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A new approach for the electrophoretic detection of apoptosis

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ABSTRACT

Apoptotic cell death is often characterized by internucleosomal cleavage of genomic DNA, which exhibits a distinctive ladder upon electrophoresis. However, techniques used for the isolation and detection of DNA to demonstrate laddering may not be sufficiently sensitive, particularly when cleaved DNA is present at modest levels. We propose a new approach for isolating total cellular DNA using a silica-based resin that improves the resolution of DNA laddering. In addition, we introduce a rapid DNA labeling method that can increase the sensitivity of detecting DNA laddering. Each of these methods can be used for DNA from cell cultures or tissues.

Apoptosis is a form of cell death distinct from coagulative necrosis in which cells are deleted in a focal, type-specific manner under certain physiological or pathological conditions (1,2). This process is characterized morphologically by cell shrinkage, membrane blebbing, and nuclear condensation, after which cells are fragmented and phagocytosed by neighboring cells (3). During apoptosis, these morphological changes are often accompanied by internucleosomal cleavage of genomic DNA, which when electrophoresed shows a characteristic ladder consisting of 180 bp multimeric bands (4).

Although in some experimental models apoptotic DNA fragmentation is widespread and easily detected, in other cases apoptosis may be found in only a limited number of cells and, consequently, DNA fragmentation may be masked by necrotic and/or undegraded DNA. In the latter case, conventional ethidium bromide staining of DNA may not be sufficiently sensitive to reveal a classical DNA ladder. Southern blot hybridization has been used in such cases to detect low levels of apoptotic DNA fragmentation; however, hybridization is time-consuming, requires the use of specific DNA probes, and may give rise to high background signals (5). Alternatively, the sensitivity of DNA electrophoretic analysis can be increased by enzymatic radiolabeling of DNA fragments by terminal deoxynucleotidyl transferase (TdT) or the Klenow fragment of DNA polymerase I (6,7).

Here we present a new approach to detect internucleosomal DNA cleavage in both *in vitro* and *in vivo* models of apoptosis. This method involves the gentle isolation of genomic DNA with a silica-based resin, and, where greater sensitivity is required, the labeling of internucleosomal fragments by *Taq* DNA polymerase.

To demonstrate the utility of this method in an *in vitro* model of apoptosis, we used primary cultures of cerebellar granule cells, in which apoptosis can be readily induced by withdrawal of serum (8) and/or deprivation of depolarizing concentrations of potassium (9). Cerebellar granule cells were prepared from 8 day old rat pups as described previously (10), except that cells were plated onto poly-lysine coated 60 mm tissue culture dishes at a density of 1.25×10^6 cells per ml and at a volume of 3 ml per dish. On *in vitro* day 7, the culture medium was replaced with fresh medium devoid of serum or supplemented potassium, and incubated at 37°C for 14 h.

To isolate total DNA, the cells of one dish were washed with 3 ml PBS and lysed *in situ* with 2 ml of 7 M guanidine hydrochloride. The lysate was transferred to a 10 ml culture tube containing 1 ml Wizard Minipreps DNA Purification Resin (Promega), mixed gently, and centrifuged at 2000 *g* for 3 min. The resin–DNA pellet was resuspended in 3 ml of washing solution (90 mM NaCl, 9 mM Tris–HCl pH 7.4, 2.25 mM EDTA, 55% ethanol) and drawn by vacuum through a 3cc syringe attached to a Wizard Minicolumn (Promega). The column was washed twice with 3 ml of washing solution and dried by centrifugation over a microfuge tube at 5000 *g* for 5 min. To elute the DNA, 50 µl TE buffer pH 8.0 were added, followed by incubation for 5 min at room temperature and centrifugation over a new microfuge tube at 5000 *g* for 5 min. Residual RNA was removed by addition of 1 µg RNase A and incubation at 37°C for 30 min.

We compared the electrophoretic ladder of resin-isolated DNA with that of DNA isolated by two other commonly used methods (11,12). After ethidium bromide staining, all three samples exhibited the characteristic banding pattern of internucleosomally-cleaved DNA (Fig. 1); however, the most prominent ladder was observed in the resin-extracted sample. This result may be due to the omission of a phenolic extraction and agitation step which can shear DNA and cause background electrophoretic smearing. In addition, our technique does not include a precipitation step in which low molecular weight DNA fragments may be lost. Finally, because of the apparent differences in the elution rates of different molecular weights, resin-isolated samples may show an enriched fraction of internucleosomally-cleaved DNA.

While apoptosis induced in cultures of cerebellar granule cells is widespread, other models with more heterogeneous cell populations may contain only limited numbers of apoptotic cells. One such example is the delayed neuronal death observed after fluid percussion-induced traumatic brain injury in rats. This commonly used model of experimental brain injury has been

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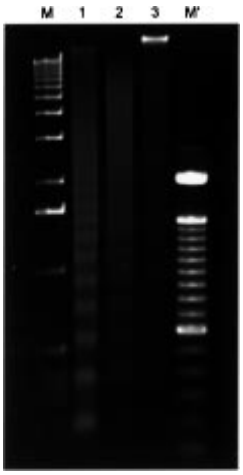

Figure 1. Internucleosomal DNA fragmentation in cerebellar granule cells. DNA was isolated from sister cultures using three different isolation techniques. Sample 1 was prepared by our DNA isolation method as described in the text. Sample 2 was prepared by incubation in 10 mM EDTA/0.2% Triton X-100, extraction with buffer-saturated phenol (pH 8.0) and phenol:chloroform:isoamyl alcohol, and precipitation in isopropanol (ref. 11). Sample 3 was prepared by lysis in 100 mM EDTA/0.5% sodium dodecyl sulfate/20 µg/ml RNase A, incubation with proteinase K, extraction with buffer-saturated phenol (pH 8.0), and precipitation in ethanol (ref. 12). Aliquots from each sample (400 ng) were loaded onto a 1% agarose gel in TBE running buffer, electrophoresed at 5 V/cm, and stained with ethidium bromide (0.5 µg/ml). M and M′ represent 1 kb and 100 bp molecular weight markers, respectively (Gibco BRL). As discussed in the text, sample 1 appears to produce the most prominent ladder.

employed extensively in our laboratory for pharmacological and neurochemical studies of secondary neuronal injury (13,14). In brief, a craniotomy is made over the left parietal cortex, and a transient deformation of the underlying brain is induced by a pressure pulse transmitted through a saline-filled reservoir that is connected to the craniotomy site via a luer-loc adapter. This model simulates many features of human concussive brain injury and demonstrates both necrotic (15) and apoptotic (16) cell death.

To extract genomic DNA from each injured or control ipsilateral cortex, 50–100 mg tissue were gently homogenized with a Dounce-type pestle in 200 µl PBS. One ml of 7 M guanidine hydrochloride was added, and the suspension was centrifuged at 10 000 *g* for 15 min to remove cellular debris. The supernatant was then mixed with 3 ml of Wizard Minipreps Resin, and this suspension was drawn by vacuum through a Wizard Midicolumn (Promega). After washing the column with 10 ml of washing solution, the procedure continued as described above.

Conventional gel electrophoretic analysis of resin-isolated DNA from the cortices of injured animals did not reveal any evidence of apoptosis by ethidium bromide staining, despite the optimization of imaging conditions (Fig. 2A). To increase the sensitivity of detection, DNA was radiolabeled by *Taq* polymerase. This application of *Taq* polymerase is based on both its template- and non-template-dependent activities; that is, polymerization at 3′ recessed ends and nicks, and preferential deoxyadenosine addition at 3′ blunted ends, respectively (17).

To label with *Taq* polymerase, 1 µg of DNA was added to each 20 µl labeling mixture which contained finally 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 10 mM $MgCl_2$, 2 µCi [α-^{32}P]dATP (3000 Ci/mmol), and 1 U *Taq* DNA polymerase. Reactions were incubated at 72°C for 20 min and terminated by the addition of gel loading buffer. After electrophoresis, the DNA

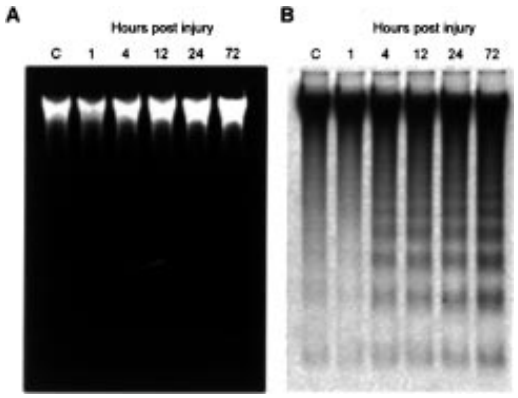

Figure 2. Internucleosomal DNA fragmentation in rat ipsilateral cortex after lateral fluid percussion-induced traumatic brain injury. Lane markings denote time after injury or sham-operated control (lane C). Samples were loaded onto a 1.5% agarose gel in TBE running buffer and electrophoresed at 4 V/cm. (**A**) 10 µg DNA per sample were visualized by ethidium bromide fluorescence under optimized imaging conditions. (**B**) 1 µg DNA per sample was radiolabeled with *Taq* polymerase according to our method as described in the text and was visualized by autoradiography. Despite containing 10-fold less DNA than the ethidium bromide-stained samples, the radiolabeled samples produce increasingly prominent ladders after injury.

was visualized by autoradiography, which revealed prominent DNA ladders (Fig. 2B).

In conclusion, the techniques presented here provide a simple alternative for detecting apoptotic DNA fragmentation. Because both methods combined take less than 2 h to complete, they can be used, either separately or in combination, for the rapid screening of apoptosis in tissues and cultured cells.

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