Improvement of Separation Method of Fragmented DNA from an Apoptotic Cell DNA Sample for the Quantitation Using Agarose Gel Electrophoresis

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In order to quantify fragmented DNA extracted from apoptotic cells, we devised a separation method which condenses fragmented DNA into a small band, separating it from larger-size DNA with agarose gel electrophoresis. Calf thymus DNA and standard fragmented DNA were loaded onto 1.0% gel for 0.5, 1.0, 1.5 and 2.0 cm length, and onto 0.7, 1.0, 1.5 and 2.0% of gels for 1 cm length. DNA was then extracted from gel slices with the UltraClean™ 15 DNA Purification Kit, and estimated by measuring fluorescence intensity using Hoechst No.33258 dye. DNA recovery from the gel showed constant values regardless of the amount of loaded DNA up to 1 μg/assay, and a plot of loaded DNA amounts vs. the DNA amount yielded resulted in a straight line in any gel concentration used. Our results show the best conditions to estimate DNA fragmentation rates in apoptotic cells in which fragmented DNA was separated from thymus DNA by loading on 1.0% gel for 1.0 cm length. We used our method to estimate fragmentation rates in DNA fractions extracted from apoptotic human cervical fibroblast, amnion epithelial and chorion laeve trophoblaster cells by stimulation with actinomycin D. The results show that DNA fragmentation rates in these cells were consistent with the electrophoretic patterns of the DNA samples shown by their photographs.

Key words fragmented DNA; apoptosis; agarose gel electrophoresis

During vertebrates embryogenesis, programmed cell death (or apoptosis) is an important event in tissue formation and organogenesis. Generally, the biochemical parameters listed for cell death were a lactate dehydrogenase assay, a TdT-mediated dUTP nick end-labeling assay, a staining of nuclear DNA with Hoechst No.33258 dye and a TUNEL assay, and fragmented DNA quantification as follows. A hallmark of apoptosis is internucleosomal fragmentation of nuclear DNA, occurring relatively early during cell death as a result of induced nuclease activity. This process of specific DNA fragmentation leads to oligonucleosomes of different but defined length, i.e., multiples of 180—200 bp corresponding to DNA content/nucleosome. After agarose gel electrophoresis of extracted cellular DNA, apoptotic cell death reveals the appearance of a typical DNA ladder. This laddering is usually not found when looking at DNA from intact cells or cells killed by necrosis that contain only high molecular weight DNA or a smear of randomly degraded DNA. Some methods for quantifying DNA fragmentation rates have been reported previously. These methods extract only fragmented DNA from cells, use radio-isotope labeling of fragmented DNA, stain blot DNA with a stable probe after electrophoresis, use the ELISA technique with anti-nucleosomal antibodies or analyze gel photographs after electrophoresis densitometrically.

We are currently studying the mechanism of apoptotic cell death which occurs in human fetal membrane tissues at normal parturition. During our examination, a simpler method for evaluating the extent of apoptotic cells death was needed. We have designed an improved method for the evaluation of the degree of DNA fragmentation from recovered DNA after agarose gel electrophoresis. It is possible to say that our improved method is not only convenient and simple, but also correct.

MATERIALS AND METHODS

Materials Bis-benzimide (Hoechst No.33258) and Calf thymus DNA were purchased from Sigma Chemical Co., (MO, U.S.A.) and Worthington Biochemical Corporation, (NJ, U.S.A.), respectively. A Ready-Load™ 100 bp DNA ladder and an UltraClean™ 15 DNA Purification Kit were purchased from Gibco BRL, Life Technologies Inc., (NY, U.S.A.) and Mo Bio Laboratories, Inc., (CA, U.S.A.), respectively.

Agarose Gel Electrophoresis of DNA and Extraction from Gel Calf thymus DNA and a Ready-Load™ 100 bp DNA ladder were used as the native-size DNA and fragmented DNA standards, respectively. Each 20 μl of various concentrations of DNA solution was electrophoresed on 1.0% of the gel for 0.5, 1.0, 1.5 and 2.0 cm length, and loaded onto 0.7, 1.0, 1.5 and 2.0% of the agarose gel for 1 cm length. Small portions of the gel containing the DNA bands were the cut out. DNA was extracted from these gel slices using the UltraClean™ 15 DNA Purification Kit according to its instruction manual. Recovered DNA content was determined by the method using benzimide described below.

Determining DNA Content Measurement of DNA content was according to a modified Yui’s method. Ten microliters of DNA solution was put into a 96 well ELISA plate, which is a black and flat bottom type plate, and 40 μl of the assay buffer (10 mM Tris–HCl, pH 7.4, containing 1 mM EDTA-2Na and 2.1 mM NaCl) were added. The DNA solution was then mixed with 100 μl of Benzimide (Sigma Chemical) solution resolved in the assay buffer at 5 μg/ml. Fluorescence was measured on a Fluoroskan II (Dainippon, Osaka, Japan).
RESULTS AND DISCUSSION

Each solution of calf thymus DNA (0.5 μg) and the Ready-Load™ 100 bp DNA ladder (0.5 μg), and their mixture (total 1.0 μg) were electrophoresed on 1.0% agarose gel for various lengths of about 0.5, 1.0, 1.5 and 2.0 cm. As shown in Fig. 1, for every migration except 0.5 cm (Figs. 1 (a)—(c)), two DNA separated well. In order to effectively extract DNA from gel slices, fragmented DNA must be separated from non-fragmented DNA and the DNA then concentrated as small a band as possible. Accordingly, these DNA standards and their mixture were loaded on various concentrations of agarose gels, 0.7, 1.0, 1.5 and 2.0% for 1 cm length, as shown in Fig. 2. While calf thymus DNA showed a smear profile on the 0.7% gel, the fragmented DNA formed a small band. On 1.0% gel, calf thymus DNA showed little smear and the fragmented DNA also formed a smaller band. On 1.5 or 2.0% gel, calf thymus DNA formed a compact and small band, however, fragmented DNA did not form a small band, furthermore, the two standards did not separate distinctly on these gels.

Next, we examined the DNA yields from the gel after electrophoresis under the conditions described. To prepare the calibration curve and examine recoveries of both DNAs from the agarose gel, various amounts of up to 1.0 μg of both DNA were loaded on 0.7, 1.0, 1.5 and 2.0% agarose gels for about 1 cm length, individually. Small portions of each gel containing DNA bands as shown by the UV-illuminator were cut out. DNA extracted from gel slices was mixed with Hoechst No.33258 dye, and the fluorescence intensity of the DNA–dye mixture was measured, as shown in Fig. 3. Simultaneously, the intensities of 0—1.0 μg of calf thymus DNA–dye mixture were measured in order to prepare a reference calibration curve, shown as a dotted line in Fig. 3 (a). From the calibration curve, the yields for both standard DNAs were estimated and plotted against fluorescence intensities. Plots of loaded DNA amount vs. the fluorescence intensity gave straight lines in all gel concentrations 0.7% (a), 1.0% (b), 1.5% (c) and 2.0% (d). All plots showed that ladder DNA yields were better than those of calf thymus DNA. Averages of the percent of DNA yields were calculated and shown in Table 1. With calf thymus DNA, the yield average was 50—62%, and with ladder DNA was 77—100%. In any case, the higher the gel concentration, the better DNA yield was.

The fact that the yield average of calf thymus DNA was remarkably low revealed that this method may be well suited for the separation and estimation of large-size DNA molecules. Given the fragmentation rate of DNA extracted from apoptotic cells, we believe that only fragmented DNA was extracted from the agarose gels, and that the resulting fragmentation rate was calculated from both DNA extracted from the gel and total DNA loaded on the gel. Accordingly, various amounts of DNA standards mixture were also loaded and only fragmented DNA was extracted from the gels. The calibration lines for amounts of ladder DNA extracted from the gels against fluorescence intensities from each gel concentration are shown in Fig. 4. The results show that yield averages were lower than those of individual DNA, and that the relations revealed straight lines using any gel concentration except 0.7% gel.

To estimate fragmentation rates of DNA samples extracted from apoptotic cells, the samples were loaded on 1% agarose gel for 1 cm length. We instanced three DNA samples for analyzing by our method, which were extracted from three types of cultured cells, amnion-epithelial cells and...
chorion trophoblast cells prepared primarily from human fetal membranes, and human uterine cervical fibroblast (HCF), induced to undergo apoptosis by stimulation with actinomycin D. After electrophoresis, small portions of the gel containing DNA bands which were bigger than a 100 bp standard DNA band and smaller than the calf thymus DNA band, were cut out. In Fig. 5(B)-(a), the profile of 1.0 cm flow of electrophoresis of HCF DNA on 1% gel was shown as an example for representing a gel portion using to extract fragmented DNA which was marked and indicated as "Ext."
Fig. 5. Fragmented DNA Quantitation of Non-apoptotic Amnion-Epithelial Cells (A), and Apoptic Human Uterine Cervical Fibroblast and Chorion-Trophoblast Cells (B and C)

(a) Amnion epithelial cells

(b) DNA fragmentation rate

(c) Chorion trophoblast cells

(a) Agarose gel electrophoresis

(b) DNA fragmentation rate

(c) Agarose gel electrophoresis

(a) and (b) in each figure show profiles of agarose gel electrophoresis and DNA fragmentation rates quantified with triple loading for a sample by our method. Arrows and values described in (a) indicate the nucleotide length (bp) of bands. Electrophoretic profiles of HCF for 1.0 cm length on 1.0% gel are shown in (B)-(a). St1, St2 and St3 show profiles of calf thymus DNA, Ready-Load™ 100 bp DNA ladder and these DNA standard mixture, respectively. Ext. describes the portion of agarose gel used for extraction of fragmented DNA.
that DNA fragmentation is used as an easy indication of the precise and reproducible evaluation of apoptotic cell death levels, our quantitation method may be useful as a simple and effectual one.

Estimating DNA fragmentation simply by analyzing photographs of the same 1 cm flow gel described in Fig. 5(B)-(a), showed that the fragmentation rates of HCF DNA were less than 10% of non-treated cells but were at extremely higher rates of about 33%, 55% and 43% of 0.1, 0.5 and 1.0 μg/ml Act.D-treated cells, respectively. We consider that the significant difference between the two values obtained by our method and the simple image analysis system may be attributed to the quantitative shortness of the photographic negative and the positive contrast of a stained gel, because from the results of DNA yields from gel, reliability of the reference calibration curves of extracted DNA and quantitation obtained by our method, it is possible to be estimate that the yield of fragmented DNA of HCF can not be more than 10% in any Act.D-treated cells.

REFERENCES