GENETICS

Quantitative Evaluation of DNA Fragmentation

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A new method for evaluation of DNA fragmentation in tissue is based on computer-aided densitometric standardization of the sum of optical densities of DNA fragments visualized by electrophoresis (180-2500 b. p.) in comparison with the density of high molecular weight DNA in the same sample. The method allows evaluation of DNA fragmentation, an acknowledged marker of apoptosis, in the fetal rat brain and detection of increased DNA fragmentation during stress and under the effect of glucocorticoids.

Key Words: *apoptosis; DNA fragmentation; ontogenesis; brain*

Programmed cell death (apoptosis) responsible for elimination of excessive or damaged cells is an obligatory component of both normal development and pathological processes [3]. The final stage of apoptosis is characterized by internucleosomal DNA cleavage yielding fragments divisible by 180 b. p. [9] laddering on agarose gels stained with ethidium bromide [8]. The presence of fragmented DNA is a reliable and incontrovertible evidence of apoptosis. This sign is widely used as a qualitative marker of cell death [5,6]. In cells entering the apoptosis pathway at different time and in tissues, where elimination of excessive cells is normal, *e.g.* developing brain [1,10], evaluation of apoptosis intensity by the presence of DNA fragments is difficult and requires special approaches improving sensitivity of this analysis (TUNEL analysis or Southern blot hybridization). We propose a method of quantitative evaluation of DNA fragmentation for detection of changes in the intensity of programmed cell death.

MATERIALS AND METHODS

DNA was isolated routinely [2]: tissue sample was homogenized in a buffer containing 100 mM NaCl, 20 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 0.5% sodium dodecyl sulfate and incubated for 10 h at 55°C with 0.5 mg/ml proteinase K (SibEnzim). DNA was extracted with phenol-chloroform mixture (pH 8.0), precipitated with isopropyl alcohol, and dissolved in TE buffer (pH 7.5). DNA concentration and residual protein concentrations were evaluated at 260 and 280 nm, respectively (SF-26 spectrophotometer).

Aliquots of DNA preparations (1.5-8.25 μg) were analyzed by electrophoresis in 1.5% agarose in Tris-acetate buffer with ethidium bromide (0.5 μg/ml). The thickness of the gel was 5 mm, pouch volume 15 mm^3. Electrophoresis was carried out at 4.5 V/cm for 1.5 h. Molecular weight marker was applied to one row in each gel (1 μg/μl, 100 b. p., SibEnzim). DNA fluorescence in UV light (300 nm) was scanned using a BioDoc II system (Biometra GmbH). Optical density was evaluated and the results were digitized using Scion Image software.

The possibility of using this method for detection of fragmented DNA in tissues, where apoptosis is a normal process, and for detecting differences in DNA fragmentation after exposure to factors stimulating cell death was evaluated in DNA preparations isolated from adult rats and from the brain of 20-21-day fetuses. The rats were kept under standard vivarium conditions. The day of detection of semen in vaginal smears was considered as the first day of gestation.

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We used 20-day fetuses from intact females and females treated with hydrocortisone (5 mg/100 g, 0.2 ml) 6 h before sacrifice and 21-day fetuses from females injected with 0.5 ml 0.9% NaCl on days 16 and 18 of gestation (weak stress).

The significance of differences between the groups was evaluated using Student's *t* test.

RESULTS

In the brain of intact 20-21-day rat fetuses we detected low-molecular-weight DNA fragments, which were absent in adult animals (Fig. 1). The presence of fragmented DNA in the brain of intact fetuses is a result of programmed death of brain cell during prenatal ontogeny. According to different estimations, 10 to 50% initially formed neurons undergo apoptosis during the formation of the nervous system in mammalians [7,10]. Cell elimination in the nervous system of rodents is most intensive during embryogenesis and the first week of life; this process leads to stabilization of cell number and connections between cells [1,4,7]. Injections of normal saline or hydrocortisone to pregnant females led to an increase in the number of DNA fragments in fetal brain (detected by electrophoresis, Fig. 1).

Quantitative evaluation of DNA fragmentation was carried out by computer-assisted densitometry of electrophoregrams using Scion Image software (Fig. 2). The degree of DNA fragmentation was estimated as the sum of optical density of DNA fragments in selected areas standardized for the density of high-molecular-weight DNA (50,000 b. p.) in the same sample (left peak on densitograms, Fig. 2, *a*).

The degree of fragmentation is estimated with consideration for high-molecular fraction present in each sample (internal standard), which is an important advantage of our method: the degree of DNA fragmentation does not depend on the amount of DNA applied to the row in a wide range. In our experiments (i.e. at certain row capacity) the amount of fragmented DNA detected in the sample was virtually constant at DNA load of 1.5 to 4.5 μg per row. A constant positive correlation (r=0.87; p<0.001) between the amount of high-molecular-weight DNA and its fragments detected by densitometry was observed for this range. Further increase in DNA load disturbs this relationship (for gel parameters used in our work) because optical density of high-molecular fraction attained plateau (row overload).

Repeated measurements of DNA fragmentation for the same samples carried out under optimal condi-

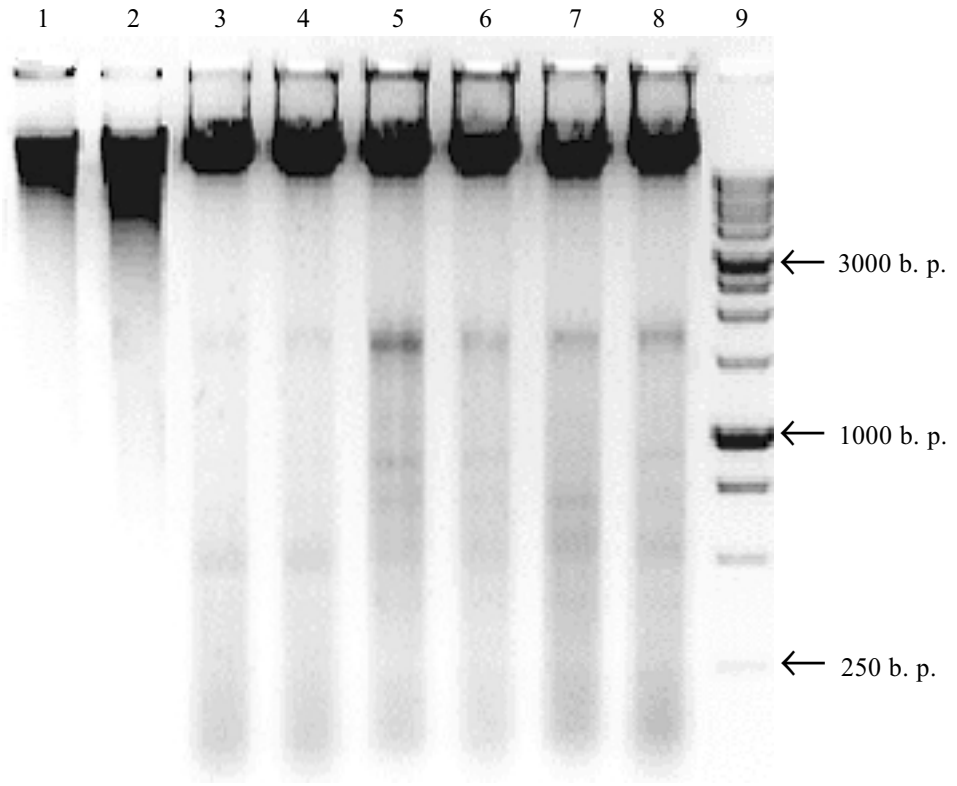

Fig. 1. Electrophoregram of DNA preparations from rat brain. 1, 2) adult animals; *3*, *4*) fetuses from intact females; *5*, *6*) 21-day fetuses from females injected with normal saline on days 16 and 18 of pregnancy; *7*, *8*) 20-day fetuses of females 6 h after injection of hydrocortisone; *9*) molecular weight marker. B. p.: base pairs.

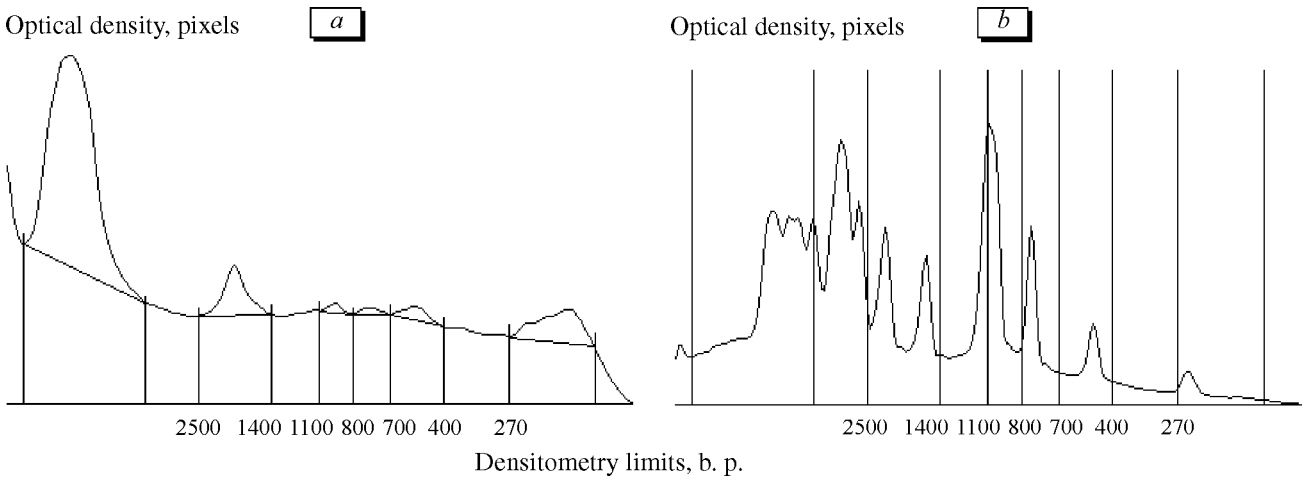

Fig. 2. A profile of optical density of DNA preparation (*a*) and molecular weight marker (*b*) plotted using Scion Image software. Vertical lines restrict areas selected for densitometry. The line connecting the peak bases (*a*) cuts away the background fluorescence and is used for estimation of peak areas by the Scion Image software.

tions (2-4.5 μg DNA per row) gave reproducible results and were in good correlation (r=0.79; p0.007); the error of repeated measurements did not surpass 12-15%.

The proposed method for evaluation of DNA fragmentation is highly sensitive and detects increased DNA fragmentation in the brain of rat fetuses after stress or hydrocortisone treatment. In our experiments 13.19±1.77% DNA detected in the brain of intact fetuses (n=8) were fragments containing less than 2500 b. p. Stress induced by injections of normal saline to females on days 16 and 18 of gestation led to a significant increase in the content of fragmented DNA in the brain of their 21-day fetuses to 23.20±2.46 and 23.3±4.3%, respectively, (n=6 and n=4). Exogenous stress hormone hydrocortisone also notably enhanced DNA fragmentation in the brain of 20-day fetuses as soon as 6 h postinjection. It is noteworthy that even after thorough standardization of the amount of DNA applied to the row it varies within a wide range, and therefore visual evaluation of the gels (Fig. 1) is not sufficient for detecting true differences.

Hence, a simple, reproducible, and sensitive method for quantitative evaluation of DNA fragmentation in tissues is proposed. This method allows evaluation of DNA fragmentation in cells entering apoptosis asynchronously in accordance with their own program of development, which was demonstrated for embryonic rat brain. In addition, this method detects enhanced DNA fragmentation in the brain of rat fetuses exposed to stress and glucocorticoids.

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