2714–2715 Nucleic Acids Research, 1994, Vol. 22, No. 13

A rapid and sensitive assay for the detection of DNA fragmentation during early phases of apoptosis

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Received February 9, 1994; Revised and Accepted April 24, 1994

Apoptosis, or physiologic cell death, is an endogenous cellular process whereby senescent, DNA-damaged or diseased cells are eliminated from the body. Classically, apoptotic cells are identified by distinct morphological criteria in histological preparations (1). Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180–200 basepairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis (2). Recently, however, it has become apparent that the 'ladder' formation in lymphocytes is a very late and probably terminal event in a highly regulated biochemical and molecular cascade of events leading to activation of endonucleases, DNA degradation and cell death (3). Additional evidence suggests that apoptosis in cells of epithelial or mesenchymal origin may not involve DNA degradation into oligonucleosomal multimers and that different forms of the endonuclease appear to be active in different cell types (3,4). Research interest in the initial signal transduction pathway leading to the activation of the nuclear endonuclease(s) has escalated because it has become apparent that an understanding of the regulation and nature of the apoptotic endonuclease (5), has major implications for the genesis and prevention of cancer (5,6), teratogenesis (1) and normal and abnormal cell differentiation (7).

In order to define the signal transduction pathway leading to 'programmed cell death', sensitive detection of initial endonuclease-mediated DNA strand breaks is an essential first step. Agarose gel electrophoresis is not capable of detecting low frequency breaks in low numbers of apoptotic cells. Recent evidence suggests that endonucleolytic cleavage into 300 or 50 kb fragments *precedes* internucleosomal DNA fragmentation and may underlie alterations in chromatin structure and nuclear condensation during the earliest phase of apoptosis (3). Presently, pulsed field gel electrophoresis (PFGE) is the only available method for detection of 300 and 50 kb fragments; however, it is time consuming and requires the use of specialized equipment. Although the electrophoretic pattern of high molecular weight DNA fragmentation is clearly distinguishable, PGFE does not quantitate the rate of fragmentation. Other methods to detect DNA fragmentation are based on end-labelling *in vitro* (8), or *in situ* (9) or on nick translation (10), but these methods lack sensitivity for low frequency strand break detection and therefore are of limited use for the evaluation of events and signals that mediate initiation of apoptosis. We present here an original assay capable of sensitive detection of low frequency DNA strand breaks present in high molecular weight DNA. The method is a modification of the random oligonucleotide primed synthesis (ROPS) assay which is well known to generate uniformly labelled DNA with high specific activity (11a). In the assay, single-stranded DNA fragments generated by endonuclease-mediated strand breaks are initially separated by a denaturation step. During the reassociation process, the DNA fragments serve as primer and the excess high molecular weight DNA serves as template. As a result, the [^{32}P]dNTP incorporation initiated by the Klenow enzyme will be proportional to the number of breaks generated and will reflect the relative rate of strand break accumulation. The assay provides a high level of sensitivity not attainable with other available methods.

To demonstrate the sensitivity of the ROPS assay and its applicability to the kinetics of apoptosis, we activated the Ca/Mg-dependent endonuclease in fresh thymocyte cell suspensions by dexamethasone exposure. We then followed the accumulation initial DNA strand breaks with the ROPS assay before evidence of DNA fragmentation was detectable on agarose gel electrophoresis. Rat thymocyte cell suspensions were prepared from freshly excised tissue and the apoptosis endonuclease was activated by exposure to 10^{-6} M dexamethasone as described by Compton and Cidlowski (12). DNA was extracted from thymocytes utilizing the Ausubel protocol which is recommended for isolation of very high molecular weight (>120 kb) DNA (11b). Approximately 10^7 cells were extracted by incubation in 300 μl digestion buffer (containing 100 mM NaCl, 10 mM Tris–HCl, pH 7.9, 25 mM EDTA, 0.5% SDS, and 0.3 mg/ml proteinase K) for 15 hours at 50°C. With gentle manipulation, the DNA was purified with phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous phase extracted with diethyl ether to remove residual phenol. The RNA was digested with μg/ml DNase-free RNase for 1 hour at 37°C. To avoid ethanol precipitation, the DNA was dialyzed overnight against an excess of TE buffer (10 mM Tris–HCl, pH 7.9, 1 mM EDTA). Just prior to ROPS reaction, DNA was denatured by exposure at 100°C for 5 min and then cooled on ice. In the reaction, the mixture contained: 0.25 μg heat denatured DNA, no additional primer, 0.5 μl [^{32}P]dCTP (3000 Ci/mmol), 0.05 mM of dGTP, dATP and dGTP, 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol, 0.5 unit Klenow enzyme (New England Biolabs), in a total volume of 25 μl. After incubation 30 min at room temperature, the reaction was stopped by the addition of an equal volume of buffer containing 12.5 mM EDTA, 10 mM Tris–HCl, pH 7.5, 100 mM NaCl. The samples were subsequently passed through Bio-Spin 30 columns (Bio-Rad) to

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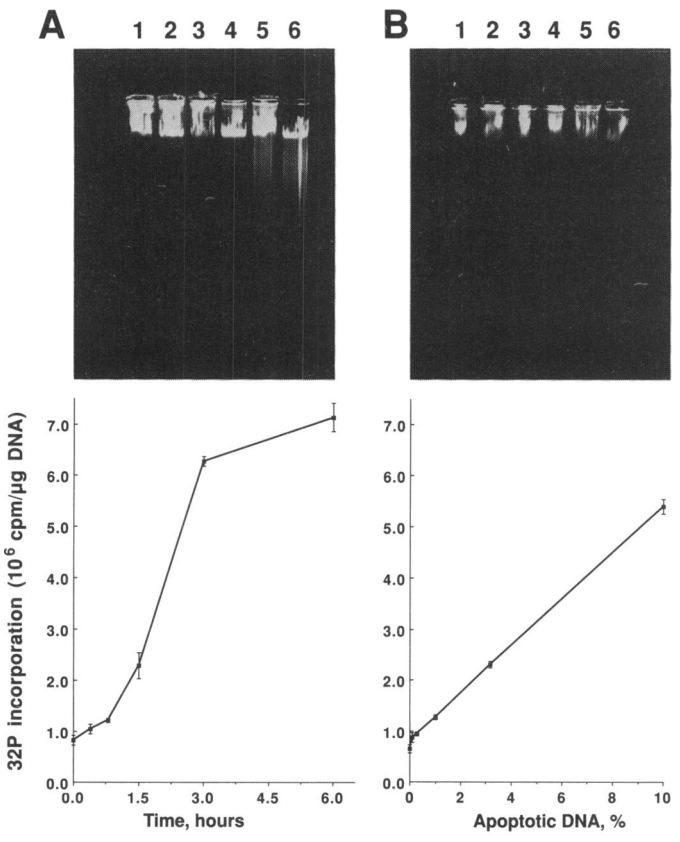

Figure 1. Kinetics of DNA fragmentation in dexamethasone-treated rat thymocytes (from 0.4 hour to 6 hours) as detected by the ROPS assay is presented in the lower half of Panel A. The values are the means ± SEM. In the upper half of the panel, the electrophoretic mobility of DNA of the corresponding ROPS samples in agarose gel in lanes 1–6 is presented. In Panel B, the dose response characteristics of the incremental addition of 0.1 to 10% predigested oligosomal DNA (from lane 6, Panel A) to high molecular weight DNA (from lane 1, Panel A) is quantified with the ROPS assay in the lower half with the corresponding sample electrophoretic mobility in 1% agarose gel in the upper half.

remove unincorporated precursors. The radioactivity in eluates was quantified in a Packard scintillation counter Model 1900TR.

In figure 1, the kinetics of endonuclease-mediated DNA fragmentation in dexamethasone-treated thymocytes is quantified with the ROPS assay and is presented in Panel A. In Panel B, the dose response characteristics of the incremental addition of endonuclease-digested oligonucleosomal DNA fragments to high molecular weight DNA derived from lane 1 is presented. The agarose gel mobility of the DNA derived from each of the six samples subjected to the ROPS assay is presented in corresponding lanes 1–6 directly above the ROPS data. Presented in this manner, it is apparent that the ROPS assay is capable of detecting the earliest strand breaks before visibility on agarose gel electrophoresis. It should be noted that no DNA fragmentation was detected in thymocytes incubated without dexamethasone (data not shown). The sigmoidal [^{32}P]dCTP accumulation of endonuclease-mediated breaks in Panel A and the linear dose response characteristics between 0.1 and 10% oligosomal DNA addition in Panel B confirm the sensitivity and reproducibility of the method. Because a DNA denaturation step is included in the assay, single strand as well as double strand DNA breaks are detectable. A direct comparison with the nick translation assay (with exclusion of the denaturation step) demonstrated that the ROPS assay resulted in approximately a 100-fold increase in [^{32}PP]dCTP incorporation (data not shown).

The methodology presented provides quantitative and sensitive method to detect the generation of low frequency DNA breaks present in a minor subpopulation of cells during the earliest stages of apoptosis when most DNA is still very high molecular weight. We demonstrate the both high molecular weight DNA fragments induced by nuclear endonuclease (Panel A) and 200 bp oligomers (Panel B) are substrate for the ROPS reaction. The potential applications of this methodology are numerous and include: a) endpoint measurement for the identification of activation signals for the endonuclease(s) during apoptosis; b) rate of nuclear endonuclease-mediated strand break accumulation in different cell types and under different conditions; and c) temporal detection of rare single strand and double strand 3′OH breaks in high molecular weight DNA induced by agents such as topoisomerase II, type II restriction enzymes, gamma radiation, and bleomycin. Finally, creative application of this methodology will provide a better understanding of the sequence of temporal sequence of events and the mechanisms of DNA degradation during apoptosis.

ACKNOWLEDGEMENTS

This research was supported in part by an appointment (A.G.B.) to the Postgraduate Research Program at the National Center for Toxicological Research administered by Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Food and Drug Administration. Research support was also provided by a Research Grant (no. CN-73B) from the American Cancer Society (S.J.J.).

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