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Short communication

Separation of oligonucleosomal DNA fragments from apoptotic animal cells using a triad of Sephacryl columns

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Abstract

The degradation of DNA, resulting in formation of oligonucleosomal fragments, is a characteristic feature of apoptosis, i.e., the process of programmed cell death. In this work we have developed a method for exact determination of the proportion of fragmented DNA in an apoptotic cell population. To this end we employed Sephacryl gel chromatography matrices and UV detection of DNA concentration. The disturbing effect of low-molecular-mass UV-absorbing contaminants was eliminated by insertion of a Sephacryl S-200 HR pre-column. Optimum resolution of DNA samples isolated from apoptotic cells was achieved using a triad of Sephacryl S-200 HR, Sephacryl S-500 HR and Sephacryl S-1000 SF columns.
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1. Introduction

Increasing attention is being paid, both on the cell physiological and on the biochemical levels, to the investigation of apoptosis, i.e., to a process of programmed cell death of animal cells. The formation of distinct DNA fragments of oligonucleosomal size, i.e., multiples of ~180 base pairs (bps) in length, has been identified as a crucial biochemical hallmark of this process [1]. Electrophoresis in agarose gels has become a popular tool for detection of fragmented DNA in biological studies, because it allows one to visualize the characteristic ladder of oligonucleosomal pattern. The problem of quantitation of DNA fragments is solved mostly by labelling of DNA. This can be achieved either biosynthetically using bromodeoxyuridine or radiolabelled thymidine, or enzymatically by end labelling of isolated DNA [2]. However, the fact that intact DNA and the largest fragments enter the gel incompletely, and manifest themselves as a thick band at the edge of the well or near the origin [1–3], represents an obstacle to perfect quantification of the fragmented DNA fraction.

A method of exact quantitative evaluation of genomic DNA degradation during the process of apoptosis, independent of the conclusions based on counting morphologically altered cells by microscopy, is still to be achieved. In our previous work we demonstrated separation of fragmented DNA from intact DNA by gel chromatography, on a low-resolution level, using Sepharose CL-2B [4,5]. This prompted us to undertake a further development of the gel chromatographic DNA analysis and to investigate chromatography matrices enabling higher resolution of the DNA fragment mixture.

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2. Experimental

2.1. Cells

Mouse hybridomas PVA 187 and HE 95 were grown in an iron-rich protein free medium [4]. HeLa cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. An immortalized keratinocyte line HPV-G (kindly donated by Dr. C. Mothersill, Dublin Institute of Technology, Dublin, Ireland) was cultured in DMEM–F 12 (1:1) medium supplemented with 10% fetal bovine serum. All cell lines were grown in T-flasks in media volumes of 5 to 50 ml. The relative number of apoptotic cells in the harvested cell populations was assessed by counting the cells displaying apoptotic morphology, i.e., shrunken cells with ruffled membranes under light microscopy. The morphological difference between round-shaped live cells and shrunken apoptotic cells was quite marked [6]. The relative number of necrotic cells, in the case of cell suspension heated to 60°C, was assessed by counting slightly swollen cells the membrane of which was permeable for trypan blue.

2.2. Isolation of mono- and dinucleosomes

Supernatant from the culture of PVA 187 hybridoma was concentrated by ultrafiltration (Ultrafiltration Cell 2000 Amicon, membrane PM-10 with cutoff of M_r 10 000) to 1/20 of the original volume and chromatographed on Sephacryl S-300 HR under conditions described earlier [7]. The contents of the tubes corresponding to mono- and dinucleosomes were pooled separately and rechromatographed.

2.3. Isolation of DNA

DNA from cells, from the culture fluid or from mono- and dinucleosomes was isolated using the procedure of Neiman et al. [8]. Briefly, the sample was treated by proteinase K in the presence of sodium dodecyl sulphate at 50°C, RNA was split by pancreatic RNAse and DNA was precipitated by isopropanol. In this mild procedure the phenol-extraction step was entirely eliminated. Electrophoretic control of the size of DNA molecules was carried out under standard conditions in 2.2% agarose gel.

2.4. Gel chromatography

An FPLC system including single path monitor UV-1, Sephacryl chromatography media and XK 16 columns (40 cm×16 mm I.D.) from Pharmacia Biotech (Uppsala, Sweden) were used for all analyses. The following columns were packed, with flow adaptors on both sides, and used either separately, in tandem or in triad: Sephacryl S-200 HR (bed height 125×16 mm I.D.), Sephacryl S-500 HR (bed height 280×16 mm I.D.), Sephacryl S-1000 SF (bed height 280×16 mm I.D.). The solvent for all separations was 1 *M* ammonium acetate. The samples were diluted to a volume 200 μl and applied to the column(s) through a sample loop. The solvent was pumped at the flow velocity 60 ml/h, i.e., 30 cm/h. The absorbance of the effluent was recorded by the UV-1 monitor at 254 nm. The settings were: UV-1 monitor at 0.1 absorbance, recorder at 5 mV.

In order to normalize the chromatography data, the void volume V_0 was defined operationally as the elution volume of intact DNA. Similarly, the volume of the stationary phase (V_s) was defined using the elution volume of thymidine. The formula for the distribution coefficient K_d [$K_d=(V_e-V_0)/V_s$] was used for characterizing the relative positions of the fragments on the chromatogram.

3. Results and discussion

Sephacryl S-500 HR is the largest-pore gel matrix available in high-resolution (HR) quality. Its resolution power for DNA molecules was tested with the DNA fragments isolated from mononucleosomes (~180 bps) and from dinucleosomes (~360 bps). The K_d values found with these samples on the Sephacryl S-500 HR column were 0.53 and 0.40 for the mono- and dinucleosomal DNA, respectively. The resolution of these two fragments (Fig. 1) could not be further improved by lowering the flow-rate.

Final DNA preparations obtained with the use of the enzymatic procedure [8] are contaminated with a fraction consisting of (ribo)nucleotides and small peptides. This mixture of UV-absorbing contaminants manifests itself as a broad irregular peak. Although the K_d of this peak is close to the value 1.0, the ascending part may slightly overlap with the

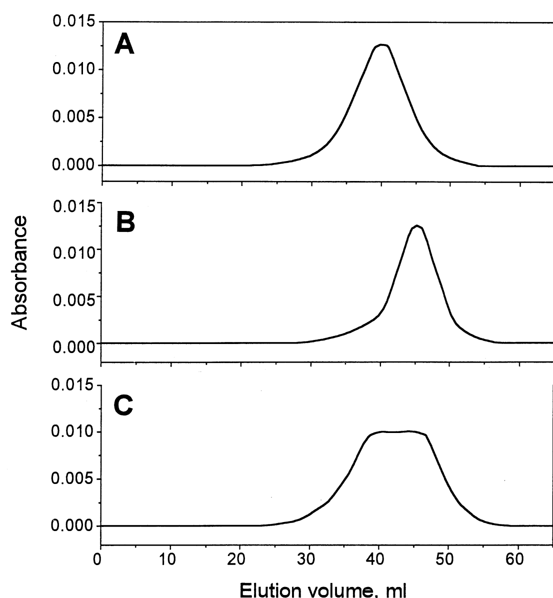

Fig. 1. Chromatography profiles of mono- and dinucleosomal DNA fragments on Sephacryl S-500 HR. (A) Dinucleosomal DNA (16 μg). (B) Mononucleosomal DNA (16 μg). (C) Mixture of mono- (8 μg) and dinucleosomal DNA (8 μg). Chromatography conditions are described in detail in Section 2.4.

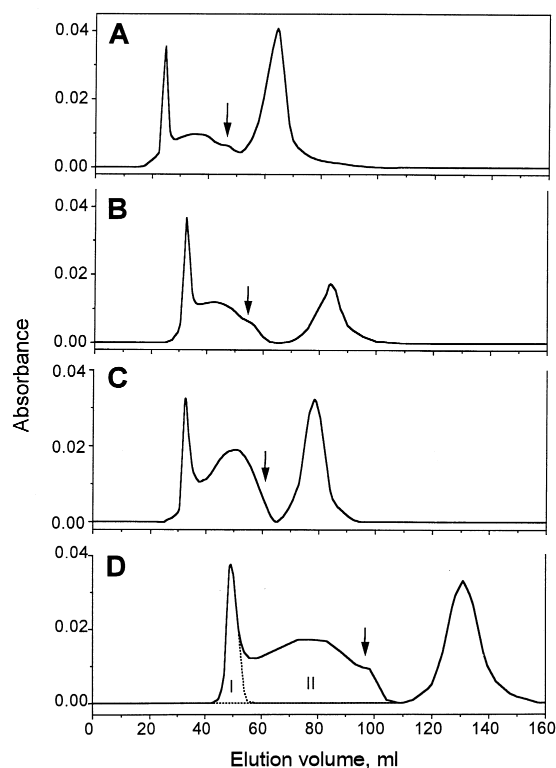

Fig. 2. Chromatography profiles of DNA from overgrown HE 95 hybridoma cells (83% apoptotic cells) on various column systems. DNA from $3 \cdot 10^6$ cells was applied to each run. (A) Sephacryl S-500 HR column. (B) Sephacryl S-500 HR column with a Sephacryl S-200 HR pre-column. (C) Sephacryl S-1000 SF column with a Sephacryl S-200 HR pre-column. (D) Triad system of Sephacryl S-200 HR, Sephacryl S-500 HR and Sephacryl S-1000 SF. The arrow indicates V_e of the mononucleosomal DNA fragment. The area below the broad peak of fragments (II in panel D) represents 82% of total DNA area. The area labelled I represents the intact DNA.

descending part of the mononucleosomal peak and may impair an accurate measurement of the area of DNA fragments (Fig. 2A). The disturbing effect of these contaminants was eliminated by insertion of a Sephacryl S-200 HR pre-column (Fig. 2B). The effective K_d value of mononucleosomal DNA in the tandem Sephacryl S-200 HR+Sephacryl S-500 HR system decreased to 0.41, as compared to 0.53 obtained without the pre-column.

DNA analysis on a tandem Sephacryl S-200 HR+ Sephacryl S-1000 SF system yielded a chromatography profile characterized by a reduced overlap of DNA fragments and intact DNA (Fig. 2C). In a separate experiment the K_d value of mononucleosomal DNA was found to be 0.53. Optimum resolution combining the advantages of Sephacryl S-500 HR and Sephacryl S-1000 SF was obtained using the triad of all columns (Fig. 2D). The K_d value of mononucleosomal DNA in this triad system was 0.50. The low-molecular-mass contaminants were fully separated from the analyzed DNA sample, and the improved resolution of DNA species allowed the measurement of areas below the peaks (Fig. 2D). The recovery of DNA collected from individual fractions was more than 95%.

The triad system was applied to the analysis of DNA in various cells and in the culture supernatant. Mouse hybridoma cells were found to respond to irradiation by enhanced DNA fragmentation (Fig. 3A and B). In accord with our previous observation [4] DNA from necrotic hybridoma cells did not display any significant increase of fragments (Fig. 3C). The

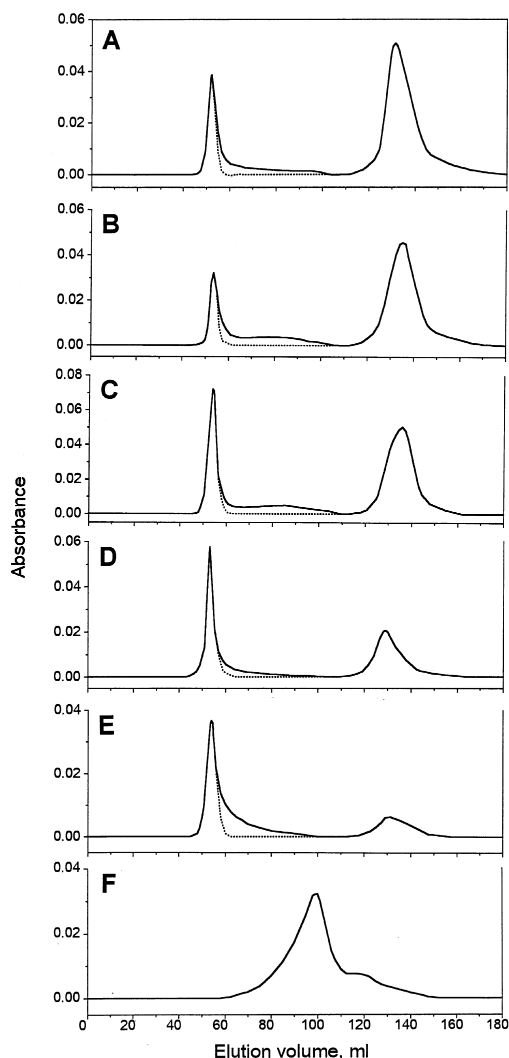

Fig. 3. Chromatography profiles of DNA from various cells on a triad of columns (Sephacryl S-200 HR+Sephacryl S-500 HR+Sephacryl S-1000 SF). The proportion of apoptotic or necrotic cells was estimated as indicated in Section 2.1. The fraction of fragmented DNA was determined as shown in Fig. 2D. (A) DNA from $2\cdot10^6$ exponentially growing hybridoma PVA 187 cells; 88% viable cells, 12% apoptotic cells; 31% DNA fragmented. (B) DNA from $3\cdot10^6$ PVA 187 hybridoma cells after X-ray treatment (5 Gy); 50% viable cells, 50% apoptotic cells; 51% DNA fragmented. (C) DNA from $4\cdot10^6$ PVA 187 cells after heating to 60°C for 5 min.; 19% viable cells, 16% apoptotic cells, 65% necrotic cells; 37% DNA fragmented. (D) DNA from $3\cdot10^6$ overgrown detached HeLa cells; 100% apoptotic cells. (E) DNA from $2\cdot10^6$ HPV-G cells after 40 h incubation with 5% dimethylsulfoxide in the medium; 100% apoptotic cells. (F) DNA from PVA 187 hybridoma culture fluid; 32% viable cells, 68% apoptotic cells.

HPV-G cells made apoptotic by a chemical agent displayed virtually no absorbance at the position of the mononucleosomal fragment (Fig. 3D), similarly with the profile of DNA from apoptotic HeLa cells (Fig. 3E). This finding is in accord with the notion that DNA in cells derived from epithelial tissues does not yield oligonucleosomal fragments, but is cleaved into large fragments [50 and 300 kilobases (kbs)] during cell death [9]. The tailing of the main DNA peak in the HeLa cell sample (Fig. 3E) is likely to represent relatively large fragments differing from typical oligonucleosomes. In the hybridoma culture fluid the mononucleosomal DNA fragment (V_e=97 ml, K_d=0.50) represented the most abundant component of the sample (Fig. 3F). The small ill-resolved peak with V_e~120 ml (K_d~0.75) was found only in the DNA from the culture supernatant. It might be identical with the 100 bp DNA fragment observed in some hybridoma cultures [10]. The profiles illustrated in Fig. 3 were in fair agreement with the electrophoretic patterns of these samples (Fig. 4). The typical oligonucleosomal ladder was pronounced in DNA from hybridoma cells (lanes 2 and 3).

The column chromatographic analysis of DNA fragmentation has been developed to serve in fundamental studies of apoptosis, in which the accurate picture of the state of DNA at a given time of a cell population, or of a piece of tissue, is the dominating demand. At first sight, the working volume of the triad of columns seems to be inadequately large for a microanalytical method. In our experience the large volume of the columns proved to be an advantage. Due to high viscosity of DNA solutions only very diluted DNA samples (below 250 μg/ml) could be applied without impairing the quality of the peak resolution. The size of the DNA sample, required for a single run, corresponds to DNA from 1 to $3\cdot10^6$ of cells. The standard deviation (S.D.) of the method in the range of ~5% (n=3 to 5), documents a fair reproducibility of the analysis.

The method overcomes the necessity of biosynthetic labelling of the analyzed DNA. The biosynthetic labelling sets limits to the experimental design, because the label has to be added many hours before the DNA sample is isolated from the cells. Moreover, the biosynthetic labelling is hardly applicable in the assessment of apoptotic DNA fragmentation

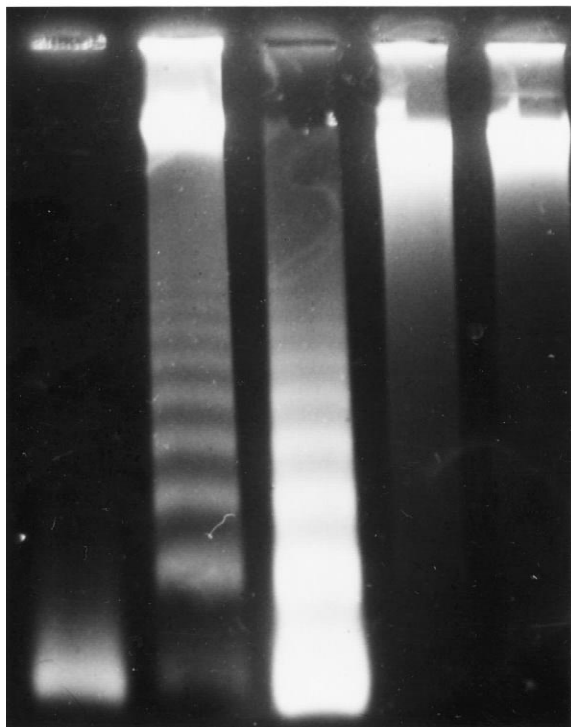

Fig. 4. Agarose gel electrophoresis of DNA from various cells. The DNA bands are visualized with ethidium bromide. Lane 1: size marker, mononucleosomal DNA fragment (3 μg). Lane 2: PVA 187 cells after after X-ray treatment (Fig. 3B). Lane 3: DNA from PVA 187 supernatant (Fig. 3F). Lane 4: apoptotic HeLa cells (Fig. 3D). Lane 5: apoptotic HPV-G cells (Fig. 3E).

occurring in tumours excised from experimental animals, as well as in large-scale bioreactor cell cultures.

Acknowledgements

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