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Quantitation of DNA fragmentation in apoptosis

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Apoptosis is a form of programmed cell death characterized by cytoplasmic condensation, plasma membrane blebbing and nuclear pycnosis, leading to nuclear DNA breakdown into multiples of ~200 bp oligonucleosomal size fragments. The detection of apoptosis in cultured cells relies heavily on techniques involving the extraction of nuclear DNA and characterization of such oligonucleosomal ladders by gel electrophoresis. These techniques are often tedious and time-consuming and provide qualitative rather than quantitative results. A simple, reproducible and rapid technique based on the hypotonic lysis of cells followed by the selective precipitation of unfragmented, high-molecular weight DNA by polyethylene glycol (PEG) 8000 is described. Fragmented DNA remains in the supernatant and can be used directly for agarose gel electrophoresis or quantitation using the fluorescent dye Hoechst 33258 without any further manipulation. DNA fragments in the range of 20–300 kb, an early sign of cellular apoptosis (1) that precedes the generation of low molecular weight oligonucleosomal fragments, are also retained in the supernatant following PEG precipitation. Such fragments are easily detected by the Hoechst assay allowing for early detection of apoptosis in cultured cells. In addition, the percentage of DNA fragmentation in cultured cells detected by this assay exhibits a direct correlation with the percentage of apoptotic nuclei present in these cultures, scored morphologically after propidium iodide staining.

Hypotonic lysis of cultured cells and removal of high molecular weight DNA by centrifugation, followed by the detection of DNA remaining in solution, is an extremely rapid and sensitive way to measure DNA fragmentation; however, it is not highly reproducible, presumably due to inefficient precipitation of the bulk nuclear DNA following cell lysis. In fact, a number of independent determinations of fragmented DNA in apoptotic cultures (HL-60 cells treated with 1 µm camptothecin for 24 h) gave a mean of 248 ng with a standard deviation of ±44 ng of fragmented DNA per 5×10^5 cells. Therefore, to eliminate the incomplete precipitation of the bulk unfragmented DNA, PEG was used to effect a selective precipitation of high molecular weight DNA (2). A concentration of 2.5% PEG and 1 M NaCl (a range of 1–10% PEG and 0–2M NaCl was explored) was found to be optimal for the selective precipitation of high molecular weight DNA from solutions containing both high and low molecular weight DNA. A repeat of the above determinations in the presence of PEG resulted in a mean of 285 ng with a standard deviation of ±8 ng of DNA per 5×10^5 cells.

Following optimization of the precipitation conditions, lysates of apoptotic cells were prepared as described below (see 'PEG/Hoechst fragmentation assay'). Direct analysis of the precipitated supernatants from control and apoptotic cultures by gel electrophoresis through a 1.5% agarose gel revealed an oligonucleosomal ladder of fragmented DNA in apoptotic cultures (Fig. 1A). Note the complete precipitation of DNA from control cultures and the clear laddering pattern of apoptotic culture supernatants treated with PEG. These results indicate that DNA fragmentation can be analyzed directly following PEG precipitation, obviating the need for phenol and/or protease treatment of the samples.

Although DNA fragmentation into oligonucleosomal ladders is characteristic of apoptosis, recent evidence indicates that not all cells undergo such extensive DNA fragmentation (1,3). In fact, fragmentation of DNA into kilobase-size fragments appears to be an early event in apoptosis, preceding the complete digestion of DNA into multiples of nucleosomal size fragments (4). Therefore, it was important to determine whether such fragments were precipitated or retained in the supernatant following precipitation with 2.5% PEG, 1 M NaCl. The retention of such fragments in the supernatant would make this assay suitable for the detection of apoptosis in cell cultures that do not undergo extensive DNA fragmentation, or prior to the generation of oligonucleosomal ladders. To assess the retention of kilobase-size DNA fragments after PEG precipitation, DNA isolated from control and apoptotic cultures was precipitated with 1–3% PEG and the supernatants were resolved by pulsed field gel electrophoresis through a 1% agarose gel in 0.5× TBE buffer (5) using the BioRad CHEF-DR II system with the following parameters: 185 V; initial time, 10 s; final time, 200 s; start ratio of 1; run time, 20 h. As shown in Figure 1B, the unfragmented, control cell DNA was completely precipitated at 2% and 3% PEG whereas DNA fragments ranging in size from 20 to 300 kb could be seen in the apoptotic cell extracts at these PEG concentrations. Of interest, fragments in the range of 500 kb remained in the supernatant even after precipitation with 1% PEG confirming the initial assumption that incomplete precipitation of unfragmented DNA by simple centrifugation interferes with the measurement of fragmented DNA.

To assess the degree of correlation between the percentage of DNA fragmentation detected by the above assay and the percentage of nuclei undergoing DNA fragmentation, apoptotic cultures of HL-60 cells were grown as before and were divided into two aliquots. Cells from the first aliquot were fixed with ice-cold 70% ethanol on ice for 1 h and then washed once in PBS. The pellet was incubated with 50 µl PBS containing 0.25 mg/ml RNase A and 50 µg/ml propidium iodide at room temperature for

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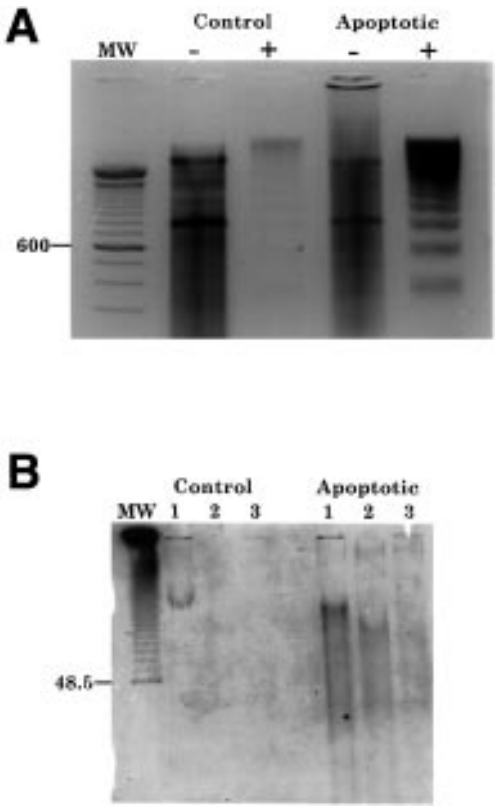

Figure 1. (**A**) Supernatants of control and apoptotic cultures analyzed by agarose gel electrophoresis following lysis and centrifugation at 16 000*g* in the presence (+) or absence (–) of PEG/NaCl. MW, 100 bp DNA ladder. (**B**) Pulse field gel electrophoresis of HL-60 cells treated with camptothecin for 3 h to generate DNA fragments in the range of 20–300 kb. Control and apoptotic cell lysates were precipitated in the presence of the indicated amount of PEG/NaCl and the supernatants were analyzed by PFGE. MW, 48.5 kb DNA ladder.

15 minutes in the dark. Approximately 600 nuclei per pellet were scored using a Zeiss Axiophot fluorescence microscope (Zeiss, Germany) using a 40× objective (Fig. 2, open circles) and the percentage of apoptotic nuclei determined. The amount of DNA in the second aliquot was determined for both the pellet fraction as well as the supernatant fraction, and the percentage of fragmented DNA remaining in the supernatant was calculated (Fig. 2, closed circles). As can be clearly seen in Figure 2, the percentage of DNA fragmentation detected by selective PEG precipitation and Hoechst fluorescence correlates closely with the percentage of apoptotic nuclei present in each sample. A slightly higher percentage of apoptotic cells is detected by Hoechst fluorescence than by a direct counting of the number of apoptotic nuclei. This deviation is most likely due to the fact that nuclei that have undergone extensive DNA fragmentation are not detectable following propidium iodide staining, whereas their fragmented DNA is easily detected with the Hoechst assay. Thus, the present assay is probably more sensitive in determining the percentage of apoptosis in cultured cells than direct scoring of apoptotic nuclei. In conclusion, this is a sensitive, simple and reproducible assay of DNA fragmentation in apoptosis that should find general utility in this expanding field.

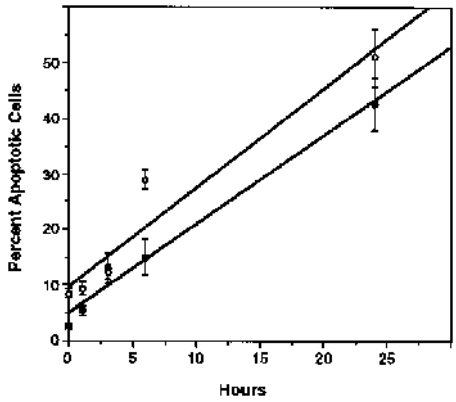

Figure 2. HL-60 cells treated with camptothecin for the times indicated. Samples were removed at each time point and lysed in the presence of PEG/NaCl and subsequently analyzed using Hoechst 33258 to determine the percentage (mean ± SD, n = 3) of fragmented DNA (○), or stained with propidium iodide and scored for apoptotic morphology to determine the percentage (mean ± SD, n = 3) of apoptotic nuclei (●).

PEG/Hoechst fragmentation assay

Approximately 5×10^6 cells were lysed by the addition of 600 µl of DNA fragmentation lysis buffer (0.1% Triton X-100, 5 mM Tris–HCl, pH 8.0, 20 mM EDTA). Following addition of PEG and NaCl to a final concentration of 2.5% and 1 M respectively, samples were placed on ice for 10 min and then centrifuged at 16 000 *g* for 10 min at room temperature. The supernatants were removed and the concentration of DNA was determined by adding an equal volume of Hoechst dye solution (0.2 µg/ml Hoechst 33258 in PBS, pH 7.4), prepared fresh from a 10 mg/ml stock solution, to an aliquot of the supernatant. The stock is stable for >6 months at –20°C whereas the diluted dye is stable for ~2 weeks at –20°C. After a 20 min incubation at room temperature, the fluorescence of the samples was determined at 360 nm excitation, 460 nm emission on a Ratio-2 System Fluorometer (Optical Technologies Devices Inc., Elmsford, NY). Alternatively, the supernatants could be ethanol precipitated and used directly for agarose gel electrophoresis (see Fig. 1).

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