ELSEVIER

Journal of Chromatography A, 871 (2000) 321–330

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Capillary electrophoresis of DNA damage after irradiation: apoptosis and necrosis

María Teresa Valenzuela*, María Isabel Núñez, María Rosario Guerrero, Mercedes Villalobos, José Mariano Ruiz de Almodóvar

Departamento de Radiología y Medicina Física, Universidad de Granada, UMIM, Hospital Clínico Universitario ''San Cecilio'', Avda. de Madrid 9, 18071 Granada, Spain

Abstract

Apoptosis is a type of cellular death but also directly regulates tumorigenesis through different gene expression. This phenomenon is often used as end-point in studies of radio- and chemosensitivity of cancer cells. Restriction DNA fragments have been separated quickly, efficiently and successfully by capillary gel electrophoresis (CGE). In this study CGE has been applied to distinguish between the discrete pattern of degraded DNA produced by apoptosis and randomized DNA breaks produced by ionizing radiation. The influence of different variables has been discussed and an example of fast separation by CGE of the apoptotic fragments produced by UV light treatment is shown.

Keywords: DNA

1. Introduction

One of the most important challenges in cellular and molecular studies is to be able to quantify what until now has been described in qualitative terms. Cell homeostasis is regulated by a balance between proliferation, growth arrest and cell death. Apoptosis and necrosis have been described as two different forms of cell death, which can be easily distinguished by the distinctive changes that take place within the affected cells. Necrosis is a pathological form of cell death usually caused by an acute cellular injury and it is typified by irregular clumping of chromatin without significant change in its distribution, rapid cell swelling and lysis. In contrast, apoptosis is characterized by the early activation of endogenous proteases leading to cytoskeletal disruption, cell shrinkage, membrane blebbing, nuclear chromatin condensation, maintenance of cytoplasmic membrane integrity until late in the process, lack of an inflammatory reaction ''in vivo'' and a typical sort of degradation of the DNA into fragments with the size of oligonucleosomes. In consequence the fragment size is determined by the link between a certain number of nucleosomes that remain together (from 1 to *n*) [1,2]. DNA electrophoresis, used in this work as standard method, has been widely used for identification of this process and the development of the so-called ''ladder'' in agarose gels has come to be regarded as a biochemical hallmark of the process [2,3].

Although both modalities of cell death (apoptosis and necrosis) can be induced by cytotoxic stress there are several cellular factors which determine the

*Corresponding author. Tel.: +34-958-242-864; fax: +34-958-249-953.

E-mail address: mteresa@goliat.ugr.es (M.T. Valenzuela)

0021-9673/00/$ – see front matter
PII: S0021-9673(99)01245-5

class of cytolysis produced in response to DNA damage, i.e., necrosis supercedes the metabolic program of apoptosis when the cell is overwhelmed by the toxin at high concentration [4]. The induction of apoptosis occurs in mammalian cells not only as an integral part of development processes, but additionally as a response to steroid stimulation, viral infection and exposure to environmental carcinogens. Importantly, many mammalian cells undergo apoptosis following exposure to γ-irradiation. Moreover, radiation, hormonal, heat or chemically induced apoptosis may be the primary regulator of tumor cell death in cancer therapy [5,6].

In our previous work [6] we have compared the results obtained with four different methods used to detect, and in some way quantify, apoptosis: three based on the detection of DNA fragmentation (in situ tailing, the comet assay and DNA ladder formation) and the fourth the counting of the floating population. The results obtained were very different. DNA ladder formation related well to the in situ tailing but was not in agreement with the proportion of floating cells or with the results obtained in comet assays. Considerable caution should be exerted in the interpretation of the data obtained because the distinction between the degraded DNA produced by apoptosis and necrosis may be difficult by some particular techniques.

Capillary electrophoresis applied to the biological systems is emerging as a new bioanalytical method with the advantages of fast analysis time, automation, on-column injection and detection, reproducible analysis and high resolving power for the separation of double-stranded (ds) DNA fragments [7–9].

The velocity of the DNA fragment separated by capillary gel electrophoresis (CGE) is dependent on two physics conditions, electric field (E) and temperature (T), and one biochemical feature, the size of the DNA strand (S). This is because as a first approximation the total charge of DNA molecule (Q) can be assumed to be proportional to its number of base pairs (bp) [10].

Theoretically the mobility of a DNA fragment under a constant electric field (E) in CGE can be expressed as:

$$\ln \frac{1}{V} = \ln \frac{k}{ES} + \frac{E_a}{RT} \quad (1)$$

where V is the steady-state velocity of the particle movement calculated as the ratio between the capillary length and the migration time, k is a factor related to the net polyanion charge (Q), S is the size of the DNA fragment and also related to Q, E_a the activation energy for the viscous flow, T is the absolute temperature and R the universal gas constant [8].

In this study we present our results which focused on the search for a better method to perform quantitative studies on cell death after treatment with genotoxic agents. Analysis of DNA damage by a CGE method is compared with those obtained in conventional agarose gel-based electrophoretic systems. The main aim of the present study was to address and optimize the possibilities of CGE in resolving DNA fragments produced after apoptosis or necrosis in an irradiated human tumor cell line.

2. Experimental

2.1. Cell culture

A human lymphoma cell line named U937 was used in this study. It was grown in RPMI medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin (100 units ml^{-1}) and streptomycin (0.1 mg ml^{-1}). All cells were incubated at 37°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Freedom from mycoplasma contamination was checked regularly by testing with Hoechst 33528 dye (Sigma).

2.2. Radiation treatment

U937 cells in exponential growth were irradiated in all the experiments. Apoptotic death was induced after a 5-min treatment with UV light. At the end of UV-irradiation cells were reincubated in complete culture medium and standard conditions during 0, 2 and 3 h. Necrosis was studied after treatment with 15, 30, 45 Gy of γ-irradiation. Cells were irradiated using a ^{60}Co source at a dose rate of 1.67 Gy min^{-1} and after the treatment the initial damage was studied. Three separate experiments were done to study each cell death type.

2.3. DNA extraction

At the end of each incubation period after radiation, cells were collected, centrifuged for 10 min at 1500 rpm and washed twice with phosphate-buffered saline (PBS). The pellet was resuspended in 500 μl of lysis buffer (20 m*M* Tris–HCl, pH 7.3, 2 m*M* EDTA, 10 m*M* NaCl, 0.4% Triton X-100) and incubated at 4°C for 15 min. We then added 20 μg ml^{-1} RNAase and 100 μg ml^{-1} proteinase and the mixture was incubated at 37°C overnight. The DNA was extracted by adding an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) (Sigma) and after separating the aqueous phase by centrifugation (10 min at 3500 rpm), DNA was precipitated by adding 0.1 volume of 3 *M* sodium acetate and two volumes of ethanol at −20°C. DNA was washed twice using 70% ethanol and finally resuspended in Tris–EDTA buffer (0.1 *M* Tris–HCl, pH 8, 10 m*M* EDTA). DNA concentration and purity was quantified by spectrophotometric determination at wavelengths of 260 and 280 nm [11].

2.4. Analysis of DNA fragmentation pattern

Two different methods based on the detection of DNA fragmentation have been used:

2.4.1. DNA gel electrophoresis

The DNA samples (20 μl each) were electrophoretically separated using a Minicell System (Bio-Rad Labs., Hercules, CA, USA) in 1% agarose gel containing ethidium bromide (0.5 μg ml^{-1}). DNA was visualized with an UV transilluminator, and the gels were photographed.

2.4.2. Capillary gel electrophoresis

Separations were carried out by CGE on a P/ACE 5000 instrument from Beckman (Beckman Instruments, Palo Alto, CA, USA) in the reversed polarity mode. For laser-induced fluorescence (LIF) detection an argon-ion laser source (Beckman Instruments) was used with excitation at 488 nm and emission at 520 (for fluorescein) or 530 nm (for EnhanCE intercalator). Data were collected and analyzed using P/ACE Station Software version 1.0 (Beckman Instruments). Analysis of DNA fragments was conducted using the eCAP dsDNA 1000 kit and coated capillaries (27, 37, 47 or 57 cm×100 μm I.D.) filled with the DNA buffer supplied according to the manufacturer's recommendations (Beckman Instruments). The fluorescent intercalator EnhanCE was added to the buffer (0.4 μg ml^{-1}). Run temperature was set at 20, 30, 40 or 50°C depending on the experiment. Before each run the capillary was rinsed 3 min with eCAP dsDNA 1000 buffer. Then DNA samples, without further preparation after the extraction, were injected for 10 s at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). Marker standards were used for confirmation of peak identity and size determination: ΦX-174 Hea III fragments ranged from 72 to 1353 bp (dsDNA 1000 test mix, Beckman Instruments), λDNA/Hind III fragments ranged from 125 to 23 130 bp (Beckman Instruments) and AmpliSize Molecular Ruler ranged to 50 to 2000 bp (Bio-Rad Labs.). The volume of sample injected was estimated by CE expert (Beckman Instruments). The calibration curve for a 57-cm length capillary is shown in Fig. 1.

3. Results

3.1. DNA patterns analyzed by capillary gel electrophoresis

We have performed CGE of DNA fragmented by apoptosis using coated capillaries. The profile described by the relative fluorescence unit (RFU) emitted vs. time closely reproduces the ''laddered'' pattern seen in agarose gel-based electrophoresis. Fig. 2 exhibits the separation for DNA fragmented by apoptotic degradation or by 45 Gy of γ-irradiation using conventional agarose gel electrophoresis and CGE. This second even is representative of the induction of randomized DNA breaks. In contrast each of apoptotic DNA fragments are produced by enzymatic breaks at the linker regions between nucleosomes. In these experiments at least fragments that include from one to six nucleosomes and their linkers could clearly be distinguished in the CGE method. The expected mean sizes for these were approximately 166, 332, 498, 664, 830, 996, 1162 and 1328 bp. As in agarose-based electrophoresis, in CGE the small molecules are detected quicker because they migrate faster than larger molecules.

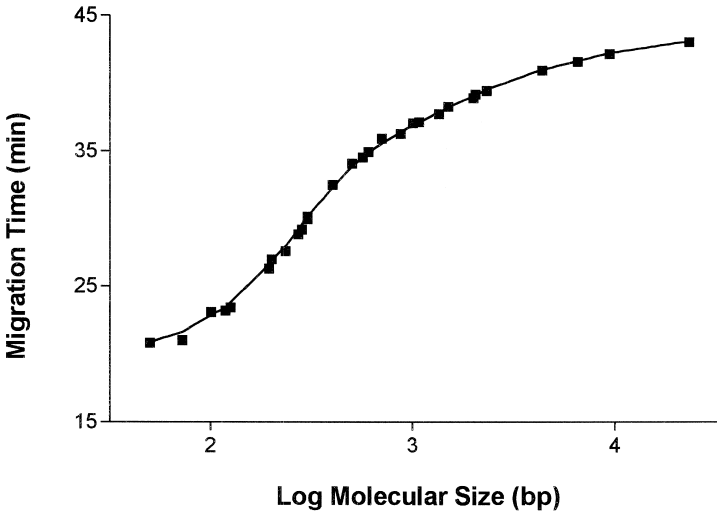

Fig. 1. Relationship between DNA size and migration time for three different molecular markers (total range for DNA size: 50–23 130 bp). Conditions: capillary total length 57 cm, 20°C, eCAP dsDNA 1000 buffer, 5 s injection.

After repeated runs the migration time for each peak does vary but the difference was less than 15 s when the capillary was 57 cm long (relative error <0.005) and less than 7 s when it was 27 cm long (relative error <0.005). This indicates a well-behaved reproducibility of the system to measure this phenomenon.

Taking into account the variables included in Eq. (1) we can propose a fast method to separate DNA fragments whatever their origin was. The separation can be performed in less than 15 min at 20°C in a 27 cm long capillary (Fig. 2A). The parameters that describe the apoptotic pattern are shown in Table 1.

3.2. Effect of temperature on DNA fragment separation

Fig. 3 compares the separation of U937 apoptotic DNA (5 min treated with UV light and reincubated for 2.5 h under standard conditions before the extraction) by CGE at different temperatures. The migration time of the DNA fragments decreases as temperature increases and a good separation of fragments corresponding to the first six nucleosomes is seen. The logarithm of migration time for different DNA fragments (corresponding to the first six nucleosomes and two fragments belonging to the AmpliSize Molecular Ruler marker – 50 and 2000 bp) is plotted in Fig. 4 as a function of the reciprocal temperature. All data were fitted by linear regression and the slope (E_a/R), y-intercept, correlation coefficient and P values are shown in Table 2. It can be seen that all the slopes, parameter proportional to the activation energy, are very similar decreasing slightly when the molecular mass is higher.

3.3. Effect of capillary length on DNA fragment separation

Fig. 5 shows the linear relationship between the migration time and the molecular mass of nucleosome fragments when we used capillaries with different lengths (27, 37 and 57 cm). Two fragments of the molecular marker (50 and 2000 bp) were also included in this graph. Using a 27-cm-length capillary the total time necessary to get the electropherogram is reduced to 33% with respect to the time using a 57-cm-length capillary.

3.4. Effect of voltage applied on DNA fragment separation

Fig. 6 shows the effect of the applied electric field on the migration velocity of apoptotic DNA fragments. Two fragments of the molecular mass marker (50 and 2000 bp) were also included in this graph. In agreement with Eq. (1) a linear relationship was

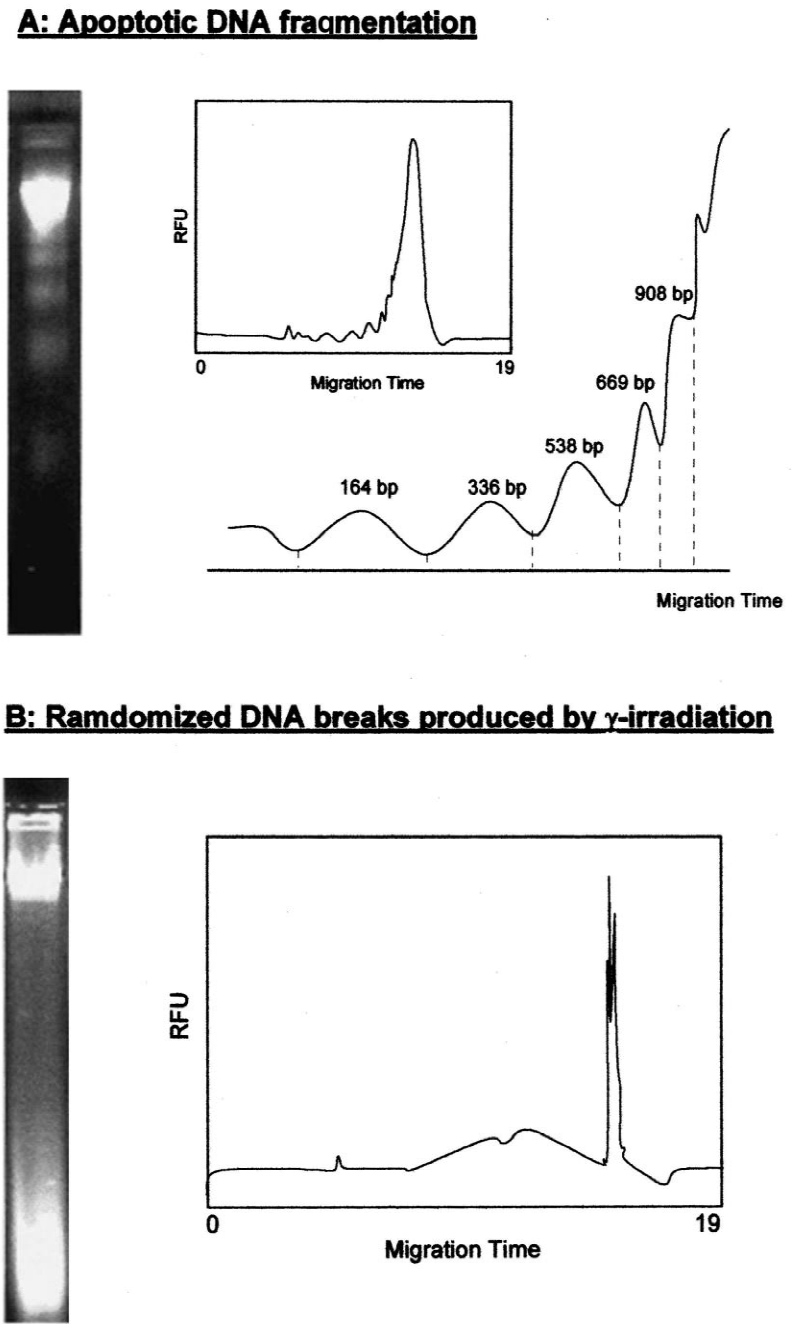

Fig. 2. Agarose gel electrophoresis and CGE electropherogram of DNA extracted from U937 cells. (A) Cells 5-min treatment with UV light and reincubated in complete culture medium 2 h (apoptotic DNA). A detail of the separation of nucleosome fragments is shown; (B) DNA pattern after 45 Gy γ-irradiation. In both methods fragments were identified using molecular mass markers as reference. Conditions: capillary total length 27 cm, 20°C, eCAP dsDNA 1000 buffer, 10 s injection. Time scale in min.

Table 1
Estimation of mean size of apoptotic DNA fragments by CGE[a]

Expected size (E)	Observed size (O), mean value (range)	Ratio (O/E)
1 nucleosome (≈166 bp)	164 (84–251)	0.98
2 nucleosomes (≈332 bp)	336 (289–440)	1.01
3 nucleosomes (≈498 bp)	538 (440–630)	1.08
4 nucleosomes (≈664 bp)	669 (630–803)	1.01
5 nucleosomes (≈830 bp)	908 (803–980)	1.09
6 nucleosomes (≈996 bp)	1097 (980–1196)	1.10

[a] Conditions: capillary total length 27 cm, eCAP dsDNA 1000 buffer, 10 s injection. Observed values are compared to expected ones.

found between the logarithm of the reciprocal migration velocity value and the logarithm of the reciprocal voltage applied in the range of DNA fragments studied. The slopes obtained were very similar and close to one in all the cases.

The electrophoretic migration velocity data as a function of the size of apoptotic DNA fragments at different voltages has been studied too. For this analysis the net charge of the DNA was directly substituted by the base-pair number in Eq. (1) because the total electrostatic force on the DNA molecule can be assumed to be constant per unit length. The total charge of the molecule is proportional to the number of base pairs and therefore one can use this parameter instead of the net charge [8,10]. When we consider all the fragments included in an interval ranged between 50–2000 bp a non-linear relationship is seen between the reciprocal absolute base pair number and the logarithm of the migration velocity measured (data not shown). This relationship fits to a linear equation if the 50 bp point is excluded from the analysis. The correlation coefficient was $r \geq 0.99$ and P value, $P \leq 0.0001$ for all the voltages used (data not shown).

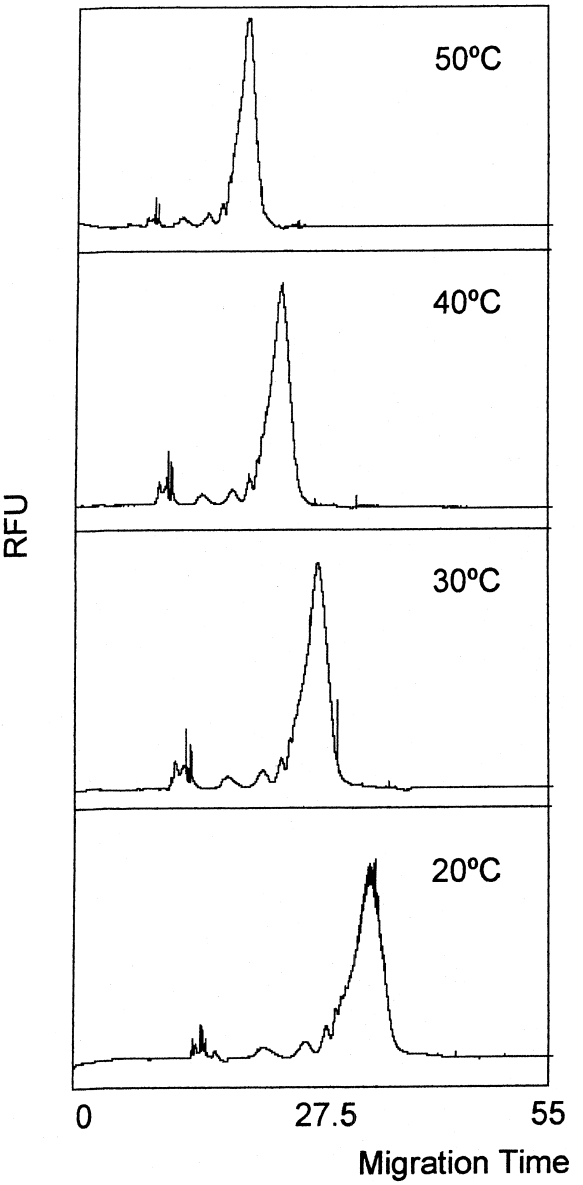

Fig. 3. Separation of apoptotic DNA fragments by CGE at different temperatures. Conditions: capillary total length 57 cm, eCAP dsDNA 1000 buffer, 10 s injection. Time scale in min.

4. Discussion

Because the capillary column has a large surface-to-volume ratio, the high applied voltages used in CGE produce rapid separation. Many types of DNA molecules with a specific molecular mass [molecular markers, restriction fragments, polymerase chain reaction (PCR) fragments] have been separated quickly, efficiently and successfully [7,9,12]. They appear as very thin bands in agarose gels and as sharp and well-defined peaks in the electropherogram. In this study we have investigated the optimal conditions to distinguish between the discrete pattern of degraded DNA produced by apoptosis and ran-

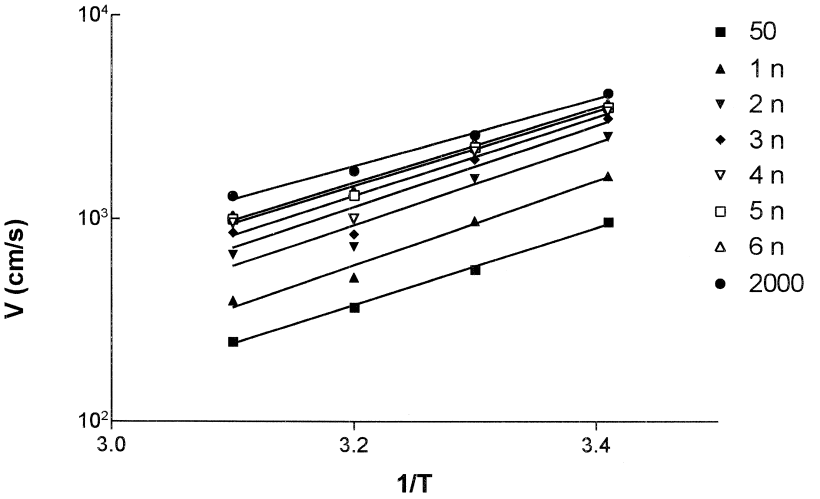

Fig. 4. Relationship between the velocity of apoptotic DNA fragments and the reciprocal temperature. Two fragments of the molecular marker (50 and 2000 bp) were also included in this graph. Lines correspond to different sized DNA fragments: from top to bottom, 2000, ≈996 (six nucleosome fragments), ≈830 (five nucleosome fragments), ≈664 (four nucleosome fragments), ≈498 (three nucleosome fragments), ≈332 (two nucleosome fragments), ≈166 (one nucleosome fragment) and 50 bp. Conditions as in Fig. 3.

domized DNA breaks produced by ionizing radiation treatment using CGE.

First of all we have separated and identified apoptotic DNA fragments which are the result of a double-strand cleavage of nuclear DNA at the linker regions between nucleosomes [1,2]. Each nucleosome consists of two full turns of DNA wound around as octameric histone core plus the adjacent ''linker DNA'' (≈83 nucleotide pairs per turn). The apoptotic fragments have a size determined by the link between a certain number of nucleosomes that remains together and appears as broad bands in a ''laddered'' pattern in agarose gels (Fig. 2A). In CGE these fragments include a particular region into the electropherogram. Their qualitative analysis and identification was possible using the position of the well-defined fragments of DNA mass markers as calibration curve under the conditions previously described. Table 1 gathers the results obtained for each apoptotic fragment: mean size and the interval corresponding to each one. The calibration accuracy has been estimated through the ratio between the observed and the expected value. In the six fragments analyzed, the ratio was always ≤ 1.10. This mean that the dispersion is reasonably low ($\leq 10\%$).

The effect of different variables such us temperature or capillary length were studied to optimize the apoptotic DNA fragment separation. When we considered the effect of temperature in DNA fragment separation we could see a decrease of the migration

Table 2
Effect of temperature in DNA fragment separation

	E_a/R	ln (k/ES)	r	P value
50 bp	1.90±0.08	−3.45±0.24	0.998	0.001
1 nucleosome (≈166 bp)	2.07±0.21	−3.80±0.69	0.999	0.010
2 nucleosomes (≈332 bp)	2.02±0.38	−3.47±1.24	0.967	0.033
3 nucleosomes (≈498 bp)	2.00±0.48	−3.29±1.58	0.946	0.054
4 nucleosomes (≈664 bp)	1.93±0.40	−3.02±1.30	0.960	0.040
5 nucleosomes (≈830 bp)	1.84±0.16	−2.67±0.51	0.993	0.007
6 nucleosomes (≈996 bp)	1.84±0.16	−2.66±0.51	0.993	0.007
2000 bp	1.65±0.10	−1.97±0.32	0.996	0.003

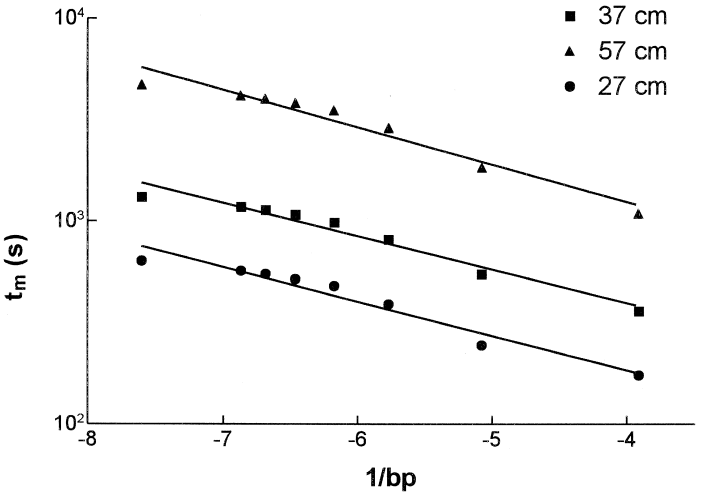

Fig. 5. Effect of the capillary length (57, 37 and 27 cm) on the migration time. Different nucleosome fragments and two fragments with 50 and 2000 bp have been plotted in this graph. Conditions: 20°C, eCAP dsDNA 1000 buffer, 10 s injection.

time as temperature increases (Fig. 3). In spite of the high temperature and faster migration, it is important to point out that the resolving power is enough to distinguish not only the restriction fragments of DNA molecular mass marker as described by other authors [8] but also apoptotic fragments. Because of the temperature effect on viscosity of the separation buffer the increase in migration velocity found was expected. Besides the linear relationship between the migration time for different DNA fragments and the reciprocal temperature was in good agreement with Eq. (1) where the term E_a/R describes the migration properties of the DNA fragment. This parameter that is proportional to the activation energy is very similar for all the fragments decreasing slightly when the molecular mass is higher. It could be explained

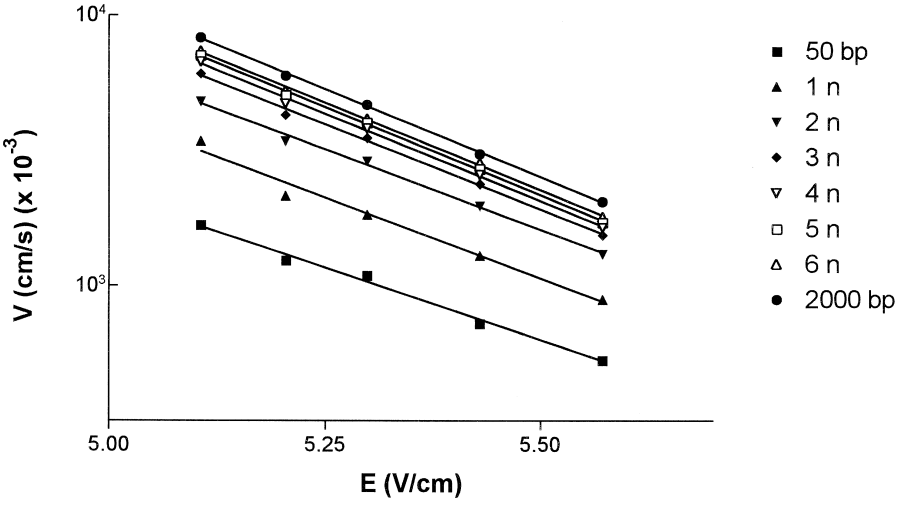

Fig. 6. Effect of the applied electric field on the migration velocity of apoptotic DNA fragments. Two fragments of the molecular marker (50 and 2000 bp) were also included in this graph. Lines correspond to different sized DNA fragments: from top to bottom, 2000, ≈996 (six nucleosome fragments), ≈830 (five nucleosome fragments), ≈664 (four nucleosome fragments), ≈498 (three nucleosome fragments), ≈332 (two nucleosome fragments), ≈166 (one nucleosome fragment) and 50 bp. Conditions as in Fig. 3.

by possible conformational DNA changes with elevated temperature [9,13]. These changes might be more important in larger fragments.

The migration time is also influenced by the capillary length (Fig. 5). The manipulation of the capillary length affects the speed without altering highly the resolving power not only for DNA restriction fragments but also for different apoptotic DNA fragments.

Differences in sample properties, such as shape, size or net charge, lead to differences in electrophoretic mobility which provide the basis of electrophoretic separation are also pointed out studying the effect of voltage applied in DNA fragment separation (Fig. 6). In general, the electrophoretic mobility is independent of field strength. However, it was found that high field density in the high-molecular-mass range leads to field-dependent mobility in the electrophoresis of DNA [14,15]. These effects are believed to arise from the orientation and stretching of the coiled configuration of DNA by the increased applied voltage [10,14]. Under our experimental conditions there is an apparent linear relationship between velocity and voltage used. This relationship includes implicitly information about the apparent shape of DNA molecule as previously described by Guttman [16]. Moreover we have found in all the electrical conditions used that below 72 bp the velocity of the double stranded DNA molecules is less than the predicted by Eq. (1) (data not shown). Because of partial melting, this result might be due to the apparent increase in the size of the low-molecular-mass fragments which leads to anomalous migration [14].

In addition it has previously described that the apparent length of the DNA molecule increases with complexation of intercalating ligand as ethidium bromide which increases the intrinsic viscosity of DNA [14]. Therefore the increased apparent length and rigidity of DNA would certainly tent to change migration properties of the complex. In the CGE with laser induced fluorescence detection the fluorescent intercalator EnhanCE is used. Then the increase in the apparent length of DNA and the variation in the migration time could be reinforced by the presence of this compound.

In consequence modifications of temperature and/or electric field can allow not only the separation of DNA fragments but also an instrumental approach to the ''apparent'' shape of different DNA molecules under the conditions chosen.

Another important aspect of CGE was the small injection volume used for each run ($\approx$0.3 μl, 10 s injection in a 27-cm-length capillary at 20°C). This is extremely important when sample amount is limited, id, when only a small number of cells is available for analysis. This is the case when dealing with biological material in short supply. Apoptosis is a type of cellular death but also directly regulates tumorigenesis though different gene expression [2,4,5]. This phenomenon is often used as end-point in a lot of studies of radio and chemosensitivity of cancer cells. Routine analysis could be achieved for the characterization of apoptotic DNA within 15 min without intense labor in an automated fashion using CGE as an instrumental approach to agarose gel electrophoresis. CGE not only affords rapid and automated analysis but also the possibility to quantify the induced damage on the cellular DNA by different treatments. To quantify each apoptotic fragment resolved by CGE one must have a well-behaved reproducibility of the migration time that in our conditions is observed. Further experiments which are now been pursued at our laboratory are needed to quantify DNA damage. Then, the differences in cellular sensitivity to different treatments could be also detected comparing the electropherogram patterns observed.

Acknowledgements

This work was supported by the Comisión Interministerial de Ciencia y Tecnología through the project PM97-0185 and by the Fundación Ramón Areces through the project entitled ''Apoptosis y Cáncer de Mama: Estudio Clínico-Experimental de Moléculas Relacionadas con los Mecanismos de la Resistencia a la Terapéutica''. M.T.V. is supported by a grant from the University of Granada (Beca de Perfeccionamiento de Doctores, plan propio 1997) and M.R.G. by a grant from the Ministerio de Educación y Cultura (AP95 24210266). The capillary electrophoresis system P/ACE 5000 (Beckman Instruments, Palo Alto, CA, USA) was funded by a

special grant of Fondo de Investigaciones Sanitarias (FIS-1998).

References

[1] J.F. Kerr, A.H. Wyllie, A.R. Currie, Br. J. Cancer 26 (1972) 239.
[2] M.B. Kastan, C.E. Canman, C.J. Leonard, Cancer Metastasis Rev. 14 (1995) 3.
[3] A.H. Wyllie, Br. J. Cancer 67 (1993) 205.
[4] B.W. Steward, J. Natl. Cancer Inst. 86 (1994) 1286.
[5] J.M. Lee, A. Berstein, Cancer Metastasis Rev. 14 (1995) 149.
[6] E. Siles, M. Villalobos, L. Jones, R. Guerrero, J.J. Eady, M.T. Valenzuela, M.I. Núñez, T.J. McMillan, J.M. Ruiz de Almodóvar, Br. J. Cancer 78 (1998) 1594.
[7] A. Guttman, A.S. Cohen, D.N. Heiger, B.L. Karger, Anal. Chem. 62 (1990) 37.
[8] A. Guttman, N. Cooke, J. Chromatogr. 559 (1991) 285.
[9] A.S. Cohen, D. Najarian, J.A. Smith, B.L. Karger, J. Chromatogr. 492 (1989) 585.
[10] R.C. Cantor, C.L. Smith, M.K. Mathew, Annu. Rev. Biophys. Chem. 17 (1988) 287.
[11] J. Sambrook, in: N. Ford, C. Nolan, M. Ferguson (Eds.), Molecular Cloning. A Laboratory Manual, 2nd Edition, Cold Spring Laboratories Press, 1989, p. E.5.
[12] T.A. Felmlee, P.S. Mitchell, K.J. Ulfelder, D.H. Persing, J.P. Landers, J. Capil. Electrophoresis 2 (1995) 125.
[13] N.C. Stellwagen, Biochemistry 22 (1983) 6186.
[14] A. Guttman, N. Cooke, Anal. Chem. 63 (1991) 2038.
[15] E.M. Southern, Anal. Biochem. 100 (1979) 319.
[16] A. Guttman, Appl. Theor. Electrophoresis 3 (1992) 91.