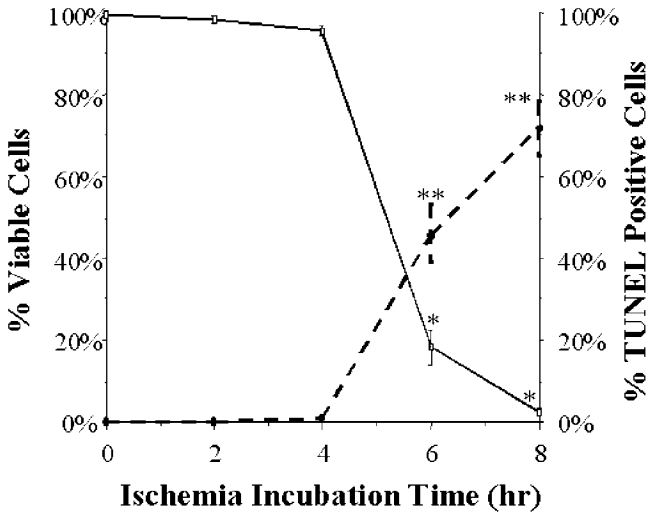

Fig. 2. Statistical analysis of cell viability (solid line) and DNA fragmentation (dashed line) in astrocytes during ischemic insult. Data are expressed as a percentage as the mean ±SEM from 6 individual experiments. For each experiment, 9 random fields were counted in each culture. * and ** signifify $P \leq 0.05$ compared with the control value at 0 h.

Lau and Yu (2001), with minor modifications. Briefly, meninges-free cortices were cut into small cubes (<1 mm^3) and suspended in modified Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Life Technologies, NY). After being mechanically dissociated, the cell suspension was sieved through nylon filters (Spectrum Medical Industries, TX). Fetal bovine serum (FBS) (Hyclone, UT) was added to 10% (v/v) and the mixture seeded in 35-mm Falcon tissue culture dishes (Becton Dickinson Labware, NJ). All cultures were incubated at 37°C with 5%/95% CO_2/air (v/v) and 95% humidity. Cultures became confluent in around 2 weeks. They were used for experiments after 4 weeks.

Ischemia Model

Ischemia was induced using an anaerobic chamber (model 1029, Forma Scientific, OH) (Juurlink and Hertz, 1993; Yu and Lau, 2000; Ho et al., 2001; Lau and Yu, 2001). Glucose- and serum-free DMEM was made anoxic by aeration with 99.95% N_2 for 30 min, and subsequently saturated with 5% CO_2 in N_2 for a further 20 min. The cultures and the anoxic glucose-free DMEM were transferred to the anaerobic chamber saturated with 85% N_2/10% H_2/5% CO_2. The cultures were washed 3 times with the anoxic glucose-free DMEM. This model is different from those used by Juurlink and Hertz (1993) in having only 800 μl of the incubation medium in 35-mm dish during the ischemia incubation. This modification reduces the extracellular volume, allowing the accumulation of toxic metabolites that is an important component contributing to physiological ischemia damage (Yu et al., 1995). The cultures were subsequently wrapped with parafilm, to prevent evaporation, and were kept in an incubator inside the ischemic chamber at 37°C. Postischemia was created by returning the cultures to a normal culture incubator with fresh medium at the end of the designated period of ischemic incubation.

Cell Viability Assay

Cell viability assay was performed with a LIVE/DEAD® Eukolight™ Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). Ischemic cultures were rinsed with phosphate-buffered saline (PBS) and subsequently incubated with viability assay solution containing 4 μM calcein-AM and 4 μM ethidium homodimer in DMEM with 10% (v/v) FBS at 37°C. After incubation, the cells were rinsed with PBS and the cell death pattern examined under a fluorescent microscope (Axiophot, Zeiss, Germany). The extent of cell death was calculated by counting stained cells in 9 random fields from 6 culture dishes.

Annexin V Staining

An Annexin-V-FLUOS Staining Kit (Boehringer-Mannheim, Germany) was employed to detect the translocation of phosphatidylserine from the inner plasma membrane to the outer membrane of cells. Cells were washed once with PBS. Reaction mixture containing 1:1:20 (v/v/v) of Annexin-V-fluorescein labeling reagent, propidium iodide (PI) and Hepes buffer was added to the cells, which were incubated for 15 min at room temperature. The cultures were observed under a fluorescent microscope (excitation at 488 nm) with a 515-nm filter.

TUNEL Assay

The TUNEL technique was performed with an In Situ Cell Death Detection Kit, Fluorescein (Boehringer-Mannheim). The ischemic cultures were fixed with 4% (v/v) paraformaldehyde in PBS for 30 min, and subsequently incubated for 2 min at 4°C with 0.1% (v/v) Triton® X-100 in 0.1% (w/v) sodium citrate in PBS. After rinsing, cultures were incubated in the TUNEL reaction mixture for 75 min at 37°C, then examined under a fluorescent microscope. The positive controls used in this study included apoptosis induced in astrocytes by 200 U/ml DNase I (Boehringer-Mannheim), 1 mM 2-chloroadenosine, and 1 μM staurosporine (Abbracchio et al., 1995; Mangoura and Dawson, 1998).

DNA Laddering Studies

Total genomic DNA from astrocytes was extracted with the Apoptotic DNA Ladder Kit (Boehringer-Mannheim). The ischemic astrocytes were scraped

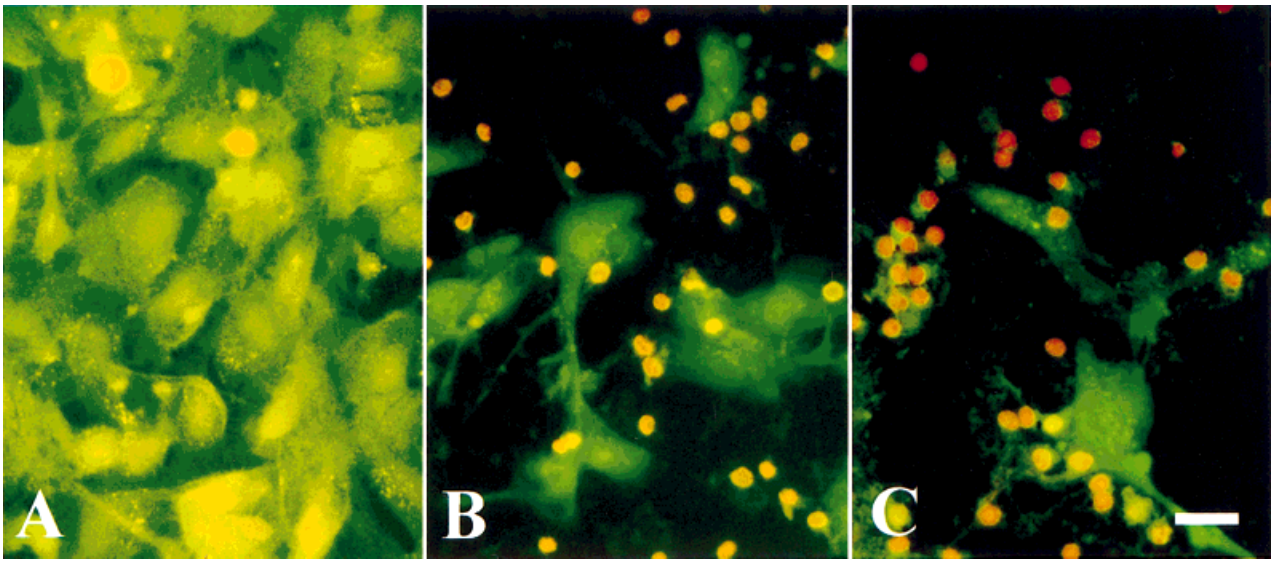

Fig. 3. Viability/cytotoxicity assay of astrocytes during ischemia. Live cells appear yellowish green, while the nuclei of dead cells appear orange-red. Most of the cells were alive after 4 h of ischemia (**A**). At 6 h (**B**) and 8 h (**C**) of ischemia, most of the astrocytes in the culture were dead. Scale bar = 12 μm.

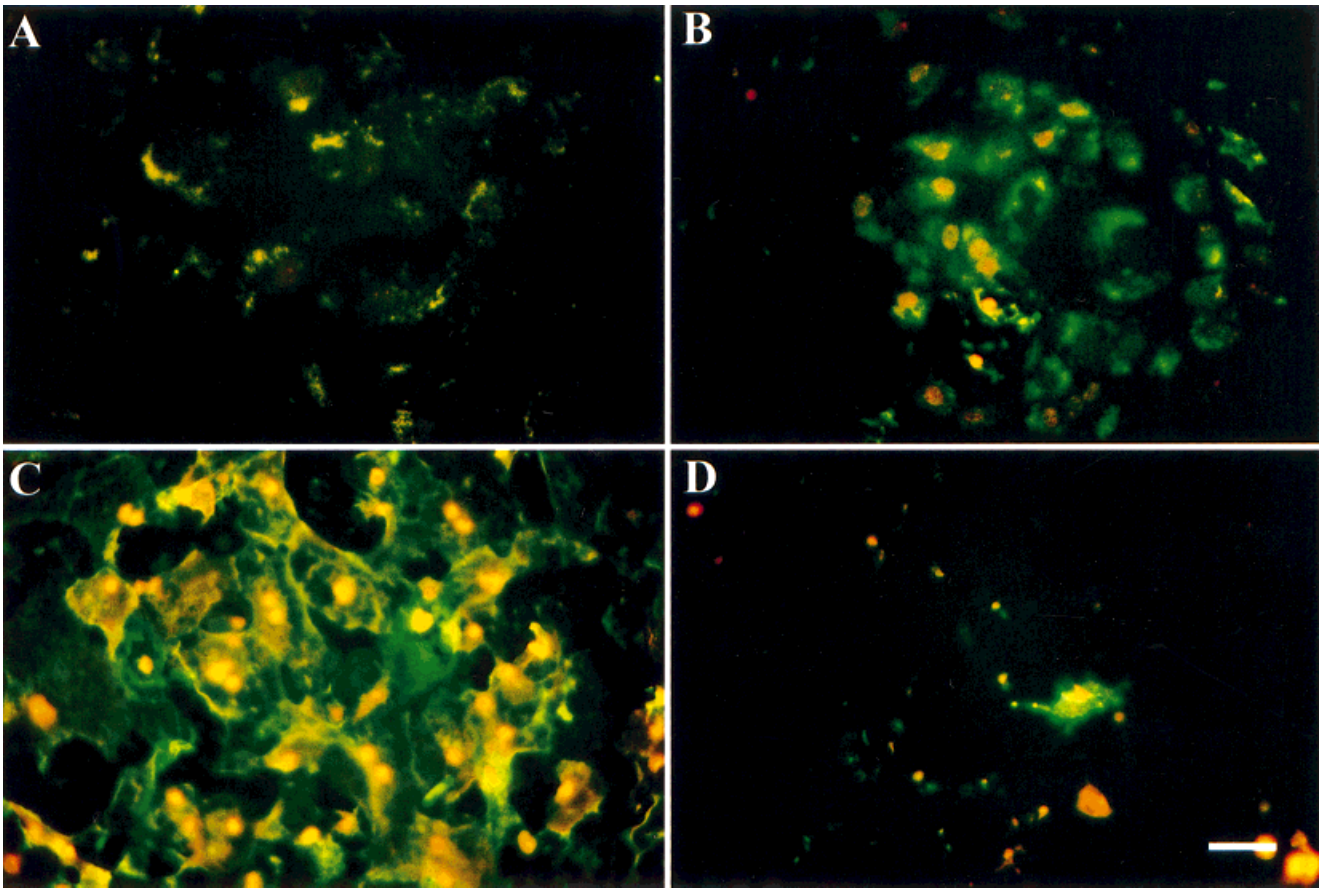

Fig. 4. Annexin V staining of primary astrocytes during ischemia. At 0 h of ischemia, only light background staining could be detected (**A**). Increased staining was observed from 4 h of ischemia (**B**, 4 h; **C**, 6 h). The orange nuclei indicates incorporation of propidium iodide in cells that have lost membrane integrity. Staurosporine-treated HeLa cells served as a positive control (**D**). Scale bar = 20 μm.

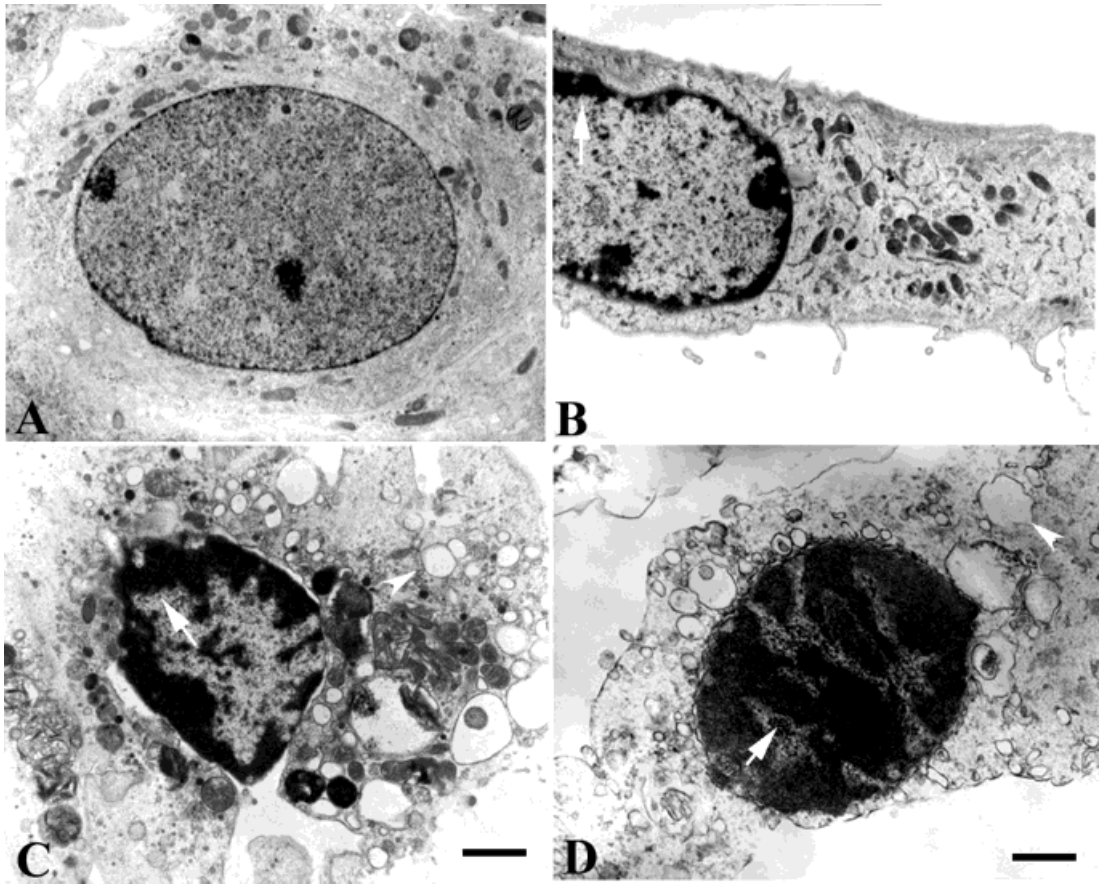

Fig. 5. Electron micrographs showing ultrastructural details in ischemic astrocytes at different time points. A normal astrocytic nucleus appears homogenous and oval-shaped (**A**). From 4 h of ischemia, chromatin was condensed and delineated abutting the inner surface of the nuclear envelope (**B**, 4 h; **C**, 6 h; arrows). Condensed chromatin (C,D, arrowhead), vacuoles (C,D, concave arrowheads), shrunken nucleus, and swollen mitochondria were found in 8 h ischemic astrocytes. All these distinct features are typical of cells undergoing apoptosis during ischemia. Scale bar = 3 μm in A-C and 2.5 μm in D.

from the dish and lysed in 200 μl of lysis buffer [6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, pH 7.4, and 20% (v/v) Triton® X-100] at 72°C for 10 min. The lysates were then mixed with a one-fourth volume of isopropanol. The mixture was transferred to a filtering column and centrifuged for 1 min at 8,000*g*. The column was rinsed twice with washing buffer (2 mM Tris-HCl, pH 7.5, 20 mM NaCl). Prewarmed elution buffer was used to elute the captured DNA. The eluted DNA was concentrated by ethanol precipitation, and the final DNA pellet was dissolved in elution buffer.

Electron Microscopy Studies

Cultures were fixed with 2.5% (v/v) glutaraldehyde (Structure Probe, PA) in 0.1 M cacodylate buffer (pH 7.4) containing cacodylic acid sodium salt (Structure Probe). They were then postfixed with 1% (w/v) osmium tetroxide (Structure Probe). The fixed cultures were dehydrated with a series of increasing concentrations of ethanol until they were totally dehydrated in absolute ethanol. Cells were detached with propylene oxide and were then infiltrated with Spurr's embedding medium, containing vinyl cyclohexene dioxide (Electron Microscopy Sciences, PA), polyglycol diepoxide/epoxy resin (Structure Probe), nonenyl succinic anhydride (Electron Microscopy Sciences) and dimethylaminoethanol (Electron Microscopy Sciences). The cells were left to polymerize at 70°C overnight. The grid was prepared by cutting the polymerized block with an ultramicrotome and was then examined under a transmission electron microscope (Hitachi H-7100FA Electron Microscope, Japan).

Measurement of Caspase Activity

The caspase-1 and -3 activities in astrocytes were measured by a CaspACE™ Assay System (Promega, Madison, WI).

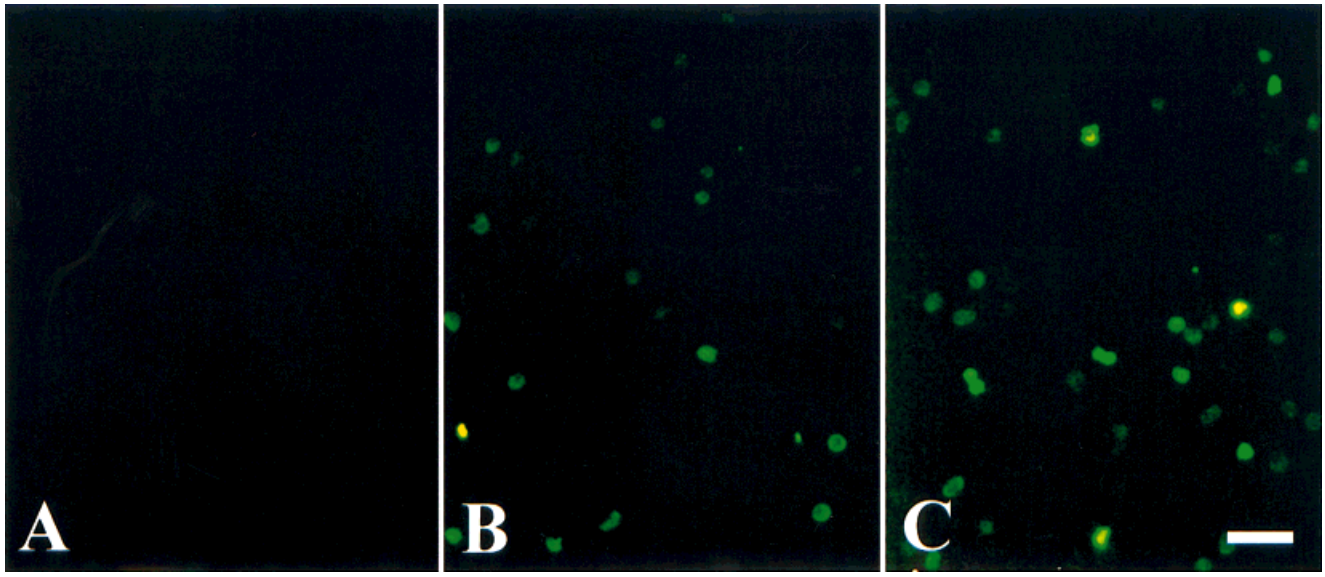

Figure 6.

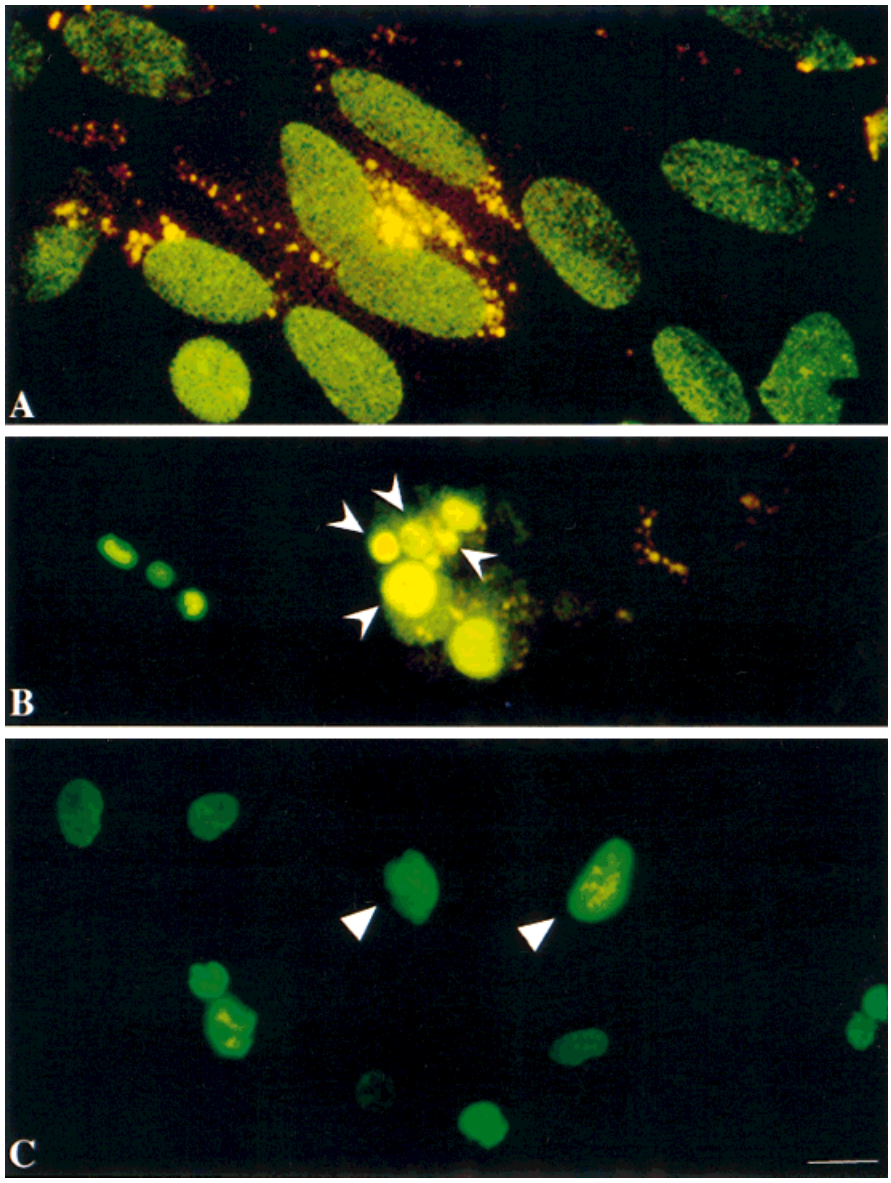

Figure 7.

Statistical Analysis

Mean values of cell death and apoptotic TUNEL-positive cells in injured astrocytes were compared with those in the control cultures, using a Student's *t*-test. A P value of <0.05 was considered statistically significant. All values were represented in the form of mean ±SEM.

RESULTS

Morphological Features and Cell Death of Ischemic Astrocytes

Astrocytes appeared as a confluent layer of cobblestones before ischemic incubation (Fig. 1A). After 2 h of ischemic incubation, the cytoplasm became granulated, and many nuclei became visible (Fig. 1B). The density of granulation increased with the length of ischemia incubation. When ischemia was extended to 4 h (Fig. 1C), the cell membranes began to lose their integrity. Nuclear shrinkage became more pronounced and many nuclei became pyknotic. More than 95% of the attached astrocytes were alive at this stage, as most of the cells appeared green in the cell viability assay (Figs. 2, solid line, 3A). Some pyknotic nuclei formed a highly condensed dark mass and were surrounded by a phase-bright ring of cell debris and cytoplasmic remains (Fig. 1C–E). These cells were presumed to have died from necrosis. They were removed during the staining procedure. At 6 h (Fig. 1D) and 8 h (Fig. 1E) of ischemia, most of the astrocytes were dead and the highly condensed nuclei appeared as scattered small dark spots in the culture dish. More condensed nuclei surrounded by a bright ring were observed. The size of these condensed nuclei was about one-fourth that of the control. Most of the nuclei appeared orange-red (Fig. 3B,C). A cell count indicated that over 80% and 95% of the ischemic astrocytes were dead at 6 and 8 h of ischemia, respectively (see Fig. 2). In these cultures, a small number of astrocytes were alive and scattered among the dead cells. They appeared phase dark (Fig. 1E) and with hypertrophic cytoplasmic processes (Fig. 3B,C).

Fig. 6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) of fragmented DNA in a primary culture of astrocytes during ischemia. TUNEL-positive nuclei were not detected until 6 h of ischemia. **A:** 4 h. **B:** 6 h. **C:** 8 h. Scale bar = 30 μm.

Fig. 7. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of primary cortical astrocytes during DNase I, 2-chloroadenosine and ischemia treatment. **A:** Normal astrocytes were treated with DNase I and served as a positive control for TUNEL staining. **B:** Astrocytes were treated with 1 mM 2-chloroadenosine and served as a positive control for apoptosis in astrocytes. Nuclei were fragmented into several pieces (concave arrowheads). **C:** Astrocytic nuclei at 8 h of ischemia were TUNEL-positive. Most nuclei remained intact but were severely condensed compared with the normal astrocytic nuclei in A (arrowheads). Astrocytic nuclei appeared to be different under chemical and pathological conditions. Scale bar = 10 μm.

Annexin V Staining

Loss of the asymmetrical arrangement of the plasma membrane was taken as an early indicator of astrocytic commitment to apoptosis during in vitro ischemia. Annexin V positively-stained astrocytes appeared at 4 h of ischemia (Fig. 4B). Most of the cell membranes were stained green without the incorporation of PI in the nuclei, indicating that these cells were intact. However, from 6 h of ischemia, the membrane integrity was lost and incorporation of PI produced orange-yellow fluorescence inside the nuclei (6 h, Fig. 4C; 8 h, data not shown). HeLa cells treated with 1 μM of staurosporine served as a positive control (Fig. 4D).

Electron Microscopy Studies

Electron microscopy studies indicated that ischemic astrocytes underwent apoptotic cell death. Chromatin was observed evenly distributed in a normal astrocytic nucleus (Fig. 5A). All organelles appeared intact. As ischemia was prolonged to 4 h (Fig. 5B) and beyond (Fig. 5C,D), the astrocyte nuclear chromatin became apoptotic-like, i.e. compacted and segregated into sharply delineated masses lying against the nuclear envelope (Fig. 5B,C,D). Swollen mitochondria (Fig. 5C) and vacuoles (Fig. 5C,D; concave arrowhead) were observed in the cytoplasm. The integrity of the nuclear membrane was lost and massive granules were observed inside the cytoplasm. All intracellular organelles were disorganized.

TUNEL, DNA Laddering, and Caspase Activity Studies

DNA fragmentation was investigated using the in situ detection technique, TUNEL. Ischemic astrocytic nuclei were not positively stained with TUNEL before 6 h of ischemia (Fig. 6A), even though at 4 h of ischemia some nuclei were already exposed, and the cytoplasmic membranes had disintegrated (Fig. 1C). DNA fragmentation was readily detected starting from 6 h of ischemia (Fig. 6B). A cell count indicated that almost 50% of the astrocytic nuclei were positively stained with TUNEL (Fig. 2, dashed line). At 8 h of ischemia, over 70% of the astrocytic nuclei were TUNEL positive (Fig. 6C; Fig. 2, dashed line).

2-Chloroadenosine-treated astrocytes were employed as a positive control for apoptotic astrocytes. Astrocytes treated with 1 mM 2-chloroadenosine for 2 days underwent apoptosis, as TUNEL-positive nuclei were clearly observed (Fig. 7B). The degree of cell damage, cell detachment and the number of TUNEL-positive nuclei increased with the incubation time. DNase I-treated astrocytes served as a positive control for TUNEL labeling (Fig. 7A). In a comparison of nuclear staining and nuclei shape after DNase I (Fig. 7A), 2-chloroadenosine (Fig. 7B) and ischemia (Fig. 7C) treatment,

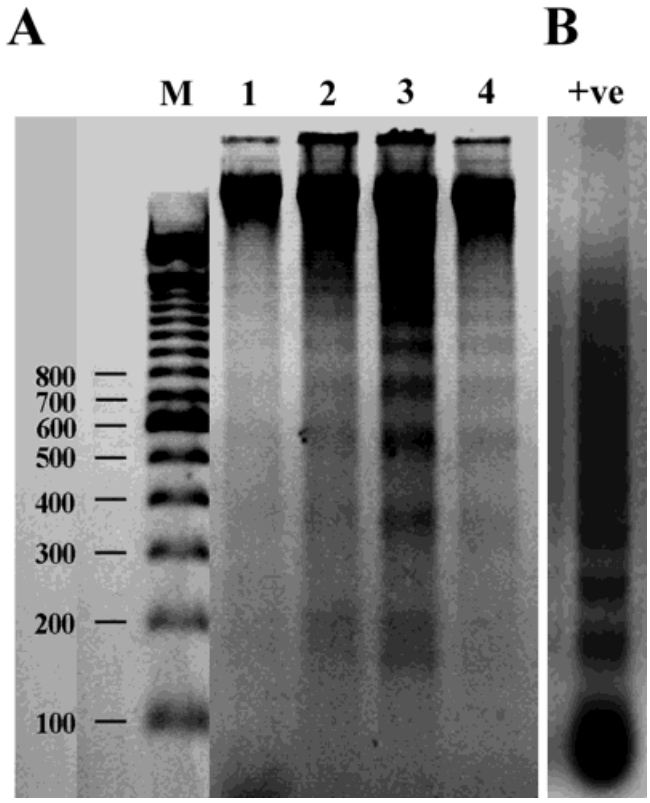

Fig. 8. DNA fragmentation in ischemic astrocytes is internucleosomal. **A:** The DNA laddering pattern observed with agarose gel electrophoresis began to appear from 5–8 h of ischemia. **M**-marker: **lane 1**, 5 h; **lane 2**, 6 h; **lane 3**, 7 h; **lane 4**, 8 h. **B:** DNA extract from staurosporine-induced apoptotic HeLa cells also showed a DNA laddering pattern, which served as a positive control.

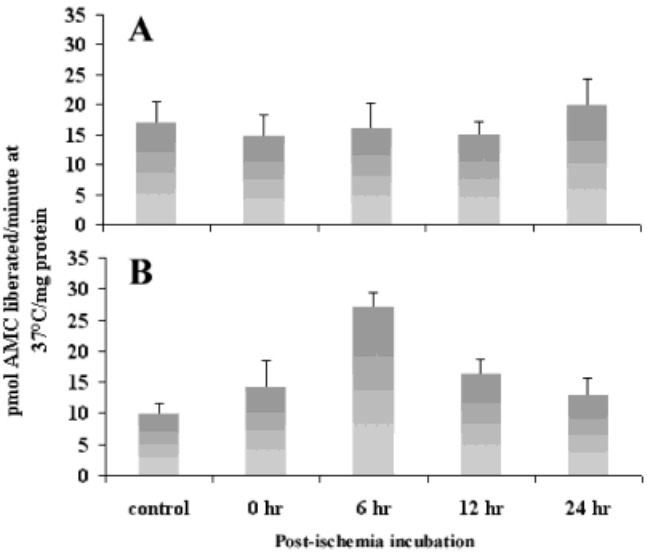

Fig. 9. Measurement of caspase activities under postischemia in astrocytes subjected to 4 h of ischemia treatment. **A:** Caspase-1 activity. **B:** Caspase-3 activity. 0 h meant that astrocytes completed 4 h of ischemia and began postischemia incubation. Control was cultures without ischemia incubation.

DNase I was found to produce a homogeneous TUNEL staining with normal nuclei shape. Interestingly, 2-chloroadenosine-treated apoptotic astrocytic nuclei tended to fragment (Fig. 7B) into several pieces, which is uncommon during ischemia (Fig. 7C). Nuclei in astrocytes under ischemia showed shrinkage (Fig. 7C).

In order to confirm that TUNEL staining reflects nucleosomal DNA fragmentation, we analyzed genomic DNA extracted from the lysates of ischemic astrocytes. DNA fragments, creating a laddering pattern with each band separated by approximately 180 bp, were readily detected from 5 h of ischemia (Fig. 8A, lanes 1–4). The increasing extent of the DNA laddering pattern from 5 h to 7 h of ischemia paralleled the number of apoptotic astrocytes during ischemia as shown by the TUNEL-positive cell count (Fig. 3). DNA ladders obtained from 1 μM staurosporine-treated HeLa cells (for 12 h) were used as a positive control.

Caspase-1 and caspase-3 activity was monitored for 24 h after a 3-h ischemic insult. Compared with a normal control, caspase-1 activity did not change under ischemia or under postischemia (Fig. 9A). The caspase-3 activity increased 50% immediately after the ischemic insult, and doubled after 6 h ($P < 0.05$). Caspase-3 activity returned to the level seen immediately after the ischemic insult after 12 h and declined further after 24 h under postischemia (Fig. 9B).

DISCUSSION

It is generally believed that fulminant insult to the nervous system results in necrosis, while more subtle insults result in delayed apoptosis. This hypothesis may hold in in vivo model systems, such as focal cerebral ischemia (Lipton, 1999). This is probably not true for the culture model used in this study, where the insult was created uniformly, the injury was cumulative, and the severity depended on the duration of exposure. The results indicate that some astrocytes died from necrosis but the majority died from apoptosis, as indicated by TUNEL staining and the morphology of mitochondria. This may indicate that ischemia can trigger both apoptosis and necrosis in astrocytes in this experimental model.

If the two death pathways are indeed independent, the critical question becomes why the process of astrocytic death within the same culture follows different pathways during ischemia insults. One possibility is that the astrocyte populations in the culture are composed of heterogeneous subtypes that have not yet been recognized (Shao and McCarthy, 1993; Klein and Fricker, 1992). The other possibility is that portions of the astrocytes in the culture were at different energy states at the time of the insult. This second possibility is supported by a study of kidney tubule cells, which showed a very strong relationship between the percentage decrement in ATP and the cell death pathway

taken (Lieberthal et al., 1998). Apoptosis proceeded when ATP levels were >25% and necrosis when levels were 15%. It has been shown previously that the ATP levels in astrocytes decline under hypoxia (Yu et al., 1989; Gregory et al., 1990) and ischemia (Sonnewald et al., 1994) incubation. Injury in this culture ischemia model was cumulative. Due to the lack of reperfusion, cells committed to apoptosis at an early phase of ischemia would have no opportunity to replenish their ATP levels, resulting in irreversible damage and necrosis. The electron microscopy study revealed that the astrocyte mitochondria were swollen in astrocytes after 6–8 h of ischemia. There are contradictions in the reported features of mitochondria under apoptosis in that the mitochondria are morphologically normal and then swollen, whereas they appear swollen in necrotic cells (Petit et al., 1996). The apoptotic-necrotic-like cell death supports the hypothesis that astrocytes under this model of ischemia could not complete apoptosis due to an energy failure, thus died of necrosis. Thus necrosis, in this model, may be a fallback pathway if apoptosis is somehow not available or could not be completed due to an energy failure. It is apparent that an insult can spawn more than one mode of death in astrocytes. Furthermore, in many cases, one astrocyte will manifest signs of more than one mode of cell death.

The positive control for apoptosis was astrocytes treated with 2-chloroadenosine (Ceruti et al., 1997). Although DNase-treated and ischemic astrocytes both showed positive TUNEL staining, the shape of the nucleus of astrocytes in cultures treated with 2-chloroadenosine, DNase, and ischemia appeared to be different, indicating that DNA fragmentation and the nucleic response of astrocytes could be different as the result of different stimuli. Although the biochemistry of apoptotic death is not completely understood, some useful identifying characteristics have been identified (Kerr et al., 1995; Gold et al., 1994). All morphological and biochemical data from this study showed that a substantial portion of the ischemic astrocytes in culture did undergo apoptosis when they committed to death. From 4 h of ischemia (≤8 h, which was the longest incubation time investigated), both phase contrast and electron microscopy indicated that many ischemic astrocytes died by exhibiting distinct features of apoptosis, such as nuclear condensation, cytoplasmic shrinkage, and chromatin aggregation. Another unique feature of apoptosis observed in this model was the binding of Annexin V at 4 h of ischemia indicating the translocation of phosphatidylserine to the outer plasma membrane—a process targeting cells for phagocytosis (Walton et al., 1997). The other feature of apoptosis studied was DNA damage, as measured by TUNEL and DNA electrophoresis. DNA damage was most apparent in astrocytes from 5 h of ischemia. This validated the observation of Petito et al. (1998) that glial apoptosis contributes to the DNA ladders of apoptotic oligonucleosomes found in postischemic brain. The activation of caspase-3 provides some of the strongest biochemical evidence for apoptosis in the ischemic astrocytes.

Astrocytes might die as a secondary consequence of neuronal death (Lee et al., 1999). Our studies indicate that a major portion of astrocytic death was due to apoptosis in the culture and without the presence of neighboring neurons. This implies that astrocytes possess all the necessary machinery to die by apoptosis.

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