An experiment on apoptosis induced by polyamine adducts produced in the presence of serum amine oxidase

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Abstract

A two-session experiment is proposed to train students with an easy, economic and educative procedure to detect the typical DNA ladder produced in many apoptotic events. The procedure is accurate enough to provide an easy way to compare degrees of damage in DNA caused by different treatments. © 2000 IUBMB. Published by Elsevier Science Ltd. All rights reserved.

1. Introduction

Apoptosis, first described by Kerr et al. [1], has become one of the most intensively studied biological phenomena in recent years. This phenomenon can be induced by normal physiological processes and by multiple nonphysiological stimuli, including oxidative stress as well as by chemotherapeutic agents. A great deal of information concerning apoptosis is now available and these days it is generally agreed that the use of only a single method for apoptotic cell detection is not really acceptable. Electron microscopy is perhaps the “gold standard” and there are others (e.g. TUNEL) that are widely used. A large number of commercial “ready-to-use” kits are now available and they offer a powerful tool for the research laboratory, since they allow the study of specific aspects related to apoptosis, e.g. caspase activation, protein expression (as p53, Bcl-2, …), etc. However, these methods usually require qualified staff and expensive instruments, such as a flow cytometer [2] or a fluorescence microscope, which are not always available.

There are many mechanisms involved in apoptosis and several changes appear in apoptotic cells but frequently one of the most relevant features of these cells is the activation of nucleases that cleave chromosomal DNA into high molecular weight and/or low molecular weight oligonucleosomal DNA fragments. These DNA fragments can be resolved by electrophoresis on agarose gels where they are observed as a typical ladder of 200 base pairs or multiples thereof. Thus, although this is not currently the reference method for apoptosis detection in the research laboratory, learning a protocol for isolation of apoptotic DNA may be a very interesting experience for a Biochemistry student, since basic molecular biology techniques are used. In this report we describe a simple, cheap and quite easy protocol for the selective isolation of oligonucleosomal fragments from apoptotic cells. After DNA isolation, only a simple agarose gel electrophoresis followed by an ethidium bromide staining is needed for DNA ladder visualization. Other protocols are available for complete cellular DNA isolation [3] but they are more difficult to carry out with satisfactory results.

2. Experimental

Oligonucleosomal fragment isolation (this is the procedure of Centeno et al. [4] with slight modifications). Treated and control cells (at least $10^6$ cells) are washed with phosphate-buffered saline and collected. The cell pellet is frozen in liquid nitrogen and suspended in 0.5 mL 5 mM Tris-HCl, pH 7.4, containing 20 mM EDTA plus 25 µL 10% (v/v) Triton-X-100. Samples are incubated with continuous gentle shaking at 4°C for 1 h and then centrifuged at 15,000 g for 15 min at 4°C. Afterwards, the supernatants are treated with 0.5% sodium-dodecyl sulfate (SDS) plus proteinase K (0.1 mg/ml) for 3 h at room temperature. The next step is a phenol–chloroform (1 : 1) extraction followed by a treatment
with ribonuclease IA (15 μg/mL) for 1 h at 37°C. A final phenol–chloroform extraction is performed and the DNA present in the aqueous phase is precipitated by the addition of 0.1 vol 3 M sodium acetate, pH 5.2, and 3 vol of absolute ethanol and incubation at −20°C. The samples were finally centrifuged at 15,000 g for 15 min at 4°C and the DNA pellets washed with 70% ethanol, air dried and suspended in sterile redistilled water.

Agarose gel electrophoresis: Samples are diluted using 6× loading buffer (10 mM EDTA, 0.25% bromophenol blue, 50% glycerol) and electrophoresed at 6 V/cm on a 1% agarose gel in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8). Once the electrophoresis is complete, the gel is stained with ethidium bromide [CAUTION] (1 μg/mL), destained and visualized by UV fluorescence.

3. Suggestions for a laboratory practical on apoptotic DNA isolation and evaluation for undergraduate students

The procedures described above are easy to carry out and can be performed by students in a two-day session. On the first day, students are provided with the theoretical background, including a brief explanation of the reason why each step described in the protocol must be performed, and they carry out the oligonucleosomal fragment isolation, from treated and control cell pellets previously prepared and stored at −80°C by the instructors. On the second day, they carry out the agarose gel electrophoresis, staining, destaining and UV fluorescence visualization. They observe results and discuss them. Our experience with students leads us to advise that the laboratory practical proposed should be designed for a small group of Biochemistry students (e.g. 10–12 maximum), familiar with working in the Biochemistry laboratory. They must know how to use micropipettes for example. Instructors should advise students that they are going to use dangerous reagents (phenol and ethidium bromide must be manipulated with care, wearing gloves and in a chemical hood, etc) and equipment (electrophoresis power supply, UV transilluminator [face mask to be worn]). It is suggested that students prepare an agarose gel with “wide wells” so that wasting the samples when loading them can be avoided. Apart from these details, with a small group of students, we had a really formative experience with satisfactory results.

Cell culture facilities are required for this experiment and several models can be used. For example, growth-factor-dependent cell lines enter apoptosis following removal of the growth-promoting agent from the culture medium [5]. Many cell lines undergo apoptosis when culture medium conditions are altered, e.g. removal of serum or overgrowing cultures to produce high cell densities. Finally, apoptosis can also be induced by exposure to a wide range of cytotoxic agents, e.g. human leukemia HL-60 cells treated with 2 μg/mL camptothecin for 3 h [2].

Treatment of different cell lines with polyamines also induces apoptosis [6]. The polyamines, spermine, spermidine and putrescine, are essential for the normal growth and differentiation of eukaryotic cells, and their intracellular levels are tightly regulated [7]. Bovine serum contains amine oxidases which act on spermidine or spermine to produce aminoaldehydes, NH₃ and H₂O₂. The products of this reaction are cytotoxic [6]. Aminoguanidine is an inhibitor of bovine amine oxidases [8] and therefore, adding polyamines and/or aminoguanidine to cell cultures in the presence of bovine serum is an easy and illustrative model for the practical work proposed. However, the teacher must finally choose the best model, according to cell lines and/or material availability. In what we propose the teacher must treat and prepare.

Fig. 1. Nucleosomal fragmentation in mouse C57 mast cells after different treatments. Untreated cells (lane 1); cells treated with 10 μM spermidine for 6 h (lane 2); cells treated with 5 μM spermine for 6 h (lane 3).
4. Results

In Fig. 1, oligonucleosomal DNA fragments are observed following both spermidine (spd) and spermine (spm) treatments. Signal intensity after spermine treatment was greater than for spermidine, which is in agreement with the damage caused to the C57 mast cells by both compounds at the concentrations used. In fact, IC\textsubscript{50} values for them, measured by the MTT method [9], were 4 and 18 \(\mu\text{M}\), respectively. These data suggest that the method proposed is sensitive enough to compare degrees of damage caused to the cells by different treatments, since equal volumes (equivalent to equal numbers of cells) were loaded in the agarose gel. Furthermore, when treatments were carried out in the simultaneous presence of 1 mM aminoguanidine, a very weak signal was obtained [10], demonstrating the protective effect of this amine which inhibits bovine amine oxidases and therefore prevents aldehyde and \(\text{H}_2\text{O}_2\) generation by polyamines oxidation.

Several conclusions can be reached from the present work. With the practical method proposed, students can learn to perform a protocol for DNA isolation which is a basic molecular biology procedure, and they easily may discern between apoptosis and necrosis. Moreover, the model described allows different degrees of damage to be caused to many cell lines, and our method could therefore be useful for comparing these degrees of damage. Thus, with a good design, students can learn how cells can be sensitive to physiological agents under specific conditions and how to evaluate these situations.

References