Detection of Early and Late Stage of Apoptosis with Field Inversion Gel Electrophoresis

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Introduction

One of the hallmarks of apoptosis is the breakdown of chromatin, usually first into intermediate 300 kbp and 50 kbp fragments and eventually into nucleosome multimers and individual nucleosomes. When these DNA breakdown products are separated on a gel, a ladder pattern is observed. We describe here a modification of a previously published procedure by Walker and coworkers[1] which permits, on a single gel, under one set of conditions, the simultaneous visualization of both the intermediate and large (50 and 300 kbp) fragments and the nucleosome monomers and multimers formed during apoptosis. This procedure provides a means for assessing apoptotic DNA fragmentation before more detailed analyses are carried out.

Typically when preparing DNAs for pulsed field gel electrophoresis (PFGE), the host cells are first embedded in agarose and all manipulations of the cells are done within the agarose plugs. This prevents shearing or mechanical breakage of the DNA during manipulations. Unfortunately, smaller fragments like nucleosomes and nucleosome multimers usually diffuse from the agarose plugs during the digestion and washing steps. One of the approaches suggested by Walker and colleagues was to first embed the cells in agarose and to recover the diffused DNA by addition of salt and ethanol. Another suggested approach was to mix the cell suspension with an equal volume of 2% SDS and to load the resulting lysate directly into the wells of an agarose gel. Walker *et al.* resolved the DNAs by the sequential combination of conventional and pulsed field gel electrophoresis.

Sample Preparation

1. A 25 cm long gel consisting of 1.2% Pulsed Field Certified Agarose (Bio-Rad) in 0.5x TBE (45 mM Tris, 45 mM borate, 1 mM EDTA) was cast and submerged in 0.5x TBE running buffer.
2. A photodynamic process which can induce apoptosis within 30 minutes was used.[2] Murine leukemia P388 cells were photodamaged with SnET2, an agent that photosensitizes mitochondria and lysosomes,[3] irradiated with 660 nm light under conditions which reduced cell viability by 50%, then incubated in fresh medium for 0–120 minutes at 37 °C.
3. After incubation, the cells were resuspended in 25 µl of phosphate buffered saline (PBS) containing 1 mg/ml RNase and then loaded directly into wells of the agarose gel containing 25 µl of 2% sodium dodecyl sulfate (SDS). The wells were then sealed with a 1% solution of low melting point agarose.
4. The gel was incubated at room temperature for 30 minutes to permit cell lysis.

Electrophoresis Conditions

1. The gel was placed in a FIGE Mapper cell and the buffer was circulated at 14 °C for 60 minutes before electrophoresis was initiated. There was no need for preliminary conventional gel electrophoresis at low voltage since the DNA can enter the gel without formation of the tight knots described by others.[4]
2. Electrophoresis was conducted at 14 °C in 0.5x TBE. The total run time was 19 hours with forward and reverse voltages of 150 V. The forward switch time increased linearly from 5.4 seconds to 54 seconds, and the reverse switch time increased linearly from 1.8 seconds to 18 seconds over the 19 hour run time.
3. Two sets of markers were run on the gel: a mixture of lambda fragments, lambda DNA and lambda concatemers, and a set of low molecular weight DNA fragments ranging from 154 to 2,176 bp.

Results

We found in comparing the two methods mentioned above that we retained significantly more of the nucleosome multimers and monomers when the cells were incubated in the SDS solution as opposed to recovering them after they diffused from the agarose plug (data not shown). Although the SDS method was superior, we also found that we could lessen the amount of DNA breakage by pipeting the cells directly into the wells already containing 2% SDS. Even gentle pipeting of the naked DNA resulted in some non-specific fragmentation.

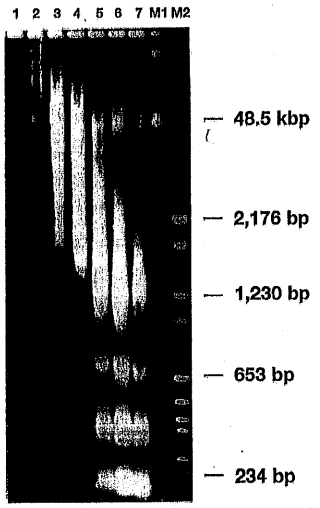

Fig. 1. Separation of DNA fragments. Lane 1, untreated cells; lanes 2–7, treated cells lysed at specified times after PDT: Lane 2=10 minutes, Lane 3=20 minutes, Lane 4=30 minutes, Lane 5=45 minutes, Lane 6=60 minutes, Lane 7=90 minutes. Lane M1= Lambda DNA markers; Lane M2=154–2,176 bp markers.

Figure 1 shows the resolution of all the marker bands (lanes M1 and M2). Lane 1 indicates the lack of degradation of DNA in a control cell sample. As early as 10 minutes after irradiation of photosensitized cells, DNA fragmentation was detected (lane 2). Further fragmentation was observed after 20 minutes (lane 3) with a pattern of nucleosome multimers observed within 30 minutes (lane 4), and more clearly after 45 minutes (lane 5), 60 minutes (lane 6) and 90 minutes (lane 7). In lanes 5, 6 and 7, a 50 kbp band is visible as are nucleosome and nucleosome multimers of approximately 200, 400 and 600 bp.

Discussion

We have presented a method which represents a significant savings in time over previously published procedures for the monitoring of early and late stages of apoptosis. The entire procedure can be carried out within 24 hours and permits simultaneous detection of both 50 kbp and nucleosomal DNA fragments. We can thereby assess the progression of apoptosis as a function of time. The modified procedure will also be useful to determine whether an inhibitor affects early or late stages of the apoptotic process. While this procedure lacks the fine resolution of nucleosome oligomers that can be obtained by conventional gel electrophoresis, it does permit an analysis of the progress of chromatin fragmentation during apoptosis.

References

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